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INVESTIGATING SEROTONIN RECEPTOR EXPRESSION IN SINGLE
HOMOLOGOUS NEURONS UNDERLYING INDEPENDENTLY EVOLVED AND
SPECIES-SPECIFIC BEHAVIORS

by

ARIANNA N. TAMVACAKIS

Under the Direction of Paul S. Katz, Ph.D.

ABSTRACT

Serotonin (5-HT) receptors modulate neuronal and synaptic properties, altering the functional output of neural circuits. Changing the functions of a neural circuit can alter behavior. Over evolutionary time, species differences in neuromodulation could allow for species-specific behaviors to evolve. To investigate this idea, this dissertation compared neuromodulatory receptor gene expression underlying species-specific swimming behaviors in sea slugs.

The sea slug *Tritonia diomedea* (Mollusca, Gastropoda, Nudipleura, Nudibranchia), performs a rhythmic dorsal-ventral (DV) escape swim behavior. The behavior is controlled by a central pattern generator (CPG), composed of a small number

of large, identifiable neurons. During swimming, 5-HT enhances the synaptic strength of a neuron in the swim CPG, called C2. In contrast, the nudibranch *Hermisenda crassicornis* does not swim in this manner. It has C2 homologues, and 5-HT is present, however, 5-HT does not modulate C2 synaptic strength. *Pleurobranchaea californica*, a Nudipleura species belonging to a sister clade of Nudibranchia, swims with DV flexions, although in this species swimming varies within individuals. 5-HT enhances *Pleurobranchaea* C2 homologue synaptic strength in swimming animals, only. Phylogenetic analysis showed that *Tritonia* and *Pleurobranchaea* independently evolved DV-swimming. Thus, there is a correlation between independently evolved swimming and serotonergic modulation of C2 homologues. It was hypothesized that 5-HT receptor differences in C2 neurons underlie species-specific swimming and modulation.

To test this hypothesis, 5-HT receptor genes were identified in each species. A total of seven receptor subtypes, from five gene families, were found to be expressed in the brains of each species. Using single-cell quantitative PCR (qPCR), 5-HT receptor expression profiles were determined in C2 homologues. Genes known as 5-HT_{2a} and 5-HT₇ were expressed in C2 homologues from *Tritonia* and swimming *Pleurobranchaea*, only. Single-neuron transcriptome sequencing verified these results. The expression profiles of neuromodulatory receptor genes in single, homologous neurons correlated with species-specific swimming and modulation. The results illustrate how differences in neuromodulatory gene expression may alter the functional output of homologous neural structures, shedding light on a means by which neuromodulation can alter the brain to facilitate the evolution of species-specific behaviors.

INDEX WORDS: Evolution, Mollusc, Neuromodulation, Serotonin, Receptor, Behavior, Next-Generation Sequencing, Transcriptomics

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

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April 2017

DEDICATION

This is dedicated to my family, and especially to my wonderful husband, PC.

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Thank you to my advisor, Dr. Paul Katz, for his guidance, and to my committee members for their support and advice. Thank you also to the members of my lab and my department, for their assistance and generosity.

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1 INTRODUCTION

1.1 General Introduction

Animal behaviors evolve through species differences in the nervous system. The nervous system is evolutionarily constrained in many ways, however, so that large-scale anatomical or functional changes happen rarely (Striedter 2005). The evolutionary changes that more commonly shape behaviors must therefore be more subtle. One way that such subtle changes can occur is through species-specific functional changes to existing neural structures. Functional changes can occur through the actions of neuromodulators, such as serotonin (5-HT). Neuromodulators are chemicals that can change neuronal physiological properties or responses. Neuromodulation can therefore change the function of a neural circuit by modifying its component neurons, and thus alter the behavior output of that circuit. Thus, evolutionary changes in the neuromodulatory properties of neural circuit components could result in species differences in behavior (Katz and Harris-Warrick 1999).

My dissertation hypothesizes that species differences in neuromodulatory receptors expressed by identified, homologous single neurons underlie species-specific behaviors. It also examines whether similar, independently evolved behaviors involve parallel neuromodulatory gene expression at the level of single cells. I examined these hypotheses in three species of sea slugs, two of which use serotonergic neuromodulation in species-specific swimming behaviors. Comparing neuromodulatory gene expression within homologous neural circuit components can help explain how behaviors evolved among closely related species, as well as general neural mechanisms of behaviors across species. Information from my work can help us better understand how evolution shaped the brain to create the diversity of animals and behaviors we see today.

1.2 Identifiable and Homologous Neurons

Identifying and classifying neurons has been an important part of neuroscience for over a century. What are now considered classical methods of neuron identification formed Ramon y Cajal's "Neuron Doctrine," which first stated that neurons are discrete and identifiable cells (López-Muñoz et al. 2006; Ramon Y Cajal 1894). The identification of individual neurons has allowed researchers to connect synapses and track groups of neurons that connect to one another to form circuits, providing vital tools for the study of the neural basis of behavior, neural development, and neurological disease. Over the past 50 years, identified neurons have been especially important in examining neural circuits controlling behaviors in invertebrates.

Neurons can be reliably classified based on several characteristics. Traditionally, characterizations of neurons have been done using morphology and anatomical location, but more recently neurons have been classified by studies of single gene expression, synaptic properties, physiology, and the neurotransmitters they release (Bullock 2000; Hudson et al. 2010). In the past few years, single neuron transcriptome profiles have been used as an additional tool to identify neuronal subpopulations via large-scale gene expression comparisons (Cadwell et al. 2016; Crocker et al. 2016; Fuzik et al. 2016; Gokce et al. 2016; Lake et al. 2016; Usoskin et al. 2015; Zeisel et al. 2015).

Identifying and characterizing neuron types and their synapses has allowed scientists to pinpoint synaptic chains of neurons within the brain, and thereby create maps of neural circuits controlling behaviors (Hudson et al. 2010). Although advances in technology have made neural circuit identification easier for researchers studying mammals, the dense and intricate connectivity of the vertebrate brain makes identifying neurons and their connectivity difficult in vertebrate species.

For this reason, invertebrate identified neurons have been popular in studies involving neural circuits controlling behaviors. Because brains of many invertebrate species are relatively small, behaviors are often controlled by a small number of distinct neurons. This fact has allowed researchers to characterize entire neural circuits controlling several behaviors. Some examples are the *Tritonia diomedea* swim motor pattern circuit (Dorsett et al. 1969; Hume et al. 1982; Katz 1998; Katz and Frost 1995a; Katz and Frost 1995b; Katz et al. 1994; Willows and Hoyle 1969), the gill withdrawal reflex circuit in *Aplysia californica* (Carew and Kandel 1973; Castellucci et al. 1970; Kupfermann et al. 1971; Leonard and Edstrom 2004), the gastric mill and pyloric circuits in crustaceans (Mulloney and Selverston 1974; Selverston et al. 2009), or neural-controlled heartbeat in leeches (Calabrese et al. 2016; Calabrese and Peterson 1983; Maranto and Calabrese 1984; Shafer and Calabrese 1981).

While identifying neurons in the brains of individuals within a species has been useful, evolutionary neuroscientists have gone a step beyond, finding that individual neurons can sometimes be identified across species as well (Bullock 2000; Croll 1987b). These neurons are homologous, that is, they are found in the brains of related species, share common characteristics, and are predicted to have been present in the common ancestor of those species.

Homologous neurons have been defined by comparing across species, examining characteristics such as anatomical location, developmental origin, morphology, and biochemical and genetic makeup. For example, the Mauthner cells are a large pair of neurons found across species of teleost fish. They are located in the hindbrain, connected by gap junctions to other cells, and contain an axon cap formed by glia which help conduct electrical signals (Korn and Faber 2005). They control the fish C-start escape reflex, which

helps fish to quickly evade a predator or other potential danger. Those characteristics have been conserved across almost all teleost species, as well as some amphibians, likely because the morphological characteristics of the neurons allow for an extremely fast response, an important tool for survival (Zottoli 1978). In another example, researchers identified a set of three serotonergic homologous cells present across all lophotrochozoan larvae, which during development innervate the prototroch (Hay-Schmidt 2000). A large neuron found in the cerebral ganglia of sea slugs, called C₁, is believed to be homologous within gastropod molluscs; it is serotonergic and is involved in feeding across species (Croll 1987a; Malyshev and Balaban 2011; Weiss and Kupfermann 1976).

Homologous neurons are not necessarily functionally analogous, because function can diverge over evolutionary time. Functional differences can be compared across species by examining different aspects of the neural circuitry. For example, synaptic differences in homologous mechanosensory neurons known as P-cells correlate with species differences in body movements of two leech species, *Hirudo verbana* and *Erpobdella obscura* (Baltzley et al. 2010). The modulatory proctolin neurons (MPNs) in the crab, *Cancer borealis* (Nusbaum and Marder 1989a; Nusbaum and Marder 1989b) and the GABA neurons 1 and 2 (GN_{1/2}) in the lobster *Homarus gammarus* (Cournil et al. 1990) are homologous neurons with differing peptidergic signals that produce opposite effects on the species-specific gastric mill rhythm (Meyrand et al. 2000).

A group of three homologous neurons known as the Dorsal Swim Interneurons (DSIs) has been studied in several species of gastropod molluscs. Across species, they are serotonergic, and cluster bilaterally in the cerebral ganglia. They function in relation to movement, although different species use the neurons in species-specific ways, and in some species the DSIs have been identified as having a multifunctional role (Newcomb

and Katz 2009). In the anaspid sea slug, *Aplysia californica*, the DSI homologues are known as the Posterior Cell Cluster (PCC), and function to increase the strength of the central pattern generator controlling muscular wave crawling (Jing et al. 2008; Katz et al. 2001). DSI homologues, known as CPTs, in the nudibranch *Hermisenda crassicornis* act to cause foot retraction, but do not contribute to crawling (Tian et al. 2006). In the nudibranch, *Tritonia diomedea*, DSI neurons activate ciliary crawling (Popescu and Frost 2002) and swimming (Getting 1981; Getting et al. 1980; Katz and Frost 1995a; Katz and Frost 1995b; Katz et al. 1994). In the more distantly related Nudipleura species *Pleurobranchaea californica*, the DSI homologues were found to have similar functions (Jing and Gillette 2000; Lillvis and Katz 2013; Newcomb et al. 2012). In *Pleurobranchaea*, the DSIs have also been characterized as facilitating directional avoidance (Jing and Gillette 2003), feeding, and arousal (Jing and Gillette 2000). The sea slugs *Clione limacina* and *Melibe leonina* both swim, although *Clione* swims by flapping parapodial wings, and *Melibe* swims by moving its body laterally. *Clione* swimming is controlled in part by DSI homologue CR-SP activation, which mediates wing flapping strength (Satterlie and Norekian 1995). In *Melibe*, the DSI homologues activate swimming (Newcomb and Katz 2009). Thus, these species have homologous neurons in their brains, but those neurons control species-specific locomotive behaviors. This likely occurs because of small species-specific differences in the synaptic wiring of the circuits of which the DSIs are a part.

The cases of the leech P-cells, the crustacean projection neurons, and the molluscan DSI neurons mentioned above are examples of homologous neurons that share some characteristics, like morphology and anatomical position, but differ in their function. The differences are likely due to subtle physical variations between the

homologues, like synaptic connectivity and modulatory inputs, and through evolutionary adaptations in gene expression between species, for example species differences in ion channel or neurotransmitter receptor gene expression between homologues (Clark et al. 2008; Grashow et al. 2010; Hamood and Marder 2014; Marder et al. 2014). The observation that functional differences occur between identified, homologous neurons has advanced a more modern view than that of the time of Ramon y Cajal's "Neuron Doctrine," that although neurons are discrete, identifiable cells within the nervous system, their connections and genetic identity allow them to be flexible in their functional output, within and between species (Bullock et al. 2005).

1.3 Species Differences in Neural Circuits Underlying Species-Specific Behaviors

Behaviors are controlled by groups of neurons that synapse together to form circuits. Because those circuits can be made up of homologous neurons, some researchers have postulated that neural circuits are conserved, and from that idea they reasoned that the behaviors those circuits control may also be well conserved (Tierney 1995). However, others have proposed that small changes within a circuit can result in dramatically different behaviors (Katz and Harris-Warrick 1999), opposing Tierney's theory. Evidence against Tierney's theory can be observed in closely related species that exhibit different behaviors, which are reflective of differences at the levels of sensory, motor, and interneurons in the nervous system.

Between species, changes in sensory neuron circuit organization have evolved to reflect changing sensory structures and ecological habitats. An example of this is found in the insectivores, namely within a clade comprising hedgehogs, moles, and some shrews

(Stanhope et al. 1998). Hedgehogs use a combination of visual, auditory, and somatosensory cues to hunt, while moles and shrews do not rely heavily on vision, and moles have evolved a specialized Eimer's organ to detect prey underground (Catania 2005; Catania and Remple 2005). In each species, studies of neuromorphology and electrophysiological recordings have revealed somatosensory cortical organization that is specialized to reflect these disparate modes of sensing (Catania 2005; Catania et al. 2000; Catania and Kaas 2001). These species differences in sensory neuron circuitry reflect the role of evolution in shaping neural mechanisms underlying species-specific behaviors.

Motor circuits containing homologous neurons also show species differences. Hexapods, decapod crustaceans, and scorpions use inhibitory motor neurons as part of their neural circuits for walking, which are believed to be homologous (Harzsch et al. 2005; Wiersma 1941; Wolf and Harzsch 2002b). However, scorpion legs have up to twelve inhibitory motor neurons per legs, whereas hexapods and decapods have only one or two (Wolf and Harzsch 2002a; Wolf and Harzsch 2002b). Comparing across these three species, the number of inhibitory motor neurons was found to positively correlate with leg size, indicating that homologous motor neurons changed in number as leg size evolved (Wolf 2014).

Comparisons of homologous interneurons in molluscs have also yielded species differences at the level of neural circuits. In gastropod sea slugs, different forms of swimming have been described at the level of the individual homologous neurons that control the behaviors. One form, which is only found among the nudibranchs, is left-right (LR) swimming, in which animals move their body laterally to propel themselves through the water. LR swimming appears to have evolved in several nudibranch lineages (Goodheart et al. 2015; Newcomb et al. 2012). However, comparisons of the swim neural

circuits in two LR-swimming species, *Melibe leonina* and *Dendronotus iris*, have shown that different species use different sets of interneurons in their swim neural circuits. Swim Interneuron 1 and 2 (Si1 and Si2) comprise part of the central pattern generator (CPG) controlling swimming in *Melibe*, while another neuron called Si3 is not involved in swimming. In contrast, *Dendronotus* Si2 and Si3 homologues are parts of its swim CPG (Sakurai and Katz 2016), while Si1 homologues are not involved in the swim motor pattern (Sakurai et al. 2011). One caveat to this research is that whether the Si2s are homologous between these two species is unclear, however (Sakurai and Katz 2016). Regardless, the two species have evolved to produce the same behavior, but use disparate homologous neurons as part of their swim neural circuits.

1.4 Neuromodulatory Mechanisms Underlying Species Differences in Behavior

While individual neurons can be conserved across species, changes in their functions over evolutionary time result in species-specific behaviors (Katz and Harris-Warrick 1999). These evolutionary changes are reflective of the nervous system's flexibility. This flexibility is produced through the actions of substances released by neurons, called neuromodulators.

Major neuromodulators in vertebrates and invertebrates include biogenic amines, like 5-HT, dopamine, octopamine, histamine, adrenaline/epinephrine, and noradrenaline/norepinephrine, as well as acetylcholine, some neuropeptides, and the gaseous transmitter nitric oxide (NO). Neuromodulators often act as “volume transmitters,” meaning that they are released to diffuse to several different neurons. Neuromodulatory chemicals change the properties of a neuron or synapse by activating

corresponding receptors in post-synaptic neurons, which in turn cause second messenger activation and downstream signaling changes within the cell. These effects change the post-synaptic neuron output, and its role within a neural circuit, altering the behavioral output of that circuit (Kupfermann 1979). Within an individual animal, small variations in properties related to neuromodulatory transmitter release or receptor activation could result in behavior modification. Species differences in these properties over evolutionary time are one way that species-specific behaviors can evolve (Katz and Harris-Warrick 1999).

Neuromodulators have been implicated in several comparisons of species-specific behaviors. In genomic comparisons of two baboon species, dopamine-related gene differences correlated with species differences in social behaviors and aggression (Bergey et al. 2016). NO-synthase (NOS) is used as a neuromodulator in a variety of species-specific behaviors in sea slugs, like feeding in *Pleurobranchaea* (Hatcher et al. 2006; Korneev et al. 1998), the gill-withdrawal reflex in *Aplysia* (Antonov et al. 2007; Newcomb et al. 2012), and metamorphosis in the nudibranch *Phestilla sibogae* Bergh (Bishop et al. 2008). Distinct arginine-vasotocin and 5-HT neuromodulatory activity controls species-specific aggression in homologous brain regions of two species of electric fish (Silva et al. 2013).

Another series of experiments showed a causal relationship between species-specific modulation and pair bonding in rodents. Larry Young and other researchers showed that monogamous prairie voles and non-monogamous montane voles differed in expression of neuromodulatory vasopressin receptor subtype V1A in the ventral forebrain (Hammock and Young 2002; Young et al. 1999). They went on to show that upregulation of that receptor could cause the non-monogamous montane to behave like the

monogamous prairie voles (Lim et al. 2004), meaning that changes in expression of a single neuromodulatory gene resulted in species differences in behavior.

1.5 Neural and Genetic Comparisons of Species with Similar, Independently Evolved Behaviors

Analogous behaviors, like analogous anatomical structures, can evolve independently in disparate species (Darwin 1859; Johnson et al. 2010; Katz 2011; York and Fernald 2017). That is, two different species may evolve to behave in the same way. Some examples include independently evolved courtship songs in frogs (Leininger and Kelley 2015; Tobias et al. 2011) and knuckle-walking in arboreal and terrestrial primates (Kivell and Schmitt 2009). Eusociality has evolved independently between bee species (Michener 1974), across insects (Nowak et al. 2010; Wilson 1971; Wilson and Holldobler 2005), and twice in mole rats (Burland et al. 2002; Faulkes et al. 1994; Jarvis 1981). Such behaviors could have evolved through convergent or parallel means (Striedter and Northcutt 1991). In “Origin of Species,” Darwin described independent evolution as being like “two men [who] have sometimes independently hit on the very same invention” (Darwin 1859).

Homologous neural or genetic mechanisms have been identified in several examples of independently evolved behaviors. Bats and cetaceans both use sonar, and both exhibit similar mutations in a protein called prestin, which was implicated in amplifying high-frequency sound (Liu et al. 2010). Neural connections and the genes that mediate their development are similar in vocal learning-related brain areas among songbird, parrot, and hummingbird species with independently evolved vocal learning

behaviors (Wang et al. 2015). In these examples, homoplasious behaviors were produced in different species using homologous neurons and genes.

On the other hand, there are examples of analogous behaviors that evolved with different underlying mechanisms. Weakly electric fish species use electric pulses to communicate. When their electric organs and skeletal muscles were examined, a sodium channel normally expressed in the muscle of non-electric fish was found to be expressed only in the electric organs of electric species. This expression was found in two distinct lineages of electric fish, the African mormyrids and the South American gymnotiforms, which evolved electric signaling independently. When the amino-acid structures of the sodium channel genes were examined in each species, the gymnotiform and mormyrid gene orthologues were found to have different functional changes: in the gymnotiforms, the sodium channel amino acid residues forming the “ball” of the inactivation loop were mutated, while in the mormyrids, the amino acid motif making up the receptor site for the inactivation loop was mutated (Zakon et al. 2006). Thus, evolution produced the same behaviors, using parallel evolution of neural components but with different, convergent protein functions.

Cephalopods, insects, and mammals are three taxa that are capable of complex learning and memory formation. Comparisons of the vertical lobe in cephalopods, the mammalian hippocampus, and the insect mushroom bodies have yielded insight into a universal organization plan that facilitates this ability (Buzsaki et al. 1990; Capaldi et al. 1999; Hochner 2010; Schurmann 2016; Shomrat et al. 2015; Young 1988). The organizational structures of these regions are similar, although it is unclear if this organization evolved by convergence or through an ancient ground plan in these species (Farris 2008a; Farris 2008b; Strausfeld et al. 2009; Wolff and Strausfeld 2015).

Regardless, the structural similarities between the groups points to the importance of neural circuit architecture in producing complex behaviors, and their comparisons have yielded biological mechanisms that are important for the evolution of plasticity and learning (Hochner 2010).

1.6 Dissertation Summary

In the above review of the literature, I have shown specific examples of ways in which homologous neurons and neuromodulators contribute to species-specific behaviors. In some of these studies, however, the effects of neuromodulators were not studied at the level of single cells. In other studies, while single-cell data were available, comparisons were not made between independently evolved behaviors. My dissertation combines these aspects in the study of gene expression in single neurons controlling species-specific and independently evolved behaviors.

The evolution of swimming in sea slugs provides an opportunity to dissect the neural mechanisms underlying species-specific, independently evolved behaviors at the level of single neurons. *Tritonia diomedea* is a sea slug that performs a rhythmic dorsal-ventral (DV) flexion escape swim in response to a predator or noxious stimulus. The neural circuit that controls this behavior has been characterized (Getting 1981; Getting et al. 1980; Katz 1998; Newcomb et al. 2012; Willows and Hoyle 1969) and is composed in part by neurons called C2 and DSI (Getting et al. 1980). The swim motor pattern is activated when DSI releases 5-HT onto post-synaptic C2, VSI, and motor neurons; 5-HT modulates C2 and VSI synapses with motor neurons (Katz and Frost 1995a; Katz et al. 1994; McClellan et al. 1994).

Several studies on the effects of serotonergic modulation of C2 have elucidated its exact mechanisms. 5-HT release from DSI neurons directly enhances C2 excitability and modulates its synapses pre-synaptically. These actions enhance C2 transmitter release. This pre-synaptic enhancement is found at all known C2 synapses (Katz and Frost 1995a; Katz and Frost 1995b; Katz et al. 1994). C2 excitability is also enhanced by the 5-HT precursor 5-hydroxytryptophan (5-HTP) (Fickbohm and Katz 2000). DSI stimulation or a brief 5-HT puff results in increased calcium signaling in C2 neurites, indicating that serotonergic stimulation of C2 may occur via receptors that activate changes in intracellular calcium (Hill et al. 2008). DSI produces both fast and slow responses in C2. The fast EPSP response was believed to be produced by ionotropic receptors, because of the synaptic activity timing and actions of imipradine. The slow response was believed to be metabotropic, because it is blocked by the 5-HT metabotropic receptor antagonist methysergide. This research led to the conclusion that C2 may express both metabotropic and ionotropic receptors that respond to 5-HT (Clemens and Katz 2001; Katz and Frost 1995a; Katz and Frost 1995b). However, the only known ionotropic 5-HT receptor, 5-HT₃, has only been identified genetically in chordates (Reeves and Lummis 2002), and there are instances of fast-acting G-protein coupled receptors (GPCRs) (Chen 2005; Ferguson and Caron 1998; Najafi et al. 2012).

One aspect of serotonin's effects on C2 that had not been investigated is the identity of the 5-HT receptors that it expresses. Five subtypes were previously identified in *Aplysia*, *Lymnaea*, and other molluscs (Barbas et al. 2005; Barbas et al. 2003; Barbas et al. 2002; Kawai et al. 2011; Lee et al. 2009; Mapara et al. 2008b; Nagakura et al. 2010; Panasophonkul et al. 2009; Patocka et al. 2014), but none have been identified in *Tritonia* or closely related species.

Tritonia is a member of the clade, Nudibranchia (Mollusca, Gastropoda, Nudipleura) (Goodheart et al. 2015). Recent work on the phylogeny of the Nudibranchia and Nudipleura has informed our understanding of how DV swimming behaviors evolved. The nudibranch clade includes over 2000 species, most of which do not swim (Figure 1-1). *Pleurobranchaea californica*, a Nudipleura species that is an outgroup to Nudibranchia, performs DV swimming that is analogous to the behavior seen in *Tritonia* (Jing and Gillette 1999; Newcomb et al. 2012). The few Nudipleura species that do perform DV-swimming are not closely related to one another. It is therefore most parsimonious to assume that *Tritonia* and *Pleurobranchaea* evolved swimming independently (Newcomb et al. 2012).

C2 neurons are found in all Nudipleura species examined. They share a common set of characteristics: they are single large white neurons located bilaterally in the cerebral ganglion, their axons project contralaterally to the pedal ganglia, they are immunoreactive for Small Cardioactive Peptide (SCP) and FMRFamide, they are electrically coupled, and at rest they exhibit few action potentials but regular excitatory post-synaptic potentials (EPSPs) (Lillvis et al. 2012). These common features uniquely identify the neuron in the brain of each species and therefore suggest that they are homologous.

In *Pleurobranchaea*, C2 (A1) acts as part of the swim neural circuit. 5-HT released from *Pleurobranchaea* DSI homologues, also known as AS1-4 (Jing and Gillette 1995; Jing and Gillette 1999), modulates C2 neurons in a way that is similar to the neural mechanism in *Tritonia* (Lillvis and Katz 2013). Thus, in *Tritonia* and *Pleurobranchaea*, parallel neuromodulatory mechanisms underlie homoplasious swimming behaviors.

Similar 5-HT receptor expression profiles may underlie the parallel neural mechanisms controlling swimming in these two species.

A third Nudipleura species, *Hermisenda crassicornis*, which is more closely related to *Tritonia* than *Pleurobranchaea* is, cannot perform DV-swimming and its C2 homologues are not modulated by 5-HT, although serotonergic DSI homologues are present (Lillvis and Katz 2013). Species differences in C2 5-HT receptor expression may play a role in species differences in DV-swimming and C2 synapse modulation.

In *Pleurobranchaea*, unlike *Tritonia*, swimming capability varies on an almost daily basis. That is, an individual *Pleurobranchaea* will swim one day, but not the next. Variability in serotonergic modulation of C2 correlated with swimming (Lillvis and Katz 2013). It is possible that individual variability in 5-HT receptor expression in *Pleurobranchaea* C2 homologues is an underlying cause of the individual differences in swimming behavior.

Using this background, my thesis tested the hypothesis that C2 homologue 5-HT receptor expression differences underlie species- and individual- differences in swimming behavior. I predict that independently evolved behaviors are accompanied by similar 5-HT receptor expression. This hypothesis was tested at several levels. At the level of species differences in behavior, DV-swimmer *Tritonia* and non-DV-swimmer *Hermisenda* C2 homologues can be compared. *Tritonia* can also be compared with *Pleurobranchaea*, to determine whether independently evolved behaviors utilize orthologous 5-HT receptor genes. Finally, C2 homologue gene expression can be compared between swimming and non-swimming *Pleurobranchaea*, to determine whether individual variability can be tracked to gene expression in single neurons.

My thesis examined two specific aims: 1) identify 5-HT receptors in whole-brain tissue from each of the three species mentioned; and 2) determine 5-HT receptor expression profiles in C2 neuron homologues from each species.

A lack of genetic data from molluscs was a major hindrance to achieving the above aims, because no genetic information on 5-HT receptor sequences was available for these species. In Chapter 2, I sequenced the transcriptome for the *Hermissenda crassicornis* brain, along with help from post-doctoral fellow and co-author Adriano Senatore. By BLAST-mining the *Hermissenda* transcriptome, along with other transcriptomes created by our lab, I identified Nudipleura orthologues of the five previously identified molluscan 5-HT receptor subtypes (Nagakura et al. 2010). I identified two additional subtypes: 5-HT2b, which was previously only identified in arthropods (Clark et al. 2004; Gasque et al. 2013); and 5-HT6, which was previously only identified in vertebrates (Peroutka and Howell 1994).

I used the 5-HT receptor subtype gene sequences identified in Chapter 2 to execute a single-neuron quantitative PCR (qPCR) study of C2 homologues in chapter 3. I found that there were differences in 5-HT receptor expression by C2 neurons between DV-swimmer *Tritonia* and non-DV-swimmer *Hermissenda*. Independently evolved DV-swimmer *Pleurobranchaea* C2 homologues shared expression of 5-HT2a and 5-HT7 with *Tritonia*. I also found that there was a difference in 5-HT receptor expression between swimming and non-swimming *Pleurobranchaea*. These results are summarized in Chapter 4, in light of what is known about 5-HT receptor evolution and the evolution of neuromodulator-mediated behaviors.

1.7 Figure Legend

Figure 1-1: Abbreviated Phylogeny of Nudipleura. Phylogenetic tree shows selected species from Nudibranchia and Nudipleura. Species swimming behavior is shown in parentheses: dorsal-ventral (DV), left-right (LR), asymmetrical swimmer (AS), flapping (F), non-swimmer (NS), unknown (?). The phylogeny is based on Goodheart et al. 2015 and Newcomb et al. 2012.

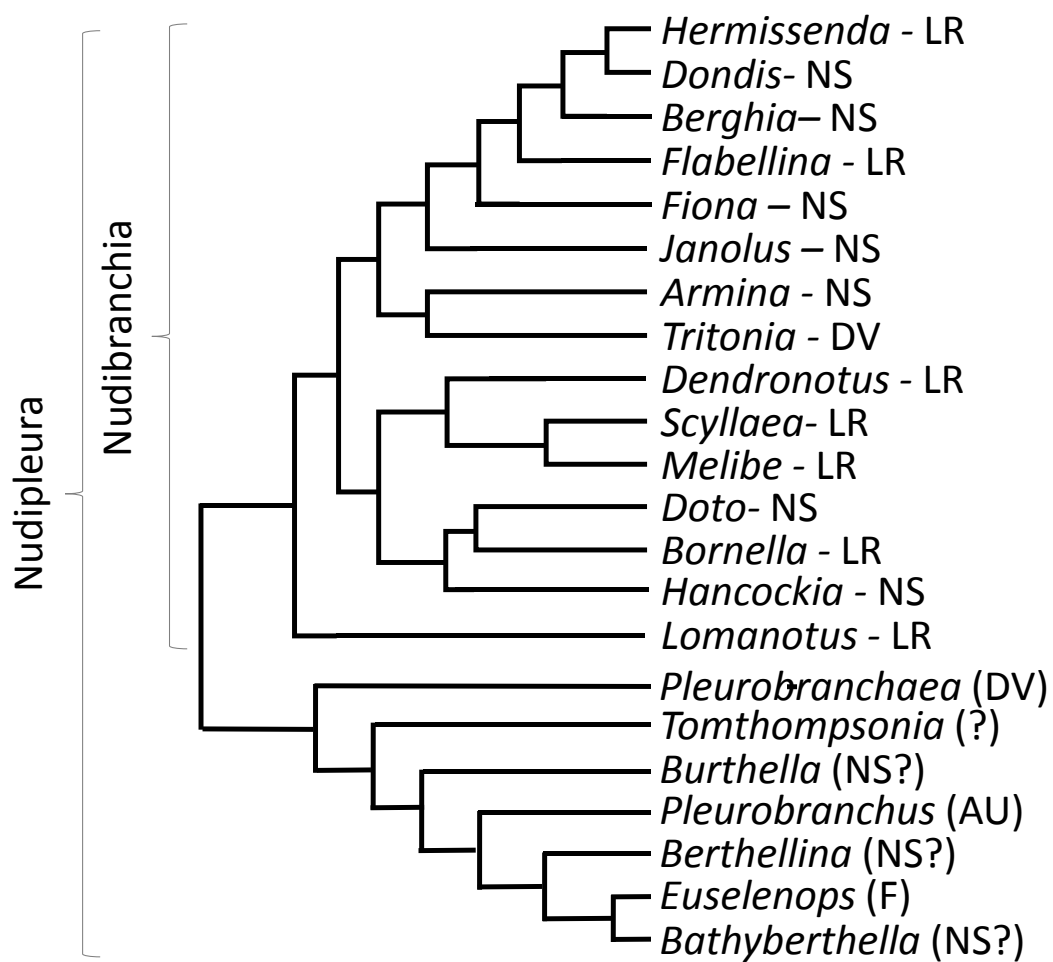


Figure 1-1: Abbreviated Phylogeny of Nudipleura

2 IDENTIFICATION OF 5-HT RECEPTOR SUBTYPE GENES EXPRESSED IN THE NUDIBRANCH BRAIN

2.1 Introduction

This chapter summarizes work to identify genes coding for 5-HT receptor subtypes in whole brain samples from *Tritonia* and *Hermissenda*. Identification of 5-HT receptor subtype was a major component of the overarching aim to identify 5-HT receptors underlying DV-swimming. Seven subtypes were found in the brains of both species, including two that were previously unidentified in molluscs. The gene sequences identified in this chapter were used in the receptor expression comparisons in the remainder of the dissertation.

To reach a broader audience with these findings, the 5-HT receptor gene sequences were published with respect to their impact on the molluscan learning and memory research community. The publication included, in addition to the information on 5-HT receptors, single-gene phylogenies on other learning and memory-related genes, and information on the *Hermissenda* whole-brain transcriptome. The publication is included here as the second thesis chapter. It was originally published as “Identification of genes related to learning and memory in the brain transcriptome of the mollusc, *Hermissenda crassicornis*” by A.N. Tamvacakis, A. Senatore, P.S. Katz, (2015) in *Journal of Learning and Memory*, Nov 16;22(12):617-21.

2.2 Abstract

The sea slug *Hermissenda crassicornis* (Mollusca, Gastropoda, Nudibranchia) has been studied extensively in associative learning paradigms. However, lack of genetic information previously hindered molecular level investigations. Here, the *Hermissenda*

brain transcriptome was sequenced and assembled *de novo*, producing 165,743 total transcripts. Homologues of 95 genes implicated in learning were identified. These included genes for a serotonin receptor and a GABA-B receptor subunit, which had not been previously described in molluscs, as well as an adenylyl cyclase gene not previously described in gastropods. This study illustrates the *Hermisenda* transcriptome's potential as an important genetic tool in future learning and memory research.

2.3 Background

Nervous systems of molluscs have been a focus of neuroscience research for many decades, yet their study has been impeded by lack of genetic information. The nudibranch *Hermisenda crassicornis* exhibits a simple form of associative learning, which has been studied extensively: it can learn to associate light changes with vestibular stimulation (Crow & Alkon, 1978). The neural correlates of *Hermisenda*'s associative memories formed during light-movement pairings have been uncovered (Alkon, 1980; Crow & Alkon, 1980; Britton & Farley, 1999; Tamse et al., 2003; Cavallo et al., 2014). *Hermisenda* has also been the subject of investigations related to many other aspects of neuroscience, including sensory and motor neuron physiology (Crow & Tian, 2004; Nesse & Clark, 2010; Jin & Crow, 2011; Crow et al., 2013) and the evolution of behaviors and neurotransmitter systems (Lillvis et al., 2012; Newcomb et al., 2012; Lillvis & Katz, 2013). A limited number of molecular-level studies have been performed on *Hermisenda* in these areas (Nelson & Alkon, 1988; Crow et al., 1997). Broader-scale genetic experiments, such as identification of genes or specific genetic isoforms that produce proteins involved in *Hermisenda* associative learning, have been impossible without more extensive genetic information specifically from *Hermisenda*.

Transcriptomes have been sequenced in a small number of other gastropod species, including neuronal transcriptomes from *Aplysia californica* (Moroz et al., 2006; Fiedler et al., 2010; Heyland et al., 2011), *Aplysia kurodai* (Lee et al., 2008; Choi et al., 2010), *Lymnaea stagnalis* (Bouetard et al., 2012; Sadamoto et al., 2012), and *Tritonia diomedea* (Senatore et al., 2015), which have been used in studies of learning and other aspects of neuroscience. The field of molluscan neuro-genetics is growing thanks in large part to recent advances in sequencing technologies, which allow larger amounts of transcriptomic information to be sequenced at lower costs compared with technologies available a few years ago.

In this study, we describe the brain transcriptome of the sea slug *Hermisenda crassicornis*. The transcriptome was sequenced from central nervous system tissue mRNA, which included the cerebropleural ganglia, pedal ganglia, optic ganglia, eyes, and statocyst hair cells. Shotgun *de novo* assembly generated 165,743 total transcripts. To illustrate its usefulness as a genetic tool, we have identified homologues of genes related to learning and memory from the *Hermisenda* transcriptome, including genes that were not previously identified in molluscs.

2.4 Methods

The central nervous system, consisting of the cerebropleural ganglia, pedal ganglia, optic ganglia, the eyes and the statocysts, was dissected from thirty-one *Hermisenda crassicornis* specimens, weighing between 1 g and 3.6 g (Monterey Bay Abalone Co.). The tissue was immediately flash-frozen in liquid nitrogen for storage at -80°C. RNA was extracted with the RNeasy Universal Plus Midi-Kit (Qiagen). RNA concentration and quality were determined by Nanodrop and Bioanalyzer 2100 (Agilent). Beckman

Genomics isolated mRNA and reverse-transcribed to cDNA, which was then PCR-amplified and barcoded for sequencing. Paired-end sequences were generated by an Illumina HiSeq 2500 high-throughput sequencer.

Raw Illumina transcript reads were analyzed by FastQC (Barbraham Bioinformatics) for transcript quality, and then trimmed to remove low quality reads using Sickle (<https://github.com/najoshi/sickle>). The remaining reads were then *de novo* assembled by Trinity software (Haas et al., 2013) (version r20140717) on the VELA high performance computer system at Georgia State University, which consists of four IBM System x3850 X5 servers running Linux. Trinity assembly was run using FASTQ sequence type, with 140 GB Jellyfish memory and 40 processors. Assembly statistics were generated using TrinityStats.pl. RSEM (Li, 2011) was used to evaluate transcript expression levels. TransDecoder (Haas et al., 2013) was used to identify candidate predicted protein coding regions within the transcripts, specifying a minimum length of 99 amino acids. A small amount of cross-contamination of cDNA from molluscan, plant, and insect species occurred during Beckman sequencing. Transcripts identified as matching a non-*Hermisenda* mollusc species were removed after transcriptome assembly, using a previously published filtering method (Senatore et al., 2015). Plant and insect transcripts were not removed, but are believed to represent a very small amount of transcripts.

BLAST+ version 2.2.29 (Camacho et al., 2009) was used either as locally installed software or on VELA. Nucleotide databases were generated from the published *Lymnaea stagnalis* transcriptome (Sadamoto et al., 2012) and *Aplysia californica* NCBI mRNA collection (Taxid: 6500) on February 22nd, 2014, whereas the *Tritonia diomedea* transcriptome shotgun assembly database (Senatore et al., 2015) was generated by our

lab. Protein databases were generated from SwissProt on May 24th, 2014 and from RefSeq on July 14th, 2014. These databases were searched by querying both the transcriptome and its TransDecoder-predicted proteins and then analyzing the results with Microsoft SQL. Functional annotations of gene ontology (GO) terms (Ashburner et al., 2000) were done using BLAST2GO (<http://www.blast2go.com/b2ghome>). KEGG (Kanehisa & Goto, 2000) pathways containing enzymes involved in learning or memory processes were then identified using the BLAST2GO interface.

Individual genes coding for proteins whose function is related to learning or memory were identified in the *Hermisenda* transcriptome by BLAST search using single gene queries from molluscan SwissProt or NCBI published genes. *Hermisenda* orthologue identity was confirmed by BLAST against NCBI or UniProtKB/SwissProt databases. Select identified gene sequences were translated to predicted amino acid open reading frames and aligned using ClustalW (Larkin et al., 2007). For membrane bound proteins, predicted transmembrane domain regions were identified using Phobius (Käll et al., 2004). For *Hermisenda* and *Tritonia* genes used in phylogenetic tree generation, predicted proteins from serotonin (5-HT) receptor sequences (see below) generated from whole brain or whole body cDNA cloning and sequencing were used. *Hermisenda* and *Tritonia* dopamine receptor protein sequences were predicted from transcriptome sequences. A 15 amino acid-long predicted protein was deleted from the *Aplysia* dopamine receptor D2 protein sequence during alignment. GABA-B receptor and adenylyl cyclase (AC) predicted proteins were generated directly from transcriptome sequences. Unrooted phylogenetic trees were created by Maximum Likelihood with Jones-Taylor-Thornton model using MEGA6.06 software (Tamura K, 2011). Branch

supports were provided using 500 bootstrap replicates. Gene identification numbers for all published genes are listed in Supplemental Table 2-3.

To clone 5-HT receptor genes, primers were designed against transcriptome 5-HT receptor sequences. Primers for 5-HT receptors are listed in Supplemental Table 2-4. Whole brain RNA was extracted as described above. cDNA was reverse-transcribed using SuperScript IV (Invitrogen). PCR amplification of 5-HT receptor genes was performed using *Taq* DNA polymerase, buffer, dNTP, and magnesium chloride from ThermoFisher. DNA bands were excised using a Zymoclean Gel DNA Recovery Kit (Zymoresearch), ligated using pGEMT Easy (Promega), and transformed into JM109 competent cells (Promega). Resulting colonies were isolated and plasmids were extracted using a GenElute plasmid mini-prep kit (Sigma). Plasmids with inserts were sequenced by a 3730xl DNA Analyzer (Life Technologies). Resulting sequences were verified against NCBI and UniProtKB/SwissProt databases. Three or more sequences resulting from separate PCRs, as well as the transcriptome sequence, were aligned using ClustalW to determine the most likely sequence for each gene.

Plasmids from *Hermisenda* 5-HT_{2a}, as well as 5-HT transporter and pedal peptide 3 precursor, were *in vitro* transcribed to make synthetic RNA using the Ambion MegaScript RNA synthesis kit (ThermoFisher). Copy number was determined using resulting synthetic RNA Nanodrop concentration and sequence information for that gene. RNA was serially diluted to volumes calculated to contain standard amounts of each RNA strand, and reverse-transcribed using SuperScript IV (Invitrogen). Absolute qPCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR system, using Perfecta SYBR green with low Rox (Quanta Biosciences). Whole brain cDNA was compared in triplicate against the RNA standards. No-reverse transcriptase controls and no-template

controls were subtracted from resulting values. To ensure that only a single amplification product was quantified, melt curves were determined for each qPCR trial, and resulting PCR products were run on a 1% agarose gel (not shown).

2.5 Results

Paired-end sequencing of *Hermissenda* mRNA generated 109M 100bp-reads. Low quality reads were trimmed using Sickle (Joshi and Fass, 2011), and reads were assembled by *de novo* assembly using Trinity (Haas et al., 2013). The 165,743 total transcripts, or contigs, were grouped in the assembly into 99,944 Trinity Chrysalis components (e.g. comp1, comp2, etc.) and 115,126 Trinity Butterfly components (e.g. comp1_c0, comp1_c1 etc.). We consider the Chrysalis components as the completely non-redundant transcript groupings, since Butterfly components originated from Chrysalis-derived De Bruijn graphs, some of which subsequently partitioned during Butterfly due to low read support (Haas et al., 2013). The average contig length of the *Hermissenda* transcriptome was 778.81 base pairs (bp) (Table 2-1). The largest contig was 35,109 bp in length. Proteins were predicted *in silico* by TransDecoder. More than half of the predicted proteins with complete open reading frames (ORFs) were 300 amino acids (aa) or longer, indicating that the transcriptome assembly contains a large number of genes coding for full-length proteins (Supplemental Figure 2-4A). Of the total number of TransDecoder predicted proteins, 4912 proteins were complete, 3515 were missing the 5' end, 1851 were missing the 3' end, and 5404 were missing both the 5' and 3' ends (Supplemental Figure 2-4A inlay).

To compare the *Hermissenda* brain transcriptome with previously published genetic information, BLAST searches were run against published data sets. Matching

components were then filtered to select the best hit per component (BHPC). The *Hermisenda* transcriptome yielded 20,152 non-redundant BHPC matches with E-values below $1e-6$ by tBLASTx (i.e. protein level alignment) against a database created from *Tritonia diomedea* brain transcriptome mRNA and 16,995 matches against an *Aplysia californica* mRNA data set (Supplemental Figure 2-4B). The invertebrate RefSeq protein dataset matched the highest number of *Hermisenda* translated nucleotide transcripts, compared with mammalian and non-mammalian vertebrate RefSeq protein datasets, as well as the SwissProt protein dataset (Supplemental Figure 2-4C). These BLAST results indicate that the *Hermisenda* transcriptome is complete in its genetic coverage relative to other published transcriptome assemblies.

Using translated protein BLAST results from the comparison with the SwissProt protein dataset, components were filtered to remove hits with E-values of $1e-3$ or greater, in order to reduce potential redundant matches or matches made by chance alone. The 12,081 resulting transcripts were subsequently uploaded to BLAST2GO (Conesa et al., 2005), which used a cut-off value of $1e-6$ to infer homology to SwissProt database sequences, followed by InterProScan, GO-SLIM, and Enzyme Code Mapping. The resulting 8,916 genes were assigned gene ontology (GO) terms (Ashburner et al., 2000). GO assignments were generated for each of the three major GO branches at BLAST2GO Level 2: molecular function, biological process, and cellular component (Supplemental Figure 2-5A). GO terms were similar at level 2 compared with the previously published *Lymnaea stagnalis* (Sadamoto et al., 2012) and *Tritonia diomedea* (Senatore et al., 2015) assemblies.

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000), enzymatic pathways were mapped via BLAST2GO-KEGG: 103 biological pathways

were identified. These pathways incorporated 1,143 transcriptome sequences, including 579 enzyme sequences. Seventeen pathways were identified as containing one or more enzymes related to learning (Supplemental Figure 2-5C). Together, these analyses indicate that the *Hermissenda* transcriptome contains a large amount of genetic information, meaning that it can be used as a new tool in the study of molluscan learning.

Several intracellular mechanisms have been identified as mediating light-turbulence associative learning in *Hermissenda*. Hair cells activated by turbulence release gamma-aminobutyric-acid (GABA) through a mechanism mediated by adenylyl cyclase (AC), protein kinase A (PKA), and calcium (Alkon et al., 1993; Tamse et al., 2003). GABA release activates type B photoreceptor metabotropic GABA receptors, and when paired with light stimulation enhances photoreceptor response. Serotonin (5-HT) stimulation from interneurons paired with light also enhances photoreceptor responses. Within the photoreceptors, the light-turbulence pairing and subsequent GABA and 5-HT release activates pathways involving IP₃/DAG, protein kinase C (PKC), and mitogen activated protein kinase (MAPK), and elevation of arachidonic acid (AA) (Supplemental Figure 2-6). This leads to an increase in calcium within photoreceptors, which amplifies their excitability and is a correlate of memory storage (Blackwell, 2006; Blackwell & Farley, 2008). The specific genes underlying these protein interactions have not, however, been identified previously in *Hermissenda*. We have used the proposed model for *Hermissenda* associative learning as a basis to identify genes that code for proteins related to these changes (Supplemental Table 2-2), and have highlighted three categories of genes using phylogenetic analyses here.

5-HT modulates *Hermissenda* photoreceptors and interneurons during paired light and turbulence associative learning (Jin et al., 2009). Therefore, we investigated 5-

HT receptor genes in *Hermisenda*. Four families of 5-HT receptors genes (families 1, 2, 4, and 7) had been previously identified in molluscs (Nagakura et al., 2010) and a fifth family (family 6) had been predicted from the *Aplysia* genome (XP_005105784.1). The 5-HT1 gene has undergone a duplication event in molluscs, resulting in 5-HT1a and 5-HT1b receptors (Nagakura et al., 2010). No 5-HT receptor genes had been previously identified in *Hermisenda* or other nudibranchs.

The *Hermisenda* brain transcriptome revealed putative homologues of each of the previously identified receptor genes, 5-HT1a, -1b, -2, -4, and -7. It also uncovered a 5-HT6 homologue, which had previously not been identified as expressed from mRNA in any invertebrate, to our knowledge. A maximum likelihood phylogenetic tree shows that all of the families cluster with other known receptor genes (Figure 2-1A). As previously shown, 5-HT2 receptors clustered with dopamine D2 receptors, whereas 5-HT6 genes were more closely related to dopamine D1 receptors (Nagakura et al., 2010; Mustard et al., 2005; Spielman et al., 2015).

We confirmed the existence of the 5-HT6 gene in the *Aplysia* (aplysiagenetools.org) and *Tritonia* brain transcriptomes. DNA plasmids were generated by three or more independent PCR reactions, followed by cloning to pGEM-T Easy vectors. Each plasmid was sequenced, and sequences were aligned using ClustalW to verify that sequence identity matched between the transcriptomes and the cDNA. *Hermisenda* 5-HT 1a, 1b, 4, 6, and 7 receptor genes were sequenced. *Tritonia* 5-HT 1a, 1b, 4, 6, and 7 receptor genes were also sequenced. The resulting PCR-generated DNA sequences were ~98% identical to the transcriptome sequences by ClustalW alignment (data not shown).

The transcriptome also contained a novel isoform of the 5-HT₂ family, referred to here as 5-HT_{2b}. The previously identified molluscan 5-HT₂ type gene will be referred to as 5-HT_{2a} here. The 5-HT_{2b_{Herm}} gene fragment predicted a 335 amino acid-long protein containing transmembrane domains 1-5, only, and aligned by BLAST to arthropod 5-HT_{2b} receptors. The 5-HT_{2a_{Herm}} gene was fragmented between two contigs: comp74520_co_seq1 predicted a 314 amino acid protein containing transmembrane domains 1-5, while comp77190_co_seq1 predicted a 383 amino acid protein, which coded for intracellular loop 5 and transmembrane domains 6-7. To our knowledge, 5-HT_{2b} has not been previously identified in any other mollusc. To be assured that the gene is molluscan, we identified 5-HT_{2b} homologues using BLAST searches in the *Aplysia* (aplysiagenetools.org) and *Tritonia* brain transcriptomes. Phylogenetic analysis and BLAST comparisons revealed that the molluscan 5-HT_{2b} receptor clustered more closely with the arthropod 5-HT_{2b} gene than with the molluscan 5-HT_{2a} gene. Note that although the 5-HT₂ receptors in invertebrates and vertebrates form a cluster, the nomenclature for 5-HT₂ subtypes in vertebrates and invertebrates does not correspond because the genes diverged in each group after the protostome/deuterostome split (Peroutka and Howell, 1994).

Interestingly the crustacean 5-HT_{2b} protein contains a highly derived DRF-motif, which causes constitutive activity of the associated G-protein activation site (Clark et al., 2004), instead of a DRY-motif G-protein activation site, which is more common in 5-HT receptors. The DRF-motif was also found in the molluscan 5-HT_{2b} predicted proteins (Figure 2-1C), which might be further indication that the 5-HT_{2b} gene evolved before the lophotrochozoan-ecdysozoan split. Alternatively, it could indicate parallel evolution of G-

protein activation site motifs. The identification of a 5-HT_{2b} gene may provide useful information about the functions of 5-HT receptors in molluscan learning.

We PCR amplified, cloned, and sequenced 5-HT_{2a} and 5-HT_{2b} genes from cDNA generated from *Hermissenda* and *Tritonia* brains and from *Hermissenda* whole-body cDNA. In *Hermissenda*, the initial PCR amplification indicated that 5-HT_{2a} and 2b genes were more highly expressed in whole-body cDNA compared with brain cDNA. This indicates that while 5-HT_{2a} and 2b are expressed at very low levels in the *Hermissenda* brain, they are expressed at higher levels elsewhere in the body. RSEM (Li, 2011), a program that estimates relative abundance of genes in the transcriptome by providing transcripts per million (TPM) values, indicated that these genes were in very low abundance in the brain (see RSEM Relative Transcriptome Abundance, Supplemental Table 2-2; *Tritonia* RSEM abundance not shown). To verify the RSEM data, we compared 5-HT_{2a}_{Her} (Table 2-2, TPM 3.38) with the *Hermissenda* 5-HT transporter (Table 2-2, TPM 166.17), and the *Hermissenda* Pedal peptide 3 precursor (contig ID comp64571_co_seq1, TPM 1264.32). Using absolute real-time quantitative PCR (qPCR), we found that there was a strong correlation between the RSEM-predicted expression level in the transcriptome and the amount measured from whole brain tissue by qPCR (Supplemental Figure 2-7).

GABA is another neurotransmitter that modulates light-movement paired learning (Schultz & Clark, 1997; Blackwell, 2002). No GABA receptor genes had been previously described from *Hermissenda*. Metabotropic GABA type B receptor subunits 1 (GABA-BR1) and 2 (GABA-BR2) were predicted from the *Aplysia* genome (XM_005092746.1 and XM_005109637.1) and a GABA-BR1 subunit gene was described in the *Tritonia* brain transcriptome (Senatore et al., 2015). The BR1 and BR2 subunits most likely function as

heterodimers, as they do in other species (Kammerer et al., 1999). A GABA-BR3 subunit was identified in *Drosophila* (Mezler et al., 2001), but had not been previously identified in any molluscs. We identified GABA-BRs in the *Hermisenda* transcriptome, and verified sequences by tBLASTx or BLASTx against NCBI and UniProt/SwissProt databases. GABA-BR1 genes were identified in the *Hermisenda*, as well as the *Aplysia* brain transcriptomes. GABA-BR2 genes were identified in *Hermisenda*, *Tritonia*, and *Aplysia* transcriptomes. A GABA-BR3 subunit was also identified in the brain transcriptomes of each of the three species, which aligns more closely with the *Drosophila* GABA-BR3 subunit than it does with molluscan GABA-BR1 or GABA-BR2 subunits (Figure 2-2). The function of the GABA-BR3 gene in *Drosophila* is unknown, however (Mezler et al., 2001), so we cannot predict whether this gene is involved in molluscan learning, although the newly identified GABA-BR1_{Herm} and GABA-BR2_{Herm} genes most likely play roles in mediating *Hermisenda* light-vestibular stimulation pairing.

Adenylyl cyclase (AC) is part of the second messenger system required for memory formation, and is involved in changes within the pre-synaptic hair cells that occur during *Hermisenda* associative learning (Tamse et al., 2003). In *Aplysia*, four AC genes had been previously identified from families known as AC1, AC5/6, AC2/4/7, and AC9 (Sossin & Abrams, 2009; Lin et al., 2010). Of those genes, the AC1 family is calcium-sensitive, and is involved in learning (Abrams, 1985). Homologues of each of the four AC genes were identified in the *Hermisenda* and *Tritonia* transcriptomes (Figure 2-3). The *Hermisenda* and *Tritonia* AC genes were fragmented into several contigs, which spanned portions of the C1 region or transmembrane spanning domains. It may be that the transcriptome assembly did not detect the entire genes, since it would be unlikely that all known gene homologues are truncated in these species. The fragmentation of the

molluscan genes may have caused the resulting phylogenetic tree in Figure 2-3 to indicate that *Drosophila* and rat AC genes are more closely related to one another than they actually are. *Hermisenda* and *Tritonia* AC genes were named here using the naming convention previously published for *Aplysia* (Sossin & Abrams, 2009).

In addition to the four previously identified AC genes, a fifth family, known as AC3, has been predicted from *Aplysia* genomic DNA (NCBI reference XM_005108194.1) and was also identified in the *Lottia gigantea* genome (Sossin & Abrams, 2009), but had not been identified as being expressed as mRNA in molluscs. The *Hermisenda* transcriptome contained a homologue of the AC3 gene. A similar gene was also found in the *Aplysia* transcriptome, although no AC3 gene was found in the *Tritonia* transcriptome (Figure 2-3). *Tritonia*'s missing AC3 gene may be explained by an incomplete transcriptome, but could also be because the orthologue was lost in that species.

2.6 Discussion

In conclusion, we have sequenced and *de novo* assembled the *Hermisenda crassicornis* brain transcriptome, generating 165,743 total transcripts. Using BLAST annotations of the assembled transcriptome and its TransDecoder-predicted proteins, the *Hermisenda* transcriptome was found to be highly similar in gene content to other published transcriptomes. BLAST searches against SwissProt and RefSeq, in combination with BLAST2GO annotation, revealed a large number of transcripts that are likely homologues of previously published genes. Although a portion of the transcripts revealed fragmented genes, TransDecoder analysis showed that at least 50% of predicted proteins greater than 300 amino acids in length were full length proteins. As assembly methods

continue to improve, greater assembly coverage may be attained using this transcriptome, therefore we have published the raw transcriptome data to NCBI BioProject PRJNA270545, and have published a BLAST-searchable assembly at <http://neuroscience.gsu.edu/blast/>.

To illustrate its power as a tool for learning and memory research, we selected genes that produce proteins involved in learning and mined their homologues from the *Hermisenda* transcriptome. We identified 95 genes by BLAST analysis as homologues of learning-related genes. Of those, we highlighted three families of genes by phylogenetic analysis: 5-HT receptors, GABA-B receptors, and ACs. Within each gene family, one or more genes were identified that are novel for molluscs. Their identification indicates that the *Hermisenda* transcriptome is not only able to identify homologues of *Aplysia* learning and memory genes, but can advance the field of molluscan learning and memory studies by identifying genes previously not described in molluscs. Gastropod molluscs are useful research subjects because their brains contain relatively simple neural circuits composed of identifiable neurons, but their value as a genetic research model has been limited to a few species. The *Hermisenda* transcriptome will increase knowledge of the neural correlates of learning by providing new genetic information and a basis for gene manipulation that was not previously possible, therefore making it a beneficial tool for learning and other molluscan research in the future.

2.7 Figure and Table Legends

Figure 2-1: 5-HT Receptor Phylogenetic Relationships. (A) Maximum likelihood phylogenetic tree showing relationships between receptor subtypes. Bootstrap values are shown for each node. Subunits were aligned using transmembrane regions 1-5 and 6-7,

or 1-5 only for fragmented receptor subtypes. Receptors that were identified from *Hermisenda* or *Tritonia* are shown in bold and marked with an asterisk. Receptors that were identified as novel in molluscs (5-HT2b) or invertebrates (5-HT6) are shown in bold and underlined text. The dopamine receptors D1 and D2 are also shown, as they are part of the superfamily of aminergic receptors. (B) 5-HT receptor model showing seven transmembrane domain regions with location of predicted ligand binding site and G-protein activation site shown. (C) 5-HT2b ClustalW alignment shows location of predicted ligand binding conserved cysteine residue, shaded in light grey, and predicted G-protein activation site shaded in light grey for DRY motif, dark grey for DRF motif. Species abbreviations: *Hermisenda crassicornis* (*Herm*), *Tritonia diomedea* (*Trit*), *Aplysia californica* (*Aply*), *Drosophila melanogaster* (*Dros*), *Rattus norvegicus* (*Rat*), *Procambarus clarkii* (*Proc*).

Figure 2-2: Phylogenetic Relationships between GABA-B Receptor Subunits.

(A) Diagram of GABA-B receptor subunits, with predicted GABA-BR_{3Herm}. The seven transmembrane domains, heterodimeric C-terminals, and venus-flytrap motif N-terminals are shown. (B) Maximum likelihood phylogenetic tree of GABA-B receptor subunits. Subunits were aligned using transmembrane regions 1-7. Bootstrap values are shown for each node. Receptor subunits identified for *Hermisenda*, *Tritonia*, or *Aplysia* are shown in bold and marked with an asterisk. Receptor subunits that were identified as novel in molluscs are shown in bold and underlined text. Species abbreviations: *Hermisenda crassicornis* (*Herm*), *Tritonia diomedea* (*Trit*), *Aplysia californica* (*Aply*), *Drosophila melanogaster* (*Dros*), *Rattus norvegicus* (*Rat*).

Figure 2-3: Phylogenetic Relationships between Adenylyl Cyclase (AC)

Genes. Maximum likelihood phylogenetic tree showing relationship between AC families. Bootstrap values are shown for each node. Genes identified as novel for *Hermisenda* and *Tritonia* are shown in bold and marked with an asterisk. Genes that were identified as novel in gastropods are shown in bold underlined text. Fragments of the *Hermisenda* and *Tritonia* genes, and the *Aplysia* AC3 gene, were identified from their transcriptomes and aligned against full length homologues from *Aplysia*, *Drosophila*, and rat to determine their orientation. Most fragments aligned to the C1 region of the gene or to one of the transmembrane domain spanning regions. For previously published species, full-length proteins were used in the alignment. Species abbreviations: *Hermisenda crassicornis* (*Herm*), *Tritonia diomedea* (*Trit*), *Aplysia californica* (*Aply*), *Lottia gigantea* (*Lott*), *Drosophila melanogaster* (*Dros*), *Rattus norvegicus* (*Rat*).

Supplemental Figure 2-4: TransDecoder and BLAST Comparisons. (A)

TransDecoder-predicted peptides from the *Hermisenda* transcriptome assembly. Predicted proteins are separated to show numbers of predicted complete and incomplete open reading frames. Inlay shows cumulative histogram of TransDecoder predicted proteins. There were 4912 complete sequences, 3515 that were missing the 5' end and 1851 that were missing the 3' end. Most of the fragments with just internal sequences are under 200 amino acids in length. (B) BLAST comparisons of the *Hermisenda* transcriptome assembly are shown against published mRNA databases from *Tritonia*, *Aplysia*, and *Lymnaea* with E-values less than $1e-6$, using as query *Hermisenda* transcriptome (white bars, tBLASTx) or *Hermisenda* predicted peptides (light grey bars,

tBLASTn). (C) BLAST comparisons of the *Hermissenda* transcriptome assembly are shown against published protein databases with E-values less than $1e-6$, using as query *Hermissenda* transcriptome cDNA (dark grey bars, BLASTx) or *Hermissenda* predicted peptides (black bars, BLASTp). Protein database abbreviations: Invertebrate RefSeq database (Invert RS), Mammalian RefSeq database (Mamm RS), Non-mammalian vertebrate RefSeq database (Non-mamm vert RS), and SwissProt database (SwissProt). See supplemental methods for database details.

Supplemental Figure 2-5: GO-terms Mapped to the *Hermissenda* Transcriptome. (A) Gene ontology (GO) comparisons were done at BLAST2GO level 2 for molecular functions, biological processes, and cellular processes. (B) Selected additional GO-terms are shown from levels 3 to 8. (C) Selected KEGG-pathway predicted enzymes related to learning and memory were calculated using BLAST2GO.

Supplemental Figure 2-6: Model of *Hermissenda* Associative-Learning Pathways, Including Gene Products Predicted From the *Hermissenda* Transcriptome. Genes identified in Supplemental Table 2-2 were used to re-create an associative learning pathway model that has been previously described (Tamse et al., 2003; Blackwell & Farley, 2008). Solid black lines represent known pathways, dashed lines indicate hypothesized pathways. Gene and metabolic product abbreviations: 5-HT (serotonin), GABA (gamma-aminobutyric acid), DAG (diacylglycerol), IP₃ (inositol triphosphate), IP₃R (inositol triphosphate receptor), AC (adenylyl cyclase), cAMP (cyclic adenosine monophosphate), PLC (phospholipase C), PLA₂ (phospholipase 2), AA (arachidonic acid), RyR (ryanodine receptor), PKC (protein kinase C), MAPK (mitogen-

activated protein kinase), MEK (ERK-activating kinase), PKA (protein kinase A), Ca (calcium).

Supplemental Figure 2-7: Comparison of RSEM Values and qPCR-Derived Absolute Copy Numbers for Three *Hermissenda* Genes. 5-HT_{2a}, 5-HT transporter, and Pedal peptide 3 precursor gene synthetic RNA standards were used to determine the absolute copy number for each gene in whole brain cDNA. The absolute copy numbers for each gene correlated with their RSEM transcripts per million reads (TPM) values with an r^2 value of 0.9999436. Error bars represent standard deviation.

Table 2-1: *Hermissenda crassicornis* Brain Transcriptome Assembly Statistics. The *de novo* assembly of trimmed reads. (A) Transcriptome size and content. (B) Contig size and quality based on entire assembled transcriptome, or (C) based on longest isoform per component.

Supplemental Table 2-2: Select Learning- Related Genes With Homologues Identified In The *Hermissenda* Brain Transcriptome. Molluscan genes related to learning or memory were selected from the UniProt/SwissProt database and BLAST searched against the *Hermissenda* transcriptome. *Hermissenda* gene homologues identified are listed as their contig identification number, and include their transcript length in base pairs (bp), their predicted protein open reading frame (ORF) length in amino acids (aa), and their RSEM-estimated relative abundance in the *Hermissenda* transcriptome as transcripts per million mapped reads (TPM) value. The predicted protein length for each identified gene was compared against published full length genes

from other species: if the published gene was 100 or more amino acids longer than the *Hermisenda* predicted gene, then the *Hermisenda* gene was considered a fragment, and was labeled next to the ORF length in the table. If multiple fragments were identified as being part of the same gene, then their contigs are listed in single cells.

Supplemental Table 2-3: Gene Identification Numbers for Previously Published Genes. The gene identification number and the database location it was retrieved from are listed.

Supplemental Table 2-4: Primer Sequences for 5-HT Receptor Verification from Whole Brain cDNA.

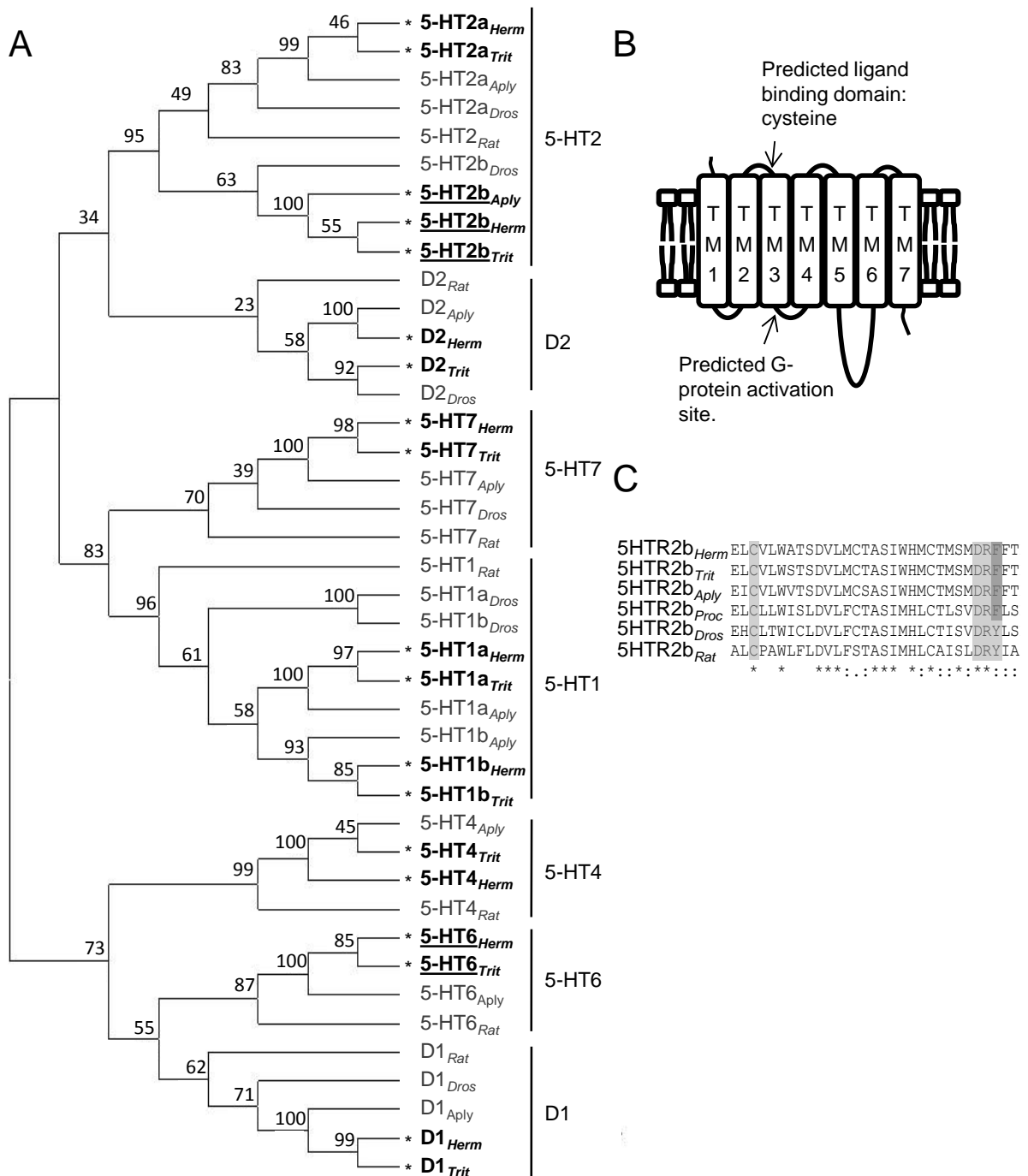


Figure 2-1: 5-HT Receptor Phylogenetic Relationships.

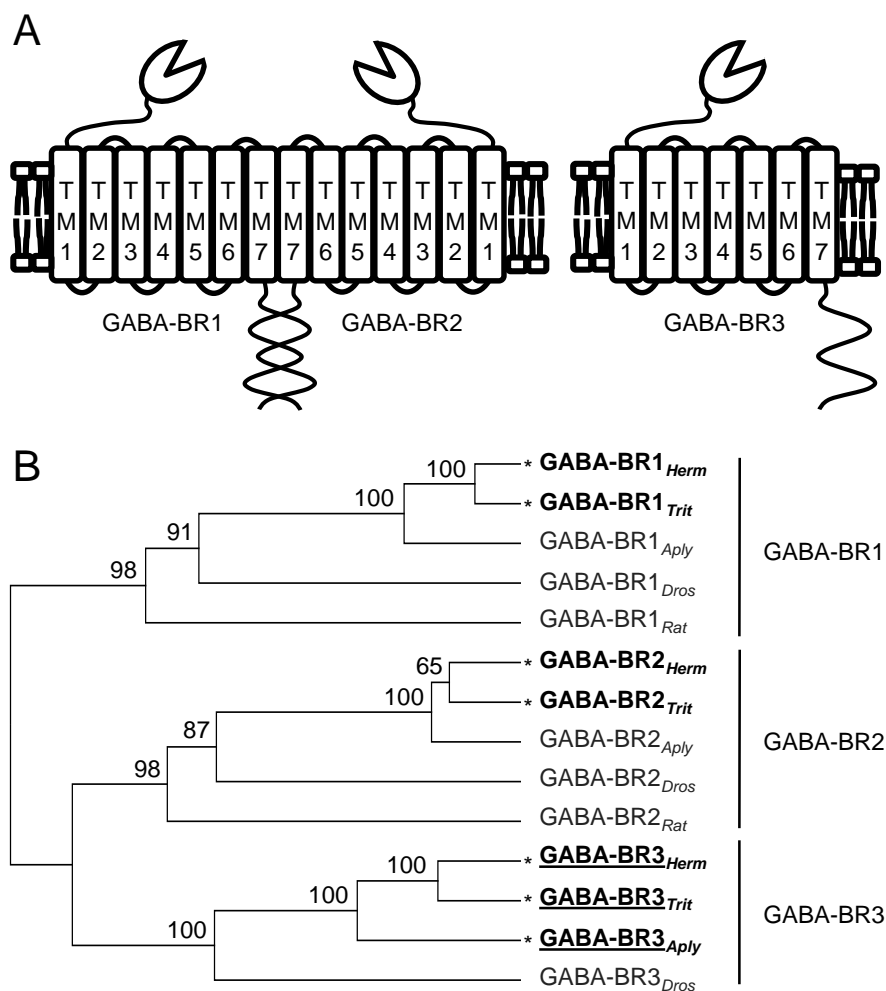


Figure 2-2: Phylogenetic Relationships between GABA-B Receptor Subunits.

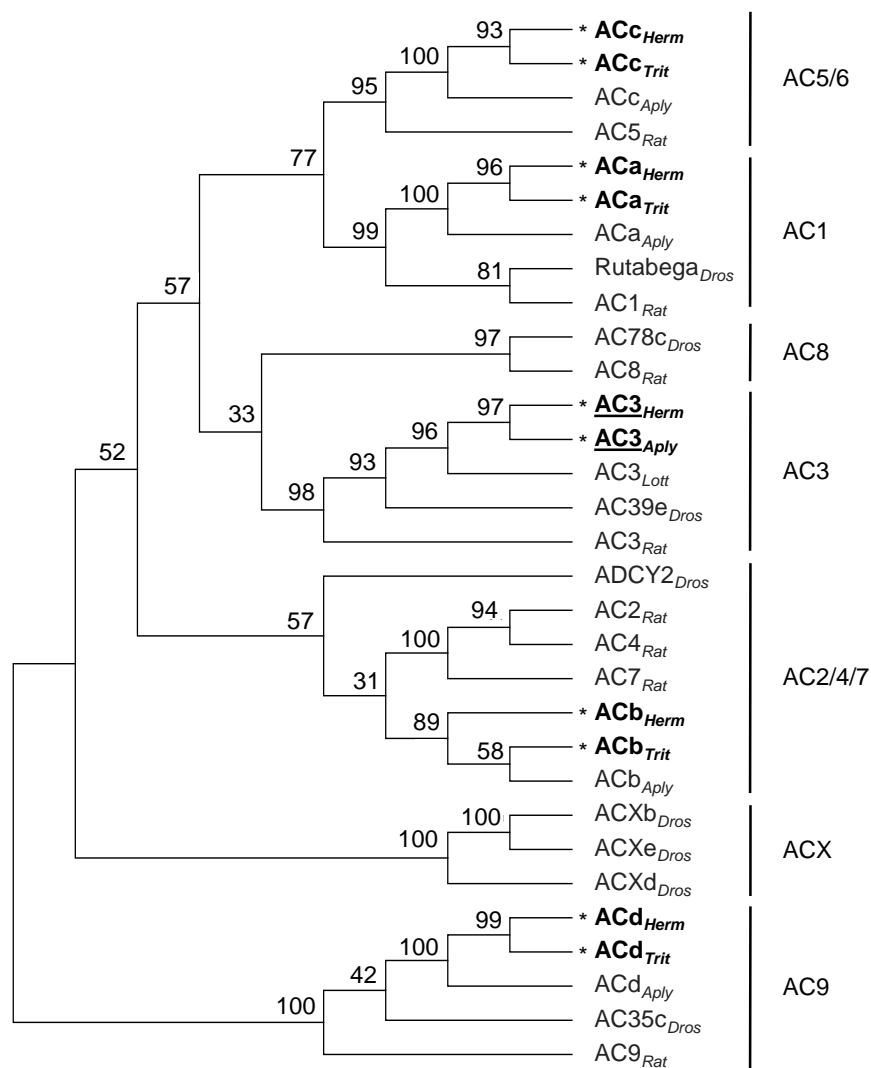
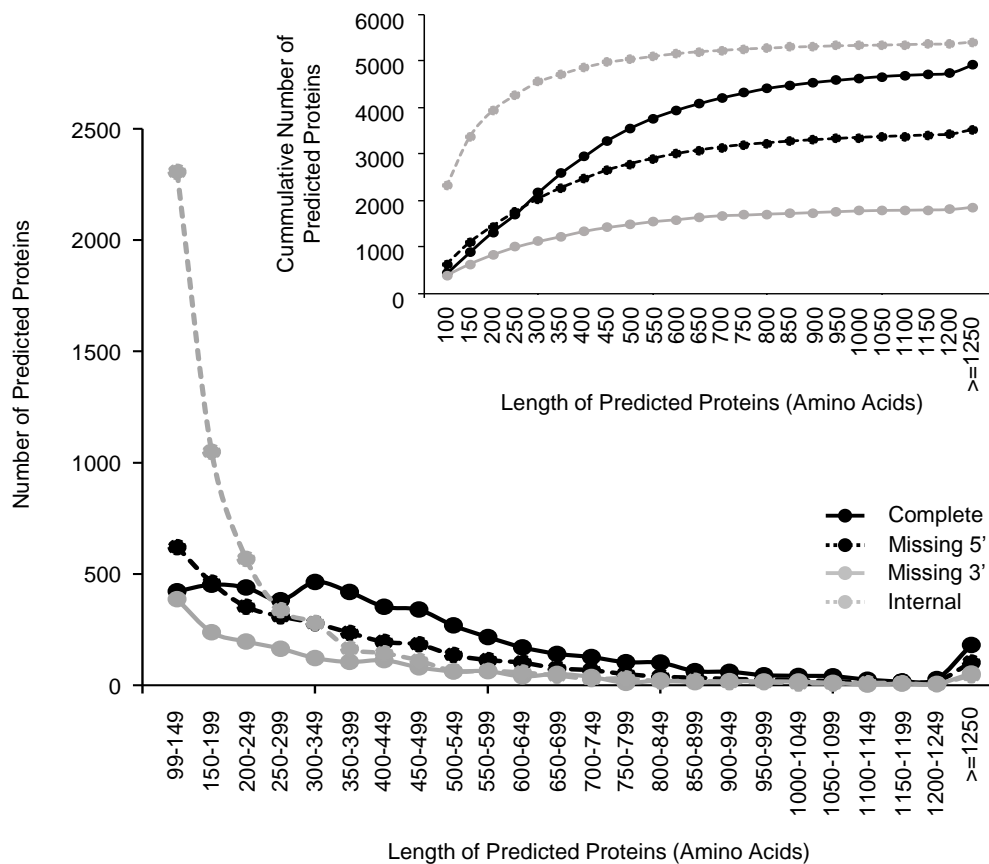
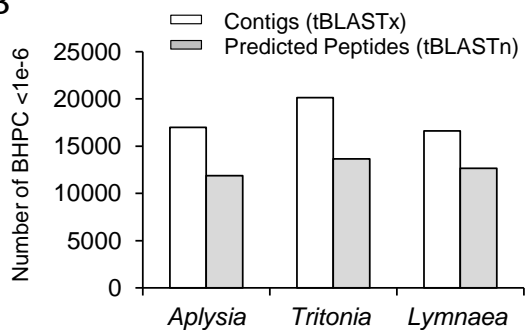


Figure 2-3: Phylogenetic Relationships between Adenylyl Cyclase (AC) Genes.

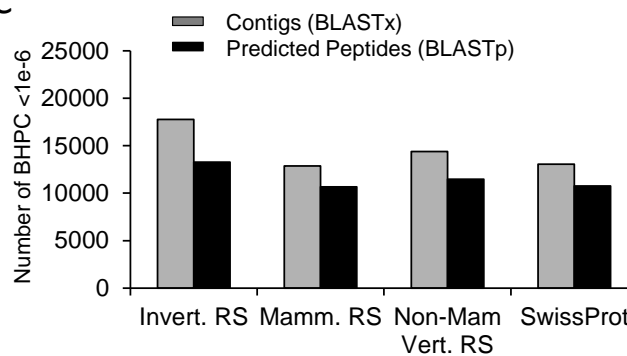
A



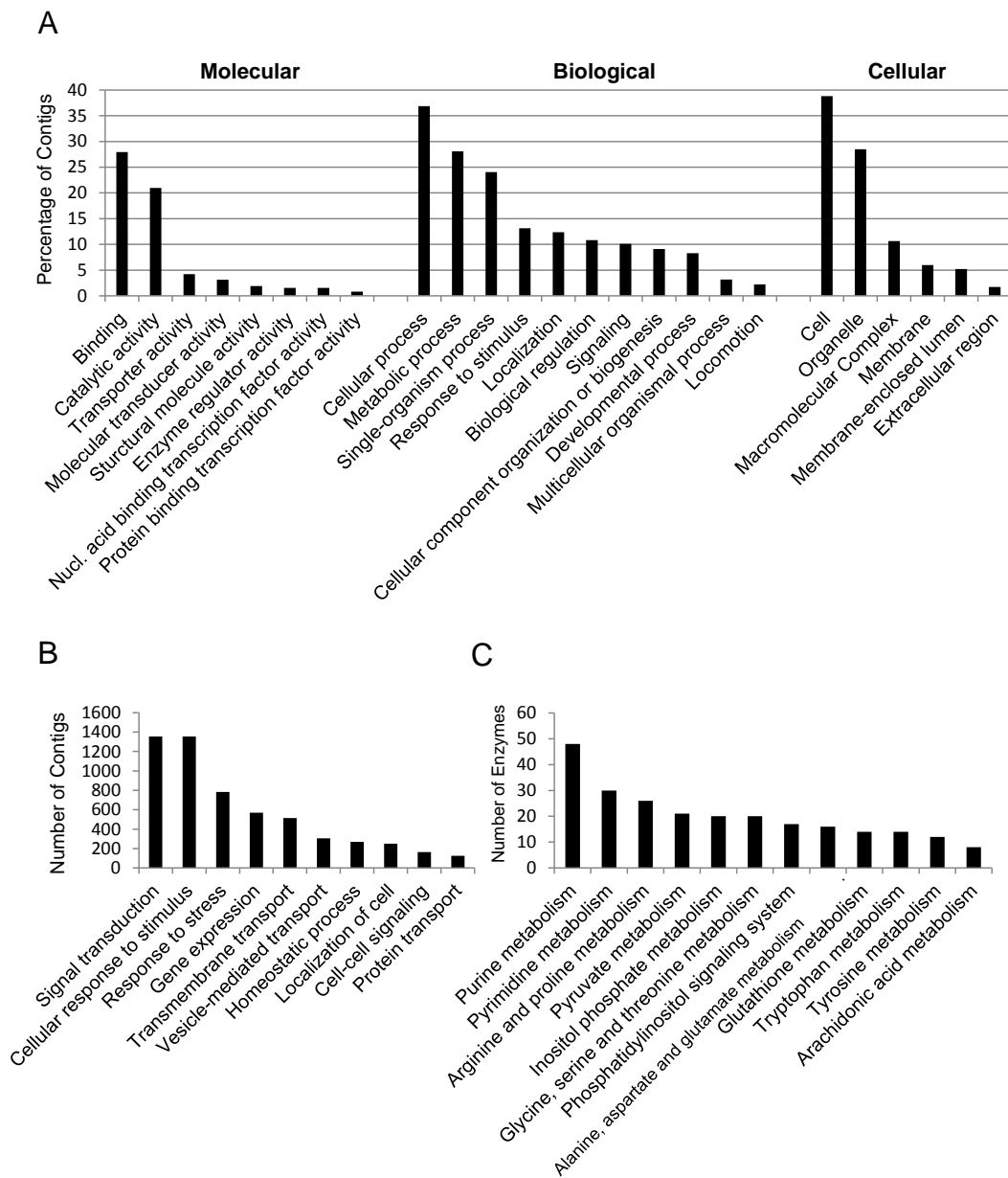
B



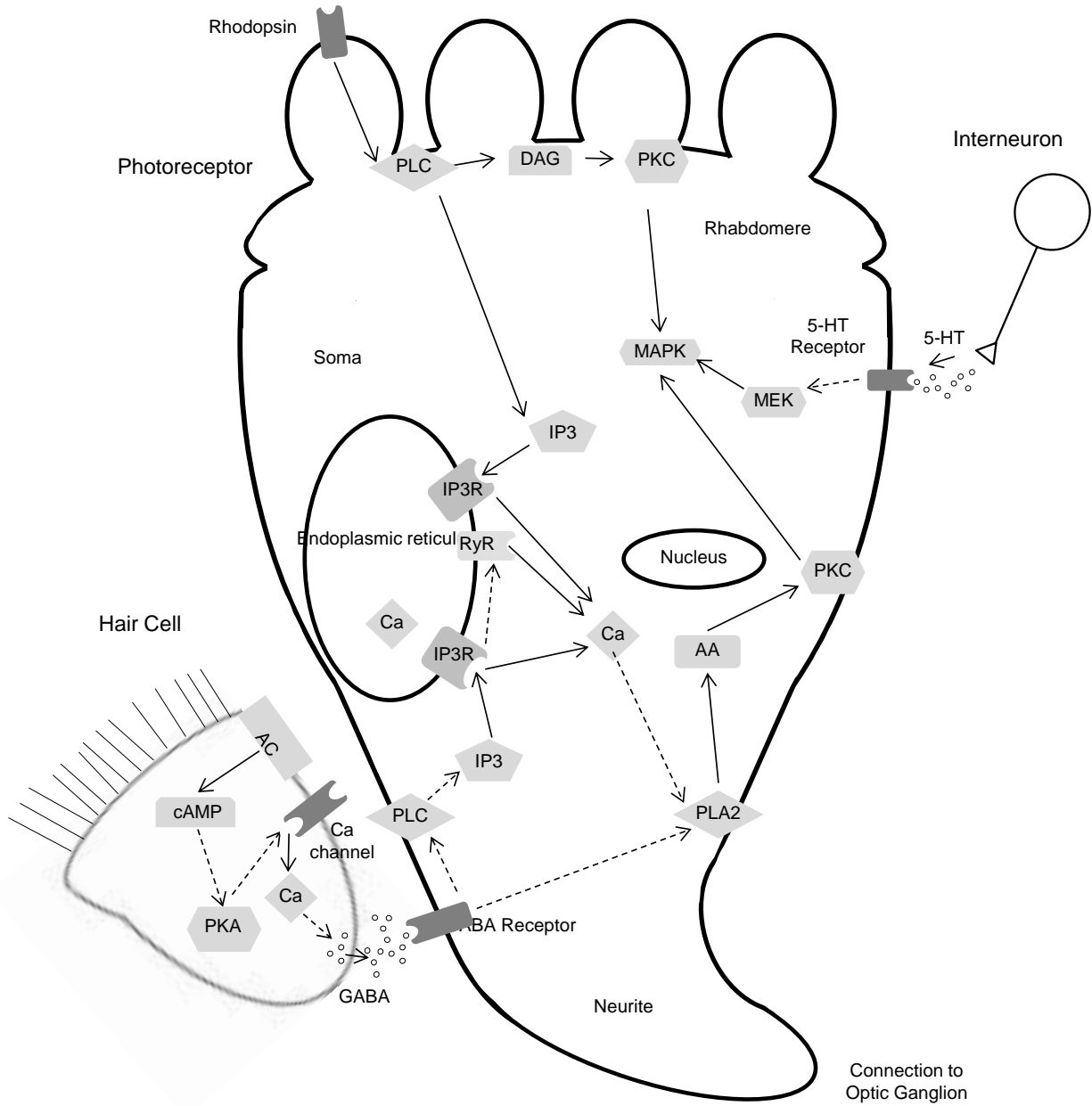
C



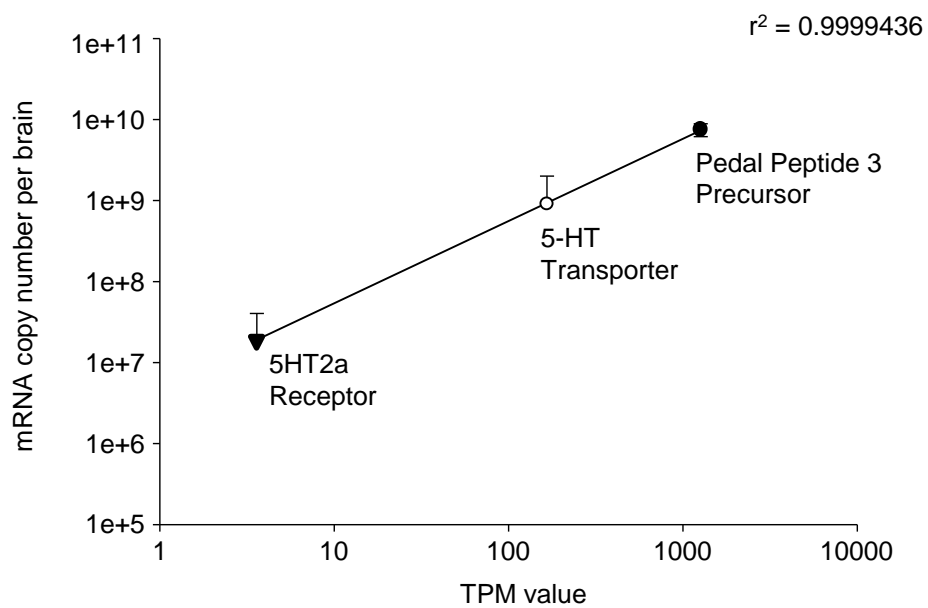
Supplemental Figure 2-4: TransDecoder and BLAST Comparisons.



Supplemental Figure 2-5: GO-terms Mapped to the *Hermisenda* Transcriptome.



Supplemental Figure 2-6: Model of Hermissenda Associative-Learning Pathways, Including Gene Products Predicted from the Hermissenda Transcriptome.



Supplemental Figure 2-7: Comparison of RSEM Values and qPCR-Derived Absolute Copy Numbers for Three Hermissenda Genes.

Table 2-1: *Hermissenda crassicornis* Brain Transcriptome Assembly Statistics

(A) Description of Transcripts	
Total transcripts:	165,743
Total Chrysalis components:	99,944
Total Butterfly components:	115,126
Percent GC:	39.51

(B) Based on All Transcripts (bp)	
Median contig length:	389
Average contig:	778.1
Total assembled bases:	128,965,468
Contig N ₁₀ :	4,851
Contig N ₂₀ :	3,341
Contig N ₃₀ :	2,483
Contig N ₄₀ :	1,880
Contig N ₅₀ :	1,400

(C) Based on Longest Isoform per Sub-Component (bp)	
Median contig length:	329
Average contig:	604.5
Total assembled bases:	69,593,541
Contig N ₁₀ :	3,831
Contig N ₂₀ :	2,566
Contig N ₃₀ :	1,842
Contig N ₄₀ :	1,300
Contig N ₅₀ :	903

Supplemental Table 2-2: Select Learning- Related Genes with Homologues Identified in The Hermisenda Brain Transcriptome.

Protein names	Contig ID number	Contig Length (bp)	Predicted Protein ORF Length (aa)	RSEM Relative Transcriptome Abundance (TPM)
Serotonin receptor 5HT1a	comp75907_co_seq1	2484	522	2.54
Serotonin receptor 5HT1b	comp80902_c1_seq2	1908	544	4.19
Serotonin receptor 5HT2a	comp74520_co_seq1, comp77190_co_seq1	1229, 1544	314 (fragment), 383 (fragment)	3.38, 3.81
Serotonin receptor 5HT2b	comp79654_c1_seq1	1582	335 (fragment)	0
Serotonin receptor 5HT4	comp77096_c1_seq1	3988	381	1.59
Serotonin receptor 5HT6	comp87935_co_seq1	3647	347	6.53
Serotonin receptor 5HT7	comp76525_c1_seq1	2819	522	1.87
GABA receptor B subunit 1	comp88149_c3_seq4	2761	838	1.94
GABA receptor B subunit 2	comp81756_c2_seq2	2812	877	0.95
GABA receptor B subunit 3	comp82183_co_seq1	2398	783	2.38
GABA receptor A subunit alpha	comp78604_c1_seq1	1946	490	39.38
	comp87192_co_seq3	3824	449	11.97
	comp85609_co_seq1	2074	629	9.35
GABA receptor A subunit beta	comp77774_co_seq2	2331	503	13.66
Dopamine D1 receptor	comp78910_co_seq1	2561	421	3.84
Dopamine D2 receptor	comp72330_c1_seq1	1770	355	17.28
Glycine receptor subunit	comp87365_c3_seq3	2913	451	1.9
	comp84820_co_seq5	2383	428	8.5
	comp86462_co_seq1	3095	439	8.54
	comp85632_co_seq2	1514	405	15
	comp85691_co_seq6	1686	485	3.61
	comp83920_co_seq4	1448	322	2.02

NMDA-like glutamate receptor subunit 1	comp88311_co_seq1	6328	990	16.38
NMDA-like glutamate receptor subunit 2b/epsilon	comp84136_co_seq1	3790	985	2.8
	comp81259_c3_seq3	3969	963	1.81
	comp87786_c2_seq1	3771	842	8.24
	comp84692_c1_seq1	3102	632 (fragment)	3.81
NMDA-like glutamate receptor 3A subunit	comp80220_c1_seq2	4045	1095	0
	comp85214_c1_seq11	6637	1676	2.68
AMPA-like glutamate receptor subunit 3 or 4	comp77836_c3_seq7	3732	929	17.4
AMPA-like glutamate receptor subunit 1 or 2	comp85099_co_seq6	3266	920	5.86
AMPA-like glutamate receptor subunit, class unclear	comp87697_co_seq1	2152	919	11.94
	comp84942_c2_seq1	711	230 (fragment)	4.67
	comp85436_c2_seq1	3844	905	23.5
Kainate-like glutamate receptor subunit, class unclear	comp87624_co_seq12	3723	760	0.17
	comp81488_co_seq5	2774	709	1.97
	comp88219_co_seq1	7096	918	14.76
	comp53416_co_seq1, comp73915_co_seq2	1113, 714	364 (fragment), 214 (fragment)	1.8, 1.38
	comp87976_co_seq2	3766	922	9.42
	comp79834_co_seq1	1724	510 (fragment)	3.01
	comp71700_co_seq1	1278	382 (fragment)	1.68
	comp86103_co_seq1	2171	580 (fragment)	9.56
	comp45483_c1_seq1	546	147 (fragment)	0.45
Ionotropic glutamate receptor, class unclear	comp77775_co_seq1	3591	901	21.42
Metabotropic glutamate receptor, class unclear	comp89428_co_seq1, comp64312_co_seq1	1281, 959	336 (fragment), 279 (fragment)	2.04, 1.8

	comp77788_co_seq1	3418	909	0
	comp93998_co_seq1, comp72401_c1_seq1	294, 1976	61 (fragment), 539 (fragment)	.79, 1.96
	comp64725_co_seq1	2399	772	1.28
	comp80942_c1_seq1	2710	833	14.63
	comp68453_co_seq1	1650	425 (fragment)	2.27
TRP channel	comp84209_co_seq6	2870	953	3.28
	comp86024_co_seq1, comp85557_co_seq3	2347, 1559	618 (fragment), 511 (fragment)	3.91, 4.63
	comp72133_c1_seq1	1096	345 (fragment)	1.11
Ryanodine receptor	comp85614_c1_seq8	11566	2818	4
	comp86886_c1_seq3	3789	1235 (fragment)	5.99
IP3 Receptor	comp84922_co_seq3	6123	800 (fragment)	5.92
	comp88019_c1_seq5, comp86034_co_seq5	2867, 5033	935 (fragment), 1381 (fragment)	3.58, 0.88
	comp85064_co_seq3	4710	1350	2.84
Serotonin Transporter	comp80597_c1_seq1	4027	627	166.17
Glycine Transporter, Na and Cl dependent	comp76784_co_seq1	2772	677	13.45
	comp87032_co_seq2	3414	743	5.53
	comp88337_co_seq3	6252	631	0.52
	comp87711_co_seq7	6641	519	7.88
	comp81128_c2_seq1	2737	602	6.7
	comp66058_co_seq1	1933	617	1.51
Taurine Transporter	comp82773_co_seq1	5110	426	21.58
GABA transporter, Na and Cl dependent	comp82343_c2_seq6	2757	522	1.85
	comp87809_c1_seq2	2033	627	6.25
Catecholamine transporter, Na and Cl dependent	comp80019_co_seq2	2560	579	3.35
	comp93073_co_seq1, comp34995_co_seq1, comp53751_co_seq1	950, 346, 1042	269 (fragment), 103 (fragment),	1.08, 1.08, 1.68

			114 (fragment)		
Amino acid transporter	comp88051_co_seq1, comp86739_co_seq4	5068, 1727	1571, 311 (fragment)	6.03,	4.85
Adenylyl cyclase ACa	comp72421_co_seq1, comp78525_co_seq1, comp86305_c3_seq1	2336, 699, 1910	406 (fragment), 127 (fragment), 577 (fragment)	1.45,	4.17, 1.59
Adenylyl cyclase ACb	comp85926_c1_seq4	953	291 (fragment)	1.04	
Adenylyl cyclase ACd	comp88080_c2_seq1	1953	529 (fragment)	4.92	
Adenylyl cyclase ACc	comp7502_co_seq1	354	101 (fragment)	0.73	
Adenylyl cyclase AC3	comp33277_co_seq1	548	165 (fragment)	0.82	
Guanylyl cyclase	comp88355_co_seq3	7331	1620	8.6	
	comp80491_c1_seq2	3525	916	0.66	
Synapsin	comp79643_co_seq1	3138	495	36.49	
Calcium/calmodulin- dependent serine protein kinase (CASK)	comp80189_co_seq8	1484	393	0.42	
CaM kinase II	comp84598_c1_seq7	3830	487	12.88	
CaM kinase I	comp81905_c1_seq1	1495	365	24.07	
P38 MAP kinase	comp84061_co_seq2	2651	357	13.29	
cAMP-dependent PKA subunit	comp78774_c4_seq1	1776	352	87.93	
G-protein coupled receptor protein kinase	comp78088_c1_seq6	3542	575	6.2	
PKC delta-type	comp74846_co_seq1	1409	316	2	
PKC atypical	comp83489_c1_seq11	2173	616	3.78	
Calcium-independent protein kinase C	comp79370_c5_seq1	2437	742	22.74	
PKC alpha-type	comp86858_co_seq5	1670	533	7.42	
serine/threonine protein kinase H1	comp59268_co_seq1	3658	452	2.59	
serine/threonine protein kinase chk2	comp83090_c1_seq3	2683	481	3.16	
RAC serine-threonine kinase	comp88229_co_seq1	5348	486	10.34	
Diacylglycerol kinase zeta	comp86914_co_seq9	5831	1496	1.1	

Diacylglycerol kinase theta	comp78773_co_seq1	4526	944	3.03
Diacylglycerol kinase beta	comp76021_co_seq1	2551	813	3.26
Diacylglycerol kinase delta	comp87731_co_seq6	2405	598	1.17
Diacylglycerol kinase epsilon	comp87949_co_seq3	3106	526	5.55

Supplemental Table 2-3: Gene Identification Numbers for Previously Published Genes.

Gene Name	Gene Identification #	Database
5-HT1a _{Trit}	comp44433_co_seq1	<i>Tritonia diomedea</i> transcriptome assembly
5-HT1b _{Trit}	comp64711_co_seq1	<i>Tritonia diomedea</i> transcriptome assembly
5-HT2a _{Trit}	comp74599_co_seq1, comp58370_co_seq1	<i>Tritonia diomedea</i> transcriptome assembly
5-HT2b _{Trit}	comp67836_co_seq1	<i>Tritonia diomedea</i> transcriptome assembly
5-HT4 _{Trit}	comp56135_co_seq1	<i>Tritonia diomedea</i> transcriptome assembly
5-HT6 _{Trit}	comp57581_co_seq1	<i>Tritonia diomedea</i> transcriptome assembly
5-HT7 _{Trit}	comp70307_c2_seq2	<i>Tritonia diomedea</i> transcriptome assembly
5-HT1a _{Aply}	AF041039	NCBI
5-HT1b _{Aply}	AF372526	NCBI
5-HT2a _{Aply}	HM187583.1	NCBI
5-HT2b _{Aply}	comp88174_c8_seq3	<i>Aplysia californica</i> transcriptome assembly
5-HT4 _{Aply}	HM187584.1	NCBI
5-HT6 _{Aply}	comp79116_co_seq1	<i>Aplysia californica</i> transcriptome assembly
5-HT7 _{Aply}	FJ477896.1	NCBI
5-HT1a _{Dros}	NM_166322.2	NCBI
5-HT1b _{Dros}	NM_079065.6	NCBI
5-HT2a _{Dros}	NM_001104214.3	NCBI
5-HT2b _{Dros}	NM_001300309.1	NCBI
5-HT7 _{Dros}	NM_079860.3	NCBI
5-HT1 _{Rat}	NM_012585.1	NCBI
5-HT2 _{Rat}	NM_017254.1	NCBI
5-HT4 _{Rat}	NM_012853.1	NCBI
5-HT6 _{Rat}	NM_024365.1	NCBI
5-HT7 _{Rat}	NM_022938.2	NCBI
5-HT2b _{Proc}	EU131666.1	NCBI
D1 _{Trit}	comp12659_co_seq1	<i>Tritonia diomedea</i> transcriptome assembly
D2 _{Trit}	comp66454_c1_seq1	<i>Tritonia diomedea</i> transcriptome assembly
D1 _{Aply}	XP_005100403.1	NCBI

D2 _{Aply}	XP_005099999.1	NCBI
D1 _{Dros}	NP_001262563.1	NCBI
D2 _{Dros}	NP_001014760.2	NCBI
D1 _{Rat}	NP_036678.3	NCBI
D2 _{Rat}	D2_XP_008764413.1	NCBI
GABA-BR1 _{Trit}	comp69887_c11_seq1	<i>Tritonia diomedea</i> transcriptome assembly
GABA-BR2 _{Trit}	omp70543_c5_seq4	<i>Tritonia diomedea</i> transcriptome assembly
GABA-BR3 _{Trit}	comp67894_co_seq2	<i>Tritonia diomedea</i> transcriptome assembly
GABA-BR1 _{Aply}	comp79706_co_seq3	<i>Aplysia californica</i> transcriptome assembly
GABA-BR2 _{Aply}	comp83693_c1_seq2	<i>Aplysia californica</i> transcriptome assembly
GABA-BR3 _{Aply}	comp88599_c1_seq14	<i>Aplysia californica</i> transcriptome assembly
GABA-BR1 _{Dros}	Q9V3Q9	SwissProt/UniProtKB
GABA-BR2 _{Dros}	Q9Y133	SwissProt/UniProtKB
GABA-BR3 _{Dros}	Q9VPS7	SwissProt/UniProtKB
GABA-BR1 _{Rat}	Q9ZoU4	SwissProt/UniProtKB
GABA-BR2 _{Rat}	O88871	SwissProt/UniProtKB
ACa _{Trit}	comp70707_co_seq6	<i>Tritonia diomedea</i> transcriptome assembly
ACb _{Trit}	comp31797_co_seq1	<i>Tritonia diomedea</i> transcriptome assembly
ACc _{Trit}	comp60825_co_seq3	<i>Tritonia diomedea</i> transcriptome assembly
ACd _{Trit}	comp70550_c1_seq3	<i>Tritonia diomedea</i> transcriptome assembly
ACa _{Aply}	NM_001204606.1	NCBI
ACb _{Aply}	NM_001204726.1	NCBI
ACc _{Aply}	NM_001204659.1	NCBI
ACd _{Aply}	NM_001204733.1	NCBI
AC3 _{Aply}	comp64659_co_seq1	<i>Aplysia californica</i> transcriptome assembly
AC3 _{Lott}	XP_009050770.1	NCBI
Rutabega _{Dros}	M81887.1	SwissProt/UniProtKB
AC78 _{C_{Dros}}	Q9Y2Vo	SwissProt/UniProtKB
AC39 _{e_{Dros}}	O96306	SwissProt/UniProtKB
AC35 _{C_{Dros}}	Q9VXu0	SwissProt/UniProtKB
ADCY2 _{Dros}	Q9VW60	SwissProt/UniProtKB
ACXb _{Dros}	Q9NJA1	SwissProt/UniProtKB

<i>ACXd_{Dros}</i>	Q9NJ94	SwissProt/UniProtKB
<i>ACXe_{Dros}</i>	Q9VV038	SwissProt/UniProtKB
<i>AC1_{Rat}</i>	NM_001107239.1	NCBI
<i>AC2_{Rat}</i>	NM_031007.1	NCBI
<i>AC3_{Rat}</i>	NM_130779.2	NCBI
<i>AC5_{Rat}</i>	NM_022600.1	NCBI
<i>AC9_{Rat}</i>	XM_008774618.1	NCBI

Supplemental Table 2-4: Primer Sequences for 5-HT Receptor Verification from Whole Brain cDNA.

Gene Name	Sense Primer (5'-3')	Antisense Primer (5'-3')
5-HT1a _{Herm}	GTGCCACGATTCAAGTAATTGG	ATAGAATAGCATAGTGCAATCTTAGTC G
5-HT1b _{Herm}	CCATGATGAGTAACGTCACGCTACCG	CAGGAGAGTCGTTTCGATGATGCGTC
5-HT2a _{Herm}	GCTTTAACGGAACGGACGGTGATTTCG	CAGGGGAGGAATAAGTTTTACGGAGGA G
5-HT2b _{Herm}	CACGCACCACTACAACAACATCATCC TC	GGTCATCGGGAAAATCCTGTTCGTGTG
5-HT4 _{Herm}	CCAACACAGCGGCCCCACC	CCATGGCAAGTCGCTCTATGGTTAGG
5-HT6 _{Herm}	GCAGATTCCCAGTAGAGTGGTCAACG	CATAGCATCACCGCTGTCCCTAACC
5-HT7 _{Herm}	CCCCTTCCAATCAATCGGTTG	CCCGACACAACACGGAGTCT
5-HT1a _{Trit}	CCAGACTACATTCAACCTAAACCAGA GC	GTGGTCAGTTTATGCCTCTGGCTTG
5-HT1b _{Trit}	GGTAATGGTAGTTTAATCAGCGTG	GTTTCGGTAAACAGTTAAAGCTCTAG
5-HT2a _{Trit}	CAGGGTCAAGGAACTCTCGGCAATC	GATTTCGGAGATCCACAAGCAGTAAGTC G
5-HT2b _{Trit}	CCCATTTGCTGAGAGGTCTGTCTG	CAGATGGCACCCCTTAATCGCTATTGAT GG
5-HT4 _{Trit}	CAATAGCGACATTGTGATCCTTGCGC C	GCGGAGGTGGAGGAACACGAAAAG
5-HT6 _{Trit}	GCCTTTCTCCAGACGCCTGCTG	GGGTGGCTTCTGCAGATTTAAGAATTA TC
5-HT7 _{Trit}	GACTATCACTCTCACCGATTCTAACAC GG	CCCTAGATAACCCAGCGCGAATATTTTA C
5-HT Transporter _{Herm}	CTTCATCACCACCATCATCTCCACCAC	GTCTCTGGGAGTGAGGTTGAAGTCC
qPCR 5-HT Transporter _{Herm}	TCTATGGCGTGGAGCGATTCTG	GAAGAAGACGACGACGATGAAGATG
Pedal Peptide 3 Precursor _{Herm}	TGAACACCTTGCGAATCATCCTGG	CTATTTCAAGGCTCATACGGGCTTCAAG G
qPCR Pedal Peptide 3 Precursor _{Herm}	GAACACCTTGCGAATCATCCTGG	GGCTCATACGGGCTTCAAGGATT

3 SPECIES- AND INDIVIDUAL- DIFFERENCES IN SEROTONIN RECEPTOR EXPRESSION IN HOMOLOGOUS SINGLE NEURONS CORRELATES WITH SWIMMING BEHAVIORS IN SEA SLUGS.

3.1 Abstract

Species differences in neuromodulation can facilitate evolution of behaviors. This study tested this idea by examining correlations between species-specific gene expression in single neurons and species- and individual-differences in behavior. In the Nudibranch sea slug *Tritonia diomedea* (Mollusca; Gastropoda; Heterobranchia; Nudipleura), serotonin (5-HT) is necessary for the activation of a dorsal-ventral (DV) escape swim behavior and modulation of a neuron that comprises part of the swim motor pattern circuit, called C2. Neurons homologous to C2 are present across the Nudipleura. Previous research found that in the Nudibranch, *Hermisenda crassicornis*, C2 homologues are not modulated by 5-HT and no DV swimming occurs. A third Nudipleura species, *Pleurobranchaea californica*, independently evolved DV swimming, with intrinsic neuromodulation of its C2 homologues by 5-HT playing a role in the generation of its swim motor pattern. *Pleurobranchaea* is a variable swimmer, meaning an individual animal swims some days but not others; C2 modulation was previously found to correlate with this behavioral variability. We tested the hypothesis that expression of neuromodulatory receptor genes underlies species-specific and individual DV swimming behaviors. Using single C2 neuron absolute quantitative PCR (qPCR), we found that 5-HT receptor expression in C2 homologues correlates with swimming behaviors. Furthermore, we found that independently evolved behaviors shared expression patterns for orthologous 5-HT receptor genes in C2 homologues. These results support the idea that neuromodulatory gene expression can facilitate behavioral evolvability.

3.2 Introduction

Katz and Harris-Warrick (1999) proposed that species differences in behavior could arise through differences in neuromodulatory signaling. Neuromodulation changes aspects of neuronal physiology, such as action potential firing frequency, membrane potential, or synaptic strength, effectively changing the outputs of neural circuits (Harris-Warrick 2011; Kupfermann 1979; Marder 2012). Neuromodulators, such as serotonin (5-HT), perform these actions by activating corresponding receptors. Thus, species differences in neuromodulatory receptor expression might underlie differences in species-typical behaviors. Furthermore, individuals within a species also show variability in their behaviors. Individual variability in behavior might be accounted for by individual differences in neuromodulatory receptor expression, as is hypothesized for species-level differences.

Species differences are seen in swimming behaviors among Nudipleura sea slugs (Mollusca; Gastropoda; Heterobranchia). The nudibranch *Tritonia diomedea* swims by producing alternating dorsal and ventral (DV) body flexions. In contrast, the nudibranch *Hermisenda crassicornis* lacks this swimming behavior. However, the more distantly related *Pleurobranchaea californica* does swim with DV body flexions. Based on the phylogeny of Nudipleura (Goodheart et al. 2015) and the distribution of swimming behaviors, (Newcomb et al. 2012) we concluded that *Tritonia* and *Pleurobranchaea* evolved DV swimming independently.

The neural basis for DV swimming in *Tritonia* has been well-studied (Dorsett et al. 1973; Willows et al. 1973). The central pattern generator (CPG) underlying swimming consists of the identified neurons C2, DSI, and VSI, which fire bursts of action potentials that drive the swimming behavior (Figure 3-1a). The DSIs are serotonergic and enhance

the synapses made by C2 and VSI by presynaptically increasing neurotransmitter release (Katz and Frost 1995b). In C2, this is accompanied by an increase in spike-evoked Ca^{2+} influx (Hill et al. 2008). This “intrinsic neuromodulation” is essential for production of the swim motor pattern (Calin-Jageman et al. 2007; Katz 1998; Katz et al. 1994). The 5-HT receptor antagonist methysergide blocks DSI’s neuromodulatory actions on C2 synapses (Katz and Frost 1995a). Methysergide, the mammalian 5-HT₂ family antagonist, blocks the production of the swim motor pattern in the isolated brain and prevents the animal itself from swimming (McClellan et al. 1994). Furthermore, application of 5-HT to the isolated brain is sufficient to evoke a swim motor pattern and injection of the animal with 5-HT evokes a swim motor pattern (McClellan et al. 1994). Serotonergic neuromodulation of C2 synaptic strength plays a central role in the production of the swim motor pattern in *Tritonia*.

Although *Hermisenda* does not produce a DV swim motor pattern (Figure 3-1b), it has neurons that are homologous to DSI and C2. The DSIs are identified by 5-HT immunoreactivity and their position in the brain (Newcomb et al. 2006; Newcomb and Katz 2007; Newcomb and Katz 2009). C2 is identified by its white soma in the cerebral ganglion, which is about 80 μm in diameter and immunoreactive to the neuropeptides FMRFamide and Small Cardioactive Peptide (SCP), and its contralaterally-projecting axon (Lillvis et al. 2012). However, unlike in *Tritonia*, neither DSI nor 5-HT modulates C2 synaptic strength in *Hermisenda*. Furthermore, application of 5-HT to the isolated *Hermisenda* brain fails to evoke a swim motor pattern, and injection of *Hermisenda* with 5-HT does not cause the animal to swim (Lillvis and Katz 2013). Thus, behavioral differences between *Tritonia* and *Hermisenda* could be caused by differences in the expression of particular 5-HT receptors in C2.

Pleurobranchaea also has homologs of both DSI and C2, known as As1-3 and A1 respectively, which are identified by the same criteria as in *Tritonia* and *Hermisenda* (Jing and Gillette 1995; Jing and Gillette 1999; Lillvis et al. 2012). Furthermore, as in *Tritonia*, these neurons are members of its DV swim CPG and fire rhythmic bursts of action potentials during the swim motor pattern (Figure 3-1c) (Jing and Gillette 1999). In this paper, we will refer to the neurons using the *Tritonia* nomenclature. In *Pleurobranchaea*, DSI and 5-HT enhance the strength of C2 synapses. Additionally, 5-HT enhances swimming following injection in live *Pleurobranchaea* or bath application to isolated brain preparations. Methysergide blocks swimming in live *Pleurobranchaea* (Lillvis and Katz 2013). *Pleurobranchaea* and *Tritonia* appear to have evolved DV swimming behaviors independently, but their underlying neuromodulatory mechanisms evolved in parallel.

Unlike in *Tritonia*, swimming in *Pleurobranchaea* is not consistent; the same animal varies in its propensity to swim on subsequent days (Jing and Gillette 1995; Jing and Gillette 1999; Lillvis and Katz 2013). Similarly, some isolated brain preparations do not exhibit bursting activity typical of a swim motor pattern (Figure 3-1d). Furthermore, the extent of enhancement of C2 synaptic strength is correlated with the number of burst cycles in the motor pattern (Lillvis and Katz 2013). Thus, serotonergic neuromodulation plays a central role in swimming in *Pleurobranchaea* and the modulation correlates with individual variability in swimming behavior.

The 5-HT receptor subtypes expressed by C2 homologues are not identified. Seven receptor subtypes, from five families of 5-HT receptors, have been identified in whole brain tissue of *Aplysia*, *Tritonia*, and *Hermisenda* (Nagakura et al. 2010; Tamvacakis et al. 2015) (Supplemental Figure 3-4). Using their gene sequences, we examined the

expression of 5-HT receptor genes in C2 neurons from *Tritonia*, *Hermisenda*, and *Pleurobranchaea*. We hypothesize that differences in 5-HT receptor expression among C2 homologues underlies species and individual differences in swimming behavior.

3.3 Methods

Animals

Tritonia diomedea was collected by Living Elements LLC (Vancouver, B.C.), *Hermisenda crassicornis* and *Pleurobranchaea californica* were collected by Monterey Abalone Co. (Monterey, CA). All animals were housed at 10°C in recirculating artificial seawater (ASW, Instant Ocean). Individual *Tritonia* were anaesthetized before dissection using cold temperature, and the other species were anaesthetized using 0.33 M magnesium chloride.

Whole-Brain RNA Extraction and cDNA Production

For whole-brain RNA extraction, brains were dissected and cleaned of connective tissue, flash-frozen using liquid nitrogen, then stored at -80°C. RNA extraction was performed using the RNeasy Plus Universal Mini Kit (Qiagen). RNA extracts were quantified using Nanodrop (Thermo Fisher). RNA was reverse transcribed to cDNA using Superscript IV (Thermo Fisher) following manufacturer's instructions.

5-HT Receptor Plasmid Cloning

Species-specific primers were designed using transcriptome-derived 5-HT receptor sequences from *Tritonia* (Senatore et al. 2015), *Hermisenda* (Tamvacakis et al. 2015), and a previously unpublished *Pleurobranchaea* transcriptome (NCBI SRA TBD).

The primers (Eurofins) are listed in Supplemental Table 3-1a. PCR was performed to amplify putative 5-HT receptor genes from whole-brain cDNA from each species using *Taq* polymerase (Thermo Fisher). PCR products were gel purified using the Qiaquick Gel Extraction Kit (Qiagen). Resulting DNA was ligated using T4 DNA ligase and inserted into JM109 competent cells (Promega). Cloned plasmids were extracted using GenElute Plasmid Mini-prep Kit (Sigma Aldrich). Plasmids were sequenced on a 3730 DNA Analyzer (Thermo Fisher), and sequences were aligned against transcriptome sequences using MUSCLE (Edgar 2004) to verify gene identity.

Phylogenetic Analysis of 5-HT Receptors

A phylogenetic tree was created using methods described previously for *Tritonia* and *Hermissenda* receptors (Supplemental Figure 3-4) (Tamvacakis et al. 2015). Previously unpublished *Pleurobranchaea californica* 5-HT receptor sequences were determined from a whole brain transcriptome, and sequences were confirmed by plasmid cloning. MUSCLE alignment followed by maximum likelihood tree construction with bootstrapping was done using MEGA6 software (Tamura K. 2013).

Synthetic RNA Production for qPCR Standards

Plasmid DNA sequences for each species-specific 5-HT receptor subtype were linearized with T7- or Sp6-oriented enzyme digests (New England Biosciences) and gel-verified. Digested plasmids were purified using phenol-chloroform extraction and alcohol precipitation, and quantified using a Nanodrop 2000C (Thermo Fisher). Synthetic RNA was produced using either T7 or Sp6 MegaScript Kit (Thermo Fisher) and purified. RNA was quantified using Nanodrop. The copy number for each 5-HT receptor synthetic RNA

sample was calculated using the gene-specific molecular weight and RNA concentration (Fronhoffs et al. 2002). RNA standards were then serially diluted and individually reverse transcribed to cDNA using SuperScript IV (Thermo Fisher).

Single-Neuron Isolation

Following initial dissection, the brain, which consists of the fused cerebral, pleural, and pedal ganglia, was stored in either artificial sea water (Instant Ocean) or normal saline (NS) (420 mM NaCl, 10 mM KCl, 10 mM CaCl₂, 50 mM MgCl₂, 11 mM d-glucose, and 10 mM HEPES, pH 7.5). The connective sheath was removed using fine scissors and forceps. C2 was visually identified as the white neuron in the anterolateral cerebral ganglion (Getting 1977; Lillvis et al. 2012; Taghert 1978).

Intracellular recordings were made using 10-30 M Ω glass microelectrodes connected to an IX2-700 Intracellular Amplifier (Dagan). Extracellular recordings and stimulation of body wall nerves were made by placing them in polyethylene tubing connected to a Model 1700 differential amplifier (A-M Systems). Signals were digitized using the Micro1401 and Spike2 software (Cambridge Electronic Design Limited).

To identify C2, cells were recorded intracellularly and a swim motor pattern was evoked by stimulating a body wall nerve with 20–35 V at 5 Hz for 3 s. C2 neurons from *Tritonia* were not isolated if a swim motor pattern consisting of two or more bursts could not be evoked, an indication that the C2 cell or another part of the swim neural circuit was damaged. In *Pleurobranchaea*, C2 neurons were isolated from bursting and non-bursting preparations; individual preparations were defined as “swimming” if two or more bursts could be evoked, and “non-swimming” if one or fewer bursts was stimulated (Figure 3-1). Contralateral C2 neurons are electrically coupled to each other in *Tritonia*,

Hermisenda, and *Pleurobranchaea* (Getting 1981; Getting et al. 1980; Jing and Gillette 1995; Jing and Gillette 1999; Lillvis et al. 2012b; Taghert PH 1978). Electrical coupling was tested in some *Hermisenda* and non-swimming *Pleurobranchaea* preparations when participation in the swim motor pattern could not be used to help identify C2.

After identification of C2, the brain was bathed in 0.2% Protease IX for five min and washed with ASW or NS. Individual C2 neurons were removed with forceps, and a suction pipette and placed in a 1.5 ml tube containing distilled water and RNaseOut (Thermo Fisher).

C2 neurons were discarded from isolation if they appeared damaged at any point during isolation, or did not meet species-specific physiological hallmarks of C2 neurons as previously described (Lillvis et al. 2012). Neurons were not isolated if the central ganglia appeared damaged. C2 neurons that appeared to have other neurons attached to them were also discarded. After removal from the brain, C2 neurons were put in tubes and visually inspected under a light microscope to verify that cells were present and intact in the tube.

Single-Neuron cDNA Synthesis for Quantitative PCR

Neurons used for qPCR were isolated as described above and were directly transcribed to cDNA using Superscript IV (Thermo Fisher). The cells were added to a mixture of distilled water and RNaseOut and frozen at -80°C. A master mix was created following the Superscript IV manufacturer's protocol, and aliquots were added directly to the RNA. The RNA was annealed and cooled on ice. The annealed RNA was then divided by volume equally into two tubes. One tube received reverse transcriptase enzyme plus additional Superscript IV reagents (+RT). The other tube served as the without reverse

transcriptase control (-RT control) and received a volume of distilled water in place of the enzyme volume. The RNA samples were then reverse-transcribed following manufacturer's instructions.

mRNA Quantification Using Single-Neuron and Whole Brain Absolute qPCR

Absolute qPCR was performed for individual genes from each species. qPCR-specific primers were designed against each gene orthologue (Supplemental Table 3-1b). For 5-HT_{2a} in *Tritonia* and *Hermisenda*, two sets of primers were used to measure mRNA expression at multiple locations along the 5-HT_{2a} mRNA. Other gene orthologues were measured with one primer pair. RNA standards were prepared as described above, and were run alongside cell cDNA, -RT control samples, and without-template control samples on every qPCR trial. C2 samples were run as either single cells, or pooled cells from multiple animals; raw copy numbers were divided by starting cell amount. Whole brain tissue was run with 150 ng RNA calculated per one triplicate sample. All C2 and whole brain samples, standards, and controls were run in triplicate using Perfecta SYBR Green Supermix with ROX or Low ROX (Quanta Bio) on an Applied Biosystems 7500 or StepOne Plus qPCR machine.

Absolute mRNA copy number values were calculated using the standard curve generated during each trial. If amplification occurred in the -RT samples, it was subtracted from the +RT samples. Trials were omitted if the standard curve efficiency was calculated to be outside the range of 85 to 110%. A melt curve was run on every trial, and samples were omitted if a double peak was detected or if samples showed multiple bands in an agarose gel (Sigma Aldrich) following the qPCR trial. mRNA copy numbers were analyzed using SigmaPlot v10 (Systat Software Inc.).

3.4 Results

A Subset of 5-HT Receptors Were Expressed In Tritonia C2 Neurons

We measured 5-HT receptor subtype gene expression from single *Tritonia* C2 neurons. Only four of the seven receptor subtypes previously identified from whole brain transcriptomes were reliably expressed in *Tritonia* C2 neurons. 5-HT2a and 5-HT7 receptor subtypes both had median expression well above 200 copies per cell (Figure 3-2). Within these two data sets, variability in copy number across samples was high, with 5-HT2a showing a range between 152 and 655 copies, and 5-HT7 ranging between 209 and 611 copies. 5-HT1b consistently expressed at fewer than 200 copies per cell. One out of the five *Tritonia* C2 neurons that were tested expressed the 5-HT1a receptor, and three out of six *Tritonia* samples expressed 5-HT2b. The other subtypes, 5-HT4 and 5-HT6 were not detected in *Tritonia* C2 neurons. All seven of the 5-HT receptor subtypes were measured in whole brain tissue using qPCR, however, indicating that the qPCR primers were able to amplify the gene of interest when it was present (Supplemental Figure 3-5).

Hermisenda C2 Homologues Expressed a Different Subset of 5-HT Receptors than Tritonia

When comparing single neuron 5-HT receptor expression in *Tritonia* with that in *Hermisenda*, there were clear species differences (Figure 3-2). In *Hermisenda*, C2 expressed 5-HT4 and 5-HT6 receptors at greater than 200 copies per cell. These genes were not expressed in C2 in *Tritonia*. In *Hermisenda*, C2 also expressed 5-HT1a and 1b below 200 copies per cell, which is similar to what was observed in *Tritonia*. *Hermisenda* C2s did not express 5-HT2a receptors, which were highly expressed in *Tritonia*. Only one *Hermisenda* sample tested showed low expression of 5-HT7, the others did not express

that gene. Furthermore, *Hermisenda* did not express 5-HT_{2b}, which was expressed in some *Tritonia* C2 samples. Thus, there was a clear species difference in the expression of 5-HT receptors in homologous neurons that differ in their responses to 5-HT.

5-HT Receptor Subtypes Were Identified In Pleurobranchaea Whole-Brain Tissue

Before testing which 5-HT receptor subtypes *Pleurobranchaea* C2 homologues expressed, we identified the receptor genes from the *Pleurobranchaea* whole brain transcriptome. All seven molluscan 5-HT receptor subtype orthologues were identified (Supplemental Table 3-2). To confirm their sequence identities, we created consensus sequences using plasmid DNA for each receptor subtype, generated from whole-brain tissue. The *Pleurobranchaea* 5-HT receptor subtype sequences clustered with other subtypes from each 5-HT receptor family (Supplemental Figure 3-4).

Receptor Expression Differed In Swimming and Non-Swimming Pleurobranchaea

Since *Pleurobranchaea* does not reliably swim, we partitioned samples from this species based on the motor pattern produced at the time that the C2 sample was taken. If the motor pattern consisted of more than a single burst, it was considered a “swimmer” (Figure 3-1c). On the other hand, if stimulation of the body wall nerve produced one or fewer bursts, the individual was categorized as a “non-swimmer” (Figure 3-1d).

Receptors 5-HT_{2a} and 5-HT₇ were expressed in *Tritonia* C2, but not in the *Hermisenda* homologues. 5-HT_{2a} was expressed in the C2s of swimming *Pleurobranchaea*. Only one sample from the non-swimming *Pleurobranchaea* group showed low expression of 5-HT_{2a}, calculated at 45 mRNA copies (Figure 3-3a). There was a large degree of variability in the amount expressed in individual C2 samples from

swimming individuals, from 100 to 1276 copies, a greater range in individual expression levels than that observed in any of the *Tritonia* or *Hermissenda* C2 samples measured.

5-HT₇ receptor expression was also compared in swimmers and non-swimmers. 5-HT₇ receptors were highly expressed in swimming *Pleurobranchaea*, except for one sample, which did not express this gene (Figure 3-3b). Variability in expression was highest in this group, with a range of 0 to 2446. In one of the seven non-swimmers measured, C2 homologues expressed 5-HT₇. Overall, the results showed that swimming *Pleurobranchaea* expressed two of the same receptor subtypes measured in *Tritonia*. The remaining five subtypes were not tested in this species, however.

Small Cardioactive Peptide Precursor Was Expressed in C2 Homologues from Each Species

Measuring 5-HT receptor subtype gene expression in C2s revealed several differences between homologous neurons. To determine if there were any gene expression similarities, we measured the expression of the gene for the precursor of SCP. C2 homologues are immunoreactive against SCP in all three species (Lillvis et al. 2012), and were therefore expected to contain the precursor genes. The gene for SCP was expressed in C2 neurons from all three species (Supplemental Figure 3-6). For SCP measurements from *Pleurobranchaea*, C2 neurons from swimming and non-swimming individuals were used and are presented as one group. There was no difference in SCP expression between *Pleurobranchaea* swimmers and non-swimmers. There were differences in the amounts expressed between species, however.

3.5 Discussion

Our results provide evidence that species differences in expression of neuromodulatory receptor genes in single homologous cells facilitates species-specific behaviors. Using single-neuron qPCR, 5-HT receptors and SCP were measured in C2 homologues from three species of Nudipleura sea slugs. *Tritonia* and *Hermisenda* C2 homologues differed in their expression patterns of 5-HT receptor subtypes, correlating with species differences in C2 synapse modulation by 5-HT and swimming capability. *Pleurobranchaea* C2 homologues extracted from swimming individuals shared 5-HT receptor subtype expression with *Tritonia*. Overall, these results indicate a role for neuromodulatory genes in facilitating species-specific behaviors, as well as individual variability in behaviors.

Individual C2 Neurons Expressed Many Receptor Subtypes

C2 homologues expressed multiple 5-HT receptor subtypes in *Tritonia*, *Hermisenda*, and *Pleurobranchaea*. In *Tritonia* C2 neurons, three subtypes were consistently measured across samples, while *Hermisenda* C2 neurons consistently expressed four subtypes. C2 homologues of swimming *Pleurobranchaea* expressed two subtypes. The function of each receptor subtype in these cells is currently unknown, but the identification of multiple subtypes points to the possibility that C2 is a multifunctional neuron.

Neurons can have multiple functions. In addition to their role in DV swimming, C2 neurons in *Tritonia* may be involved in foot cilia-mediated crawling (Snow 1982), while *Pleurobranchaea* C2 homologues inhibit feeding command neurons, possibly to inhibit feeding behavior when an escape response is necessary (Jing and Gillette 1995; Jing and

Gillette 2000). The multiple 5-HT receptor subtypes identified in C2 homologues may facilitate C2's multiple functional roles in the brain by activating different downstream pathways.

The expression of multiple neuromodulatory receptors is a feature that has been observed in several single-neuron studies. In the gastropod mollusc *Aplysia californica*, pleural ganglion sensory neurons express at least three 5-HT receptor subtypes, identified with single cell PCR (Nagakura et al. 2010). In crustaceans, single identified neurons have been studied with respect to their neuromodulatory receptor expression. The pyloric dilator (PD) pacemaker cells, for example, have been shown to express multiple 5-HT receptor subtypes, an acetylcholine receptor (Katz and Harris-Warrick 1989; Katz and Harris-Warrick 1990; Zhang and Harris-Warrick 1994), and dopamine receptors (Oginsky et al. 2010), using a combination of pharmacology, immunohistochemistry, and single-cell PCR.

Tritonia Swimming May Be Controlled Through 5-HT_{2a} and 5-HT₇ Receptor-Mediated Mechanisms

Serotonergic modulation is an important characteristic of DV swimming in *Tritonia*, a behavior that has been studied for over 30 years. With recent advances in technology, we can now dissect the neural circuit controlling swimming down to genes expressed in single cells. The single-neuron qPCR experiments from this study measured reliable expression of 5-HT_{1b}, 5-HT_{2a}, and 5-HT₇ in *Tritonia* C2 neurons, pointing to their potential roles in *Tritonia* swimming and serotonergic modulation of C2.

Other evidence published previously supports the findings presented here. Methysergide, a mammalian 5-HT_{1/2} family antagonist, blocked swimming in both

Tritonia and *Pleurobranchaea* (Lillvis and Katz 2013; McClellan et al. 1994). Additionally, it reduced DSI-mediated modulation of C2 when bath applied to isolated *Tritonia* brain preparations (Katz and Frost 1995a). It is possible that methysergide acts on 5HT_{2a} receptors in *Tritonia* and *Pleurobranchaea* C2 homologues, consistent with its actions on orthologous receptors in mammals. However, methysergide binding selectivity may differ between members of different animal phyla, as has been reported previously with respect to receptor pharmacology (Dumitriu et al. 2006; Tierney 2001). While methysergide preferentially binds to 5-HT receptors in invertebrates and vertebrates, it may not by itself be a reliable means of determining receptor subtype identity in non-mammalian species.

Tritonia C2 neurons were reported to exhibit increased calcium signaling in neurites following 5-HT bath application, indicating that a 5-HT receptor linked to calcium was being stimulated (Hill et al. 2008). 5-HT₂ family receptors, such as 5-HT_{2a}, have conserved G-protein coupling to the IP₃/DAG pathway, which increases intracellular calcium when stimulated. Furthermore, *Tritonia* C2 neurons exhibit fast and slow responses to DSI-released 5-HT. The multiple responses were predicted to be due to different receptor types expressed by C2 neurons (Clemens and Katz 2001; Katz and Frost 1995a; Katz and Frost 1995b). Thus, the finding that *Tritonia* C2 expressed multiple receptor subtypes, including at least one that is predicted to increase intracellular calcium, is consistent with previously published research.

C2 Homologue 5-HT Receptor Expression was Species-Specific

While both *Tritonia* and *Hermisenda* C2 neurons expressed multiple 5-HT receptor subtypes, the identity of those subtypes was species-specific. Although the exact role played by each subtype in C2 is currently unknown, the expression of 5-HT_{2a} and 5-

HT7 in C2 in *Tritonia*, but not *Hermisenda*, indicates that one or both of these subtypes likely contributes to species-specific modulation and swimming capability. On the other hand, *Hermisenda* C2 homologues expressed 5-HT4 and 6, which were not observed in *Tritonia* C2 neurons. The functions of 5-HT receptors in *Hermisenda* C2 homologues are currently unknown. It is unclear if the presence of 5-HT4 and 5-HT6 actively prevents C2 serotonergic modulation and swimming ability, or if they are present for other purposes.

Each 5-HT receptor subtype is descended from a conserved family of G-protein coupled receptors, and each has a predicted downstream second messenger pathway (Nichols and Nichols 2008). Family 1, 4, 6, and 7 receptors affect adenylyl cyclase (AC), family 1 receptors are Gi-coupled and inhibit AC activity, whereas family 4, 6, and 7 receptors are Gs-coupled and increase AC activity. Family 2, which has two known subtypes (5-HT2a and 2b) in arthropods and molluscs, is predicted to be coupled to Gq, which activates the IP3/DAG pathway. *Tritonia* and swimming *Pleurobranchaea* C2 homologues expressed 5-HT2a according to qPCR, while the non-swimming *Pleurobranchaea* and *Hermisenda* did not. It is possible that the presence of the IP3/DAG pathway activation is an underlying cause of DV swimming capability in both species.

While the experiments described here showed species-specific expression of 5-HT receptors, the SCP precursor gene was expressed across C2 homologues, regardless of species. SCP was previously identified as a hallmark of C2 neurons, and an indication of their homology across species (Lillvis et al. 2012). The presence of SCP mRNA in C2 homologues from each species confirmed the identity of the cell, and showed that there are gene markers found across homologue cells, regardless of species. It is likely that

there are many more gene expression similarities shared by these homologous cells. As a next step to the current study, measuring broad scale gene expression through a technique such as single-cell RNA sequencing is predicted to show many more similarities, as well as species differences.

Previous research has found receptor expression differences between homologous neurons from different species. The LP neurons of crabs, for example, respond to 5-HT (Zhang and Harris-Warrick 1994), proctolin (Zhao et al. 2011), and CCAP (Weimann et al. 1997), while their homologues in lobsters respond to DA through D1Rs (Zhang et al. 2010), mGLUR agonists (Perez-Acevedo and Krenz 2005), and orcokinin (Li et al. 2002). Species differences in the genes expressed by homologous neurons is expected, given the fact that homologous neurons often have species-specific functions (Katz 2011).

Independently Evolved Swimming Behaviors, Parallel Evolution of Neuromodulatory Genes

Most Nudipleura species do not exhibit DV swimming. Furthermore, based on phylogeny, it appears that *Tritonia* and *Pleurobranchaea* do not share a most recent common ancestor that exhibited this behavior and thus likely evolved DV swimming independently (Newcomb et al. 2012). Both species use serotonergic modulation of C2 homologues, an example of parallel evolution of a neural mechanisms underlying convergent behaviors (Lillvis and Katz 2013). The shared 5-HT_{2a} and 5-HT₇ expression in C2 homologues from swimming *Pleurobranchaea* and *Tritonia* indicates that the neuromodulatory responses of C2 homologues in these two species also evolved in parallel.

It was previously proposed that serotonergic modulation transformed C2 and other neurons from a latent Nudipleura circuit into the functional swim motor pattern circuit observed in *Tritonia* and *Pleurobranchaea* (Lillvis and Katz 2013). In fact, it may be that the presence of 5-HT_{2a} and/or 5-HT₇ is the “switch” in this evolutionary transformation. A similar phenomenon has been well characterized in species-specific mating behaviors in voles: the neuromodulatory vasopressin receptor V1a is expressed in the ventral pallidum of monogamous prairie voles, but not the closely related, non-monogamous montane voles (Hammock and Young 2002; Nair and Young 2006; Young et al. 1999; Young and Wang 2004). In this example, the non-monogamous voles could become monogamous when the V1a receptor was exogenously expressed in their ventral pallidum (Lim et al. 2004). Although the functional roles of the 5-HT receptors are currently unknown in C2 homologues, the correlation between their expression and independently evolved swimming behaviors shown here presents a tantalizing possibility that manipulating 5-HT_{2a} or 5-HT₇ expression in non-swimmer C2 neurons might cause DV swimming to occur, while knocking down their expression in DV-swimmers may reduce or eliminate the swim motor pattern.

Individual Variability in Behavior Correlated with Gene Expression Differences

DV swimming varies daily in *Pleurobranchaea*, as measured using both *in vivo* behavioral assays and *in vitro* fictive swimming (Jing and Gillette 1995; Jing and Gillette 1999; Lillvis and Katz 2013). This variability was found previously to correlate with serotonergic enhancement of C2 synaptic strength (Lillvis and Katz 2013). In the present study, 5-HT receptor expression was found to correlate with fictive swimming. While the presence of mRNA in a cell does not necessarily indicate that the corresponding protein

will also be present (Maier et al. 2009), the correlation of 5-HT receptor mRNA expression and swim motor pattern bursts by C2 in *Pleurobranchaea* points to a genetic mechanism that may at least partially facilitate the mechanism by which C2 and other neural components become functional as the swim motor pattern circuit (Lillvis and Katz 2013).

The *Pleurobranchaea* 5-HT receptor genes tested in this study were 5-HT2a and 5-HT7. *Pleurobranchaea* whole brain tissue expressed all seven known molluscan 5-HT receptor subtypes, however (Supplemental Figure 3-4). It is possible that, like *Tritonia* and *Hermisenda*, other 5-HT receptor genes are expressed in *Pleurobranchaea* C2 neurons. Will the full *Pleurobranchaea* C2 5-HT receptor profile include 5-HT1b, like *Tritonia* and *Hermisenda*? Or will a different subset of receptors be expressed? If other receptors are expressed, will their expression amounts vary with swimming as 5-HT2a and 5-HT7 did? Additional experiments will be necessary to answer these questions.

Variability in gene expression has been shown to correlate with individual variability in behaviors in other species. Individual variability in neural circuit function correlating with individual neuron properties and gene expression has been studied in the crab, *Cancer borealis*, where individual variability in ion channel and other gene expression in single cells correlates with pyloric rhythms (Goaillard et al. 2009; Hamood and Marder 2014; Schulz et al. 2006; Schulz et al. 2007; Shruti et al. 2014; Temporal et al. 2012; Temporal et al. 2014). In this system, variability likely ensures robustness of the neural circuit output to disturbances. However, there is no difference in the functional output of the circuit.

C2s Showed Within- And Between-Species Variability in Amount of 5-HT Receptor mRNA Expressed

There was variability in C2 expression of some receptor subtypes within each species that was not tied to known behavioral or neurophysiological variability. 5-HT7 was expressed in one of six *Hermisenda* C2 samples, and in one non-swimming *Pleurobranchaea* sample. 5-HT1a was expressed in one *Tritonia* C2 neuron sample, while it was below the level of qPCR detection in the other five samples tested for that gene. Similarly, 5-HT2b was expressed in half of the *Tritonia* C2 neurons tested. In the remaining qPCR trials, there was variability in the amounts of each subtype expressed between samples from the same species. This variability could be due to natural fluctuations in the amount of mRNA for a given gene, which occurs randomly in many cell types (Ozbudak et al. 2002; Raser and O'Shea 2005). On the other hand, variable ion channel gene expression correlates with bursting properties of stomatogastric neurons (Goaillard et al. 2009), indicating the possibility that the observed variability in 5-HT receptor mRNA in C2 homologues may have as yet undiscovered functional consequences. It is also worth noting that the animals used in this study were wild-caught adults. They may have exhibited varying mRNA expression because of their individual experiences in the wild before ending up in a laboratory tank.

Individual Variability in 5-HT Receptor Gene Expression May Be Due To Intracellular mRNA Cycling

In all eukaryotic cells, mRNA molecules are transcribed in the nucleus, translated to proteins by ribosomes, and then degraded as part of normal cell cycling. It is therefore to be expected that mRNA amounts would wax and wane over time, causing the variability

in qPCR measurements observed in these experiments. There was some variability between almost all of the samples, but *Pleurobranchaea* C2 samples were the most variable in all three genes measured, 5-HT2a, 5-HT7, and SCP.

In other systems, 5-HT receptor mRNA is variable in its expression, in ways that correlate with specific behaviors. In the neural circuit controlling song production in birds, microRNAs alter seasonal expression of 5-HT and other receptors (Larson et al. 2015). Estrogen produced during reproductive phases causes a reduction in 5-HT receptor mRNA in mussel gonads (Cubero-Leon et al. 2010). In non-human primates and rats, 5-HT receptors and other 5-HT-related genes vary with stress in several brain regions (Bethea et al. 2013; Bethea et al. 2005; Centeno et al. 2007; Holmes et al. 1995). 5-HT receptor mRNA was found to vary with circadian rhythm and hibernation periods in rodents (Naumenko et al. 2008; Volgin et al. 2013). The variability in 5-HT receptor expression observed in *Nudipleura* C2 homologues may be due to some as yet undocumented cellular process that is also found outside the Mollusca.

Perhaps some aspect of mRNA cycling in *Pleurobranchaea* C2 homologues resulted in the increased variability observed. If this were the case, then over time there would be periods where there were few 5-HT receptor proteins expressed at C2 synapses in *Pleurobranchaea*, which could cause temporary loss of DV swimming and serotonergic modulation, leading to the previously observed individual variability in this species.

Conclusion

Measuring 5-HT receptor gene expression in single neurons controlling independently evolved DV swimming uncovered a correlation between neuromodulatory gene expression and behavior. Neuromodulation has been hypothesized to be a means of

functionally repurposing a tissue or cell to facilitate the evolution of species-specific behaviors (Katz and Harris-Warrick 1999). The identification of 5-HT_{2a} and 5-HT₇ in C2 neurons from two DV swimming species can help in our understanding of how neuromodulators can facilitate adaptation of existing network structures, and is an example of a potential neural mechanism underlying the evolvability of behaviors.

3.5 Figure and Table Legends

Figure 3-1: Swim Motor Patterns in Three Nudipleura Species. Intracellular microelectrodes impaled C2 neurons from *Tritonia* (a), *Hermisenda* (b), and swimming (c) and non-swimming (d) *Pleurobranchaea*. C2 responses to body wall stimulation (black arrow) are shown. Only *Tritonia* and swimming *Pleurobranchaea* show action potential bursts, forming the swim motor pattern.

Figure 3-2: 5-HT Receptor Expression in C2 from *Tritonia* and *Hermisenda*. 5-HT receptor expression was measured using absolute qPCR to determine mRNA copy number in individual C2 homologue samples from each species. Individual samples are shown as open circles within whisker plots for *Tritonia* (light gray) and *Hermisenda* (dark gray). The boxes represent the range from 25% to 75% of sample expression levels. The median is represented by a black line in each box. When the standard error of the mean was beyond the 25% to 75% range, it is represented as grey error bars.

Figure 3-3: 5-HT_{2a} and 5-HT₇ Receptor Expression in C2 from *Pleurobranchaea* with Variable Swimming Behaviors. 5-HT_{2a} (a) and 5-HT₇ (b) receptor expression was measured in *Pleurobranchaea* C2 homologues, which were

categorized as “Swimmers” (hatches) and “Non-swimmers” (light dots). Individual samples are represented as open circles on each graph. The boxes represent the range from 25% to 75% of sample expression levels. The median is represented by a black line in each box. When the standard error of the mean was beyond the 25% to 75% range, it is represented as grey error bars.

Supplemental Figure 3-4: Phylogenetic Tree of 5-HT and Dopamine Receptor Subtypes. Maximum likelihood phylogeny showing seven 5-HT receptor subtypes spanning five families, with dopamine D1 and D2 receptors included because they fall within the same ancestral grouping. Bootstrap values represent percentage of predicted replicates at each node. Receptor subunits were aligned using conserved transmembrane domain regions.

Supplemental Figure 3-5: Whole-brain Expression of 5-HT Receptors. qPCR measured 5-HT receptor subtype expression from whole brain tissue in *Tritonia* (a), *Hermisenda* (b), and *Pleurobranchaea* (c).

Supplemental Figure 3-6: Small Cardioactive Peptide (SCP) Gene Expression in C2 Homologues. SCP gene expression was measured in C2 homologues from *Tritonia* (light gray), *Hermisenda* (dark gray), and *Pleurobranchaea* (white), using absolute qPCR. Individual samples are represented as open circles on each graph. The boxes represent the range from 25% to 75% of sample expression levels. The median is represented by a black line in each box. When the standard error of the mean was beyond the 25% to 75% range, it is represented as grey error bars.

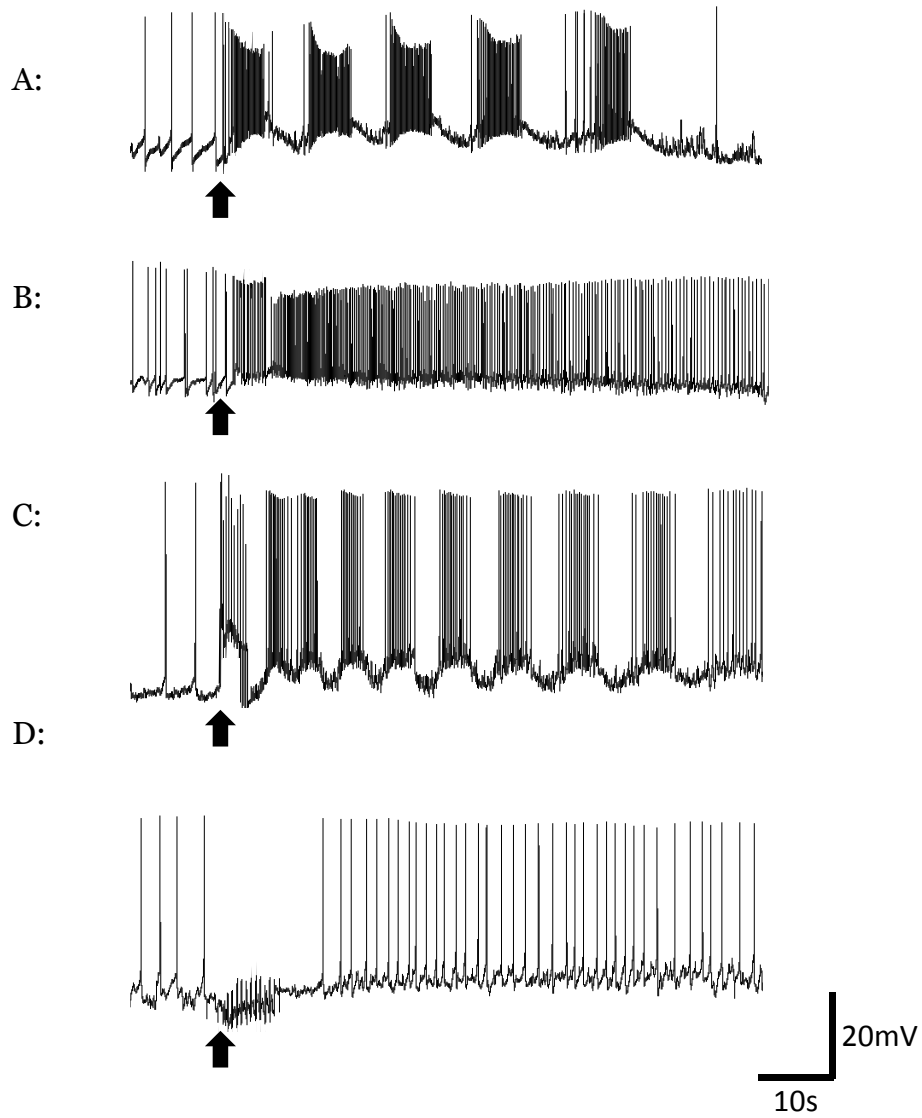


Figure 3-1: Swim Motor Pattern in Three Nudipleura Species.

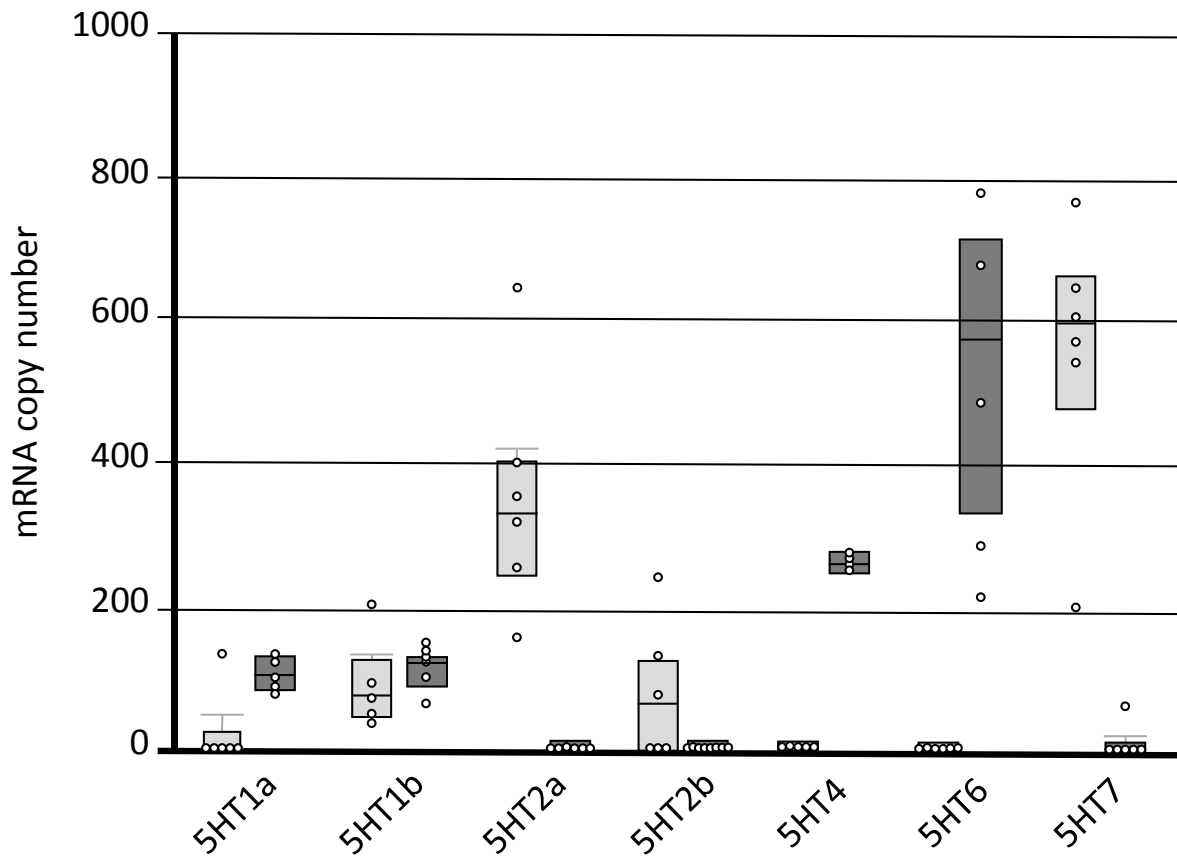
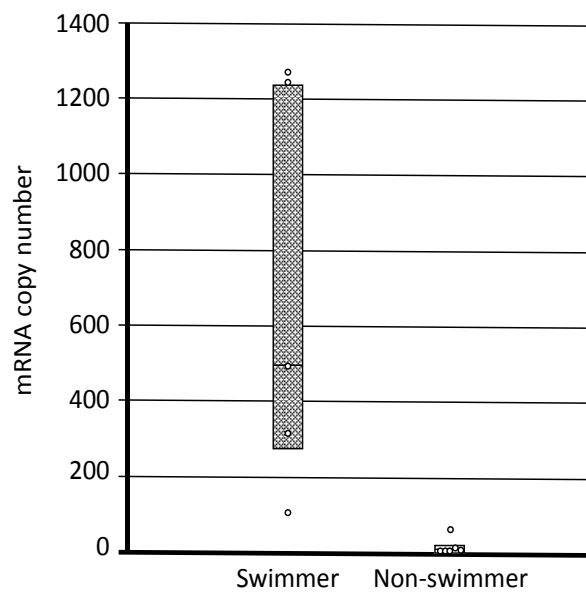


Figure 3-2: 5-HT Receptor Expression in C2 from Tritonia and Hermissenda

A:



B:

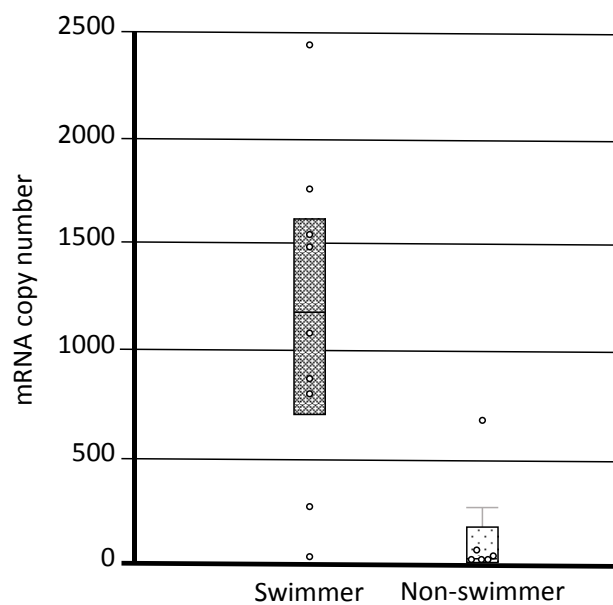
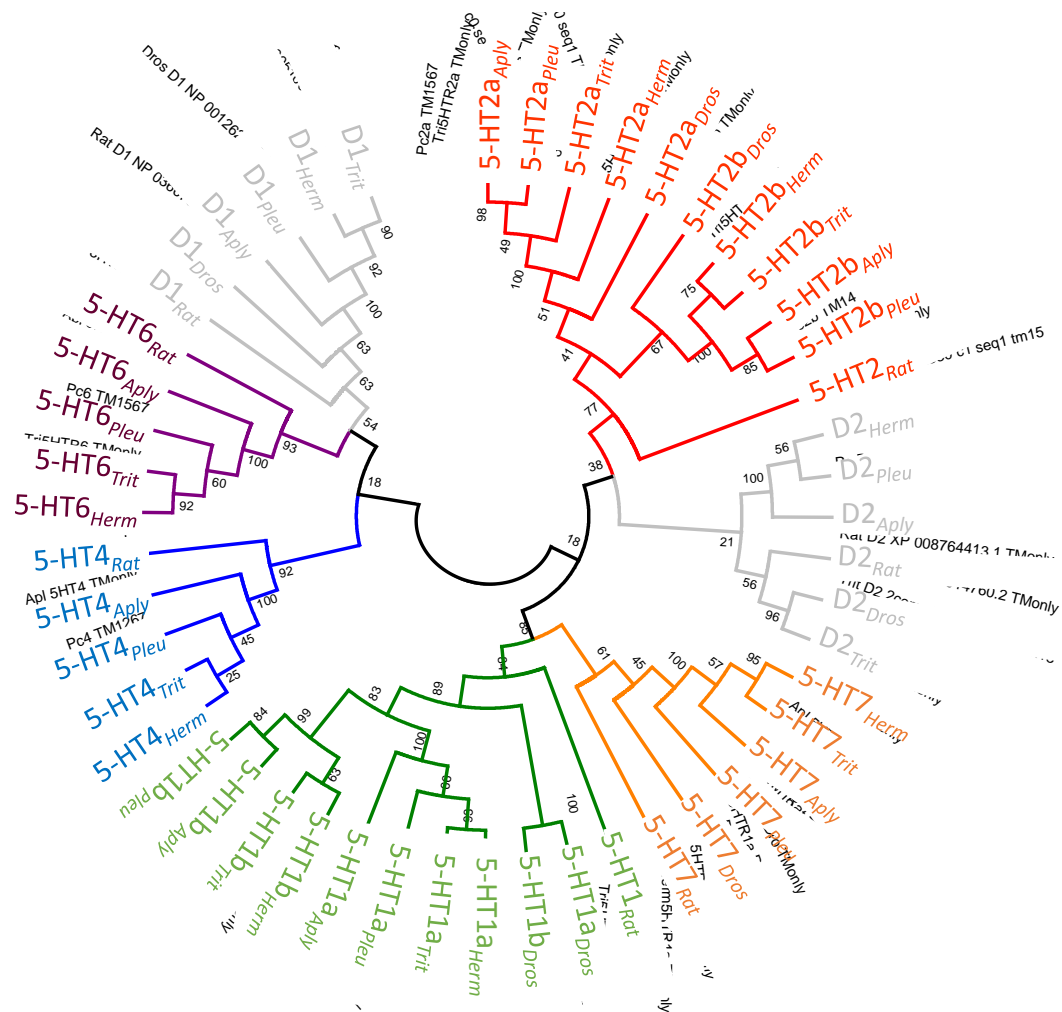
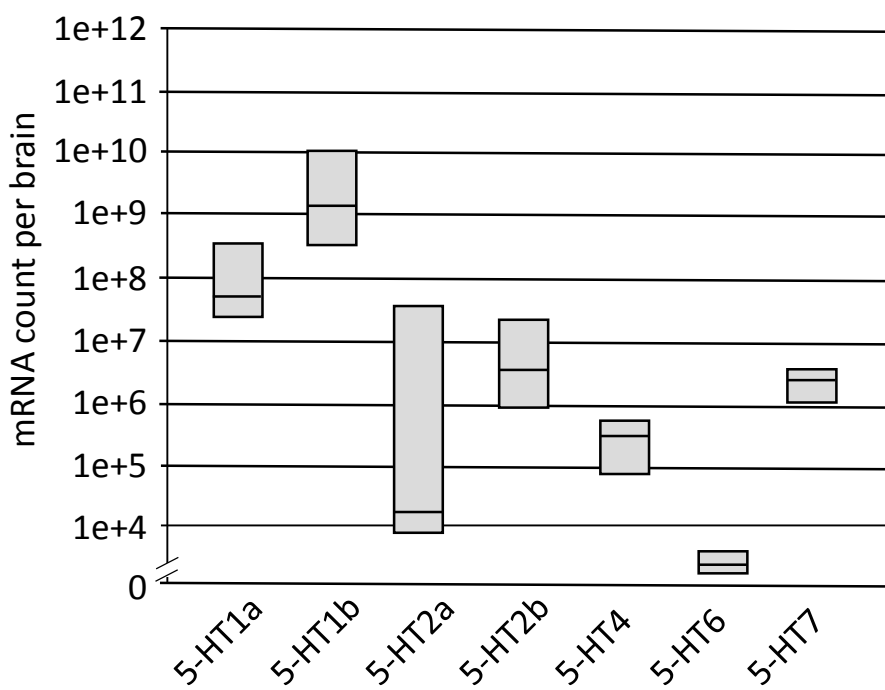


Figure 3-3: 5-HT_{2a} and 5-HT₇ Receptor Expression in C₂ from Pleurobranchaea with Variable Swimming Behaviors.

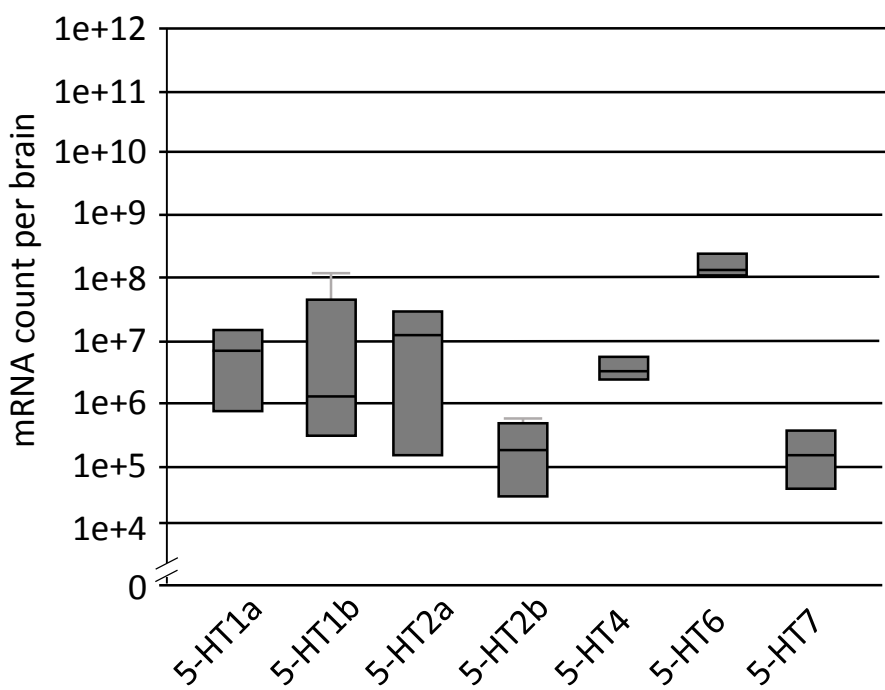


Supplemental Figure 3-4: Phylogenetic Tree of 5-HT and Dopamine Receptor Subtypes.

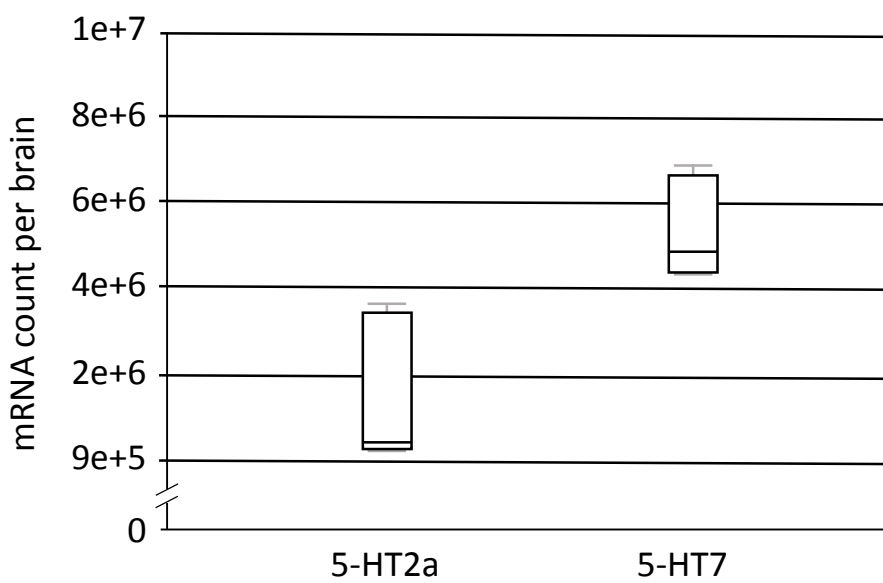
A:



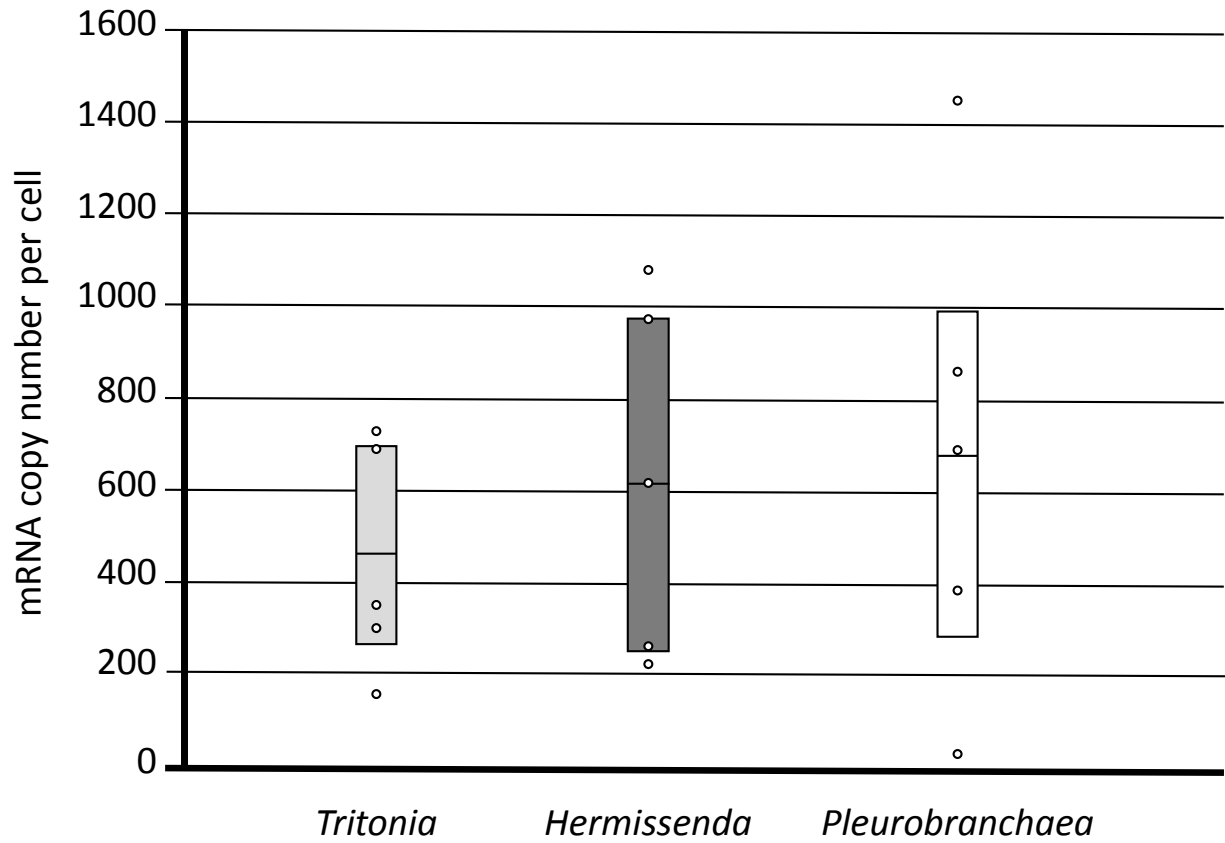
B:



C:



Supplemental Figure 3-5: Whole-Brain Expression of 5-HT Receptors.



Supplemental Figure 3-6: Small Cardioactive Peptide (SCP) Gene Expression in C2 Homologues.

Supplemental Table 3-1: Primers for Cloning (A) and Quantitative PCR (B)

A:

Gene	FW Primer	RV Primer
5-HT1a _{Trit}	CCAGACTACATTCAACCTAAACCAGAGC	GTGGTCAGTTTATGCCTCTGGCTTG
5-HT1b _{Trit}	GGTAATGGTAGTTTAATCAGCGTG	GTTCGGTAAACAGTTAAAGCTCTAG
5-HT2a _{Trit}	CAGGGTCAAGGAACTCTCGGCAATC	GATTCGGAGATCCACAAGCAGTAAGTCG
5-HT2b _{Trit}	CCCATTTGCTGAGAGGTCCTGTCTG	CAGATGGCACCCTTAATCGCTATTGATGG
5-HT4 _{Trit}	CAATAGCGACATTGTGATCCTTGCGCC	GCGGAGGTGGAGGAACACGAAAAG
5-HT6 _{Trit}	GCCTTTCTCCAGACGCCTGCTG	GGGTGGCTTCTGCAGATTTAAGAATTATC
5-HT7 _{Trit}	GACTATCACTCTCACCGATTCTAACACGG	CCCTAGATAACCCAGCGCGAATATTTTAC
5-HT1a _{Herm}	GTGCCACGATTCAAGTAATTGG	ATAGAATAGCATAGTGCAATCTTAGTCG
5-HT1b _{Herm}	CCATGATGAGTAACGTCACGCTACCG	CAGGAGAGTCGTTCGATGATGCGTC
5-HT2a _{Herm}	GCTTTAACGGAACGGACGGTGATTCTG	CAGGGGAGGAATAAGTTTTACGGAGGAG
5-HT2b _{Herm}	CACGCACCACTACAACAACATCATCCTC	GGTCATCGGGAAAATCCTGTTTCGTGTG
5-HT4 _{Herm}	CCAACACAGCGGCCCCACC	CCATGGCAAGTCGCTCTATGGTTAGG
5-HT6 _{Herm}	GCAGATTCCCAGTAGAGTGGTCAACG	CATAGCATCACCGCTGTCCCTAACC
5-HT7 _{Herm}	CCCCTTCCCAATCAATCGGTTG	CCCGACACAACACGGAGTCT
5-HT2a _{Pleu}	GACACCTTCCACTCCAGCAACAT	TACGGCTCCATCTTCGCTTTCTTCAT
5-HT7 _{Pleu}	GAGACCAGGTCACCATGACCAGC	CACACTGCCGGTACACACGGG
SCP _{Trit}	GTTGTCTCGAGGTCAGGCTTGATAC	GTTGTCTACTCAATGTGTGTTTCCTGACG
SCP _{Herm}	TCCAGCCCGAAGTCCAACAGTAATG	CCAACCACCATCCCGTCTATTGTAG
SCP _{Pleu}	CAAGCAGAACCCTCCAAGACACAATG	GTTCGGCATTTCATCACAGTTTGAAGG

B:

Gene	FW Primer	RV Primer
5-HT1a _{Trit}	GATTTCAATGGATGTCTTGTGCTGC	GCATTCTACTGTCCTCTGATTCTCA
5-HT1b _{Trit}	CAGCCAGGTATGGTTTCTCCAC	GAAACGAAGTGCCAGGCGGATT
5-HT2a _{Trit}	ATTACGGCTCTCACCCGAAGAAG	CGATTCCACGAACGACAAGCAG
5-HT2a _{Trit}	CAGGGTCAAGGAACTCTCGGCAATC	GATTCGGAGATCCACAAGCAGTAAGTCG
5-HT2b _{Trit}	GTTATGCCCTTTGGAATGGTAGTAG	ATGGGACTCGTAGATAGGACAG
5-HT4 _{Trit}	CGTGTTTGGAATAGCGTGGTCATT	CAGACAGATAAACAGCAACGACTG
5-HT6 _{Trit}	CAGAACCGTCTCCAACCTCTTTAT	GAGCATTGTGTCATCAGCTTC
5-HT7 _{Trit}	GACTATCACTCTCACCGATTCTAACACGG	CCCTAGATAACCCAGCGCGAATATTTTAC
5-HT1a _{Herm}	GTGCCACGATTCAAGTAATTGG	ATAGAATAGCATAGTGCAATCTTAGTCG
5-HT1b _{Herm}	CCAACGGAAATAGCAATACCAACAT	CGAGTATCGTTCTGGGACTGTTC
5-HT2a _{Herm}	GCTTTAACGGAACGGACGGTGATTTCG	CAGGGGAGGAATAAGTTTTACGGAGGAG
5-HT2a _{Herm}	CACGCACCACTACAACAACATCATCCTC	GGTCATCGGGAAAATCCTGTTTCGTGTG
5-HT2b _{Herm}	CGCTAACCTCATCATTCTGTGC	CCGAACATGCGTGTGTGGG
5-HT4 _{Herm}	GTCCCAATCCTGACCGTGTT	CATTTTCCATCTTTCTTGTCTCGCC
5-HT6 _{Herm}	CAGCAGCACCTTGAGAACCAT	GCGATCATAACGCCTCTACG
5-HT7 _{Herm}	CCCCTTCCAATCAATCGGTTG	CCCGACACAACACGGAGTCT
5-HT2a _{Pleu}	CGAGCACTTCATCATCTACGGC	TCCACCAGCCGCCGTAAC
5-HT7 _{Pleu}	GAGACCAGGTCACCATGACCAGC	CACACTGCCGGTACACACGGG
SCP _{Trit}	GAAATGACAATGCCCCGAGCAAC	CACGGCATCAGTGTCTGTGTG
SCP _{Herm}	GAGAATGTTGTGGCATCGGACTC	ACCCAGTTCCTGACCAAG
SCP _{Pleu}	GTCCAAAGCCGACGCATCG	CGACGAGAAACAGGACGGC

Supplemental Table 3-2: Pleurobranchaea 5-HT Receptor Transcriptome Gene Identification Numbers.

Gene	Gene Identification #
5-HT1a _{pleu}	comp77412_co_seq1
5-HT1b _{pleu}	comp71667_co_seq4
5-HT2a _{pleu}	comp68325_c1_seq1 and comp70123_c2_seq3
5-HT2b _{pleu}	comp53876_co_seq1
5-HT4 _{pleu}	comp159323_co_seq1 and comp48526_c1_seq1
5-HT4 _{pleu}	comp43137_co_seq1
5-HT6 _{pleu}	comp68387_c1_seq1
5-HT7 _{pleu}	comp73047_c11_seq1

4 CONCLUSION

4.1 General Discussion

DV-swimming is a behavior found in only a handful of the more than 3000 Nudipleura species that are currently known, and is believed to have not been present in the common ancestor of this clade (Goodheart et al. 2015; Newcomb et al. 2012). It is therefore most parsimonious to assume that it evolved independently in *Tritonia* and *Pleurobranchaea*. In both species, swimming behaviors are controlled in part by the neuromodulatory action of 5-HT on C2 homologues, an example of parallel evolution of a biological mechanism underlying analogous behaviors (Katz and Frost 1995a; Lillvis and Katz 2013). This dissertation addresses two main questions related to this phenomenon: 1) what 5-HT receptors are expressed in the brains of Nudipleura sea slugs, and 2) which of those receptors are expressed in C2 homologues from *Tritonia*, *Pleurobranchaea*, and non-DV swimmer *Hermisenda*? The answers to these questions increase our understanding of the evolution of biogenic amine receptors across animal phyla, and illustrate a biological mechanism by which neuromodulatory receptor genes may facilitate the evolution of species-specific behaviors.

The findings presented in this dissertation provide support for a proposed biological mechanism by which species-specific behaviors can occur, namely that species-differences in neuromodulation can result in species-specific behavior (Katz et al. 1999). This dissertation examined this idea with respect to neuromodulatory 5-HT receptor expression. The results of the dissertation showed that similar receptor expression correlates with species-specific, independently evolved swimming behaviors. Below, I will review literature on 5-HT receptor evolution and homologous cells, two subjects which have implications for the results of this dissertation.

4.2 Serotonin Receptor Evolution

Introduction

The previous chapters of this thesis contributed to our understanding of 5-HT receptor evolution by identifying 5-HT receptor subtype genes in molluscs, and characterizing their expression patterns in single homologous cells from three species. This portion of the concluding chapter discusses these findings with respect to what was previously known about 5-HT receptor evolution.

5-HT is an ancient signaling molecule from a class of neurotransmitters known as biogenic amines. It is currently theorized that 5-HT first evolved as early as 2 billion years ago, in an ancestor of eukaryotes (Levine 1980; Turlejski 1996). Because it evolved so early, it is present in a diverse array of extant organisms, from single-celled eukaryotes, to almost all living animals and many plants (Pelagio-Flores et al. 2011; Turlejski 1996; Walker and Holden-Dye 1991; Wojtaszek 2003). 5-HT is synthesized from tryptophan through a series of enzymatic reactions. The chemical preferentially acts on 5-HT receptors, which first evolved over 700 MYA (Peroutka and Howell 1994). The first three families of 5-HT receptor genes were characterized in the 1980s (Bradley et al. 1986). In the approximately 30 years following this discovery, the repertoire of known 5-HT receptors has grown exponentially both in terms of species in which they are expressed, and in the number of families and subtypes.

Today, 5-HT receptors have more identified families and subtypes than any other biogenic amine receptor known (Nichols and Nichols 2008). Seven families of G-protein coupled 5-HT receptors (GPCRs) have been identified in bilaterians (Hoyer and Martin 1996; Nichols and Nichols 2008; Peroutka 1994; Peroutka and Howell 1994). Within some of these families, different subtypes have evolved independently in different phyla.

Despite the divergence in subtypes, however, the GPCR families share common amino acid structures, G-protein coupling, and pharmacological traits (Hen 1993; Nichols and Nichols 2008).

5-HT receptors are involved in a vast number of different biological processes. They regulate learning and memory in the hippocampus, amygdala, and cerebellum in mammals (Glikmann-Johnston et al. 2015; Stiedl et al. 2015; Zhang and Stackman 2015), and may work similarly in single neurons that form memories in *Aplysia* (Barbas et al. 2003; Dumitriu et al. 2006; Lee et al. 2009). They are activated during aggressive postures and in dominance hierarchy formation in crayfish (Momohara et al. 2013; Tierney and Mangiamele 2001; Yeh et al. 1996). They control swimming through their activation and modulation of synapses in sea slugs (Katz 1998; Katz et al. 1994; Lillvis and Katz 2013; Newcomb et al. 2012). They are activated shortly after mammalian spinal cord injury, and may contribute to the individual variability in healing observed in humans following traumatic nervous system injury (Ghosh and Pearse 2014; Nardone et al. 2015; Navailles et al. 2013; Slawinska et al. 2014). They are important in human cognition, and their dysfunction may be at the core of human mental disorders: mutations in human 5-HT_{2c} have been correlated with occurrence of schizophrenia and depression (Chagraoui et al. 2016; Fakhoury 2016; McCreary and Newman-Tancredi 2015; Samuels et al. 2016). These examples show that 5-HT receptor function has been characterized in a diverse array of behaviors across many animal species. The diverse functions 5-HT plays are relevant to the evolutionary history of its receptors.

The evolutionary origin of 5-HT receptors has been described previously (Nichols and Nichols 2008; Peroutka 1994; Peroutka and Howell 1994). The findings from chapter 2, as well as other recently identified 5-HT receptor genes, support the prevailing theory

of 5-HT receptor origin, however they add new information to our understanding of how these receptors evolved. Studying the origin of this gene family, and the potential reasons for the diversity of its members, can impact our understanding of 5-HT-mediated behaviors and disorders.

Vertebrate and invertebrate 5-HT receptors shared a common ancestor

The 5HT receptor families in vertebrates and invertebrates share a common origin (Peroutka 1992; Peroutka 1993; Peroutka 1994). The currently accepted theory is that a single ancient receptor diverged into three super families, known now as 5-HT₁, 5-HT₂, and 5-HT₆. This divergence occurred after metazoans diverged to become cnidarians and bilaterians, but before the separation of protostomes and deuterostomes (Table 4-1). The Nudipleura 5-HT receptor sequences identified in Figures 2-1 and 3-4 fall in phylogenetically with receptors from other animal phyla, because their sequences cluster together with other receptors from the same family, rather than with sequences from more closely related animal species. Their identification and phylogenetic characterization in Chapter 2 supports the single 5-HT receptor origin theory.

The theory of a common ancestor of all 5-HT receptors was first presented over two decades ago (Peroutka 1993; Peroutka 1994; Peroutka and Howell 1994). Peroutka and Howell analyzed rates of amino acid substitution between 5-HT receptor families from diverse phyla, including vertebrates, insects, and molluscs. They compared amino acid substitution rates from extant 5-HT receptor protein sequences with estimated dates of divergence between species, to estimate the age of the receptor gene divergence. Peroutka and Howell's research calculated that the first 5-HT receptor evolved between 700 and 900 million years ago (MYA) (Peroutka and Howell 1994), after metazoans

diverged to form bilaterians and cnidarians (Park et al. 2012). At approximately 700 to 800 MYA, the authors concluded that the primordial 5-HT receptor differentiated into super-families 5-HT1, 5-HT2, and 5-HT6. At approximately 600 to 700 MYA, the 5-HT1 super family further diverged, forming the extant families 1, 5, and 7. At approximately 600 MYA, the ancient ancestor of bilaterians diverged to form protostomes and deuterostomes (Erwin and Davidson 2002). Consequently, the extant 5-HT receptors are believed to share a common origin, even between distantly related phyla, and were present in the common ancestor of deuterostomes and protostomes. No extant 5-HT receptor, however, represents the original ancestral genes, because the individual genes have undergone mutations for up to hundreds of millions of years.

Although there has been a large amount of divergence in the genetic sequences of 5-HT receptors in the past 600 million years, amino acid features that are essential to 5-HT receptor functioning have remained conserved. One prominent characteristic of all known 5-HT receptors is the conserved cysteine residue in the extracellular loop of the receptor between transmembrane domains two and three, which is believed to be involved in securing the helices together in a loop and in ligand binding (Nichols and Nichols 2008; Wurch and Pauwels 2000). This feature is found in all known 5-HT receptor amino acid sequences, including the Nudipleura receptors identified in Chapter 2 (Nichols and Nichols 2008; Tamvacakis et al. 2015). Another conserved feature is the G-protein activation site, which activates binding to GTP. It is represented as the amino acid motif DRY or ERY in almost all 5-HT GPCRs (Nichols and Nichols 2008), with the exception of the 5-HT2b subtypes found in molluscs and arthropods (Clark et al. 2004; Tamvacakis et al. 2015). These are two examples of the phylogenetic commonalities of 5-HT receptors, which are believed to maintain their basic function.

Until the early 2000s, genetic data from many invertebrate species was severely lacking, so while the theory of 5-HT receptor evolution explained above was accepted, it was based on a relatively limited sample size of species. As more and more invertebrate genetic information became available, however, analyses showed phylogenetic relationships between invertebrate and vertebrate 5-HT receptors with better resolution. More recent publications showed that invertebrate and vertebrate receptor proteins fall into the same 5-HT receptor families predicted by Peroutka and Howell. For example, a given 5-HT receptor gene is more closely related to members of its gene family in distantly related animal phyla than it is to different genes in closely related species (Barbas et al. 2003; Dacks et al. 2013; Dass and Sudandiradoss 2012; Mapara et al. 2008a; Mustard et al. 2005; Nagakura et al. 2010; Nichols and Nichols 2008; Paluzzi et al. 2015; Spielman et al. 2015; Tamvacakis et al. 2015). One exception to the rule appears to be opossums, and possibly all marsupials. Opossum 5-HT_{2a} and 5-HT₇ do not cluster with the current classification (Dass and Sudandiradoss 2012). It may be that recent evolutionary events have allowed amino acid substitutions in this species after its divergence with placental mammalian species, but it is unclear whether these changes result in functional changes in the receptors. Another exception is the 5-HT₃ receptor gene, an ionotropic receptor found only in vertebrates, which according to phylogenetic analysis most likely evolved from existing nicotinic acetylcholine receptors (Maricq et al. 1991; Reeves and Lummis 2002). Overall, even with the recent advancements in gene sequencing technology, the single 5-HT receptor ancestor idea proposed in 1994 by Peroutka and Howell is still supported.

The early research on 5-HT receptor evolution estimated that the first 5-HT receptor evolved after the bilaterian-cnidarian split, approximately 600 MYA. This would

predict that cnidarian nervous systems do not express a 5-HT receptor-like gene. While cnidarian nervous systems do possess biogenic amine-like chemicals (Anctil and Bouchard 2004) and GPCRs (New et al. 2000), they seem to rely more on neuropeptide signaling than other phyla do (Grimmelikhuijzen et al. 2002). Using radio-ligand binding assays, a group of researchers showed that polyps of the sea pansy *Renilla koellikeri* bind 5-HT (Dergham and Anctil 1998; Hajj-Ali and Anctil 1997). This research indicates that a cnidarian receptor may be present that recognizes 5-HT. However, no subsequent study has shown expression of any genes that are phylogenetically similar to 5-HT receptors, indicating that the cnidarian 5-HT response may be due to a protein that does not share ancestry with the extant bilaterian receptors. Thus, the idea that 5-HT receptors evolved after bilaterians and cnidarians split is still supported.

Pharmacological studies have also attempted to characterize 5-HT receptors, but these have been performed mainly on vertebrate receptor orthologues. Exogenous agonists and antagonists, which are often developed against specific mammalian 5-HT receptor subtypes, cannot be used to identify invertebrate 5-HT receptor orthologues. Selection pressure on extant 5-HT receptor families has resulted in divergence of the genes (Dumitriu et al. 2006; Tierney 2001). The invertebrate receptors all still preferentially respond to 5-HT (at least, those species-specific subtypes that have been functionally characterized respond), but beyond that, pharmacological characterization is difficult. For example, an agonist against a mammalian 5-HT₇ receptor cannot reliably identify 5-HT₇ receptors in a mollusc or arthropod, because divergence of the amino acid sequences between these phyla has caused changes in the conformation of the receptor proteins. Thus, invertebrate and vertebrate 5-HT receptors are pharmacologically distinct, despite their phylogenetic similarities. That is why the majority of 5-HT receptor

identification done today uses phylogenetic analysis of amino acid sequences, a much more accurate means of identifying homology between invertebrate and vertebrate genes.

Although each family was originally classified by the similarity of their protein structures, the families each have specific and conserved G-protein coupling, and each receptor family's activation results in specific downstream activity (Nichols and Nichols 2008). For example, 5-HT₂ family receptors are coupled to G_q/G₁₁ G-proteins and activate an intracellular activity pathway involving IP₃/DAG. In the description of the *Aplysia* 5-HT₂ receptor, Nagakura et al. (2010) show that the *Aplysia* 5-HT_{2a} gene has these same coupling and IP₃/DAG pathway.

The phylogeny of 5-HT receptors presented in Chapters 2 and 3 recapitulates the previously published GPCR phylogenies, even with the addition of the newly identified molluscan 5-HT receptor genes 5-HT_{2b} and 5-HT₆. Overall, it is clear that the single origin of 5-HT receptors theory originally proposed by Peroutka and Howell is upheld.

Phylogenetic Analyses of Selected Specific Molluscan 5-HT Receptor Subtypes

The evolutionary relationships between specific receptors identified over the last two decades support Peroutka and Howell's theory of a common ancestor of 5-HT receptor subtypes (Tierney 2001). Here, the theory will be applied to 5-HT₁, 5-HT₂, and 5-HT₆ family genes.

After the protostome/deuterostome split, the 5-HT receptor genes continued to diverge within the newly evolving species. 400 to 500 MYA, differentiation of the mammalian 5-HT₁ orthologue occurred, leading to mammalian subtypes 1a, 1b, 1d, 1e, and 1f (Peroutka 1994). These mammalian receptor subtypes are not considered as being homologous to the invertebrate type 1x receptors. Instead, the 5-HT_{1a} and 1b subtypes

found in molluscs and arthropods diverged from the ancestral 5-HT₁ family receptor independently in each phyla, resulting in molluscan-specific and arthropod-specific 5-HT_{1a} and 1b paralogs. The phylogeny presented in Chapter 2 supports this idea: the Nudipleura 5-HT_{1a} and 1b subtypes are more closely related to one another than to the *Drosophila* 1a and 1b receptors (Figure 4-1a). Thus, we can conclude that the divergences seen in the invertebrate 5-HT₁ genes are phyla-specific gene duplication events.

The 5-HT₂ family genes have also duplicated independently. The vertebrate 5-HT₂ receptor differentiated into the subtypes we call 5-HT_{2a}, 2b, and 2c between 500 and 600 MYA (Peroutka 1993). Recent research has shown that there are two 5-HT₂ subtypes expressed by arthropods (Clark et al. 2004; Dacks et al. 2013). Research presented in Chapter 2 provided the first evidence that there are two 5-HT₂ family subtypes expressed in the molluscan brain. According to the phylogenetic tree presented in Chapter 2, the molluscan 5-HT_{2a} and 2b subtypes are more closely related to their arthropod counterparts than they are to one another, indicating that, unlike the molluscan specific duplication event in the 5-HT₁ family, the 5-HT₂ subtypes diverged from one another before the ecdysozoan/lophotrochozoan split. The molluscan/arthropod 2a and 2b receptors are not phylogenetically similar to the vertebrate 5-HT_{2x} subtypes, indicating that these two groups diverged within the 5-HT₂ gene family (Figure 4-1b). An alternative explanation is that the 5-HT₂ duplication event occurred independently in each arthropods and molluscs, and the sequences converged in such a way that they appear similar.

This alternative explanation is less parsimonious given the existence of a special G-protein binding sight shared by arthropod and molluscan 5-HT_{2b} receptors. Most GPCRs contain a G-protein activation site coded by amino acids DRY or ERY. The 5-HT_{2b}

receptors in arthropods, however, were found to have an altered site coded by amino acids DRF, resulting in altered G-protein activity (Clark et al. 2004). The *Nudipleura* and *Aplysia* 5-HT_{2b} receptors were found to contain the same DRF motif, although the *Melibe* 5-HT_{2b} receptor was found to have the more common DRY. The shift between tyrosine (Y) and phenylalanine (F) is a single nucleotide, so it may be that the 5-HT_{2b} genes evolved independently across groups. Furthermore, NCBI data sets revealed no 5-HT_{2b} orthologue in other lophotrochozoans. It is also important to note that the 5-HT_{2b} receptor has not been functionally characterized in any mollusc, although it was in crustaceans and insects (Clark et al. 2004; Dacks et al. 2013). Despite this point, we make the assertion that the gene coding for 5-HT_{2b} evolved in the common ancestor of arthropods and molluscs, because of the shared DRF motif and phylogenetic tree distribution.

5-HT₆ was proposed to be one of the three ancestral 5-HT receptor families (Peroutka 1994). However, more recent 5-HT receptor phylogenies call this idea in to question: 5-HT₄ family genes, which were not included in the Peroutka publication, may be ancestral to 5-HT₆. More current publications show 5-HT₄ and 6 as sister groups, so that the exact identity of the ancestor is unclear (Nichols and Nichols 2008; Spielman et al. 2015; Tamvacakis et al. 2015). A focus on the evolutionary history of these specific receptors would better determine the true ancestral state.

The 5-HT₄ and 5-HT₆ genes are missing from several large extant phyla. They have only been characterized in vertebrates and molluscs (Nichols and Nichols 2008; Tamvacakis et al. 2015). Perhaps the gene was lost in other species, and retained in only these two phyla. It could have been lost and regained in either vertebrates or molluscs. Or, it could be that one or the other phylum independently evolved a receptor that is

highly similar to the extant 5-HT6. An examination of 5-HT6 amino acid substitution rates in extant species, and potential pseudogenization in species that do not express the receptor, could help answer these questions. While a 5-HT4/6 ancestor was undoubtedly one of the three ancestral families, the amino acid sequence of the ancestral receptor could be better understood with a more thorough investigation.

5-HT Receptor Subtype Diversity

Individual 5-HT receptor subtypes are more numerous than any other major biogenic amine receptor class, possibly excluding trace amine-associated receptors (TAARs) (Nichols and Nichols 2008). This is because several of the receptor gene families contain multiple subtypes. The 5-HT1 family, for example, contains up to five subtypes in vertebrates. Two subtypes evolved in insects, and two in molluscs, through independent gene duplication events (Nagakura et al. 2010). In the 5-HT2 family, multiple subtypes again evolved independently in different species (Clark et al. 2004; Dacks et al. 2013; Tamvacakis et al. 2015). The 5-HT5 family contains at least one gene duplication, resulting in 5-HT5a and 5b (Grailhe et al. 2001; Rees et al. 1994). In contrast, there has been little to no documented evidence of gene duplications in families 4, 6, or 7 (Nichols and Nichols 2008), although one study has identified 5-HT4 subtypes in humans (Bach et al. 2001). It appears that some 5-HT receptor gene families more likely to contain multiple receptor subtypes.

Gene duplication events likely led to the evolution of many of the additional subtypes. The molluscan 5-HT1a and 1b subtypes, for example, resulted from tandem gene duplication (Nagakura et al. 2010). The two genes are located next to one another on a chromosome in the *Aplysia* genome (taxonomic ID 6500). Other tandem gene

duplications likely allowed for subtype divergence amongst other species, like the whole-genome duplication event in vertebrates (Herculano and Maximino 2014). An examination of genomic 5-HT receptors and their potential pseudogenes would illustrate the number of 5-HT receptors that evolved through these types of events.

Comparing the divergence rates of 5-HT1 and 2 families with the other families might illustrate why some receptor families have more subtypes than others. Positive selection on gene families 1 and 2, or negative selection on the other families, could be inferred by comparing amino acid substitution rates. Although this would not explain *why* those families have diverged into numerous subtypes, it would explain how it happened through the course of evolution.

If there is increased positive selection on these receptors, then there may be a greater diversity of them than previously known. 5-HT receptors from the more diverse families may have been gained and lost several times due to mutations resulting in additions and deletions. This has been shown to be the case for opsins, in which nine families of opsin genes have been identified in bilaterians, and four families are shared between bilaterians and cnidarians (Ramirez et al. 2016). Olfactory receptors have undergone multiple gene duplications and losses, as illustrated by the variable identity of olfactory receptor genes across closely related species (Nei et al. 2008; Niimura 2012; Niimura and Nei 2007). One interesting theory that has come from this is the idea that the high rates of amino acid substitutions has brought about orphan receptors (Ramirez et al. 2016).

Some of those gains and losses may have resulted in orphaned 5-HT receptors, as well. It may also mean that there is greater diversity in the specific DNA and amino acid sequences of the extant subtypes between individuals of the same species. Although we

did not observe this in molluscan 5-HT receptor DNA sequences, it would be interesting to know if such a diversity exists in species with more subtypes per family, as is the case in humans. If it does, does that contribute to the vast variability of heritable mental disorders, responses to brain trauma, and drug addiction in humans? This has been postulated for human OXTR (Brune 2012), and could occur in 5-HT receptors as well.

Conclusion

The evidence summarized above describes what is currently known about how 5-HT receptors evolved. This research shows that 5-HT receptors evolved from a single ancient receptor that was present before the divergence of protostomes and deuterostomes. The receptors have since diverged greatly from one another, and are today essential to neural functioning in almost all bilaterian animals. It is possible that many important 5-HT-mediated behaviors occur the way that they do because of subtle changes in the evolution of the receptor sequences. If so, then understanding how 5-HT receptor subtypes evolved across species can help us better understand neural mechanisms of behavior in light of evolution.

4.3 Evolution of Cell Types across Species

Introduction

In Chapter 3, gene expression in homologous C2 neurons was compared using qPCR, and differences in 5-HT receptor expression were found to correlate with species-specific behaviors. This finding illustrates an important point about homologous cells: that while they share a common ancestor, they do not necessarily share common functions or gene expression in extant organisms. In this section, I will discuss

homologous single cells beyond those found in the relatively closely related Nudipleura, to explain what they can tell us about the evolution of the cell, with respect to recent advances in RNA-Seq and cellular fingerprinting technologies.

Homologous Cells, Again

Homologous neurons were described in Chapter 1 as identifiable cells that are found across species, share some common traits, and are predicted to have been present in a common ancestor. According to the theory of common descent (Darwin 1859), however, all neurons and other cell types are descended from a common ancestor, so technically all cells are homologous at some level. Instead, the term is used to describe more recent evolutionary events, which allows us to distinguish between the differentiated cell types within and between species. The term “homologous cell” is therefore different from that of “sister cell,” which indicates a cell that was derived from the same ancestor but then diverged to form a new cell type (Arendt 2008). Comparing cells that are considered homologous between extant species can help explain how cells diversified and specialized over evolutionary time.

Cells are complex and highly diversified from one another, yet they share commonalities because they descended from a common ancestor. If cells descended from a common ancestor, then it follows that they would be subject to principles of evolution that are similar to those found to occur for single genes (Arendt 2008). Arendt (2008) proposes that a major property of cellular evolution is ancestral multifunctionality, followed by increasing specialization and diversification. One example of this is the proposed ancient ancestor of the neuron, which is believed to have been a cell with multiple roles in sensing the external environment and secreting chemicals to

communicate with neighboring cells by paracrine signaling (Marlow and Arendt 2014; Striedter GF et al. 2013). This ancestral cell diversified into the neuron types known today.

The proposed ideas of how cells evolve is similar to that observed in single genes, but single-gene evolutionary studies are more common. For example, HOX genes are believed to have been multifunctional in the ancestors of bilaterians (Force et al. 1999), then were subject to gene duplications and asymmetric divergence in functions (Holland et al. 2017).

The idea that cells evolve in ways that are similar to single gene evolution has existed for several years, yet it has been difficult to test (Arendt 2008). Cellular homology was traditionally characterized using microscopy, but conclusions became difficult with less closely related species. With recent advances in technology, however, it is now possible to compare broad scale gene expression across single cells from much more distantly related species. This could allow for the potential to identify suites of gene markers that identify a homologous cell across species, and would lead to the creation of phylogenies for cells, like those for species or individual genes. This could tell us how cells evolved across more distantly related species, and how differences in homologous cells can lead to species-specific traits.

Single-Cell Transcriptomics

Using RNA-Seq, microarray, and other molecular fingerprinting techniques, large-scale gene expression comparisons are now being used to determine the evolutionary relationships between cells. Next-Gen sequencing applied to single cell types has the potential to identify homologous cells based on their gene expression.

In Appendix A of this dissertation, we attempted a pilot study of single-neuron RNA-Seq, to compare gene expression between homologous C2 neurons from four species. The datasets produced were used to compare 5-HT receptor expression across species. Other studies have performed RNA-Seq on single cell types to look more broadly at the molecular expression signatures of extant cells. In comparing maternal and fetal placental cells using single-cell RNA-Seq, a recent study found that specific gene markers could reliably identify specific cell types (Nelson et al. 2016). In a study of mouse retinal cells, a technique called Drop-Seq was used to sequence individual cell types in the retina. The authors found that the molecular fingerprinting of those cells separated them reliably in to cell classes that were predicted based on previous morphological and single-gene expression data (Macosko et al.). In embryonic rats and mice, gene expression in pyramidal neurons and hippocampal neurons was compared, and common gene markers were identified that were shared by the cell types between species. The same study also identified significant variability in gene expression across repeated samples compared with controls (Dueck et al. 2015). The authors hypothesized that the gene expression variability may be under regulatory control, and that such variability may be necessary for functioning in large, complex tissues like the brain. The technology used to perform these studies is relatively new, and as the number of studies undertaken increases we can expect to see more species-comparisons of single cell transcriptomes, with further investigations into gene expression variability.

Comparing homologous cells from different species using single-cell transcriptome sequencing is the next step in understanding how cells evolved. In many systems, this comparison is limited to embryonic development, when there are fewer numbers and types of cells. The adult molluscan nervous system is composed of large,

identifiable cells that can be compared across species, however, making it a viable alternative to embryonic-only studies. Furthermore, because several neurons in the Nudipleura brain have been functionally characterized, their functions can be compared at the level of species-specific single cell transcriptomes.

Conclusions

In this dissertation, I have used the study of gene expression in homologous neurons to make inferences about how behaviors evolved. The study of homologous cells has broader utility, however. It allows for an understanding of biology and evolution at a different level from that of genes or larger tissues, which are more common for evolutionary biologists to compare. Cells have evolved just as genes have, and may be subject to evolutionary principles that are similar to those for individual gene evolution (Arendt 2008). Understanding how cell types evolved can help explain how more complex systems like the nervous system evolved, which in turn can lead to a better understanding of the diversity of animals and behaviors seen today.

4.4 Dissertation Conclusion

The results of this dissertation illustrated 5-HT receptor expression across Nudipleura species, and correlated specific receptor subtype expression in single neurons with species-specific swimming behaviors. This work aligns with previously published research on the evolution of 5-HT receptors, and work on 5-HT modulation of DV swimming in Nudipleura sea slugs.

The dissertation also addresses the comparison of homologous cells with respect to their receptor expression. While there were commonalities in some of the biogenic

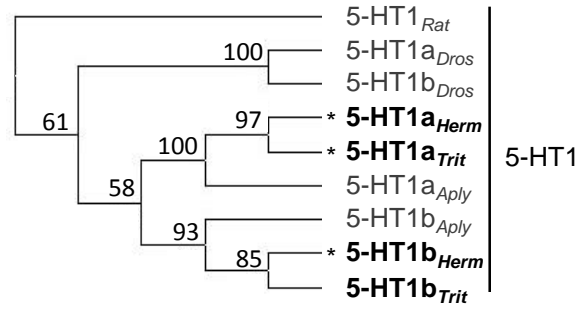
amine receptors identified between *Tritonia* and *Hermisenda* C2 neurons as summarized in Appendix A, there were also species-differences in both 5-HT receptors and dopamine receptors. While the exact functions of these species-differences in receptor expression are currently unknown, the differences in some specific 5-HT receptors correlated with DV swimming and modulation. The observed differences between C2 homologues presented here may be reflective of a more widespread phenomenon, in which homologous cells express different genes, yielding species-specific functions. Neuromodulatory receptor expression plasticity in homologous neurons could explain many other species-differences in behavior. Studying these subtle changes in gene expression can expand our view of the neural mechanisms and evolution of behavior.

4.5 Figure and Table Legends

Figure 4-1: Updated Phylogeny of 5-HT Receptors. The 5-HT1 family receptors (a) show phylum-specific gene duplications resulting in 5-HT1a and 5-HT1b in Nudipleura representatives, *Tritonia* and *Hermisenda*. The 5-HT2 family receptors (b) split into two subtypes, 5-HT2a and 5-HT2b.

Table 4-1: 5-HT Receptor Evolution Timeline. Timeline shows estimated number of years of 5-HT receptor evolution. Based on Peroutka and Howell, 1994.

A



B

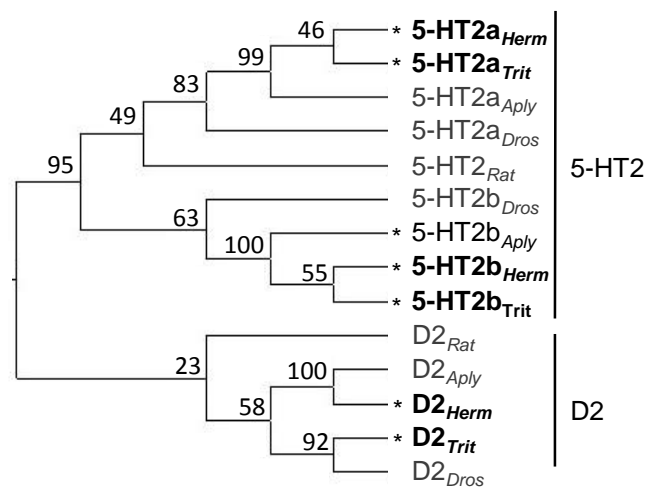


Figure 4-1: Updated Phylogeny of 5-HT Receptors

Table 4-1: 5-HT Receptor Evolution Timeline

Years	Event
1,000,000,000	Life begins
1,200,000,000	Plant and animal differentiation occurs
	First GPCR
900,000,000	Bilaterian/cnidarian split
	Ancestral 5-HT receptor
800,000,000	Ancestral 5-HT receptor differentiates to 5-HT1, 5-HT2, and 5-HT6
700,000,000	5-HT5, 5-HT7 differentiation occurs
600,000,000	Deuterostome/protostome split
500,000,000	Further differentiation of phyla-specific subtypes
400,000,000	Fish/amniotic vertebrate differentiation occurs
240,000,000	Dinosaurs evolve
90,000,000	Differentiation of mammalian receptors
65,000,000	Dinosaurs disappear in fossil record
4,000,000	Humans appear in fossil record
60	5-HT discovered
25	First 5-HT receptor genes sequenced

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5 APPENDIX

Appendix A: Single Cell Transcriptome Sequencing

5.1 Introduction

C2 homologues from DV and non-DV swimming species were compared in Chapter 3 using qPCR to determine whether 5-HT receptor genes expression differences correlated with swimming. To go beyond a single gene family, we sequenced the transcriptomes of single C2 neurons from each of four species in a pilot study to establish cell isolation and sequencing methodology. This project will allow for a broad scale comparison of gene expression in homologous neurons with different behavioral functions.

5.2 Methods

Animals

Tritonia diomedea were collected by Living Elements Ltd. (Vancouver, BC). *Hermisenda crassicornis*, *Dendronotus iris*, and *Pleurobranchaea californica* were collected by Monterey Abalone Co (Monterey, CA). Animals were housed at 10° C in artificial salt water. Animals were dissected in Normal Saline (NS) (see chapters 2 and 3). All dissections were done between 24 hours and two weeks following animal delivery.

Recording

Tritonia and *Pleurobranchaea* C2 neurons were recorded from during body wall nerve stimulation to determine swimming capability. *Hermisenda* and *Dendronotus* C2 neurons were recorded to determine left-right coupling using a series of 3 nA electrical

pulses to the contralateral cell. Following recording, electrodes were carefully removed from the soma to prevent damaging the cells.

Isolation

A 0.2% Protease IX (Sigma Aldrich) solution in normal saline was bath applied for five minutes to the brains following recording. Following this incubation, the brains were washed with filtered artificial salt water (FSW). A second wash of FSW was done before cell removal, to remove as much contaminating genetic material from the bath as possible. A glass electrode attached to a suction tube and syringe was placed near the C2 neuron. Cells were removed from the ganglia, pulled into the electrode, and placed in a micro-centrifuge tube containing 8ul distilled water and 2ul RNaseOut (Invitrogen). One or two C2 neurons per animal were considered as one “sample”. Cells were either frozen at -80°C immediately, or were taken immediately to the first step of the cDNA synthesis process.

Eyes were removed during initial dissection for *Tritonia* and *Pleurobranchaea*, and from the ganglia following C2 isolation for *Hermisenda* and *Dendronotus*. They were handled and stored as described for C2 homologues.

Transcriptome assembly and gene identification

Single cell and eye cDNA was synthesized using the Clontech SmartSeq v4 Ultra-Low Input RNA Kit (Takara). Libraries were prepared and indexed using the Nextera XT DNA Library Preparation Kit and 96-Sample Index Kit (Illumina). Qubit (Thermo Fisher) and Bioanalyzer (Agilent) analyses were used to judge quantity and quality of cDNA outputs during both protocols. cDNA samples were sequenced on a Hi-Seq2500

(Illumina) with a read depth of 10 million reads per sample. Sequencing reads as fastq files were analyzed using FastQC (Andrews 2010) via BaseSpace analysis (Illumina). J. Boykin assembled the fastq files using Trinity (Grabherr et al. 2011) for both single-sample fastq files and concatenated files. BLAST+ version 2.4.0 (Camacho et al. 2009) was used to identify genes with queries from whole-brain transcriptomes of the same species. NCBI tBLASTx was used to confirm phylogenetic identity of each identified gene. MUSCLE (Edgar 2004) was used to align individual sequences.

5.3 Results

The initial sequencing results were analyzed for quality control, and 94% of reads were found to have a Q-score above Q30 (Figure 5-1a), indicating that the initial reads were of good quality and that sequence base calls were likely accurate. Additionally, alignments were done for select individual genes from the C2 concatenated transcriptomes with their whole-brain transcriptome counterpart. The alignments were more than 94% identical between the two. However, many of the genes selected for this analysis were fragmented in the C2 transcriptome, meaning that only the 5', 3', or middle of the gene was present in the C2 databases (Figure 5-1b).

The concatenated and single-sample C2 transcriptomes were BLAST searched for 5-HT receptors. The results are summarized in Table 5-1. One single-sample *Hermisenda* C2 assembly showed expression of a 5-HT_{2a}-like gene. The expression of this receptor subtype conflicted with the data reported in chapter 3 using qPCR. When the *Hermisenda* C2 5-HT_{2a}-like gene was BLAST searched on NCBI, it aligned with both molluscan muscarinic acetylcholine receptors (E-value 9e-25) and several insect 5-HT_{2a} receptors (E-value 2e-19 and greater). When it was aligned with its *Hermisenda* whole-

brain counterpart, the alignment quality was poor (28%) (Figure 5-2a). When aligned against 5-HT2a orthologues from other species, the *Hermissenda* C2 gene also showed poor alignment (Figure 5-2b and 5-2c). In contrast, other genes from the datasets showed good alignment. For example, the *Hermissenda* C2 orthologue for 5-HT1b showed 94% alignment to its whole-brain counterpart (Figure 5-2d). In addition to 5-HT2a, 5-HT7 was measured in one qPCR trial testing *Hermissenda* C2 homologues, but was not measured in any other qPCR or sequencing trial.

5-HT receptors represented in the concatenated assembly were often not present in the single-sample assemblies. For example, only one of the five *Tritonia* C2 assemblies showed expression of 5-HT1b, and one of five assemblies showed expression of 5-HT7. Similarly inconsistent patterns were seen for all of the seven 5-HTR subtypes tested (Table 5-3).

The concatenated transcriptome databases from *Tritonia*, *Hermissenda*, and *Pleurobranchaea* were also BLAST searched for a number of other genes. The gene precursor for Small Cardioactive Peptide (SCP) was found to be expressed in the C2 transcriptomes from all three species. In molluscan whole brain tissue, long and short isoforms of SCP had been previously identified (Perry et al. 1999). The C2 transcriptome SCP gene was found to align to the short isoform of the gene in all three species (Figure 5-3).

The molluscan brain has at least three dopamine receptor subtypes, known as D1, D2, and DInv (Nagakura et al. 2010a; Perry et al. 1999). D2 was expressed in all three species, but *Tritonia* alone expressed DInv, and *Hermissenda* expressed D1 (Table 5-4).

Finally, a number of other G-protein coupled receptors (GPCRs), were expressed in the C2 transcriptomes (Table 5-5). These receptors were expressed in both *Tritonia*

and *Hermisenda* concatenated C2 transcriptomes. The receptors were identified during 5-HT receptor BLAST searches, because they are phylogenetically similar to the 5-HT receptor genes used as BLAST queries. Each of the reported non-5-HT receptors was verified using NCBI tBLASTx. The *Pleurobranchaea* and *Dendronotus* C2 transcriptomes were not analyzed for these receptors, and the single-sample assemblies were not searched.

5.4 Discussion

The C2 transcriptome study was a pilot effort to determine the parameters necessary for deep sequencing. Determining read depth, number of samples, number of cells per sample and other parameters is crucial to create a database that accurately reflects all of the genes expressed in a given tissue type, in this case single neurons. While other single-neuron transcriptome studies have reported their parameters (Dueck et al. 2015; Kadakkuzha et al. 2013; Moroz and Kohn 2013; Wang and Song 2017), many of those studies used single-neuron populations, or single neurons that were much smaller or larger than *Nudipleura* C2 cells, meaning that there was no study similar enough to our aims for us to replicate. We chose to sequence 10 million reads per sample. We also chose to sequence C2 samples containing only one or two neurons, from the same animal, in order to attempt to capture individual variability. Finally, we chose to sequence up to five samples per species, because we were constrained by the number of species and cost of an additional lane in the sequencer.

While the initial quality control metrics (as in Figure 5-1a) indicated good quality sequencing, the fragmented nature of many individual genes (as in Figure 5-1b) and the variability between individual assemblies for low copy number 5-HT receptor genes

indicated that in subsequent experiments a greater depth of reads should be attempted. This could be achieved by pooling C2 neurons from multiple animals, using fewer samples per lane to allow for more reads per sample. The fragmented nature of the genes also indicates that making assumptions about the presence or absence of genes could lead to false positives or false negatives.

The conflict between the C2 transcriptome and qPCR results on the *Hermissenda* 5-HT2a receptor subtype expression could have several potential explanations. It is possible that it conflicted because of individual variability in the animals sequenced. Using qPCR, 5-HT1a was measured by qPCR in two *Tritonia* C2 neuron samples, and 5-HT7 was expressed in one *Hermissenda* C2 sample and one non-swimming *Pleurobranchaea* sample (see chapter 3). Thus, there is some variability in the 5-HT receptor subtype profile by qPCR, for a yet unknown reason. This variability could represent a biological phenomenon, reflected in the C2 transcriptomes. However, the *Hermissenda* C2 5-HT2a-like gene did not align well with orthologues from the *Hermissenda* whole brain transcriptome, or from other molluscan databases, although the *Hermissenda* C2 contig was labeled as most similar to 5-HT2a according to BLAST search of the C2 assembly. The alignment shows that the C2 gene is poorly aligned to other 5-HT2a orthologues. NCBI BLAST search showed that it was weakly related to a molluscan acetylcholine receptor, and insect 5-HT2a receptors. These results could indicate that the *Hermissenda* 5-HT2a-like gene is a pseudogene that is not functional in its current mRNA form. However, the most likely explanation is that it is an assembly artifact, given its low alignment percentage, poor E-values, and its presence in only one individual C2 transcriptome database. This conclusion led me to omit them from the transcriptome 5-HT receptor subtype tables 5-1, 5-2, and 5-3.

The SCP precursor gene was identified in the C2 transcriptome databases. The SCP gene is known to code for two isoforms, SCP-A and SCP-B (Lloyd et al. 1987; Mahon et al. 1985). Furthermore, the SCP gene has two isoform in molluscan whole-brain tissue, a short and long isoform. The *Aplysia* SCP precursor gene published on Uniprot is the short isoform (Uniprot ID P09892). According to its Uniprot entry, the short isoform codes for both SCP-A and SCP-B proteins through a post-translation cleavage event. It can be inferred from this information that the short and long isoforms of the gene found in Nudipleura likely both code for SCP-A and -B. Thus, the finding that the short SCP isoform is expressed in the C2 transcriptomes does not allow for any conclusion about which form(s) of the protein C2 might express.

Some dopamine receptor subtypes also showed species-specific expression in C2 homologues, using the concatenated datasets. D2 was expressed across species, and between swimming and non-swimming *Pleurobranchaea*, while D1 was found only in *Hermisenda* and DInv was found in *Tritonia*. There could be several explanations for this. It is possible that D2 is conserved across Nudipleura C2 homologues. The dopamine receptor subtypes D1 and DInv could have been ancestral in Nudipleura and lost in *Pleurobranchaea*, or evolved in Nudibranchs, only. D1 or DInv could have been lost and gained within different Nudibranchs. There could be several other phylogenetic explanations. It could also be that the transcriptome sequencing did not detect dopamine receptor subtypes in one or more C2 samples, however. Finally, because the receptor sequences are fragmented, positive identification is more difficult: it is possible that while the fragments currently available align with portions of different dopamine receptor subtypes, a more complete sequence generated from additional sequencing may yield a different result. More samples and more species would help clarify this discrepancy. Of

note, the *Aplysia* dopamine receptors B1 and B2 were found in neither the whole brain transcriptomes nor the C2 transcriptomes, supporting the theory that they are specific to *Aplysia* (Nagakura et al. 2010a).

Non-5-HT biogenic amine receptors and other GPCRs were also identified through BLAST searches. These receptors were identified because of their gene sequence similarities to 5-HT receptors, which were used as BLAST queries, which means that other receptors may be present in the C2 transcriptomes but are as yet unaccounted for. The biogenic amine receptors Alpha-1x adrenergic receptors, two octopamine receptor subtypes, and one histamine receptor subtype were expressed in both *Hermissenda* and *Tritonia* concatenated C2 assemblies. Because their expression is shared between a swimming and non-swimming species, it is unlikely that they play a role in swimming. It would be interesting to understand the other roles that C2 plays in the brain and how the modulation from these neurotransmitters affects this cell type. An orphan receptor, GPR83, was expressed. The identification of this receptor could make C2 a useful tool to determine GPR83s function, through knockdown experiments, for example. Finally, several neuropeptide receptors were identified. Some of these were uncharacterized, according to NCBI. C2 could again provide a useful tool to functionally characterize these neuropeptide receptors. Overall, these findings indicate that C2 is likely modulated by a number of other chemicals besides 5-HT, and points to its multifunctional role within the brain (Jing and Gillette 1995; Jing and Gillette 2000; Snow 1982).

The initial C2 transcriptomes represent the first study comparing single homologous cells with different functions across species that we know of. Although these datasets may be greatly improved with more sequencing efforts, they are a good first step

to understanding how homologous single cells differ across species, and how those differences relate to species-specific behaviors.

5.5 Figure and Table Legends

Figure 5-1: Single-Cell Transcriptome Quality Control Illustrations. FASTQC (1a) was used to predict quality scores following initial sequencing. Individual genes, like the *Tritonia* 5-HT7 gene shown (1b), were performed using MUSCLE with genes from the concatenated C2 transcriptome files and the whole brain transcriptome files from the same species.

Figure 5-2: *Hermissenda* C2 5-HT2a Alignments. A gene expressed in *Hermissenda* C2 concatenated and C2-4 individual assemblies was identified as most similar to 5-HT2a by BLAST. The gene sequence used in alignments a-c is from the concatenated file (HcC2_2a). The gene was aligned against the *Hermissenda* whole-brain transcriptome (a), the *Tritonia* whole brain transcriptome (b), and the *Aplysia* published 5-HT2a orthologue. For comparison (d), the *Hermissenda* C2 concatenated assembly 5-HT1b gene (HcC2_1b) is aligned with its whole-brain transcriptome orthologue.

Figure 5-3: C2 and Whole Brain SCP Sequence Alignment. MUSCLE was used to align the short and long SCP gene sequences identified in whole brain tissue with the single sequence identified from the C2 transcriptome.

Table 5-1: 5-HT Receptors Identified from Concatenated Assembly.

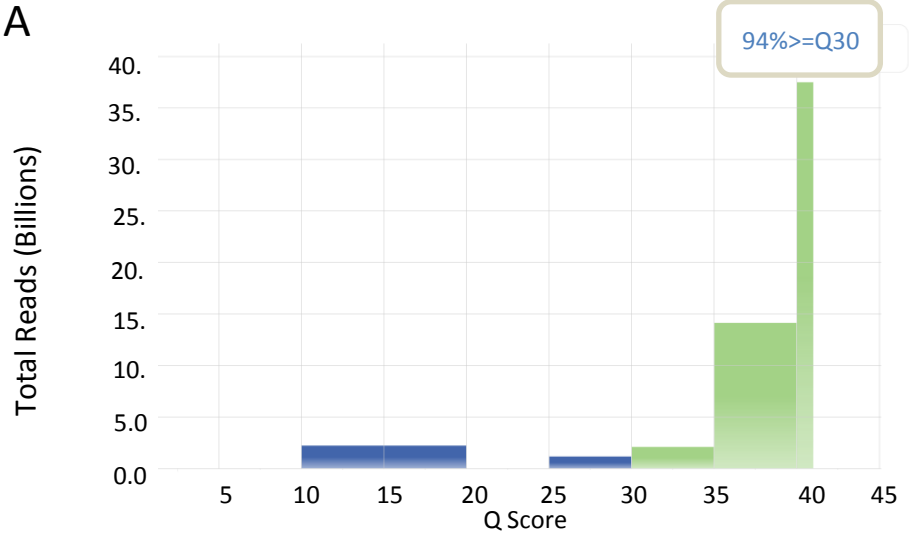
Table 5-2: Comparison of *Tritonia* and *Hermisenda* 5-HT Receptor Expression in qPCR and Concatenated Assembly.

Table 5-3: Comparison of 5-HT Receptor Expression in Individual C2 Assemblies. Each of the five individual C2 assemblies for *Tritonia* (a) and *Hermisenda* (b) were searched using BLAST to examine expression of 5-HT receptor genes across non-concatenated assemblies. The results are compared to the concatenated assembly (C2-concat) and to the qPCR results for each species.

Table 5-4: Identification of Dopamine Receptors in C2 Homologues. Dopamine receptors D1, D2, and the invertebrate-specific DInv were BLAST searched in each of the concatenated transcriptome assemblies.

Table 5-5: Other Biogenic Amine Receptors Identified in the Concatenated *Tritonia* and *Hermisenda* Assemblies. Several other GPCRs were identified in the concatenated assemblies. Select identified receptors were listed here.

A



B:

```

Td7_WB      MTSIPAPVVVTPPFVSTILNGSDNILVPYDVNASSIVTTTINYVSNVTTMVANVTNPSSN
Td7_C2      -----

Td7_WB      VTNGTTEIQQPPIPYQVWEQIVIALILGVLIICTLIGNSLVCMSVAIVKRLQSPSNLLIV
Td7_C2      -----

Td7_WB      SLAVADLCVGLFVMPFAAVLQVYGSWVLGSVVCDMWTTVDVLLCTSSILNLCAISVDRYF
Td7_C2      -----

Td7_WB      VITQPPFRYAMKRTPKRMGLMVLVFWTLSSVVCIPPVFGWKSEHQKYNCMISNDLGYQIYA
Td7_C2      -----MKRTPKRMGLMVLVFWTLSSVVCIPPVFGWKSEHQKYNCMISNDLGYQIYA
                *****

Td7_WB      TLCAFYLPLFVMIFVYFKIWRVSSKIARQEAQSKIGSFDKGAEFQLGRPSHDSGDSNLLA
Td7_C2      TLCAFYLPLFVMIFVYFKIWRVSSKIARQEAQSKIGSFDKGAEFQLGRPSHDSGDSNLLA
                *****

Td7_WB      NGTTKEGGVANGDDDGSIEILQKKPEFEKLNKRRFTIRSLLRHPKSSISKDSKATKTLG
Td7_C2      NGTTKEGGVANGDDDGSIEILQKKPEFEKLNKRRFTIRSLLRHPKSSISKDSKATKTLG
                *****

Td7_WB      IIMGCFTLCWLPPFFILALVKTFCLECEVPMALDNILMWLGYTNSFLNPVIYARFNREFRT
Td7_C2      IIMGCFTLCWLPPFFILALVKTFCLECEVPMALDNILM-----
                *****

Td7_WB      PFKEILLFRCRGINRRMRSESYVEQYGPVASHRDSLRTTDTVVRYN SQQTMTVTVGN GS
Td7_C2      -----

Td7_WB      ANGSRHTE SRI
Td7_C2      -----

```

Figure 5-1: Single-Cell Transcriptome Quality Control Illustrations

A:

Hc2a_WB -----MDNIFTGSGSDVIFNLNDTISGGFHNMMNMMNSSFN-----
HcC2_combined_2a SYPSSITTTTIYNNNNNFTNNITHHYNNIILNGTITSIQSYNNITQSSGGGILPAGAV
 .*:..... *:. . . : * .:..*: :*

Hc2a_WB ---GTDGDSTSPADLY-----VMMVLLMAPLVLFGIAGNTLVILAISLERLQNT
HcC2_combined_2a GTEGLDELGLTPSSLPLPASQKGQPLKWGMLALTIIILCTAVGNLLVCLAVCWERRLQNM
 * * . :*:.* * : * :: :*: .** ** **:. *****:*

Hc2a_WB NYFLLSLAVTDLLVSLIVMPFSIINMITGRWLFGLLLCDFVTSVDMCTSSIFHLCFIS
HcC2_combined_2a NYFLMSLAIADFLVSLVMPLGM-----
 ::~*:***:***:~:

Hc2a_WB AERYIGIRYPLWSKNKSKRVLLKIVLVWTLAFAVTSPISILGVVQEQNILVDGRCVLSN
HcC2_combined_2a -----

Hc2a_WB EYFIIYGSIFAFVIPLSIMVIMYILTVRMLNHQAKLCHKRRGEDGEVEPMIRRSTSSKHR
HcC2_combined_2a -----

Hc2a_WB SWQGRNKFYGGGGEFQTSFRNHDLKEHILDCSGNFHLQPNMYDCGGGG
HcC2_combined_2a -----

B:

Td2a_WB MLLMAPLVVFGIAGNTLVILAISLEKRLQNVNTNYFLLSLAVTDLLMSLIVMPFSIINVFT
HcC2_combined_2a -----

Td2a_WB GRWLFGALVCDFFVTSVDMCTSSIFHLCTISTERYIGIRYPLWSKNKSKRLVLLKIVLV
HcC2_combined_2a -----

Td2a_WB WTLAIAITSPITVLGVVREENVLVHGECVLSNDHFIIYGSIFAFYIPLTIMVLMYILTVR
HcC2_combined_2a -----SYSSITTTTIYNNNNNFTNNITHHYNNIILNGTITSIQS-----
 : .*.*. : :~*:~. :. :. :~*:~* ~*~ :

Td2a_WB MLSKQAKMCSSRRGNDSEGEPMIRRSTSSKSWRGRPKYYGNSLRSPENKIMSSGGLNP
HcC2_combined_2a -----YNNITQS-----
 **.* ~*~

Td2a_WB HYFELTTRGNARPHFKASSRHATLNHGGMNGDNGGGGGSGNSGGGGSGVISGGSPIA
HcC2_combined_2a -----SGGILPAGAVGTEG-----
 .*** ~*~*~*

Td2a_WB KRNTINVISTINNNKSNQGVLKSNKNGKNNVRRSRNGVLFDAITCGGNRSGGVENRSNA
HcC2_combined_2a -----

Td2a_WB NSSEHQYCNGHSSDLGSGKDDGNRQDPLSSSPSHDQKHLKDLVRKHHVALRAANILLMK
HcC2_combined_2a -----LDELGLTPS-----
 * * . :**

Td2a_WB KVDQSPNPMHLVPTTATTKTSTVLTTTPPSSTKASIRRDNSVKTEQKATKVLGVVFMIFV
HcC2_combined_2a -----SLLPASQKGQPLKWGMLALTIIILCTAVGNLLVCLA
 : *.*.. :~*:~ . . * ..* :~*:~ :

Td2a_WB VCWAPFFTVNILTVCDRCKFEPTLITVFVWLGYSSTLNPIIYTFNIFRNFTFKLLC
HcC2_combined_2a VCWERRLQ-----MTNYFLMSLAIADFLVSLVMPLGM-----
 *** : : *~*:~ : .* ~*~:

Td2a_WB CRYRLLHRARRNSNLIRKENGIMTSNCTSYSTTTNLISSNCCSNNSNLSASNKASTSS
 HcC2_combined_2a -----

Td2a_WB VMDESNC
 HcC2_combined_2a -----

C:

Ac_5HTR2a_published MSDNDPWSLRPPGLASAGREEGEGEDILGMNKSARNTGAGHRHRHNKHNLGALSEASA
 HcC2_combined_2a -----

Ac_5HTR2a_published DGAAGGGGAGHGNVDLNRGSNYAASASSPAADFRDYSRDIGVKGEFQDALKESSAFNSSA
 HcC2_combined_2a -----SY PSSITTTT
 . . * : : : : :

Ac_5HTR2a_published LYDRLLRGGPRSDFPVSAPRRVIGDGGDDFFGLPGSNGTVSDDASGLFSGMVKNPMRQN
 HcC2_combined_2a IYN-----NN
 : * : : *

Ac_5HTR2a_published SQFPLSNNTYSISDLDLGSAKSDPIDSTIPVHYTEYDIMNLGGGGGDRGGGGGGVVG
 HcC2_combined_2a NNFTTNNITHYNNIILN-----GTITSIQSYNNITQSSGGGILPAGAVG-----
 . : * . * * : . : : * . * * . : * : : . . * * * . * . *

Ac_5HTR2a_published GYSPGTQDLYDIHANNDATFYRDVYDDENDA EYQVNHILPINADEGSKTPHFDAEFFPE
 HcC2_combined_2a -----

Ac_5HTR2a_published SAGDSEDNLSFNNTLLNPSSGGVFTNPKNDAASYSFSFSPSWSASPPTGAGTSDVFSNSQP
 HcC2_combined_2a --TEGLDELGLTPSSLLPASQ-----
 : . * : * : . : * * : *

Ac_5HTR2a_published QPVPNFDDLHLHNLTLRGGSEFNITPLVDPEYTNVLFQGTRLAPDYDYSIVAHAAANNS
 HcC2_combined_2a -----

Ac_5HTR2a_published SIFGGNITDLLMADGGREDEYTWISILMMAPLVVFGVAGNTLVILAI SLEKRLQNVNTNYFL
 HcC2_combined_2a -----KGQPLKWGMLALTLIILCTAVGNLLVCLAVCWERRLQNMNTNYFL
 : : . * : * : : : : . * * * * : . * : * * * : * * * * :

Ac_5HTR2a_published LSLAVTDLLVSLIVMPFSIINVFTGRWLFGLLLCDFVTS DVL MCTSSILHLCTISLERY
 HcC2_combined_2a MSLAIADFLVSLVMPLGM-----
 : * * * : * : * * * : * * * : :

Ac_5HTR2a_published IGIRYPLWTKNKSkrvLLKIVLVWTIALAITSPITVLGVVRAQNVLVDDVCVVNNEHFV
 HcC2_combined_2a -----

Ac_5HTR2a_published IYGSIFAFFLPLAIMILMYALTVRMLNKQARLCQTRRADDGEGEPMIRRSTSRRNWQGRR
 HcC2_combined_2a -----

Ac_5HTR2a_published KFYGREVLSATPSCCDPRSGGGGGGDSGGGSGGVLSFHQRYP LGGGVSRHNTIPLYHN
 HcC2_combined_2a -----

Ac_5HTR2a_published SHHHHHHHNHNHNHQQHHRNHNSSYAHNNYLP LDRHHQIERRETANCCGVGVVDWG
 HcC2_combined_2a -----

```

Ac_5HTR2a_published      GGGEPKRLRELVRKHHVAVKAANILLKRDGQQQQQQQQHHQQRGQGGVQGPSSGHHG
HcC2_combined_2a        -----

Ac_5HTR2a_published      GHRGSTSSSTNYSVRRDNSVRTEQKASKVLGVVFMIFVVCWAPFFTVNILTVLCTSCRF
HcC2_combined_2a        -----

Ac_5HTR2a_published      EPTLITAFVWLGYVSSTLNPIIYTIFNNIFRITFIKLLCCRYRLLHRARSSSHMTGLRNG
HcC2_combined_2a        -----

Ac_5HTR2a_published      ILACNAFCPAPLAVQTSNSNVTNSTLHDESHC
HcC2_combined_2a        -----

D:

Hc1b_transcriptome      MMSNVTLPSETISDMSITPTTAAKIMISTLVSNIKRLNDSINESTSFSIAAAGGSSISP
HcC2_combined_1b        -----

Hc1b_transcriptome      GVLGTSADMIDKSNNGSNTNMAATGSGSSSSSGGGSNGSNVGVTDGEIPWWEFSVYSQEH
HcC2_combined_1b        -----

Hc1b_transcriptome      LIVTSIVLGLFVLCCIIGNCFVIAAVILERSLHNVANHLIVSLAVADLMVAVLVMPLSVV
HcC2_combined_1b        -----

Hc1b_transcriptome      SEISTDWFLDQEVCDMWISVDVLCCTASILHLVAISLDRYWAVTGIDYIRKRSKRILLM
HcC2_combined_1b        -----

Hc1b_transcriptome      IFTVWVVALFISIPPLFGWRDPKNDSITGMCIISQDKGYTIFSTVGAFYLPMLMMIY
HcC2_combined_1b        -----

Hc1b_transcriptome      AKIYVVAKSRIKDKFHKRLHKKRTEETLVSSPKTEYSVVNDCNGCGENNSPATENGKK
HcC2_combined_1b        -----GINAEYCGENNSPATENGKK
                          : *      *****

Hc1b_transcriptome      KRRAPFKSYGCSRPERKKRGNNKQNSEGNNHNGVNGNSTDRLQHLTVIEPDAFTSGHN
HcC2_combined_1b        KRRAPFKSYGCSRPERKKRGNNKQNSEGNNHNGVNGNSTDRLQHLTVIEPDAFTSGHN
                          *****

Hc1b_transcriptome      DEAKLAMLDTAHTNSSTPSHNPQALDNYNFQRNKEKLELKRERKAARTLAIITGAFIICW
HcC2_combined_1b        DEAKLAMLDTAHTNSSTPSHNPRLR-----
                          ***** : **

Hc1b_transcriptome      LPFFIVALIGPFIHQEIPAFVGSFILWLGYFNSSLNPIIYTIFSPEFRSAFHKILFGKYR
HcC2_combined_1b        -----

Hc1b_transcriptome      RVVR
HcC2_combined_1b        ----

```

Figure 5-2: C2 and Whole Brain SCP Sequence Alignment.

```

Td_WB_long      MEMTMPRATVSLTLLFVI ICTVDAMNYLAFPRMGRSDLTRVKRHVDMPWNMIFPRKRGGM
Td_C2_SCP       MEMTMPRATVSLTLLFVI ICTVDAMNYLAFPRMGRS-----
Td_WB_short     MEMTMPRATVSLTLLFVI ICTVDAMNYLAFPRMGRS-----
                *****

Td_WB_long      LPENFIFPRKRGSMLPGNFIFPRKRQNGYLAFFPRMGRSQAKAGTAE AIDTECCGIGLKSE
Td_C2_SCP       -----GYLAFFPRMGRSQAKAGTAE AIDTECCGIGLKSE
Td_WB_short     -----GYLAFFPRMGRSQAKAGTAE AIDTECCGIGLKSE
                *****

Td_WB_long      FAVSDDGKEELHNICTASVSVCC EGLRELADEKPNGVVYSMCVPDVSKMYPSSYNKLR L
Td_C2_SCP       FAVSDDGKEELHNICTASVSVCC EGLRELADEKPNGVVYSMCVPDVSKMYPSSYNKLR L
Td_WB_short     FAVSDDGKEELHNICTASVSVCC EGLRELADEKPNGVVYSMCVPDVSKMYPSSYNKLR L
                *****

Td_WB_long      LTK
Td_C2_SCP       LTK
Td_WB_short     LTK
                ***

```

Figure 5-3: C2 and Whole Brain SCP Sequence Alignment.

Table 5-1: 5-HT Receptors Identified from Concatenated Assembly.

	Tritonia C2	Hermisenda C2	Pleurobranchaea (swimmer) C2	Pleurobranchaea (non-swimmer) C2
5-HT1a	✓	✓	✗	✗
5-HT1b	✓	✓	✓	✗
5-HT2a	✓	✗	✓	✗
5-HT2b	✓	✗	✗	✗
5-HT4	✗	✓	✗	✗
5-HT6	✗	✓	✗	✗
5-HT7	✓	✗	✓	✗

Table 5-2: Comparison of Tritonia and Hermissenda 5-HT Receptor Expression in qPCR and Concatenated Assembly.

	Tritonia C2 transcriptome	Tritonia C2 qPCR	Hermissenda C2 transcriptome	Hermissenda C2 qPCR
5HT1a	✓	1/5	✓	✓
5HT1b	✓	✓	✓	✓
5HT2a	✓	✓	✗	✗
5HT2b	✓	3/6	✗	✗
5HT4	✗	✗	✓	✓
5HT6	✗	✗	✓	✓
5HT7	✓	✓	✗	1/7

Table 5-3 Comparison of 5-HT Receptor Expression in Individual C2 Assemblies.

A:

	TdC2-1	TdC2-2	TdC2-3	TdC2-4	TdC2-5	TdC2-concat	TdC2-qPCR
5HT1a	✓	✗	✗	✗	✗	✓	1/5
5HT1b	✗	✗	✓	✗	✗	✓	✓
5HT2a	✗	✗	✓	✗	✗	✓	✓
5HT2b	✓	✗	✗	✗	✗	✓	3/6
5HT4	✗	✗	✗	✗	✗	✗	✗
5HT6	✗	✗	✗	✗	✗	✗	✗
5HT7	✓	✗	✗	✗	✗	✓	✓

B:

	HcC2-1	HcC2-2	HcC2-3	HcC2-4	HcC2-5	HcC2-concat	HcC2-qPCR
5HT1a	✓	✗	✗	✗	✗	✓	✓
5HT1b	✓	✗	✗	✗	✗	✓	✓
5HT2a	✗	✗	✗	✗	✗	✗	✗
5HT2b	✗	✗	✗	✗	✗	✗	✗
5HT4	✓	✓	✗	✗	✓	✓	✓
5HT6	✗	✓	✗	✓	✓	✓	✓
5HT7	✗	✗	✗	✗	✗	✗	1/7

Table 5-4: Identification of Dopamine Receptors in C2 Homologues.













	Tritonia C2	Hermisenda C2	Pleurobranchaea C2 (swimmer)	<i>Pleurobranchaea</i> C2 (non-swimmer)
D1				
D2				
DInv				

Table 5-5: Other Biogenic Amine Receptors Identified in the Concatenated Tritonia and Hermissenda Assemblies.

Additional receptors identified in <i>Tritonia</i> and <i>Hermissenda</i> concatenated C2 transcriptomes
Alpha-1x adrenergic receptor
Histamine H1 receptor
Muscarinic acetylcholine receptor
Multiple neuropeptide receptors
Octopamine receptors 1 and 2
Orexin receptor
Orphan GPCR83