Characterization of Three Putative Monoamine Oxidase Genes in *Caenorhabditis elegans*

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This thesis entitled
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ABSTRACT

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Characterization of Three Putative Monoamine Oxidase Genes in Caenorhabditis elegans

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Monoamine neurotransmitters like dopamine and serotonin regulate neuronal function and behavior in animals. In vertebrates, monoamine oxidase (MAO) degrades monoamines. We investigated the function of three amx genes, putative MAO genes in the nematode Caenorhabditis elegans. We hypothesized that if the amx genes encode MAOs, deletion mutants would have a partial or complete absence of MAO activity, causing an increase in monoamines levels, altered monoamine modulated behaviors and hypersensitivity to exogenous monoamines. Using glyoxylic acid induced fluorescence, we quantified dopamine and serotonin in the mutants but did not find any significant differences. However, the amx mutants exhibited abnormalities in some monoamine modulated behaviors and increased sensitivity to exogenous dopamine and serotonin. Finally and interestingly, we found that Pamx-1::GFP transgenic lines suggested that amx-1 was expressed predominantly in chemosensory neurons.

Approved: ______________________________________________________________

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TABLE OF CONTENTS

Abstract ............................................................................................................................... 3

Acknowledgements ........................................................................................................... 4

List of tables ..................................................................................................................... 7

List of figures ................................................................................................................... 8

Chapter 1 Introduction .................................................................................................... 10

1.1 Monoamine neurotransmitters .............................................................................. 10

1.2 Monoamine catabolism and monoamine oxidases .............................................. 11

1.3 Caenorhabditis elegans as a model organism ....................................................... 13

1.4 Monoamines in Caenorhabditis elegans ............................................................... 13

1.5 Putative monoamine oxidases genes in C. elegans ............................................. 14

1.6 Our hypotheses ...................................................................................................... 15

Chapter 2 Characterization of monoamine levels in amx mutants ......................... 20

2.1 Introduction ............................................................................................................ 20

2.2 Experimental ......................................................................................................... 21

2.3 Results and discussion ......................................................................................... 27

Chapter 3 Analysis of amx-1 expression profile in transgenic amx-1 nematodes ....... 40

3.1 Introduction .......................................................................................................... 40

3.2 Experimental ........................................................................................................ 41

3.3 Results and discussion ....................................................................................... 44
Chapter 4 Characterization of monoamine dependent behavior and drug sensitivity in *amx* mutants

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction</td>
<td>53</td>
</tr>
<tr>
<td>4.2 Experimental</td>
<td>55</td>
</tr>
<tr>
<td>4.3 Results and discussion</td>
<td>59</td>
</tr>
<tr>
<td>Summary and conclusions</td>
<td>85</td>
</tr>
<tr>
<td>Bibliography</td>
<td>90</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>Primers for ( amx ) genes</td>
<td>31</td>
</tr>
<tr>
<td>Table 2.</td>
<td>Dopamine intensities for all induced fluorescence experiments</td>
<td>34</td>
</tr>
<tr>
<td>Table 3.</td>
<td>Serotonin intensities for all induced fluorescence experiments</td>
<td>35</td>
</tr>
<tr>
<td>Table 4.</td>
<td>Body bends in a minute in methyl cellulose solutions</td>
<td>67</td>
</tr>
<tr>
<td>Table 5.</td>
<td>Body bends in 20 seconds in dopamine solutions</td>
<td>76</td>
</tr>
<tr>
<td>Table 6.</td>
<td>Immobilization in 90 mM dopamine solution</td>
<td>78</td>
</tr>
<tr>
<td>Table 7.</td>
<td>Body bends in 20 seconds in serotonin solutions</td>
<td>80</td>
</tr>
<tr>
<td>Table 8.</td>
<td>Immobilization in 8.5 mM serotonin solution</td>
<td>82</td>
</tr>
<tr>
<td>Table 9.</td>
<td>Summary of behavior results</td>
<td>84</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Monoamine neurotransmitters in <em>C. elegans</em></td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Major routes for catabolism of dopamine in vertebrates</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Model of dopamine trafficking</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>MAO homologs in <em>C. elegans</em></td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>Primers for amx genes</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>PCR products amplified from nematode genomic DNA</td>
<td>33</td>
</tr>
<tr>
<td>7</td>
<td>Diagrams of <em>C. elegans</em></td>
<td>36</td>
</tr>
<tr>
<td>8</td>
<td>Diagrams of CEP and NSM neurons in the head</td>
<td>37</td>
</tr>
<tr>
<td>9</td>
<td>Immunolocalization of serotonin in the NSM neurons</td>
<td>38</td>
</tr>
<tr>
<td>10</td>
<td>Glyoxylic acid induced fluorescence in wild-type vs. amx mutant</td>
<td>39</td>
</tr>
<tr>
<td>11</td>
<td>Anti-GFP, anti-VMAT and anti-DAT in the heads of Pamx-1:: GFP transgenic strains</td>
<td>48</td>
</tr>
<tr>
<td>12</td>
<td>DiI staining in the head</td>
<td>49</td>
</tr>
<tr>
<td>13</td>
<td>DiI staining in the heads of Pamx-1:: GFP transgenic strains</td>
<td>50</td>
</tr>
<tr>
<td>14</td>
<td>Anti-GFP and anti-ChAT in the heads of Pamx-1:: GFP transgenic strains</td>
<td>51</td>
</tr>
<tr>
<td>15</td>
<td>Anti-GFP and anti-ChAT in the tails of Pamx-1:: GFP transgenic strains</td>
<td>52</td>
</tr>
<tr>
<td>16</td>
<td>Movement in methyl cellulose</td>
<td>68</td>
</tr>
<tr>
<td>17</td>
<td>Movement in food</td>
<td>69</td>
</tr>
<tr>
<td>18</td>
<td>Movement in sephadex bead suspension</td>
<td>70</td>
</tr>
<tr>
<td>19</td>
<td>Pharyngeal pumping</td>
<td>71</td>
</tr>
</tbody>
</table>
Figure 20. Egg-laying. ..................................................................................................... 72
Figure 21. Embryos \emph{in utero}. .................................................................................. 73
Figure 22. Embryos age \emph{in utero}. ........................................................................... 74
Figure 23. Survival curves for wild-type vs. mutant nematodes. ................................. 75
Figure 24. Movement in exogenous dopamine. ......................................................... 77
Figure 25. Immobilization in 90 mM dopamine.......................................................... 79
Figure 26. Movement in exogenous serotonin ......................................................... 81
Figure 27. Immobilization in 8.5 mM serotonin ...................................................... 83
CHAPTER 1 INTRODUCTION

1.1 Monoamine Neurotransmitters

Monoamine (MA) neurotransmitters play a central role in regulating neuronal function and modulating behavior throughout the animal kingdom (reviewed in Purves et al. 2001). These neurotransmitters are derivatives of amino acids; they include the catecholamines, dopamine (DA), epinephrine (adrenaline) and norepinephrine (noradrenaline), as well as histamine, serotonin (SER), phenylethylamine, tyramine (TYR), and octopamine (OCT). In humans and other vertebrates, DA has been associated with learning, memory, motivation, pleasure, motor activity, and other behaviors. SER regulates general arousal, appetite, mood, sleep and anger, while epinephrine and norepinephrine are involved in the stress-response and arousal. Many psychological disorders, including depression, Parkinson’s disease and schizophrenia, have been associated with defects in MA metabolism (Nagatsu 2004). For example, Parkinson’s disease is a major neurodegenerative disease caused by progressive loss of dopaminergic neurons that result in motor dysfunction and eventual death. Drugs that alter SER metabolism such as the SSRIs (selective serotonin re-uptake inhibitors) are commonly prescribed for patients with clinical depression (Purves et al. 2001). MAs are also important in regulating neuronal function and behavior in invertebrates such as crustaceans and Drosophila (Sloley 2004). In nematodes, DA, SER, OCT and TYR (Figure 1) are present and modulate many behaviors (see Chapter 4).
1.2 Monoamine Catabolism and Monoamine Oxidases

MAs are synthesized from amino acids by specific synthetic enzymes and then packaged into synaptic vesicles by vesicular monoamine transporters (VMATs) (reviewed in Cooper et al. 2002). When the MA containing neurons are stimulated, calcium-dependent vesicle fusion releases MAs into the extracellular space. The MAs can then bind to specific membrane receptors (pre-synaptic, post-synaptic, or distant). MAs generally activate G-protein coupled receptors rather than opening ion channels, causing a variety of cellular changes such as alterations in cAMP, Ca\(^{2+}\), or inositol triphosphate (IP3) levels. MAs may be transported back into pre-synaptic cells by re-uptake transporters for reuse or degradation by enzymes, or they may diffuse away in the extracellular space. A schematic of DA trafficking is shown in Figure 2; all MAs have a similar trafficking pathways.

MAs are degraded in vertebrates by enzymes such as monoamine oxidases (MAOs) (reviewed in Shih et al. 1999) and catechol-O-methyl-transferase (COMT) (reviewed in Cooper et al. 2003). Figure 3 shows major catabolic routes for DA degradation in vertebrates. COMT degrades catecholamines; it is found in the post-synaptic neurons, mostly associated with microsomes and plasma membrane. MAO degrades most of the neuronal MAs in vertebrates. It is associated with the outer membrane of mitochondria in pre-synaptic, MA containing neurons. There it degrades any newly synthesized or recycled MA that is not packaged into vesicles. In vertebrates, two genes encode two slightly different enzymes, MAO-A and MAO-B (reviewed in Shih 1999, Nagatsu 2004). Both enzymes require flavin adenine dinucleotide (FAD) as a
cofactor to catalyze the oxidative deamination of MAs. MAO-A preferentially oxidizes SER and norepinephrine while MAO-B preferentially oxidizes phenylethylamine and benzylamine. In the vertebrate brain, MAO-A is found in catecholaminergic (dopamine, noradrenaline, and adrenaline containing) neurons and MAO-B in serotonergic and histaminergic neurons and glia. MAO-B in serotonergic cells acts to degrades all MAs other than SER. Non-neuronal tissues such as liver and kidney predominantly contain MAO-B while placenta and thyroid contain MAO-A.

MAO catalytic activity has a critical role in regulating the release and degradation of MAs. MAO-A knockout mice have high levels of SER and show behavioral differences such as aggression, which are consistent with elevated SER levels (Cases et al. 1995, Chen et al. 2007). MAO-B knockout mice do not show any changes in DA levels and show few behavioral changes (Nagatsu 2004, Shih 2004). MAO-A deficiency in humans has been linked to antisocial personality disorder and increased aggression (Chen et al. 2006, Alia-Klien et al. 2008). Other MA related clinical conditions in humans include depressive illness and Parkinson’s disease. MAO-A inhibitors are used as anti-depressants; blocking MAO-A’s activity leads to higher levels of SER, an important factor in regulating mood (reviewed in Purves et al. 2001). MAO-B inhibitors, which cause an increase in DA levels, are used in treatment of Parkinson’s disease (Riederer et al. 2007).

Invertebrates use a variety of enzymatic routes to breakdown monoamines. MAO and MAO-like activities have been shown in echinoderms, arachnids, cephalopods, bivalves, and platyhelminthes (reviewed in Sloley 2004). In insects and
pseudocoelomates, N-acetylation rather than oxidative deamination is the preferred catabolic pathway for monoamines (MAs). The MA degradation in nematodes is discussed below.

1.3 *Caenorhabditis elegans* as a Model Organism

*C. elegans* has been recognized as a powerful and simple model organism (Brenner 1974; Aamodt 2006). These nematodes are small, only 1.5 mm long, and are translucent. These can be easily grown in the laboratory on agar plates spread with bacteria. They have a short generation time of 3 days, and a large brood size of over 200 offspring. They reproduce either by means of mating between hermaphrodites and males or by self-fertilization of hermaphrodite. The nematodes have a simple cellular organization (WormAtlas 2008). They have 959 somatic cells, which includes 302 neurons with known location and morphology. MA mutants can be generated with ease and studied to assay behavioral and nervous system function. Hence, in *C. elegans* studying MAs is tractable (Brenner 1974, WormAtlas 2008), making it an appropriate model for the present study.

1.4 Monoamines in *Caenorhabditis elegans*

*C. elegans* contains DA, SER, OCT, and TYR but no functional epinephrine and norepinephrine (reviewed in Chase *et al.* 2007). The pathway of synthesis, packaging, and release followed by degradation or diffusion is similar for all monoamines (Figure 2). Out of 302 neurons, there are approximately 25 MA containing neurons, including 8 DA mechanosensory neurons (Sulston *et. al.* 1975), 12 SER motor or interneurons (Horvitz *et al.* 1982; Desai *et al.* 1988; Duerr *et al.* 1999); 2 TYR motor neurons, and 2 OCT
interneurons (Alkema et al. 2005). Different MAs modulate various behaviors including egg-laying, pharyngeal pumping, learning, and locomotion (reviewed in Chase et al. 2007). Specific MA modulated behaviors are discussed in detail in Chapter 3.

1.5 Putative Monoamine Oxidases Genes in *C. elegans*

In *C. elegans* and other nematodes, biochemical assays have indicated the presence of monoamine metabolizing MAO and aryl-alkylamine N-acetyltransferase activities (reviewed in Isaac et al. 1996). The proteins that possess these activities are not known. Vertebrates contain two similar monoamine oxidases, MAO-A and MAO-B, each with a flavin-containing amine oxidase domain (PFAM PF01593) that is shared with polyamine oxidases. There are several genes with a similar domain in *C. elegans*, including the three *amx* genes *amx-1, amx-2, amx-3* and the unnamed genes F55C5.6 and C24G6.6 (WormBase 2008). AMX-2 (protein) is the most similar to vertebrate neuronal MAOs (41% similarity to human MAO-A), while AMX-1 is more similar to the highly related vertebrate flavin-containing amine oxidase domain containing protein 1 (36% similarity). AMX-3 and F55C5.6 are both more similar to polyamine oxidase 1 than vertebrate MAOs and C24G6.6 is similar to PAOX peroxisomal oxidase (Dr. Duerr, personal communication). We requested deletion mutants for all of these genes from the *C. elegans* Gene Knockout Consortium (http://celeganskoconsortium.omrf.org/); they used PCR screening of mutants to identify deletions of the *amx-1, amx-2, and amx-3* genes. Figure 4 shows the gene models (in purple), the location of the monoamine oxidase protein domains (in brown) and the deletions present in the mutants (in red). The figure also shows the homology with *C. briggsae* (a sister species of *C. elegans*); low
percentage homology is indicated in gray and high percentage in blue (Kent et al. 2000). The gene models represent predictions of the genes from a variety of sources including computational prediction and mRNA sequencing.

1.6 Our Hypotheses

We hypothesized that one, MAO activity is important for MA degradation in *C. elegans* and two, one or more of the *amx* genes encode MAOs. Therefore, we predicted the *amx* deletion mutants would have a partial or complete absence of MAO activity and an increased level of one or more MAs, depending on the specificity of the particular AMX enzyme. For the triple mutant with deletions of all three genes, we expected an increase in all MAs. Therefore, we examined MA levels in the *amx* mutants using histochemical techniques. We also tested the mutants for any alterations in MA dependent behaviors. Finally, we characterized expression of the *amx-1* gene to see if it is localized in MA containing cells. The results of these tests will provide evidence on whether or not the *amx* genes encode MAOs that are important for MA degradation in *C. elegans*. 
Figure 1. Monoamine neurotransmitters in *C. elegans*. 
Figure 2. Model of dopamine trafficking. The schematic shows a synapse. In the presynaptic cell (left), dopamine is synthesized from tyrosine, which is converted to L-DOPA by tyrosine hydroxylase (TH). Aromatic amino acid decarboxylase (AADC) converts L-DOPA to dopamine. The vesicular MA transporter then packages the DA into synaptic vesicles. When vesicles fuse, DA is released and can bind to G-protein coupled receptors (DAR) on the pre- or post-synaptic cells. DA may be then taken up by a membrane transporter (DAT), diffuse away or be degraded. In vertebrates, DA may be degraded by monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT) (not shown, see Figure 3) (Figure by Dr. Duerr).
Figure 3. Major routes for catabolism of dopamine in vertebrates
Figure 4. MAO homologs in *C. elegans*. Top bars show location; hash=100 bp. Underneath are gene models (purple), amine oxidase domain (PF01593, brown), and deletion alleles (red). *C. briggsae* lines indicate low (gray) or high (blue) percentage homology (modified from Wormbase 2008).
CHAPTER 2 CHARACTERIZATION OF MONOAMINE LEVELS IN *AMX* MUTANTS

2.1 Introduction

*Monoamine Cells in C. elegans Hermaphrodites*

*C. elegans* hermaphrodites have approximately 959 cells. These include 8 dopaminergic neurons, 4 CEPs and 2 ADEs in the head and 2 PDEs in the body and tail (Sulston *et al.* 1975). CEPs are mechanosensory cells with cell bodies near the nerve ring (the ‘brain,’ or major synapse-rich region of the head) and processes extending to the tip of the nose. ADEs and PDEs also have mechanosensory endings in the outer body (White *et al.* 1986, WormAtlas 2008). Twelve SER motor or interneurons (Horvitz *et al.* 1982) have also been identified; the bilaterally symmetric pairs of the NSMs, HSNs, ADFs, AIMs and the unpaired neurons I5, VC4, VC5 and RIH neurons (reviewed in Chase *et al.* 2007). We have focused on the NSMs, neurosecretory motor neurons that have somas and processes in the pharynx. SER is thought to be released from the NSMs in response to food (White *et al.* 1986, WormAtlas 2008). Finally, two TYR motor neurons and two OCT interneurons have been identified (Alkema *et al.* 2005).

We hypothesized that the *amx* genes encode MAOs and that the *amx* deletion mutants would have inefficient MA degradation and increased levels of one or more MAAs. We used two techniques, antibody staining and glyoxylic acid induced fluorescence, to examine DA and SER in specific cells. High performance liquid chromatography is another technique that can be used to quantify the levels of
monoamines and metabolites in whole nematodes homogenates (Sanyal et al. 2004, Kuwahara et al. 2006); however, levels in specific cells can not be quantified. This technique has not been used in the present work.

We used indirect immunofluorescence with anti-SER antibodies to examine SER levels in the NSM cells in the pharynx. This technique allows detection of approximately two-fold change in the levels of SER. Quantification of levels of other MAs like DA, OCT and TYR can not be done with this technique because specific antibodies to these MAs that work in *C. elegans* are not available.

For quantification of DA and SER, we used glyoxylic acid induced fluorescence (Duerr et al. 1999). Glyoxylic acid treatment produces fluorescent derivatives of some monoamines. In particular, derivatives of DA (and related compounds) will fluoresce green and derivatives of SER will fluoresce yellow; TYR and OCT derivatives do not fluoresce. This technique is considerably easier to perform than indirect immunofluorescence, but it has less sensitivity. Since monoamine related compounds may fluorescence when treated with glyoxylic acid, it is important to identify the position of the DA cells in the head (the CEPs and ADEs) and the SER cells in the pharynx (the NSMs) while quantifying the monoamines.

### 2.2 Experimental

*C. elegans Strains and Strain Construction*

Strains were maintained as described by Brenner (1974), at 20°C on NGM agar spread with OP50 *E. coli* as a food source. Wild-type nematodes were *C. elegans* strain Bristol N2. Mutant strains used were *amx-1(ok659)* RM2350, *amx-2(ok 1235)* RM2356
and \textit{amx-3(Ok1868)} RM2373. The original deletions were made by the \textit{C. elegans} Gene Knockout Consortium and obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN, USA). These deletions were made by random mutagenesis; we verified their specificity by amplification of the genomic DNA using polymerase chain reaction (PCR) and DNA sequencing.

To eliminate undesired background mutations introduced by the mutagenesis process, our laboratory out-crossed the strains six times with wild-type nematodes. \textit{amx-1} and \textit{amx-2} were out-crossed by Nanda Filkin; \textit{amx-3} mutant was out-crossed by Tami Coursey and me. One round of out-crossing is done as follows (Ahringer 2006). A cross was set up between three N2 wild-type males and one mutant hermaphrodite. For all mating, males were generated by heat shock (30°C) for 6 hours (in \textit{C. elegans} hermaphrodites are XX and males are XO and can be generated by non-disjunction of the sex chromosome, Hart 2006). After a successful cross (progeny consisting of half males and half hermaphrodites), 10 hermaphrodite F1 progeny were isolated and allowed to reproduce through self fertilization. The progeny of each F1 were tested for heterozygosity using PCR (for primer information see Table 1 and Figure 5, primers made by Invitrogen, Carlsbad, CA). Twenty progeny of heterozygous F1 were selected and allowed to propagate through self-fertilization. Their progeny (F3 generation) were tested for homozygosity of the deletion using PCR.

Generation of the double mutant \textit{amx-2; amx-1} and triple mutant \textit{amx-2; amx-1; amx-3} were done in a similar fashion. First, \textit{amx-1} was mated with \textit{amx-2} (by Melissa
LaBonty in our laboratory) to produce the double mutant \textit{amx-2; amx-1}. Then I crossed the \textit{amx-3} males with \textit{amx-2; amx-1} hermaphrodites to produce the \textit{amx} triple mutant.

\textit{Serotonin Antibody Staining}

The antibody staining was done using the standard procedure as described below (Duerr 2006).

\textit{Fixation}: One to ten plates of a mixed population of well-fed nematodes were washed using M9 saline (0.02 M K$_2$HPO$_4$·7H$_2$O, 0.02 M KH$_2$PO$_4$, 0.08 M NaCl, 0.01 M MgSO$_4$ in dH$_2$O) and collected in a 1.5 ml tube. These were spun at 400 g for 2-3 minutes. Supernatant was discarded and saline rinses were repeated 3-4 times to remove all traces of bacteria. Nematodes were then washed with distilled water and spun for 2 minutes at 400 g. The supernatant was discarded, leaving 50-100 \textmu l of solution with nematodes. These were put on ice for 2-3 minutes before adding 1 ml fix solution (4% formaldehyde made fresh in 0.1 M Na$_2$PO$_4$, pH 7.2). Tubes were then alternatively frozen in a dry ice-90\% alcohol bath and defrosted in hot water to crack their cuticles. The tubes were then incubated on a rocker at 4\degree C for 24 hours. To remove the fixative, tubes were spun at high speed, supernatant was discarded and 3-5 rinses with PBST (PBS = 1.4 M NaCl, 0.02 M KCl, 0.1 M Na$_2$HPO$_4$·7H$_2$O, 0.02 M KH$_2$PO$_4$, 0.03 M sodium azide; PBST = PBS + 0.5\% Triton X-100) were done.

\textit{Reduction}: Nematodes were placed in freshly made \textbeta-mercaptoethanol solution (5\% \textbeta-mercaptoethanol, 1\% Triton X-100, 120 mM Tris pH 7.0) and shaken at 37\degree C for 2 hours. These were then spun at 1,000 g for 3 minutes, supernatant was removed and 8-9 PBST rinses were done.
Collagenase treatment: Nematodes were transferred to 0.5 ml tubes and were shaken at 37°C in 50-100 μl collagenase solution [2,000 units/ml collagenase type VII (Sigma #C-0773) in 100 mM Tris, 1 mM CaCl$_2$, 0.1% Triton X-100 pH 7.4]. The treatment was stopped after 24-36 hours, when many but not all adult nematodes were broken in half. Tubes were then kept on ice for 2 minutes and spun at 400 g for 2 minutes. Nematodes were then rinsed with PBS 2 times.

Antibody staining: For blocking non-specific protein binding, 5-10 μl of fixed worms were added to 250 μl block [10% donkey serum in antibody buffer (PBS with 0.5% Triton X-100, 1 mM EDTA, 0.1% BSA, 0.05% sodium azide)] and shaken for 1 hour at room temperature. Tubes were spun at 1,000 g for 2 minutes and block was removed. 1:500 rabbit anti-serotonin antibody (ImmunoStar Inc., Hudson, WI) in antibody buffer was added and shaken overnight at 4°C followed by a spin at 400 g for 2 minutes. Supernatant was discarded and tubes were topped with antibody buffer and shaken for 15 minutes at room temperature. This was repeated twice. After discarding the supernatant, 1:1000 Cy3-donkey anti-rabbit (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in antibody buffer was added and tubes were shaken in the dark overnight at 4°C. Tubes were spun at 400 g for 2 minutes to remove secondary antibody solution and rinsed three times for 15 minutes each with antibody buffer at room temperature with gentle shaking. Antibody buffer was then replaced with PBS. For mounting the nematodes, the tubes were spun at 400 g for 2 minutes then PBS was removed. A small (10 μl) drop of nematodes was placed on a clean slide and 10 μl of mounting medium [20 mg/ml n-propyl gallate, 30 mM Tris pH 9, 70% glycerol, 2 μg/ml
DAPI (4', 6-diamidino-2-phenylindole)] was added. N-propyl gallate is an anti-oxidant used to preserve fluorescence and DAPI stains DNA. A cover slip was placed on top of the sample and sealed with two to three layers of nail polish. The slides were stored at 4°C in the dark.

*Laser Confocal Microscope*

The slides were viewed with the Zeiss LSM scanning confocal microscope, using filters appropriate for Cy-3 (red; excitation 633 nm and emission >650 nm) and DAPI (blue; excitation 364 nm and emission 385-470 nm).

*Glyoxylic Acid Induced Fluorescence*

To make pre-dipped slides for adhering nematodes, commercial poly-lysine slides (Erie Scientific Company, Portsmouth, NH) were dipped in a poly-lysine solution (Sigma Aldrich, St. Louis, MO) (1 mg/ml Sigma P1524 poly-L-lysine, 0.1% sodium azide) as follows. Slides were dipped in poly-L-lysine for 5 minutes with gentle shaking. The slides were then dried in a 60°C oven for at least an hour; slides were stored at 4°C.

Adult hermaphrodites were selected 48 hours prior to the assay and grown at 20°C. Approximately 20 individual nematodes were picked and transferred to a drop of 20 μl water on a pre-dipped poly-lysine slide. An untreated commercial poly-lysine slide was placed on top and the sample was compressed slightly. These slide ‘sandwiches’ were kept on dry ice for 10 minutes to freeze the nematodes. Slides were then pulled apart to break open the cuticle of the worms, leaving a fraction of the nematodes adhered to the stickier pre-dipped bottom slide. The bottom slide and adherent nematodes were immediately dipped in ice-cold glyoxylic acid solution (0.2 M sucrose, 0.2 M monobasic
KH$_2$PO$_4$, 0.1 M glyoxylic acid, final pH 7.4) for 7.5 minutes. The slides were dried under blowing cool air for 30 minutes, then covered with five drops of light mineral oil (Sigma Aldrich, St. Louis, MO) and incubated for 2.5 minutes on a 102°C hot plate and covered with cover slips. The slides were stored in at 4°C in the dark for up to one day before viewing. After successful treatment, from 3-5 individuals with clear morphology in the head were produced on each slide.

**Confocal Microscope Settings**

The slides were viewed with the Zeiss LSM scanning confocal microscope, using two sequential scans and lasers and filters appropriate for ‘green’ DA (excitation 514 nm and emission >530 nm) and ‘red’ SER (excitation 364 nm and emission >475 nm).

**Image Collection and Analysis**

The images for each genotype were obtained in parallel with wild-type nematodes processed and imaged on the same day. A 150 micron by 150 micron area was scanned for each image. Since the nematodes were significantly compressed during the procedure, the stack size of each z-series varied from 5 microns to 8 microns. At the beginning of each day, amplifier gain for the confocal detector was adjusted such that the specific signal in control (wild-type) slides did not saturate the detectors (<255 intensity) and the background of the slide was approximately zero. This was necessary due to the day-to-day variability of the induced fluorescence technique.

To obtain a semi-quantitative comparison of the fluorescent intensities of different nematodes, maximum projections of confocal series were analyzed using Image J (Rasband 2007). DA images and SER images were analyzed separately. For analysis of
DA, a region of interest (ROI) 50 pixels wide by 50 pixels long (14.6 by 14.6 microns) was manually placed over the ventral portion of the nerve ring, including parts of the processes of the CEPs, ADEs, and PDEs. For SER a narrow ROI 70 pixels wide by 15 pixels (20.5 by 5.9 microns) long was placed over a portion of the NSM processes in the pharynx. In each image, a background region of the same size adjacent to the nerve ring, but not containing any neuron specific fluorescence, was also selected (see Figure 8 for an example of ROIs). A corrected mean intensity was calculated as \( \text{Xcorrected} = (\text{mean intensity ROI}) - (\text{mean intensity background}) \). Five or more images for each genotype were analyzed from different days. Xcorrected values for mutants were compared with Xcorrected values for wild-types on the same day.

**Statistical Analysis**

Wilcoxon paired rank test was used to compare the means of different genotypes on different days. A p-value of <0.05 was considered significant.

2.3 Results and Discussion

The \( amx \) deletion mutants, obtained from the *C. elegans* Gene Knockout Consortium, were out-crossed six times with the wild-type strain and tested for specific \( amx \) deletions after each cross. Using specific primers (Table 1 and Figure 5), genomic DNA from each of the mutants was amplified using PCR and deletions confirmed by the size or absence of specific products. The band sizes obtained in wild-type nematodes for each gene primers are shown in Figure 5. For the \( amx-1 \) deletion, primers 105 and 108 gave no product since primer 108 was in the deletion, while primers 102 and 105 gave a 433 bp product when the deletion was present and a 3kb product in wild-type nematodes.
For the *amx*-2 deletion, primers 176 and 207 gave no PCR product since 207 was in the deletion, while primers 175 and 204 gave a 600 bp product when the deletion was present and a 2.2 kb product in wild-type nematodes. For the *amx*-3 deletion, primers 226 and 231 gave no product since primer 226 was in the deletion, while primers 231 and 233 gave a 400 bp product when the deletion was present and a 2.1 kb product in wild-type nematodes. Figure 6 shows an example of a gel of PCR products from the fourth round of out-crossing of the *amx*-3 deletion, compared with controls (wild-type, the original *amx*-3 deletions strain, and a mixture of wild-type and *amx*-3 deletion nematodes to mimic a heterozygous stock).

Figure 8 shows the location of specific DA and SER neurons in *C. elegans*. We did not detect any significant difference in SER through anti-serotonin antibody staining in *amx* mutants. However, staining with anti-serotonin antibodies was quite variable in my hands and I had only one successful staining experiment among four attempts. Note also that we observed significant levels of nonspecific staining in the nerve ring, pharynx bulbs and the body of nematodes with this specific batch of commercial antibody (Figure 9).

We did not observe any significant differences in DA levels in the *amx* mutants using glyoxylic acid induced fluorescence (see Table 2; Figure 10). (Experiment number 9 was done by Dr. Duerr.) For all images, a maximum projection of a confocal z-series was made and a region of interest (ROI) including a portion of the CEP, ADE and PDE processes in the nerve ring was determined. For analysis, corrected mean intensity values ($X_{corrected} = \text{mean intensity in ROI} - \text{mean intensity in background}$) were calculated for
each nematode. The results of the wilcoxon sign-rank test comparing wild-type and mutant nematodes on each day indicated a p-value for > 0.05 for all mutants. However, we found that the $X_{\text{corrected}}$ values for wild-type nematodes were very variable, with up to a 4-fold variation on one day (e.g., N2 DA $X_{\text{corrected}} = 8.8$ to 50.6 on one day, where the maximum intensity is 255). Such high variability in wild-type nematodes indicates that the technique is very variable and hence it would be very difficult to detect moderate changes in DA levels in mutants.

Similar results were obtained for SER levels (see Table 3). Unfortunately, the variability in SER $X_{\text{corrected}}$ for wild-type nematodes was high, with a two fold variation for wild-type nematodes on any one day. As with DA levels, an increase in SER level was seen in SER $X_{\text{corrected}}$ values for all $amx$ mutants: $amx$-1 (51.6+/- 25.3), $amx$-2 (56+/- 26.4), $amx$-3 (45.8+/-25.4) and the triple $amx$ mutant (54.7+/- 30.7). However, there were no statistically significant changes (Table 3; Figure 10).

Together, although the DA and SER levels were higher in $amx$ mutants, no statistically significant changes were detectable. Our hypothesis was that any single $amx$ mutant ($amx$-1, $amx$-2 or $amx$-3) might have DA or SER levels similar to wild-type nematodes because of specificity of particular MAOs (AMXs) for individual MAs or compensation for loss of one $amx$ gene by up-regulation of other $amx$ genes (as is seen with MAO-A and MAO-B in vertebrates, Shih et al. 1999) or COMT. We did expect to see differences in MA levels in the $amx$ triple mutant. However, no significant increase in levels was present. The lack of observed changes in MA levels could have been due to any of a number of factors. In MAO-A knockout mice there is a nine-fold increase in
circulatory SER while in MAO-B knockout mice there is no detectable change in MA levels (Shih 2004). In nematodes, absence of MAOs may also cause similar or minor changes in MAs that were not detected in the present study. It is also possible that the relative contribution of the MAOs to MA degradation was small enough that loss of MAOs did not lead to the >4 fold difference in MA levels that could be detected with the techniques used in the present study. In addition, regulation of endogenous MA levels, e.g., decreased MA synthesis, in the amx mutants may have compensated for decreased MA degradation.
Table 1

*Primers for amx genes*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer no.</th>
<th>Location</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>amx-1</td>
<td>108</td>
<td>III: 3825046-065</td>
<td>GAC TTG GAC TCT CTC TAC AC</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>III: 3825922-940</td>
<td>TAC GTA GCA TCA CCA TCC G</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>III: 3822872-889</td>
<td>TGA CAA CCG ATG CTT CTC T</td>
</tr>
<tr>
<td>amx-2</td>
<td>176</td>
<td>I: 12771893-912</td>
<td>TCT CAG CAA ATG GAC ACT GC</td>
</tr>
<tr>
<td></td>
<td>204</td>
<td>I: 12763165-84</td>
<td>GCT GTG CCA ATT CCG ACA</td>
</tr>
<tr>
<td></td>
<td>175</td>
<td>I: 12769109-28</td>
<td>CAG CCT CAA CCA CCT TTT GT</td>
</tr>
<tr>
<td></td>
<td>207</td>
<td>I: 12761793-812</td>
<td>CCC ACC CAA ATT GTG CGA G</td>
</tr>
<tr>
<td>amx-3</td>
<td>226</td>
<td>V: 20896670-90</td>
<td>CGA GGT TTA CAA CTT GGT CG</td>
</tr>
<tr>
<td></td>
<td>231</td>
<td>V 20894955-74</td>
<td>GAA TTC TCG CGC ACG TGA G</td>
</tr>
<tr>
<td></td>
<td>233</td>
<td>V: 20897035-54</td>
<td>CTT ATC GCC GAT ATC GTC CG</td>
</tr>
</tbody>
</table>

The Table lists the primers used for verifying the specific deletions in each of the *amx* mutants. The chromosome number, location and sequence are also listed. See Figure 5 for the gene maps.
Figure 5. Primers for \textit{amx} genes. For out-crossing and construction of \textit{amx} double and triple mutants, the above primers were used. For chromosomal location and sequence of primers, see Table 1.
Figure 6. PCR products amplified from nematode genomic DNA. This photograph shows the results from the fourth out-crossing of the amx-3 gene deletion strain. Lanes 1 and 6 have wild-type DNA as a positive control, 2 and 7 have a mixture of wild-type and amx-3 deletion mutant DNA, 3 and 8 have amx-3 deletion mutant DNA as negative control, and lanes 4 and 9 have the DNA amplified from the fourth out-crossed amx-3 deletion mutants. Promega 1 KB ladder is in lane 5.
Table 2

Dopamine intensities for all induced fluorescence experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>N2</th>
<th>amx-1</th>
<th>amx-2</th>
<th>amx-3</th>
<th>amx triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57.8 (n=1)</td>
<td>61.4 (n=2)</td>
<td>67.3 (n=3)</td>
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<td>42.3 (n=3)</td>
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<tr>
<td>2</td>
<td>20.6 (n=2)</td>
<td>27.8 (n=3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>34.2 (n=2)</td>
<td>-</td>
<td>51.0 (n=1)</td>
<td>60.8 (n=2)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>37.2 (n=3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>28.1 (n=2)</td>
</tr>
<tr>
<td>5</td>
<td>28.2 (n=6)</td>
<td>-</td>
<td>-</td>
<td>36.9 (n=2)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>46.9 (n=3)</td>
<td>-</td>
<td>52.8 (n=4)</td>
<td>-</td>
<td>47.1 (n=6)</td>
</tr>
<tr>
<td>7</td>
<td>36.6 (n=1)</td>
<td>-</td>
<td>25.8 (n=1)</td>
<td>55.0 (n=1)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>45.9 (n=5)</td>
<td>69.3 (n=2)</td>
<td>76.4 (n=3)</td>
<td>63.9 (n=6)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>15.2 (n=2)</td>
<td>19.3 (n=2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

% of N2       | 100 | 123 (n=3) | 132 (n=4) | 136 (n=4) | 107.7 (n=5) |

The table shows the results of glyoxylic acid induced fluorescence. The mean DA X\text{corrected} value (intensity in region of interest – background intensity) for each strain for individual experimental days is listed. “n” denotes the number of nematodes that were imaged and analyzed to get the mean $X_{\text{corrected}}$ on each day. Percentage of N2 was calculated as $X_{\text{corrected}}$ mutant / $X_{\text{corrected}}$ N2. “-” denotes that on that specific experimental day the mutant was not imaged. Experiment number 9 was done by Dr. Duerr.
Table 3

*Serotonin intensities for all induced fluorescence experiments*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>N2</th>
<th><em>amx-1</em></th>
<th><em>amx-2</em></th>
<th><em>amx-3</em></th>
<th><em>amx triple</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.6 (n=1)</td>
<td>41.1 (n=2)</td>
<td>38.7 (n=2)</td>
<td>-</td>
<td>20.8 (n=2)</td>
</tr>
<tr>
<td>2</td>
<td>33.7 (n=2)</td>
<td>64.0 (n=3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>20.0 (n=2)</td>
<td>-</td>
<td>-</td>
<td>23.5 (n=2)</td>
<td>26.4 (n=2)</td>
</tr>
<tr>
<td>4</td>
<td>48.7 (n=4)</td>
<td>-</td>
<td>73.3 (n=2)</td>
<td>60.7 (n=3)</td>
<td>73.5 (n=3)</td>
</tr>
<tr>
<td>% of N2</td>
<td>100</td>
<td>182 (n=2)</td>
<td>157 (n=2)</td>
<td>121 (n=2)</td>
<td>124 (n=3)</td>
</tr>
</tbody>
</table>

The table shows the results of glyoxylic acid induced fluorescence. The mean SER $X_{corrected}$ values (intensity in region of interest – background intensity) for each strain for individual experiments. “n” denotes the number of nematodes that were imaged and analyzed to get the mean $X_{corrected}$ on each day. Percentage of N2 was calculated as $X_{corrected}$ mutant / $X_{corrected}$ N2. “-” denotes that on that specific day the mutant was not imaged.
Figure 7. Diagrams of *C. elegans*

A) Adult hermaphrodite. The body of an adult hermaphrodite shows pharynx (green) in the head, intestine (purple), gonads (blue), uterus and anus (©WormAtlas 2008)

B) Diagram of the head. The head contains over half of the neurons (cell bodies in blue). Neuronal processes (green) include the nerve ring, a synapse rich region, ventral nerve cord (VNC) and dorsal nerve cord (DNC). The pharynx and a portion of gut (in dark grey) are also shown. subs= sublateral neuronal processes. (Diagram made by Dr. Duerr)
Figure 8. Diagrams of CEP and NSM neurons in the head (©WormAtlas 2008). The neurons are dark pink, the pharynx is green and a part of gut is light pink. The diagrams show a region of the head approximately 250 microns wide.

A) The four CEPs (dorsal left, dorsal right, ventral left and ventral right) have dendrites extending to the tip of the snout and axons in the nerve ring. A portion of the nerve ring where the processes of the CEPs, ADEs and PDEs (not shown) are located was chosen as the region of interest (ROI) for analysis. For analysis of induced fluorescence, a square 50 pixels by 50 pixels within the oval area was used.

B) One of the two NSMs (left and right). A portion of the process of NSMs was chosen as the region of interest (ROI) for analysis. For analysis of induced fluorescence, a rectangle 70 pixels by 15 pixels within the oval area was used.
Figure 9. Immunolocalization of serotonin in the NSMs. Hermaphrodites were stained with anti-SER antibodies (in red). Images are maximum projections of confocal z-series. Each image is 100 microns wide and includes the two bulbs of pharynx (see diagrams in Figures 7 and 8).

A) Diagram of the head of a nematode showing the region between two bulbs of pharynx. The soma of one of the NSMs is shown in pink in the first bulb (©WormAtlas2008).
B) The head of a wild-type nematode with NSM cell bodies (arrows) visible in the first bulb of pharynx.
C) The head of an amx triple mutant with NSM cell bodies (arrows).
Figure 10. Glyoxylic acid induced fluorescence in wild-type vs. amx mutants. All images are maximal projections of confocal z-series. No significant differences were detected in DA (green) and SER (red) levels. Images are 100 microns wide.

A) Diagram of head of a nematode showing the region between the two bulbs of pharynx. The CEPs (dopaminergic neurons) are shown in red. NSMs (serotonergic neurons) are not shown. Images B-F show the same region of the head (©WormAtlas2008).

B) Wild-type nematode. DA (green) and SER (red) are indicated by arrows. The bacteria inside the pharynx are also fluorescent (yellow).

C) amx-1 mutant
D) amx-2 mutant
E) amx-3 mutant
F) amx triple mutant
CHAPTER 3 ANALYSIS OF AMX-1 EXPRESSION PROFILE IN TRANSGENIC AMX-1 NEMATODES

3.1 Introduction

Transgenic nematodes can be created by microinjection of DNA into the gonads of adult hermaphrodites (Mello et al. 1991; Evans 2006). The transgenic offspring typically carry large extra-chromosomal arrays that contain many copies of the injected DNA; some of the arrays are stable and may be inherited. Transgenic strains provide a rapid and inexpensive method to investigate the expression of genes of interest. The transgenic construct that we used consisted of the putative upstream promoter region of the gene of interest \((amx-1)\) fused with the coding region of green fluorescent protein (GFP). Thus, GFP expression should provide a preliminary indication of where AMX-1 is normally expressed (Boulin 2006).

To generate the DNA used for injection, a PCR-fusion method (Hobert 2002) was used to produce \(Pamx-1::\text{GFP}\). The construct included 1.8 kb of DNA upstream of the start of the \(amx-1\) coding region. To aid in the identification of transgenic progeny, the DNA was co-injected with the pRF4 plasmid, which encodes a mutant collagen (\(rol-6\)) that results in a dominant roller phenotype (Boulin 2006). The transgenic lines were generated by Dr. Janet Duerr; I analyzed three independent lines. Note that the transgenic expression pattern may not exactly reproduce the endogenous gene’s expression since it may not contain all of the promoter. In addition, it is in a multi-copy array and is not in its normal chromosomal location; finally, it can be lost during development, causing variability in siblings of in a single strain (Mello et al. 1995).
Our initial examination of transgenic nematodes found that *Pamx-1:: GFP* was expressed in neurons, including in the head ganglia and tail, as well as some cells inside the pharynx. To identify the neurons, we looked at the expression of GFP in nematodes stained with different neuron-specific markers. To determine if *amx-1* was expressed in MA containing cells, we fixed and stained transgenic nematodes with antibodies against GFP, VMAT (vesicular monoamine transporter) and DAT (dopamine transporter) (Duerr *et al.* 1999). VMAT is present in the synaptic vesicles of MA cells and DAT is present in the plasma membrane of DA cells. We also stained the three transgenic lines with another intracellular marker - DiI. This fluorescent dye is highly lipophilic and does not penetrate the cuticle. However, it does stain the plasma membrane of specific sensory neurons with cilia exposed to the exterior environment of the nematode (Hedgecock *et al.* 1985; Aamodt 2006).

3.2 Experimental

*Transgenic Lines*

We examined three transgenic lines, N41c, H69 and A2b, created by Dr. Duerr. The transcriptional fusion construct included a 1.8 kb region upstream of the start of *amx-1* gene fused to a nuclear localization signal in frame with the coding sequence of GFP in N41c and A2b; H69 lacked the NLS. In the first step, *Pamx-1* and GFP were amplified using specific primers. One of the primers for *Pamx-1* had a short region overlapping with one of the GFP primers to facilitate fusion in the second round of PCR. *amx-1* was amplified using primer 192 (III: 3820574-93; 1807 bp upstream of *amx-1* start) and primer 105 [III: 3825922-940 RC (reverse complement from the last *amx-1* exon) fused
to a sequence overlapping with primer 128]. For amplification of a nematode optimized GFP, we used plasmid L2463 (a gift of A. Fire, Stanford University) with primer 128 (upstream of GFP start 2-25) and primer 133 (downstream of GFP 1875-1900 RC). For fusion PCR, primer 193 (nested inside primer 192 at III: 3820578-99) and primer 134 (nested inside primer 133 at L2463 1850-1876RC) were used. For injections, 10-20 ng/μl *amx-1* and 40-100 ng/μl *rol-6* DNA was used. Roller transgenic progeny were isolated and tested for the presence of GFP by PCR analysis and fluorescent microscopy.

**DiI Staining**

A 1 mM DiI (1,1',di-octadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) suspension was prepared by adding 2 mg of DiI to 1ml of M9 saline (see experimental Chapter 1) and vortexing. For staining, healthy adult nematodes were added to 50 μl DiI suspension for 2.5 hours. The nematodes were then transferred to a blank NGM agar plate for 30 minutes. This helped the nematodes get rid of excessive DiI on their cuticle. The nematodes were then placed in a drop of water on agar pads. (Agar pads were made by dropping 20 μl of 2% agar solution on a glass slide followed by drying in 60 degrees oven for at least one hour.) In some cases, 10 mM levamisole was used in the drop of water to decrease the motility of the nematodes; however, levamisole slowly quenched the fluorescence. Levamisole is a potent cholinergic agonist that binds to acetylcholine receptors present in body-wall muscle and causes hyper-contracted paralysis (Rand 2007). A cover slip was placed on the slide and the slide was sealed with nail polish.
Antibody Staining

Staining was done with antibodies to GFP (green fluorescent protein), VMAT (vesicular monoamine transporter), and DAT (dopamine transporter) or with antibodies to GFP and ChAT (choline acetyltransferase, a marker for cholinergic neurons). The standard procedure of staining was followed (Duerr 2006).

Freeze-crack and fixation. 20-25 healthy adult hermaphrodites were placed in a drop of water on the center of a treated poly-lysine slide (see Chapter 2). An untreated poly-lysine slide was placed on top and compressed slightly and this “sandwich” was placed on dry ice for 15 minutes. Slides were then swiftly pulled apart. Slides were immediately immersed in ice cold methanol for 2 minutes and then transferred to ice cold acetone for 4 minutes.

Antibody incubation. Slides were rinsed in PBS, then transferred to block for 1 hour at room temperature or overnight at 4°C. Slides were incubated in primary antibody diluted in antibody buffer at 4°C overnight with gentle rocking. One set of primary antibodies included 1:1000 rabbit anti-GFP (BD Clontech living colors anti-GFP); 1:500 goat anti-VMAT (Duerr et al. 1999) and 1:500 chicken anti-DAT (generated against C-terminal region peptide “QRVTMPYRKPNQTE” of C. elegans DAT coupled to KLH, Dr. Duerr, personal communication). A second set of primaries included 1:1000 rabbit anti-GFP and 1:500 mouse anti-ChAT (Duerr et al. 2008). After primary incubation, slides were transferred to PBS for a minute followed by 3 x 20 minutes washes with antibody buffer. Slides were then transferred to secondary antibody solution for 4 hours in the dark at room temperature. Secondary mixtures were 1:1000 Alexa 488 donkey anti-
rabbit (Molecular probes, Eugene, OR), 1:1000 Cy3 donkey anti-chicken (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 1:1000 Cy5 donkey anti-goat (Molecular Probes, Eugene, OR) or 1:2000 Alexa 488 donkey anti-rabbit and 1:2000 Cy3 donkey anti-mouse (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Slides were then rinsed in PBS for a minute followed by 3 x 20 minutes rinses with antibody buffer, then PBS for 5 minutes. For mounting, 20 µl of mounting medium (see Chapter 2) containing the DNA stain DAPI was used. A 24X60 mm cover slip was placed over the sample and sealed with two to three layers of nail polish. The slides were then stored in the dark until viewing.

_Laser Confocal Microscope_

The slides were viewed with the Zeiss LSM scanning confocal microscope. For living nematodes, we used laser lines and filters appropriate for GFP (excitation 488 nm, emission 505-545 nm) and DiI (excitation 514 + 543 nm, emission >560 nm). Antibody stained nematodes were imaged with the following confocal settings for DAPI (excitation 364 nm, emission >475 nm), Alexa 488 (excitation 488 nm, emission 505-545 nm), Cy-3 (excitation 514 + 543 nm, emission >560 nm) and Cy-5 (excitation 633nm, emission >650 nm).

3.3 Results and Discussion

For studying the expression pattern of _Pamx-1:: GFP_, we analyzed three different transgenic lines, A2b, H69, and N41c. This allowed us to look for consistency in expression pattern between different lines. Since expression pattern can also differ between individual nematodes of a single strain (see introduction), we looked at multiple
nematodes of each line. In live nematodes of all the three transgenic lines (A2b, H69, and N41c), \textit{Pamx-1::GFP} expression was seen in cells in the head and tail, but not the body. The intensity of GFP fluorescence and the number of fluorescent cells varied, but in general, there were 25 cells in the head and 7 cells in the tail. To determine if \textit{Pamx-1::GFP} was expressed in MA cells, we stained the three transgenic lines with anti-GFP, anti-VMAT and anti-DAT antibodies. We analyzed 6-10 individuals of each of three strains. As seen in Figure 11, the dopaminergic CEP neurons (and ADEs and PDEs; not shown) did not express \textit{Pamx-1::GFP}. It was more difficult to determine whether there was co-localization of VMAT and GFP, because VMAT is present on the synaptic vesicles in the processes of cells and GFP is synthesized in the cell bodies. However, we did not detect any obvious co-localization of VMAT and GFP.

To identify what kinds of cells expressed GFP, we used another cellular marker, the lipophilic dye DiI. DiI stains a subset of the amphid neurons in the head, the left and right ASK, ADL, ASI, ADF, ASH and ASJ. These are putative chemosensory neurons, with plasma membranes that are exposed to the external environment through a pair of small openings in the tip of the snout. Ten other pairs of amphid neurons (AFD, AIA, AIB, AIY, AIZ, ASE, ASG, AWA, AWB, AWC) do not contact the external environment and are not stained by DiI. The amphid neurons as a whole are thermo-, chemo-, mechano- and odor-sensory neurons (reviewed in WormAtlas 2008). These neurons contain neurotransmitters other than monoamines, such as glutamate. Figure 12 (from WormAtlas 2008) shows the subset of amphid neurons and IL1 and IL2 cells which stain with DiI calcium acetate. For our expression analysis, we looked at heads of
5-8 individuals of each of 3 transgenic strains. *Pamx-1:: GFP* was expressed in some but not all of the DiI positive amphid neurons in the head (see Figure 13). One pair of DiI and GFP positive cells was identified as the ASJs, due to their relatively ventral and posterior location. In addition, GFP and DiI positive cells included three pairs of IL2 neurons, also identifiable from their location; this identification was confirmed below using anti-GFP and anti-ChAT antibody staining. In addition, a cluster of four bilaterally symmetrical DiI and GFP positive amphid cells was identified in all three lines. These cells were difficult to identify uniquely identify because of their extreme proximity to each other, but were among the pairs of ASK, ASH, ADF, ADL and ASI neurons. We also saw faint GFP in an average of two cells inside the pharynx.

In the tail, two pairs of cells showed bright GFP expression in all three transgenic strains. These cells also stained with DiI, so we were able to identify them as the bilateral pairs of PHA and PHB phasmid neurons. These phasmid neurons are chemosensory neurons involved in chemorepulsion in nematodes (WormAtlas 2008). PHB contains low levels of serotonin (Sawin *et al.* 2000). There was an average of three other neurons in the tail that expressed faint GFP. In addition to these cells, *Pamx-1:: GFP* was expressed in the anal sphincter muscle (see Figure 15). The anal sphincter is a saddle shaped specialized muscle in nematodes which is involved in the defecation (WormAtlas 2008).

To further determine the identity of the GFP positive cells, we compared anti-GFP with an antibody to a marker of cholinergic (acetylcholine utilizing) neurons. Over one-third of the 302 neurons in *C. elegans* are cholinergic, including 51 identified motor neurons and 15 identified interneurons in the body and tail (Duerr *et al.* 2008) and
approximately 30 partially identified motor neurons or interneurons in the head (Dr. Duerr; personal communication). Using anti-ChAT and anti-GFP antibody staining, we analyzed 4-5 individuals of each of three strains and found that approximately 20 of ChAT positive neurons in the head expressed Pamx-1:: GFP. This staining confirmed the expression of Pamx-1:: GFP in the six IL2 neurons (which are stained by DiI and are cholinergic, see Figure 14). Staining was also seen in two pairs of cells that, by location, appeared to be non-DiI positive amphid neurons. None of the cholinergic motor neurons in the body or tail was GFP positive.

Together, our results indicate that Pamx-1:: GFP was expressed predominantly in chemosensory neurons. Only one pair of GFP positive cells, the pair of phasmid PHB neurons, are thought to contain monoamine (they may contain serotonin, Sawin et al. 2000). The rest of the GFP positive cells use other neurotransmitters like glutamate, acetylcholine, or neuropeptides (reviewed in WormAtlas 2008). The expression analysis is consistent with a role for amx-1 in the function of chemosensory neurons, rather than a MAO function. Results of behavioral assays in amx-1 mutant are consistent with the findings of the expression studies; MA modulated behaviors are generally normal in amx-1 mutants (see Chapter 4 and final summary).
Figure 11. Anti-GFP, anti-VMAT and anti-DAT in the heads of *Pamx-1:: GFP* transgenic strains
A) Diagram of the head. The head contains over half of the neurons (cell bodies in blue and neuronal processes in green, diagram made by Dr. Duerr). Images B and C show approximately the same region.
B) Anti-GFP (green); anti-VMAT (blue), and anti-DAT (red) in strain N41c. The dopaminergic CEP neurons (arrow) and the VMAT positive cells did not express *Pamx-1:: GFP*. The image is a single section of a z-series and is 100 microns wide.
C) Anti-GFP, anti-VMAT and anti-DAT in a single section in strain A2b. The image is 100 microns wide.
Figure 12. DiI staining in the head. A subset of the amphid neurons and the IL1 and IL2 neurons and their (labial) processes are labeled with this dye in a lateral view of the head. Similar staining was done in the present study (see figure 13) L=left, VL=ventral left. (©WormAtlas 2008).
Figure 13. DiI staining in the heads of Pamx-1::GFP transgenic strains. DiI (red) labels a subset of the chemosensory amphid neurons. A and B are maximum projections and C and D are individual sections of different nematodes. Images are 120 microns wide.

A) Strain N41c ventral view. Many DiI-positive (red) amphid neurons expressed GFP (green). GFP positive and DiI-negative cells were also present in these clusters.

B) Strain A2b. Amphid cell bodies include GFP+DiI+, GFP+DiI-, and GFP-DiI+ cells.

C) Strain H69. A single section (lateral view) to show overlap between GFP and DiI in a pair of IL2s (arrow). GFP is present predominantly in the cell body (nucleus and cytosol) and DiI stains the plasma membrane.

D) Strain H69. A single section (ventral view) to show overlap between GFP and DiI in some amphid somas.
Figure 14. Anti-GFP and anti-ChAT in the heads of Pamx-1::GFP transgenic strains. Each image is a maximum projection of z-series and is 100 microns wide.

A) Strain A2b. Some of the cluster of amphid neurons and IL2 (arrows) expressed Pamx-1::GFP (green) and ChAT (red, labels cholinergic neurons), but most ChAT positive neurons are not GFP positive.

B) Strain H69. Maximal projection.

C) Strain H69 strain. A single section showing overlap of GFP and ChAT in some cells.
Figure 15. *Pamx-1:: GFP* expression in tails of *Pamx-1:: GFP* transgenic strains. Each image is maximum projection of z-series and is 100 microns wide.

A) A diagram of the tail showing the phasmid neurons (©WormAtlas 2008).

B) DiI staining in the tail. PHA and PHB neurons expressed *Pamx-1:: GFP* (green), DiI (red) stains the membrane of these neurons.

C) Anti-ChAT and anti-GFP antibody staining in the tail. PHA, PHB and the anal sphincter muscle expressed GFP (green). Other cholinergic neurons (red) were not GFP positive.
CHAPTER 4 CHARACTERIZATION OF MONOAMINE DEPENDENT BEHAVIOR AND DRUG SENSITIVITY IN AMY MUTANTS

4.1 Introduction

Monoamine Dependent Behaviors in C. elegans

In C. elegans, DA, SER, OCT, and TYR act by binding specific receptors on neurons and muscles to affect egg-laying, eating (pumping of the pharynx to ingest food), learning and locomotion (reviewed in Chase et al. 2007). DA signaling in C. elegans can be inhibitory or stimulatory depending upon the DA receptors present on the pre- or post-synaptic cell; a single cell may have both excitatory and inhibitory receptors. There are at least four G-protein coupled DA receptors, DOP-1 through DOP-4. These receptors may stimulate GOA-1 (a member of the Gi/o class) and negatively regulate transmission through changes in diacylglycerol (DAG) and diacylglycerol kinase (Bastiani et al. 2006). For example, well-fed animals show a “basal slowing response” in the presence of bacteria or small sephadex beads which is mediated by DA acting on the DOP-3 receptor (Sawin et al. 2000, Chase et al. 2004, Kindt et al. 2007). DOP-1 is involved in tap habituation, which is a form of learning and memory (Sanyal et al. 2004, Kindt et al. 2007).

SER is important for some starvation dependent changes in behavior (Sawin et al. 2000). GOA-1 is one of the downstream targets of SER (Bastiani et al. 2006), but SER can also directly activate ion channels. When starved animals encounter food, they exhibit a larger than basal slowing response termed the “enhanced slowing response” that is dependent upon serotonin and the MOD-1 serotonin-gated chloride channel (Weinshenker et al. 1995, Sawin et al. 2000). SER stimulates egg-laying, predominantly
by release of SER from the HSN neurons, which activates GOA-1 in muscles and other neurons. SER synthesis mutants (tph-1) and ser-1 receptor mutants are egg-laying defective, but can still lay eggs in a partially regulated fashion (Weinshenker et al. 1995, Schafer 2006), indicating that SER release is not solely responsible for this behavior. Egg-laying and pharyngeal pumping are regulated in opposite directions by exogenous SER versus OCT and/or TYR, suggesting that these compounds function physiologically as antagonists (Horvitz 1982, Alkema 1995, Suo et al. 2006, Wragg et al. 2007).

We hypothesized that if amx mutants have defective degradation of MAs, then they might show differences in MA-dependent behaviors. Therefore, we examined known MA-dependent behaviors in individual and amx triple mutants. Behavioral analysis also gave us a tool to see if changes in the individual amx mutants were consistent with changes in specific monoamines.

Effect of Serotonin Signaling on Longevity in C. elegans

SER signaling has been implicated in negatively regulating longevity in C. elegans (Golden et al. 2007, Petrascheck et al. 2007). The drug miniaserin blocks SER-4 (SER receptor) as well as SER-3 (OCT receptor) to increase longevity (Petrascheck et al. 2007). These effects are linked to dietary restrictions. Since MAs are involved in regulating lifespan, we examined longevity in the amx mutants. We hypothesized that amx mutants might have an increased level of SER and might have a short lifespan.

Sensitivity to Exogenous Dopamine and Serotonin

If monoamine degradation is defective in the amx mutants, the mutants might be more sensitive to exogenous DA and SER than normal nematodes. To test this
hypothesis, we examined the response of mutant nematodes to DA and SER in two movement assays. Normally, exogenous SER or DA cause decreased speed of locomotion followed by gradual paralysis. We compared the rate of movement in relatively low concentrations of SER and DA and immobilization in a fairly high concentration of DA or SER in normal versus amn mutant nematodes.

4.2 Experimental

Behavior Analyses

Well-fed adult hermaphrodites (12-48 hours past the last larval stage, L4) grown at 20° C were used in all behavioral assays. In general, movement assays were done on slightly younger nematodes (12-18 hours post L4) since these young adults have relatively few embryos in utero and a large number of embryos in utero can impact movement. Older, fully mature adults were used to count embryos in utero. N2 (wild-type nematodes) were assayed in parallel with all mutants. In general, three or more groups of ten worms each were assayed on different days. After transfer of individual worms for testing, acclimatization periods of 1-2 minutes were given before counting began, to allow the hermaphrodites to recover from being moved.

Movement in methyl cellulose: Adult hermaphrodites (L4s picked 18 hours before testing) were placed in 20 mg/ml methyl cellulose (Sigma Aldrich, St. Louis, MO) in M9 saline (see Chapter 2). After 1-2 minutes of acclimatization, body bends were counted for one minute. Methyl cellulose polymers of different lengths and thus different viscosities were used to test nematode movement in solutions with similar osmolarity and salts.
Movement on food: Adult hermaphrodites (L4s picked 18 hours before testing) were placed on NGM plates spread with bacteria (food). After acclimatization for 2 minutes, body bends were counted for one minute. In order to avoid any effects of variations in the thickness or status of the bacteria, NGM plates made and spread with *E. coli* at the same time were used for assays of mutants and matched wild-type nematodes.

Sephadex movement assay: Sephadex beads (Sephadex™ G-50 superfine 20-50 μm; Amersham Biosciences, Sweden) were suspended in saline (65 mg in 1 ml M9) in petri dishes. Petri dishes were kept on a rocker for at least 30 minutes until the beads were well-hydrated and dispersed. Adult hermaphrodites (L4s picked 18 hrs before testing) were placed in the Sephadex bead suspension. Nematodes are 1.5 mm long and approximately 50-100 μm wide; hitting the 20-50 μm beads during movement provide a gentle mechanical stimulus. After acclimatization for 2 minute, body bends were counted for a minute.

Pharyngeal pumping: Adult hermaphrodites (L4s picked 18 hrs before testing) were placed on NGM plates with food. After acclimatization for 2 minutes, the number of pharyngeal pumps was counted for a minute. This assay was done by Nanda Filkin, a member of laboratory

Egg-laying assay: Adult hermaphrodites (24 hours after the L4 larval stage) were individually placed on NGM plates covered with bacteria and the number of eggs laid in 2.5 hours was counted. A relatively long period was used, since egg-laying occurs in cycles of approximately 20 minutes duration (Schafer 2005).
Embryos in utero and embryo age: Adult hermaphrodites (L4 larva picked 48 hours before assay) were placed in ~5 μl drops of bleach solution (1:1 10 N NaOH and 5% bleach) on chilled agar plates or on glass slides. The bleach dissolved the entire body of the animal except the fertilized embryos, which were surrounded by a tough egg shell. The number of fertilized embryos in utero was counted for each individual. To determine the age of the oldest embryo in utero, mounting medium containing the nuclear stain DAPI (see Chapter 2) was applied before putting on a cover slip. The age of the oldest embryo was recorded as class I: < 16 cells, class II: 16-32 cells and class 3: > 32 cells using a Nikon Optiphot-2 epi-fluorescence microscope.

Drug Sensitivity

Movement in dopamine: Adult hermaphrodites (L4 larva picked 18 hours before assay) were individually placed in 100 μl dopamine solution in a 96 well plate. A range of concentrations of DA, 30 mM, 50 mM and 80 mM were used. (DA was dissolved in 1 ml M9 saline plus 1 μl 1M glacial acetic acid, pH adjusted to 7.2). After 1-2 minutes of acclimatization, body bends were counted for 20 seconds.

Immobilization in dopamine: Adult hermaphrodites (L4 larva picked 18 hours before assay) were placed in 90 mM DA and their state of mobility was recorded every minute for the first five minutes and every 5 minutes for the next 20 minutes.

Movement in serotonin: Adult hermaphrodites (L4 larva picked 18 hours before assay) were individually placed in 100 μl serotonin solution (serotonin was dissolved in 1 μl of 1 M HCL plus 1 ml M9, pH was adjusted to 7.2) in a 96 well plate. A range of concentrations, 4 mM (1.6 mg/ml), 8.5 mM (3.3 mg/ml), and 15 mM (6 mg/ml) were
used. All solutions were made fresh. After 1-2 minutes of acclimatization, body bends were counted for 20 seconds. Note that the cuticle is relatively impermeable to MAs, so the effective internal concentration is thought to be approximately 1/1000th of the external concentration (Srinivasan et al. 2008).

*Immobilization in serotonin:* Adult hermaphrodites (L4 larva picked 18 hours before assay) were placed in 100 μl 8.5 mM SER solution (made as above). The state of mobility was recorded every minute for the first five minutes and every 5 minutes for the next 20 minutes.

*Lifespan Assay*

L4 hermaphrodites were transferred to NGM plates with bacteria. These were kept at 20°C; the L4 stage was counted as day 0. Adults were frequently transferred to fresh plates during the first few days to remove them from their offspring. After ten days, the adults stopped laying many offspring and became more fragile, so they were transferred less frequently. Hermaphrodites were considered dead when they did not move any portion of their body, even in response to touch.

*Statistical Analysis*

One way ANOVA analysis was done for most behavioral assays and immobilization assays. For DA and SER movement assays, two-way ANOVA was done. This was followed by a post-hoc test, the Tukey multiple comparison test. In all cases, p<0.05 was considered significant.
4.3 Results and Discussion

**Characterization of Behavioral Changes in Mutants**

Different MAs interact to modulate behavior in *C. elegans*. The behavioral changes seen in different assays were varied in *amx* mutants. These results are discussed in following section and summarized in a summary table (Table 9).

*Movement in methyl cellulose.* This assay assesses rapid and continuous movement and reflects the state of overall health of the mutants. All *amx* mutants were relatively healthy; they showed normal maximum rates of movement in liquid and showed normal slowing of motion in methyl cellulose solutions of increasing viscosity (Figure 16). Other members of lab have studied *cat-1*, a mutant that lacks the vesicular monoamine transporter and should be deficient in neuronal signaling by all monoamines. This mutant also shows normal slowing in increasingly viscous solutions. This suggests that mutants with abnormal MA signaling were healthy and that monoamines (and AMX proteins) are not important for regulating the rate of movement in homogeneous liquids.

*Movement in food.* *amx-2* and *amx-3* mutants showed significantly larger decreases in locomotion in food than normal (p<0.001). Wild-type nematodes display 55 body bends in a minute in food. The results for the mutants (values as percentage N2) are shown in Figure 17. DA neurons are thought to be mechanosensory, mediating decreased locomotion in response to mechanical stimuli, including food (Sawin *et al.* 2000; Kindt *et al.* 2007). SER also decreases locomotion of starved nematodes in the presence of food (Sawin *et al.* 2000). The effects of TYR and OCT in this assay have not been well-characterized. If DA levels were high in *amx* mutants, then the mutants would be
expected to show increased slowing in food, such as that observed in amx-2 and amx-3 mutants. Since we did not detect any large changes in endogenous DA levels in amx mutants using induced fluorescence (Chapter 2), these behavioral changes may be due to changes in MAs undetected with the induced fluorescence method. It is also possible that they are due to MA independent changes in behavior.

Movement in sephadex. Wild-type nematodes show more rapid movement in liquid than on NGM plates; they averaged 186 body bends in a minute in a suspension of sephadex beads. amx-3 and amx triple mutants moved slightly but significantly more slowly than normal nematodes in the bead suspension (p<0.001; Figure 18). However, amx-1 mutants moved normally and the amx-2 mutant moved slightly but significantly faster than normal.

DA mediates slowing of motion in response to gentle mechanical stimuli similar to ones provided by bacteria or superfine sephadex beads (Sawin et al. 2000). SER does not appear to be important for the decrease in locomotion in the presence of beads (Sawin et al. 2000), probably because beads only provide mechanical stimulation as opposed to the mechanical and chemical stimulation provided by bacteria. The effects of TYR and OCT on this behavior are not known; however, cat-1 mutants, with defective packaging of all MAs, move faster in sephadex (Nanda Filkin; personal communication). In amx-3 and amx triple mutants, the slowing of locomotion is consistent with increased levels of DA in these mutants. As in food, amx-1 mutants had normal locomotion in beads, consistent with no significant changes in behaviorally relevant MAs (and consistent with our Pamx-1:: GFP expression studies). The behavior of the amx-2 mutant was not
expected. It is possible that this mutant had increased OCT and TYR as well as increased DA. These MAs may regulate behaviors in opposite ways. Further investigation of possible roles for OCT and/or TYR in regulating this behavior may provide an explanation for the observed difference between the movement in food and sephadex suspensions. For example, to reveal the roles of TYR and OCT, mutants in which OCT and TYR are not synthesized can be studied for behavioral modulations and compared with doubles with amx mutants.

**Pharyngeal Pumping.** Wild-type nematodes normally pump their pharynxes at a rate of 233 pumps per minute when ingesting food (see Figure 19). *amx*-2 mutants had a significantly decreased rate of pumping (p<0.001); *amx*-3 mutants had a significantly increased rate and *amx*-1 and amx triple mutants pumped at a normal speed.

Different MAs affect pumping differently; exogenous SER promotes pharyngeal pumping, while OCT and TYR inhibit pumping (Horvitz 1982; Alkema *et al.* 2005). *cat-I* (VMAT deficient) mutants, in which all MA neurotransmission may be deficient, exhibit slow pumping (18 % slower than normal; Duerr *et al.* 1999). Since MAs positively and negatively regulating pharyngeal pumping, the higher or lower rates seen in different *amx* mutants could result from varied effects of the different AMX proteins on different MAs. *amx*-2 mutants show an increase in pumping, which might have been caused by increased OCT and TYR. Note that *amx*-2 mutants also move faster in sephadex beads, which again could be effect of increased OCT and TYR. On the other hand, increased pumping rates in *amx*-3 mutants are consistent with increased SER levels.
Egg-laying Behavior. *amx-2* and *amx-3* and *amx* triple mutants laid significantly fewer eggs (embryos) in a given interval than wild-type hermaphrodites (see Figure 20). *amx-1* mutants showed normal egg-laying. The role of MAs in regulating this behavior is complex; SER stimulates egg-laying, while OCT and TYR inhibit egg-laying (Horvitz 1982, Alkema *et al.* 2005). Therefore, if AMX proteins differentially degrade different MAs, a variety of behavioral changes might be observed in the mutants.

We also looked at the number and age of embryos in a mature hermaphrodite (see Figures 21 and 22). We found that *amx-2* and *amx-3* mutants contained lower number of embryos than wild-type nematodes: *amx-2* (21.6 +/- 3.8% below normal), *amx-3* (27.5 +/- 2.1% below normal) and *amx* triple (9.7 +/- 3.3% below normal). The age of the oldest embryo in utero was also examined (Figure 22). The ages of the oldest embryos held in utero in these same mutants, *amx-2*, *amx-3* and the *amx* triple, were younger than normal. Thus, most of the *amx* mutants had fewer embryos in utero and the oldest embryo in utero was at a younger stage, consistent with the mutants laying embryos prematurely. Most *amx* mutant also laid fewer eggs than wild-type nematodes in a given period. It would be of interest in the future to determine whether or not these mutants had normal overall fecundity.

**Lifespan Assay**

All of the *amx* mutants had normal lifespans, as assayed in three separate experiments (Figure 23). The p-values for comparisons of survival curves for wild-type vs. *amx-1* was 0.9, for *amx-2* was 0.8, for *amx-3* was 0.6, and for the *amx* triple was 0.1. Thus, if these mutants have higher than normal levels of MAs, it does not significantly
negatively impact their longevity. Note that OCT and TYR may interact with SER to affect longevity, so modification of multiple MAs may have complex effects on longevity (Golden et al. 2007, Petrascheck et al. 2007).

**Drug Sensitivity**

To look at sensitivity of *amx* mutants to exogenous MAs, we placed them in solutions of DA and SER and monitored their movement. The assays were done in a range of concentrations of DA and SER and at different time points. Normal nematodes move slower in MA solutions and eventually become immobilized (Schafer et al. 1995).

We examined acute changes in mobility after one minute in DA (0 mM, 20 mM, 50 mM, 60 mM and 90 mM) by recording the body bends for 20 seconds (Table 5; Figure 24). There were significant differences between wild-type and the *amx-2, amx-3* and *amx* triple mutants (two-way ANOVA, F=7.3, df= 4, p<0.0001). Tukey multiple comparison test revealed that *amx-2, amx-3* and *amx* triple mutants were different from wild-type at a significance level of p<0.05. There was also a significant slowing in movement for all genotypes with increasing concentration of DA (two-way ANOVA, F=541.8, df=3, p<0.0001). One-way ANOVA (p<0.05) showed that at *amx-3* was significantly more sensitive to DA at 20, 50, 60 and 90 mM while *amx-2* were more sensitive at 60 mM and *amx* triple mutant at 60 and 90 mM.

To assay the long term effects of DA, we selected a DA concentration (90 mM) at which approximately half (0.47 +/-0.2) of wild-type nematodes were immobilized at 25 minutes (Table 6; Figure 25). This allowed us to test whether the *amx* mutants were hyper- or hypo- sensitive to long-term application of DA. We found that the *amx-2, amx-
3 and amx triple mutants were slightly but insignificantly sensitive to DA. Unexpectedly, amx-1 was resistant to DA; amx-1 mutants were significantly different from wild-type at 15, 20 and 25 minutes (p<0.05). Together, the short term and long term DA sensitivity assays revealed that all amx mutants except amx-1 were generally more sensitive to DA than wild-type, although this difference was only statistically significant for a subset of concentrations of DA at immediate time points. The reasons for insensitivity of amx-1 mutants to DA were not clear. Since Pamx-1::GFP expression pattern (see Chapter 3) suggests that amx-1 gene may have a function in chemosensory neurons, insensitivity to DA might have resulted from an improper sensory abilities in these mutants.

To examine SER sensitivities, we looked at the short term effects of 0 mM, 4 mM, 8.5 mM and 15 mM SER solutions on movement (Table 7; Figure 26). We found that SER inhibited movement in amx-2 mutants significantly more than in wild-types, all other mutants were normal (two-way ANOVA, F=3.0, df=4, p<0.05). Tukey multiple comparison test also revealed that the amx-2 mutants were more sensitive to SER than wild-type at a significance level of p<0.05. One-way ANOVA showed that amx-2 was significantly different at 8.5 mM. To assay the long term effects of SER, we choose 8.5 mM SER because approximately half (0.7 +/- 0.1) of the wild-type nematodes were immobilized at 25 minutes (Table 8; Figure 27). amx-2 mutants were more immobile at 5 and 15 minutes than wild-type nematodes (p<0.05). amx-1, amx-3 and amx triple mutants had normal immobilization patterns. In summary, there was a significantly higher sensitivity to SER in amx-2 mutants that was consistent with inefficient SER degradation.
Other mutants did not show increased sensitivity, which implied that amx mutants responded to exogenous SER in similar ways as wild-type nematodes.

In summary, our behavior analysis results suggest that amx-1 mutants have normal MA modulated behavior (see table 9). This is consistent with the expression pattern of Pamx-1::GFP (in chemosensory cells, see Chapter 3); both lines of evidence indicate that AMX-1 is not a vertebrate-like MAO. amx-2 and amx-3 and amx triple mutants do show changes in specific MA modulated behavior. These alterations in behaviors are complex and are consistent with involvement of multiple MAs in each behavior. As discussed above, movement of well-fed nematodes in food is modulated by DA, but not SER. The roles of OCT and TYR in this behavior are unknown. amx-2 and amx-3 mutants slow more than normal in food, which is consistent with an increase in DA. DA also mediates slowing in sephadex bead suspensions. Interestingly, amx-2 mutants did not slow, in fact they move faster than normal while the amx-3 mutants move slower than normal. Opposite results of these two behaviors in amx-2 mutants may indicates that other MAs like OCT and TYR interact with DA to modulate this behavior; we can test this in the future using mutants defective in OCT or TYR synthesis.

Other behaviors like pharyngeal pumping and egg-laying are known to be stimulated by SER and inhibited by OCT and TYR. amx-2 mutants show a decrease in pharyngeal pumping and egg-laying while amx-3 mutants show increased pumping and decreased egg-laying. One possibility is that amx-2 had high OCT and TYR that interfered with DA modulated (movement in sephadex beads) and SER modulated (pumping and egg-laying) behavior (Table 9). amx-3 may have higher SER since it shows
increased pharyngeal pumping. However, the modulation of most behaviors is complex and it is difficult to identify specific MA changes in a mutant (Table 9).

Overall, we saw behavioral changes in *amx-2, amx-3* and *amx* triple but not *amx-1* that were consistent with changes in regulation of MAs. Together, our behavior results are consistent with abnormal MA levels resulting from changes in MAOs. In case of the triple mutant, behaviors like movement in food and pharyngeal pumping were normal, which may reflect the opposing effects of different MAs on these behaviors and thus relatively small behavioral changes when all of the MAs are improperly regulated.
Table 4

*Body bends in a minute in methyl cellulose solutions*

<table>
<thead>
<tr>
<th>Viscosity</th>
<th>N2</th>
<th>amx-1</th>
<th>amx-2</th>
<th>amx-3</th>
<th>amx triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9</td>
<td>192 +/- 1.1</td>
<td>190 +/- 0.8</td>
<td>190 +/- 1.8</td>
<td>190 +/- 2.0</td>
<td>194 +/- 2.0</td>
</tr>
<tr>
<td>15 cp</td>
<td>190 +/- 1.2</td>
<td>188 +/- 1.5</td>
<td>188 +/- 1.8</td>
<td>188 +/- 1.1</td>
<td>188 +/- 0.5</td>
</tr>
<tr>
<td>25 cp</td>
<td>182 +/- 1.9</td>
<td>183 +/- 2.8</td>
<td>178 +/- 1.1</td>
<td>166 +/- 2.3</td>
<td>178 +/- 2.0</td>
</tr>
<tr>
<td>400 cp</td>
<td>160 +/- 1.2</td>
<td>157 +/- 1.2</td>
<td>158 +/- 0.8</td>
<td>158 +/- 1.5</td>
<td>156 +/- 6.1</td>
</tr>
<tr>
<td>1500 cp</td>
<td>134 +/- 2.0</td>
<td>138 +/- 2.1</td>
<td>134 +/- 1.8</td>
<td>130 +/- 1.1</td>
<td>136 +/- 0.6</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

The table lists the mean number of body bends recorded in a minute for three groups of 10 nematodes each +/- SEM for all strains in different solutions of methyl cellulose. See Figure 16 for graph.
Figure 16. Movement in methyl cellulose. Nematodes were placed in methyl cellulose solutions of varying viscosity and body bends were counted for a minute. All of the amx mutants showed normal maximal locomotion and a normal decrease in locomotion with increasing viscosity. Error bars are SEM for at least 3 days of experiments, 10 nematodes per experiment. cp = centipoises of viscosity
Figure 17. Movement in food. Nematodes were placed on plates spread with bacteria and body bends were counted for one minute. Locomotion in food was significantly decreased in amx-2 and amx-3 mutants. The graph shows percent of wild-type (N2) values. Note the Y-intercept is 60 to emphasize the differences in the mutants. Error bars indicate SEM [n= 3 groups of 10 nematodes each for all strains; * p<0.001].
Figure 18. Movement in sephadex suspensions. Nematodes were placed in a suspension of superfine sephadex beads and body bends were counted for a minute. The amx-2 mutants moved slightly faster while amx-3 and amx triple mutants moved slightly slower than normal. The graph shows percent of wild-type (N2) values. Note the Y-intercept is 80 to make the small differences in mutants more visible. Error bars indicate SEM [n= 3 groups of 10 nematodes each for all strains except that n=6 for amx-1; * p<0.001].
Figure 19. Pharyngeal pumping. Nematodes were placed on plates spread with bacteria and pharyngeal pumps were recorded for a minute. The $amx$-2 mutants showed a slight but significant decrease while $amx$-3 mutants showed a slight but significant increase. The graph shows percentage of wild-type (N2) values. Note the Y-intercept is 80 to make the differences in mutants more visible. Error bars indicate SEM [n=3 groups of 10 nematodes each for all strains; * p<0.001]. This assay was done by Nanda Filkin.
Figure 20. Egg-laying. Nematodes were placed on plates spread with bacteria and the numbers of embryos laid in 2.5 hrs were counted. The *amx-2*, *amx-3* and *amx* triple mutants showed significantly decreased egg-laying. This is consistent with fewer embryos in *utero* in these mutants (see Figure 21). The graph shows percentage of wild-type (N2) values. Error bars indicate SEM [n=3 groups of 10 nematodes each for all except n= 4 for *amx-1*, *p<0.01].
Figure 21. Embryos in utero. amx-2, amx-3 and amx triple mutants show fewer than normal embryos in utero. This is consistent with fewer number of eggs laid (see Figure 20). The graph shows percentage of wild-type (N2). The error bars indicate SEM. [n=3 groups of 10 nematodes each for all except n=4 for amx-1, * p<0.05].
Figure 22. Embryo age in utero. The oldest embryos in \textit{amx}-2, \textit{amx}-3 and \textit{amx} triple mutants were younger than normal. This is consistent with fewer number of eggs laid and carried in utero (see Figures 20 and 21). G= gastrulating. [n=3 groups of 10 nematodes].
Figure 23. Survival curves for wild-type vs. mutant nematodes. In each graph, the wild-type N2 survival curve (black) is plotted against the survival curve for a mutant (colored). Three different trials were performed for each mutant. All mutants exhibited normal lifespan.
Table 5

*Body bends in 20 seconds in dopamine solutions*

<table>
<thead>
<tr>
<th>Dopamine</th>
<th>N2</th>
<th>amx-1</th>
<th>amx-2</th>
<th>amx-3</th>
<th>amx triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>64.0 +/- 1.1</td>
<td>66.7 +/- 0.8</td>
<td>66.7 +/- 1.8</td>
<td>66.7 +/- 2.0</td>
<td>64.7 +/- 2.0</td>
</tr>
<tr>
<td>20 mM</td>
<td>33.9 +/- 0.3</td>
<td>33.1 +/- 1.4</td>
<td>32.5 +/- 1.2</td>
<td>31.1 +/- 0.9*</td>
<td>32.8 +/- 1.0</td>
</tr>
<tr>
<td>50 mM</td>
<td>26.6 +/- 0.4</td>
<td>23.4 +/- 1.3</td>
<td>22.6 +/- 2.3</td>
<td>24.6 +/- 0.6*</td>
<td>27.1 +/- 1.3</td>
</tr>
<tr>
<td>60 mM</td>
<td>22.5 +/- 1.2</td>
<td>23.4 +/- 0.4</td>
<td>20.4 +/- 0.3*</td>
<td>20.1 +/- 0.3*</td>
<td>20.2 +/- 0.9*</td>
</tr>
<tr>
<td>90 mM</td>
<td>19.6 +/- 0.9</td>
<td>16.3 +/- 1.5</td>
<td>18.1 +/- 0.1</td>
<td>16.4 +/- 0.1*</td>
<td>14.5 +/- 0.9*</td>
</tr>
<tr>
<td>n=</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

The table lists the mean number of body bends recorded in a minute +/- SEM for all strains in dopamine solutions. * denotes a significant difference (p<0.05) from wild-type. See Figure 24 for graph.
Figure 24. Body bends in dopamine solutions. Nematodes were placed in 0, 20, 50, 60 or 90 mM DA and body bends were counted for 20 seconds. The \textit{amx-2}, \textit{amx-3} and \textit{amx} triple mutants were slightly but significantly different from the wild-type nematodes. The error bars indicate SEM (two way ANOVA, p<0.05). See data in Table 5.
Table 6

*Immobilization in 90 mM dopamine solution*

<table>
<thead>
<tr>
<th>Minute</th>
<th>N2</th>
<th>amx-1</th>
<th>amx-2</th>
<th>amx-3</th>
<th>amx triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9 +/- 0.1</td>
<td>1.0 +/- 0.0</td>
<td>0.9 +/- 0.1</td>
<td>0.9 +/- 0.1</td>
<td>0.9 +/- 0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.9 +/- 0.1</td>
<td>1.0 +/- 0.0</td>
<td>0.9 +/- 0.1</td>
<td>0.8 +/- 0.1</td>
<td>0.8 +/- 0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.9 +/- 0.1</td>
<td>0.9 +/- 0.0</td>
<td>0.8 +/- 0.1</td>
<td>0.8 +/- 0.1</td>
<td>0.8 +/- 0.1</td>
</tr>
<tr>
<td>4</td>
<td>0.8 +/- 0.1</td>
<td>0.9 +/- 0.0</td>
<td>0.8 +/- 0.1</td>
<td>0.7 +/- 0.2</td>
<td>0.7 +/- 0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.8 +/- 0.2</td>
<td>1.0 +/- 0.0</td>
<td>0.8 +/- 0.2</td>
<td>0.6 +/- 0.2</td>
<td>0.7 +/- 0.2</td>
</tr>
<tr>
<td>10</td>
<td>0.7 +/- 0.2</td>
<td>0.9 +/- 0.0</td>
<td>0.6 +/- 0.3</td>
<td>0.5 +/- 0.2</td>
<td>0.7 +/- 0.2</td>
</tr>
<tr>
<td>15</td>
<td>0.6 +/- 0.2</td>
<td>0.9 +/- 0.1*</td>
<td>0.5 +/- 0.3</td>
<td>0.4 +/- 0.2</td>
<td>0.3 +/- 0.2</td>
</tr>
<tr>
<td>20</td>
<td>0.5 +/- 0.2</td>
<td>0.9 +/- 0.0*</td>
<td>0.5 +/- 0.4</td>
<td>0.2 +/- 0.2</td>
<td>0.3 +/- 0.2</td>
</tr>
<tr>
<td>25</td>
<td>0.5 +/- 0.2</td>
<td>0.9 +/- 0.1*</td>
<td>0.4 +/- 0.3</td>
<td>0.2 +/- 0.2</td>
<td>0.3 +/- 0.2</td>
</tr>
<tr>
<td>n = 6</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

The table lists the mean fraction of nematodes moving in a 90 mM DA solution at a given time interval +/- standard deviation for 3-5 groups of 10 nematodes each. * denotes a significant (p<0.05) difference from wild-type. See Figure 25 for graph.
Figure 25. Immobilization in 90 mM dopamine solution. Nematodes were placed in a 90 mM dopamine solution and their state of mobility was recorded for 25 minutes. *amx-1* mutants were resistant to DA, while *amx-2, amx-3* and *amx triple* mutants were similar to wild-type. Error bars indicate standard deviation [n=3 for all] Note that the scale of x-axis is non-linear. See data in Table 6.
Table 7

*Body bends in 20 seconds in serotonin solutions*

<table>
<thead>
<tr>
<th>Serotonin</th>
<th>N2</th>
<th>amx-1</th>
<th>amx-2</th>
<th>amx-3</th>
<th>amx triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>64.0 +/- 1.1</td>
<td>66.7 +/- 0.8</td>
<td>66.7 +/- 1.8</td>
<td>66.7 +/- 2.0</td>
<td>64.7 +/- 2.0</td>
</tr>
<tr>
<td>4 mM</td>
<td>34.8 +/- 0.9</td>
<td>33.6 +/- 1.1</td>
<td>32.0 +/- 1.4</td>
<td>32.6 +/- 0.2</td>
<td>32.4 +/- 1.6</td>
</tr>
<tr>
<td>8.5 mM</td>
<td>23.8 +/- 0.1</td>
<td>23.1 +/- 1.0</td>
<td>21.4 +/- 0.2*</td>
<td>22.5 +/- 0.2</td>
<td>22.1 +/- 0.6</td>
</tr>
<tr>
<td>15 mM</td>
<td>19.9 +/- 0.3</td>
<td>19.9 +/- 1.1</td>
<td>19.5 +/- 0.5</td>
<td>20.5 +/- 0.5</td>
<td>20.6 +/- 0.3</td>
</tr>
<tr>
<td>n=</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

The table lists the mean number of body bends in 20 seconds +/- SEM for all strains in serotonin solutions. * denotes a significant difference (p<0.05) from wild-type. See Figure 26 for graph.
Figure 26. Body bends in serotonin. Nematodes were placed in 0, 4, 8.5 or 15 mM SER and body bends were counted for 20 seconds. The amx-2 mutants were significantly different from the wild-type nematodes. Error bars indicate SEM (* denotes a significant difference (p<0.05) from wild-type). See data in Table 7.
Table 8

*Immobilization in 8.5 mM serotonin solution*

<table>
<thead>
<tr>
<th>Minute</th>
<th>N2</th>
<th>amx-1</th>
<th>amx-2</th>
<th>amx-3</th>
<th>amx triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
</tr>
<tr>
<td>2</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
</tr>
<tr>
<td>3</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
</tr>
<tr>
<td>4</td>
<td>1.0 +/- 0.0</td>
<td>1.0 +/- 0.0</td>
<td>0.9 +/- 0.2</td>
<td>0.9 +/- 0.1</td>
<td>1.0 +/- 0.1</td>
</tr>
<tr>
<td>5</td>
<td>1.0 +/- 0.0</td>
<td>0.9 +/- 0.1</td>
<td>0.8 +/- 0.1*</td>
<td>0.9 +/- 0.2</td>
<td>0.9 +/- 0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.9 +/- 0.1</td>
<td>0.8 +/- 0.1</td>
<td>0.8 +/- 0.1</td>
<td>0.8 +/- 0.3</td>
<td>0.8 +/- 0.1</td>
</tr>
<tr>
<td>15</td>
<td>0.9 +/- 0.1</td>
<td>0.7 +/- 0.1</td>
<td>0.6 +/- 0.1*</td>
<td>0.7 +/- 0.2</td>
<td>0.7 +/- 0.1</td>
</tr>
<tr>
<td>20</td>
<td>0.8 +/- 0.1</td>
<td>0.6 +/- 0.2</td>
<td>0.6 +/- 0.1</td>
<td>0.7 +/- 0.2</td>
<td>0.6 +/- 0.2</td>
</tr>
<tr>
<td>25</td>
<td>0.7 +/- 0.1</td>
<td>0.6 +/- 0.1</td>
<td>0.6 +/- 0.2</td>
<td>0.6 +/- 0.2</td>
<td>0.6 +/- 0.2</td>
</tr>
</tbody>
</table>

n = 3 3 3 3 3

The table lists the mean fraction of nematodes moving in an 8.5 mM SER solution at a given time interval +/- standard deviation for three groups of 10 nematodes each. * denotes a significant (p<0.05) difference from wild-type. See Figure 27 for graph.
Figure 27. Immobilization in Serotonin. Nematodes were placed in 8.5 mM serotonin and their state of mobility was recorded over time. *amx-2* had a higher fraction of immobilized nematodes at 5 and 15 minutes (p<0.05). Note that the scale of the X-axis is non-linear. See data in Table 8.
**Table 9**

*Summary of behavior results*

<table>
<thead>
<tr>
<th>Behavior</th>
<th>MA effects</th>
<th>N2</th>
<th>amx-1</th>
<th>amx-2</th>
<th>amx-3</th>
<th>amx triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bends in food</td>
<td>DA ↓ OCT/ TYR?</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>↓</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SER none</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bends in sephadex</td>
<td>DA ↓ OCT/ TYR?</td>
<td>+</td>
<td>+</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>SER none</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pumping</td>
<td>DA?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SER↑ OCT/TYR↓</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>↑</td>
<td>+</td>
</tr>
<tr>
<td>Egg-laying</td>
<td>DA?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SER↑ OCT/TYR↓</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Bends in DA</td>
<td>DA ↓</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Paralysis in DA</td>
<td>DA ↓ insensitive</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bends in SER</td>
<td>SER↓</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Paralysis in SER</td>
<td>SER↓</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

↓ = decrease; ↑ = increase; + = wild-type; ? = unknown
Summary and Conclusions

Monoamine oxidase (MAO) is an important enzyme for MA metabolism in many animals (Shih et al. 1999). In the present study, we investigated the function of three MAO homologs in *C. elegans* encoded by the *amx* genes (see Chapter 1). We hypothesized that *amx* deletion mutants would have a deficiency in MAO activity and inefficient MA degradation. We further hypothesized that this deficiency would lead to increased levels of endogenous MAs and accompanying altered MA-modulated behaviors.

We quantified the levels of DA and SER in specific neurons using glyoxylic acid induced fluorescence. Although, we detected an overall increase in levels of the DA and SER in *amx* mutants, these changes were not statistically significant. It should be noted that the variability in the detected DA and SER levels in wild-type nematodes was very high (6 folds in some experiments), which made detection of low or moderate changes in MAs in the mutants very difficult (see Chapter 2). In comparison, MAO-A knockout mice show a nine-fold increase in SER levels, while MAO-B knockout mice have virtually normal levels of DA and SER (Cases et al. 1995, Shih 2004). There might be similar or minor changes in mutant nematodes that were undetectable with the techniques used in the present study. High pressure liquid chromatography (not used in this study) can be used to quantify levels of MAs and MA metabolites in whole nematode extracts in smaller quantities (femtomoles). However, MA levels in specific cells can not be detected with this technique.
To model the normal expression of amx-1, we determined the expression pattern of \textit{Pamx-1:: GFP} (see Chapter 3). Transcriptional constructs consisted of 1.8 kb upstream of the start of \textit{amx-1} (predicted to contain most of the \textit{cis}-regulating region) and most of the \textit{amx-1} coding sequence (to include any regulatory introns) fused to a GFP coding sequence (Boulin 2006). We found that \textit{Pamx-1:: GFP} was expressed in the head and tail of three independent transgenic lines (made by Dr. Duerr). To identify the cells, we used antibody staining and other neuron-specific staining. In vertebrates, MAO-A and MAO-B are present in MA cells (Shih \textit{et al.} 1999). To see if MA cells expressed \textit{Pamx-1:: GFP}, we stained nematodes with antibodies to GFP, VMAT (vesicular monoamine transporter; present in all MA cells) and DAT (dopamine transporter; present only in DA cells). We found that most dopaminergic and serotonergic cells did not express \textit{Pamx-1:: GFP}. Staining the transgenic strains with DiI (which stains a subset of chemosensory amphid and phasmid neurons) revealed GFP was expressed in some of the amphid cells in the head (including the pair of ASJ neurons) and two pairs of phasmid neurons (PHA and PHB) in the tail. Expression was also present in the six IL2 inner labial sensory neurons. We saw faint GFP in an average of 2 unidentified cells inside the pharynx. Antibody staining with anti-ChAT (choline acetyltransferase; present in cholinergic cells) confirmed that the expression of \textit{Pamx-1:: GFP} in approximately 20 cholinergic cells, including the six IL2 neurons. Overall, the expression pattern of \textit{amx-1} was consistent with a role in chemosensory neurons and was not consistent with a role as a vertebrate-like MAO. In the future, the expression patterns of \textit{Pamx-2:: GFP} and \textit{Pamx-3:: GFP}
can be analyzed to determine if one or both of these amx genes encode a vertebrate-like MAO expressed in MA neurons.

Consistent with the Pamx-1:: GFP expression pattern, we found the amx-1 mutant exhibited largely normal MA-modulated behaviors (Table 9). The only significant difference we found was in the MA sensitivity assays, where amx-1 was significantly resistant to the mobility inhibiting effects of exogenous DA, but showed normal sensitivity to SER. Note that this is opposite to the effect predicted for a mutant with defective degradation of DA. It is possible that changes in the sensory neurons in amx-1 mutants contribute to this insensitivity. Chemosensation plays an important role in regulating the overall motility of nematodes (Bargmann 2006). In total, our results indicate that AMX-1 may not function as MAO but may rather have a role in the normal function of a subset of chemosensory neurons.

The amx-2 mutants showed specific changes in MA dependent behaviors as follows (summarized in Table 9, Chapter 4). Slowing in food (chemical and mechanical stimulation) and slowing in sephadex beads suspension (mechanical stimulation) are thought to depend upon DA, but not SER; the effects of TYR and OCT are not known. We found that amx-2 moved significantly slower in food, but it moved significantly faster in sephadex (perhaps due to more OCT or TYR). We also looked at behaviors that are modulated by SER and OCT and TYR in opposite ways. Pharyngeal pumping and egg-laying is stimulated by SER and inhibited by OCT and TYR; the role of DA has not been described. amx-2 showed a decrease in pharyngeal pumping and egg-laying consistent with increased levels of OCT and TYR. Finally, amx-2 mutants were slightly more
sensitive than wild-type to exogenous SER and DA. Overall, the behavior of amx-2 mutants was most consistent with decreased degradation of DA, OCT, and TYR.

*amx-3* mutants showed a decrease in locomotion rate in food as well as in sephadex suspensions, which is consistent with increased DA. This mutant also was very slightly sensitive to exogenous DA. *amx-3* showed an increase in pharyngeal pumping consistent with an increase in SER levels. For reproduction assays, we found similar results as obtained for *amx-2*. None of the behavioral assays was indicative of increased levels of OCT or TYR (see summary table 9, Chapter 4).

The *amx* triple mutant showed wild-type movement in food and pumping, but did show a significant decrease in locomotion in sephadex bead suspensions and in egg-laying. This mixed behavior might have resulted from changes in degradation of different MAs that affect the same behavior in opposite ways. In addition, some possible chemosensory defects in *amx-1* mutants may have interacted with the *amx-2* and *amx-3* mutations in unpredictable ways. Testing *amx-2: amx-3* double mutants might help clarify our observations.

Together, the results of present study indicate that one or both of *amx-2* and *amx-3* genes but not *amx-1* may encode vertebrate-like, neuronal-active MAO in *C. elegans*. Specific future studies of *amx* mutants should improve our understanding of the normal function of these genes. Potential studies would include examination of chemotaxis and thermotaxis studies in *amx-1* mutants. For future investigation of *amx-2* and *amx-3* genes, analysis of *Pamx-2:: GFP* and *Pamx-3:: GFP* expression patterns and behavioral...
analysis of amx-2: amx-3 double mutants, together or in combination mutants that disrupt TYR or OCT synthesis may provide more revealing insights into the functions of these two genes.
BIBLIOGRAPHY


