Neuropeptides amplify and focus the monoaminergic inhibition of nociception in Caenorhabditis elegans

Vera M. Hapiak

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Neuropeptides Amplify and Focus the Monoaminergic Inhibition of Nociception in Caenorhabditis elegans

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biology

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Nutritional status can dramatically modulate olfaction and nociception. To better understand how food availability modulates olfactory responses, we examined the nutritionally-dependent, tyraminergic inhibition of aversive behavior mediated by the two nociceptive ASH sensory neurons in *C. elegans*. Tyramine (TA) and octopamine (OA) activate adrenergic-like signaling in invertebrates and are released during starvation. TA is released both synaptically and humorally and abolishes the food or 5-HT stimulation of ASH-mediated aversive responses through the Gαq-coupled TA receptor, TYRA-3. TA and TYRA-3 stimulate the release of a complex mix of “inhibitory” monoamines, including OA and dopamine (DA), and neuropeptides from an array of additional neurons. For example, TYRA-3 stimulates the release of neuropeptides encoded by *nlp-1*, *nlp-14* and *nlp-18* from axons of the two ASI sensory neurons and both Gαq and Gαs signaling in the ASIs are required for TA inhibition. The ASI neuropeptides required for TA inhibition are distinct from the ASI neuropeptides required for OA inhibition, suggesting that individual monoamines can stimulate the release of distinct subsets of ASI neuropeptides. The ASI neuropeptides activate receptors on downstream post-
synaptic partners of the ASIs located throughout the sensory-mediated locomotory circuit. For example, neuropeptides encoded by \textit{nlp-1} activate NPR-11 on the AIA and AIY interneurons and neuropeptides encoded by \textit{nlp-14} activate NPR-10 on the ADL and ASK sensory neurons, suggesting that ASI peptidergic signaling is largely synaptic (or perisynaptic), in contrast to tyraminergic signaling that appears to be humoral. Together, these studies highlight the complexity of TA inhibition, with TA activating widespread global signaling cascades, and suggest that signaling from a complex “humoral soup” of monoamines is amplified and focused by the more localized synaptic (perisynaptic) release of neuropeptides to define nutritional state in the modulation of a wide variety of behaviors, including sensory-mediated locomotory decision-making. On a more general level, these studies emphasize the potential for neuropeptides to focus the more widespread monoaminergic activation involved in resetting complex neuronal circuits in all organisms.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIA</td>
<td>Amphidial interneuron</td>
</tr>
<tr>
<td>AIB</td>
<td>Amphidial interneuron</td>
</tr>
<tr>
<td>AIG</td>
<td>Amphidial interneuron</td>
</tr>
<tr>
<td>AIZ</td>
<td>Amphidial interneuron</td>
</tr>
<tr>
<td>ASH</td>
<td>Amphidial sensory neuron</td>
</tr>
<tr>
<td>ASI</td>
<td>Amphidial sensory neuron</td>
</tr>
<tr>
<td>ASK</td>
<td>Amphidial sensory neuron</td>
</tr>
<tr>
<td>AWB</td>
<td>Amphidial sensory neuron</td>
</tr>
<tr>
<td>BCS</td>
<td>Bovine calf serum</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived growth factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CORT</td>
<td>Corticosterone</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>daf</td>
<td>Dysregulation of dauer arrest mutants</td>
</tr>
<tr>
<td>DCV</td>
<td>Dense core vesicle</td>
</tr>
<tr>
<td>DGK</td>
<td>Diacylglycerol kinase</td>
</tr>
<tr>
<td>DiD</td>
<td>1,1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>EAT-4</td>
<td>BNPI vesicular glutamate transporter</td>
</tr>
<tr>
<td>EGL-3</td>
<td>Preprotein convertase</td>
</tr>
<tr>
<td>EGL-21</td>
<td>Carboxypeptidase E</td>
</tr>
<tr>
<td>EGL-30</td>
<td>Gαq subunit</td>
</tr>
<tr>
<td>EPI</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma–aminobutyric acid</td>
</tr>
<tr>
<td>GAL</td>
<td>Galanin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLR-1</td>
<td>Glutamate-gated ion channel</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GSA-1</td>
<td>Gαs subunit</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
</tr>
</tbody>
</table>
IBMX
3-isobutyl-L-methylxanthine

LDCV
Large dense core vesicle

MAO
Monoamine oxidase inhibitor

MAPK
Mitogen-activated protein kinase

MOD-5
Serotonin reuptake transporter

NE
Norepinephrine

NET
Norepinephrine reuptake transporter

NGM
Nematode growth media

NLP
Neuropeptide-like protein

NMDA
N-methyl-D-aspartate

NPF
Neuropeptide F

NPR
Neuropeptide receptor

NPY
Neuropeptide Y

NMSs
Neurosecretory motorneurons

OA
Octopamine

OCTR-1
Octopamine receptor 1

PBS
Phosphate-buffered saline

PVN
Paraventricular nucleus

PDE-4
5'-cAMP phosphodiesterase

PKA
Protein kinase A

PKC
Protein kinase C

PCR
Polymerase chain reaction

RIA
Ring Interneuron A

RNAi
RNA interference

ser
Serotonin receptor

sNPF
Short neuropeptide F

SNRI
Serotonin-norepinephrine reuptake inhibitors

SSRI
Selective serotonin reuptake inhibitor

SV
Synaptic vesicle

TA
Tyramine

TAAR
Trace amine receptor

TBH-1
Tyramine-β-hydroxylase

TDC-1
Tyrosine decarboxylase

TGF-β
Transformer growth factor Beta

ttx
Thermotaxis defective

tyra
Tyramine receptor
UNC-13 ............... DAG-binding protein
UNC-31 .................. Ortholog of human calcium-dependent activator protein for secretion

5-HT ....................... Serotonin
List of Symbols

\[ \alpha \quad \text{Alpha} \]
\[ \beta \quad \text{Beta} \]
\[ \gamma \quad \text{Gamma} \]
Chapter 1

Introduction

Monoamines and neuropeptides function as complex neuromodulators in the regulation of many physiological processes and key behaviors in both vertebrates and invertebrates, including development, appetite, sleep, reproduction, homeostasis, and longevity, as well as cognition and memory, olfaction, and motor function (Zeitz et al., 2002; Lai et al., 2003; Libersat and Pflueger 2004; Georgescu et al., 2005; Tiesjema et al., 2007; Doly et al, 2008; Przegalinski et al., 2008). Both can act alone or often in concert as regulators of signaling pathways; specifically, monoamines and neuropeptides can act as neurotransmitters or neurohormones to initiate fast synaptic events mediated by ion channels, or slow-acting modulatory effects mediated primarily by G-protein-coupled receptors. Increasingly, the dissection of many disease states including depression and chronic pain suggests that multiple monoaminergic and peptidergic signaling systems are extensively intertwined, can have regulatory influence on one another and reveal that in many cases, it is the balance of signaling between the two that is altered.

Given the complexity of the mammalian nervous system, defining the interactions between different neural networks and their modulation has been difficult and limited. In fact, in many cases, the use of “simpler” invertebrate model organisms, such as
Caenorhabditis elegans, Drosophila and Aplysia, has been key to identifying and understanding the specific roles of neuromodulators in the regulation of individual behaviors. Many invertebrate models have defined neuronal assemblies underlying specific behaviors consisting of fewer neurons than those in vertebrates, and in many cases, activity (i.e., Ca$^{++}$ transients) in specific neurons can be causally linked to behavior, in addition to fewer neurons and mapped neuronal circuitries. Moreover, many conserved molecular pathways and cellular mechanisms are shared in mammals and invertebrates. For example, recent work has implicated a role for the monoamine OA, the invertebrate equivalent to noepinephrine (NE) in mammals, in the modulation of sensory-mediated aversive responses in C. elegans, highlighting the utility of the C. elegans model system, with the OA inhibition of aversive responses in C. elegans mimicking the adrenergic modulation of pain in vertebrates (Mills et al., 2011; Komuniecki et al., 2012). The present study aims to dissect the role of TA signaling in the nutritional modulation of aversive responses and locomotory behavior in C. elegans.

1.1 Monoamines and neuropeptides as co-modulators for global signaling cascades in mammals and invertebrates

Although monoamines and neuropeptides modulate many behaviors in both invertebrates and mammals, much still remains to be learned about how monoamines and neuropeptides interact to regulate neural circuits and behavior. As noted above, both can function as neurotransmitters or neurohormones to activate ionotropic receptors (ligand-gated ion channels) and either excite or inhibit a target cell. Similarly, both can activate metabotropic receptors (G-protein-coupled receptors) to activate intracellular signaling
pathways and secondary messengers, such as cyclic nucleotides, inositol 1,4,5-triphosphate, or diacylglycerol. However, activation of these intracellular signaling pathways may have broader, under-appreciated implications for drug design, as growing evidence suggests that monoamines and neuropeptides may lead to the release of additional neuromodulators and activation of additional receptors, thus initiating larger, more global signaling cascades.

Often monoamines and neuropeptides work in concert to affect behavior. Many neuropeptides coexist with monoamines in neurons in the brain (Hokfelt et al., 1986, 1987) and in some cases are copackaged/released from the same vesicles (Merighi 2002; Yang et al., 2002; Burnstock 2004; Vega et al., 2010). Although monoamines and neuropeptides may work independently, activating their respective receptors, each may also act in a dependent fashion; for instance, a neuropeptide may act presynaptically to modulate the release of a monoamine or pre-/postsynaptically to modulate monoamine receptors and vice versa. In the mammalian brain, two main monoaminergic neurotransmitters, serotonin (5-HT) and NE, have been major targets for drug design, as they interact with a wide array of neuropeptides and modulate feeding behavior, mood, and sensation and the exploitation of invertebrate model systems, such as C. elegans, has greatly aided our understanding of monoamine/neuropeptide interactions in disease states such as obesity, depression and pain.

Obesity is associated with various disease states, including Type II diabetes, coronary heart disease, and hypertension and involves the complex regulation by several monoamines and neuropeptides in the management of satiety and metabolism. Feeding behavior is influenced by both stimulatory and inhibitory inputs from α2-noradrenergic,
serotonergic, and peptidergic systems (Clifton and Kennett 2006; Magalhaes et al., 2010). For example, in mammals both NE and epinephrine (EPI) elicit feeding through the hypothalamic paraventricular nucleus (PVN) via $\alpha_2$ adrenoreceptors (Leibowitz 1989; Kyrkouli et al., 1990). NE increases appetite and both NE and EPI can alter the levels of corticosterone (CORT), vasopressin and glucose that exacerbate the effects of NE and EPI and potentiate feeding (Leibowitz 1988, 1989). In contrast to NE and EPI, 5-HT suppresses food intake via 5-HT$_{1B}$ and 5-HT$_2$ receptors (Simansky 1996; Leibowitz and Alexander 1998). In general, NE/EPI and 5-HT are thought to act antagonistically in the hypothalamus to regulate food intake, but often are influenced and interact with signaling mediated by several neuropeptides, including Neuropeptide Y (NPY) and galanin (GAL).

As noted below, a similar situation exists in *C. elegans* where TA and OA often act antagonistically to 5-HT in the modulation of many nutritionally-sensitive behaviors.

Both NPY and GAL modulate feeding via distinct peptide monoamine interactions. For example, NPY is one of the most abundant neuropeptides in the central nervous system and is involved in many behaviors, including learning and memory, anxiety, as well as appetite (Silva et al., 2002; Beck 2006). Like NE, NPY increases food intake by potentiating the rate of eating, as well as the duration of the eating response and can stimulate the release of additional modulators, such CORT and vasopression to potentiate the response (Liu et al., 1994; Kakui and Kitamura 2007). GAL is also an abundantly expressed neuropeptide that coexists with GABA, 5-HT, substance P and, importantly, NE in many regions of the brain. GAL is involved in pain, reproduction, sleep as well as increased appetite (Karlsson and Holmes 2006). However, although NPY appears to function independently of monoamines, GAL acts, at least in part, through the release of
endogenous NE and subsequent activation of $\alpha_2$ receptors in the PVN (Leibowitz 1989). Whereas NPY signaling is unaffected by the presence of $\alpha_2$ receptors antagonists or inhibition of NE/EPI synthesis, the stimulatory action of GAL on feeding is dependent on functional $\alpha_2$-noradrenergic receptors and endogenous NE in the PVN. GAL (and NE) stimulatory feeding behavior could be 1) blocked with $\alpha_2$- but not $\alpha_1$- antagonists, 2) enhanced with $\alpha_2$ receptor agonists such as clonidine or antidepressant drugs that target/promote NE release, and 3) attenuated with inhibitors of endogenous NE synthesis (Kyrkouli et al., 1990). GAL/NE interactions have also been suggested in other systems, where GAL stimulates the secretion of growth hormone or lutenizing hormone-releasing hormone via $\alpha$-adrenergic signaling (Lopez and Negro-Vilar 1989; Murakami et al., 1989). These data highlight the concept that monoamines are capable of initiating the activation of more global signaling cascades where the release/effects of one neuromodulator (i.e., GAL stimulatory feeding behavior) is dependent on the release/activation of cognate receptors of another neuromodulator (i.e., endogenous NE activating $\alpha_2$-noradrenergic receptors).

Feeding behavior in *C. elegans* has been equated with the rate of pharyngeal pumping or fat storage and is also extensively regulated by the interaction of stimulatory and inhibitory monoaminergic/peptidergic signaling cascades. Feeding depends on pharyngeal pumping, is regulated by an intrinsic pharyngeal nervous system consisting of 20 neurons and is influenced by feeding history (well-fed vs starvation) and neurohormones released throughout the nervous system (White et al., 1986; Franks et al., 2006; Luedtke et al., 2010). For instance, when re-exposed to food, starved animals temporarily feed faster than well-fed animals (Avery and Horvitz 1990). Several
downstream signaling cascades are thought to regulate pharyngeal pumping following starvation including MAPK (mitogen-activated protein kinase) signaling following activation of pharyngeal muscarinic receptors, as well as signaling from amino acids such as leucine, valine, alanine and glutamate which activate the AIYs and AIBs either directly or through the metabotropic glutamate receptors, MGL-1/2, to enhance/inhibit the starvation response via release of other neuromodulators (such as the neuropeptide, FLP-18) (You et al., 2006; Kang and Avery 2009). Indeed, many of the same neurotransmitters implicated in appetite in mammals have been implicated in the regulation of feeding in *C. elegans*. Similar to *E. coli* OP50, 5-HT can significantly increase pharyngeal pumping, whereas both OA and TA (the invertebrate equivalents of NE/EPI) inhibit pharyngeal activity (Avery and Horvitz 1990; Rogers et al., 2001). Moreover, tyramine β-hydroxylase (*tbh-1*) and tyrosine decarboxylase (*tdc-1*) mutants that lack OA and OA /TA, respectively, exhibit defects in the inhibition of pharyngeal pumping and mutant animals for the TA receptor, SER-2, display an altered TA-dependent regulation of 5-HT-stimulated pharyngeal pumping (Rex et al., 2004; Alkema et al., 2005). In addition, a number of FMRFamides (FLPs) or neuropeptide-like proteins (NLPs) regulate the activity of pharyngeal muscle and/or behaviors associated with feeding (de Bono and Bargmann 1998; Rogers et al., 2001; Li 2005; Papaioannou 2008; Cohen et al., 2009; Chalasani et al., 2010).

Monoamines and neuropeptides also regulate starvation-induced quiescence in *C. elegans*. Interestingly, wild type *C. elegans* feed and move constantly in the presence of a bacterial food source, yet when fasted and then re-fed, undergo a state of quiescence where feeding and locomotion are inhibited. Quiescence is equated with satiety in
mammals as it shares the standard behavioral characteristics (cessation of meals, reduction of locomotion, and sleep) and common signaling mechanisms including peptide signals such as insulin (Wilding 2002; Wynne et al., 2005; Pliquett et al., 2006). In *C. elegans*, quiescence behavior is mediated by the EGL-4 cGMP-dependent kinase that functions downstream of insulin (via *daf-2*) and TGF-β (via *daf-7*) in sensory neurons including the ASI (You et al., 2008). Apart from its role in quiescence, the ASI-expressed DAF-7 TGF-β ligand can regulate pharyngeal pumping via signaling cascades involving TA and OA (Greer et al., 2010). In the presence of food, DAF-7 is released and activates its cognate receptors DAF-1/4 on the RIM and RIC interneurons. Activation of DAF-1/4 inhibits DAF-3 and TA and OA are not released from the tyraminergic RIMs or octopaminergic RICs and as a result, pharyngeal pumping increases. In the absence of food however, DAF-7 is not released and DAF-1/4 receptors are not activated. DAF-3 can now signal, allowing TA and OA release from the RIMs and RICs, respectively, to activate SER-2 and SER-3 receptors expressed on pharyngeal neurons to reduce pharyngeal pumping.

The dysregulation/imbalance of monoaminergic/peptidergic interactions is also linked to both depression and pain and signaling molecules and orthologous receptors involved in these phenomenon have been studied in *C. elegans*. Indeed, extensive studies have sought to determine the molecular mechanisms associated with depression and pain using mammalian and invertebrate models to identify improved drug targets. Depression and pain share common brain structures, neuronal pathways and neurotransmitters/neuromodulators, including 5-HT and NE, the main targets for the pharmacological management of these conditions. Depression has been classically linked
with deficiencies in 5-HT and NE. For example, drugs, such as serotonin-norepinephrine reuptake inhibitors (SNRIs) that block serotonin/norepinephrine reuptake transporters (SERTs/NETs) or monoamine oxidase inhibitors (MAOIs) that prevent monoamine degradation, can increase synaptic concentrations of these neurotransmitters and result in alleviation of the symptoms of depression. However, depression is not due simply to monoamine deficiency, as inhibition of 5-HT or NE synthesis fails to simulate classic depressive symptoms in normal individuals, and often noradrenergic and serotonergic antidepressants display limited efficacy, numerous side effects and lack of amelioration of depression, even following the rapid onset of monoamine reuptake inhibition (Delgado et al., 1990; Delgado 2004). Rather, depression involves an interactive matrix of monoamines and neuropeptides that can act on one another and maintain homeostasis with both stimulatory and inhibitory modulation. For instance, noradrenergic and serotonergic neurons can modulate each other with activation of $\alpha_1$- and $\alpha_2$-adrenoreceptors increasing/decreasing the firing rates of 5-HT neurons within the hypothalamus and activation of 5-HT$_1$ receptors decreasing the firing rates of NE neurons (Mongeau et al., 1997). Moreover, depression involves the hypoactivity of 5-HT, NE, and DA and hyperactivity of gamma-aminobutyric acid (GABA) and glutamate. Hyperactive signaling of GABA and glutamate may presynaptically inhibit 5-HT/NE release and contribute to 5-HT/NE deficiency (Palucha 2006; Drago et al., 2012). Additionally, neuropeptide levels are also altered in certain brain regions with hyperactivity of substance P, corticotropin-releasing hormone, and thyrotropin-releasing hormone and hypoactivity of neuropeptide Y and galanin (Werner and Covenas 2010).

Interestingly, although worms do not exhibit “depression,” *C. elegans* has served as
a beneficial genetic model to elucidate the molecular mechanisms of depression as it shares many orthologous genes involved in human disease and allows whole-animal pharmacology. Notably, the SSRI fluoxetine (Prozac) can increase serotonergic signaling in *C. elegans* by inhibiting the serotonin re-uptake transporter encoded by *mod-5* and alter multiple behaviors, including locomotion and egg-laying, by targeting serotonin receptors as well as other unidentified targets possibly including neuropeptide signaling (Ranganathan et al., 2001; Dempsey et al., 2005; Hapiak et al., 2009; Kullyev et al., 2010). The use of *C. elegans* has highlighted that monoamines, such as 5-HT can have both excitatory and inhibitory inputs into the same process (for instance, egg-laying) and that it is the balance of signaling between different 5-HT receptor subtypes that dictates behavior (Hapiak et al., 2009). Indeed, a similar observation is paralleled in mammals where the involvement of 5-HT receptors in the antidepressant-like effects of SSRIs is complex and involves the orchestrated stimulation and blockade of different 5-HT receptor subtypes (Carr and Lucki 2011). In addition, it is clear that some *C. elegans* serotonergic neurons do not synthesize 5-HT themselves, but instead rely on the uptake of humoral 5-HT, suggesting the SSRIs may actually decrease serotonergic signaling from these neurons (Kullyev et al., 2010; Jafari et al., 2011). Whether a similar phenomenon is operative in mammals remains to be determined.

Pain is closely linked with the pathology of depression and shares a common neurochemical pathway involving 5-HT and NE signaling. In the brain stem, 5-HT and NE modulate pain transmission with both facilitory and inhibitory inputs through ascending and descending neural pathways. Upon sensation and awareness of pain, serotonergic and norepinephrinergic descending neurons that project from the brain stem
into the dorsal cord of the spinal cord suppress both acute and chronic pain through multiple mechanisms (Millan 2002; Pertovaara 2006). Importantly, these effects of 5-HT and NE are dependent on receptor subtypes and their distribution (i.e., tissue localization). For instance, 5-HT acts via different receptor subtypes, with 5-HT\textsubscript{1A} receptors inhibiting the excitability of spinothalamic excitatory interneurons and 5-HT\textsubscript{1B/D} receptors inhibiting neurotransmitter release from primary afferents (Millan 2002). Likewise, NE released from descending pathways can suppress pain through inhibitory $\alpha_2$-adrenoreceptors on afferent nociceptors and by the activation of $\alpha_1$-receptors on inhibitory peptidergic interneurons (Pertovaara 2006; Seybold 2009).

Interestingly, OA inhibition of aversive responses in \textit{C. elegans} parallels the noradrenergic modulation of pain in mammals (Mills et al., 2011; Komuniecki et al., 2012). In \textit{C. elegans}, the polymodal nociceptive ASH sensory neurons are able to sense a range of noxious stimuli, including repellent chemicals such as 1-octanol and respond by inhibiting forward locomotion and initiating reversal to escape the harmful stimuli.

Similar to the effects of NE on descending neurons in response to sustained pain in mammals, increasing noxious stimulus (100% 1-octanol) in \textit{C. elegans} 1) activates the octopaminergic SER-6 receptor to cause the release of “inhibitory” neuropeptides (that act on additional receptors to modulate locomotion) and 2) potentially cause OA release from the octopaminergic RIC interneurons (to possibly dampen and prevent ASH over-stimulation via a negative feedback mechanism; Mills et al., 2011).

\textbf{1.2 The role of tyramine as a neuromodulator in mammals and invertebrates}
In both vertebrates and invertebrates, monoamines represent a small, but important, group of bioactive neuroactive chemicals synthesized through decarboxylation of amino acids. In addition to neurotransmitters shared by both phyla (DA, 5-HT, histamine), vertebrates preferentially synthesize two biogenic amines, the catecholamines noradrenaline (norepinephrine) and adrenaline (epinephrine), whereas invertebrates synthesize TA and OA. In contrast to the adrenergic/noradrenergic system, little is known of the roles of TA or OA in vertebrates. Due to its low abundance, TA is regarded as a “trace amine” in mammals, and although altered trace amine levels have been implicated in various human disorders (such as depression, Parkinsonism, hypertension, and schizophrenia) and TA-binding trace amine-mediated GPCRs have been identified (TAAR1 and TAAR4), whether TA is a physiologically relevant signaling molecule in mammals is unclear (Borowsky et al., 2001; Bunzow et al., 2001; Burchett and Hicks 2006; Premont et al., 2001; Liberles and Buck 2006). Ingestion of TA-rich foods and use of monoamine oxidase (MAO) inhibitors can compromise monoamine metabolism and increase levels/release of TA, as well other monoamines potentially masking the endogenous roles of these neuromodulators. Moreover, the family of mammalian trace amine receptors is phylogenetically and functionally distinct from invertebrate OA or TA receptors (Lindemann and Hoener 2005; Wragg et al., 2007).

In invertebrates, tyraminergic and octopaminergic signaling have been better characterized (especially in insects) and are involved in a range of physiological processes. As in mammals, OA is generated by the hydroxylation of tyramine on the β-carbon of the side chain by tyramine β-hydroxylase (TβH) and OA re-uptake systems isolated from various insect species share similar structural and pharmacological features.
with re-uptake transporters in vertebrates (Cavney and Donly 2002; Malutan et al., 2002). Due to their potential as insecticidal targets, i.e., OA agonists can alter locomotion and cause leaf walk-off, OA receptors and OA signaling pathways have been extensively studied in many invertebrates and have broad roles in appetite reinforcement in honeybee and flies, and behaviors such as aggression, locomotion, egg-laying, food-seeking, and sleep as well as synaptic functions (Hammer et al., 1998; Schroll et al., 2006; Schwaerzel et al., 2003; Saraswati et al., 2004; Zhou et al., 2008; Monastirioti et al., 1996; Suo et al., 2006; Crocker et al., 2010; Breen et al., 1983, respectively). Much less is known of the role of TA, as until recently TA was considered only as a biosynthetic intermediate of OA, but has since been recognized as an independent neurotransmitter in its own right in invertebrates. In several invertebrate species, specific neurons are tyraminergic (Stevenson and Spoerhase-Eichman 1995; Nagaya et al., 2002; Alkema et al., 2005; Kononenko et al., 2009), and multiple TA-specific receptors have been identified and pharmacologically/behaviorally characterized (Blenau et al., 2000; Rex et al., 2004, 2005; Cazzamali et al., 2005; Evans and Maqueira 2005; Huang et al., 2009). Most importantly, in a number of invertebrate species, TA-dependent physiological and behavioral changes that are distinct from OA have been identified for locomotion (Saraswati et al., 2004; Alkema et al., 2005), reproduction (Donini and Lange 2004; Alkema et al., 2005; Hirashama et al., 2007), flight initiation/maintenance (Brembs et al., 2007), olfactory avoidance (Kutsukake et al., 2000; Wragg et al., 2007), and feeding (Nisimura et al., 2005; Rex et al., 2005).

In *C. elegans*, TA is a bona fide neuroactive chemical with distinct roles in modulating an array of behaviors. In *C. elegans*, TA is synthesized from tyrosine by a
tyrosine decarboxylase (TDC-1) and tyramine β-hydroxylase (TBH-1) adds a hydroxyl group to form OA. Importantly, the localization, phenotypic analysis of the enzymes involved in the TA/OA biosynthetic pathway (tbh-1 and tdc-1) and identification of multiple TA receptors, suggests that TA and OA also have distinct roles in C. elegans (Alkema et al., 2005; Rex et al., 2004, 2005). Based on immunohistochemical staining, both TDC-1 and TBH-1 proteins are expressed in the RIC interneurons and the gonadal sheath cells, whereas TDC-1 only is expressed in the RIM motor neurons and four UV1 (uterine) cells, suggesting that the RIMs and UV1 cells are tyraminergic (Alkema et al., 2005). Indeed, tdc-1 mutants have behavioral defects not shared by tbh-1 mutants; tdc-1, but not tbh-1, mutants are hyperactive for egg-laying, display defects in reversal behavior and fail to suppress head oscillations in response to anterior touch (Alkema et al., 2005). In general, TA and OA oppose the action of 5-HT to suppress many 5-HT-stimulated behaviors, including pharyngeal pumping, egg-laying, and aversive responses to noxious stimuli (Horvitz et al., 1982; Nicaris and Avery 2003; Wragg et al., 2007). In C. elegans, three G-protein coupled TA receptors, SER-2, TYRA-2, and TYRA-3, and one TA-gated Cl− channel, LGC-55, have been identified and at least partially characterized. Like tbh-1 and tdc-1 mutant animals that are defective in the inhibition of pharyngeal pumping, SER-2 mutants display altered TA-dependent regulation of 5-HT-stimulated pharyngeal pumping (Rex et al., 2002, 2004; Alkema et al., 2005). TYRA-2 is expressed in a number of amphid neurons (ASE, ASG, ASH, and ASI) and the ALM mechanosensory neuron suggesting that TYRA-2 may modulate sensory-mediated and/or mechanosensory behaviors (Rex et al., 2005). TA also inhibits head movements and forward locomotion (by inducing long backward runs) through activation of LGC-55 to coordinate escape
responses to nematophagous fungi (Pirri et al., 2009; Maguire et al., 2011). TYRA-3 has been implicated in sensory-mediated foraging behavior and the modulation of a sensory-mediated locomotory behavior, the avoidance of the repellent, 1-octanol (Bendesky et al., 2011; Wragg et al., 2007). The present study is designed to dissect the role of TA and TYRA-3-mediated signaling in the modulation of *C. elegans* aversive responses and locomotory behaviors.

### 1.3 Significance

As noted above, olfactory and gustatory responses are modulated by nutritional status in most organisms and food availability can modulate a myriad of behaviors including dietary preference, food searching and nociception. For example, in mammals, olfaction is used to find food and initiate feeding; conversely, food availability/nutritional status can modulate olfaction and ultimately behavioral decisions. Nutritional status modulates odor detection in fasting rodents, primates, and humans, leading to the increased perception of food-related odors. Similarly, olfactory sensitivity is higher before a meal and when paired with aversive odors, results in the increased avoidance of aversive stimuli more often in fasted rather than satiated animals, suggesting that fasting increases olfactory sensitivity (Mulligan et al., 2002; Yeomans 2006; Aime et al., 2007). Monoamines and neuropeptides, such as neuropeptide Y (NPY), orexin and leptin, modulate olfaction in a nutritional state-dependent manner. For example, NPY significantly and transiently increases the amplitude of electro-olfactogram recordings in fasted, but not fed rats, and these effects can be mimicked by specific NPY-receptor agonists (Negroni et al., 2012). Likewise, orexin or leptin can behaviorally mimic the
effects of fasting or satiety in rats, i.e., orexin increases and leptin decrease locomotory activity, sniffing, and olfactory bulb activation (Fos expression) (Julliard et al., 2007; Prudhomme et al., 2009).

Similarly, in invertebrates such as Drosophila and C. elegans, feeding state also regulates olfaction. As in mammals, olfactory preferences for a food odor (3-methylthiopropanol) are significantly increased in starved Drosophila and food odors significantly increase food intake. Starvation also dramatically alters gene expression in Drosophila, with transcriptional increases and decreases observed for ~415 and ~723 genes, respectively, based on whole genome microarrays (Farhadian et al., 2012). Drosophila feeding behavior is also directly modulated by at least three different neuropeptides, including hugin-PK associated with decreased feeding, and neuropeptide F (NPF) and short neuropeptide F (sNPF) associated with increased food intake. Hugin-PK and NPF modulate gustatory responses (i.e., evaluation of food quality and feeding initiation) and sNPF interacts with the insulin signaling pathway to co-modulate olfactory receptor neurons to control olfactory sensitivity (Shen and Cai 2001; Wu et al., 2003; Melcher and Pankratz 2005; Root et al., 2011). Starvation enhances food search by increasing the presynaptic expression of the receptor for sNPF that reduces insulin signaling in olfactory neurons (with presynaptic neuron activity measured by Ca^{++} influx) (Root et al., 2011).

Food availability also modulates a host of sensory-mediated behaviors in C. elegans, including olfaction and nociception-dependent changes in locomotion. C. elegans contain 14 pairs of amphid and phasmid sensory neurons that sample the environment to mediate either attraction or repulsion to a variety of stimuli. Indeed, dozens of sensory-mediated locomotory behaviors have been described and the circuit
involved in nutritionally-driven locomotory decision-making has been analyzed extensively (Chao et al., 2004; Gray et al., 2005; Chalasani et al., 2007; Harris et al., 2009, 2010, 2011). Importantly, most, if not all, sensory-mediated locomotory behaviors in *C. elegans* are extensively modulated by food availability and nutritional status. In general, the sensory neurons mediating aversive responses, including the ASHs, synapse directly on the forward and backward command interneurons that function as a “bistable switch” controlling locomotory decision-making (Chalfie et al., 1985; Zheng et al., 1999; Brockie et al., 2001; Tsalik and Hobert 2003; Gray et al., 2005). Presumably, these nociceptive sensory neurons preferentially stimulate the backward command interneurons (AVA, AVD, AVE) and inhibit the forward command interneurons (AVB, PVC) to initiate a rapid reversal. In contrast, most of the sensory neurons mediating attraction do not synapse directly on the command interneurons and instead, in common with sensory neurons mediating aversion, synapse onto 4 pairs of highly interconnected interneurons (AIA, AIB, AIY, AIZ) that play key roles in modulating locomotory transitions. For example, Gray et al. (2004) and others laser-ablated each pair of these sensory and key interneurons individually and examined spontaneous locomotory behavior (short reversal, long reversal and omega turns) at various times after removal from food (Zheng et al., 1999; Tsalik and Hobert 2003; Wakabayashi et al., 2004; Alkema et al., 2005; Piggott et al., 2011). They concluded that the AWC and ASK sensory neurons were primarily responsibiy for the increased reversal behavior observed when animals were removed from food, i.e., ablation of the AWCs and ASKs dramatically decreased the frequency of short/long and long reversals, respectively, after removal from food, suggesting that these two neurons responded to a “food signal.” In addition, ablation of the AIB interneurons
dramatically decreased the frequency of spontaneous reversal off food, suggesting that the AIBs stimulated reversal, while ablation of the AIY interneurons or RIM motorneurons increased the frequency of reversal off food, suggesting that the AIYs and RIMs inhibited reversal. Additional studies designed to examine the relationship between the AWCs and the AIBs/AIYs, using the calcium sensor, G-CaMP to monitor changes in intracellular calcium as an indicator of neuronal activity, have demonstrated that the AWCs are off neurons, i.e., they respond to odor removal, and that AWC signaling activates the AIBs through the AMPA-like glutamate receptor, GLR-1 and inhibits the AIYs through the glutamate-gated Cl⁻ channel, GLC-3 (Chalasani et al., 2007, 2010). Since the AWCs activate the AIBs and the RIMs are the major downstream synaptic partner of the AIBs, these results suggest that the AIBs inhibit RIM signaling, an observation that was subsequently confirmed by direct electrophysiological analyses and the identification of the glutamate-gated Cl⁻ channel, AVR-14, on the RIMs (Piggott et al., 2011). Recent work has demonstrated that two distinct pathways are involved in increasing the rate of spontaneous reversal; one involves the activation of the AVA backward command interneurons directly and the second involves the AIBs and RIMs (Piggott et al., 2011). Activating the AIBs should inhibit the RIMs and stimulate reversal, as outlined above. However, the “disinhibition” of this second pathway leading to the RIMs is complex and involves more than simply the command interneurons, as animals with the AVA/AVD/AVE command interneurons ablated still spontaneously reverse, albeit at a dramatically reduced rate. In contrast, ablation of the command interneurons AVA/AVD/AVE along with the AIBs completely abolishes spontaneous reversal.
In the present study we propose to examine the role of food availability on the modulation of a simple, well-described, sensory-mediated locomotory behavior in *C. elegans*, reversal in response to the volatile repellent, 1-octanol, that is mediated by the two polymodal, nociceptive ASH sensory neurons. As noted above, the ASHs synapse on both the forward and backward command interneurons and the AIBs and are extensively modulated by food availability (Figure 1). For example, when animals are on food, 5-HT is released humorally from the two NSM neurosecretory motorneurons and dramatically decreases the time taken to reverse in response to dilute 1-octanol (Chao et al., 2004; Harris et al., 2009). In contrast, off food neuronal TA and OA are released from the two RIM motorneurons and RIC interneurons, respectively, and abolish any 5-HT stimulation (Wragg et al., 2007). Importantly, food availability, as mediated by these monoamines, not only modulates the time taken to reverse, but also locomotory decision-making post-initiation (Harris et al., 2011). For example, off food the animals reverse, execute an omega turn and move away from the source of the aversive stimulus. In contrast, on food reversal is shortened and the animals continue to move forward after reversal is complete, regardless of the intensity of the initiating stimulus. Together, these data strongly suggest that nutritional status/food availability profoundly modulates nociception and its associated aversive behavior. The receptors mediating 5-HT stimulation and OA inhibition have been functionally localized and describe a complex, humorally-translated, decision-making “circuit” involving an array of interacting sensory and interneurons (Figure 1). For example, three distinct 5-HT receptors are essential for food or 5-HT stimulation that modulate not only signaling from the ASHs directly, but also ASH-mediated circuits involved in head movement and forward locomotion,
suggesting that 5-HT stimulation requires the coordinate interaction of multiple locomotory circuits (Harris et al., 2009). Similarly, OA inhibition involves multiple OA receptors that not only inhibit ASH signaling directly, but at higher levels of ASH stimulation trigger a cascade of neuropeptides that modulate input from sensory neurons mediating either attraction or repulsion, suggesting that overall reversal dynamics involves the integration of multiple sensory signals (Mills et al., 2011).

In the present study, we have demonstrated that TA released from the two RIM motorneurons in response to nutritional status activates a global monoaminergic/peptidergic signaling cascade, with receptors localized at all levels of the sensory-mediated locomotory circuit, including sensory neurons, interneurons, motorneurons and muscle. TA inhibition of the food or 5-HT-stimulation of ASH-mediated aversive responses requires the Gαq-coupled TA receptor, TYRA-3 that stimulates the release of a complex mix of inhibitory monoamines and neuropeptides from an array of additional neurons. Interestingly, the TA/OA inhibition of ASH-mediated aversive responses appears to be largely, if not exclusively humoral, i.e., the tyraminergic RIMs do not synapse on the dopaminergic, octopaminergic or ASI neurons activated by TYRA-3, and the octopaminergic RICs do not synapse directly on the ADL, AWB, or ASI neurons activated by OA and the OA receptor, SER-6. ASI peptides encoded by three different genes, nlp-1, nlp-14 and nlp-18 are required for TA inhibition. The TA-dependent release of nlp-14 peptides from the axons of the ASIs requires TYRA-3 and both ASI Gαq and Gαs signaling, as has recently been described for neuropeptide release from Drosophila motorneurons (Shakiryanova et al., 2011). Peptides encoded by nlp-1 and nlp-14 activate the NPR-11 and NPR-10 receptors on the AIA/AIY and ASK/ADL
neurons, respectively. In contrast to tyraminergic or octopaminergic signaling from the
RIMs or RICs that appears to be largely humoral, neuropeptidergic signaling from the
ASIs in response to either TA or OA appears to be largely synaptic (or perisynaptic), as
the four neuropeptide receptors identified to date that respond to ASI peptides function
on the AIA/AIY interneurons or the ASK, ASER or AWC sensory neurons, all of which
are downstream synaptic partners of the limited ASI synaptic circuit.
Figure 1-1. ASH-mediated aversive circuit. Number of synapses noted in parentheses. Green, 5-HT receptors; blue, dopamine receptors; red, octopamine receptors.
Chapter 2

Materials and Methods

2.1 Materials
All culture reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA), DMEM from Media-tech (Herdon, VA) and neurochemicals from Sigma (St. Louis, MO). $[^3\text{H}]-\text{inositol (20 Ci/mmol)}$ was purchased from Perkin Elmer (MA, USA), yttrium silicate (YSi) scintillation proximity assay (SPA) beads from GE Healthcare (USA) and cAMP kits obtained from Assay Designs (Ann Arbor, MI). A $C. \text{elegans}$ cDNA pool was purchased from OriGene Technologies (Rockville, MD) and additional cDNA pools were constructed from mixed stage $C. \text{elegans}$ mRNA using standard techniques. Green fluorescent protein (GFP) reporter constructs were obtained from Andy Fire (Carnegie Institute of Washington, Washington, DC).

2.2 Nematode growth and strains
General techniques for the culture and handling of worms have been described (Brenner, 1974). The N2 Bristol WT isolate of $C. \text{elegans}$ was used for all studies. All animals were raised at 20°C under uncrowded conditions. Strains obtained from the
Caenorhabditis Genetics Center (University of Minnesota, MN) include tyra-3(ok325), nlp-1(ok1469), nlp-1(ok1470), nlp-3(tm3023), nlp-5 (ok1981), nlp-14(tm1880), nlp-18(ok1557), c43c3.2(ok1387), co2h7.2(ok2068), t27d1.3(ok1626), c48e5.1(ok1387), t07d4.1(ok2575), fshr-1(ok778), t27d1.3(ok1626), npr-10(ok1442), c49a9.7(ok1620), gnrr-1 (ok238), y58g8a.4(ok1583), f35g8.1(ok527), tag-49(ok381),c56g3.1(ok1439) and f41e7.3(tm1497), f14f4.1(tm2243), nlp-7(tm2184), nlp-9(tm3572), nlp-24(tm2105), npr-9(tm1652), npr-10(tm1568), tkr-1(tm1765), zc412.1(tm1504), c10c6.2(tm1583), t07d10.2(tm2765), w05b5.2(tm2974), y39a3b.5(tm3082), c30f12.6(tm3210), t07d12.2(tm2765), t22d1.12(tm1498), c16d6.2(tm1782), c56g3.1(tm1553), were received from the National Bio-Resources Project (Tokyo Women’s Medical University, Tokyo, Japan). All mutants were backcrossed with the N2 Bristol strain at least 4x before use in assays or crosses. Mutant npr-11 (ok594) and CX8912 npr-11; kyEx1686 (gcy-28(d)::npr-11::gfp) animals were kindly provided by Dr. Cori Bargmann (Rockefeller University, New York, NY).

2.3 Behavioral assays

Responses to 1-octanol were assayed as previously described (Chao et al., 2004; Harris et al., 2010). All experiments used age-matched, well-fed young adults grown at 20°C on standard nematode growth medium (NGM), seeded with E. coli strain OP50. Fourth-stage larvae were picked 24 hrs before testing. NGM plates were prepared the morning of the experiment by adding either 5-HT (4 mM) and/or TA/OA (4 mM) to liquid NGM before pouring. Dilute (30%) 1-octanol was prepared daily using 100% ethanol (vol/vol). Briefly, a hair (Loew-Cornell 9000 Kolinsky 8 paintbrush) was melted
into the end of a P200 pipette tip and the blunt end of the hair was dipped in 1-octanol and placed in front of an animal exhibiting forward sinusoidal locomotion immediately after the animal had exhibited a reversal (Chao et al., 2004; Harris et al., 2010). Time to reverse was recorded and assays were terminated after 20 s, as wild type animals spontaneously reverse ~ every 30s (Zhao et al., 2003; Chao et al., 2004). For assays in the absence of food or exogenous 5-HT, animals were first transferred to intermediate (non-seeded) plates and left for 1 min, then transferred to assay plates and tested after 10 min. For assays in the presence of food or 5-HT ± TA/OA, animals were transferred to plates and assayed after 20 or 30 min, respectively. Twenty to 25 animals were examined for each strain and condition. Reversal frequency was assayed as described previously (Tsalik and Hobert 2003; Dernovici et al., 2006). All experiments used age-matched, well-fed young adults grown at 20°C on standard nematode growth medium (NGM), seeded with *E. coli* strain OP50. Fourth-stage larvae were picked 24 hrs before testing. NGM plates were prepared the morning of the experiment and were seeded with or without OP50. Well-fed animals were transferred to NGM plates for 30 sec, then transferred to assay plates (± food) for 1 min and assayed. Reversal frequency was scored as the number of times an animal reversed within 3 min (Pierce-Shimomura et al., 1999). Locomotion was assayed as described previously (Sawin et al., 2000). Briefly, synchronized young adult animals were transferred to bacteria-free NGM to prevent bacterial carryover and then to NGM plates with/without OP50 and body bends/20 sec were counted after 1 min.

All assays were performed in triplicate (at least three different lines assayed in three separate experiments) and double-blind to eliminate any potential investigator
subjectivity. Data is presented as a mean ± SE (n = 3) and analyzed by 2-tailed Student’s \( t \) test. P values were indicated as follows: *, P<0.001.

2.4 Molecular biology and transgenesis

cDNA or genomic regions corresponding to the entire coding sequences of \( tyra-3, nlp-1, nlp-14, nlp-18, npr-10, npr-11, \) and \( f41e7.3 \) were amplified by PCR and expressed under cell-selective promoters where indicated. Selective expression in CEPs, RICs, ASIs, AIYs, ASJs and ADLs was achieved using the \( dat-1, tbh-1, gpa-4, ttx-3, sra-9 \) and \( sro-1 \) promoters, respectively. Neuron-selective rescue constructs were created by overlap fusion PCR (Hobert 2002). Transcriptional and translation fusion constructs for \( receptor::gfp \) localization were created by PCR fusion. PCR products were pooled from at least 3 separate PCR reactions and coinjected with a selectable marker (\( myo-3::gfp, unc-122::rfp \) or \( F25B3.3::gfp \)) and carrier DNA into gonads of \( C. elegans \) wild type or mutant animals by standard techniques (Kramer et al, 1990; Mello and Fire 1995).

2.5 Generation of neuron-selective RNAi constructs

Neuron-selective RNAi constructs were created as previously described (Esposito et al., 2007). A neuron-selective promoter was fused to unique exon rich regions of the target gene. Exon rich regions were amplified using a forward and reverse primer to create template A by PCR fusion. Neuron-selective promoters were amplified using a forward primer with reverse (sense) or reverse (antisense) primers to create templates B and C, respectively. Templates A and B were then fused using a forward internal promoter primer and a reverse internal target gene primer to create the sense construct
(Product D). Templates A and C were fused using the forward internal promoter primer and the forward internal target gene primer to create the antisense construct (Product E). At least 3 products were pooled and sense and antisense transgenes were microinjected at 25-100 ng/µL, with 30 ng of myo-3::gfp or F25B3.3::gfp. Multiple transgenic lines were examined for each RNAi.

2.6 cDNA cloning and sequencing

Based on sequence identity to invertebrate TA and OA receptors, tyra-3 (m03f4.3) was identified as a candidate gene encoding a putative TA receptor. Using the C. elegans database, specific primers (TYRA-3F and TYRA-3R) were designed against the tyra-3a open reading frame (TYRA-3F: 5’-ATATAAGCTTATGGCGCGCTTGGCGGC-3’; TYRA-3R: 5’-TCTAGATTAAGATATTGATGCTGATGATTTTCTTG-3’). Restriction sites for additional subcloning are italicized and the start and stop codons are underlined. Using a C. elegans cDNA library prepared from all life stages as a template, a ~1.7 kb band was obtained, isolated, subcloned into the PCR® 2.1 vector (Invitrogen, Carlsbad, CA, USA), and sequenced (Plant-Microbe Genomics Facility, Columbus, OH, USA). The ~1.7 kb cDNA was then subcloned into pFLAG-CMV2 (Sigma, St. Louis, MO, USA).

2.7 Phosphoinositide turnover and cAMP levels

Phosphatidyl inositol (PI) turnover was assayed as described previously (Brandish et al., 2003; Tejada et al., 2006). Briefly, COS-7 cells were seeded into 12-well plates and transiently transfected with pFLAG-tyra-3a (500 ng) using Lipofectamine 2000™
(Invitrogen, Carlsbad, CA, USA) at a DNA:Lipofectamine ratio of 1 ug:3 uL. Twenty-four hr after transfection, cells were washed twice with 0.5 mL phosphate-buffered saline (PBS) and 0.5 mL of inositol-free (IF) DMEM supplemented with 25 mM L-glucose, 4 mM L-glutamine, 10% BCS and [\(^{3}\)H]Ins (10 µCi/mL) was added to each well to a final concentration of 1 µCi/mL. Cells were radiolabeled overnight and then incubated for 1 hr with ligand prepared in HBSS buffer supplemented with 10 mM LiCl and 20 mM HEPES at 37°C. Incubations were stopped by rapid removal of media and the addition of 350 µl of ice-cold 50 mM formic acid followed by incubation at RT for 20 min. YSi-SPA beads (1 mg/80 µl in water) were added to separate wells followed by the addition 20 µl of the formic acid cell extract. The plate was sealed with Topseal A, shaken for 1 hr at 4°C, and after 2 hr at 4°C radioactivity was determined using a TopCount NXT system. Activity is presented as percentage activation above basal.

For cAMP determination, COS-7 cells were transiently transfected with tyra-3a::pFLAG-CMV (200ng) in 12-well plates 48 hours before the assay. Twenty-four hours after transfection, cells were washed once with phosphate-buffered saline (PBS), and Dulbecco’s modified Eagle medium (DMEM) media (minus serum) was added. Twenty-four hours after the media change, reaction mixes containing 300 uM 3-isobutyl-L-methylxanthine (IBMX), 10 uM paraglyline HCl with or without the tested ligands were added to the cells and incubated for 15 min at 37°C. Reactions were terminated by aspiration of media and addition of 300 uL 0.1 M HCl. cAMP levels were quantified using the Assay Designs enzyme-linked immunosorbent assay (ELISA) cAMP kit and protein concentration by the method of Bradford (Biorad, Hercules, CA, USA) using bovine serum albumin as a standard (Bradford 1976).
2.8 Microscopy and image analysis

Transcriptional and translational transgenes for *tyra-3a::gfp*, *tyra-3a::tyra-3a::gfp*, and *npr-10::npr-10::gfp* were generated by PCR fusion (Hobert, 2002). PCR products were pooled from at least 3 separate PCR reactions and co-injected with a selectable marker (*unc-122::rfp* or *rol-6*) by standard techniques. Uptake of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine (DiD) to identify a subset of amphid sensory neurons capable of dye uptake was assayed as described (Herman and Hedgecock, 1990). Briefly, a stock solution (1mM) of DiD (Molecular Probes/Invitrogen Labeling and Detection) was diluted 1:200 in M9 buffer. L4 animals were incubated in 100 µl of diluted DiD for 1 h at RT, transferred to a fresh NGM plate seeded with OP50 and allowed to crawl on the bacterial lawn for 1-2 h to destain and were then placed on agarose pads with 20 mM sodium azide for visualization. At least three transformed lines were analyzed for *gfp* fluorescence and DiD staining using an Olympus IX81 confocal microscope.

Neuropeptide release was assayed by imaging decreases in ASI::NLP-14::GFP fluorescence or coelomocyte uptake as described previously (Sieburth et al., 2007; Speese et al., 2007; Shakiryanova et al., 2011; Hu et al., 2011). Briefly, L4 *nlp-14* or *tyra-3 nlp-14* mutant animals that stably expressed a rescuing *gpa-4::nlp14::gfp* transgene were incubated on freshly prepared NGM plates containing OP50 or 10 mM TA/OA for 1 or 2 h, then placed on agarose pads with 20 mM sodium azide and imaged using an Olympus IX81 confocal microscope. Images of 0.4µm optical thickness of
either ASIL/ASIR or the posterior coelomocyte oriented laterally were captured with Olympus Fluoview FV1000 software and fluorescence intensity was quantified using Volocity software (Improvision). Images were cropped to contain the coelomocyte, ASI soma, axonal process, or whole neuron and fluorescent objects within the images were identified automatically by intensity. A uniform threshold value was used for all experiments and object intensities were quantified and summed for each image with small objects containing less than 3 voxels (non-specific background) eliminated. The arbitrary fluorescent unit of each measurement was standardized to the average fluorescent unit of wild-type for a given experiment and presented as relative fluorescence intensity. Data presented as a mean ± SE and analyzed by two-tailed Student’s t test **P < 0.05, ***P < 0.02 significantly different from wild type animals examined under identical conditions.
Chapter 3

Results

3.1 The TA receptor, TYRA-3, is involved in the TA inhibition of food or 5-HT-stimulated aversive responses mediated by the ASH sensory neurons

Aversive responses mediated by the two ASH sensory neurons are modulated extensively by nutritional state. For example, backward locomotion initiated by the volatile repellent, 1-octanol, is modulated independently by the monoamines, serotonin (5-HT), dopamine (DA), tyramine (TA) and octopamine (OA), with food or 5-HT decreasing the time taken to initiate an aversive response (change from forward to backward locomotion) in the presence of dilute 1-octanol (30%) from about 10 to 5 sec (Chao et al., 2004; Harris et al., 2009). In contrast, TA and OA, which appear to be released after removal from food, abolish this food or 5-HT-dependent “stimulation” of the aversive response (Wragg et al., 2007). Preliminary studies have suggested that the putative TA receptor, TYRA-3, was essential for this TA “inhibition,” so to confirm this observation and characterize the mechanism of TA inhibition further, 1-octanol avoidance was quantified in animals with null alleles for the three TA-dependent G-protein-coupled TA receptors that we identified previously and a unique TA-gated Cl⁻ channel, LGC-55 (Wragg et al., 2007; Figure 2). As noted previously, TA did not inhibit
the 5-HT stimulation of aversive responses in tyra-3 null animals, although these animals
still exhibited wild type responses off food or in the presence of 5-HT (Figure 2; Wragg
et al. 2007). As predicted, the TA inhibition in the tyra-3 null animals could be rescued
by the expression of a full-length tyra-3 genomic transgene that included about 5 kb of
the predicted tyra-3a promoter and the overexpression of this tyra-3 transgene in wild
type animals significantly inhibited the 5-HT stimulation of aversive responses (Figure
3).

TA also inhibited the more rapid aversive responses observed at increased levels
of ASH stimulation (i.e., 100% 1-octanol; about 10 vs 5 sec in wild type animals) and
this TA inhibition required lgc-55, but not tyra-3, suggesting that LGC-55 may be
modulating the ASH-mediated aversive circuit directly and TYRA-3 the circuit involved
in translating nutritional state into the modulation of locomotory behavior (Figure 4).
Figure 3-1. TYRA-3 is involved in aversive responses to dilute 1-octanol. Aversive behavior to 30% 1-octanol was assayed in wild type and mutant animals after incubation in the absence of food or in the presence of exogenous 5-HT (4 mM) with/without TA (4 mM), as described in Methods. Black bars, wild type or null animals. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. *P< 0.001, significantly different from wild type animals under identical test conditions.
Figure 3-2. TYRA-3 is essential for the TA inhibition of 5-HT-stimulated aversive responses to dilute 1-octanol. Aversive behavior to 30% 1-octanol was assayed in wild type, mutant, and transgenic animals after incubation in the presence of exogenous 5-HT (4 mM) with/without TA (4 mM), as described in Methods. Black bars, wild type or null animals; gray bars, animals expressing the indicated transgene. tyra-3+, a full length tyra-3 genomic construct that includes about 5 kb of the promoter upstream of the tyra-3a isoform. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. *P< 0.001, significantly different from wild type animals under identical test conditions.
Figure 3-3. LGC-55 is essential for the TA inhibition of aversive responses to 100\% 1-octanol. Aversive behavior to 100\% 1-octanol was assayed in wild type and mutant animals after incubation in the absence of food or the presence of exogenous TA (4 mM), as described in Methods. Black bars, wild type or null animals. Data are presented as a mean ± SE and analyzed by two-tailed Student’s t test. *P< 0.001, significantly different from wild type animals under identical test conditions.
3.2 TYRA-3 functions in dopaminergic, octopaminergic and peptidergic neurons to inhibit the food or 5-HT stimulation of aversive responses to dilute 1-octanol

Based on data in Wormbase, TYRA-3 appears to be expressed as multiple isoforms from multiple promoters (Figure 5). Previously, Bendesky et al. (2011) examined tyra-3 expression using 4.9 kb upstream of the predicted tyra-3b ATG. In the present study, we constructed tyra-3a transcriptional and rescuing translational gfp transgenes that contained about 5 kb upstream of the predicted tyra-3a ATG that included the all of the sequence used by Bendesky et al. (2011) to functionally localize tyra-3 expression (Figure 5). The expression of a tyra-3a::tyra-3::gfp transgene rescued TA inhibition in tyra-3 null animals and GFP fluorescence was observed in the ASK, ADL, AIM, AVA, BAG, CEP, OLQ and SDQR neurons (Figures 3 and 6). These results are in agreement with those reported previously for the tyra-3b::gfp transcriptional fusion, but include additional neuronal expression in the RIC and ASI neurons, probably due to the presence of additional intronic regions in the transgene (Bendesky et al., 2011). To identify the neurons involved in the TYRA-3-dependent TA inhibition of 5-HT stimulated aversive responses, neuron-selective RNAi was used to knockdown tyra-3 expression. Interestingly, the RNAi knockdown of tyra-3 in either the eight dopaminergic neurons, the two octopaminergic RIC interneurons or the two peptidergic ASI sensory neurons abolished the TA inhibition of 5-HT stimulation (Figure 7). In contrast, RNAi from an array of additional promoters had no effect on TA inhibition, and the RNAi knockdown of ser-5, a gene known to be essential for 5-HT stimulation, from these same promoters had no effect on the 5-HT stimulation of aversive responses (Figure 7; data not shown). Together, these data demonstrate the absence of RNAi
spreading using this neuron-selective knockdown approach and suggest that TYRA-3 functions in multiple neurons to inhibit the 5-HT stimulation of aversive behavior.

To confirm these RNAi results, we attempted to rescue TA “inhibition” in *tyra-3* null animals, by expressing a *tyra-3::gfp* from these same promoters. Surprisingly, *tyra-3* expression in either the dopaminergic, octopaminergic or ASI neurons rescued TA inhibition (Figure. 8). Initially, these results were surprising. How could *tyra-3* RNAi knockdown in one pair of neurons in wild type animals abolish TA inhibition, while expression in a different pair of neurons rescue TA inhibition in *tyra-3* null animals? We have also observed this phenomenon previously for the OA inhibition of aversive responses to 100% 1-octanol and discussed this apparent paradox extensively in a recent commentary (Mills et al., 2012). Monoamines appear to be released both tonically and acutely in response to internal and environmental cues and activate a variety of extrasynaptic receptors on multiple neurons, suggesting that a changing “humoral” soup of monoamines may, at least in part, define “behavioral state.” The composition of this “soup” is dependent on contributions from multiple neurons, suggesting that small increases or decreases in ligand release from any one pair of neurons has the potential to alter signaling. Indeed, the Bargmann lab has recently demonstrated that only 2-fold changes in the expression level of *tyra-3* in the BAG and ASK sensory neurons can have profound effects on an animal’s sensory-mediated decision to leave a food patch (Bendesky et al., 2011). These observations suggest that any overexpression in rescued animals has the potential to compensate for the absence of release from other neurons. This concept may also be potentially extrapolated to neural circuits that are modulated by multiple inputs, i.e., the loss of one modulator can be masked by the overexpression of...
another, i.e., neural circuits are not necessarily analogous to enzymatic pathways with one rate-limiting step when operating at saturation. These data suggest that the activation of TYRA-3 stimulates the release of DA, OA and neuropeptides that together, in total, inhibit the food or 5-HT stimulation of ASH-mediated aversive responses. Indeed, the inhibition of aversive responses by the exogenous addition of either DA or OA has been demonstrated previously and cat-2 or tbh-1 null animals, that lack the rate-limiting enzymes for DA and OA biosynthesis, respectively, respond to dilute 1-octanol more rapidly than wild type animals off food (Wragg et al., 2007).

Based on these observations, we predicted that ablation of either the dopaminergic, octopaminergic or ASI neurons should also yield animals that respond more rapidly to dilute 1-octanol than wild type animals off food. Indeed, the ablation of the CEP/ADEs, RICs or ASIs by the neuron-selective expression of EGL-1 that encodes a programmed cell death activator increased basal aversive responses off food and abolished or significantly reduced TA inhibition, suggesting that signaling from these neurons was indeed inhibiting aversive responses to dilute 1-octanol (Figure 9; Conradt and Horvitz 1998). Interestingly, based on fluorescence from DiD staining, it appeared that although the (ASI) gpa-4::EGL-1 expressing animals still exhibited more rapid basal aversive responses off food, many still contained a single ASI neuron, suggesting that ASI signaling might be dosage-dependent. Together, these data strongly suggest that TA stimulates the release of DA, OA, neuropeptides that together inhibit the ASH-mediated aversive circuit.
Figure 3-4. TYRA-3 encodes for three predicted G-protein-coupled TA receptors.

A portion of the genomic region of Chromosome X indicates the relative lengths for the tyra-3 transcriptional, rescuing translational, and genomic transgenes used. All tyra-3 isoforms are predicted to encode seven transmembrane receptors with different N-termini that result in receptors with varying extracellular N-terminal tails. Only tyra-3a and tyra-b have been confirmed by direct sequencing. (This figure has been modified from Bendesky et al., 2011.)
Figure 3-5. TYRA-3 is expressed in the octopaminergic RIC, dopaminergic CEP, and peptidergic ASI sensory neurons. (A-F) N2 animals expressing a tyra-3a::gfp transgene (A-C) or tyra-3 (ok325) mutant animals expressing a full-length tyra-3a::tyra-3::gfp translational fusion containing 5kb upstream of the predicted tyra-3a ATG and sequence encoding for the full length tyra-3 gene fused to GFP at the C-terminus (D-F) (green) were incubated with DiD (red) to stain amphid and phasmid neurons for identification and colocalization (yellow). A) Anterior portion of a tyra-3a::gfp expressing animal. B-C) Inset from Panel A of DiD stained neurons. D) Anterior portion of a tyra-3a::tyra-3::gfp expressing tyra-3 (ok325) mutant animal. E-F) Inset from Panel D of DiD stained neurons.
Figure 3-6. TYRA-3 functionally localizes to dopaminergic, octopaminergic and peptidergic neurons to mediate TA inhibition of aversive responses to 1-octanol.

Aversive responses to 30% 1-octanol were assayed in wild-type animals expressing neuron-selective tyra-3 RNAi in the presence of exogenous 5-HT (4 mM) and TA (4 mM). Black bars, wild type or null animals; grey bars, RNAi in wild type animals with promoters indicated. Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *P <0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-7. Neuron-selective expression of TYRA-3 in the dopaminergic, octopaminergic and ASI peptidergic neurons restores TA inhibition in *tyra-3* null animals to wild type levels. Aversive responses to 30% 1-octanol were assayed in *tyra-3* null animals expressing neuron-selective::tyra-3::gfp transgenes in the presence of exogenous 5-HT (4 mM) and TA (4 mM). Black bars, wild type or null animals; grey bars, animals expressing a transgene in *tyra-3* null animals. Data presented as mean ± SE and analyzed by two-tailed Student’s *t* test. *P* <0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-8. Signaling from the dopaminergic CEP, octopaminergic RIC and peptidergic ASI neurons inhibits aversive responses to dilute 1-octanol. Wild type animals expressing neuron-selective::egl-1::gfp transgenes were assayed for aversive responses to 30% 1-octanol in the absence of food and in presence of exogenous 5-HT (4 mM) and TA (4 mM). Black bars, wild type or null animals; grey bars, wild type animals expressing a transgene. Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *$P<0.001$, significantly different from wild type animals examined under identical conditions.
3.3 TYRA-3 stimulates the release of multiple inhibitory peptides from the ASIs.

As demonstrated above, TYRA-3 expression in the ASIs is essential for TA inhibition. To identify the role of TYRA-3 in modulating ASI function, we first used ASI RNAi to knockdown the expression of *eat-4*, a vesicular glutamate transporter required for glutamatergic signaling in many sensory neurons, and *egl-3*, a proprotein convertase required for the processing of most neuropeptides (Lee et al., 1999; Husson et al., 2006). Although this eat-4 RNAi has previously been used to successfully knockdown expression and alter behavior in the ASH, ADL and AWC sensory neurons (Mills et al., 2011), its expression in the ASIs had no effect on aversive responses, suggesting that ASI glutamatergic signaling was not involved in TA inhibition (Figure 10). In contrast, animals expressing ASI::egl-3 RNAi exhibited more rapid aversive responses off food and were not inhibited by TA, supporting our hypothesis that TYRA-3 activated ASI peptidergic signaling (Figure 10). However, since EGL-3 may also be involved in the post-Golgi transport of vesicles that contain not only peptides, but also GPCRs and ion channels, we examined the role of individual ASI peptide-encoding genes (Cool et al., 1997; Lou et al., 2005). The ASIs express a number of peptide-encoding genes, some of which are involved in the OA inhibition of aversive responses (Figure 11; Mills et al., 2011). To identify the ASI peptide-encoding genes involved in TA inhibition, we assayed animals with putative null alleles for each gene. *nlp-1, nlp-14* or *nlp-18* null animals exhibited more rapid aversive responses off food than wild type animals and were not inhibited by TA, suggesting that neuropeptides from all three genes were essential for TA inhibition (Figure 11). Interestingly, the ASI peptide-encoding
genes involved in TA inhibition were distinct from those involved in the OA inhibition of aversive responses \((nlp-6, nlp-7, nlp-9;\) Mills et al., 2011). As predicted, the TA inhibition of aversive responses could be rescued in each of these peptide null animals by the expression of the appropriate genomic or ASI-specific transgene (Figure 12). In addition, the ASI::RNAi knockdown of \(nlp-1, nlp-14\) or \(nlp-18\) also abolished the TA inhibition of 5-HT-stimulated aversive responses, but had no effect on the OA inhibition of aversive responses to 100% 1-octanol (Figure 13). Interestingly, animals overexpressing either \(nlp-1, nlp-14\) or \(nlp-18\) moved more slowly on or off food, based on body bends/min, and responded more slowly to dilute 1-octanol in the presence of 5-HT than wild type animals (Figures 12 and 14), suggesting that since these neuropeptides are released from multiple neurons they may also play a broader role in locomotory decision-making. Indeed, the effects of \(nlp-14\) overexpression may be especially robust as \(nlp-14\) encodes multiple similar or identical copies of the same neuropeptide (Figure 15). Together, this data suggests that TYRA-3 stimulates the release of an array of “inhibitory” neuropeptides from the ASIs that modulate not only 1-octanol avoidance, but also locomotory behavior in general. Interestingly, the \(nlp-1, nlp-14\) and \(nlp-18\) null animals exhibited more rapid aversive responses off food, while the \(tyra-3\) null animals did not, suggesting that multiple inputs may be regulating neuropeptide release from the ASIs or indeed, from other neurons expressing these same neuropeptides.
Figure 3-9. ASI peptidergic signaling is essential for the inhibition of aversive responses to dilute 1-octanol. Wild type animals expressing ASI::egl-3 or ASI::eat-4 RNAis were assayed for aversive responses to 30% 1-octanol in the absence of food and in presence of exogenous 5-HT (4 mM) with/without TA (4 mM). Black bars, wild type or null animals; hatched bars, RNAi in wild type animals. Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *P <0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-10. The TA inhibition of 5-HT-stimulated aversive responses to dilute 1-octanol requires neuropeptides encoded by *nlp-1*, *nlp-14* and *nlp-18*. Wild type, mutant and transgenic animals expressing cell-specific RNAi were assayed for aversive responses to 30 and 100% 1-octanol in the absence of food and in the presence of exogenous OA (4 mM) or 5-HT (4 mM) with/without TA (4 mM). Black bars, wild type or null animals; hatched bars, RNAi in wild type animals. Data presented as mean ± SE and analyzed by two-tailed Student’s *t* test. *P <0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-11. The overexpression of neuropeptides encoded by nlp-1, nlp-14 and nlp-18 dramatically inhibits aversive responses to dilute 1-octanol. Wild type, mutant and transgenic animals expressing genomic or ASI-specific transgenes were assayed for aversive responses to 30% 1-octanol in presence of exogenous 5-HT (4 mM) with/without TA (4 mM). Black bars, wild type or null animals; grey bars, wild type or null animals expressing a transgene. Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *P <0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-12. Neuropeptides encoded by *nlp-1*, *nlp-14* and *nlp-18* from the ASI sensory neurons are essential for the TA inhibition of 5-HT-stimulated aversive responses to dilute 1-octanol. Wild type, mutant and animals expressing cell-specific RNAi were assayed for aversive responses to 30% and 100% 1-octanol in the presence of exogenous 5-HT (4 mM) and TA (4 mM) or OA (4 mM), respectively. Black bars, wild type or null animals; hatched bars, RNAi in wild type animals. Data presented as mean ± SE and analyzed by two-tailed Student’s *t* test. *P* < 0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-13. Animals overexpressing nlp-1, nlp-14 or nlp-18 have reduced locomotory rates compared to wild type animals. Wild type, mutant and animals expressing genomic transgenes were assayed for body bends in the presence and absence of OP50. Black bars, wild type or null animals; grey bars, null animals expressing a transgene. Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *P <0.001, significantly different from wild type animals examined under identical conditions.
**NLP-1:** 20.1kDa; isoelectric point: 10.19
MKATFVACLCLVIASAHDLLKPR \textit{MDANAFRMSFGKR} SVSNPAEAKR \textit{MDPNAFRMSFGKR} SAEQNEQANK EDKATSQLDYDDTKEEMKR \textit{MDANAFRMSFGKR} SDAHQADDDQVEYVNDPSLPECKR \textit{MDANAFRMSFGKR} VNLDPNSFRMSFGKR STVGYNLARYEYFVGLGRR

**NLP-6:** 9.6kDa; isoelectric point: 9.6
MLSFSRLAFVLLVSACVMDAPKQMVFGFKR SMADISDMPEDVPMKR YKPRSFAMGFGKR AAMRSFN MGFGKR SAEHQIDYEDMSADLEEIPFIQKRRLIMGLGRR

**NLP-7:** 14.1kDa; isoelectric point: 9.64
MYKAALLVVLFGVASQITSA \textit{LYLKQA} DDDPRMFTSSFGKR SAISEPQAYPKSYRAIRIQRSMDLDPPRL \textit{MTMSFGKR} MILPDLHRTYMDKRGSDI DDPYFLFSNRLT CRC

**NLP-9:** 14.8kDa; isoelectric point: 9.74
MDRFATRFIALVLLQLQGSI \textit{FAPIAE} GGAPEDVDDRRELEKR GGRARFYGFYNAGSKR DAOAALPYYLYEK RGGGRAFHNANLRF DKR GGGRAFAGSWSPYLERFYDKRSYVFSNDYY

**NLP-14:** 23.7kDa; isoelectric point: 10.15
MLHLVLLVALSSAVTAPRRA \textit{ALDL} GSGGFGFKR ALNSLDGAGFGFKR ALNSLDGQGFGR FEKR ALDGLD GAGFGFKR ALNSLDGAGFGFKR ALDGLDGSFGFGR FEKR ALNSLDGAGFGFKR ALNSLDGQGFGR FEKR ALDGLDGSFGFGR FEKR ALNSLDGAGFGFKR ALDGLDGAGFGFKR ALNSLDGQGFGR FEKR ALDGLDGAGFGFKR ALNSLDGAGFGFKR ALNSLDGAGFGFKR ALNSLDGQGFGR FEKR ALNSLDGAGFGFKR ALNSLDGAGFGFKR ALNSLDGQGFGR FEKR ALDGLDGAGFGFKR ALNSLDGAGFGFKR ALNSLDGQGFGR FEKR ALD

**NLP-18:** 11.2kDa; isoelectric point: 10.1
MNANVSYIVYFLSLVCISAAQLHADSGATEV DGIV DKR \textit{SPYRAFAFA} KR SDEENLDFLEKR AYGFACKR \textit{SPYRTF} \textit{AFAK} \textit{RASPYGFAFA} KR GQFSSFA

**Figure 3-14.** The ASI-expressed genes involved in TA and OA inhibition of aversive responses encode multiple putative neuropeptides. Predicted protein sequences of the TA and OA-related neuropeptide-encoding genes are listed with the predicted mature neuropeptides indicated in (red). Note that some genes encode for multiples copies of the same or similar neuropeptides. Neuropeptides that have been biochemically isolated are noted in italics. Additional peptides (highlighted in grey) were not identified previously, but may also be released as mature neuropeptides, as dibasic cleavage motifs (KR) flank their N- and C-termini.
3.4 Both $G_{\alpha q}$ and $G_{\alpha s}$ signaling in the ASIs are required for TA inhibition.

To better understand the role of TYRA-3 signaling in modulating neuropeptide release from the ASIs, we examined 1) G-protein signaling in cells heterologously expressing TYRA-3, 2) the role of ASI G-protein signaling in modulating TA inhibition and 3) the role of TA on the release of peptides encoded by nlp-14 from the ASIs.

COS-7 cells transiently expressing TYRA-3 exhibited a marked TA-dependent increase in phosphoinositide turnover, with TA increasing IP$_3$ turnover from 2.5-7-fold with an EC$_{50}$ of $70.8 \pm 7.4$ nM (Figure 16). In contrast, OA and DA had minimal and no effect on IP$_3$ turnover (Figure 16A). Basal IP$_3$ responses were unchanged by transient expression of TYRA-3 and TA had no effect on IP$_3$ turnover in untransfected cells (data not shown). In contrast, cAMP levels did not change in cells incubated under similar conditions (data not shown). Together these data suggest that the TYRA-3 coupled to $G_{\alpha q}$, in agreement with similar reports for a closely-related Drosophila TA receptor, DmTyrR2, that TA-specifically activates Ca$^{++}$-dependent Cl$^-$ channels when heterologously expressed in Xenopus oocytes (Cazzamali et al., 2005).

To examine ASI G-protein signaling directly, we used a neuron-specific RNAi approach to either selectively increase or decrease ASI $G_{\alpha q}$ (EGL-30) and $G_{\alpha s}$ (GSA-1) signaling, as we have described previously for the ASHs (Harris et al., 2010). The ASI RNAi knockdown of gsa-1 ($G_{\alpha s}$) or egl-30 ($G_{\alpha q}$) dramatically reduced TA inhibition (Figure 17). Conversely, increasing either ASI $G_{\alpha s}$ or $G_{\alpha q}$ signaling by the ASI RNAi knockdown of either pde-4 that encodes a phosphodiesterase responsible for cAMP degradation or dgk-1 that encodes a diacylglycerol kinase responsible for the catabolism of diacylglycerol, a key intermediate in $G_{\alpha q}$ signaling, significantly reduced the 5-HT
stimulation of aversive responses (Figure 17). Interestingly, the ASIs are the only neurons that secrete DAF-7, a TGFβ orthologue that plays a key role in the formation of resistant dauer larvae during starvation, suggesting that stimulating ASI LDCV release might lead to constitutive dauer formation (Ren et al., 1996; Inoue and Thomas 2000). However, no dauer formation was observed in these animals, in agreement with recent reports indicating that although egl-30 mutations affect daf-7 expression, they have no apparent effect on dauer formation (Myers 2012). Together, these results suggest that both Gα₉ and Gαₛ signaling in the ASIs are required for TA inhibition and agree with recent reports from Drosophila motorneurons where the OA-evoked neuropeptide release required the synergistic interaction of PKA and ER calcium signaling (Shakiryanova et al., 2011).

To examine ASI peptide release directly, we expressed an ASI::nlp-14::gfp transgene in nlp-14 null animals that functionally rescued TA inhibition (Figures 12 and 18A). Robust GFP fluorescence was found in the ASI cell body, as well as about 15 reproducible puncta in the ASI axonal processes (Figure 18A). Since the ASIs only contain about 7-9 synapses, at least some of these puncta were extrasynaptic or alternatively, since many appeared to be paired could possibly have been perisynaptic (White et al., 1986; Crump et al., 2001). As noted below, the receptors for many of the ASI-encoded peptides have been functionally localized to neurons that are postsynaptic to the ASIs, suggesting the most ASI peptidergic signaling would be synaptic. To examine the effects of TA directly on peptide release, we first attempted to exploit a coelomocyte uptake assay previously described to indirectly measure peptide release, by monitoring the long-term accumulation of GFP in coelomocytes, large scavenger cells
found in the pseudocoelomic fluid (Sieburth et al., 2007; Speese et al., 2007; Hu et al., 2011). Unfortunately, coelomocyte fluorescence in these ASI::nlp-14::gfp expressing animals was low and inconsistent, as has recently been described for other C. elegans neuronally-expressed peptides (Hu et al., 2011). For example, although NLP-21::YFP or DAF-28::mCherry (that encodes an insulin-like molecule) released from the DA motor neurons, are rapidly accumulated by the coelomocytes and have been correlated with neuropeptide release (Sieburth et al., 2007; Lee and Ashrai 2008), no coelomocyte fluorescence could be detected from the release of NLP-12::YFP from the DVA neuron (Hu et al., 2011). The authors have proposed that the coelomocytes do not have access to the NLP-12::YFP, either because NLP-12::YFP cannot leave the DVA neuron or perhaps because it is rapidly accumulated or degraded by another cell type. A similar situation may be operating in the ASIs with NLP-14::GFP, since we predict that most of the ASI peptide release is synaptic and not necessarily released into the pseudocoelomic fluid where the coelomocytes would have access to the peptides.

In contrast, TA acutely decreased NLP-14::GFP fluorescence in the ASIs, suggesting that TA either reduced the synthesis or increased the degradation of nlp-14 encoded peptides, or stimulated their release (Figure 18B). For example, TA incubation for 1 and 2 hrs reduced ASI::NLP-14::GFP fluorescence by 24% and 51%, respectively (Figure 18B). No differences in GFP fluorescence were observed between right and left ASIs before or after TA exposure (Figure 18B). As predicted, OA had no effect on NLP-14::GFP fluorescence, even though OA appears to stimulate the release of a different subset of ASI peptides and TA had no effect on ASI::NLP-14::GFP fluorescence in tyrA-3 null animals (Figure 18B). Most importantly, the stimulatory effects of TA appeared to
Figure 3-15. Tyramine stimulates phosphoinositide metabolism in COS-7 cells expressing TYRA-3a. (A) COS-7 cells transiently transfected with TYRA-3a-pFLAG were labeled with $[^3\text{H}]$-inositol and incubated with various monoamines at [10 µM]. Phosphatidylinositol turnover was assayed from formic acid lysates and $[^3\text{H}]$-inositol phosphates isolated YSi SPA beads. Values are expressed as percentage stimulation above basal levels. Data are representative of at least three independent experiments performed in triplicate. (B) COS-7 cells transiently transfected with TYRA-3a-pFLAG were labeled with $[^3\text{H}]$-inositol and incubated with TA at a range of concentrations. Phosphosinositide turnover was assessed as described in (a). Values are expressed as percentage stimulation above basal levels. Data are representative of at least three independent experiments performed in triplicate.
Figure 3-16. Both G\(\alpha_q\) and G\(\alpha_s\), signaling are required for the TA inhibition of aversive responses to dilute 1-octanol. Wild type, mutant and transgenic animals expressing cell-specific RNAi were assayed for aversive responses to 30% 1-octanol in the presence of exogenous 5-HT (4 mM) with/without TA (4 mM). Black bars, wild type or null animals; hatched bars, RNAi in wild type animals. Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *\(P < 0.001\), significantly different from wild type animals examined under identical conditions.
Figure 3-17. TA, but not OA, stimulates a TYRA-3-dependent decrease in NLP-14::GFP fluorescence from the ASI sensory neurons. L4 nlp-14 or tyra-3 nlp-14 mutant animals, expressing a rescuing ASI::nlp-14::gfp transgene, were incubated on freshly prepared NGM containing OP50 or 10 mM TA or OA (1 or 2 hr) and imaged immediately for ASI::NLP-14::GFP fluorescence. (A) Confocal Z-stack of the anterior portion of a nlp-14 mutant expressing ASI (gpa-4)::nlp-14::gfp with individual puncta indicated (red). (B) Animals expressing ASI::nlp-14::gfp were imaged and NLP-14::GFP fluorescence from whole neurons, soma or axonal processes was quantified using Volocity software and normalized and presented as relative ASI::NLP-14::GFP fluorescence. Data was normalized to total ASI::NLP-14::GFP fluorescence in the absence of ligand, presented as a mean ± SE and analyzed by two-tailed Student’s t test **P <0.05, ***P<0.02 significantly different from wild type animals examined under identical conditions.
be confined to the axonal processes and not the soma, supporting a direct role of TYRA-3 in peptide release.

Together these data suggest the TYRA-3 activates ASI Gαq signaling to stimulate the synaptic release of nlp-14 encoded neuropeptides.

3.5 Peptides encoded by nlp-1 and nlp-14 activate NPR-11 and NPR-10, respectively, to mediate TA/TYRA-dependent inhibition

To tentatively identify the GPCRs activated by peptides encoded by nlp-1, nlp-14 and nlp-18, we took an epigenetics approach and examined TA inhibition in predicted neuropeptide receptor null animals. Putative null alleles are available for over 50 predicted peptide receptors. We have examined each of these receptor null animals in a variety of behavioral assays to develop a “behavioral fingerprint” that can be associated with animals containing null alleles of each of the peptide-encoding genes, on the assumption that peptide and cognate receptor null animals would exhibit similar phenotypes, as we have described previously for peptides encoded by nlp-3, nlp-8 and nlp-9 (Harris et al., 2010; Mills et al., 2011). Initially we examined predicted peptide null animals that exhibited wild type responses to food and 5-HT that were also inhibited by OA (Figure 19). Animals carrying putative null alleles for npr-11, f41e7.3 and c53c7.1 (npr-10) were not inhibited by TA (Figure 19). NPR-11 was previously identified as a receptor for peptides encoded by nlp-1 further supporting the validity of our approach (Chalasani et al., 2010). To identify the individual receptors for these peptide encoding genes, nlp-14 or nlp-18 were overexpressed in each of the receptor null backgrounds, on the assumption that the dramatic inhibition of 5-HT-stimulated aversive responses
observed by *nlp-14* or *nlp-18* overexpression in wild type animals would be absent or significantly reduced in animals lacking the cognate receptors, as we have described previously for the identification of peptides activating NPR-15, NPR-17 and NPR-18 (Harris et al., 2010; Mills et al., 2011). The overexpression of a *nlp-14* genomic transgene in NPR-10 animals failed to inhibit 5-HT stimulation, suggesting that NPR-10 encoded a receptor for peptides encoded by *nlp-14* (Figure 20). Together, this data suggests that peptides encoded by *nlp-1* activate NPR-11 and *nlp-14* activates NPR-10 during the TA inhibition of 5-HT-stimulated ASH-mediated aversive responses.

To functionally localize NPR-10 and NPR-11, we undertook the same promoter::GFP, neuron-selective RNAi and rescue approach described above for TYRA-3. *npr-11::gfp* expression in the AIA and AIY interneurons has been reported previously (Chalasani et al., 2010). Indeed, *npr-11* RNAi knockdown in the AIA or AIY interneurons of wild type animals abolished TA inhibition, while the expression of NPR-11 in the AIAs or AIYs rescued TA inhibition in *npr-11* null animals (Figure 21). Expression patterns for *npr-10* have not been reported previously, so we constructed a *npr-10::gfp* transgene that was expressed robustly in body wall muscle and to a lesser extent in the ASI, ADL, and ASK sensory neurons, the RIA interneurons and a number of additional neurons (Figure 22). The neuron-selective RNAi knockdown of *npr-10* in the ADLs and ASKs, but not the ASIs, RIAs or body wall muscle of wild type animals significantly reduced TA inhibition of 5-HT-stimulated aversive responses (Figure 22).

Together, these data suggest that neuropeptides encoded by *nlp-1* activate NPR-11 in the AIA and AIY interneurons and those encoded by *nlp-14* activate NPR-10 in the ADL and ASK sensory neurons.
Figure 3-18. Three predicted neuropeptide receptors, npr-11, npr-10 and f41e7.3, are not inhibited by TA for 5-HT-stimulated aversive responses to dilute 1-octanol. Putative neuropeptide receptor null mutants were assayed for aversive responses to 30% 1-octanol in the presence of exogenous 5-HT (4 mM) and TA (4 mM). Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *P < 0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-19. *nlp-14* overexpression phenotypes are absent in *npr-10* null animals, suggesting that NPR-10 may be a receptor for *nlp-14*-encoded neuropeptides. Wild type and neuropeptide receptor null mutants expressing genomic transgenes were assayed for aversive responses to 30% 1-octanol in the presence of exogenous 5-HT (4 mM). White bar, wild type animals; grey bars, wild type or null animals expressing a *nlp-18* genomic transgene; black bars, wild type or null animals expressing a *nlp-14* genomic transgene. Data presented as mean ± SE and analyzed by two-tailed Student’s *t* test. *P* <0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-20. NPR-11 functions in the AIA and AIY interneurons to modulate the TA inhibition of 5-HT-stimulated aversive responses to dilute 1-octanol. Wild type, mutant and animals expressing either cell-specific npr-11 RNAi or npr-11::gfp transgenes were examined for aversive responses to 30% 1-octanol in the presence of exogenous 5-HT (4 mM) and TA (4 mM). Black bars, wild type or null animals; hatched bars, RNAi in wild type animals; grey bars, null animals expressing a transgene. Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *P <0.001, significantly different from wild type animals examined under identical conditions.
Figure 3-21. NPR-10 functions in the ADL and ASK sensory neurons to modulate the TA inhibition of 5-HT-stimulated aversive responses. (A-D) N2 animals expressing a npr-10::gfp transgene (green) incubated with A-C) DID (red) or D) co-expressing ttx-3::, npr-9:: or glr-3::rfp for identification of A1Y, A1B and RIA neurons, respectively. E) Aversive responses to 30% 1-octanol were assayed in wild type animals expressing neuron-selective RNAi in the presence of exogenous 5-HT (4 mM) and TA (4 mM). Black bars, wild type or null animals; hatched bars, RNAi in wild type animals with promoters for ASK, ADL, ASI, RIA and body wall muscle indicated. Data presented as mean ± SE and analyzed by two-tailed Student’s t test. *P < 0.001, significantly different from wild type animals examined under identical conditions.
Chapter 4

Discussion

The present study has demonstrated that the TA inhibition of the food or 5-HT stimulation of aversive responses mediated by the two ASH sensory neurons is complex and involves two distinct TA receptors, the TA-gated Cl\(^-\) channel, LGC-55, and the Go\(_q\)-coupled TA receptor, TYRA-3, that activates much more global monoaminergic and peptidergic signaling cascades. TDC-1, the tyrosine decarboxylase required for TA biosynthesis, is expressed neuronally in the two RIM motorneurons and two RIC interneurons (Alkema et al., 2005). The RICs also express tyramine \(\beta\)-hydroxylase, TBH-1, and synthesize OA from TA, suggesting that the two RIMs are probably the major neuronal source of TA, although the UV1 uterine cells also express tdc-1 and could be an additional source of humoral TA (Alkema et al., 2005). In general, TA antagonizes the effects of food or 5-HT on most key behaviors, including, feeding, pharyngeal pumping, locomotion, egg-laying and a host of additional sensory-mediated processes that respond to nutritional modulation (Horvitz et al., 1982; Nicaris and Avery, 2003; Rex et al., 2004; Alkema et al., 2005; Wragg et al., 2007). As noted below, tyraminergic signaling has the potential to modulate each of these behaviors independently, depending on the expression of individual excitatory or inhibitory TA receptors (Rex et al., 2002, 2004, 2005; Pirri et al., 2009; Hapiak and Komuniecki, unpublished).
The present study has demonstrated that distinct TA receptors are involved in the TA inhibition of ASH-mediated aversive responses, depending on the degree of ASH stimulation. For example, the Gαq-coupled TA receptor, TYRA-3, is required for the TA inhibition of food or 5-HT-stimulated aversive responses to dilute 1-octanol and the TA-gated Cl⁻ channel, LGC-55, is required for the TA inhibition of aversive responses to 100% 1-octanol. Together, these results suggest that TYRA-3 signaling may be specifically inhibiting the nutritionally-activated circuits stimulating ASH-mediated aversive responses, as discussed more fully below. Interestingly, the OA inhibition of aversive responses exhibits similar complexity, with different OA receptors involved in responses to different levels of ASH stimulation, OA levels and/or food availability (Mills et al., 2011). For example, the OA inhibition of the food or 5-HT stimulation of aversive responses to 30% 1-octanol requires the ASH-expressed OCTR-1, while the OA inhibition of aversive responses to 100% 1-octanol requires a different OA receptor, SER-6, that stimulates the release of inhibitory peptides from the ADL, AWB and ASI sensory neurons (Mills et al., 2011).

Most TA and OA receptors are expressed on neurons not directly innervated by RIMs or RICs, suggesting that the majority of tyraminergic and octopaminergic signaling is humoral (Figure 23). In contrast, monoamine-dependent peptidergic signaling from the ASIs appears be synaptic (or perisynaptic), based on the localization of the key neuropeptide receptors to downstream synaptic partners of the ASIs, suggesting that a broad, humorally-translated monoaminergic signal is both amplified and focused by the synaptic (or perisynaptic) release of distinct neuropeptides (Figure 23). For example, the ASIs appear to be presynaptic to only 9 pairs of neurons, the ASKs (2 synapses), ASHs
(1), ASEs (3), RIB (1), AIZs (1), AIBs (2), AIYs (2), AIAs (3) and AWCs (4), but all of the neuropeptide receptors activated by ASI neuropeptides released in response to TA or OA have been functionally localized to at least one of these downstream neurons, i.e., NPR-11 (AIAs/AIYs), NPR-10 (ASKs/ADLs), NPR-18 (ASER) (Hapiak and Komuniecki, unpublished; Chalasani et al., 2010; Mills et al., 2011). Similarly, the OA/SER-6-dependent release of neuropeptides encoded by nlp-8 from the ADL sensory neurons that are involved in OA inhibition activates NPR-15 on the AWCs, the downstream synaptic partners of the ADLs (Figure 23; Mills et al., 2011).
Figure 4-1. TA and OA both activate more global signaling cascades to inhibit aversive responses mediated by the ASH sensory neurons. Monoaminergic activation appears to be humoral, while peptidergic activation appears to be largely synaptic (or perisynaptic). Top: neurons modulated by the humoral release of TA and OA that contribute to the monoaminergic inhibition of aversive responses. Bottom: neurons modulated by neuropeptides released from the ASIs and their major connectivities to the locomotory circuit.
4.1 Small changes in TYRA-3 signaling may have dramatic effects on locomotory behavior

Small changes in *tyra-3* expression can initiate dramatic alterations in behavior. For example, only two-fold changes in *tyra-3* expression in the ASK sensory neurons was sufficient to significantly increase an animal’s decision to remain on a food patch, based on a quantitative genetic analyses of *C. elegans* strains exhibiting different foraging behaviors (Bendesky et al., 2011). In addition, *tyra-3* expression in the BAG sensory neurons also modulated foraging behavior. Based on the apparent roles of the ASKs and BAGs in modulating leaving behavior, *tyra-3* signaling appeared to decrease ASK activity and increase the BAG activity, and this antagonistic tyraminergic modulation appeared to act synergistically to prevent leaving. This study highlights the complexity of tyraminergic modulation, i.e., small changes in TA release from the two RIMs has the potential to dramatically modulate individual locomotory behaviors. Interestingly, *tyra-3* expression in the ASKs or BAGs was not required for the TA inhibition of 5-HT-stimulated aversive responses observed in the present study, demonstrating that TA release from the RIMs can differentially modulate individual behaviors, depending on the expression and localization of individual TA receptors. TA modulation is probably even more complex, especially if antagonistic pairs of TA receptors operate in the same neurons or circuits, as described below for 5-HT, DA and OA signaling. In addition, all four TA receptors appear to be expressed as multiple isoforms, although their tissue-specificity and functional differences have yet to be characterized (Rex et al., 2002, 2004, 2005; Pirri et al., 2009; Bendesky et al., 2011; Wormbase). For example, TYRA-3
appears to be expressed from three distinct promoters as three different TYRA-3 isoforms with different N-termini in *C. elegans* (Figure 5; Wormbase). Although the potentially differential role of these isoforms in modulating *C. elegans* behavior has not been examined, similar isoforms expressed in the parasitic nematode, *Ascaris suum*, exhibit dramatic differences in stage-specific expression during larval hepato-pulmonary migration; for example, one isoform is much more abundant (> 100-fold) in fourth-stage than third-stage larvae (Komuniecki et al., 2012). Overall, these studies highlight the flexibility of individual behaviors and their rapid, monoamine-mediated modulation by transient changes in the environment.

4.2 TYRA-3 signaling may stimulate the release of both OA and DA to inhibit ASH-mediated aversive behavior.

TYRA-3 appears to stimulate both octopaminergic and dopaminergic neurons, suggesting that TYRA-3 may be stimulating the release of both DA and OA (Figure 23). Indeed, exogenous DA and OA both independently inhibit the food or 5-HT stimulation of aversive responses to dilute 1-octanol and *tdc-1, cat-2* or *tbh-1* null animals, deficient in the synthesis of TA/OA, DA or OA, respectively, all respond more rapidly to dilute 1-octanol than wild type animals, suggesting that basal TA, DA and OA signaling tonically inhibits ASH-mediated aversive responses when animals are off food (Sulston et al., 1975; Lints and Emmons 1999; Alkema et al., 2005; Wragg et al., 2007). However, it is worth noting that the octopaminergic RICs also form extensive gap junctions with the ASHs, possibly to prevent ASH overstimulation in response to elevated/chronic stimulation (i.e., 100% 1-octanol) (White et al., 1986; Mills et al., 2011). Interestingly, in
contrast to TA, both DA and OA modulate ASH signaling directly though antagonist pairs of DA or OA receptors on the ASHs. For example, exogenous OA at 4 mM abolishes the food or 5-HT stimulation of aversive responses to dilute 1-octanol through an ASH-expressed, Gαo-coupled OA receptor, OCTR-1, that appears to inhibit the release of both glutamate and stimulatory neuropeptides encoded by nlp-3 from the ASHs (Wragg et al, 2007; Mills et al., 2011). Surprisingly, exogenous OA at 10 mM does NOT inhibit food or 5-HT stimulation, as under these elevated OA levels, a second ASH-expressed OA receptor, SER-3, antagonizes OCTR-1 signaling (Mills et al., 2011). A similar antagonism appears to operate between DOP-3 and DOP-4 in the ASHs in the DA inhibition of food or 5-HT stimulation (Ezak and Ferkey 2010; Ezcurra et al., 2011). In addition to neuron-specific monoaminergic antagonism, antagonistic monoaminergic inputs also appear to modulate individual behavioral circuits. For example, 5-HT provides both excitatory and inhibitory inputs into the egg-laying circuit, with SER-1 and SER-7 stimulating egg-laying and SER-4 and MOD-1 inhibiting the process (Hapiak et al., 2009). It will be important to determine if an antagonistic TA receptor also functions on the ASIs and whether this type of antagonistic neuron-specific signaling is a generalized component of monoaminergic modulation. Indeed, both TYRA-3 and the Gαo-coupled TA receptor, SER-2, appear to be expressed in the RICs (Tsalik and Hobert 2003; Rex et al., 2004). Importantly, although expression patterns for most of the monoamine receptors have been reported using full length rescuing promoter::gfp transgenes, it appears that GFP fluorescence from these transgenes only provides minimal expression patterns for these potentially low abundance receptors. For example, both DOP-3 and DOP-4 function in the ASHs, based on the neuron-specific rescue of null
animals and RNAi knockdown in wild type animals, but no ASH expression was observed in rescuing dop-3 or dop-4 transgenes, suggesting that monoamine receptors may be much more broadly expressed that previously appreciated (Ezak and Ferkey 2010; Ezcurra et al., 2011).

4.3 TA and OA stimulate the release of distinct and non-overlapping pools of neuropeptides from the ASIs to inhibit ASH-mediated aversive behaviors.

As noted above, TA stimulates the release of ASI peptides encoded by nlp-1, nlp-14 and nlp-18 through the Goq-coupled TYRA-3 that are essential for the TA inhibition of the food or 5-HT stimulation of aversive responses to 30% 1-octanol. In contrast, OA stimulates the release of ASI peptides encoded by nlp-6, nlp-7 and nlp-9 through the Goq-coupled SER-6 that are essential for the OA inhibition of basal aversive responses to 100% 1-octanol (Mills et al., 2011). The ASI knockdown of nlp-1, nlp-14 and nlp-18 has no effect on OA inhibition and, conversely, the ASI knockdown of nlp-6, nlp-7, or nlp-9 has no effect on TA inhibition (Mills et al., 2011; present study). Superficially, these results would suggest that these two distinct sets of ASI neuropeptides function through different non-overlapping pathways. However, this view is probably naïve, with both sets of neuropeptides probably providing inputs into the overall circuit modulating locomotory decision-making and indeed, preliminary studies suggest that the over-expression of at least some of these ASI neuropeptide-encoding genes can inhibit aversive responses to both 30 and 100% 1-octanol (Hapiak and Komuniecki, unpublished). Indeed, neuropeptides encoded by nlp-9 act on receptors functionally localized to the ASER sensory neuron and recent unpublished work from our lab suggests
that glutamatergic signaling from ASER decreases basal aversive responses to 30% 1-octanol from 10 to 5 sec, based on the overexpression of eat-4 in ASER, predicted to increase glutamatergic signaling from the ASER (Mills et al., 2011; Summers and Komuniecki, unpublished). Similarly, peptides encoded by nlp-8 that are released from the ADL sensory neurons during the OA inhibition of aversive responses to 100% 1-octanol activate receptors on the AWC sensory neurons and glutamatergic signaling from the AWCs inhibits the 5-HT stimulation of aversive responses to 30% 1-octanol from 5 to 10 sec, based on eat-4 overexpression in the AWCs (Mills et al., 2011; Summers and Komuniecki, unpublished). Conversely, AWC eat-4 RNAi knockdown increases aversive responses to 30% 1-octanol from 10 to 5 sec (Mills et al., 2011). Signaling from most sensory neurons is integrated by a small group of interneurons (two AIAs, AIBs, AIYs, and AIZs) to ultimately dictate the bimodal decision to move forward or backward. Theoretically, peptidergic input has the capacity to modify the decision to move forward, backward or initiate a omega turn by altering the output from any sensory neuron that innervates the locomotory circuit (as most do), since these decisions are ultimately driven by the sum of the integrated sensory inputs and it appears that monoamine-dependent ASI peptidergic signaling observed for TA and OA “inhibition” has the potential to significantly modulate the integration of this sensory input into locomotory decision-making.

4.4 The role of TA in locomotory-decision making: understanding the circuits modulating spontaneous and sensory-evoked reversal.
Backward locomotion and reversal appear to be controlled by two distinct pathways, one involving the command interneurons that interact to dictate forward or backward locomotion directly and a second involving the AIB interneurons and RIM motorneurons that tonically inhibits spontaneous reversal, by modulating sensory inputs into the command interneurons and/or an array of additional, as yet unidentified, neurons involved in locomotion (Figure 24; Zheng et al., 1999; Brockie et al., 2001; Tsalik and Hobert 2003; Gray et al., 2005; Guo et al., 2009; Piggott et al., 2011). The ablation of both pathways is essential to completely abolish spontaneous reversal. Simplistically, spontaneous reversal can be initiated by stimulating the AVA backward command interneurons or “disinhibiting” the AIB:RIM pathway that tonically inhibits reversal, as discussed more fully below. For example, the ASHs synapse directly on the AVAs, so that glutamatergic signaling from the ASHs would be expected to initiate backward movement. Indeed, the interaction between the ASHs and the AVAs has been extensively interrogated using the Ca\(^{++}\) indicator, G-CaMP, and direct electrophysiological recording (Guo et al., 2009; Lindsay et al., 2011; Piggott et al., 2011). As predicted, ASH-released glutamate activates the AVA AMPA-like glutamate receptor, GLR-1, to depolarize the AVAs and initiate backward locomotion (Hart et al., 1995; Zheng et al., 1999; Brockie et al., 2001; Mellem et al., 2002; Guo et al., 2009; Piggott et al., 2011). Increasing the level of ASH stimulation presumably increases glutamate release and more rapidly depolarizes the AVAs, leading to more rapid reversal. The ASHs also release neuropeptides encoded by nlp-3 that can increase ASH glutamate-dependent reversal by activating receptors elsewhere in the locomotory circuit (Harris et al., 2010). The food or 5-HT stimulation of aversive responses requires nlp-3 expression
in the ASHs and ASH::nlp-3 overexpression in wild type animals increases basal aversive responses off food (Harris et al., 2010).

In contrast, spontaneous reversal (both short and long) initiated by the AIBs appears to involve the inhibition of their major synaptic partner, the RIMs (Figure 24). For example, laser ablation of the AIBs dramatically decreases spontaneous reversal off food, while ablation of the RIMs increases reversal, suggesting that the AIBs inhibit the RIMs, i.e., the RIMs inhibit reversal and the AIB inhibition of RIMs increases reversal (Piggott et al., 2011). As predicted, the reversal associated with the activation of the ASHs (and potentially the FLPs) by nose touch activates the AIBs and inhibits the RIMs and the AIB-dependent hyperpolarization of the RIMs requires the inhibitory glutamate-gated Cl\(^{-}\) channel, AVR-14, supporting an inhibitory role for the RIMs in initiating sensory-evoked reversal (Piggott et al., 2011). However, these results are surprising. The RIMs form extensive gap junctions with the AVA and AVE backward command interneurons and synaptic connections through an inhibitory TA-gated Cl\(^{-}\) channel, LGC-55, with the AVB forward command interneurons, both of which would be predicted to increase spontaneous reversal (Figure 24). Indeed, the activation of channel rhodopsin in the RIMs is accompanied by an immediate reversal, also suggesting that stimulating the RIMs stimulates reversal (Guo et al., 2009; Piggott et al., 2011). So, do the RIMs stimulate or inhibit spontaneous reversal? Interestingly, although the AIBs are stimulated by both nose touch and osmotic shock, another ASH-mediated behavior, the RIMs are inhibited by nose touch and stimulated by osmotic shock (Piggott et al., 2011). Conversely, osmotic shock suppresses head oscillations and nose touch does not, presumably because during osmotic shock the RIMs inhibit head oscillations by the
tyraminergic activation of LGC-55 on head muscle, i.e., osmotic shock activates the ASHs that, in turn, activate the AVA backward command interneurons that through gap junctions activate the RIMs, facilitating TA release from the RIMs and the tyraminergic inhibition of head oscillations through LGC-55 on head muscle (Pirri et al., 2009; Piggott et al., 2011). Indeed, as predicted, when the command neurons are ablated, osmotic shock inhibits the RIMs and fails to suppress head oscillations (Piggott et al. 2011).

Together, these studies highlight the complexity of RIM signaling and how much remains to be learned about the role of the RIMs in both spontaneous and sensory-evoked reversal behavior.

In the present study, TA abolished the food or 5-HT stimulation of aversive responses. If endogenous TA were released from the RIMs, it would imply that tyraminergic signaling from the RIMs had the potential to decrease aversive responses to dilute 1-octanol and would, at least superficially agree, with the RIM ablation data, i.e., the RIMs decrease the frequency of spontaneous reversal. However, the major inhibitory effects of TYRA-3 on aversive responses appear to be humoral and inhibit food or 5-HT stimulated, and not basal, aversive responses, suggesting that the targets of this TYRA-3 dependent, tyraminergic signaling do not involve the command interneurons directly. Whether the humoral source of TA comes from the RIMs and/or the uterine UV1 cells remains to be determined. Since the ASI peptidergic modulation of aversive behavior involves neuropeptide receptors on sensory neurons or interneurons that synapse directly on the AIBs, it is most likely that ASI peptidergic signaling modulates the AIB-mediated “disinhibitory” pathway (Figure 23). For example, ASI neuropeptides encoded nlp-8, nlp-9 and nlp-14 activate receptors directly on the ADL, AWC, ASER and ASK sensory
neurons that innervate the AIBs directly and nlp-1 activates a receptor the AIA and AIY interneurons that interact extensively with the AIBs to integrate sensory inputs (Figure 23; Mills et al., 2011).

Similar conflicting observations have also been noted about the role of the AIBs in spontaneous and sensory-evoked reversal. For example, the inhibition of the AIBs by the 5-HT gated Cl⁻ channel, MOD-1, is required for the food or 5-HT stimulation of aversive responses, implying that the AIBs inhibit ASH-mediated reversal (Harris et al., 2009, 2011). However, as noted above, ablation of the AIBs dramatically decreases the rate of spontaneous reversal, suggesting that the AIBs stimulate reversal, i.e., the AIBs stimulate the frequency of spontaneous reversal, but the MOD-1-dependent hyperpolarization of the AIBs is essential to decrease the time taken to initiate sensory-evoked reversal in the presence of food or 5-HT (Tsalik and Hobert 2003; Wakabayashi et al., 2004; Gray et al., 2005; Harris et al., 2009; Piggott et al., 2011). Clearly, these apparently contradictory observations need to be explained. For example:

- Are the decisions to reverse, the time taken to initiate reversal and the length of reversal modulated independently? Interestingly, tdc-1 and, to a lesser extent, lgc-55 null animals spontaneous reverse more frequently than wild type animals on food, and exhibit a much greater percentage of short reversals (1-2 body bends) than long reversals (3+ body bends), suggesting that TA inhibits spontaneous reversal, but, somewhat paradoxically, selectively increases the frequency of long reversals, most probably by inhibiting the forward command interneurons through LGC-55. Conversely, TA decreases the frequency of short reversals (Alkema et al., 2005; Pirri et al., 2009). On food, 5-HT levels should be elevated, so the TA-dependent inhibition of spontaneous
reversal correlates well with the TA inhibition of 5-HT-stimulated aversive responses observed in the present study and suggest that it also will be important to examine the effects of TA on the length of sensory-evoked reversals. Together, these data suggest that even a “simple” phenotype, such as spontaneous reversal, may be more complex than previously appreciated, with the potential for short and long reversals to be modulated independently (Alkema et al., 2005; Pirri et al., 2009; Harris et al., 2011).

- Can ASH glutamatergic activation of the AVAs depolarize the RIMs through AVA::RIM gap junctions under certain conditions and can signaling through these gap junctions be modulated by other neurons, such as the AIBs or SAAs, as has been described for NPR-1 in the potential nutritionally-dependent modulation of gap junctions on the RMG neurons in the modulation of locomotory behaviors? (Macosko et al., 2009)

  - Could the intensity, extent or duration of ASH stimulation differentially modulate AIB or RIM signaling?

  - Can RIM signaling through gap junctions or TA release be modulated independently?

Clearly, much also remains to be learned about the overall role of the AIBs (and their signaling to the RIMs) in modulating both spontaneous and sensory-evoked reversal, and, more specifically, about how ASI neuropeptides modulate the AIB:RIM pathway to integrate food availability with sensory-mediated locomotory decision-making.
Figure 4-2. The RIMs extensively innervate the command interneurons that dictate forward and backward locomotion. The RIMs form extensive gap junctions with the AVA and AVE backward command interneurons and activate the inhibitory tyramine-gated Cl⁻ channel, LGC-55, on the AVB forward command interneurons, both of which would be predicted to increase backward locomotion upon RIM stimulation. This diagram only highlights major connectivities; for example the SAAs also innervate the AVAs (24) and the RIMs, the SMDs (11) and head muscle (2).
4.5 The neuropeptides involved in the TA or OA inhibition of aversive responses may be packaged into different large dense-core vesicles in the ASIs.

The ASIs appear to selectively release neuropeptides in response to TA- or OA-dependent Gαq-coupled TYRA-3 or SER-6 signaling, respectively, but the mechanism of this apparent selective release is unclear. Recent work in the C. elegans RIA interneurons suggests that GPCR signaling in the axons of the RIAs can cause the local release of internal Ca\(^{++}\) from the ER and selectively stimulate the release of SVs and, potentially, large dense core vesicles (LDCVs) at individual synapses or, in the case of LDCVs, extra- or perisynaptic sites (Hendricks et al., 2012). If TYRA-3 and SER-6 were differentially localized in the ASIs, this might explain selective neuropeptide release from individual populations of ASI LDCVs. Indeed, all of the neuropeptide receptors responding to ASI neuropeptides identified to date have been functionally localized to neurons directly postsynaptic to the ASIs. In contrast, ASI neuropeptides might be selectively packaged into discrete populations of LDCVs that are independently trafficked to different locations in the ASIs, perhaps even translated at different sites, as selective mRNA axonal and dendritic trafficking and extra-somal translation has been reported for other neuronal signaling proteins (Holt and Bullock 2009; Wang et al., 2010; Swanger and Bassell 2011; Kindler and Kreienkamp 2012). Indeed, if the synthesis of the neuropeptides is temporally distinct, the neuropeptide complement of individual LDCVs might be temporally-dependent on differential rates of synthesis (or degradation?). Alternatively, the neuropeptides might be selectively proteolyzed after
release by the array of specific neprilysins differentially expressed in individual sites of neuropeptide release (Isaac et al., 2000; Turner et al., 2001; Isaac et al., 2009). Certainly, much remains to be learned about the packaging, trafficking and targeting of LDCVs in all systems, but all three mechanisms outlined above could be operative. In the dorsal root ganglion, neuropeptides are sorted and packaged into LDCVs in the trans-Golgi network and transported to afferent terminals for release (Camilli and Jahn 1990; Hokfelt et al., 2003). These LDCVs also transport a variety of other proteins, including GPCRs, ion channels, and other signaling components (Zhao et al., 2011). For example, γ-opioid receptor-1 that is activated by the anti-nociceptive opioid peptides is trafficked to afferent terminals in LDCVs where its surface expression can be rapidly induced by an array of stimuli (Zhang et al., 1998; Bao et al., 2003; Guan et al., 2005; Wang et al., 2010). The observation that LDCVs traffic more than neuropeptides has highlighted the complexity of LDCV packaging. For example, most neuropeptides are synthesized as preproproteins that are processed through a multi-step pathway involving an LDCV-localized proprotein convertase, encoded by egl-3 in C. elegans, although the extracellular processing of some proproteins by the metalloproteinases, MPP3 and MPP7, has also been reported (Fricker 1988; Smith et al., 1995; Kass et al., 2001; Lee et al., 2001; McMawley and Matrisian 2001). As predicted, C. elegans egl-3 null animals have a dramatically reduced neuropeptide composition, based on immunostaining and mass spectrometry, but are still viable, supporting the notion that neuropeptides modulate most processes, but are not essential for their overall function (Kass et al., 2001; Husson et al., 2006). Precursors of some mammalian neutrophilins, such as proNGF, are cleaved within the trans-Golgi network by a proprotein convertase, packaged into constitutive vesicles and secreted
continuously (Edwards et al., 1988; Seidah et al., 1996; Gu et al., 2001; Mowla et al., 2001; Hibbert et al., 2003). In contrast, the precursor for another neutrophilin, BNDF, is sorted into the regulated secretory pathway for secretion by a receptor-mediated mechanism, similar to the sorting of lysosomal proteins to the lysosomes (Cool et al., 1997; Zhang et al., 1999; Bicknell et al., 2001; Loh et al., 2004). This activity-dependent secretion mediates many aspects of synaptic plasticity, including long-term potentiation (LTP) (Katz and Shatz 1996; Korte et al., 1998; Mizuno et al., 2000; Lu 2004).

Neuropeptide sorting requires specific sorting motifs that have been identified in a number of neuropeptide precursors, including pro-opiomelanocortin and insulin, that interact with a membrane carboxypeptidase (CPE; EGL-21 in C. elegans) to target these proproteins to the regulated secretory pathway (Cool et al., 1995). Indeed, C. elegans egl-21 null animals also appear to have markedly deficient neuropeptide profiles (Husson et al., 2007). Similarly, the sorting of the neuropeptide tachykinins into LDCVs is a key step in their regulated secretion (Ma et al., 2008). The sorting of both the tachykinin propeptide and the mature peptide-containing sequence of protachykinin requires the polarized distribution of amino acids at the N-terminus of the proprotein that appear to serve as aggregation signals and regulate sorting efficiency (Ma et al., 2008).

Since we have hypothesized that the ASI neuropeptides released by TA may be localized to different LDCVs than those released by OA, we analyzed each of the predicted neuropeptide preprosequences for clusters of charged amino acids similar to those observed for protachykinin. Interestingly, each of the TA-dependent preproproteins (encoded by nlp-1, nlp-14 and nlp-18) had charged amino acids within ten amino acids upstream of the first dibasic cleavage site (Figure 25). In contrast, no charged amino
acids were found ten amino acids upstream of the first mature neuropeptide in the
neuropeptide proproteins simulated by OA (nlp-6, nlp-7 and nlp-9) and, more
interestingly, each of the three OA-stimulated preproproteins lacked dibasic cleavage
sites upstream of the first mature neuropeptide, in contrast to the three TA-stimulated
proproteins that possessed clear dibasic cleavage sites upstream of the mature first
peptide (Figure 25). Presumably, the signal peptidase truncated the preproproteins
encoded by nlp-6, nlp-7 and nlp-9 immediately upstream of the first mature neuropeptide,
while removal of the signal peptide from the preproproteins encoded nlp-1, nlp-14 and
nlp-18 preproproteins left an N-terminal charged sequence upstream of the first
neuropeptide in the proprotein. As predicted, signal peptidases often cleave immediately
after an alanine residue, as has been reported for the human ghrelin, gastrin and
tachykinin preproproteins and the mutation of alanine to threonine in preprovasopressin
associated with familial central diabetes insipidus markedly decreases cleavage efficiency
by the signal peptidase (Nothwehr and Gordon 1990; Ito et al., 1993; Siggaard et al.,
1999). Importantly, the identity of the N-terminal mature peptides encoded by nlp-1,
nlp-18, nlp-6, nlp-7 and nlp-9 have been confirmed by mass spectrometry (Husson et al.,
2005; Li and Kim 2008). Whether these dramatically different N-terminal upstream
regions play a role in the potential differential localization of the processed neuropeptides
stimulated by either OA or TA remains to be determined, but these observations certainly
are provocative.
TA-dependent:

NLP-1: NKATFVLACCLLVIAAVSHADLLPKRMDANAFRMSFGKR_176
NLP-14: MLHLIVLVLVALSSAHTAGRRRALDGLDSGFGFDKR_224
NLP-18: MNANVSVIVYFLSFLVLCISAQLHADSGATEVDCIVDKSPYRAFAPAKR_59

OA-dependent:

NLP-6: MLSFSLAFVLLVSACVMAMAAPQMSVFGKR_103
NLP-7: MYIKALLIVVGFVQITSAIYLQADFDPRMFTSSHGKR_121
NLP-9: MDRFATRFIALLLLVQIGSIFA祭IAGASAPENVDDRELEKR_29

Figure 4-3. The predicted N-terminal preprosequences of the genes encoding the TA- and OA-dependent ASI neuropeptides differ significantly. For example, the genes encoding the OA-dependent neuropeptides have no dibasic cleavage site upstream of the first mature neuropeptides; this initial cleavage is probably catalyzed by the signal peptidase. In contrast, the genes encoding the TA-dependent neuropeptides have dibasic cleavage sites upstream of the first mature neuropeptides and cleavage by the signal peptidase most likely leaves a charged leader on the proproteins that can potentially be used to differentially target these TA-dependent proproteins. Red: mature neuropeptide; Italic: neuropeptide sequence confirmed by direct sequencing; Blue: predicted site of cleavage by the signal peptidase indicating the absence of a dibasic cleavage site upstream of the first predicted mature neuropeptide; Orange: acidic amino acids; Green: basic amino acids.
4.6 Changes in nutritional status can also have long term effects on olfaction and its associated behaviors.

The ASIs respond directly to a variety of sensory stimuli; for example they play a key role in thermoregulation and are the only neurons that secrete the TGFβ orthologue, DAF-7, that plays a key role in the formation of resistant dauer larvae in response to starvation (Ren et al., 1996; Beverly et al., 2011). In the presence of food, DAF-7 expression is high and activates the TGFβ receptors DAF-1/4 to signal via the DAF-8 and DAF-14 Smads to inhibit the DAF-3 Smad that promotes dauer development (Thomas et al., 1993; Ren et al., 1996; Patterson et al., 1997; Patterson and Padgett 2000; Savage-Dunn 2005). However, in the absence of food, DAF-7 expression is repressed and DAF-3 signaling is initiated, ultimately leading to large-scale changes in transcription, protein composition and entry into dauer development. TA and OA also appear to be hunger signals and activate the ASIs during removal from food, suggesting that TYRA-3 and SER-6 may also have long-term effects on ASI signaling. Indeed, steady-state nlp-1, nlp-14 and nlp-18 mRNA levels are significantly elevated during starvation, when TA levels are also presumably elevated, suggesting that TA may not only stimulate neuropeptide release, but also neuropeptide synthesis (Hapiak, Korchnack and Komuniecki, unpublished). Post-aversive responses to noxious odorants also appear to be acutely modulated by food in *C. elegans* and the decision to continue forward or reverse direction after the initial backward response to a noxious odor is entirely dependent on food availability and not on the strength of the repellent stimulus, i.e., on or off food animals back away from 1-octanol, but continue forward on food and initiate an omega turn and reverse away from the noxious stimulus off food (Harris et al., 2011). Starvation also
leads to robust changes in gene expression in *Drosophila* chemosensory neurons, with starvation upregulating the expression of the neuropeptide-like receptor, sNPFR1, in the fly antenna to potentially inhibit aversive responses during starvation (Wu et al., 2005; Root et al., 2011). For example, stage-dependent attraction or aversion to sugar is mediated by NPFR1 (Wu et al., 2005). Together these observations suggest that starvation may potentially reduce or overcome aversive responses in both worms and flies and that these nutritionally-dependent changes in aversive behavior may have broad adaptive significance and modulate food preference during starvation, i.e., food-associated aversive stimuli may be less pronounced when animals are food-deprived.

### 4.7 Summary

TA released from the two RIM motorneurons in response to nutritional status activates a global monoaminergic/peptidergic signaling cascade, with receptors localized at all levels of the sensory-mediated locomotory circuit, including sensory neurons, interneurons, motorneurons and muscle (Figure 23). Interestingly, the TYRA-3-mediated inhibition of ASH-mediated aversive responses appears to be largely, if not exclusively humoral, i.e., the RIMs do not synapse on the dopaminergic, octopaminergic or ASI neurons activated by TYRA-3 and the RICs do not synapse directly on the ADL, AWB, ASI neurons activated by OA and SER-6. In contrast, synaptic cross-talk between some of the inhibitory monoaminergic neurons is apparent, as the dopaminergic CEPs synapse extensively on the octopaminergic RICs (24 synapses) that, in turn, synapse on the tyraminergic RIMs (3 synapses). In contrast to tyraminergic or octopaminergic signaling from the RIMs or RICs, peptidergic signaling from the ASIs in response to either TA or
OA stimulation appears to be largely synaptic (or perisynaptic), as the four neuropeptide receptors identified to date that respond to ASI neuropeptides function on the AIA/AIY interneurons or the ASK, ASER or AWC sensory neurons, all of which are downstream synaptic partners of the limited ASI synaptic circuit (Figure 23). Specifically, these studies have demonstrated that:

- the humoral release of TA activates the $G_{\alpha_q}$-coupled TA receptor, TYRA-3 on octopaminergic, dopaminergic, and peptidergic neurons to abolish the food or 5-HT stimulation of ASH-mediated aversive responses

- the TYRA-3 activation of the ASI sensory neurons appears to stimulate the axonal release of neuropeptides encoded by nlp-1, nlp-14, and nlp-18, whose ASI overexpression also abolishes the food or 5-HT stimulation of ASH-mediated aversive responses

- ASI neuropeptides encoded by nlp-1, nlp-14 and nlp-18 are also released from a variety of other neurons and can have more widespread effects on locomotory behavior

- the receptors for neuropeptides encoded by nlp-1 and nlp-14 function on the AIA/AIY interneurons and the ASK/ADL sensory neurons, respectively, and, as predicted, receptor overexpression also interferes with the food or 5-HT stimulation of aversive responses.

- the activation of both $G_{\alpha_q}$ and $G_{\alpha_s}$ signaling in the ASIs is essential for TA inhibition.

- the ASI neuropeptides released in response to TA and TYRA-3 (encoded by nlp-1, nlp-14 and nlp-18) differ completely from the ASI neuropeptides released in response to OA and SER-6 (encoded by nlp-6, nlp-7, and nlp-9).
• the release of TA- or OA-stimulated ASI neuropeptides appears to be synaptic (or perisynaptic), since all of the neuropeptide receptors identified to date that respond to ASI peptides are found on downstream postsynaptic partners of the ASIs. Together these data suggest that ASI neuropeptides may be:

- selectively released at different sites,

- globally released into synapses with different neuropeptide receptors (although OA does not appear to stimulate the release of neuropeptides encoded by \textit{nlp-14})

- selectively degraded by differentially-localized peptide degrading enzymes or

- differentially sequestered into different populations of LDCVs. Indeed, the prosequences of the TA and OA-dependent ASI prepropeptides differ significantly.

Together, these results suggest that nutritional signals released from a limited number of neurons are broadcast globally and amplified locally to modulate many aspects of not only locomotory decision-making, but, indeed, most, if not all, key, nutritionally-modulated, behaviors in \textit{C. elegans}. On a broader level, they highlight the obligate interaction between monoaminergic and peptidergic signaling in the complex, multi-level regulation of sensory-mediated behaviors in all organisms.
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