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A Comparative Analysis of the Neural Basis for Dorsal-Ventral Swimming in the Nudipleura

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A COMPARATIVE ANALYSIS OF THE NEURAL BASIS FOR DORSAL-VENTRAL SWIMMING IN THE NUDIPLEURA

by

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Under the Direction of Paul S. Katz

ABSTRACT

Despite having similar brains, related species can display divergent behaviors. Investigating the neural basis of such behavioral divergence can elucidate the neural mechanisms that allow behavioral change and identify neural mechanisms that influence the evolution of behavior.

Fewer than three percent of Nudipleura (Mollusca, Opisthobranchia, Gastropoda) species have been documented to swim. However, *Tritonia diomedea* and *Pleurobranchaea californica* express analogous, independently evolved swim behaviors consisting of rhythmic, alternating dorsal and ventral flexions. The *Tritonia* and *Pleurobranchaea* swims are produced by central pattern generator (CPG) circuits containing homologous neurons named DSI and C2. Homologues of DSI have been identified throughout the Nudipleura, including in species that do not express a dorsal-ventral swim. It is unclear what neural mechanisms allow *Tritonia* and

Pleurobranchaea to produce a rhythmic swim behavior using homologous neurons that are not rhythmic in the majority of Nudipleura species.

Here, C2 homologues were also identified in species that do not express a dorsal-ventral swim. We found that certain electrophysiological properties of the DSI and C2 homologues were similar regardless of swim behavior. However, some synaptic connections differed in the non-dorsal-ventral swimming *Hermissenda crassicornis* compared to *Tritonia* and *Pleurobranchaea*. This suggests that particular CPG synaptic connections may play a role in dorsal-ventral swim expression.

DSI modulates the strength of C2 synapses in *Tritonia*, and this serotonergic modulation appears to be necessary for *Tritonia* to swim. DSI modulation of C2 synapses was also found to be present in *Pleurobranchaea*. Moreover, serotonergic modulation was necessary for swimming in *Pleurobranchaea*. The extent of this neuromodulation also correlated with the swimming ability in individual *Pleurobranchaea*; as the modulatory effect increased, so too did the number of swim cycles produced. Conversely, DSI did not modulate the amplitude of C2 synapses in *Hermissenda*. This indicates that species differences in neuromodulation may account for the ability to produce a dorsal-ventral swim.

The results indicate that differences in synaptic connections and neuromodulatory dynamics allow the expression of rhythmic swim behavior from homologous non-rhythmic components. Additionally, the results suggest that constraints on the nervous system may influence the neural mechanisms and behaviors that can evolve from homologous neural components.

INDEX WORDS: Evolution, Neuromodulation, Homology, Mollusc, Electrophysiology, Synapse

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LIST OF ABBREVIATIONS

5-HT	serotonin
ASD	antiserum diluent
BWN	body wall nerve
C2	cerebral neuron 2
Ce	cerebral ganglion
CPG	central pattern generator
CPT	cerebropleural ganglion triplets
DA	dopamine
DMSO	dimethyl sulfoxide
DRI	dorsal ramp interneuron
DSI	dorsal swim interneuron
DV	dorsal-ventral
E	excitatory
E:I	excitatory and inhibitory
EOD	electric organ discharge
EPSP	excitatory postsynaptic potential
Flab	*Flabellina iodinea*
Herm	*Hermissenda crassicornis*
I	inhibitory
I_b	*Hermissenda* Interneuron B
IPSP	inhibitory postsynaptic potential
LR	left-right
JAR	jamming avoidance response
Mel	*Melibe leonina*

NO	nitric oxide
NOS	nitric oxide synthase
NSF	National Science Foundation
OT	oxytocin
PBS	phosphate buffered saline
Pd	pedal ganglion
PdN2	pedal nerve 2
PdN3	pedal nerve 3
Pl	pleural ganglion
Pleur	*Pleurobranchaea californica*
PP2	pedal-pedal commissure 2
PSP	postsynaptic potential
SCP	small cardioactive peptide
SCP_B	small cardioactive peptide B
STG	stomatogastric ganglion
Tr1	trigger neuron 1
Tri	*Tritonia diomedea*
V1aR	vasopressin 1a receptor
VSI	ventral swim interneuron

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CHAPTER 1: INTRODUCTION

General introduction

The vast amount of behavioral diversity displayed across the animal kingdom is astonishing. This would be true in a vacuum, but given the apparent evolutionary constraints in generating different behaviors across species, the diversity observed is even more surprising. These evolutionary constraints are a product of the organization of the nervous system and the role of the nervous system in producing behavior. Neural circuits produce behavior. Therefore, aside from instances where behaviors have evolved due to changes outside the nervous system, the evolution of behaviors would require changes in the properties of neural circuits. However, the neurons that make up neural circuits can be highly multifunctional, as can neural circuits themselves (Marder and Calabrese, 1996). Thus, any change to neural circuitry or its components could have ramifications beyond the change to a single behavior or process in the brain. This suggests that changes to the nervous system are generally constrained, and thus, changes in behavior too would be relatively constrained. However, behavior has clearly evolved a great deal across phyla and even across related species. This raises the question of what neural mechanisms allow for differences in behavior in related species that have similar, evolutionarily constrained brains.

One way to investigate the neural mechanisms that underlie behavioral diversity is to compare and contrast related species with similar and different behaviors. While difficult, successful comparisons of behavior at the neural level allow a glimpse into two key aspects of behavioral neuroscience and evolutionary theory. First, comparisons can highlight the neural circuit properties that are important to the functional behavioral output of a circuit and those properties that play a lesser role. Second, comparisons can demonstrate the neural circuit properties that may be more amenable to evolutionary change. Thus, such comparative studies can inform

which neural circuit differences are important and what neural circuit differences are possible given the constraints of the nervous system. Such knowledge has immediate impact on our understanding of the brain, behavior, and evolution as well as more distant ramifications in terms of developing treatments for nervous system dysfunction.

This dissertation uses a comparative approach to investigate the neural mechanisms underlying a similar independently evolved swim behavior of two Nudipleura (Mollusca, Gastropoda, Opisthobranchia) species. Most Nudipleura species do not produce this behavior but have similar nervous systems made up of homologous neurons, synapses, and neural circuits. Four possible neural differences that could underlie such species differences in behavior were examined in this dissertation: differences in 1) the number, 2) electrophysiological properties, 3) synaptic connections, and 4) neuromodulatory properties of neurons making up neural circuits. The investigations described here tested each of these possible differences at the cellular level in two species that can produce the rare swim behavior and in one to three species that cannot. The results demonstrate that species differences in the presence of neuromodulation of homologous neurons can account for the differences in swim behavior observed. Additional differences in specific synaptic connections also appear to play a role in the behavioral differences. However, other synaptic connections and electrophysiological properties of homologous neurons do not appear to play a role in the species differences in behavior. Thus, the results suggest that some electrophysiological properties and in some cases synaptic connections may be less influential on neural circuit output than key synaptic connections and the neuromodulatory dynamics within neural circuits.

With respect to evolution, the results suggest that synaptic connections can vary substantially across related species, which stands in contrast to the expected constraints on such differences. The results also suggest a role for neuromodulation in species differences in behavior, which falls in line with the expected constraints on circuit properties across species. Prior reports have suggested such a role for neuromodulation in species differences in behavior, but

the work presented here is the first to directly link differences in neuromodulation at the cellular level to species differences in natural behavior. In summary, the dissertation demonstrates an evolutionary mechanism that has functional ramifications while remaining within the apparent evolutionary constraints of nervous systems. However, the results also suggest that the evolutionary constraints of the nervous system may not be as significant as initially perceived.

The neural basis of species differences in behavior

As noted above, neural circuits produce behavior. The properties of the neurons and synapses that make up neural circuits determine the behavioral output. Neuromodulation can alter the properties of the neurons and synapses, thus altering the behavioral output of the circuits. Apart from instances where behavioral differences are accounted for by differences in peripheral biophysical properties (e.g., Wainwright, 2002), differences in the properties of neural circuits or differences in the neuromodulation of neural circuits must underlie such species differences in behavior.

Differences in neural circuit properties that could underlie species differences in behavior include differences in the number, properties, or synaptic connections of neurons. Testing for each of these differences is possible among related species that have similar nervous systems. Comparative studies of this nature have been done, and roles for these possible neural circuit differences in species differences in behavior have been documented in nematode feeding (Chiang et al., 2006) and chemosensation (McGrath et al., 2011); leech mechanosensation (Baltzley et al., 2010); insect vision (Shaw and Meinertzhagen, 1986; Shaw and Moore, 1989; Buschbeck and Strausfeld, 1997), locomotion (Wilson et al., 1982), and feeding strategies (Farris, 2008; Farris and Schulmeister, 2011); decapod locomotion (Faulkes, 2008); fish mechanosensation (Yoshizawa et al., 2010) and electrosensation (Kawasaki, 2009); bat echolocation (Neuweiler, 2003); mammalian tactile sensation (Catania, 2012) and sociality (Holmes et al., 2009); primate vision and somatosensory behavior (Kaas, 2008); and primate limb and hand

movement (Padberg et al., 2007; Kaas et al., 2012). However, in the majority of these cases it is still unclear what neural mechanisms are actually responsible for the observed species differences in behavior. Given the difficulty of understanding the neural mechanisms responsible for producing behavior in a single species, it is quite challenging to study circuitry across species and delineate the neural differences responsible for behavioral differences.

To illustrate the complexity of such an undertaking, I will briefly review the evolution of vocal learning behavior in songbirds. The songbird research community has extensively examined the neural mechanisms underlying the ability of some songbirds to learn vocalizations, and yet many crucial unknowns remain. The most obvious difference observed across species is that there is an increase in the number of neurons in seven brain nuclei that control song learning and production in the vocal learners. Such differences have been reported across species that can and cannot perform vocal learning behavior (Ball, 1994; Brenowitz, 1997) and across species that exhibit a range of song learning ability (Devoogd et al., 1993; DeVoogd, 2004; Zeng et al., 2007). Moreover, numerous studies have explicitly demonstrated the importance of song nuclei volume on vocal learning and song production across species (Mooney, 2009). Of course, with an increase in neurons must also come changes in other neural circuit properties. Differences in the synaptic connectivity of song nuclei and to vocal and respiratory motor and pre-motor neurons in the brainstem are present between vocal-learning species and non-vocal learners (Brenowitz, 1997; Wild et al., 1997; Farries, 2001; Jarvis, 2004; Wild, 2004). There is also evidence for increased synaptic transmission in the song nuclei indicating potential changes in synaptic connectivity within the nuclei and/or increases in neuromodulation within the nuclei compared to non-vocal learners (Wada et al., 2004; Meitzen et al., 2007). Additionally, species and seasonal differences in the response to steroid hormones show a relationship with the expansion of brain regions and singing ability indicating a role of neuromodulation in these processes (Brenowitz, 1997; Brenowitz et al., 1997; Wilbrecht and Kirn, 2004; Wild, 2004; Meitzen et al., 2007; Thompson et al., 2007; Meitzen and Thompson, 2008). Finally, there is evidence

suggesting that dopamine plays an important neuromodulatory role in song production and possibly in song learning that may not be present in non-vocal learning bird species (Kubikova et al., 2010; Simonyan et al., 2012).

Thus, there are clear differences in neuron number and synaptic connections associated with species differences in behavior. There are also likely differences in the neuromodulatory relationships related to differences in behavior. However, it is generally unclear how such neuromodulatory differences are incorporated into the circuits in a way that causes behavioral differences. These results mirror much of what is known about the neural mechanisms underlying species differences in behavior; differences can be found across species, but the direct connection to behavioral differences is difficult to delineate. Of particular note is the potential role of neuromodulation, which despite being repeatedly implicated as a mechanism underlying species differences in behavior has rarely been demonstrated.

The role of neuromodulation in shaping behavior

The properties of the neurons and synapses making up neural circuits can be altered by neuromodulation, thus altering the behavioral output of the neural circuits. The importance of neuromodulation for the proper function of circuitry underlying diverse processes has been reviewed extensively (Katz and Frost, 1996; Birmingham and Tauck, 2003; Kristan et al., 2005; Dickinson, 2006; Marder and Bucher, 2007; Eppinger et al., 2011; Harris-Warrick, 2011; Miles and Sillar, 2011; Bargmann, 2012; Griffith, 2012), and dysfunction in neuromodulatory signaling is heavily implicated in a number of human disease states, including drug addiction, Parkinson's, and schizophrenia (Montague et al., 2004). Moreover, in instances where neuromodulatory signaling has been studied in great detail, its pervasiveness further demonstrates how important it is for circuit function in general. For example, the neurons of the stomatogastric ganglion (STG) of decapod crustaceans produce two motor patterns in the gut. Each motor pattern is controlled by central pattern generators (CPG) composed of overlapping sets of STG neurons

(Marder and Bucher, 2007). Neuromodulators affect the output of the CPGs via effects on the sensory neurons, intrinsic neuronal properties, electrical synapses, chemical synapses, neuromuscular junctions, and the muscles themselves (Stein, 2009). Moreover, differences in the neuromodulatory input to the STG can allow multiple rhythmic outputs from the same CPG networks (Nusbaum and Beenhakker, 2002; Blitz and Nusbaum, 2011; Nusbaum and Blitz, 2012). Additionally, removing neuromodulatory input from projection neurons stops rhythmic activity in the STG. This can recover via homeostatic mechanisms, but it is clear that the neuromodulatory input is required for normal function (Thoby-Brisson and Simmers, 1998). Thus, in the STG, neuromodulatory inputs are necessary for normal circuit function and allow for multiple outputs from the same network components.

It is evident that neuromodulation can influence network output. Moreover, dynamically changing the way that neurons, synapses, or circuits are modulated can change the functional output without changing the actual structure of the neurons and synapses. Thus, neuromodulation is a prime candidate as a neural mechanism underling species differences in behavior (Katz, 1991; Katz and Harris-Warrick, 1999; Katz, 2011).

Species differences in neuromodulation related to differences in behavior

Although species differences in neuromodulation would appear to be a common mechanism underlying species differences in behavior, there are only a few studies that have directly reported this to be the case. However, there are a number of studies that suggest a role for neuromodulation in species differences in behavior. The first group of examples I will discuss fall short of directly demonstrating a role for neuromodulation in species differences in behavior but are worth reviewing in brief. Notably, I am not discussing studies in which genetic evidence alone suggests a role for neuromodulation in species differences in behavior. Only studies that provide evidence for how such differences are manifested in the nervous system are being reviewed here.

Within a species, sex or hierarchical differences in behavior provide interesting examples of neural mechanisms that can underlie differences in behavior. These examples are often due to hormonal differences, which are likely manifested as differences in the way that circuitry is modulated. However, such examples do not translate well to the sources of species differences in behavior. For example, as discussed above, vocal learning songbirds have enlarged vocal song nuclei compared to non-vocal learning species. Male songbirds typically have larger song nuclei than females of the same species as well, but these differences are regulated by hormonal differences that cause the death of song nuclei neurons during development (Wild, 2004). Species differences in song nuclei volume are due to the gain of neurons, not a loss, and thus a different neural mechanism causes the disparity in neuron number in the song nuclei. Species differences in hormonal content or regulation are perhaps more telling of a role for neuromodulation in species differences in behavior, but such differences have not yet resulted in direct examples of how neural circuits are differentially modulated to allow differences in behavior across species (e.g., Holmes et al., 2009).

There are also differences in the way that species respond to neuromodulators. For example, the neuropeptide arginine vasotocin can increase vocal call rates of the bullfrog *Rana catebeiana* (Boyd, 1994) but decrease call rates in the related species of the same genus, *Rana pipiens* (Raimondi and Diakow, 1981). This indicates that similar behaviors are regulated differently, but not that different behaviors are actually being produced due to neuromodulatory differences.

Furthermore, there are a number of studies that address a role for neuromodulation in specialized behaviors. For example, it has been shown that dopamine levels influence the characteristics of the vocalizations used for bat echolocation behavior (see Tressler et al., 2011). While the vocalizations of bats represent an interesting behavioral difference compared to most mammals, dopamine may be important for less specialized vocalization in other mammals as well. This is similar to the involvement of dopamine in vocal learning behavior of songbirds and

humans (Doupe et al., 2005; Simonyan et al., 2012). Evidence in songbirds and humans suggests a role for dopamine and basal ganglia involvement in vocal learning and production. Vocal learning is a specialized behavior that differs from the vocal behaviors of many other non-vocal learning birds and mammals. However, the role of dopamine in vocal learning may not differ from the role of dopamine and the basal ganglia in motor learning in general. Thus, it is not clear that such neuromodulation is actually involved in species differences in behavior.

Finally, there is more direct evidence of a role for neuromodulation in differences in behavior among different strains of lab mice and rats, but how such differences correspond to differences in actual natural behaviors is unclear. For example, a number of studies have been able to link neuromodulatory differences to differences in laboratory tasks or drug responses across rodent strains or between mice and rat species (e.g., Pattij et al., 2007; Gieryk et al., 2010; Pobbe et al., 2011). These tasks or drug response profiles are suggestive of natural behavior differences and are certainly worth being aware of, but will not be discussed further here because it is ambiguous as to what such differences mean in terms of actual species differences in behavior.

There are more direct examples of a role for neuromodulation in species differences in natural behavior than the types listed above. The related amphibian species *Xenopus laevis* and *Rana temporaria* exhibit differences in their response to mechanosensory stimuli shortly after hatching. *Xenopus* reliably swims, and while *Rana* will swim, it also produces a coiling behavior not observed in *Xenopus*. Noradrenaline and nitric oxide elicit the coiling behavior in *Rana*, whereas it only acts to slow swimming *Xenopus* (Merrywest et al., 2004). This suggests that species differences in the neuromodulatory response to noradrenaline and nitric oxide may play a role in the differences in response behavior observed from a mechanosensory stimulus. Moreover, there appear to be differences in nitric oxide innervation to the spinal cord between the two species that could explain how such experimental differences are manifested *in vivo* (Merrywest et al., 2004).

Most mammals display echo suppression that blocks echoes from being perceived after brief delays. However, echolocating bats show echo sensitivity that appears to be a combination of a reduction in the suppression and a general increase in echo sensitivity (Neuweiler, 2003). Descending projections from the auditory cortex to the medial geniculate nucleus and inferior colliculus modulate auditory signal processing to increase echo sensitivity in bats (Yan and Suga, 1996; Zhang et al., 1997). While descending cortical projections modulate the auditory system in non-echo locating animals as well, the modulation that effects echosensitivity appears to be a specialized feature not found in non-echosensitive mammals (Suga et al., 2002).

Weakly electric fish produce electric organ discharges (EOD) for navigation and communication. Individuals within a species will modulate the frequency of their EOD when encountering others in an effort to allow the signals to remain distinct. This mechanism is called the jamming avoidance response (JAR). In addition to individual differences in JAR behavior, there are also species differences in the frequency of the baseline EOD and in JAR behavior. One study examined the potential role of nitric oxide in the species differences in EOD and JAR behavior (Smith et al., 2001). Nitric oxide synthase (NOS) staining in the EOD pacemaker nuclei differed in a manner that correlates with behavioral differences. The species with the lowest frequency EOD, *Sternopygus macrurus*, showed weaker nitric oxide (NO) staining than three species that showed higher frequency discharges, *Apternotus leptorhynchus*, *Apternotus albifrons*, and *Eigenmannia virescens*. Thus, NO may modulate the ability to produce high frequency EODs. Cells in the EOD that stain for NOS show activity patterns in sync with the EOD rhythm, which supports the hypothesis that NO signaling may play a role in aspects of the rhythmic output (Turner and Moroz, 1995). Another possible role of the NO differences relates to species differences in their JAR. In addition to showing the least amount of NOS staining, *Sternopygus* also demonstrates little variation in its JAR. Conversely, *Apternotus* species and *Eigenmannia* show higher NO staining and more versatile JAR ability. Importantly, these correlations also fall in line with the phylogenetic relationships of these species, suggesting that the differences could

be a function of the phylogeny and not actually a contributing factor in the behavioral differences observed. Until further research is conducted, the significance of these reported correlations is uncertain.

A series of studies identified differences in serotonergic neuromodulation related to species differences in associative learning behavior. While these experiments did not test natural behavior *per se*, they did reveal a potential role for neuromodulatory differences related to behavior differences at the cellular level that are worth discussing. Serotonin neuromodulation of sensory neuron synapses has been identified as a necessary component of associative learning in the aplysiid mollusc *Aplysia californica* (Barbas et al., 2003). Furthermore, species differences in serotonin neuromodulation of sensory synapses correlates with species differences in associative learning ability in three aplysiid species including *Aplysia* (Hoover et al., 2006). However, there were no apparent differences in serotonergic signaling in the species (Marinesco et al., 2003), which suggests that differences in serotonin receptor expression or second messenger systems may underlie the differences in behavior and neuromodulatory response to serotonin.

Such species differences in the receptors receiving neuromodulatory signals appear to underlie the best studied examples of species differences in behavior related to differences in neuromodulation. These experiments focus on the role of neuromodulatory signaling underlying species differences in mammalian social behavior. A small minority of mammalian species display social monogamy, defined as a long-term association between a male and female with cooperation in parenting. Prairie voles (*Microtus ochrogaster*) are one such socially monogamous species. Male prairie voles will preferably partner with a female vole with whom they have prior experience as opposed to partnering with novel females, while other species such as the montane (*Microtus montanus*) and meadow vole (*Microtus pennsylvanicus*) do not form these preferential bonds (Shapiro and Dewsbury, 1990; Salo et al., 1993).

Socially monogamous prairie voles have higher vasopressin 1a receptor (V1aR) expression in the ventral pallidum than the non-socially monogamous montane and meadow voles

(Lim et al., 2004a; but see Ophir et al., 2008a). Importantly, over expressing the V1aR in the ventral pallidum of non-socially monogamous species causes the animals to form partner preferences. This demonstrates that the circuitry underlying social monogamy is similar across species except for V1aR expression and that this receptor difference is sufficient to account for the differences in behavior (Lim et al., 2004b). Other studies have further shown the necessity of V1aR related signaling associated with the formation and expression of the partner preference (Donaldson et al., 2010). Finally, polymorphic variations in a microsatellite promoter region of the V1aR gene correlate with the differences in behavior as well. In the vole species discussed, a longer microsatellite length was found in the monogamous versus the non-monogamous species (Hammock and Young, 2006).

Relationships between V1aR expression and social monogamy have been found outside of vole species as well. Similar patterns of V1aR expression in the ventral pallidum were associated with social monogamy in the mouse species *Peromyscus californicus* versus the non-socially monogamous mouse *P. leucopus* (Bester-Meredith et al., 1999). Moreover, expressing the vole V1aR in the non-socially monogamous mouse species in conjunction with vasopressin application increases partner preference-like behavior but does not induce an actual partner-preference formation (Young et al., 1999b). Similarity in V1aR expression patterns and behavior was also seen in the socially monogamous common marmoset versus the non-socially monogamous rhesus monkey (Young et al., 1999a). Lastly, the relationship between the V1aR microsatellite length correlated with measures of partner preference behavior in humans (Walum et al., 2008).

V1aR expression patterns also show relationships with other social behaviors in addition to social monogamy, such as singing ability in mice (Campbell et al., 2009). V1aR binding shows increased expression in the vocal production centers (periaqueductal gray and anterior hypothalamus) in the more vocal of singing mice species. Moreover, V1aR receptor binding in the anterior and laterodorsal thalamus is higher in a species of singing mouse that has a higher

need for sociospatial memory than the other species of singing mouse (Campbell et al., 2009). Additionally, an exceedingly large body of literature has emerged concerning the role of nonapeptides such as vasopressin in differences in social behaviors in fish, reptiles, birds, and other mammals including humans (see Donaldson and Young, 2008; Goodson and Thompson, 2010).

Thus, it is clear that peptide receptor expression can have a profound impact on social behavior across vertebrate taxa. But what does this mean, and how does it relate to neuromodulation? The hypothesis proposed by Young concerning species differences in social monogamy suggests that vasopressin is involved in social learning (Young and Wang, 2004). Essentially, this suggests that higher V1aR expression allows the socially monogamous species to make an olfactory association with a rewarding mating experience, whereas the non-monogamous species does not form such an association and thus does not form a partner preference. This hypothesis is further supported by the fact that blocking dopamine type-2 receptors inhibits the partner preference in socially monogamous species (Aragona et al., 2006). Thus, the model suggests that the limbic system reward circuit is differentially modulated across species, resulting in different outputs and thus different behaviors. Such a mechanism could easily apply to the myriad of examples that demonstrate species differences in social behavior associated with nonapeptide receptor expression.

Despite the prevalence of peptide receptor expression differences related to species differences in behavior across vertebrates, it is important to note that these relationships are not the only way that species can acquire such social behaviors. For example, V1aR distribution in the ventral pallidum does not predict partner preference behavior across deer mouse species (Turner et al., 2010). Similarly, while the socially monogamous prairie vole V1aR gene promoter region contains a long microsatellite region that is not present in the non-socially monogamous montane and meadow voles, this relationship is not consistent across other taxa and vole species and does not show consistency with social monogamy in prairie voles living in natural con-

ditions (Fink et al., 2006; Ophir et al., 2008b; Solomon et al., 2009; Turner et al., 2010; Mabry et al., 2011).

In summary, these extensive experiments on the role of peptide receptor expression in social behavior in vertebrates inform us in several ways. First, they suggest that what appear to be similar conserved neural circuits can produce very different outputs via differences in neuromodulatory signaling. Second, the repeated evolution of such a neural mechanism suggests that differences in neuromodulatory signaling may be more evolutionarily achievable than the other possible neural mechanisms reviewed earlier. Finally, there are other ways to achieve similar social behaviors besides manipulating vasopressin modulation. Therefore, while a similar neuromodulatory mechanism appears to have evolved independently many times to achieve a behavioral state, other options are available.

Despite the literature reviewed above on how neuromodulatory signaling may underlie species differences in behavior, there are many aspects we do not understand. For one, barring the large body of literature on nonapeptide receptors detailed above, there are few examples of a role for neuromodulation in species differences in behavior. Is this series of repeated examples a rarity, or are there many other examples waiting to be uncovered? The other important aspect that is currently unexplored is how such a difference in neuromodulation is manifested at the cellular level to produce species differences in behavior. The discussed example on learning and memory in aplysiid species is the closest representation, but those studies concerned laboratory behaviors and did not investigate physiological neuromodulatory signaling at the cellular level. This dissertation aims to fill some of these knowledge gaps by examining, at the cellular level, the role of neuromodulation – in addition to the role of neuronal properties and synapses – in species differences in natural behaviors.

Dissertation overview

As reviewed above, there is evidence that species differences in neuromodulation can play a role in species differences in behavior. However, to my knowledge, no study has demonstrated the role of neuromodulation in the evolution of natural behavior at the cellular level. Additionally, it has been shown that differences in the number, properties, or synaptic connections of neurons can play a role in species differences in behavior. However, few studies have been able to systematically investigate the role of each of these possible brain differences underlying differences in behavior at the cellular level. In some cases, this is simply a byproduct of the results. For example, several decapod crustacean lineages have lost particular escape tail-flipping behaviors. This loss was accompanied by the loss of the neurons that produce the behavior, hence studying neuronal property or synaptic differences was not a possibility (Faulkes, 2008). In most instances, however, the systems being studied have not been amenable to investigations at the cellular level. Studying members of the Nudipleura (Mollusca, Gastropoda, Opisthobranchia) clade provides an opportunity to study the role of neuron properties, synaptic connections, and neuromodulation at the cellular level in relation to the evolution of behavior.

Such studies can be conducted in the Nudipleura because the phylogeny is well defined, species throughout the phylogeny have different behaviors and similar independently evolved behaviors, the neural circuits underlying those behaviors can be characterized, and homologues of the neural circuits neurons can be found across species (Newcomb et al., 2012). The dissertation described here aimed to identify the neural mechanisms underlying the ability of two Nudipleura species to produce a similar independently evolved swim behavior that the vast majority of the Nudipleura do not express. To do this, the work focused on five species: *Tritonia diomedea*, *Melibe leonina*, *Flabellina iodinea*, *Hermissenda crassicornis*, and *Pleurobranchaea californica*. For the remainder of this introduction, I will refer to each species by its genus name. Below, I will review the phylogenetic relationships, behaviors, and what is already known about the neural circuitry underlying the behaviors of these animals. I will then briefly describe the ex-

periments that were conducted to determine the neural circuit differences that allow differences in behavior across the related species.

Nudipleura phylogeny

The phylogeny of the Nudipleura has been characterized using morphological and molecular characteristics (Bouchet et al., 2005; Newcomb et al., 2012). Unranked clades are used instead of orders, suborders, and superfamilies. Nudipleura is a monophyletic clade composed of two subclades called Pleurobranchomorpha and Nudibranchia. *Pleurobranchaea* is a member of the Pleurobranchomorpha clade. The Nudibranchia clade consists of two prominent subclades: Euctenidiacea and Cladobranchia. The remaining species in this study are in Cladobranchia. *Flabellina* and *Hermissenda* are members of the monophyletic subclade within Cladobranchia called Aeolidida. *Tritonia* and *Melibe* are members of the paraphyletic subclade called Dendronotida (Figure 1.1). Thus, the species to be investigated here differ in their relatedness. Additionally, these species differ in their behavior, which will allow comparisons across species that differ in their phylogenetic relationship to each other and in the behaviors they express.

Swim behavior

Evidence indicates that the vast majority of Nudipleura species do not swim. Crawling is the primary form of locomotion for the approximately 3000 species of Nudipleura, and only 63 of those species have been documented to swim (Newcomb et al., 2012). It is probable that more species swim than have been described, but it appears as though swimming is a rare behavior among the Nudipleura. The most prominent swim type expressed is left-right (LR) swimming. This swim behavior consists of a flattening of the body wall in the sagittal plane followed by rhythmic alternating left and right whole body flexions (Figure 1.2). A form of dorsal-ventral (DV) swimming has been documented in 17 species. This behavior consists of a flattening of the body in the medial plane followed by a series of rhythmic alternating dorsal and ventral whole

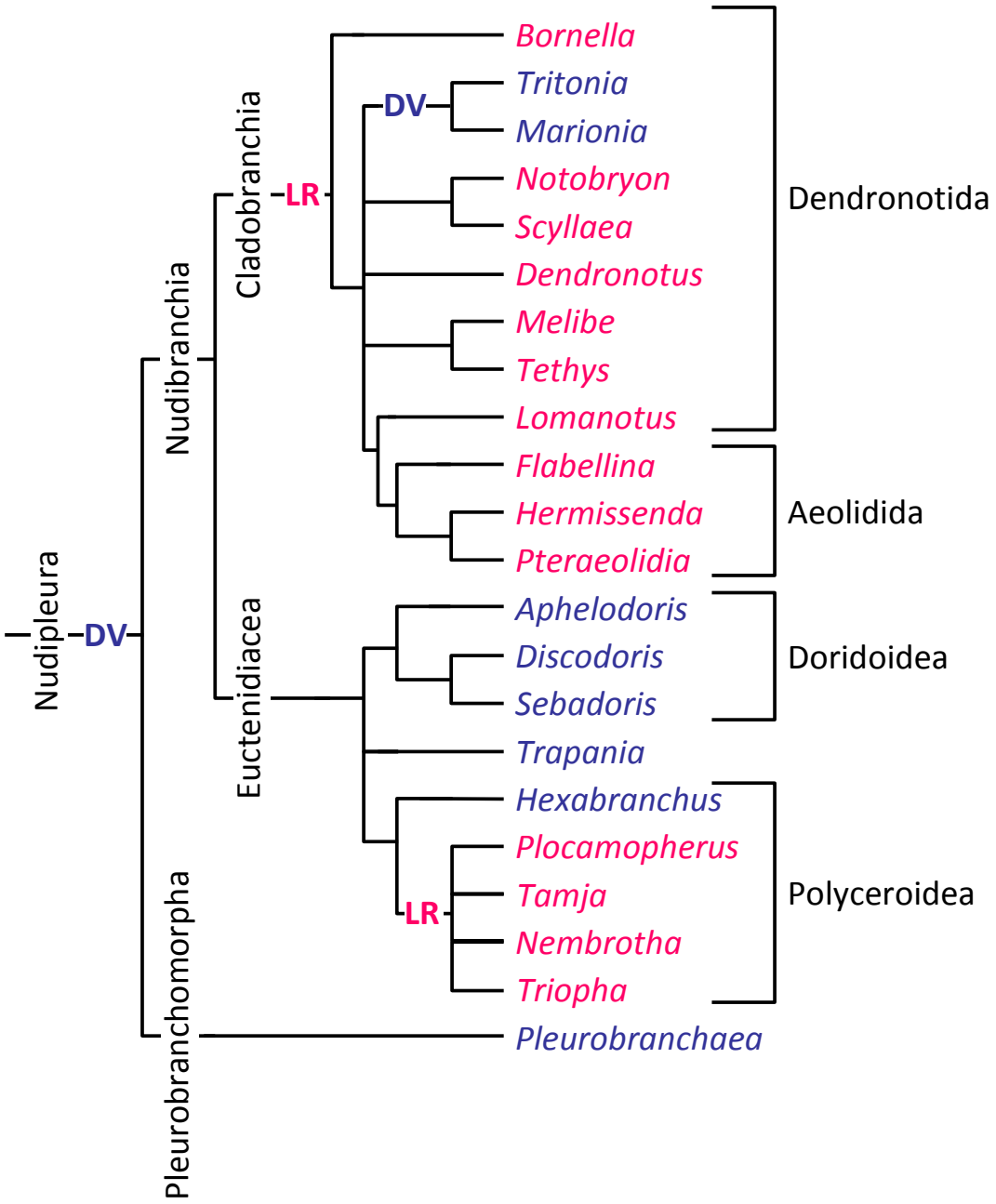

Figure 1.1: Abbreviated Nudipleura phylogeny and swim behaviors. Genus with known left-right (pink) or dorsal-ventral (blue) swimmers are listed. Clade and subclades are indicated. All clades are monophyletic except Dendronotida, which is paraphyletic. DV indicates possible origin of dorsal-ventral swimming. LR indicates possible origin of LR swimming. The most parsimonious explanations suggest that DV swimming arose independently in *Tritonia* and *Pleurobranchaea*. Figure information from (Newcomb et al., 2012).

body contractions (Figure 1.2) (Newcomb et al., 2012). *Melibe*, *Flabellina*, and *Hermissenda* express LR swims, while *Tritonia* and *Pleurobranchaea* express DV swims (Figure 1.2). This dissertation will focus on the potential neural differences responsible for the expression of DV swimming in *Tritonia* and *Pleurobranchaea* compared to the non-DV swimming species.

Evolution of the dorsal-ventral swim

Given the rarity of DV swimming among the Nudipleura, the most parsimonious explanation for the origin of DV swimming is that it repeatedly evolved independently. Alternate explanations would generally necessitate the loss of swim behaviors in hundreds of species. A combination of these hypotheses is possible as well. Regardless of whether DV swimming was present in a common ancestor of *Tritonia* and *Pleurobranchaea*, the most likely scenarios suggest that DV swimming evolved independently in *Tritonia* and *Pleurobranchaea* (Figure 1.1) (Newcomb et al., 2012). This allows us to test whether similar neural mechanisms underlie the independently evolved DV swims in the two species and whether particular neural mechanisms are more important than others for the behavior. It also allows us to test the neural differences that are present between the DV swimmers and non-DV swimmers that allow the rare DV swim behavior to be expressed. Much of the groundwork has been laid for this project, as the neural circuits underlying the DV swim of *Tritonia* and *Pleurobranchaea* have been well described.

Dorsal-ventral swimming in *Tritonia diomedea*

Swimming can be elicited in *Tritonia* by contact with a predatory sea star or from high molarity salt concentrations applied to the body wall (Willows, 1967; Hume et al., 1982). Sensory neurons called S-cells mediate this response. S-cells synapse onto Trigger Neuron 1 (Tr1) and Dorsal Ramp Interneuron (DRI) (Frost and Katz, 1996). DRI, acting as a command neuron, activates the central pattern generator (CPG) that is responsible for the rhythmic swim behavior.

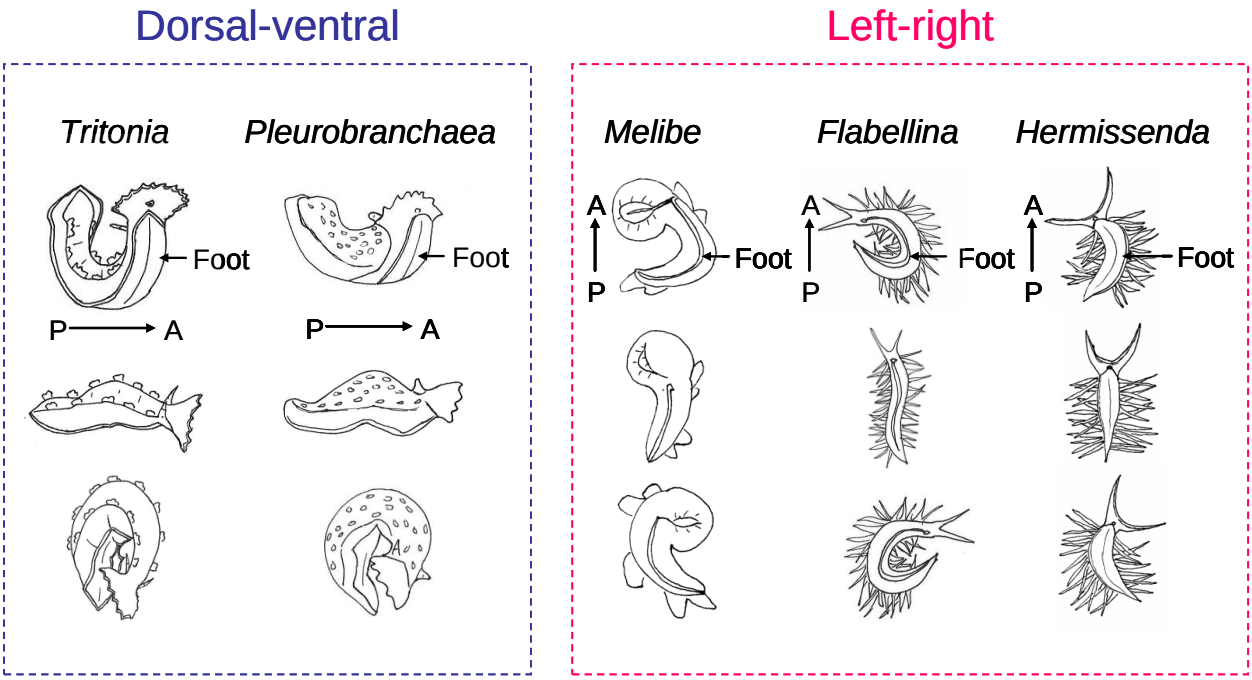

Figure 1.2: Swim behaviors of the species studied. *Tritonia* and *Pleurobranchaea* exhibit swim behaviors consisting of rhythmic dorsal and ventral whole body flexions. *Melibe*, *Flabellina*, and *Hermissenda* exhibit swim behaviors consisting of rhythmic left and right whole body flexions. A: anterior, P: posterior.

The CPG consists of just three neuron types. The Dorsal Swim Interneurons (DSI), Ventral Swim Interneurons (VSI), and Cerebral Neuron 2 (C2) (Katz, 2009).

There is one VSI in each hemisphere of the brain. Similarly, there is one C2 in each hemisphere. There are three DSIs in each hemisphere. There are differences in the properties of the DSIs in relation to each other and the other CPG neurons, but, generally, the following connections describe the CPG network accurately: DSI excites C2, while C2 elicits a fast excitation followed by a slow inhibition in DSI; DSI and VSI mutually inhibit each other; finally, VSI inhibits C2, while C2 elicits a fast excitation followed by a slow inhibition in VSI (Figure 1.3A). The CPG neurons synapse onto motor neurons that project to the musculature mediating the rhythmic movements. The CPG neurons can be monitored in the isolated brain and elicited to produce the rhythmic motor pattern via body wall nerve stimulation (Figure 1.3B).

In addition to the basic components of the swim network and CPG, there are also neuromodulatory components intrinsic to the network that appear to be necessary for the CPG to produce the functional motor output. The DSIs use serotonin (5-HT) as a neurotransmitter and as a neuromodulator (Katz and Frost, 1995b). As a neuromodulator, 5-HT can facilitate potentiation of VSI synapses that can last for several minutes, cause short term facilitation of VSI synapses lasting for several seconds, and depotentiate VSI synapses (Sakurai and Katz, 2003; Sakurai et al., 2006; Sakurai et al., 2007; Sakurai and Katz, 2009a). The effect of 5-HT on VSI synapses depends on the precise timing of DSI firing in relation to VSI firing. The role of these neuromodulatory mechanisms is not clear, though evidence suggests that the neuromodulatory mechanisms could act to increase the strength of VSI synapses during a swim while reducing the VSI effect after the swim when crawling is the key locomotor behavior (Sakurai and Katz, 2009a). 5-HT also increases the excitability of C2, reduces spike-frequency adaptation of C2, and increases the strength of C2 synapses (Katz et al., 1994a; Katz and Frost, 1995b, a, 1997). Modeling evidence suggests that this increase in C2 synaptic strength via 5-HT is necessary for

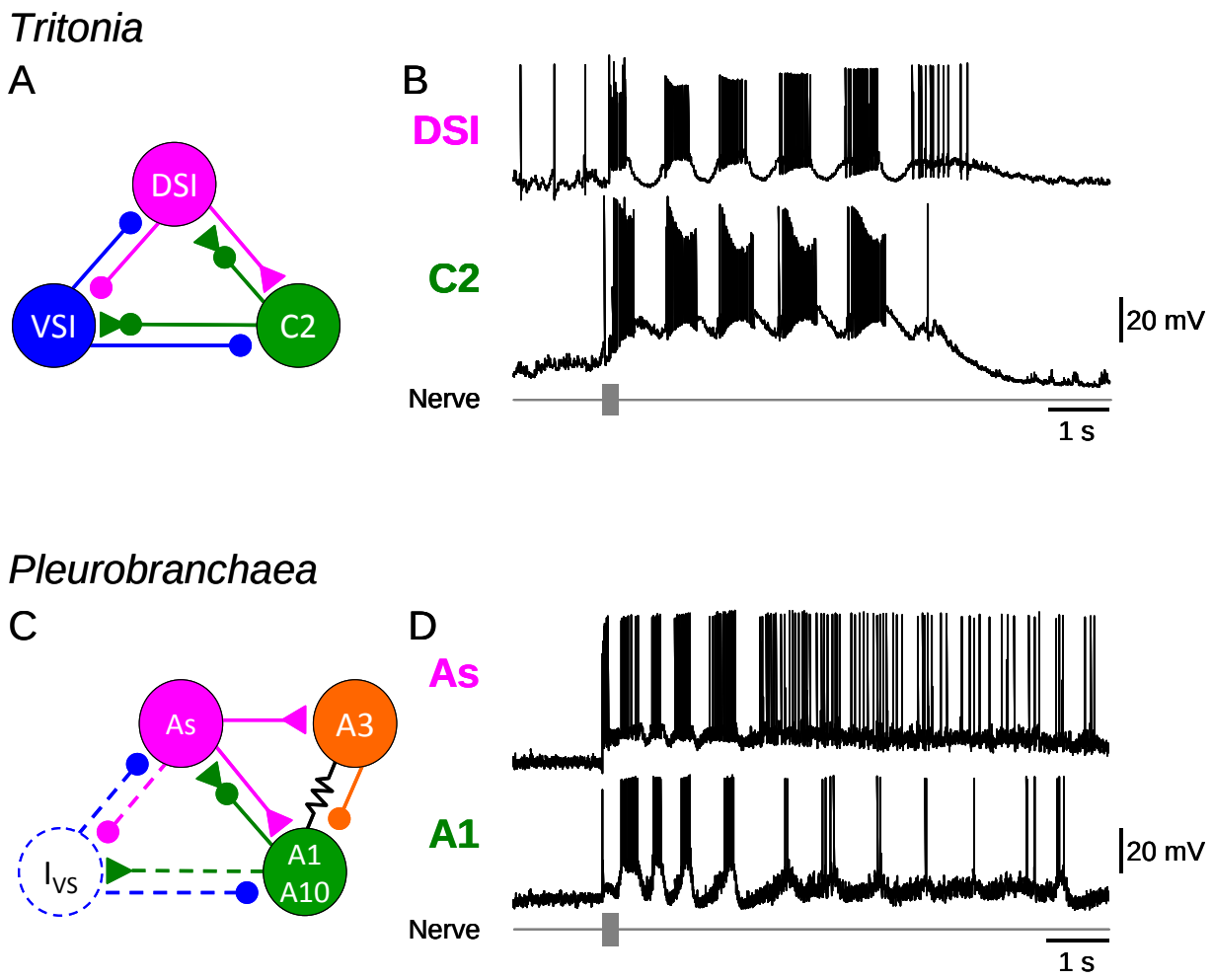

Figure 1.3: Dorsal-ventral swim CPG circuits and isolated nervous system motor patterns in *Tritonia* and *Pleurobranchaea*. A) The *Tritonia* swim CPG is composed of 3 synaptically connected neuron types. B) Stimulating a body wall nerve (grey bar) elicits the swim motor pattern as recorded in DSI and C2. C) The *Pleurobranchaea* swim CPG contains neurons that are homologous to DSI (As) and C2 (A1). I_{VS} is an unidentified neuron that is presumed to be present based on synaptic activity in the other CPG neurons. A10 and A3 are CPG neurons that have not been identified in *Tritonia*. D) Stimulating a body wall nerve elicits a swim motor pattern as recorded in As and A1. Triangles: excitatory synapses, circles: inhibitory synapses, both: multicomponent synapses; resistor: electrical coupling.

the CPG to produce the rhythmic output (Calin-Jageman et al., 2007). Moreover, blocking G-protein coupled signaling in C2, which is hypothesized to mediate the C2 synaptic modulation effect of DSI, blocks the ability of *Tritonia* to swim (Clemens and Katz, 2003). Additionally, blocking the neuromodulatory actions of 5-HT using the serotonin receptor antagonist methysergide also blocks the swim *in vivo* and in the isolated brain (McClellan et al., 1994; Katz and Frost, 1995b). Thus, evidence suggests that the modulatory mechanisms of 5-HT within the CPG are necessary for the CPG to produce the proper DV output.

In summary, the neurons, synaptic connections, and neuromodulatory mechanisms within the *Tritonia* DV swim CPG are well described. This allows us to investigate whether there are similarities and differences in these properties between species that can swim like *Tritonia* (*Pleurobranchaea*) and species that cannot (*Melibe*, *Flabellina*, and *Hermissenda*).

Dorsal-ventral swimming in *Pleurobranchaea californica*

Like *Tritonia*, *Pleurobranchaea* can also perform a DV swim (Gillette et al., 1991). The sensory pathway that initiates the *Pleurobranchaea* swim is not as well-characterized, but the CPG has been described. It contains homologues of the *Tritonia* CPG neurons DSI and C2, named As and A1 respectively (Jing and Gillette, 1995, 1999). A VSI-like cell presumably exists based on synaptic activity in As and A1 neurons, but it has not been found. There are also two additional CPG neurons found in *Pleurobranchaea* that have not been found in *Tritonia*, A10 and A3. A10 is a neuron that is very strongly coupled to A1 and therefore acts similarly to A1. A10 may act similarly to DRI in *Tritonia* in its ability to elicit the swim motor pattern. A3 is an interneuron that is also electrically coupled to A1 but also inhibits A1 and is excited by As neurons. The synaptic connections between As and A1 are similar to those observed between DSI and C2 in *Tritonia* (Jing and Gillette, 1999) (Figure 1.3C). Moreover, the CPG neurons can also

be monitored in the isolated brain in *Pleurobranchaea* and produce a rhythmic output similar to that of the *Tritonia* DV swim CPG (Figure 1.3D).

Accordingly, the neural circuits underlying the independently evolved swims in *Tritonia* and *Pleurobranchaea* show strong similarities but also differences. This suggests that certain nervous system constraints may necessitate the involvement of DSI and C2 homologues in the DV swim CPGs but that other circuit parameters can show flexibility without apparent detriment to the ability to produce the swim. Given the use of DSI and C2 homologues with similar synaptic connections in both CPG circuits, it is of interest to investigate whether both neurons are present and have similar synaptic connections in species that cannot produce a DV swim. Additionally, no neuromodulatory properties of the *Pleurobranchaea* swim CPG have been described. Given the importance of neuromodulation to the CPG output in *Tritonia*, it is of interest to investigate whether these dynamic mechanisms are also present in *Pleurobranchaea* and in non-DV swimming species.

CPG homologue identification

The first step in testing the neural mechanisms underlying the ability of *Tritonia* and *Pleurobranchaea* to produce a DV swim is to identify homologues of the CPG neurons in species that cannot produce the behavior. The *Tritonia* CPG homologues were identified in *Pleurobranchaea* thanks in large part to the fact that they were part of the rhythmic CPG oscillator in that species. Using anatomical and neurochemical characteristics, neuronal homologues can also be identified in species that do not swim like *Tritonia* and *Pleurobranchaea*. This is because the anatomical and neurochemical characteristics of neurons appear to be highly conserved across species (Pentreath et al., 1982; Croll, 1987a; Arbas et al., 1991; Bullock, 2000; Striedter, 2006; Newcomb and Katz, 2007). Such methods have been used to identify homologues of the DSI neurons across the Nudipleura. Cell body location, a characteristic axon projection, and 5-HT immunoreactivity will uniquely identify DSI homologues across species. To date, DSI homo-

logues have been identified in 9 species of Nudipleura including *Melibe*, *Flabellina*, *Hermissenda*, and *Pleurobranchaea* (Jing and Gillette, 1999; Tian et al., 2006; Newcomb and Katz, 2007). DSI homologues have also been identified in *Aplysia californica* and *Clione limacina*, two species outside of the Nudipleura (Panchin et al., 1995; Satterlie and Norekian, 1995; Fickbohm et al., 2001). These studies have shown that the anatomical position, axonal projection, and neurochemistry of DSI homologues are conserved across the Nudipleura phyla and beyond, regardless of behavior. Whether this is true of the other CPG neurons is unknown.

The functions and properties of DSI across species

The DSI neurons are multifunctional in *Tritonia* and have been shown to have multiple functions in other related species as well. In *Tritonia*, in addition to being part of the DV swim CPG, the DSIs also initiate crawling behaviors (Popescu and Willows, 1999; Popescu and Frost, 2002). The DSI homologues are also involved in crawling behaviors in *Pleurobranchaea* and *Aplysia* (Jing and Gillette, 2000; Jing et al., 2008). The DSIs can also cause foot contraction in *Hermissenda* (Tian et al., 2006). Additionally, the DSIs can facilitate feeding *Pleurobranchaea* and *Aplysia* (Jing and Gillette, 2000; Jing et al., 2008). The DSIs also modulate the swims of the LR swimming *Melibe* and the wing-like flapping swim of *Clione* (Panchin et al., 1995; Satterlie and Norekian, 1995; Newcomb and Katz, 2009). Despite the many functions of the DSIs, the basic electrophysiological properties of the DSI homologues are generally similar across species (Newcomb and Katz, 2007). There are some differences, but no differences correlate with the ability to produce a DV swim. Thus, no investigated property of the DSI homologues – aside from being part of the DV swim CPG – can account for species differences in DV swim expression.

Dissertation summary

This finally brings us to the experiments conducted for this dissertation. Because no property of the DSIs appears to account for the expression of a DV swim across species, other properties of the CPG circuit are likely to be responsible for the differences in behavior. The possible differences could be that 1) the other CPG homologues are not present in the non-DV swimming species, 2) the properties of other CPG homologues differ in relation to behavioral differences, 3) the synaptic connections of the CPG homologues differ in relation to behavioral differences, or 4) the neuromodulatory properties among the CPG homologues differ in relation to behavioral differences. This dissertation aims to test these possibilities by focusing on one of the other two *Tritonia* CPG neurons, C2.

Chapter 2 details experiments that identified homologues of C2 using anatomical and neurochemical characteristics. First the chapter describes the characteristics that uniquely identify C2 in *Tritonia* based solely on anatomy and neurochemistry. These same characteristics confirmed the C2 homologue identity of the previously identified A1 in *Pleurobranchaea*. Moreover, these characteristics also uniquely identified C2 homologues in three species that do not express DV swims: *Melibe*, *Flabellina*, and *Hermissenda*. This work was published in PLoS ONE, with my lab mate Charuni Gunaratne and my advisor Paul Katz as co-authors (Lillvis et al., 2012). Charuni performed nerve backfilling experiments that greatly aided our conclusions in the manuscript.

Chapter 3 describes experiments that focused on *Tritonia*, *Pleurobranchaea*, and *Hermissenda*. In these experiments, I tested the electrophysiological properties and synaptic connections of DSI and C2 homologues in each species in an effort to determine whether any differences correlated with the expression of the DV swim behavior. I found that there were species differences in the neuronal properties and synaptic connections that correlated with phylogeny but not with expression of the DV swim behavior. That is, there were differences between *Tritonia* and *Pleurobranchaea* even though both species can produce a DV swim. How-

ever, there were also DSI and C2 synaptic connection differences that did correlate with the ability to produce the dorsal-ventral swim behavior.

Chapter 4 summarizes work testing whether DSI modulated C2 synaptic strength in *Tritonia*, *Pleurobranchaea*, and *Hermissenda*. I found that DSI modulated C2 synaptic strength only in the species that express a DV swim. Moreover, the extent of serotonergic modulation also correlated with the strength of swimming in *Pleurobranchaea*. Finally, serotonergic neuromodulation was necessary for swimming in the DV swimming species.

These experiments demonstrate that the properties of neurons and synapses can vary across species without affecting behavior. The experiments also suggest a role for synaptic connection and neuromodulatory mechanisms underlying species differences in behavior at the cellular level. The implications of the results are detailed in each chapter and summarized in Chapter 5, which specifically discusses the results in light of our current understanding about neural circuit function and the evolution of behavior.

CHAPTER 2: NEUROCHEMICAL AND NEUROANATOMICAL IDENTIFICATION OF CENTRAL PATTERN GENERATOR NEURON HOMOLOGUES IN NUDIPLEURA MOLLUSCS

Joshua L. Lillvis, Charuni A. Gunaratne, and Paul S. Katz

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Abstract

Certain invertebrate neurons can be identified by their behavioral functions. However, evolutionary divergence can cause some species to not display particular behaviors, thereby making it impossible to use physiological characteristics related to those behaviors for identifying homologous neurons across species. Therefore, to understand the neural basis of species-specific behavior, it is necessary to identify homologues using characteristics that are independent of physiology. In the Nudipleura mollusc *Tritonia diomedea*, Cerebral Neuron 2 (C2) was first described as being a member of the swim central pattern generator (CPG). Here we demonstrate that neurochemical markers, in conjunction with previously known neuroanatomical characteristics, allow C2 to be uniquely identified without the aid of electrophysiological measures. Specifically, C2 had three characteristics that, taken together, identified the neuron: 1) a white cell on the dorsal surface of the cerebral ganglion, 2) an axon that projected to the contralateral pedal ganglion and through the pedal commissure, and 3) immunoreactivity for the peptides FMRFamide and Small Cardioactive Peptide B. These same anatomical and neurochemical characteristics also uniquely identified the C2 homologue in *Pleurobranchaea californica* (called A1), which was previously identified by its analogous role in the *Pleurobranchaea* swim CPG. Furthermore, these characteristics were used to identify C2 homologues in *Melibe leonina*, *Hermissenda crassicornis*, and *Flabellina iodinea*, species that are phylogenetically

closer to *Tritonia* than *Pleurobranchaea*, but do not display the same swimming behavior as *Tritonia* or *Pleurobranchaea*. These identifications will allow future studies comparing and contrasting the physiological properties of C2 across species that can and cannot produce the type of swimming behavior exhibited by *Tritonia*.

Introduction

The ability to reliably identify neurons in some invertebrate model systems allows individual neurons to be linked to behavior. As such, it is compelling to attempt to find homologues of identified neurons across species with similar or different suites of behavior. This allows the physiological properties of the homologues to be compared in an effort to better understand the neural basis of behavior and its evolution. It is not possible to use behavioral function to characterize neuronal homologues, however, if the behavior used to identify the neuron differs across species. Therefore, characteristics must be found that can identify homologous neurons regardless of physiological activity. Here, as a step toward comparing the physiological properties of homologous neurons in species with different behaviors, we have used neuroanatomical and neurochemical characteristics to identify homologues of an individual neuron across gastropod mollusc species within the Nudipleura clade (Figure 2.1) (Bouchet et al., 2005).

Gastropod molluscs are well suited for a comparative study of single neurons and small neural networks. The phylogeny is well documented (Wollscheid and Wagele, 1999; Wagele and Willan, 2000; Wollscheid-Lengeling E., 2001; Grande et al., 2004; Bouchet et al., 2005; Vonnemann et al., 2005) and closely related species display a variety of behaviors (Farmer, 1970; Audesirk and Audesirk, 1985; Willows, 2001). Moreover, gastropod brains have a relatively small number of neurons (5,000-10,000), which have large cell bodies (up to 1 mm in diameter) (Bullock and Horridge, 1965; Boyle et al., 1983). Many gastropod neurons can be uniquely identified based upon a suite of anatomical, neurochemical, and electrophysiological characteristics (Croll, 1987a; Bullock, 2000). Homologues of neurons in other species can be

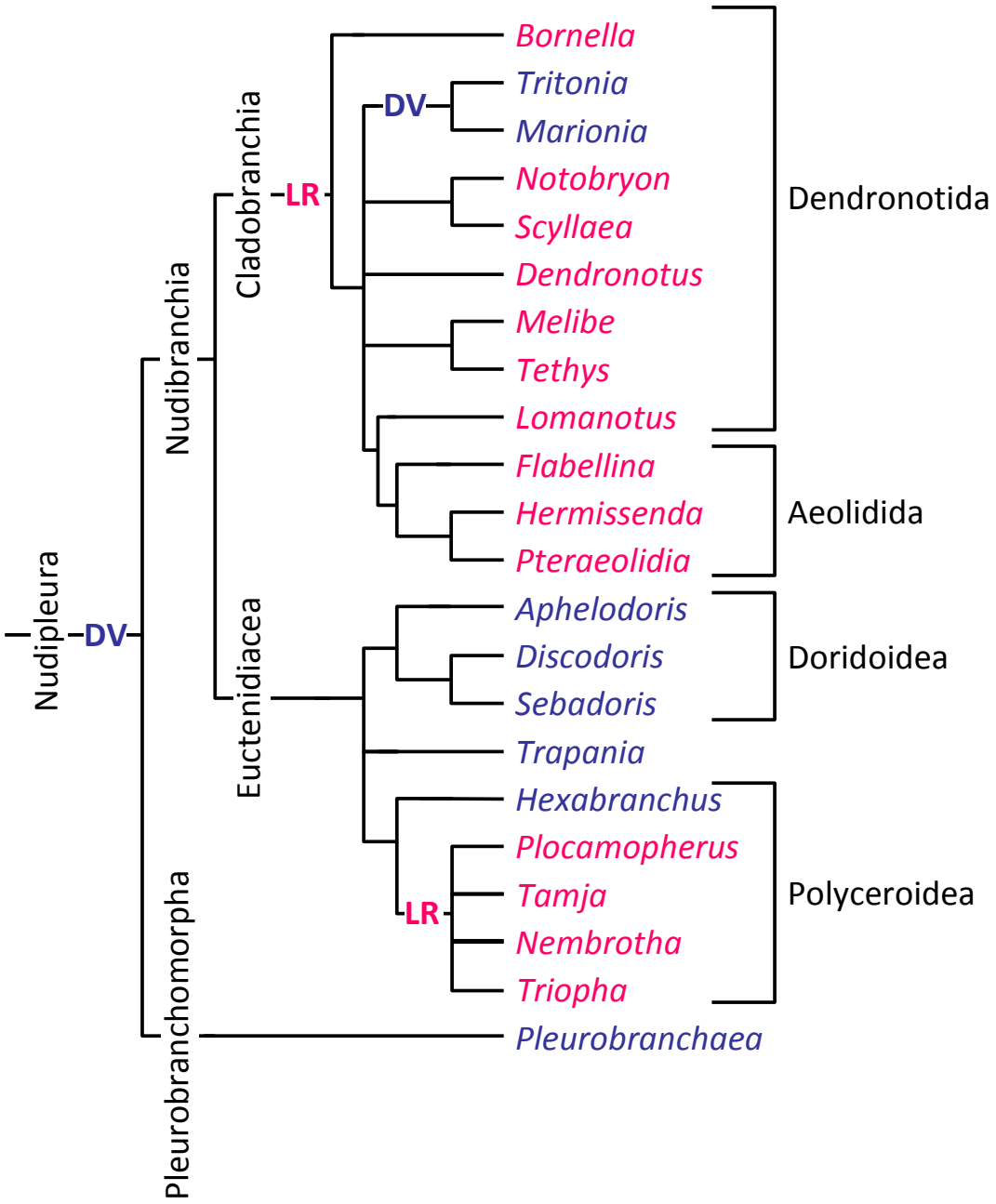

Figure 2.1: Abbreviated Nudipleura phylogeny and swim behaviors. Genus with known left-right (pink) or dorsal-ventral (blue) swimmers are listed. Clade and subclades are indicated. All clades are monophyletic except Dendronotida, which is paraphyletic. DV indicates possible origin of dorsal-ventral swimming. LR indicates possible origin of LR swimming. The most parsimonious explanations suggest that DV swimming arose independently in *Tritonia* and *Pleurobranchaea*. Figure information from (Newcomb et al., 2012).

identified using the same suite of characteristics (Weiss and Kupfermann, 1976; Granzow and Fraser Rowell, 1981; Pentreath et al., 1982; Croll, 1987a; Watson and Willows, 1992; Yoshida and Kobayashi, 1994; Jing and Gillette, 1995, 1999; Fickbohm et al., 2001; Newcomb and Katz, 2007; Wentzell et al., 2009; Sakurai et al., 2011). The argument for homology is based on parsimony; it being more likely that neurons with the same identifying characteristics in different species were present in a common ancestor than that they acquired these uniquely identifying characteristics independently (Croll, 1987a; Striedter and Northcutt, 1991). The more species that exhibit the shared characteristics, the stronger is the argument in favor of homology. The ability to identify homologues in gastropods allows comparative studies of homologous neurons across species with similar or divergent suites of behavior. Studies of this kind have been conducted to comparatively investigate the neural basis of feeding (Weiss and Kupfermann, 1976; Granzow and Fraser Rowell, 1981; Pentreath et al., 1982; Watson and Willows, 1992; Yoshida and Kobayashi, 1994; Jing and Gillette, 2000; Jing et al., 2008; Wentzell et al., 2009) and locomotor (Jing and Gillette, 1995, 1999; Newcomb and Katz, 2009; Sakurai et al., 2011) behaviors across species.

The species to which others will be compared is a gastropod mollusc within the Nudipleura clade named *Tritonia diomedea*. *Tritonia* produces a swim that consists of rhythmic, alternating dorsal and ventral whole body flexions (Dorsett et al., 1969; Willows and Hoyle, 1969; Hume et al., 1982). The central pattern generator (CPG) underlying the swim is made up of just three cell types: the Dorsal Swim Interneurons (DSI) (http://neuronbank.org/Tri0001043), Ventral Swim Interneurons (VSI) (http://neuronbank.org/Tri0002436), and Cerebral Neuron 2 (C2) (http://neuronbank.org/Tri0002380) (Getting et al., 1980b; Getting, 1981, 1983, 1989; Katz, 2009). In an effort to understand how *Tritonia* can produce a dorsal-ventral swim while most Nudipleura molluscs cannot, we wanted to identify homologues of CPG neurons across species that can and cannot swim like *Tritonia* (Figure 1.1). This will enable future studies comparing the properties of the homologous neurons. Previous work identified homologues of DSI in 11 spe-

cies based on conserved soma location, axon projection, and serotonergic immunoreactivity (Panchin et al., 1995; Satterlie and Norekian, 1995; Sudlow et al., 1998; Jing and Gillette, 1999; Katz et al., 2001; Tian et al., 2006; Newcomb and Katz, 2007; Jing et al., 2008). Here, we sought to identify another CPG neuron, C2, across species.

We have established neurochemical characteristics that allow C2 to be uniquely identified in *Tritonia* without the aid of electrophysiological measures. Using these characteristics, we have provided further evidence for homology of the previously identified A1 neuron (http://neuronbank.org/Ple0002601) in *Pleurobranchaea californica*, a species that can swim like *Tritonia* (Jing and Gillette, 1995, 1999). Furthermore, we found that the same characteristics uniquely identified C2 homologues in three Nudipleura molluscs that cannot swim like *Tritonia*: *Melibe leonina*, *Hermissenda crassicornis*, and *Flabellina iodinea*. Similar results were reported by Longley and Longley in a 1987 abstract for the Society for Neuroscience annual meeting.

Methods

Animal collection and maintenance

Tritonia and *Melibe* were collected by Living Elements (Vancouver, BC, Canada). Additional *Melibe as well as Flabellina, Hermissenda and Pleurobranchaea* were collected by Monterey Abalone Company (Monterey, California, USA).

All animals were kept in re-circulating artificial seawater (Instant Ocean) tanks at Georgia State University on a 12:12 light/dark cycle. *Pleurobranchaea* were maintained at 14ºC. All other animals were kept at 10-13ºC.

Dissection

Animals were anesthetized by chilling and/or by injecting 0.33 M $MgCl_2$ into the body cavity. The brain, consisting of the cerebral, pleural, and pedal ganglia, was removed from the animal and immediately pinned to the bottom of a Sylgard-lined chamber, which was superfused

with saline at 4°C. Physiological saline composition was as follows (in mM): 420 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 10 D-Glucose, and 10 HEPES, pH 7.4. For experiments where intracellular tracer injection was to be conducted, the cell bodies of the neurons were exposed by removing the connective tissue sheath from the surface of the ganglia with fine scissors and forceps. For nerve backfill experiments, the connective tissue sheath remained intact. The temperature was then raised to 14°C and 13°C for *P. californica* and *H. crassicornis*, respectively. The temperature was raised to 10-11°C for all other animals.

Tracer injection and whole-mount immunohistochemistry

To visualize the axonal projection of the neurons, their somata were impaled with glass microelectrodes (10-50 MΩ in resistance) filled with 2-4% Neurobiotin (Vector Laboratories) and/or 2.5% biocytin (Invitrogen) dissolved in 0.75 M KCl solution. The electrodes were connected to Axoclamp 2B amplifiers (Molecular Devices). The tracer was injected via iontophoresis for 15-120 minutes (-10 to 10 nA, 1 Hz, 50% duty cycle). The injection variability was due to variability in the electrode resistance. For low resistance electrodes, high amplitude current pulses were used for short durations of time. For high resistance electrodes, smaller amplitude current pulses were applied for longer durations. After dye injection, preparations were incubated in superfusing normal saline for 12-72 hours at physiological temperatures (see above).

For biocytin nerve backfills, the cut end of the nerve was drawn into a petroleum jelly well created on top of a Sylgard block. Several drops of distilled H_2O were added to the well and the nerve was cut again and left in H_2O for 30 seconds. The distilled H_2O was then replaced with a 2-2.5% solution of biocytin in 1 M KCl. The well was covered with more petroleum jelly to reduce evaporation of the dye and the preparation was incubated at 4º C for 2-48 hours. During this incubation, the dye transported retrogradely to cell bodies with axons in the nerve. After the incubation, the preparation was washed briefly in saline.

After dye injection or backfill incubations and saline washes, preparations were fixed for 12-24 hours in 4% formaldehyde in phosphate buffered saline (PBS, 50 mM Na_2HPO_4 in 140 mM $NaCl_2$, pH 7.2). After fixation, brains were washed (20-90 minutes) with PBS and the connective tissue sheath was removed if still present. Brains were washed twice (20-30 minutes each) with 4% Triton X-100 in PBS and then incubated for 1 hour in antiserum diluent (ASD, 0.5% Triton X-100, 1% normal goat serum and 1% bovine serum albumen in PBS). This was followed by incubation in primary antiserum with Streptavidin-Alexa Fluor 594 conjugate (1:50 - 1:200, Invitrogen) added to visualize the tracer (72-120 hours): rabbit anti-FMRFamide antiserum (Immunostar) diluted 1:1000 and/or mouse monoclonal anti-Small Cardioactive Peptide B (SCP_B; courtesy of Stephen Kempf) diluted 1:20 in ASD. Brains were then washed 5 times (1 hour each) with 0.5% Triton X-100 in PBS and then incubated 12-24 hours in goat anti-rabbit antiserum and/or goat anti-mouse antiserum conjugated to either Alexa Fluor 488, Alexa Fluor 594 (Invitrogen), or DyLight 405 (Jackson ImmunoResearch) diluted 1:00 in ASD. Next, brains were washed 5 times (1 hour each) with 0.5% Triton X-100 in PBS, dehydrated in an ethanol series, cleared in methyl salicylate, and mounted on a slide with Cytoseal 60 (Richard-Allan Scientific). Brains were kept at 4ºC for all the immunohistochemistry protocol before dehydration and all steps from fixation to dehydration were completed with gentle agitation.

Imaging

Fluorescence images were obtained using confocal microscopy (LSM 510 mounted on Axiovert 100 M microscope or LSM 700 on Axio Examiner D1 microscope, Carl Zeiss, Inc.) with a 5-20x objective. Fluorophores were excited with three lasers (405, 488, and 555 nm) and fluorescent emissions were passed through a 490 nm short-pass filter to visualize DyLight 405, a band-pass filter (505-550 nm) for visualization of Alexa Fluor 488 and a 560 nm long-pass filter to visualize Alexa Fluor 594. The thickness of each confocal section was optimized and kept consistent within a preparation. Maximal projections of confocal stacks were exported as TIFF

files and imported into Adobe Photoshop. Projections were assembled into a montage of the CNS and brightness and contrast were adjusted.

Results

Identifying C2 in Tritonia diomedea

The *Tritonia* brain consists of paired fused cerebral, pleural and pedal ganglia (Figure 2.2A, B) (Willows et al., 1973). $C2_{Tri}$ (subscripts will be used here to distinguish homologues in each species) is a bilaterally represented neuron with a white soma found on the dorsal surface of the cerebral ganglion. The soma is located in the anterior-lateral region of the ganglion near the origin of cerebral nerve 1 (CeN1), a nerve that originates on the dorsal surface of the cerebral ganglion (Willows et al., 1973) (Figure 2.2A) (nerve nomenclature is based on reference (Newcomb et al., 2006) unless indicated otherwise). $C2_{Tri}$ has a characteristic contralateral axon projection to the pedal ganglion and through the pedal commissure (PP2; pedal nerve 6), the largest of the commissure nerves that connect the left and right pedal ganglia (Figure 2.2B, D) (Getting et al., 1980b; Sakurai and Katz, 2009b). These anatomical features help to identify $C2_{Tri}$, but electrophysiological characters are often necessary to unequivocally identify the neuron. Specifically, C2 receives spontaneous, discrete excitatory post-synaptic potentials but is quiescent at rest. It is also electrically coupled to its contralateral counterpart (Getting, 1981). Finally, synaptic connections with other swim CPG members and the participation of C2 in the dorsal-ventral swim CPG unequivocally identify this neuron (Getting, 1977; Taghert and Willows, 1978; Getting, 1989). Here, we have further characterized $C2_{Tri}$ using anatomical and neurochemical measures in order to allow homologue identification in species that may not share the electrophysiological properties observed in *Tritonia*.

Neurochemical markers can greatly aid in identifying individual neurons both within and across species. Evidence suggests that $C2_{Tri}$ is peptidergic (Snow, 1982b) and our immunohistochemistry experiments support that hypothesis. We filled the $C2_{Tri}$ soma with a biotinylated

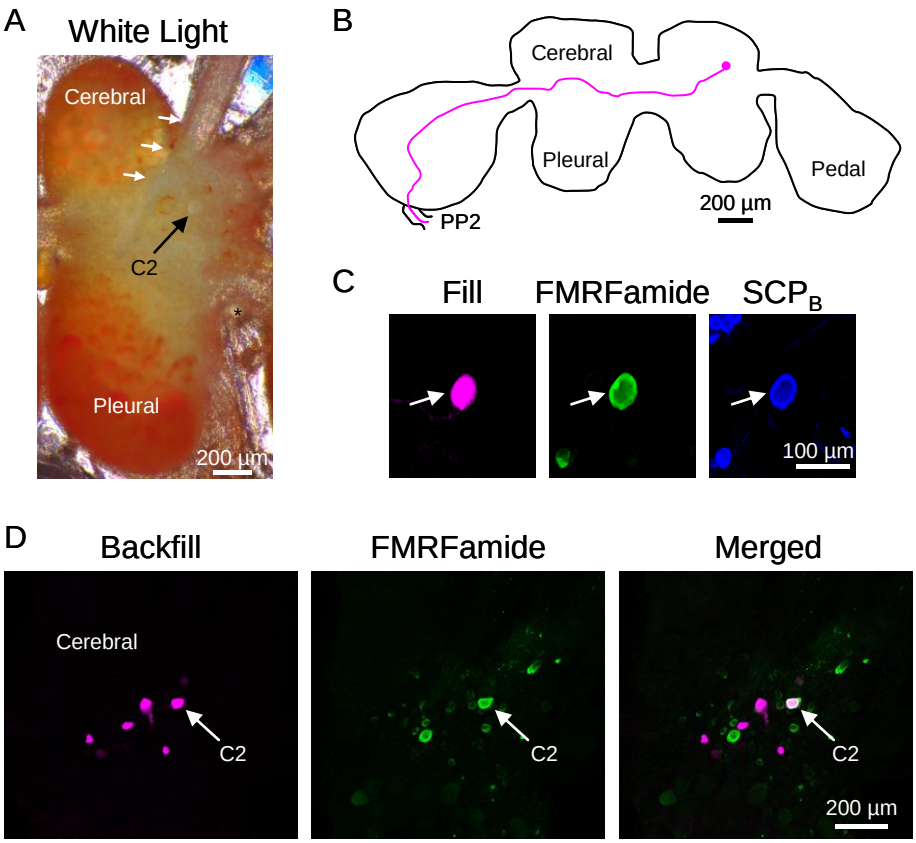

Figure 2.2: C2 characteristics in *Tritonia diomedea*. A) C2$_{Tri}$ could be identified visually due to its characteristic white soma near the origin of cerebral nerve 1 (CeN1, white arrows). An asterisk (*) labels the statocyst. Only one cerebral-pleural ganglion is shown. B) Filling the C2$_{Tri}$ soma with Neurobiotin revealed a contralateral axon projection through the anterior cerebral-pedal commissure (not shown) and into the pedal commissure (PP2). The example is a representative image in which the outline of the brain and the axon projection were traced for ease of viewing. C) C2$_{Tri}$ (arrow) was filled with biocytin (left). It was immunoreactive for both FMRFamide (middle) and SCP$_B$ (right) as shown. D) Backfilling the pedal commissure with biocytin in *Tritonia* labeled 3-4 neurons near C2$_{Tri}$ (left). Only the cerebral-pleural ganglion contralateral to the backfilled nerve is shown. FMRFamide-like immunohistochemistry labeled C2$_{Tri}$ (middle). Combining FMRFamide-like immunoreactivity with the backfill revealed just one neuron, C2$_{Tri}$, (right).

tracer and tested antisera and monoclonal antibodies raised against neuropeptides on whole brain preparations. We determined that $C2_{Tri}$ displays dual FMRFamide-like immunoreactivity (n=10) and Small Cardioactive Peptide B (SCP_B)-like immunoreactivity (n=6 somata in 5 preparations) (Figure 2.2C).

$C2_{Tri}$ could be unequivocally identified with just FMRFamide immunoreactivity in conjunction with the contralateral axon projection to PP2. Backfilling PP2 with biocytin showed that approximately four cell bodies in the vicinity of $C2_{Tri}$ share a similar contralateral axon projection to PP2 (Figure 2.2D; n=4). However of the four cell bodies, only $C2_{Tri}$ also displayed FMRFamide immunoreactivity (Figure 2.2D, n=3). Moreover, $C2_{Tri}$ was the only neuron on the dorsal surface of the cerebral-pleural ganglion that was both FMRFamide immunoreactive and projected an axon contralaterally to and through PP2. Thus, $C2_{Tri}$ can be uniquely identified without the aid of electrophysiological characters; the characteristics of a FMRFamide immunoreactive soma on the dorsal surface of the cerebral-pleural ganglion in conjunction with a contralateral axon projection into PP2 were sufficient to identify the neuron. Additional characteristics of C2 were SCP_B immunoreactivity and a contralateral axon projection through the anterior of two fiber tracts connecting the cerebral and pedal ganglia (anterior cerebral-pedal commissure) before reaching PP2. Furthermore, the characteristics of a white soma on the dorsal surface of the brain near the origin of CeN1 help to identify the neuron in the living preparation.

Identifying C2 in Pleurobranchaea californica

Pleurobranchaea is in the Pleurobranchomorpha clade of Nudipleura, which makes it the species that is most distantly related to *Tritonia* in this study (Figure 2.1) (Bouchet et al., 2005). It is the only other species investigated, however, that can produce a rhythmic, dorsal-ventral swim like that of *Tritonia* (Davis and Mpitsos, 1971) (Figure 1.1). The CPG underlying the *Pleurobranchaea* swim contains homologues of the *Tritonia* swim CPG neurons, including the homologue of C2 (Jing and Gillette, 1995, 1999), which was named A1, but will be called $C2_{Pleur}$ here

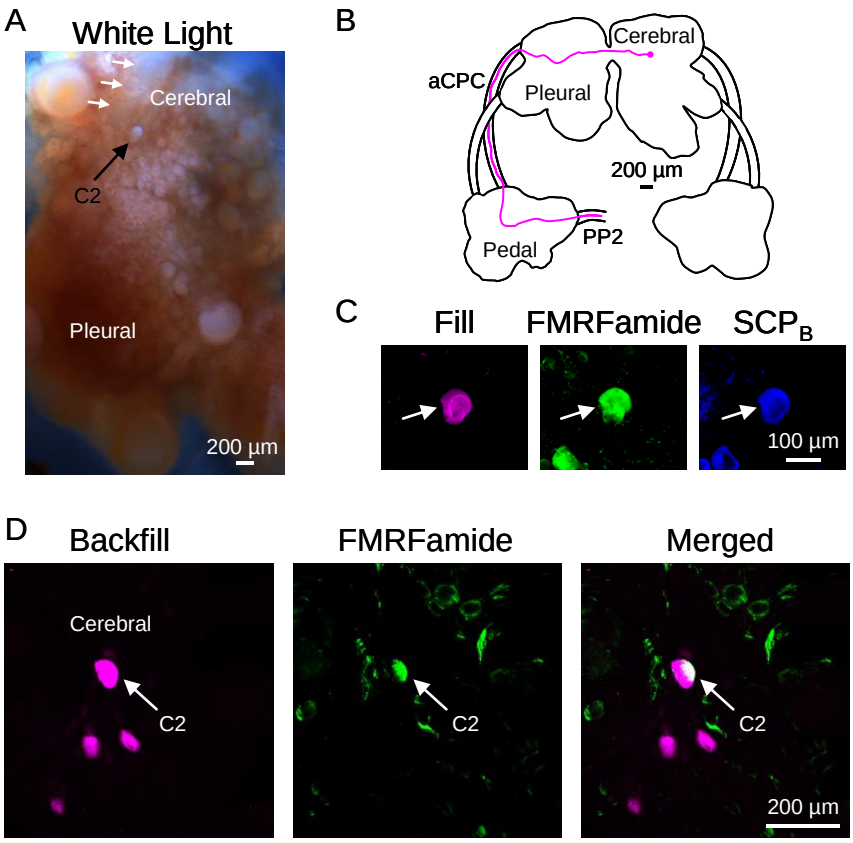

Figure 2.3: C2 characteristics in *Pleurobranchaea californica*. A) C2$_{Pleur}$ could be identified visually due to its characteristic white soma near the origin of cerebral nerve 1 (CeN1, white arrows). It was the more anterior-lateral of two white neurons near the origin of CeN1 (the more medial white neuron is not seen here). Only one cerebral-pleural ganglion is shown. B) Filling the C2$_{Pleur}$ soma with biocytin revealed a contralateral axon projection through the anterior cerebral-pedal commissure (aCPC) into the pedal commissure (PP2). The example is a representative image in which the outline of the brain and the axon projection were traced for ease of viewing. C) C2$_{Pleur}$ (arrow) was filled with biocytin (left). It was immunoreactive for both FMRFamide (middle) and SCP$_B$ (right) as shown. D) Backfilling the pedal commissure with biocytin labeled 3-4 neurons near C2$_{Pleur}$ (left). Only the cerebral-pleural ganglion contralateral to the backfilled nerve is shown. FMRFamide-like immunohistochemistry labeled C2$_{Pleur}$ (middle). Combining FMRFamide-like immunoreactivity with the backfill revealed just one neuron, C2$_{Pleur}$ (right).

for consistency. $C2_{Pleur}$ is not only part of the swim CPG, but also has a white soma near the origin of CeN1 (called the rhinophore nerve in *Pleurobranchaea*) (Figure 2.3A) and a contralateral axon projection through the anterior cerebral-pedal commissure and through PP2 (called the pedal commissure in *Pleurobranchaea*) (Figure 2.3B, D) (Jing and Gillette, 1995).

Experiments in which the $C2_{Pleur}$ soma was injected with a biotinylated tracer combined with whole brain immunohistochemistry revealed that, like $C2_{Tri}$, $C2_{Pleur}$ displayed dual FMRFamide (n=11 somata in 7 preparations) and SCP_B immunoreactivity (n=7 somata in 4 preparations) (Figure 2.3C). Moreover, combining biocytin backfills of PP2 with FMRFamide immunohistochemistry, showed that $C2_{Pleur}$ was the only neuron on the dorsal surface of the cerebral-pleural ganglion with a contralateral axon projection to and through PP2 that is also immunoreactive for FMRFamide (n=6) (Figure 2.3D). Consequently, the same anatomical and neurochemical characteristics that could uniquely identify $C2_{Tri}$ also uniquely identified C2 in *Pleurobranchaea*, the most distantly related species investigated here.

Identifying C2 in Melibe leonina

Like *Tritonia*, *Melibe leonina* is in the paraphyletic subclade Dendronotida within the Nudibranchia clade and Cladobranchia subclade of Nudipleura, making *Melibe* the species that is mostly closely related to *Tritonia* in this study (Figure 2.1) (Bouchet et al., 2005). *Melibe* can perform a swim consisting of rhythmic, left-right whole- body flexions (Lawrence and WH, 2002), but not a dorsal-ventral swim like that of *Tritonia* or *Pleurobranchaea*.

Biocytin backfills of PP2 in conjunction with FMRFamide immunohistochemistry uniquely identified a neuron in *Melibe* with the same characteristics as $C2_{Tri}$, which we have named $C2_{Mel}$ (Figure 2.4A). As in *Tritonia* and *Pleurobranchaea*, $C2_{Mel}$ was the only neuron on the dorsal surface of the cerebral-pleural ganglion with a contralateral axon projection into PP2 that was also FMRFamide immunoreactive (n=5). The soma of $C2_{Mel}$ was also white, but relatively more posterior-medial than the position of the C2 soma in *Tritonia* and *Pleurobranchaea* (Figure 2.4B).

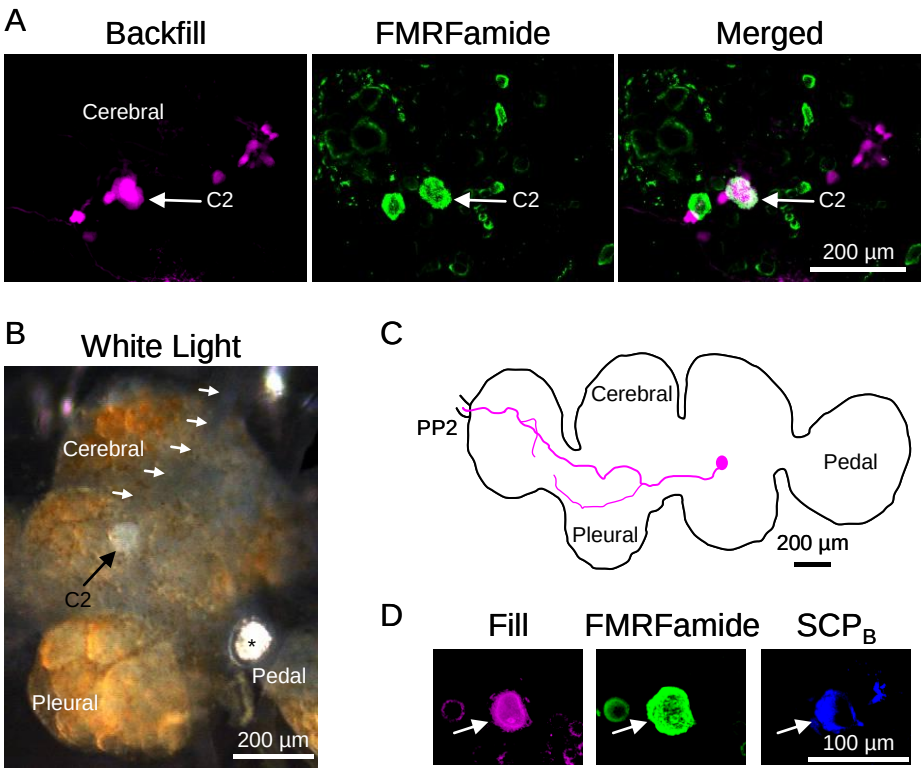

Figure 2.4: C2 characteristics in *Melibe leonina*. A) Backfilling the pedal commissure with biocytin labeled 3-4 neurons near $C2_{Mel}$ (left). Only the cerebral-pleural ganglion contralateral to the backfilled nerve is shown. FMRFamide-like immunohistochemistry labeled $C2_{Mel}$ (middle). Combining FMRFamide-like immunoreactivity with the backfill revealed just one neuron, $C2_{Mel}$ (right). B) $C2_{Mel}$ could be identified visually due to its characteristic white soma near the origin of cerebral nerve 1 (CeN1, white arrows). An asterisk (*) labels the statocyst. Only one cerebral-pleural ganglion is shown. C) Filling the $C2_{Mel}$ soma with Neurobiotin revealed a branching contralateral axon projection through the anterior cerebral-pedal commissure (not shown) and into the pedal commissure (PP2). The example is a representative image in which the outline of the brain and the axon projection were traced for ease of viewing. D) $C2_{Mel}$ (arrow) was filled with biocytin (left). It was immunoreactive for both FMRFamide (middle) and SCP_B (right) as shown.

However, the origin of CeN1 is more posterior-medial in *Melibe* as well. Thus, the $C2_{Mel}$ soma position in relation to the origin of CeN1 was similar to that of the other species tested.

Injecting a biotinylated tracer into the soma of $C2_{Mel}$ combined with FMRFamide and SCP_B immunohistochemistry confirmed that the neuron was FMRFamide (n=7) and SCP_B (n=3) immunoreactive (Figure 2.4D). Tracer injection also revealed a more detailed axon projection. While the axon projection was similar to C2 in the other species tested, in that it has a contralateral projection through the anterior cerebral-pedal commissure and then into PP2, there were additional branches not observed in the other species (n=4) (Figure 2.4C). Still, the characteristics of neurochemical staining and axon projection that identified C2 in the other species also uniquely identified $C2_{Mel}$; no other neuron exhibited these features. We therefore conclude that $C2_{Mel}$ is homologous to $C2_{Tri.}$

Identifying the C2 homologue in Hermissenda crassicornis

Hermissenda is in the monophyletic subclade Aeolidida within the Nudibranchia clade and Cladobranchia subclade (Figure 2.1) (Bouchet et al., 2005). As such, it is more closely related to *Tritonia* and *Melibe* than it is to *Pleurobranchaea*. We observed that *Hermissenda* was able to produce rhythmic, left-right whole-body flexions, however the behavior was not as robust as the swim observed in *Melibe* in that it did not cause the animal to stay suspended in the water. The response was not observed in all of the individual *Hermissenda* tested; in one trial, 6 of 11 animals produced these body flexions in response to a 100 µl puff of 5M NaCl.

As in the other species investigated, biocytin backfills of PP2 in conjunction with FMRFamide immunohistochemistry revealed that only one neuron on the dorsal surface of the *Hermissenda* cerebral-pleural ganglion had a contralateral axon projection into PP2 and displayed FMRFamide immunoreactivity (n=3) (Figure 2.5A). Filling the white soma of this neuron (Figure 2.5B), which we will now call $C2_{Herm}$, showed that it displayed dual FMRFamide (n=11) and SCP_B immunoreactivity (n=7 somata in 5 preparations) (Figure 2.5D), and a contralateral

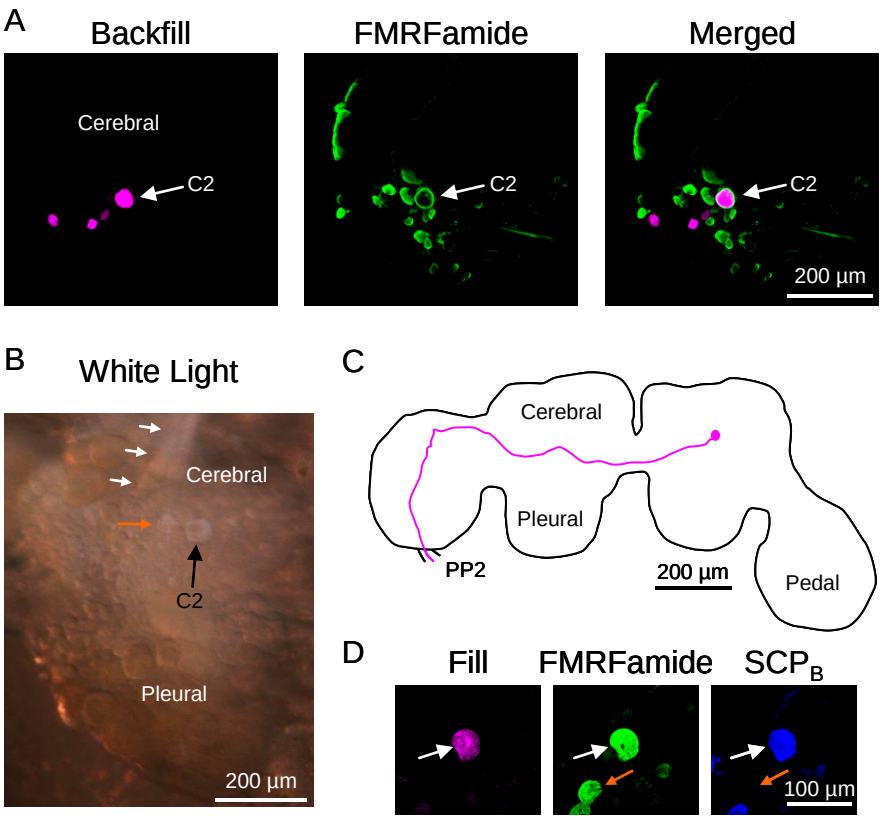

Figure 2.5: C2 characteristics in *Hermissenda crassicornis*. A) Backfilling the pedal commissure with biocytin labeled 3-4 neurons near $C2_{Herm}$ (left). Only the cerebral-pleural ganglion contralateral to the backfilled nerve is shown. FMRFamide-like immunohistochemistry labeled $C2_{Herm}$ (middle). Combining FMRFamide-like immunoreactivity with the backfill revealed just one neuron, $C2_{Herm}$ (right). B) $C2_{Herm}$ could be identified visually due to its characteristic white soma (black arrow) near the origin of cerebral nerve 1 (CeN1, white arrows). Note the white cell just medial to $C2_{Herm}$ (orange arrow). An asterisk (*) labels the statocyst. Only one cerebral-pleural ganglion is shown. C) Filling the $C2_{Herm}$ soma with biocytin revealed a contralateral axon projection through the anterior cerebral-pedal commissure (not shown) and into the pedal commissure (PP2). The example is a representative image in which the outline of the brain and the axon projection were traced for ease of viewing. D) $C2_{Herm}$ (arrow) was filled with biocytin (left). It was immunoreactive for both FMRFamide (middle) and SCP_B (right) as shown. The white cell just medial to $C2_{Herm}$ is immunoreactive for FMRFamide, but not SCP_B (orange arrow).

axon projection through the anterior cerebral-pedal commissure and into PP2 (Figure 2.5C). We conclude from this evidence that $C2_{Herm}$ is homologous to $C2_{Tri}$. It should be noted, that there was another neuron with a white soma near the origin of CeN1 that was FMRFamide immunoreactive (Figure 2.5D) and had a contralateral axon projection to the pedal ganglion. The axon did not project into PP2 and the neuron was not SCP_B immunoreactive, however (Figure 2.5D). An additional characteristic to help identify $C2_{Herm}$ from the nearby white soma in the living preparation is that $C2_{Herm}$ is the more anterior-lateral of the two white cells near CeN1 (Figure 2.5B, D).

Identifying C2 in Flabellina iodinea

Flabellina is in the Aeolidida subclade like *Hermissenda*, and thus shares the same phylogenetic relationship to *Tritonia* (Figure 2.1) (Bouchet et al., 2005). *Flabellina* demonstrates a much more robust left-right, rhythmic swimming behavior than *Hermissenda*, however (Farmer, 1970).

As in *Hermissenda*, there were two white neurons near the origin of CeN1 that displayed FMRFamide immunoreactivity (Figure 2.6A). Biotinylated tracer fills of both of these white cells revealed that only one, which we now call $C2_{Flab}$, demonstrated dual immunoreactivity for FMRFamide and SCP_B and also had a contralateral axon projection through the anterior cerebral-pedal commissure and into PP2 (n=4) (Figure 2.6B, C). As in *Hermissenda*, this was the more anterior-lateral of the two white cells (Figure 2.6A, C). Also like *Hermissenda*, the more medial white cell had a contralateral axon projection to the pedal ganglion and was FMRFamide immunoreactive but did not display SCP_B immunoreactivity (Figure 2.6C) or an axon projection into PP2. Unfortunately, PP2 in *Flabellina* was too short to effectively backfill with biocytin. However, the suite of characteristics that uniquely identify C2 in *Tritonia*, *Pleurobranchaea*, *Melibe*, and *Hermissenda* also identified $C2_{Flab}$. Therefore, we conclude that $C2_{Flab}$ is homologous to $C2_{Tri}$.

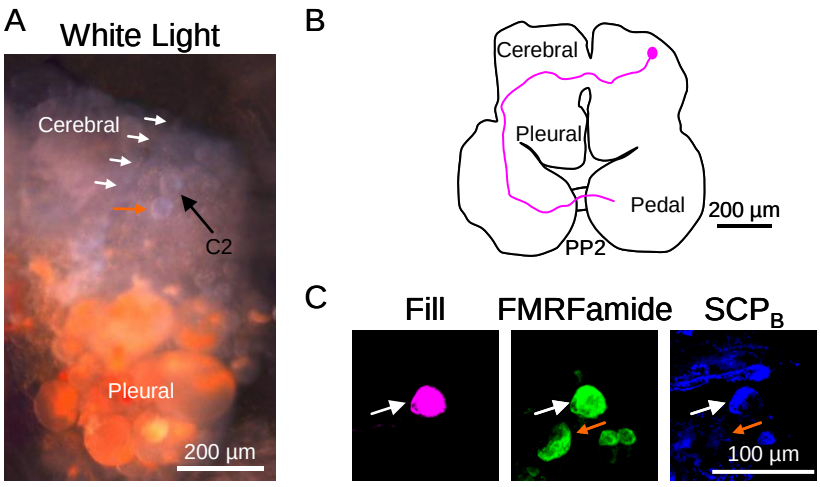

Figure 2.6: C2 characteristics in *Flabellina iodinea*. A) $C2_{Flab}$ could be identified visually due to its characteristic white soma (black arrow) near the origin of cerebral nerve 1 (CeN1, white arrows). Note the white cell just posterior-medial to $C2_{Flab}$ (orange arrow). Only one cerebral-pleural ganglion is shown. B) Filling the $C2_{Flab}$ soma with biocytin revealed a contralateral axon projection through the anterior cerebral-pedal commissure (not shown) and into the pedal commissure (PP2). The example is a representative image in which the outline of the brain and the axon projection were traced for ease of viewing. C) $C2_{Flab}$ (arrow) was filled with biocytin (left). It was immunoreactive for both FMRFamide (middle) and SCP_B (right) as shown. The white cell just medial to $C2_{Herm}$ is immunoreactive for FMRFamide, but not SCP_B (orange arrow).

Discussion

Neurochemical and neuroanatomical characteristics were found to uniquely identify C2 in *Tritonia* and homologues in four other Nudipleura molluscs independent of electrophysiological activity. $C2_{Tri}$ can be uniquely identified as a neuron having the following characteristics: 1) a white cell body near the origin of CeN1 on the dorsal surface of the cerebral ganglion, 2) FMRFamide and SCP_B immunoreactivity, and 3) an axon that projects contralaterally through the anterior cerebral-pedal commissure to the pedal ganglion and then through PP2. We showed that these same three characteristics can be used to identify the previously described C2 homologue in *Pleurobranchaea californica* (Jing and Gillette, 1995). Homology is further supported by the fact that $C2_{Tri}$ and $C2_{Pleur}$ share electrophysiological characteristics, such as activity during a swim motor pattern and synaptic connectivity to DSI homologues (Jing and Gillette, 1995, 1999). Moreover, we found that the same three anatomical and neurochemical characteristics were able to uniquely distinguish a single bilaterally represented neuron in three species that do not swim with dorsal-ventral body flexions: *Melibe leonina*, *Hermissenda crassicornis*, and *Flabellina iodinea*. Because only one neuron in all of the species investigated displayed a contralateral projection into PP2 and FMRFamide and SCP_B immunoreactivity, we conclude that these neurons are homologous. This is the most parsimonious explanation for the same set of three characteristics identifying these neurons.

FMRFamide and SCP_B

Whereas we found that C2 is immunoreactive for both FMRFamide and SCP_B, it is not clear whether C2 actually uses either peptide as a neurotransmitter. The commercially available FMRFamide antibodies are thought to stain generally for RFamides (Arg-Phe-NH_2), but not necessarily FMRFamide specifically. The SCP_B monoclonal antibody is specific for antigenic sequence (Masinovsky et al., 1988), but there is no additional molecular evidence to support the presence of native SCP_B. Regardless of whether C2 actually uses either peptide as a neuro

transmitter, the FMRFamide antisera and SCP_B monoclonal antibodies exhibited reliable staining patterns within species. The detection of C2 and its putative homologues via dual FMRFamide-like and SCP_B-like immunoreactivity in conjunction with other anatomical characters helps to uniquely identify the neuron. While it remains of interest to know which peptide(s) C2 uses as a neurotransmitter, it is beyond the scope of this study.

Previous reports have documented FMRFamide and SCP_B immunoreactive neurons in the cerebral ganglia of opisthobranchs not discussed here. Notably, FMRFamide immunolabeling has been reported in the cerebral ganglion of the nudibranch *Phestilla sibogae* (Croll et al., 2001), and in the non-Nudipleura opisthobranchs *Bulla gouldiana* (Roberts et al., 1988) and *Aplysia californica* (Soinila and Mpitsos, 1991). FMRFamide gene expression in the cerebral ganglion of *Aplysia californica* has also been demonstrated via *in situ* hybridization (Jezzini et al., 2005). SCP_B immunolabeling has been shown previously in the cerebral ganglion of *Tritonia diomedea* and *Hermissenda crassicornis* (Masinovsky et al., 1988). The staining patterns are similar to what we have seen in our experiments. That study also investigated two additional nudibranchs not discussed here: *Tritonia festiva* and *Dendronotus dalli* (Masinovsky et al., 1988). These studies reveal possible C2 homologues, but without additional information it is difficult to speculate any further on cell identity.

The $C2_{Mel}$ axon projection

$C2_{Mel}$ exhibits all of the characteristics that uniquely identify C2 in the other species tested. However, the axon projection of $C2_{Mel}$ showed additional branching that was not observed in the other species. It is possible that there was branching in the other species that was not captured with the tracer injections or that *Melibe* exhibits more axonal branching than the other species investigated here. Differences in axon projection patterns have been reported between other homologous gastropod neurons. One prominent example is the variability in axon morphology of the serotonergic cerebral cell, variously named the giant serotonin neuron, the meta-

cerebral giant cell, and the giant cerebral neuron in different species; clear homologues of these neurons have been identified and axon projections have been reported in at least 12 different gastropod species (Granzow and Fraser Rowell, 1981; Pentreath et al., 1982; Croll, 1987b). Despite the similarities that allow these neurons to be identified as homologues, the neurons exhibit major differences in axon projections including whether the axon projects bilaterally or unilaterally. Thus, the species-differences seen in this study are not unprecedented.

The C2 homologue in Hermissenda may be the previously identified I_b interneuron

Previous work in *Hermissenda* identified a bilaterally represented neuron in the anterior-lateral region of the cerebral ganglion that plays a role in ciliary crawling and foot contraction (Crow and Tian, 2004, 2009). This neuron, named the I_b interneuron (http://neuronbank.org/Her0002676) has a contralateral axon projection that closely resembles the projection pattern that we observed for $C2_{Herm}$ (Crow and Tian, 2004). Moreover, the contralateral I_b interneurons are electrically coupled (Crow and Tian, 2004) and inhibit the serotonergic CPT neurons (http://neuronbank.org/Her0002693) (Tian et al., 2006) (homologues of the *Tritonia* DSI neurons); two characteristics that are also true of $C2_{Tri}$ (Getting et al., 1980b; Getting, 1981). Despite the anatomical and physiological similarities between the I_b interneuron and $C2_{Herm}$, we were unable to rule out the possibility that they are different neurons because we do not know if I_b projects through the pedal commissure or whether it is immunoreactive for FMR-Famide and SCP_B. Based on what we know, it is equally possible that the I_b interneuron is the white neuron just medial to $C2_{Herm}$. Further experiments will be needed to determine whether the I_b interneuron is in fact $C2_{Herm}$.

Neuronal multifunctionality and central pattern generator evolution

While *Melibe*, *Hermissenda*, and *Flabellina* cannot perform a dorsal-ventral swim, they all possess C2. This indicates that C2 was present in a common ancestor to all Nudipleura molluscs, although we have not yet identified a C2 homologue in a species outside of this clade. It

is likely that dorsal-ventral swimming arose independently several times within the Nudipleura clade because of the location on the phylogenetic tree of lineages that display this behavior (Figure 1.1). Therefore, the dorsal-ventral swim CPGs must have arisen from neurons (including C2) that provided another function in the ancestral brain. This function may be conserved across Nudipleura species regardless of whether they exhibit dorsal-ventral swimming.

For example, in addition to its role in the dorsal-ventral swim CPG, the C2 homologue in *Pleurobranchaea* is also involved in the suppression of feeding behaviors (Jing and Gillette, 1995, 2000). While untested, the role of C2 in feeding behavior may be similar in other species.

A more thoroughly studied example is the role of the *Tritonia* DSIs across not just Nudipleura, but indeed more disparate opisthobranch species. These neurons are involved in feeding and locomotor or foot contraction behaviors in each of the species tested (Panchin et al., 1995; Satterlie and Norekian, 1995; Jing and Gillette, 2000; Popescu and Frost, 2002; Jing and Gillette, 2003; Tian et al., 2006; Jing et al., 2008). In *Tritonia* and *Pleurobranchaea*, the neurons are also part of the dorsal-ventral swim CPG. A separate role in crawling persists in both species, however. These examples suggest that neurons with roles in conserved behaviors can become incorporated into new circuits without losing their original function.

Now that the C2 homologues have been identified in species that cannot perform the dorsal-ventral swimming behavior, it is of interest to compare the properties of C2 and the network properties between C2 and DSI across the dorsal-ventral swimming and non-dorsal-ventral swimming species. Based on modeling and experimental studies, we know that certain network properties between C2 and DSI are crucial for producing the motor program of the dorsal-ventral swim in *Tritonia* (Calin-Jageman et al., 2007; Katz, 2009). Experiments investigating those properties will be the key to understanding what differences allow these conserved neurons to reorganize and produce novel behaviors.

Acknowledgments

The authors would like to thank Stephen Kempf and Dennis Willows for providing us with the SCP_B antibody, Birgit Neuhaus for assistance with brain imaging, and Akira Sakurai for helpful comments on this manuscript.

CHAPTER 3: COMPARATIVE ANALYSIS OF ELECTROPHYSIOLOGICAL PROPERTIES AND SYNAPTIC CONNECTIONS OF HOMOLOGOUS NEURONS RELATED TO BEHAVIOR IN NUDIPLEURA MOLLUSCS

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In preparation for publication

Abstract

Despite having brains comprised largely of homologous neural structures, related species can exhibit different behaviors. Differences in the properties of the homologous structures could underlie behavioral differences observed. To investigate such differences at the cellular level, we tested the electrophysiological properties and synaptic connections of homologous identified neurons in three Nudipleura (Mollusca, Opisthobranchia, Gastropoda) species. *Tritonia diomedea* and *Pleurobranchaea californica* express similar swimming behaviors that evolved independently. The central pattern generators (CPG) underlying swimming in both species contain homologous identified neurons. The neuronal homologues are also present in *Hermissenda crassicornis*, a species that cannot swim like *Tritonia* or *Pleurobranchaea*. We tested the electrophysiological properties, electrical coupling relationships, and synaptic connections of the homologues in each species. A number of the properties measured were similar in all three species. We also found differences in the properties, electrical coupling relationships, and functional synaptic connections of the homologues that correlated with phylogeny. This suggests that certain homologue properties can differ in *Tritonia* and *Pleurobranchaea* without detriment to the swim behavior. Additionally, we found functional synaptic differences that correlated with behavior. These differences may play a role in the ability of *Tritonia* and *Pleurobranchaea* to produce the swim behavior that *Hermissenda* does not express. Therefore, differ-

ences in some properties, electrical coupling relationships, and synaptic connections of homologous neurons may play a lesser role than other synaptic connections in species differences in behavior. Additionally, the results demonstrated that the synaptic connections of multifunctional homologous neurons differ substantially across Nudipleura species.

Introduction

Related species can express different behaviors despite having brains composed largely of homologous components. In a number of invertebrate species, individual homologous neurons can be identified across species with different behaviors. Differences in the properties and synaptic connections of the neuronal homologues could underlie the species differences in behavior. Such differences in sensory afferents and motor efferents have been reported to play a role in species differences in behavior of nematodes (Chiang et al., 2006; McGrath et al., 2011), leech (Baltzley et al., 2010), and insects (Arbas, 1983a, b; Shaw and Meinertzhagen, 1986; Shaw and Moore, 1989; Martin et al., 2011). However, electrophysiological properties and synaptic differences that play a role in species differences in behavior have not been described among identified homologous interneurons that generate motor behavior.

In fact, studies in the stomatogastric ganglion of crabs have shown just the opposite, namely that neuronal and synaptic properties can vary widely without affecting the functional output of the circuit (Prinz et al., 2004; Marder and Bucher, 2007; Marder, 2011). Similar reports have emerged from the analysis of the neurons and synapses underlying heartbeat in leech (Calabrese et al., 2011a; Roffman et al., 2012). Moreover, whereas the crustacean and leech studies described variability in neuron properties and the strength of synaptic connections within species, a synapse critical to proper swim behavior in one nudibranch mollusc species was completely absent in a related nudibranch species that exhibits a similar swim behavior (Sakurai et al., 2011). These results suggest three possibilities: individual neuron and synaptic properties do not strongly influence circuit output, redundancy within neural circuits enables differences in

neuron and synaptic properties without detriment to output, or compensatory mechanisms allow the differences without detriment to the functional output of the circuits. To further investigate a role for neuron and synapse differences in species differences in behavior, we tested the electrophysiological properties and synaptic connections of identified homologous interneurons underlying a swim behavior in two Nudipleura (Mollusca, Gastropoda, Opisthobranchia) species that can produce the behavior and one species that cannot.

Tritonia diomedea expresses a rhythmic escape swim behavior consisting of alternating dorsal and ventral whole body flexions that can be elicited by a touch from a predatory sea star (Willows, 1967; Hume et al., 1982). The neural circuit underlying this swim behavior has been well-characterized and includes a central pattern generator (CPG) consisting of just three neuron types named C2, DSI_{A-C}, and $VSI_{A,B}$ (Katz, 2009). *Pleurobranchaea californica* expresses a similar dorsal-ventral swim (Gillette et al., 1991) that appears to have evolved independently (Newcomb et al., 2012). The CPG circuit underlying swimming in *Pleurobranchaea* includes homologues of the *Tritonia* CPG neurons C2 and DSI (named A1 and As_{1-3}, respectively) (Jing and Gillette, 1995, 1999). *Hermissenda crassicornis*, like most members of Nudipleura, cannot swim in the same manner as *Tritonia* or *Pleurobranchaea* (Newcomb et al., 2012). However, a number of Nudipleura species, including *Hermissenda*, possess C2 and DSI homologues (DSI was named CPT in *Hermissenda*) (Tian et al., 2006; Newcomb and Katz, 2007; Lillvis et al., 2012). *Hermissenda* and *Tritonia* belong to the monophyletic subclade, Cladobranchia, and are more closely related to each other than to *Pleurobranchaea*. This provides an opportunity to test whether the neuronal properties and synaptic connections of the homologous CPG neurons in the three species correlate with behavior or the phylogenetic relationships of the species.

Previous reports have shown electrophysiological properties of the DSI homologues that are similar regardless of phylogeny and swim behavior, as well as electrical coupling relationships that correlate with swim behavior expressed in *Tritonia*, *Pleurobranchaea*, and *Hermissenda*. Electrophysiological properties, electrical coupling relationships, and synaptic connec-

tions of C2 have also been examined in *Tritonia* and *Pleurobranchaea*. However, no properties or synaptic connections of C2 have been investigated in *Hermissenda*.

Here we also show electrophysiological properties of the DSI homologues that are similar in all three species. Electrophysiological properties of the C2 homologues showed some correlations with the phylogeny but did not correlate with the type of swimming behavior expressed. Differences in the electrical coupling relationships and synaptic connections of the C2 homologues also showed some correlations with phylogeny. However, synaptic connections between C2 and DSI demonstrated correlations with swim behavior. The results indicate that synaptic connections and electrical coupling properties of the CPG homologues can differ across species. Moreover, some of these differences may influence species differences in behavior in the Nudipleura.

Methods

Animal collection, maintenance, and dissection

Tritonia diomedea individuals were obtained from Living Elements Ltd. (Delta, BC, Canada). *Pleurobranchaea californica* and *Hermissenda crassicornis* individuals were obtained from Monterey Abalone Company (Monterey Bay, CA, USA). Animals were maintained in artificial seawater (Instant Ocean) tanks at a fixed 12:12 light/dark cycle. *Tritonia* individuals were maintained at 11 ± 1° C. *Pleurobranchaea* and *Hermissenda* individuals were maintained at 13 ± 1° C.

Tritonia individuals were anesthetized by cooling. *Pleurobranchaea* and *Hermissenda* individuals were anesthetized by injection of 0.33 M $MgCl_2$ into the body cavity. A cut was made on the dorsal surface of the body wall near the buccal mass. The brain, consisting of the cerebral, pleural, and pedal ganglia, was removed by cutting all nerve roots. The brain was transferred to a Sylgard-lined dish where it was superfused, at a rate of ~0.5 ml/min, with artificial saline (in mM): 420 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 11 D-glucose, and 10 HEPES, pH 7.5.

Connective tissue surrounding the brain was manually removed with forceps and fine scissors while maintaining a temperature of ~4 °C to reduce neuronal activity. The temperature was raised to 10-11° C for *Tritonia* and 13-14° C for *Pleurobranchaea* and *Hermissenda* electrophysiological experiments.

Electrophysiology

Intracellular recordings were obtained using 10-80 MΩ glass microelectrodes filled with 3 M KCl connected to Axoclamp 2B amplifiers (Molecular Devices). Extracellular suction electrode recordings were obtained by drawing individual nerves into polyethylene tubing filled with artificial saline connected to an A-M Systems Differential AC Amplifier (model 1700, A-M Systems, Inc.). Both intra- and extracellular recordings were digitized (>1 kHz) with a 1401 Plus A/D converter from Cambridge Electronic Design.

In the isolated brain, body wall nerve stimulations were used to mimic swim inducing stimuli in the whole animal. The stimuli were varied in an effort to thoroughly test whether the DSI and C2 homologues were capable of producing the bursting activity that underlies dorsal-ventral swimming (1-5 s, 5-20 ms pulses, 5-20 Hz). Nerve stimuli were applied to the body wall nerve (BWN) in *Pleurobranchaea*, which was previously shown to elicit swimming in isolated brain preparations (Jing and Gillette, 1999). Stimuli were applied to Pedal Nerve 2 or 3 (PdN2/3) (Newcomb et al., 2006) in *Tritonia* and *Hermissenda*, which project to the dorsal body wall and reliably elicit swimming in *Tritonia* (Frost and Katz, 1996). We generically refer to these nerves as body wall nerves.

Input resistance tests were conducted in artificial saline. The neuron was monitored using a discontinuous current clamp (DCC) to ensure accurate membrane potential measurements without using two electrodes in the soma. Current steps (3 s, 1 nA to -5 nA) were applied and the change in membrane potential was measured. The current injected was plotted against

the corresponding change in membrane potential. Input resistance was calculated by measuring the slope of the linear regression line fit to the plotted data.

To test electrical coupling and chemical synapses, the bathing medium was switched to high divalent cation (HiDi) saline, which raises the threshold for spiking and reduces spontaneous neural firing. The composition of the HiDi saline was (in mM): 285 NaCl, 10 KCl, 25 $CaCl_2$, 125 $MgCl_2$, 11 D-glucose, and 10 HEPES (pH 7.5). To test electrical coupling, the presynaptic neuron was monitored using DCC to ensure accurate membrane potential measurements without using two electrodes in the soma. Current steps (5 s, 1 nA to -7 nA) were applied to the presynaptic neuron while the membrane potential was monitored in both pre- and postsynaptic neurons. The changes in pre- and corresponding postsynaptic membrane potential were plotted against each other. Coupling coefficients were calculated as the slope of the linear regression line fit to the plotted data. To test synaptic connections, depolarizing current pulses (10 to 20 ms pulses for 0.5 to 3 s at 10 to 20 Hz) were injected into the presynaptic neuron and the voltage response was measured in the postsynaptic neuron. The membrane potential of the postsynaptic neuron was hyperpolarized and depolarized to test for synaptic components that may be hidden at the resting membrane potential.

Neuron Identification

C2 neurons were identified preliminarily using soma position, size, and pigmentation (white). C2 is conspicuous and relatively easy to identify visually across species (Lillvis et al., 2012). Upon impaling C2 with a microelectrode, its characteristic resting activity, synaptic input, and response to a nerve stimulus further aided identification. In *Pleurobranchaea*, the $C2_{Pleur}$ axon projection was confirmed by monitoring the contralateral anterior cerebral-pedal connective and the larger of the pedal-pedal connectives (PP2) (Newcomb et al., 2006), which in this case is the anterior pedal-pedal connective, via extracellular recordings. In *Tritonia* and *Pleurobranchaea*, C2 identity could be confirmed by its involvement in the fictive dorsal-ventral swim

motor pattern. Finally, in experiments where C2 identity was not certain, the soma was filled with the biotinylated tracer biocytin (2.5% dissolved in 0.75 M KCl) (Invitrogen) or Neurobiotin (2-4% dissolved in 0.75 M KCl) (Vector Laboratories) and processed for FMRFamide and/or Small Cardioactive Peptide B (SCP_B) immunoreactivity (see below) because the axon projection in conjunction with FMRFamide and SCP_B immunoreactivity uniquely identifies C2 across species (Lillvis et al., 2012).

DSIs were identified preliminarily by soma position, size, and pigmentation (generally unpigmented, but some mottled dark pigmentation is present). There are three DSIs found in a cluster in a characteristic location in each species (Jing and Gillette, 1999; Tian et al., 2006; Newcomb and Katz, 2007). Upon impaling DSI with a microelectrode, it shows characteristic spiking activity, action potential shape, and synaptic input that allowed the neurons to be distinguished from neighboring cells. In *Pleurobranchaea*, the axon projection of DSI could be confirmed by monitoring the contralateral anterior cerebral-pedal connective. In *Tritonia* and *Pleurobranchaea*, DSI could be confirmed by its involvement in the fictive dorsal-ventral swim motor pattern. Finally, the DSI soma was filled with the biotinylated tracer biocytin or Neurobiotin after the experiment and processed for 5-HT immunohistochemistry (see below). Soma position and axon projection in conjunction with 5-HT immunoreactivity uniquely identify DSI across species (Newcomb and Katz, 2007). 5-HT immunoreactivity was used to confirm DSI identity in all *Hermissenda* preparations, and in all *Tritonia* and *Pleurobranchaea* preparations in which it was not confirmed by involvement in the fictive swim motor pattern. Hereafter we will refer to all *Tritonia* CPG homologues by the *Tritonia* name and will distinguish species using the subscripts of $_{Tri}$, $_{Pleur}$, and $_{Herm}$.

Biocytin or Neurobiotin processing and immunohistochemistry procedures were identical to those reported in Lillvis et al., 2012. Briefly, after fixation, brains were washed and incubated in one or more of the following primary antiserum: rabbit anti-FMRFamide (Immunostar) or anti-serotonin (Invitrogen) antiserum diluted 1:1000 and/or mouse monoclonal anti-SCP_B (courtesy

of Stephen Kempf) diluted in 1:20 antiserum diluent (ASD). Streptavidin-Alexa Fluor 594 conjugate (1:50-1:200, Invitrogen) was also added to visualize the biotinylated tracer. Brains were then washed and incubated in goat anti-rabbit and/or goat anti-mouse antiserum conjugated to Alexa Fluor 488, Alexa Fluor 594 (Invitrogen), or DyLight 405 (Jackson ImmunoResearch) diluted 1:100 in ASD. Brains were then washed, dehydrated, and mounted on a slide to visualize soma immunohistochemistry and axon projection.

Data acquisition, analysis, and statistics

Data acquisition and analysis were performed with Spike2 software (Cambridge Electronic Design) and SigmaPlot (Jandel Scientific). Statistical comparisons were made using a one-way analysis of variance (ANOVA) with post-hoc pair wise multiple comparisons using the Holm-Sidak method in all cases aside from tests of input resistance. The input resistance data failed the Shapiro-Wilk test of normality. Therefore, the median values were compared and the non-parametric Dunn's post-hoc pairwise multiple comparison test was used. In all cases, $p<0.05$ was considered significant. Results are expressed as the mean ± standard error of the mean (SEM) unless stated otherwise.

Results

DSI and C2 responses to fictive swim stimuli in the isolated brain

Tritonia and *Pleurobranchaea* express similar rhythmic dorsal-ventral swim behaviors. Moreover, the isolated brains of both species are capable of producing the rhythmic patterns of neural activity that underlie the swims (Dorsett et al., 1973; Jing and Gillette, 1999). The CPG neurons DSI and C2 can be monitored electrophysiologically, and produce the rhythmic bursts of neural activity that underlie the dorsal flexion portion of the dorsal-ventral swim motor pattern (Getting et al., 1980b; Jing and Gillette, 1995, 1999). We were able to replicate these results

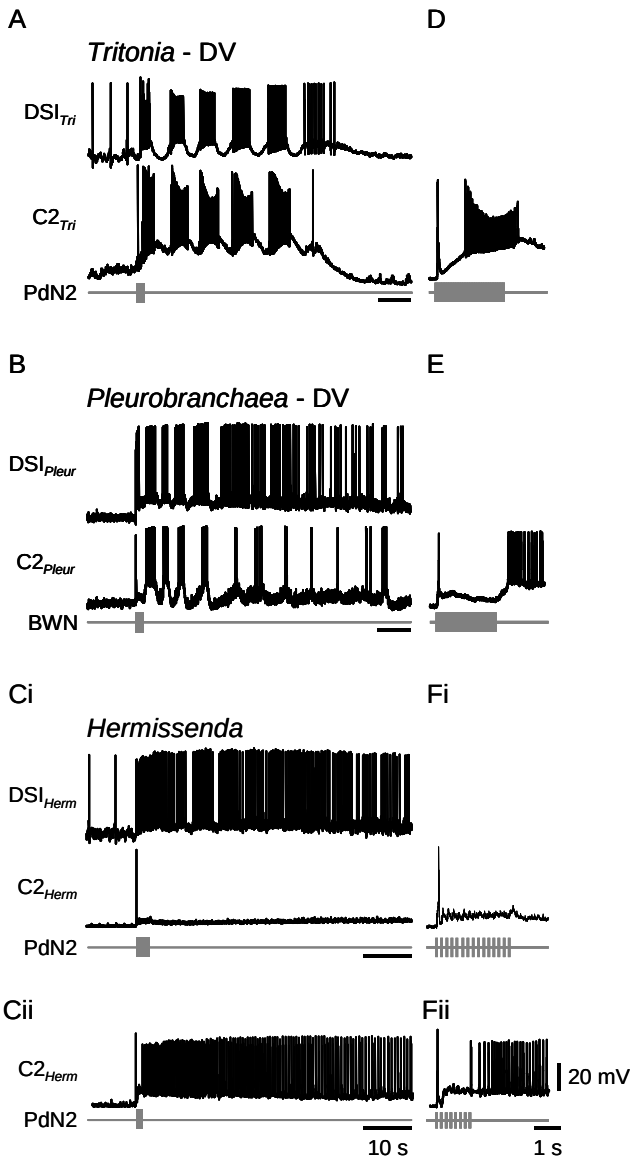

Figure 3.1: Species differences in the response to body wall nerve stimulation. *Tritonia* and *Pleurobranchaea* exhibit dorsal-ventral swimming *in vivo*, but *Hermissenda* does not. A) In the isolated brain, stimulating pedal nerve 2 (PdN2; grey bar) in *Tritonia* and B) the body wall nerve (BWN) in *Pleurobranchaea* induced rhythmic bursting responsible for the dorsal aspect of the dorsal-ventral swim in DSI and C2. Ci) In *Hermissenda*, PdN2 stimuli elicited high frequency spiking in DSI, but no rhythmic bursting. PdN2 stimuli could elicit Ci) little activity or Cii) high frequency firing in C2. D-F) The initial nerve stimulus elicited a similar response of 1-2 action potentials followed by a period of quiescence. This was true whether or not the species swam, and whether or not C2 showed high frequency firing in *Hermissenda*. Representative examples of the population are shown.

and initiate rhythmic bursting in DSI and C2 in both species by stimulating body wall nerves (Figure 3.1A, B).

Hermissenda does not express a dorsal-ventral swim behavior (Lillvis et al., 2012). Moreover, stimulating a body wall nerve in *Hermissenda* did not induce rhythmic bursting in DSI_{Herm} and $C2_{Herm}$ (Figure 3.1C). Instead, a nerve stimulus induced depolarized membrane potentials and/or increased action potential firing frequencies in both neurons. In *Hermissenda*, DSI_{Herm} always exhibited high frequency action potential firing (>2 Hz) from a body wall stimulus (n=5) (Figure 3.1Ci). $C2_{Herm}$ exhibited depolarized membrane potentials but little or no action potential firing in 14 of 28 preparations (Figure 3.1Ci). In the other 14 preparations, $C2_{Herm}$ exhibited 1 Hz or greater firing from a body wall nerve stimulus (Figure 3.1Cii). In 11 of the 14 preparations in which $C2_{Herm}$ fired action potentials at a higher frequency, a single burst of action potentials was evoked from a body wall nerve stimulus. However, repeated bursts of action potentials were not observed. The reason for the variable $C2_{Herm}$ responses to nerve stimuli is unclear. Ranges of nerve stimulation parameters were used (see Methods), but there were no correlations with the C2 response. The absence of rhythmic firing of DSI_{Herm} and $C2_{Herm}$ is consistent with the inability to produce a dorsal-ventral swim *in vivo*.

Despite the differences in response to nerve stimuli, there were some similarities. When nerve stimuli are sub-threshold to elicit rhythmic bursting in *Tritonia* and *Pleurobranchaea*, DSI and C2 exhibit elevated membrane potentials and firing frequencies (Jing and Gillette, 1995, 1999; Newcomb and Katz, 2007) that are not unlike those that we observed in *Hermissenda*. Additionally, the initial C2 response to a nerve stimulus was similar in all three species. As previously reported in *Tritonia* (Frost and Katz, 1996), a body wall nerve stimulation caused C2 to produce one or two distinct excitatory post-synaptic potentials (EPSPs) or action potentials followed by a brief period of quiescence before bursting begins (Figure 3.1D). Similar C2 responses were elicited from nerve stimuli in *Pleurobranchaea* (Figure 3.1E). In *Hermissenda*, this immediate response was observed both in preparations in which C2 did not fire subsequently

(Figure 3.1Fi) and in preparations where C2 exhibited prolonged spiking (Figure 3.1Fii). This suggests that the sensory input to C2 may be similar in all three species.

DSI electrophysiological properties

Properties of the DSI homologues were tested to determine whether they showed a relationship with swim behavior or phylogeny. The average DSI resting membrane potentials were similar in all three species at -46.2 ± 2.8 mV in *Tritonia* (n=5), -42.5 ± 1.1 mV in *Pleurobranchaea* (n=17), and -41.6 ± 3.4 mV in *Hermissenda* (n=5) ($p>0.05$). Our measurements were similar to the previously published values of -47.5 ± 0.39 in *Tritonia* (Calin-Jageman et al., 2007) and -49.7 ± 4.3 in *Pleurobranchaea* (Jing and Gillette, 1999). The spontaneous activity of the DSIs was also similar across species. The DSIs fired spontaneous action potentials in *Tritonia* (n=12), *Pleurobranchaea* (n=9), and *Hermissenda* (n=5) (Figure 3.2A-C). The spontaneous activity observed is similar to what was previously reported in all three species (Jing and Gillette, 1999; Tian et al., 2006; Newcomb and Katz, 2007).

The results demonstrate that the measured DSI electrophysiological properties were similar in all three species. Therefore, the electrophysiological properties that were measured do not correlate exclusively with behavior or phylogeny.

C2 electrophysiological properties

C2 homologues were also tested to determine whether any properties correlated with behavior or phylogeny across species. The average resting membrane potentials of C2 were similar in each species at -45 ± 2.4 mV in *Tritonia* (n=9), -45.2 ± 1.1 mV in *Pleurobranchaea* (n=23), and -43.1 ± 1.2 mV in *Hermissenda* (n=23) ($p>0.05$). The membrane potential values measured were similar to previous reports of -48.0 ± 0.46 in *Tritonia* (Calin-Jageman et al., 2007) and -48.6 ± 4.3 in *Pleurobranchaea* (Jing and Gillette, 1999).

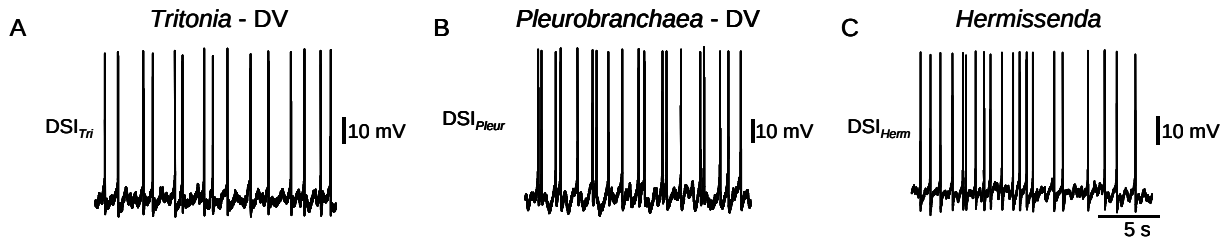

Figure 3.2: Spontaneous DSI activity across species. DSI fired spontaneous action potentials in A) *Tritonia*, B) *Pleurobranchaea*, and C) *Hermissenda*. Examples shown are representative of the population.

C2 was either quiescent or fired action potentials at <1 Hz in *Tritonia* (n=40), *Pleurobranchaea* (n=88 of 96 preparations), and *Hermissenda* (n=68 of 69 preparations) (Figure 3.3A-C). This was similar to previously published reports in *Tritonia* (Getting et al., 1980b) and *Pleurobranchaea* (Jing and Gillette, 1999). Additionally, the left and right C2 received common synaptic input in *Tritonia* (n=19), *Pleurobranchaea* (n=49), and *Hermissenda* (n=29) (Figure 3.3A-C).

It has been established that the C2 homologues in *Tritonia* (Getting, 1977) and *Pleurobranchaea* (Jing and Gillette, 1999) differ in the synaptic input received at rest. As in previous studies, *Tritonia* received excitatory input (n=40) (Figure 3.3A), while *Pleurobranchaea* primarily received inhibitory input at rest (n=78 of 96 preparations) (Figure 3.3B). *Hermissenda* was similar to *Tritonia* and received excitatory input at rest (n=63 of 69 preparations) (Figure 3.3C). Additionally, $C2_{Pleur}$ input resistance differed in *Pleurobranchaea* compared to the other species. The median input resistance of C2 was 30.58 MΩ in *Tritonia* (n=10), 59.53 MΩ in *Hermissenda* (n=11), and 15.16 MΩ in *Pleurobranchaea* (n=16). The *Pleurobranchaea* value was significantly different from both *Tritonia* ($p<0.05$) and *Hermissenda* ($p<0.05$), which were statistically similar to each other ($p>0.05$). Our input resistance measurement in *Tritonia* was somewhat different than the previously published figure of 23.3 ± 3.49 MΩ (the mean *Tritonia* input resistance here was 32.3 ± 2.38 MΩ) (Calin-Jageman et al., 2007). The lower value in the published report is likely due to the use of two microelectrodes in the soma compared to one being used in here (see Methods), as values were closer to the previously published report when two electrodes were used (data not used in these analyses).

Thus, $C2_{Herm}$ was more similar to $C2_{Tri}$ than to $C2_{Pleur}$ in the electrophysiological properties that we measured. Therefore, C2 synaptic input and input resistance correlated with phylogeny rather than behavior.

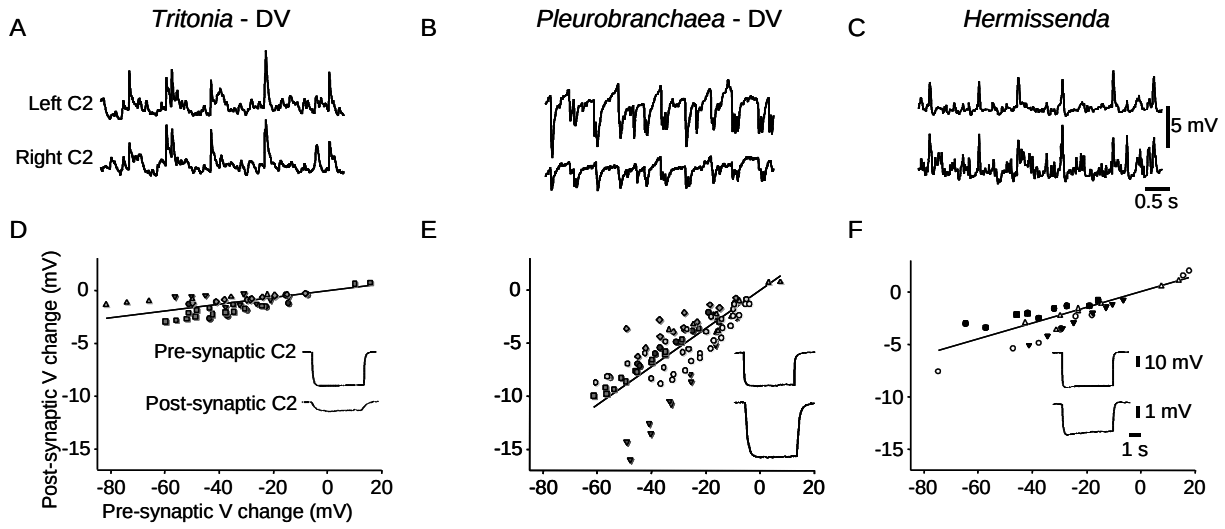

Figure 3.3: Electrophysiological properties and electrical coupling relationships of C2 across species. C2 was quiescent at rest in A) *Tritonia*, B) *Pleurobranchaea*, and C) *Hermissenda*. The left and right C2 in A) *Tritonia* and C) *Hermissenda* receive common excitatory synaptic input at rest. The left and right C2 in B) *Pleurobranchaea* receive common inhibitory synaptic input at rest. Examples in A-C are representative of the population. D-F) Electrical coupling relationships of the left and right C2 in each species. Each plot shows the relationship between the membrane potential change of the presynaptic C2 and the corresponding change in the postsynaptic C2. Symbols represent different experiments, and the lines represent the linear regression from all experiments. Insets show single examples of electrical coupling with similar presynaptic membrane potential changes on the top and the corresponding postsynaptic changes on the bottom. E) *Pleurobranchaea* shows C2-to-C2 electrical coupling that is significantly stronger than D) *Tritonia* and F) *Hermissenda*.

Electrical coupling of the contralateral C2s

It has been established that the left and right C2 are electrically coupled in *Tritonia* and *Pleurobranchaea*. In *Tritonia*, the coupling coefficient was 0.03 ± 0.006 (n=5) (Figure 3.3D), which is similar to the previously published value of 0.02 (Getting et al., 1980b). In *Pleurobranchaea*, the coupling coefficient was more than five times greater than in *Tritonia*; we measured a coupling coefficient of 0.19 ± 0.03 (n=7) (Figure 3.3E), which is similar to the previously reported value of 0.16 ± 0.014 (Jing and Gillette, 1999). We found that the C2 coupling coefficient in *Hermissenda* was intermediate between *Tritonia* and *Pleurobranchaea*: 0.09 ± 0.01 in *Hermissenda* (n=5) (Figure 3.3F) Although the left and right C2 are electrically coupled in all three species, the coupling coefficient of the C2s in *Pleurobranchaea* was significantly stronger than that of either *Tritonia* ($p<0.05$) or *Hermissenda* ($p<0.05$).Therefore, the left and right C2 coupling coefficient correlated more with phylogeny than behavior.

C2-to-C2 and C2-to-DSI synaptic connections

We observed postsynaptic responses typical of a chemical synapse in $C2_{Tri}$ when the contralateral $C2_{Tri}$ was made to fire action potentials (n=8) (Figure 3.4A). In *Pleurobranchaea*, spikes in one $C2_{Pleur}$ caused a weak excitation followed by a recruited inhibition in the contralateral $C2_{Pleur}$ (n=16) (Figure 3.4B). Such recruited inhibition can be observed in the presynaptic neuron that was stimulated as well (Jing and Gillette, 1995, 1999). In *Hermissenda*, spikes in one C2 evoked a purely depolarizing potential in the contralateral C2 as in *Tritonia*. However, the amplitude was much smaller than that in *Tritonia* (n=10) (Figure 3.4C). Recruited inhibitory feedback was never present in *Tritonia* or *Hermissenda*.

The synaptic connections between C2 and DSI were also tested in each species. In *Tritonia*, $C2_{Tri}$ had a multicomponent synapse with DSI_{Tri} consisting of a fast excitation followed by a slow inhibitory component (n=6 of 9 preparations) (Figure 3.4D). This functional synaptic connection was similar to previous reports (Getting, 1981) although we were unable to elicit any

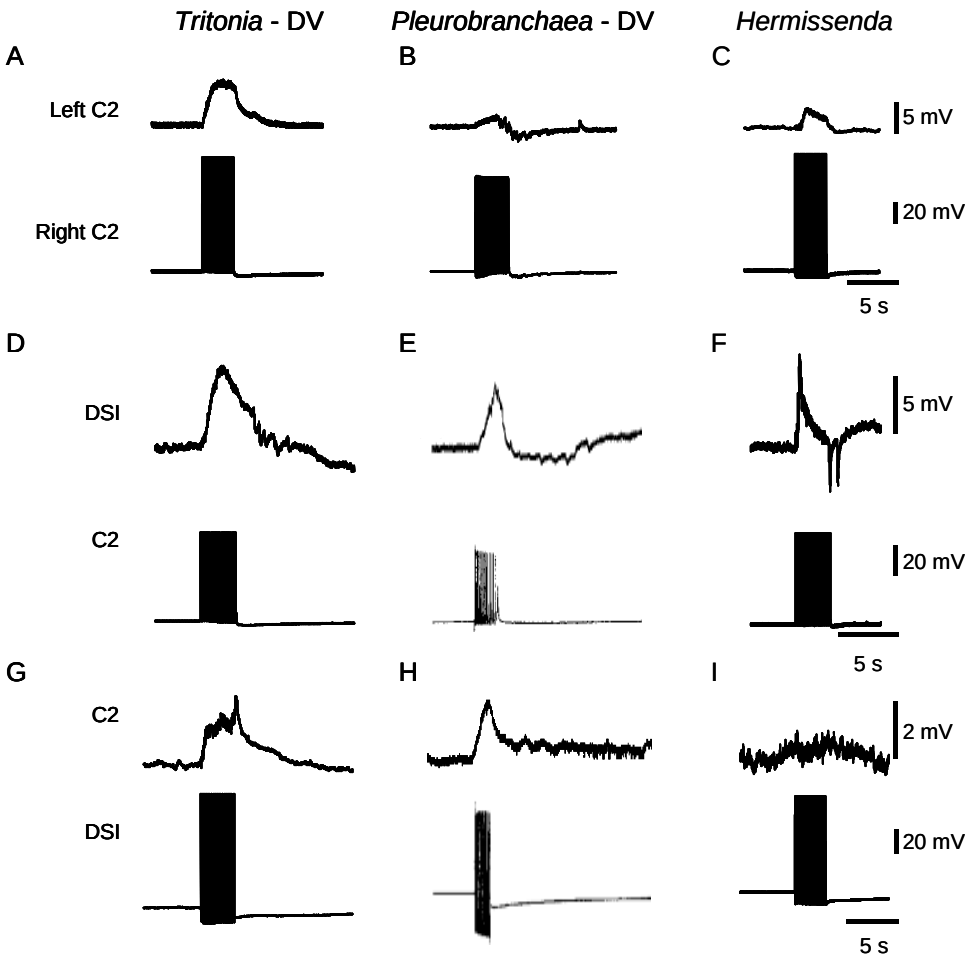

Figure 3.4: Synaptic connections of the CPG homologues across species. Stimulating the right C2 excited the left C2 in all three species, but the response was attenuated in B) *Pleurobranchaea* and C) *Hermissenda* compared to A) *Tritonia*. B) Inhibition was recruited in *Pleurobranchaea*. D-F) Stimulating C2 could excite and inhibit the ipsilateral DSI in all three species (D-F). However, the response in *Hermissenda* was not consistent. Stimulating DSI excited C2 in G) *Tritonia* and H) *Pleurobranchaea*. I) DSI did not excite C2 in the majority of *Hermissenda* preparations. *Tritonia* and *Hermissenda* examples are representative of the population. E, H) Adapted from (Jing and Gillette, 1999).

synaptic response in 3 of the 9 preparations. In *Pleurobranchaea*, it was previously reported that $C2_{Pleur}$ also forms a multicomponent synapse with DSI_{Pleur} consisting of a fast excitation followed by a slow inhibitory component (Jing and Gillette, 1999) (Figure 3.4E). However, we were unable to record synaptic connections between $C2_{Pleur}$ and DSI_{Pleur} in *Pleurobranchaea* (n=36). In *Hermissenda*, we recorded a similar $C2_{Herm}$-to-DSI_{Herm} synapse consisting of a fast excitatory component followed by a slower inhibitory component (n=2 of 5 preparations) (Figure 3.4F). However, this connection was not consistent across preparations. In 2 of 5 preparations, we recorded a $C2_{Herm}$-to-DSI_{Herm} synapse that was purely excitatory with no apparent inhibitory component. In 1 of 5 preparations, we recorded a $C2_{Herm}$-to-DSI_{Herm} synapse that was purely inhibitory with no apparent excitatory component.

Thus, the C2-to-C2 synaptic connection correlated with phylogeny. The C2-to-DSI synapse correlated with behavior to a certain extent, depending on the preparation.

DSI-to-C2 synaptic connections

In *Tritonia*, DSI_{Tri} reliably excited $C2_{Tri}$ (n=9) (Figure 3.4G). This was similar to the previously reported synapse from DSI_{Tri}-to-$C2_{Tri}$ (Getting et al., 1980b; Getting, 1981). As with the $C2_{Pleur}$-to-DSI_{Pleur} connection, we were unable to record DSI_{Pleur}-to-$C2_{Pleur}$ synapses in *Pleurobranchaea*. However, the published functional connection from DSI_{Pleur}-to-$C2_{Pleur}$ is similar to that of *Tritonia* (Jing and Gillette, 1999) (Figure 3.4H). In *Hermissenda*, an excitatory synapse from DSI_{Herm}-to-$C2_{Herm}$ was also recorded, but this was a rare example (n=1 of 5 preparations). In 4 of 5 preparations, there was no discernible synaptic connection from DSI_{Herm}-to-$C2_{Herm}$ (Figure 3.4I). Therefore, the DSI-to-C2 synaptic connection appeared to correlate with behavior, but not phylogeny.

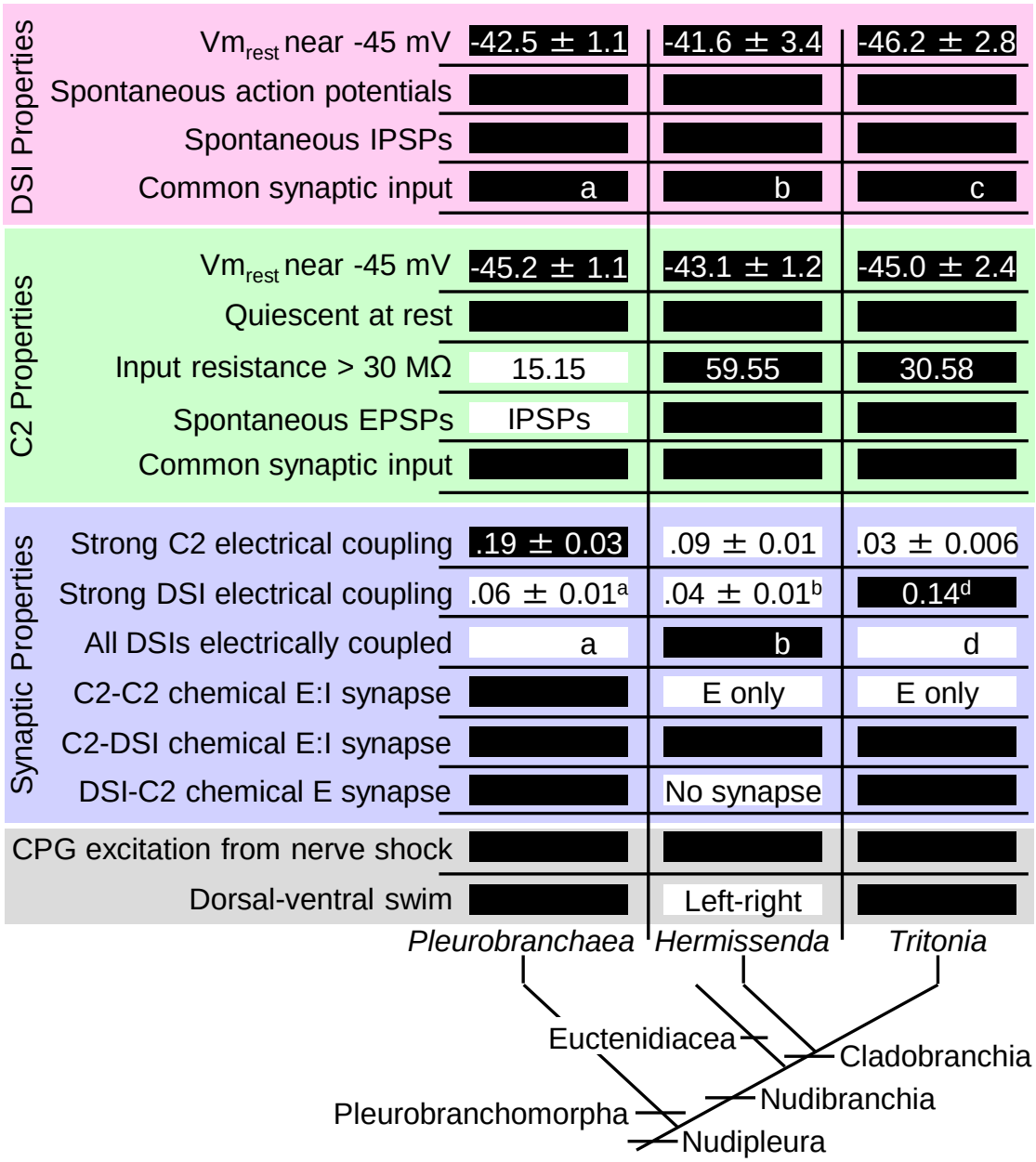

Figure 3.5: Summary of results in relation to phylogeny. Black boxes indicate the presence of the characteristic to the left. White boxes indicate absence of the characteristic. Where values were measured, they are indicated in the boxes. The results show C2 electrophysiological properties and synaptic connections that correlated with phylogeny and synaptic connections that correlated with behavior. *Tritonia* and *Hermissenda* are members of the monophyletic clade Cladobranchia. *Pleurobranchaea* is not, but is a member of the monophyletic Nudipleura clade with *Tritonia* and *Hermissenda*. References: a, (Jing and Gillette, 1999); b, (Tian et al., 2006); c, (Newcomb and Katz, 2007); d, (Getting, 1981).

Discussion

The results from previous reports and the new results reported here are summarized in relation to phylogeny in Figure 3.5. Four neuron or synaptic differences correlated with phylogeny: C2 synaptic input, C2 input resistance, the strength of left and right C2 electrical coupling, and the functional synaptic connection between the left and right C2. In all of these cases, *Tritonia* and *Hermissenda* were similar to each other but both were different from *Pleurobranchaea*. The results also show three synaptic differences that correlated with behavior: the electrical coupling relationships among the DSIs and the C2-to-DSI synaptic connection and the DSI-to-C2 synaptic connection. In each of these cases, *Tritonia* and *Pleurobranchaea* are similar to each other but both were different from *Hermissenda*. The results identify potential synaptic connection differences that may account, in part, for species differences in dorsal-ventral swim behavior.

Sensory input to the CPG homologues

Nerve stimuli elicited a similar excitatory response followed by a period of quiescence in C2 in all three species. This suggests that the initial synaptic drive from a nerve stimulus was similar regardless of swim behavior expressed.

However, after the quiescent period the C2 response differed somewhat in *Hermissenda* compared to *Tritonia* and *Pleurobranchaea*. In 50% of the preparations, $C2_{Herm}$ exhibited high frequency action potential firing, which was similar to that observed in *Tritonia* and *Pleurobranchaea* aside from the lack of rhythmic bursting. In the other 50%, $C2_{Herm}$ did not fire action potentials after the initial response. In *Tritonia* and *Pleurobranchaea*, the post-quiescent excitatory drive to C2 is provided by DSI (Frost and Katz, 1996; Jing and Gillette, 1999). This suggests that the variability in the C2 response to nerve stimuli in *Hermissenda* may be due to variability in the DSI to C2 synaptic connection. However, there was no correlation between the DSI_{Herm}-to-$C2_{Herm}$ synaptic connection and the $C2_{Herm}$ response from a nerve stimulus in the ex-

periments conducted here. It is also possible that the individual differences in $C2_{Herm}$ nerve response are due to the effectiveness of the nerve stimulus. However, DSI_{Herm} showed similar excitation from nerves stimuli regardless of how $C2_{Herm}$ responded. Therefore, the source for the variability in the nerve response is unclear. However, it does appear that the body wall nerve stimulus can effectively excite DSI_{Herm} and $C2_{Herm}$, and that neural differences aside from the sensory input to the CPG circuit are responsible for the species differences in behavior.

Inability to record DSI_{Pleur} and $C2_{Pleur}$ synapses in Pleurobranchaea

We were unable to record synaptic connections between DSI_{Pleur} and $C2_{Pleur}$ in *Pleurobranchaea*. Previous reports demonstrated that synaptic connections between the neurons are present (Jing and Gillette, 1999). We assume that the connections were present in our experiments but unrecordable in the soma, given that a lack of synapses should result in an inability to swim. The reason for our inability to record synaptic connections between DSI and C2 are unclear. One possibility is that there were differences in the populations of animals used compared to previous reports. However, this seems unlikely given our experiments were conducted over a three year period, thus supplying us with animals from multiple breeding cycles.

Another possibility is that the sites of the synaptic connections were too distant to record in the soma. The cerebral and pedal ganglia are connected by long commissures in *Pleurobranchaea* whereas the ganglia are more compact in *Tritonia* and *Hermissenda* where we had no issue recording DSI and C2 synaptic connections (see Lillvis et al., 2012 for illustrations of the brain in each species). However, Jing and Gillette would have dealt with the same issues in their experiments. Additionally, we attempted to increase action potential conductance by using saline solutions with higher calcium concentrations, but we were still unable to record synaptic connections.

Additionally, control measurements indicated that the lack of synaptic connections observed was not due to experimental error. The action potentials elicited from $C2_{Pleur}$ and DSI_{Pleur}

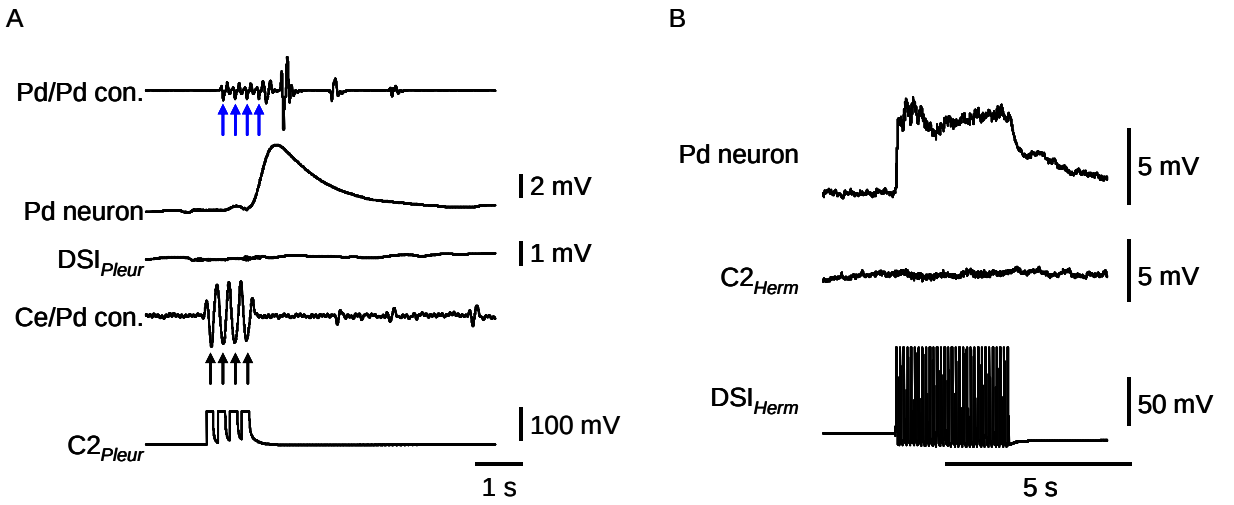

Figure 3.6: Control measurements in *Pleurobranchaea* and *Hermissenda*. A) We did not observe synaptic connections between $C2_{Pleur}$ and DSI_{Pleur} in *Pleurobranchaea*. Here, $C2_{Pleur}$ was stimulated to fire 4 action potentials. The action potential impulse was monitored on the cerebral-pedal connective (Ce/Pd con.; black arrows), no PSP was observed in DSI_{Pleur}, an EPSP was recorded with a pedal ganglion neuron (Pd neuron), and the action potential impulses were recorded on the pedal-pedal connective (Pd/Pd con.; blue arrows. B) We did not reliably observe synaptic connections between DSI_{Herm} and $C2_{Herm}$ in *Hermissenda*. Here, DSI_{Herm} was stimulated to fire a train of action potentials. There was no synapse with $C2_{Herm}$, but DSI_{Herm} did elicit an EPSPs in a pedal ganglion neuron.

stimulation in *Pleurobranchaea* could be recorded on a nerve (PP2) that is beyond the presumptive site of the $C2_{Pleur}$ and DSI_{Pleur} synaptic connection in the contralateral pedal ganglion (Figure 3.6A). Therefore, we could verify that the presynaptic neuron fired action potentials and that the action potentials propagated beyond the presumptive site of the synapses. Thus, we cannot explain the differences found here compared to the previous report.

Variability in CPG homologue synapses in Hermissenda

We report a fair amount of animal-to-animal variability in the DSI_{Herm}-to-$C2_{Herm}$ and $C2_{Herm}$-to-DSI_{Herm} synaptic connections in *Hermissenda*. This variability could be explained by two possibilities. First, the variability could be due to differences in the properties of the classes of DSI_{Herm} neurons. In *Tritonia*, $C2_{Tri}$ exhibits the fast excitatory and slow inhibitory components of its synapse with $DSI_{B/C}$, but only the inhibitory component with DSI_{A} (Katz and Frost, 1995a). In *Pleurobranchaea*, there are also different classes of DSI_{Pleur} neurons, but the synaptic connections between DSI_{Pleur} and $C2_{Pleur}$ appear to be similar for the three putative DSI_{Pleur} homologues (As_{1-3}) (Jing and Gillette, 1999). There are no published differences among the DSI_{Herm} population in *Hermissenda* (Tian et al., 2006). Still, the variability in the $C2_{Herm}$-to-DSI_{Herm} connection in *Hermissenda* could be due to recording from different classes of DSI_{Herm} neuron that have yet to be distinguished. It is also possible that different DSI_{Herm} classes have different effects on $C2_{Herm}$ in *Hermissenda* even though this has not been observed in *Tritonia* or *Pleurobranchaea*. The second possible reason for the synaptic variability observed is simply that it represents true variability across individuals. We also found variability in the $C2_{Tri}$-to-DSI_{Tri} connection in *Tritonia*, and it has been noted that the synaptic connectivity of identified neurons can vary substantially across individuals in other species (Calabrese et al., 2011b; Marder, 2011).

Furthermore, control measurements indicate that the lack of DSI_{Herm}-to-$C2_{Herm}$ synapses found in 4 of 5 experiments was not due to experimental error. In 3 of the 4 experiments where no DSI_{Herm}-to-$C2_{Herm}$ synaptic connection was observed, we were able to record DSI_{Herm} synap-

tic connections with follower neurons in the region where DSI_{Herm} and $C2_{Herm}$ presumably synapse with each other (Figure 3.6B). Additionally, $C2_{Herm}$ also had synaptic connections with these postsynaptic neurons. This indicated that DSI_{Herm} was firing action potentials and that the action potentials were conducting to the area of expected synaptic connections. Therefore, the variability observed is likely due to the possibilities posed above, not experimental error.

Similarities across species

Some properties and synaptic connections measured here and elsewhere were similar in all three species. These include the resting membrane potentials of the DSIs and C2, the spontaneous activity of the DSIs and C2, the synaptic input of the DSIs (Jing and Gillette, 1999; Tian et al., 2006; Newcomb and Katz, 2007), and in some cases the C2-to-DSI synapse. These similarities may be important to the ability to produce a dorsal-ventral swim in *Tritonia* and *Pleurobranchaea*. Alternatively, they may be important for some other unknown behavior that is present in all three species.

Differences that correlate with phylogeny

Differences in the measurements of C2 synaptic input, C2 input resistance, the strength of left and right C2 electrical coupling, and the functional chemical synapse between the left and right C2, correlated with the phylogenetic relationships of the species tested. In all of these cases, *Tritonia* and *Hermissenda* were similar to each other but both were different from *Pleurobranchaea*. There are also previously published differences between the DSI electrical coupling relationships in *Tritonia* and *Pleurobranchaea* that did not correlate with phylogeny, but are worth discussing here. The coupling coefficient of the ipsilateral $DSI_{B/C}$ neurons is 0.19 in *Tritonia* (Getting, 1981) and 0.09 ± 0005 in *Pleurobranchaea* (Jing and Gillette, 1999). The ipsilateral DSI coupling coefficient is 0.05 ± 0.008 in *Hermissenda* (Tian et al., 2006). The coupling coefficient of the contralateral $DSI_{B/C}$ neurons is 0.14 *Tritonia* (Getting, 1981) and 0.06 ± 0.007 in

Pleurobranchaea (Jing and Gillette, 1999). The contralateral DSI coupling coefficient is 0.04 ± 0.008 in *Hermissenda* (Tian et al., 2006).

The differences between *Tritonia* and *Pleurobranchaea* suggest that these properties may play a lesser role in the ability to produce a functional dorsal-ventral swim, or that other circuit mechanisms compensate for whatever negative effects such differences have on the dorsal-ventral swim in each species. Reports have shown that animal-to-animal differences in intrinsic neuron properties and synaptic connections can be present without losing the functional neural circuit output (Calabrese et al., 2011a; Marder, 2011; Roffman et al., 2012). However, such neuron property and synaptic differences are not irrelevant and can affect the circuit output (Goaillard et al., 2009; Wright and Calabrese, 2011). Additionally, manipulating the electrical coupling relationships of neurons in leech (Wright and Calabrese, 2011) and *Xenopus* (Zhang et al., 2009) can alter the phasing of rhythmic outputs. The dorsal-ventral swims of *Tritonia* and *Pleurobranchaea* are not identical. *Tritonia* tends to produce more dorsal-ventral cycles, and the *Tritonia* cycle periods are more consistent than *Pleurobranchaea* (Jing and Gillette, 1999). Therefore, the electrophysiological and electrical coupling differences reported here could play a role in the *Tritonia* and *Pleurobranchaea* dorsal-ventral swim differences observed.

Differences that correlate with behavior

Three synaptic differences found correlate with behavior: the previously reported electrical coupling relationships among the DSIs, the C2-to-DSI synaptic connection, and the DSI-to-C2 synaptic connection. That is, in each of these measurements, *Tritonia* and *Pleurobranchaea* were similar to each other but different from *Hermissenda*.

We did not test DSI electrical coupling properties here, but they have been investigated in each species. There are two classes of DSI in *Tritonia*. $DSI_{B/C}$ are indistinguishable from each other and are electrically coupled ipsi- and contralaterally, while DSI_A is coupled to the contralateral DSI_A but not to $DSI_{B/C}$ (Getting, 1981). A similar relationship is present in *Pleurobranchaea*

(between As_1 and $As_{2/3}$) (Jing and Gillette, 1999). In *Hermissenda*, a distinction between different classes of the DSIs was not made; all of the DSIs are electrically coupled to each other ipsi- and contralaterally (Tian et al., 2006). It is possible that this distinction is not present in *Hermissenda*, as there are also no apparent differences in electrical coupling among the DSI population in *Melibe leonina*, a related Nudipleura species that does not express a dorsal-ventral swim (Newcomb and Katz, 2007). The apparent lack of distinction between DSI classes in *Hermissenda* correlates with behavior, as the distinction is present in the two more distantly related dorsal-ventral swimming species. Reports in rodents (Blenkinsop and Lang, 2006; Placantonakis et al., 2006) have demonstrated that altering electrical coupling relationships of neurons can affect the ability to generate rhythmic activity. This suggests that the difference in the way that the DSI classes are coupled to each other in the dorsal-ventral swimmers compared to the apparent lack of DSI_{Herm} classes in *Hermissenda* may play a role in the ability of *Tritonia* and *Pleurobranchaea* to produce a dorsal-ventral swim that *Hermissenda* cannot produce.

The results also suggest that the difference in the C2-to-DSI and the DSI-to-C2 synaptic connections may play a role in the ability of *Tritonia* and *Pleurobranchaea* to produce the dorsal-ventral swim that *Hermissenda* does not express. Modeling studies of the *Tritonia* CPG network may be informative of the impact of the synaptic differences found in *Hermissenda* (Calin-Jageman et al., 2007). The studies indicate that the fast excitatory component of the $C2_{Tri}$-to-DSI_{Tri} synapse is important to swim generation but that the slow inhibitory component of the synapse does not influence the ability to swim. This suggests that the variability in the $C2_{Herm}$-to-DSI_{Herm} synapse in *Hermissenda* may not affect the ability of *Hermissenda* to produce a dorsal-ventral swim, as the synapse showed the excitatory component in 4 of 5 experiments.

Conversely, the modeling results suggest that the lack of an excitatory DSI_{Tri}-to-$C2_{Tri}$ synaptic connection would result in the inability to produce a dorsal-ventral swim. However, it should be noted that *Hermissenda* did not produce a dorsal-ventral swim when the DSI_{Herm}-to-

$C2_{Herm}$ synaptic connection was similar to those recorded in *Tritonia* and *Pleurobranchaea*. Therefore, while the synaptic differences may play a role in the species differences in behavior, it is likely that additional neural mechanisms play a role in the behavioral differences as well. Future experiments using dynamic clamp techniques to insert artificial or remove actual synaptic connections could be conducted to determine the importance of these synaptic connections to producing a dorsal-ventral swim motor pattern.

One possible mechanism is species differences in neuromodulation within the CPG circuit. DSI_{Tri} uses serotonin as a neuromodulator in addition to its use as a neurotransmitter (Katz and Frost, 1995b). This neuromodulation increases the excitability of $C2_{Tri}$ and the amplitude of $C2_{Tri}$-evoked synaptic potentials in *Tritonia* (Katz et al., 1994b; Katz and Frost, 1997). Evidence indicates that this modulation is necessary for swimming in *Tritonia* (McClellan et al., 1994; Katz and Frost, 1995b; Calin-Jageman et al., 2007), but it is not known whether it is present in *Pleurobranchaea* and *Hermissenda*.

Multifunctional neurons and the evolution of behavior

The CPG homologues, like many neurons, are multifunctional. In addition to being part of the dorsal-ventral swim CPG, DSI also excites motor neurons that initiate crawling in *Tritonia* (Popescu and Willows, 1999; Popescu and Frost, 2002), *Pleurobranchaea* (Jing and Gillette, 2000), and *Aplysia californica*, a distantly related species (Jing et al., 2008). DSI also facilitates avoidance turns in *Pleurobranchaea* (Jing and Gillette, 2003) and causes foot contraction in *Hermissenda* (Tian et al., 2006). Additionally DSI facilitates feeding in *Pleurobranchaea* and *Aplysia* (Jing and Gillette, 2000; Jing et al., 2008). Finally, DSI can modulate the left-right swim of *Melibe* (Newcomb and Katz, 2009) and the wing-like flapping swim of the distantly related *Clione limacina* (Panchin et al., 1995; Satterlie and Norekian, 1995). C2 suppresses feeding behavior in *Pleurobranchaea* (Jing and Gillette, 1995) and can display complex multicomponent

postsynaptic potentials (PSPs) with motor neurons that initiate crawling in *Tritonia* suggesting a role in that behavior as well (Snow, 1982a).

Because these neurons are multifunctional, it would suggest that changes to the properties of the neurons or their synapses might have effects on multiple behaviors. For this reason, it is often assumed that changes to the properties of neurons and their synapses may be constrained and would be less likely to demonstrate evolutionary change. Our results suggest that the electrophysiological properties of the neurons are generally conserved, though differences that correlate with phylogenetic relationships of the three species tested are present.

Conversely, it appears as though the electrical coupling relationships and synaptic connections of these neurons are quite variable across species. Every electrical and chemical synaptic connection examined differed in at least one species compared to the others. This suggests that the electrical coupling properties between C2s and between DSIs, the synaptic properties between C2s and between the DSIs, and the synaptic properties between C2 and DSI are not important for other behaviors or have changed in accordance with changes in behavior that we are not considering here. Alternatively, there could be redundant processes or compensatory mechanisms that account for any changes that could affect conserved behaviors. Studies in other species have shown that similar behavioral output can occur even with differences in the synaptic connections, which suggests that such redundancy and/or compensation is present in those systems (Calabrese et al., 2011a; Marder, 2011; Sakurai et al., 2011).

Thus, despite neuron and network multifunctionality, our results suggest that electrical coupling relationships and synaptic connections of homologous neurons can differ across related species. Moreover, some differences in synaptic connections found here correlate with differences in behavior. This indicates that such connections may not be as evolutionarily constrained as generally perceived, and that species differences in synaptic connections of homologous neurons could play a more prominent role in species differences in behavior than previous evidence suggests.

Acknowledgments

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CHAPTER 4: SEROTONGERGIC NEUROMODULATION INTRINSIC TO CENTRAL PATTERN GENERATORS UNDERLYING ANALOGOUS INDEPENDENTLY-EVOLVED MOTOR PATTERNS

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In preparation for publication

Abstract

Neuromodulation can dynamically alter the properties of neural circuits within an individual animal and can be necessary for circuits to produce functional behavioral output. Here we report that neuromodulation between homologous neurons is necessary to produce a functional rhythmic motor output across species. Two species of Nudipleura (Mollusca, Gastropoda, Opisthobranchia), *Tritonia diomedea* and *Pleurobranchaea californica*, produce similar swim behaviors that evolved independently. The central pattern generator (CPG) circuits underlying these behaviors contain homologous identified neurons. These neuronal homologues can also be found in *Hermissenda crassicornis*, a species that does not exhibit the swimming behavior. We show that in *Tritonia* and *Pleurobranchaea*, but not *Hermissenda*, a homologue of a serotonergic CPG neuron modulated the strength of synapses made by another CPG homologue. Furthermore, the serotonin receptor antagonist, methysergide, blocked this neuromodulation and the ability to swim. Additionally, in *Pleurobranchaea*, the robustness of swimming correlated with the extent of this synaptic modulation. Finally, injection of serotonin induced the swim behavior in *Tritonia* and *Pleurobranchaea*. These results indicate that the ability to swim like *Tritonia* and *Pleurobranchaea* is dependent upon the extent of serotonergic neuromodulation between homologous CPG neurons that are present across Nudipleura. This suggests that neuromodulation may affect behavioral evolvability by providing a mechanism that enables related

species to independently acquire analogous behaviors using homologous neural circuit components.

Introduction

The repeated evolution of similar behaviors (Foster et al., 1996; McGhee, 2011) begs the question of whether there are neural mechanisms that affect the evolvability of behavior (Kirschner and Gerhart, 1998). Behavior is produced by neural circuits whose output is dependent upon the neuronal and synaptic properties of their components. Neuromodulation can alter those properties and can be necessary for neural circuits within a species to produce functional behavioral outputs (Harris-Warrick, 2011). This suggests that differences in the way that homologous neurons and synapses are modulated may allow different functional circuit outputs, and thus behaviors in related species. Such a mechanism could be responsible for the repeated independent evolution of analogous behaviors if modulating homologous circuit components in a similar manner results in similar circuit outputs.

The Nudipleura (Mollusca, Gastropoda, Opisthobranchia) species *Tritonia diomedea* and *Pleurobranchaea californica* express similar rhythmic dorsal-ventral swim behaviors (Figure 4.1A, B) that appear to have evolved independently in each species (Newcomb et al., 2012). The central pattern generator (CPG) underlying the swim in each species contains homologous neurons named DSI_{A-C} and C2 in *Tritonia* and As_{1-3} and A1 in *Pleurobranchaea* (Getting et al., 1980b; Jing and Gillette, 1999). Homologues of these neurons have also been identified throughout the Nudipleura, including in *Hermissenda crassicornis* (Tian et al., 2006; Newcomb and Katz, 2007; Lillvis et al., 2012). *Hermissenda*, like the majority of the Nudipleura, does not exhibit a dorsal-ventral swim behavior (Figure 4.1C) despite possessing homologues for DSI (named CPT) and C2 (Newcomb et al., 2012). This and other evidence suggests that the dorsal-ventral swim CPG of *Tritonia* and *Pleurobranchaea* evolved from non-rhythmic neural circuitry present throughout the Nudipleura (Katz et al., 2001; Newcomb et al., 2012). However, the

neural mechanisms that allow the rhythmic circuit output and behavior are unknown (Katz et al., 2001).

In *Tritonia*, the serotonergic DSI modulates the strength of C2-evoked synapses (Katz et al., 1994b; Katz and Frost, 1995b). Modeling studies suggest that this neuromodulation is necessary for the *Tritonia* CPG to produce the rhythmic dorsal-ventral swim output (Calin-Jageman et al., 2007). Moreover, blocking G-protein coupled signaling in C2, which appears to mediate the DSI modulation of C2 synaptic strength, inhibits swimming (Clemens and Katz, 2003). Additionally, blocking serotonergic neuromodulation in *Tritonia* via the serotonin receptor antagonist methysergide blocks the swim behavior *in vivo* and the swim motor pattern in the isolated brain (McClellan et al., 1994).

In this study we found that DSI modulated C2 synaptic strength and that serotonergic neuromodulation was necessary to produce a dorsal-ventral swim in *Pleurobranchaea*. Additionally, we found that the extent of this modulation correlated with the strength of swimming. Finally, we found that this modulation was absent in the non-dorsal-ventral swimming *Hermissenda*. These results suggest that serotonergic neuromodulation is responsible, in part at least, for the ability of shared neural circuitry to generate the rhythmic motor pattern in certain species. This helps establish a role for neuromodulation in the evolvability of behavior at the cellular level.

Materials & Methods

Animal collection, maintenance, and dissection

Tritonia diomedea individuals were obtained from Living Elements Ltd. (Delta, BC, Canada). *Pleurobranchaea californica* and *Hermissenda crassicornis* individuals were obtained from Monterey Abalone Company (Monterey Bay, CA, USA). Animals were maintained in artificial seawater (Instant Ocean) tanks at a fixed 12:12 light/dark cycle. *Tritonia* individuals were maintained at 11 ± 1° C. *Pleurobranchaea* and *Hermissenda* individuals were maintained at 13 ± 1°

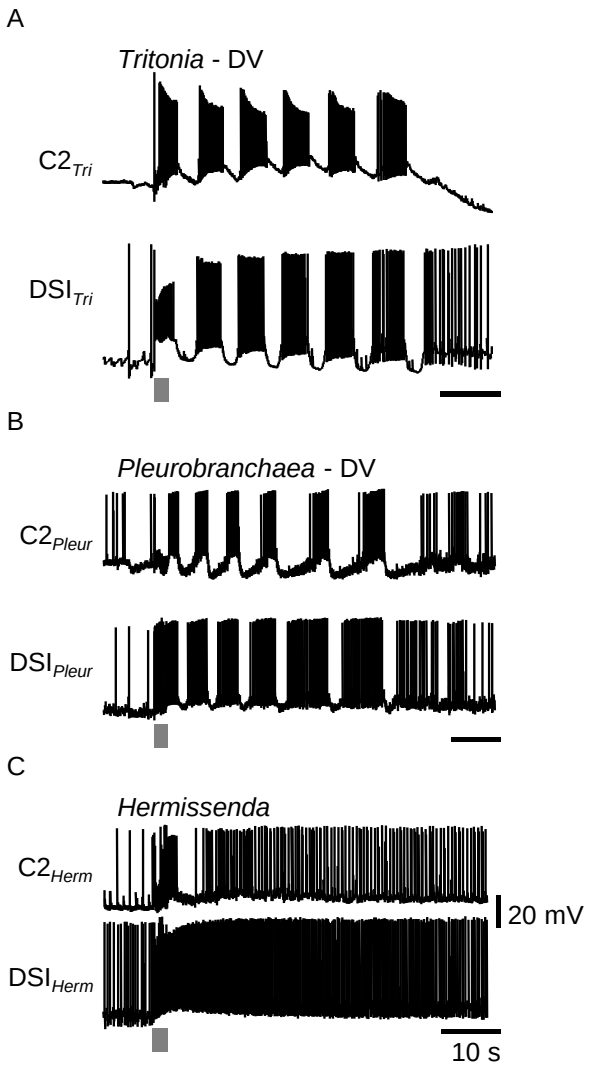

Figure 4.1: Species differences in the response to body wall nerve stimulation. *Tritonia* and *Pleurobranchaea* exhibit dorsal-ventral swimming *in vivo*, but *Hermissenda* does not. A) In the isolated brain, stimulating pedal nerve 2 (PdN2; grey bar) in *Tritonia* and B) the body wall nerve (BWN) in *Pleurobranchaea* induced rhythmic bursting responsible for the dorsal aspect of the dorsal-ventral swim in DSI and C2. C) In *Hermissenda*, PdN2 stimuli elicited high frequency spiking in DSI, but no rhythmic bursting. PdN2 stimuli could also elicit high frequency spiking in C2, and occasionally, a single burst, but no rhythmic bursting. Representative examples of the population are shown.

C.

Tritonia individuals were anesthetized by cooling. *Pleurobranchaea* and *Hermissenda* individuals were anesthetized by injection of 0.33 M $MgCl_2$ into the body cavity. A cut was made on the dorsal surface of the body wall near the buccal mass. The brain, consisting of the cerebral, pleural, and pedal ganglia, was removed by cutting all nerve roots. The brain was transferred to a Sylgard-lined dish where it was superfused, at a rate of ~0.5 ml/min, with artificial saline (in mM): 420 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 11 D-glucose, and 10 HEPES, pH 7.5.

Connective tissue surrounding the brain was manually removed with forceps and fine scissors while maintaining a temperature of ~4 °C to reduce neuronal activity. The temperature was raised to 10-11° C for *Tritonia* and 13-14° C for *Pleurobranchaea* and *Hermissenda* electrophysiological experiments.

Electrophysiology

Intracellular recordings were obtained using 10-80 MΩ glass microelectrodes filled with 3 M KCl connected to Axoclamp 2B amplifiers (Molecular Devices). Extracellular suction electrode recordings were obtained by drawing individual nerves into polyethylene tubing filled with artificial saline connected to an A-M Systems Differential AC Amplifier (model 1700, A-M Systems, Inc.). Both intra- and extracellular recordings were digitized (>1 kHz) with a 1401 Plus A/D converter from Cambridge Electronic Design.

In the isolated brain, body wall nerve stimulations were used to mimic swim inducing stimuli in the whole animal. The stimuli were varied in an effort to thoroughly test whether the DSI and C2 homologues were capable of producing the bursting activity that underlies dorsal-ventral swimming (1-5 s, 5-20 ms pulses, 5-20 Hz). Nerve stimuli were applied to the body wall nerve (BWN) in *Pleurobranchaea*, which was previously shown to elicit swimming in isolated brain preparations (Jing and Gillette, 1999). Stimuli were applied to Pedal Nerve 2 or 3 (PdN2/3) (Newcomb et al., 2006) in *Tritonia* and *Hermissenda*, which project to the dorsal body wall and

reliably elicit swimming in *Tritonia* (Frost and Katz, 1996). We generically refer to these nerves as body wall nerves.

High divalent cation saline, which raises the threshold for spiking and reduces spontaneous neural firing, was used for all modulation experiments. The composition of the high divalent cation saline was (in mM): 285 NaCl, 10 KCl, 25 $CaCl_2$, 125 $MgCl_2$, 11 D-glucose, and 10 HEPES (pH 7.5). The following protocol was used to test DSI modulation of C2 synapses. Two electrodes were inserted into the postsynaptic target of C2. One electrode was used to inject current in order to hold the membrane potential near -70 mV, though in some cases the resting potential had to be hyperpolarized to prevent action potential firing. The resting membrane potential of the postsynaptic target was held at a consistent membrane potential throughout the experiment. The other electrode monitored the membrane potential. C2 was stimulated to fire four action potentials every two minutes (four 20 ms pulses at 20 Hz). DSI was stimulated to fire 50 action potentials (fifty 20 ms pulses at 5 Hz) three seconds before every other C2 stimulus. For serotonin (5-HT) bath application experiments, C2 was stimulated to fire four action potentials every two minutes. After baseline postsynaptic potential amplitude was established, 100 μM 5-HT (Sigma Aldrich) in high divalent cation saline was superfused into the recording dish at a rate of ~1 mL/min. 5-HT was then washed out with high divalent cation saline. For experiments testing whether methysergide blocked DSI modulation, the DSI modulation of C2 protocol was used. 50 mM methysergide (dissolved in DMSO at 1:1000 total volume) (Sigma Aldrich) in high divalent cation saline was superfused into the recording dish at a rate of ~1mL/min. For the experiment testing whether methysergide blocked the *Pleurobranchaea* swim in the isolated brain, the animal was stimulated to swim every 10 minutes in artificial saline. 50 mM methysergide (in 1:1000 DMSO) in artificial saline was superfused into the recording dish at a rate of ~1 mL/min, and then washed out with artificial saline at the same rate.

Neuron Identification

C2 neurons were identified preliminarily using soma position, size, and pigmentation (white). C2 is conspicuous and relatively easy to identify visually across species (Lillvis et al., 2012). Upon impaling C2 with a microelectrode, its characteristic resting activity, synaptic input, and response to a nerve stimulus further aided identification. In Pleurobranchaea, the $C2_{Pleur}$ axon projection was confirmed by monitoring the contralateral anterior cerebral-pedal connective and the larger of the pedal-pedal connectives (PP2) (Newcomb et al., 2006), which in this case is the anterior pedal-pedal connective, via extracellular recordings. In Tritonia and Pleurobranchaea, C2 identity could be confirmed by its involvement in the fictive dorsal-ventral swim motor pattern. Finally, in experiments where C2 identity was not certain, the soma was filled with the biotinylated tracer biocytin (2.5% dissolved in 0.75 M KCl) (Invitrogen) or Neurobiotin (2-4% dissolved in 0.75 M KCl) (Vector Laboratories) and processed for FMRFamide and/or Small Cardioactive Peptide B (SCP_B) immunoreactivity (see below) because the axon projection in conjunction with FMRFamide and SCP_B immunoreactivity uniquely identifies C2 across species (Lillvis et al., 2012).

DSIs were identified preliminarily by soma position, size, and pigmentation (generally unpigmented, but some mottled dark pigmentation is present). There are three DSIs found in a cluster in a characteristic location in each species (Jing and Gillette, 1999; Tian et al., 2006; Newcomb and Katz, 2007). Upon impaling DSI with a microelectrode, the neuron shows characteristic spiking activity, action potential shape, and synaptic input that allowed the neurons to be distinguished from neighboring cells. In *Pleurobranchaea*, the axon projection of DSI could be confirmed by monitoring the contralateral anterior cerebral-pedal connective. In *Tritonia* and *Pleurobranchaea*, DSI could be confirmed by its involvement in the fictive dorsal-ventral swim motor pattern. Finally, the DSI soma was filled with the biotinylated tracer biocytin or Neurobiotin after the experiment and processed for 5-HT immunohistochemistry (see below). Soma position and axon projection in conjunction with 5-HT immunoreactivity uniquely identify DSI across spe-

cies (Newcomb and Katz, 2007). 5-HT immunoreactivity was used to confirm DSI identity in all *Hermissenda* preparations and in all *Tritonia* and *Pleurobranchaea* preparations in which it was not confirmed by involvement in the fictive swim motor pattern. DSI and C2 homologues will be referred to by the *Tritonia* name and differentiated by species using the subscripts $_{Tri}$, $_{Pleur}$, and $_{Herm}$.

Biocytin or Neurobiotin processing and immunohistochemistry procedures were identical to those reported in Lillvis et al., 2012. Briefly, after fixation brains were washed and incubated in one or more of the following primary antiserum: rabbit anti-FMRFamide (Immunostar) or anti-serotonin (Invitrogen) antiserum diluted 1:1000 and/or mouse monoclonal anti-SCP_B (courtesy of Stephen Kempf) diluted in 1:20 antiserum diluent (ASD). Streptavidin-Alexa Fluor 594 conjugate (1:50-1:200, Invitrogen) was also added to visualize the biotinylated tracer. Brains were then washed in incubated in goat anti-rabbit and/or goat anti-mouse antiserum conjugated to Alexa Fluor 488, Alexa Fluor 594 (Invitrogen), or DyLight 405 (Jackson ImmunoResearch) diluted 1:100 in ASD. Brains were then washed, dehydrated, and mounted on a slide to visualize soma immunohistochemistry and axon projection.

In vivo tests of swimming

To test for swim ability in *Pleurobranchaea*, a short 10-30 V shock was applied to the dorsal body wall. A reflex response was reliably observed from this stimulus, sometimes accompanied by the initiation of dorsal-ventral swimming. Upon arriving in the laboratory individuals were tested for swim ability approximately every day until they were used for electrophysiological experiments. Swim tests were conducted at different times to control for time-dependent effects on swim expression.

To test for the methysergide effect on swim ability in *Tritonia* and *Pleurobranchaea*, size matched individuals were selected and stimulated to swim every 30 minutes. *Tritonia* swimming was elicited by applying a 5 M NaCl salt solution to the dorsal body wall, which reliably induces

swimming. Baseline swim ability was determined for at least 4 trials. Any individual that did not show consistent swim ability was removed from the experiment. Animals that remained were injected with a 5% of total volume 10 mg/L methysergide (dissolved in 1:1000 total volume DMSO) solution in artificial seawater. Total volume of the individual was determined by sea-water displacement. The 10 mg/L measurement was based on the total volume plus the 5% injection volume. Fast green was added to the solution to ensure that the injection spread throughout the body cavity. Control injections consisted of DMSO (1:1000 total volume) with fast green in artificial seawater.

To test for the 5-HT effect on swim ability in *Tritonia*, *Pleurobranchaea*, and *Hermissenda*, size matched individuals were selected and stimulated every 30 minutes. *Hermissenda* was stimulated by applying a 5 M NaCl salt solution to the dorsal body wall, which can induce rhythmic left-right flexions (Lillvis et al., 2012). After an establishment of baseline swim ability or reaction to 5M NaCl, animals were injected with a 5% of total volume 20 mM 5-HT in artificial seawater yielding an approximate concentration of 100 μM 5-HT in the whole animal. Fast green was added to ensure the injection spread throughout the body cavity. Control injections consisted of artificial seawater with fast green at 5% of total body volume.

Data acquisition, analysis, and statistics

Data acquisition and analysis were performed with Spike2 software (Cambridge Electronic Design) and SigmaPlot (Jandel Scientific). The percentage of DSI modulation was calculated by measuring the amplitude difference between the DSI modulated C2 synapse and the C2 synapse alone on a point-by-point basis divided by the average C2 alone synaptic amplitude over the course of each experiment.

$$DSI\ modulation\ \% = mean\left(\frac{DSI + C2\ EPSP - C2\ alone\ EPSP}{mean\ C2\ alone\ EPSP}\right)$$

To determine if a relationship between swim strength and the extent of DSI_{Pleur} modulation of $C2_{Pleur}$ was present, the average percentage of DSI_{Pleur} modulation from each experiment was plotted against the number of swim cycles exhibited in *Pleuro-branchaea* on the day of the experiment. A hyperbolic equation fit the data best and yielded an R^2 value of 0.558, where y0 = 36.043, a = 151.694, and b = 0.931.

$$f = y\mathbf{0} + a * \frac{x}{b + x}$$

The percentage of modulation from 5-HT bath application was calculated by dividing the average of ten consecutive synaptic amplitude data points around the peak of the 5-HT effect by the average of the final ten consecutive synaptic amplitude data points of the baseline.

Pairwise comparisons of DSI modulation percentage in *Pleurobranchaea* were made using a t-test. Pairwise comparisons of 5-HT modulation in *Pleurobranchaea* were made using a Mann-Whitney Rank Sum test as the data failed the Kolmogorov-Smirnov test of normality. Comparisons across all groups were made using a one-way analysis of variance (ANOVA) with post-hoc pairwise multiple comparisons using the Holm-Sidak or Dunn's method. Dunn's method was used when comparing the results of 5-HT bath application where the data failed the Kolmogorov-Smirnov test of normality. In all cases where data failed the test of normality the median value of each species was compared. In all cases, $p<0.05$ was considered significant. Results are expressed as the mean ± standard error of the mean (SEM) unless stated otherwise.

Results

DSI_{Tri} and serotonin neuromodulation of $C2_{Tri}$ synapses in Tritonia

In this study, we replicated previous results (Katz et al., 1994b; Katz and Frost, 1995b) by demonstrating that DSI_{Tri} increased the amplitude of $C2_{Tri}$-evoked synapses. The DSI_{Tri} modulation is presynaptic, which allows any $C2_{Tri}$ synapse to be tested to determine the DSI_{Tri} modulatory effect (Katz and Frost, 1995a). On average, DSI_{Tri} increased $C2_{Tri}$ synaptic strength by 107 ± 26.2% (n=5) (Figure 4.2A, 4.5A). We also replicated prior results showing that bath application of 5-HT mimics the DSI_{Tri} modulation (Katz et al., 1994b; Katz and Frost, 1995b). Here, 5-HT bath application increased $C2_{Tri}$ synaptic strength by 137 ± 8% (median of 137%) (n=2) (Figure 4.2B, 4.5B). These modulation values were similar to previously published reports (Katz et al., 1994b; Katz and Frost, 1995b).

DSI_{Pleur} and serotonin neuromodulation of $C2_{Pleur}$ synapses in Pleurobranchaea

Like *Tritonia*, *Pleurobranchaea* expresses a dorsal-ventral swim. DSI_{Tri} modulation of $C2_{Tri}$ synaptic strength is critical to the swim in *Tritonia*. Therefore, we predicted that a similar neuromodulation of C2 synaptic strength would be present in *Pleurobranchaea*.

We found that our prediction was correct. On average, DSI_{Pleur} increased the amplitude of $C2_{Pleur}$-evoked synapses by 112 ± 23.6% (n=10). Additionally, bath application of 5-HT mimicked the DSI modulatory effect. Bath applying 5-HT increased the amplitude of C2-evoked synaptic strength by an average of 68 ± 16.1% (n=21). However, some experiments showed markedly reduced amounts of DSI and serotonergic neuromodulation compared to others, and thus reduced the average modulatory effect. This encouraged an investigation of the possible causes for the variable results.

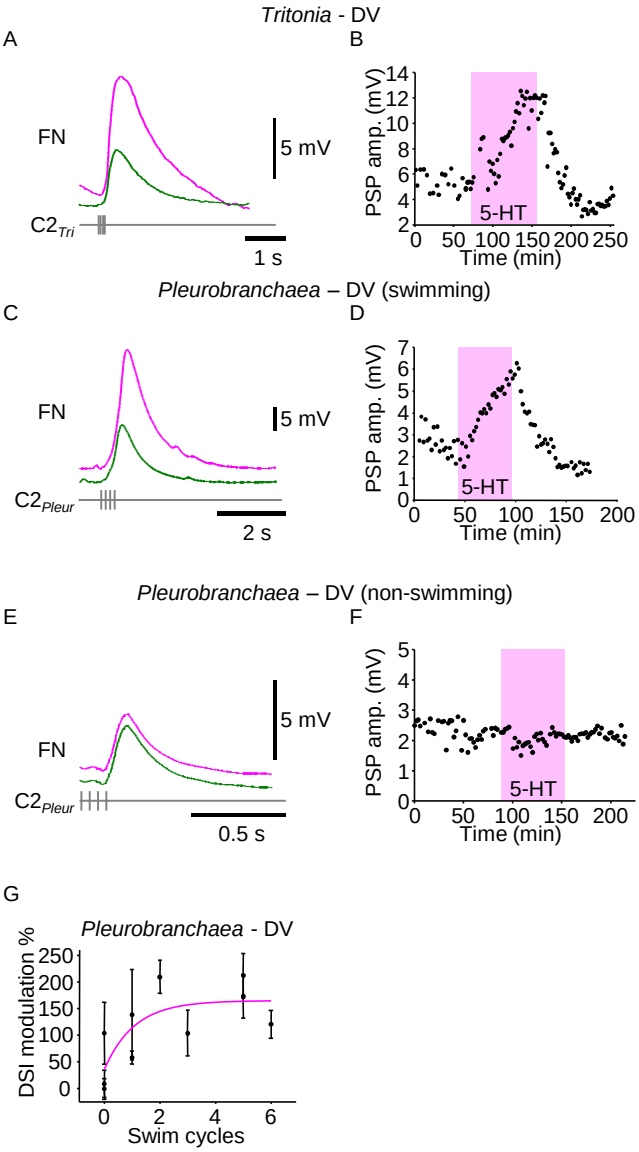

Figure 4.2: DSI and 5-HT modulation of C2-evoked synapses in *Tritonia* and *Pleurobranchaea*. A, C, E) Stimulating C2 (grey lines) elicited an EPSP (green trace) in a follower neuron in each species. Stimulating DSI 5 seconds before the C2 stimulation increased the amplitude of the C2 evoked EPSP in A) *Tritonia*, and C) swimming *Pleurobranchaea*. E) This modulatory effect was significantly reduced in non-swimming *Pleurobranchaea*. G) In *Pleurobranchaea*, the DSI_{Pleur} modulation percentage was plotted against the number of swim cycles produced. Each data point represents a single preparation. The hyperbolic equation fit the data (pink curve) with an R^2 value of 0.588. The standard deviation of the modulation throughout the course of each preparation is also represented, though the line is fit to the data points, not the spread. B, D, F) Bath applying 100 µM 5-HT mimicked the DSI modulatory effect in each species. The amplitude of the C2-evoked EPSP was plotted over time. Bath applying 5-HT increased the amplitude of this EPSP in B) *Tritonia* and D) swimming *Pleurobranchaea*. F) The modulatory effect of 5-HT in non-swimming *Pleurobranchaea* was significantly reduced. Examples in A-F are representative of the population.

Variability in Pleurobranchaea swim behavior

Unlike *Tritonia*, which produces the rhythmic motor pattern readily *in vivo* and in isolated brain preparations, *Pleurobranchaea* exhibits heterogeneity within the population in its ability to swim (Jing and Gillette, 1995, 1999). In the laboratory, 18 of 61 (30%) individual *Pleurobranchaea* tested never produced a swim *in vivo*. Of those that did swim, there was variability in their day-to-day ability to swim. The average swim duration over the life of the animal in the lab was 3.1 dorsal-ventral cycles and the average coefficient of variation was 0.80 (n=43); on some days, animals swam and on other days they did not. This variability allowed a natural experiment in which we could test whether the strength of swimming correlated with the strength of DSI_{Pleur} modulation of $C2_{Pleur}$ synapses.

DSI_{Pleur} and serotonin neuromodulation of $C2_{Pleur}$ synapses in relation to Pleurobranchaea swim ability

We found that the extent of the modulation co-varied with swim ability. DSI_{Pleur} modulated $C2_{Pleur}$ synaptic strength by only 61 ± 26.8% in individuals that swam one or fewer dorsal-ventral cycle (n=5) (Figure 4.2E, 4.5A). This was a significant reduction in DSI_{Pleur} modulation of $C2_{Pleur}$ synapses compared to modulation of 163 ± 22.3% in individuals that swam two or more cycles (t-test; $p<0.05$) (Figure 4.2C, 4.5A). DSI_{Pleur} was serotonin-immunoreactive in both non-swimming (n=5) and swimming individuals (n=5), but to ensure that the differences in modulation were not due to a difference in 5-HT released from DSI_{Pleur} we also tested whether bath application of 5-HT mimicked the DSI_{Pleur} modulation results. Indeed, bath-applied 5-HT modulation correlated with swim ability (median values of 15.4% in non-swimmers (n=10) versus 92.0% in swimmers (n=11)) (Mann-Whitney Rank Sum test; $p<0.05$) (Figure 4.2D, F; 4.5B). Moreover, plotting the strength of swimming versus the extent of DSI_{Pleur} modulation of $C2_{Pleur}$ synapses established a more nuanced association between swimming and modulation. The percentage that DSI_{Pleur} modulated $C2_{Pleur}$ synapses increased with the number of dorsal-ventral cycles ob-

served on the day of testing before leveling off at what appears to be a maximum modulation percentage near 200% (Figure 4.2G). Thus, in *Pleurobranchaea* DSI_{Pleur} modulated the strength of $C2_{Pleur}$ synapses and the extent of this 5-HT mediated neuromodulation correlated with the strength of swimming.

Serotonin receptor antagonist methysergide blocks neuromodulation

To further investigate whether the DSI_{Pleur} neuromodulation observed was mediated by 5-HT, we tested whether pharmacologically blocking 5-HT receptors disrupted DSI_{Pleur} modulation. Methysergide is a 5-HT receptor antagonist that selectively blocks DSI_{Tri} modulation of $C2_{Tri}$-evoked synapses in *Tritonia* (Katz and Frost, 1995b). We found that in *Pleurobranchaea*, methysergide reduced DSI_{Pleur} modulation of $C2_{Pleur}$ synapses. In this set of experiments, DSI_{Pleur} enhanced $C2_{Pleur}$ synapses by an average of 108 ± 18.4% before applying methysergide and 26.6 ± 11.3% after application. This was a significant reduction in modulation (Paired t-test; $p<0.05$; $n=5$) (Figure 4.3A).

Methysergide blocks swimming in Pleurobranchaea

Now that we could block the DSI_{Pleur} modulatory effects, we tested whether serotonergic modulation was necessary to produce the dorsal-ventral swim in *Pleurobranchaea*. We found that injecting methysergide into the body cavity of *Pleurobranchaea* blocked the ability to produce the dorsal-ventral swim *in vivo* (Figure 4.3B). The latency to the effect varied across individuals, but on average the swim strength was significantly reduced from baseline measurements within 2 hours of the injection ($n=6$; $p<0.05$). The swim recovered the next day. Control vehicle injections never showed a change in swim strength versus the baseline measurement ($n=6$; $p>0.05$; Figure4. 3B).

Rarely did we encounter an isolated brain preparation that was able to repeatedly and reliably produce a swim motor pattern for the time that it would take to test methysergide. How

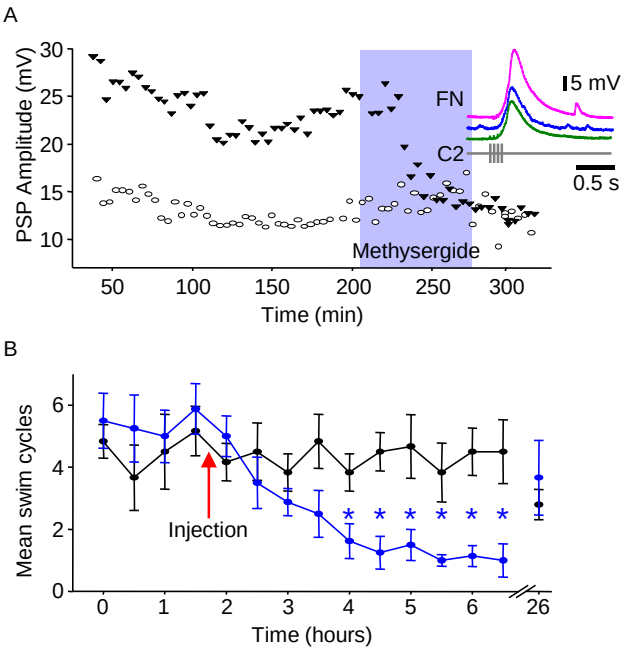

Figure 4.3: Effect of methysergide on DSI modulation and swimming in *Pleurobranchaea*. A) The amplitude of $C2_{Pleur}$-evoked EPSPs (white circles) and DSI_{Pleur} modulated $C2_{Pleur}$ -evoked EPSPs (black circles) were plotted over time. Bath application of 50 mM methysergide (blue) reduced the DSI_{Pleur} modulatory effect. Examples of the $C2_{Pleur}$ -evoked EPSP (green), the DSI_{Pleur} modulated $C2_{Pleur}$ -evoked EPSP (pink), and DSI_{Pleur} -modulated $C2_{Pleur}$ -evoked PSP in methysergide (blue trace) are shown. Examples shown are representative of the population. B) The average number of swim cycles is plotted over time. Injection of 10 mg/L methysergide (blue trace) significantly reduced the number of swim cycles; the swim recovered overnight. Vehicle control injections (black trace) did not result in the reduction of swim cycles.

ever, in one such case, bath application of methysergide also blocked expression of the swim motor pattern, which recovered after washout of the antagonist.

Therefore, as in *Tritonia*, DSI_{Pleur} modulation of $C2_{Pleur}$ was present and serotonergic modulation was necessary for *Pleurobranchaea* to produce a swim. Moreover, individual variation in swim ability correlated with the extent that DSI_{Pleur} modulated $C2_{Pleur}$ synapses. These results indicate that serotonergic modulation within the CPG circuit is necessary for expression of the dorsal-ventral swim in *Pleurobranchaea*. However, the vast majority of species in the Nudipleura do not express a dorsal-ventral swim. Therefore, we tested whether this modulation was present in a species that does not express a dorsal-ventral swim.

DSI_{Herm} and serotonin modulation of $C2_{Herm}$ synapses in Hermissenda

Hermissenda does not express a dorsal-ventral swim (Lillvis et al., 2012). Nerve stimuli that evoke swimming in the isolated brain in *Tritonia* and *Pleurobranchaea* did not elicit rhythmic bursting in DSI_{Herm} and $C2_{Herm}$ *Hermissenda*, though the DSI_{Herm} and $C2_{Herm}$ response was similar to that of *Tritonia* and *Pleurobranchaea* aside from the lack of rhythmic bursting (Figure 4.1C). We found that DSI_{Herm}'s effect on the strength of $C2_{Herm}$ synapses was significantly attenuated compared to the dorsal-ventral swimming species. The average synaptic increase produced by DSI_{Herm} was 5.1 ± 1.9%, which was significantly less than that produced by DSI in *Tritonia* ($p<0.05$) or swimming *Pleurobranchaea* ($p<0.05$) (Figure 4.4A, 4.5A) (n=6) (One-way ANOVA, Holm-Sidak pairwise comparisons). Like DSI in *Tritonia* and *Pleurobranchaea*, DSI_{Herm} is serotonergic in *Hermissenda* (Tian et al., 2006), but to ensure that species differences in modulation were not due to a difference in 5-HT released we tested whether bath-applied 5-HT mimicked the DSI_{Herm} effect. We found that bath application of 5-HT actually reduced the strength of $C2_{Herm}$ synapses (median of -6.35%, n=12) (Figure 4.4B, 4.5B). This reduction was likely a product of a decrease in $C2_{Herm}$ synaptic strength over the course of the experiments rather than a true reduction due to 5-HT. The 5-HT

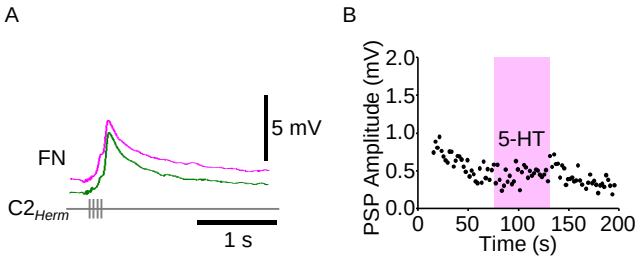

Figure 4.4: Effect of DSI stimulation and 5-HT bath application on C2 synaptic strength in *Hermissenda*. A) $C2_{Herm}$-evoked EPSP (green) is shown. Stimulating DSI_{Herm} 5 seconds before C2 did not affect the amplitude of the $C2_{Herm}$ synapse. B) Similarly, bath applying 100 µM 5-HT did not increase the amplitude of $C2_{Herm}$-evoked EPSPs. The examples shown are representative of the population.

bath application results were also significantly reduced from 5-HT bath-application results in *Tritonia* ($p<0.05$), swimming *Pleurobranchaea*, and non-swimming *Pleurobranchaea* ($p<0.05$) (Figure 4.5B) (One-way ANOVA, Dunn's pairwise comparisons).

Effect of injecting 5-HT into Pleurobranchaea and Hermissenda

In addition to serotonin modulation being necessary for swimming in *Tritonia*, injection of 5-HT into *Tritonia* reliably induces dorsal-ventral swimming (McClellan et al., 1994). We also found that 5-HT injection induced vigorous swimming in *Tritonia* (n=2) that did not occur from control seawater injections (n=2).

In *Pleurobranchaea*, 5-HT injection induced weak dorsal and ventral whole body flexions (n=4). Additionally, 5-HT injection reduced the threshold for swim induction in *Pleurobranchaea*. Whereas electrical stimulation is generally necessary to induce swimming in individual *Pleurobranchaea*, after 5-HT injection simply touching individual *Pleurobranchaea* could induce vigorous dorsal-ventral swims (n=3 of 4 preparations). No behavioral effects were observed from control seawater injections into *Pleurobranchaea* (n=4).

In *Hermissenda*, 5-HT injections did not induce dorsal-ventral swimming. Instead 5-HT injection induced noticeable movement of cerata (n=3) and could induce rhythmic left-right flexions (n=2 of 3). No behavioral effects were observed from control seawater injections (n=3).

Discussion

The results from these experiments are summarized in relation to phylogeny in Figure 4.5C. We showed that DSI modulated the strength of C2 synapses in *Tritonia* and *Pleurobranchaea*. Moreover, we showed that the extent of DSI_{Pleur} modulation of $C2_{Pleur}$ synapses correlated with the strength of swimming in *Pleurobranchaea*, which supports the hypothesis that DSI modulation of C2 synapses is important for dorsal-ventral swim expression. We also showed that the serotonin receptor antagonist methysergide significantly reduced the DSI_{Pleur}

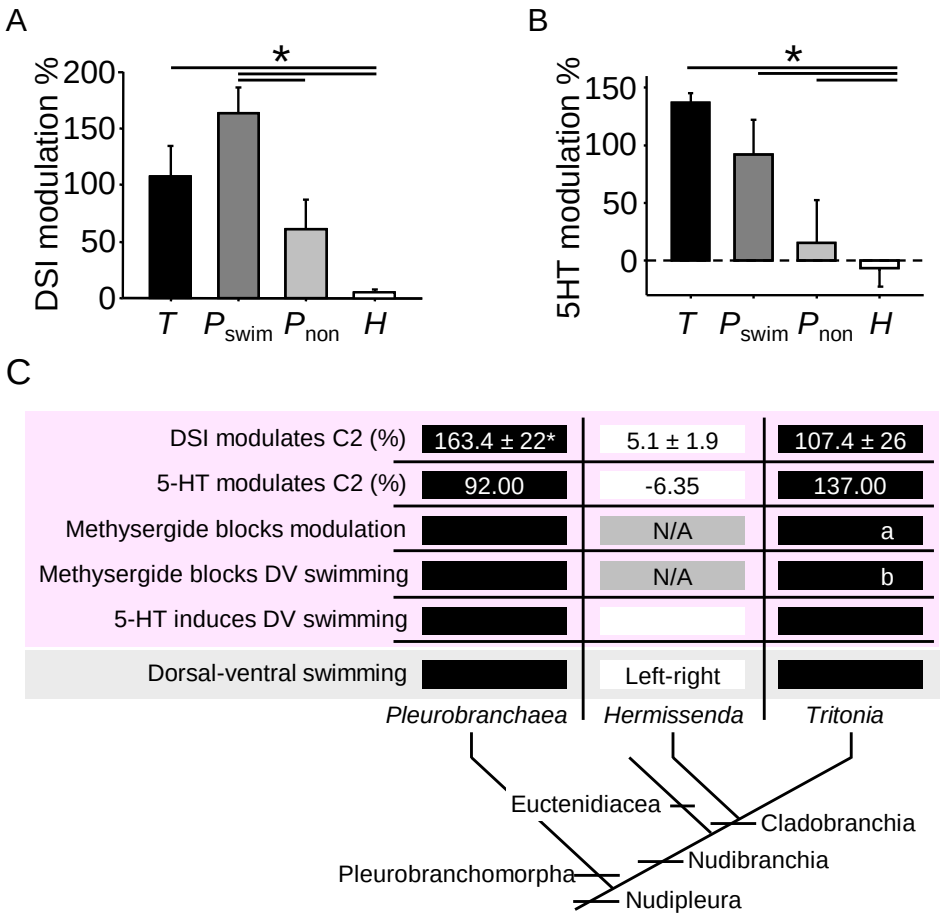

Figure 4.5: Summary of modulation results across species in relation to phylogeny. A) The average *Tritonia* ($p<0.05$) and swimming *Pleurobranchaea* ($p<0.05$) DSI modulation percentage was significantly greater than that in *Hermissenda*. Swimming *Pleurobranchaea* DSI_{Pleur} modulation percentage was also significantly greater than in non-swimming *Pleurobranchaea* ($p<0.05$). B) The median 5-HT bath application modulation percentage in *Tritonia* and swimming and non-swimming *Pleurobranchaea* was significantly greater than in *Hermissenda*. C) Black boxes indicate the presence of the characteristic to the left. White boxes indicate absence. Grey boxes indicate that the characteristic could not be tested. Where values were measured, they are indicated in the boxes. Neuromodulatory properties tested correlate with behavior. *Tritonia* and *Hermissenda* are members of the monophyletic clade Cladobranchia. *Pleurobranchaea* is not, but is a member of the monophyletic Nudipleura clade with *Tritonia* and *Hermissenda*. References: a, (Katz and Frost, 1995b); b, (McClellan et al., 1994).

modulation in *Pleurobranchaea* and inhibited swimming *in vivo* and the swim motor pattern in the isolated brain. This is similar to the effect of methysergide on DSI_{Tri} modulation (Katz and Frost, 1995b) and swimming in *Tritonia* (McClellan et al., 1994). Additionally, we showed that 5-HT injection was sufficient to produce dorsal-ventral swim like behavior in *Pleurobranchaea*, which is similar to previous results from *Tritonia* (McClellan et al., 1994). Taken together, these results indicate that DSI modulation of C2 synaptic strength via 5-HT is necessary for swimming in *Tritonia* and *Pleurobranchaea*. We then showed that this modulation was not present in the non-dorsal-ventral swimming *Hermissenda* and that injection of 5-HT did not induce dorsal-ventral swimming in *Hermissenda*. This suggests that the inability of *Hermissenda* to swim is likely due, in part at least, to the lack of serotonergic neuromodulation within the homologous CPG circuit.

Phylogenetic analysis of swim behavior in the Nudipleura suggests that the dorsal-ventral swim behaviors of *Tritonia* and *Pleurobranchaea* evolved independently (Newcomb et al., 2012). This implies that a similar neuromodulatory mechanism evolved independently in *Tritonia* and *Pleurobranchaea* allowing the expression of the rhythmic dorsal-ventral swim from non-rhythmic homologous neurons. These results suggest that neuromodulation may affect the evolvability of behavior by allowing similar behavior to independently evolve from a similar set of neural components.

Neuromodulation and differences in behavior

Despite the growing body of literature demonstrating the extreme importance of neuromodulation to proper circuit and behavioral function across phyla (Harris-Warrick, 2011), there has been relatively little neural evidence demonstrating a role for neuromodulation in behavioral differences across species. Evidence in vocal learning behavior in songbirds (Kubikova et al., 2010; Simonyan et al., 2012) and jamming avoidance response behavior in weakly electric fish (Smith et al., 2001) has suggested a role for neuromodulation contributing to species differ-

ences in these behaviors. Links between neuromodulation and species differences in behavior have also been demonstrated with echo sensitivity in bats (Yan and Suga, 1996; Zhang et al., 1997; Suga et al., 2002), frog embryo responses to mechanosensory stimuli (Merrywest et al., 2004), learning in aplysiid molluscs (Hoover et al., 2006), and in diverse laboratory behaviors of different rodent strains or between mice and rats (e.g., Pattij et al., 2007; Gieryk et al., 2010; Pobbe et al., 2011). The strongest studies to date come from the investigations of vasopressin and oxytocin receptor expression in relation to social behavior in voles (Young and Wang, 2004). This work has demonstrated that the expression patterns of such nonapeptide receptors can drastically influence social behaviors. These differences are hypothesized to result in differential modulation of otherwise conserved circuitry. Similar associations between nonapeptide receptor expression and species differences in social behavior have since been shown in fish, reptiles, birds, and other mammals including humans (see Donaldson and Young, 2008; Goodson and Thompson, 2010; Young and Flanagan-Cato, 2012).

These studies have been extremely valuable to our understanding of how differences in the way that circuits are modulated may affect behavior across species, but this is still a relatively small amount of diverse evidence for such a mechanism given that may be quite prevalent. Here, we provide direct evidence of a role for neuromodulation in the evolution of behavior. Additionally, we demonstrate how species differences in neuromodulation can be manifested at the cellular level.

Differences in modulation due to reception differences, not neuromodulator differences

Our results suggest that the species and individual differences in neuromodulation are not due to differences in the presence of 5-HT, but differences in the postsynaptic response to 5-HT. DSI is serotonergic in *Tritonia*, *Pleurobranchaea*, and *Hermissenda* and bath application of 5-HT mimicked the DSI modulation results. Additionally, species differences in the response to injection of 5-HT suggest that the behavioral differences are not due to a lack of available 5-

HT. This suggests that differences in serotonin receptor expression or intracellular responses to serotonin underlie the species and individual differences in the response to serotonin. Moreover, methysergide blocks the modulatory effect in *Tritonia* and *Pleurobranchaea* which suggests that the modulatory effect may be mediated by homologous or orthologous G-protein coupled serotonin receptors in each species (Clemens and Katz, 2003).These results are similar to studies investigating vertebrate social behavior and aplysiid learning ability, in that species differences in behavior are not correlated with the presence of the neuromodulator (Wang et al., 1996; Marinesco et al., 2003), but in the response to the neuromodulator.

A similar neuromodulatory mechanism underlying individual and species differences in behavior

Additionally, our results suggest that individual variability in behavior may be linked to the same neuromodulatory mechanism allowing species differences in behavior; the strength of *Pleurobranchaea* swimming was associated with the extent of DSI_{Pleur} neuromodulation. A similar link between polymorphic variation in the promoter region of the vasopressin 1A receptor gene and individual variability in social behavior was identified in voles as well as in human and non-human primates (Hammock and Young, 2004; Donaldson and Young, 2008; Hopkins et al., 2012). These polymorphisms can influence vasopressin receptor expression in some species and have been correlated with individual differences in behavior. This suggests that the individual variability in neuromodulation may be a mechanism upon which natural selection can act. Our results suggest a similar mechanism underlying individual and species variability in behavior, perhaps at the level of a single neuron.

Neuron multifunctionality, neuromodulation, and the evolvability of behavior

Neuromodulation can dynamically alter the output of neural circuits. This can allow neural circuits to produce different outputs without affecting neural circuit properties that are important for other behaviors. Both DSI and C2 have other behavioral functions aside from being a

part of the dorsal-ventral swim CPG. In *Tritonia*, *Pleurobranchaea*, and the aplysiid *Aplysia californica*, the DSI can initiate crawling (Popescu and Willows, 1999; Jing and Gillette, 2000; Popescu and Frost, 2002; Jing et al., 2008). DSI_{Herm} does not appear to have a role in crawling in *Hermissenda* but does elicit non-rhythmic foot contractions (Tian et al., 2006). DSI also facilitates feeding in *Pleurobranchaea* and *Aplysia* (Jing and Gillette, 2000; Jing et al., 2008). While a conserved role for crawling is not present in all of the species examined, it is possible that other functions of DSI are conserved across species. Additionally, DSI modulates the swim of *Melibe leonina*, a species that swims with rhythmic left-right flexions (Newcomb and Katz, 2009), and *Clione limacina*, a species that swims with rhythmic wing-like flapping (Panchin et al., 1995; Satterlie and Norekian, 1995). A similar effect may be present in *Hermissenda*, as 5-HT injection induced rhythmic left-right flexions. This is similar to results in *Melibe* where 5-HT injection increased the speed and duration of swim bouts (Lewis et al., 2011). However, DSI modulation is not necessary for swimming in *Melibe* and *Clione*. We did not test the role of DSI modulation on the rhythmic left-right flexions in the isolated *Hermissenda* brain. C2 suppresses feeding behavior in *Pleurobranchaea* (Jing and Gillette, 1995) and may play a role in crawling in *Tritonia* (Snow, 1982a). Such functions could be present in other species as well. Therefore, dynamic neuromodulation of DSI and C2 may allow the other behavioral functions to persist without detriment while still allowing the rhythmic dorsal-ventral swim output when needed.

In this way, neuromodulation may be a common mechanism affecting the evolvability of behavior as it can allow common neural circuits components to produce divergent outputs (Katz, 2011). The repeated observation of differential nonapeptide receptor expression associated with similar independently evolved social behaviors provides evidence for neuromodulation affecting the evolvability of behavior. Those results suggest that different species have independently acquired similar neuromodulatory mechanisms to produce similar behavior from otherwise constrained neural circuitry. We provide further evidence for such a mechanism here by showing that *Tritonia* and *Pleurobranchaea* appear to have independently acquired a similar neuromodu-

latory mechanism to produce a similar swim behavior.

In summary we have presented evidence indicating that serotonergic neuromodulation of identified homologous neurons is necessary for similar independently evolved behaviors and that this neuromodulation is absent in species that cannot produce the behaviors. This suggests that species differences in neuromodulation can allow different functional outputs from homologous neural components. Therefore, the results also suggest a role for neuromodulation in the evolvability of behavior.

Acknowledgments

The authors would like to thank Stephen Kempf for providing us with the SCP_B antibody,

CHAPTER 5: GENERAL DISCUSSION

General discussion

Less than one percent of Nudipleura species have been documented to express a DV swim. Phylogenetic analysis strongly suggests that *Tritonia* and *Pleurobranchaea* evolved the ability to produce a DV swim independently (Newcomb et al., 2012). We tested whether similar neural mechanisms evolved in each species to allow a behavior that most of their phylogenetic relatives cannot produce. This series of experiments tested three general questions with applications beyond the Nudipleura: what neural mechanisms are responsible for species differences in behavior, whether similar neural mechanisms are used to produce similar independently evolved behaviors, and how the circuit properties that are important to functional circuit output compare to properties that play a lesser role? The answer to these questions would inform our understanding of how nervous system constraints shape the evolution of behavior as well as inform our understanding of neural circuit function in general.

Chapter 2 showed that CPG neurons that underlie the swim in *Tritonia* and *Pleurobranchaea* were also present in the non-DV swimming nudibranch species *Melibe*, *Flabellina*, and *Hermissenda*. This suggested that the ability to produce the dorsal-ventral swim was not due to the gain of those neurons in the DV swimmers or the loss of those neurons in non-DV swimming species. **Chapter 3** showed that electrophysiological properties of the homologous CPG neurons were generally similar in *Tritonia*, *Pleurobranchaea*, and the non-DV swimming *Hermissenda*. This suggested that differences in the electrophysiological properties of the CPG homologues that we measured were not responsible for the ability to produce the DV swim. This chapter also showed that some synaptic connections of the CPG homologues correlated with phylogeny while other connections correlated with behavior. This suggested that certain synap-

tic connections could differ without detriment to the DV swim. It also suggested that certain synaptic connection differences may play a role in the ability of *Tritonia* and *Pleurobranchaea* to produce the DV swim compared to related species. **Chapter 4** showed that species differences in neuromodulatory properties of the CPG homologues correlated with the ability to produce the DV swim. This suggested that species differences in neuromodulation of the CPG homologues play a role in the ability to produce the DV swim.

In total, the results showed that similar neural mechanisms were used to produce similar independently evolved behaviors. We also showed that some neural and synaptic properties of homologous neurons could differ without detriment to the behavior they underlie. Moreover, we showed that synaptic and neuromodulatory differences likely enable the production of rhythmic behavior from ancestrally non-rhythmic homologous neural circuit components. Below I will review each possible neural difference that we tested and our results in light of what is currently known. Also, see Figure 5.1 for summaries of the results from these studies.

Evolution of behavior due to gain or loss of neurons

It is possible that the gain or loss of neurons can underlie differences in behavior. If neurons important for a behavior in one species were lost, barring any compensatory mechanisms, the behavior would be lost as well. Similarly, adding neurons to an otherwise conserved brain would allow for different neural circuit configurations and different behaviors. There are many studies that have shown correlations between the volume of brain regions – or even the whole brain – with selected behavioral differences with no further investigation (see Healy and Rowe, 2007 for a critique). However, there are a number of compelling studies linking differences in the size of brain regions or even differences in the presence of individual neurons with differences in behavior in insects (Strausfeld et al., 1998; Farris, 2008; Farris and Schulmeister, 2011), crustaceans (Faulkes, 2008), fish (Yoshizawa et al., 2010; Carlson et al., 2011), birds

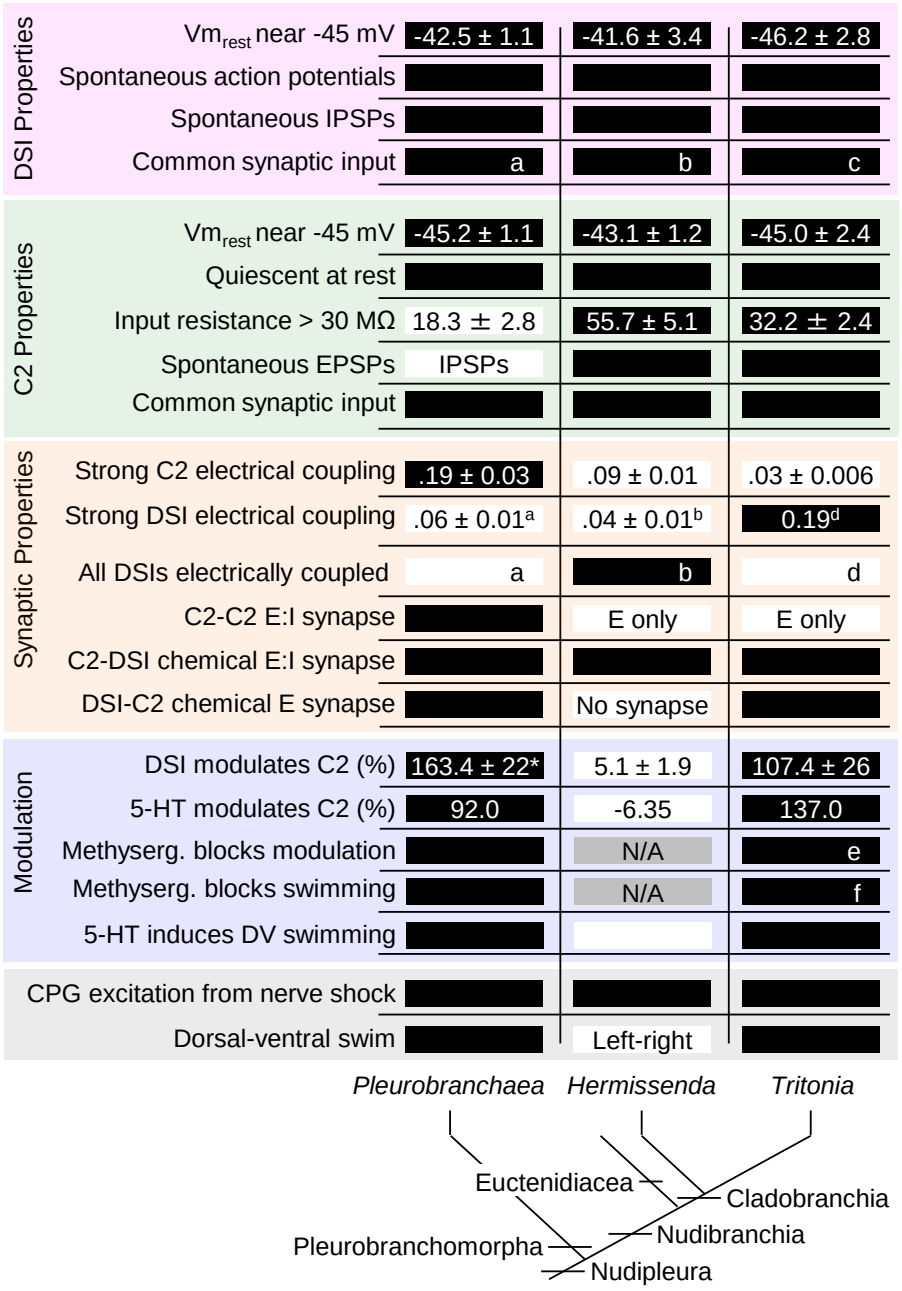

Figure 5.1: Summary of CPG homologue electrophysiological properties, synaptic connections, and neuromodulatory properties across species in relation to phylogeny. Black boxes indicate the presence of the characteristic to the left. White boxes indicate absence. Grey boxes indicate that the characteristic could not be tested. Where values were measured, they are indicated in the boxes. *Tritonia* and *Hermissenda* are members of the monophyletic clade Cladobranchia. *Pleurobranchaea* is not, but is a member of the monophyletic Nudipleura clade with *Tritonia* and *Hermissenda*. The summary shows C2 properties that correlate with phylogeny, synaptic connections that correlate with phylogeny or behavior, and modulatory properties that correlate with behavior. References: a, (Jing and Gillette, 1999); b, (Tian et al., 2006); c, (Newcomb and Katz, 2007); d, (Getting, 1981); e, (Katz and Frost, 1995b); f, (McClellan et al., 1994).

(Brenowitz, 1997; Gutiérrez-Ibáñez et al., 2009), and mammals (Catania, 2005; Padberg et al., 2005; Karlen and Krubitzer, 2007; Padberg et al., 2007; Kaas, 2008; Krubitzer et al., 2011). Many of these examples concern differences in sensory systems that lead to behavioral differences, but central neural circuitry was investigated in some cases as well.

In **Chapter 2**, we sought to test whether species differences in the presence of homologous DV swim CPG neurons in *Tritonia*, *Pleurobranchaea*, *Hermissenda*, *Melibe*, and *Flabellina* could explain the species differences in swim behavior. The nervous systems of related Nudipleura species appear to be relatively well conserved and homologous neurons have been identified across the Nudipleura and beyond (Weiss and Kupfermann, 1976; Granzow and Fraser Rowell, 1981; Pentreath et al., 1982; Croll, 1987b; Sakurai et al., 2011). However, there is evidence suggesting that there may be species differences in the number of neurons present (Newcomb et al., 2006). Therefore, it is possible that the gain or loss of DV swim CPG neurons could underlie species differences in DV swim ability.

The swim CPG circuits of both *Tritonia* and *Pleurobranchaea* contain homologues of DSI and C2 (Getting et al., 1980a; Jing and Gillette, 1999). Previous studies had identified homologues of DSI across the Nudipleura and in related clades (Panchin et al., 1995; Satterlie and Norekian, 1995; Jing and Gillette, 1999; Fickbohm et al., 2001; Tian et al., 2006; Newcomb and Katz, 2007). This indicates the presence of DSI across species is not related to species differences in the ability to produce a DV swim.

Our experiments suggest that C2 is also present across the Nudipleura, and therefore species differences in the presence of C2 are not responsible for species differences in DV swimming behavior. We established a suite of anatomical and neurochemical characteristics that uniquely identified C2 in *Tritonia* and *Pleurobranchaea*. These same characteristics also distinguished C2 in three nudibranc species that do not express a DV swim: *Melibe*, *Flabellina*, and *Hermissenda* (Newcomb et al., 2012). We are confident in our identification of the homologues because the criteria used *uniquely* identified C2 in the five species. That is, no other neu-

ron in the entire brain had the characteristics of the C2 homologues in each species. Thus, while it is theoretically possible that the C2 homologues identified are different neurons that independently acquired the same uniquely identifying characteristics, the more parsimonious explanation is that the neurons identified are homologous. It is possible that other neurons important for the expression of DV swimming in *Tritonia* and *Pleurobranchaea* are absent in non-DV swimming species and that this relates to the species differences in behavior. However, we are confident that the species differences in swimming are not due to the absence of DSI or C2. Thus, our results do not represent an example where gain or loss of neurons played a role in species differences in behavior.

Neuron multifunctionality

If DSI and C2 had no other function aside from a role in the DV CPG, one would expect the neurons might be lost over evolutionary time in species that do not express the behavior. However, DSI and C2, like many neurons, are multifunctional. The DSI homologues are also involved in crawling behaviors in *Pleurobranchaea* and *Aplysia* (Jing and Gillette, 2000; Jing et al., 2008) and cause foot contraction in *Hermissenda* (Tian et al., 2006). The DSIs can also facilitate feeding in *Pleurobranchaea* and *Aplysia* (Jing and Gillette, 2000; Jing et al., 2008). Additionally, the DSIs also modulate the swims of the left-right swimming *Melibe* and the wing-like flapping swim of *Clione* (Panchin et al., 1995; Satterlie and Norekian, 1995; Newcomb and Katz, 2009). Finally, C2 can suppress feeding in *Pleurobranchaea* (Jing and Gillette, 1995) and may play a role in crawling in *Tritonia* (Popescu and Willows, 1999; Popescu and Frost, 2002). Thus, losing the CPG homologues would not only affect DV swim ability but other behaviors as well. Because neurons can have multiple functions and changing the properties of neurons can affect multiple behaviors or neural processes, it has been hypothesized that nervous systems are more evolutionarily constrained than peripheral structures (e.g., Katz, 1991; Tierney, 1996; Katz

and Harris-Warrick, 1999). Our results demonstrating the presence of homologous neurons across species regardless of swim behavior or peripheral anatomy support this hypothesis.

Evolution of behavior due to differences in neuron properties

It is possible that differences in the electrophysiological properties of homologous neurons across species underlie behavioral differences. The constraint of neuron multifunctionality may suggest that changes to the properties of homologous neurons are rare, as changing the properties of one neuron may affect multiple behaviors. However, studies in decapod crustaceans have demonstrated that the properties of neurons can vary a great deal from animal to animal and still produce the proper behavioral output (see Marder, 2011 for a recent review). This suggests that homeostatic mechanisms within the neural circuit may compensate for the differences in neuron properties observed or that the differences measured do not play a large role in functional output. Such a result may indicate that species differences in neuronal properties may be less inhibited by nervous system constraints than the outright loss of neurons because changes in neuron properties may affect one behavior but not others due to compensatory mechanisms. Conversely, the results may also suggest that differences in neuronal properties may have a lesser effect on behavior than other neural circuit differences. However, there are examples of differences in neuron properties playing a role in behavioral differences across species. Notably, all of these differences occur in sensory reception or motor output. Such species differences leading to behavioral differences have been described in nematodes (McGrath et al., 2011), insects (Arbas, 1983a; Martin et al., 2011), crustaceans (Paul, 1991; Cronin et al., 2010), fish (Heiligenberg, 1989; Kawasaki, 2009), reptiles (Schroeder and Loop, 1976; Molenaar, 1978), and mammals (Jacobs, 2009). Given the lack of strong evidence for property differences of interneurons leading to differences in behavior, it is certainly worth studying. One likely reason for the scarcity of research in this area is that it is difficult enough determining

property differences of individual neurons in one species let alone homologous neurons in multiple species.

In **Chapter 3**, we investigated whether species differences in the electrophysiological properties of DSI and C2 may play a role in behavioral differences in *Tritonia*, *Pleurobranchaea*, and *Hermissenda*. Our results, in conjunction with previous results, suggest that the properties of the DSI and C2 homologues that we measured cannot account for the species differences in the expression of DV swim behavior. Our results demonstrated that the resting membrane potential, spontaneous activity, and synaptic input of DSI are similar in *Tritonia*, *Pleurobranchaea*, and *Hermissenda*. Previous reports likewise indicate that the DSIs receive common synaptic input in all three species (Jing and Gillette, 1999; Tian et al., 2006; Newcomb and Katz, 2007). Similarly, our results indicated that the resting potential and activity of C2 were comparable in *Tritonia*, *Pleurobranchaea*, and *Hermissenda* and that the left and right C2 receive common synaptic input in all three species. However, we found that *Pleurobranchaea* differed from *Tritonia* and *Hermissenda* in C2 input resistance and synaptic input. Therefore, while there are some differences in C2 properties across species, the differences could not explain species differences in the expression of dorsal-ventral swim behavior since *Tritonia* and *Pleurobranchaea* swim in the same manner despite the differences in neuron properties.

It is possible that differences in other properties of DSI and C2 homologues do play a role in species differences in behavior. However, our results demonstrate a great deal of electrophysiological property conservation in DSI and C2 across species. This may indicate that the measured properties are important for functions aside from swimming that may be conserved throughout the Nudipleura.

It is of interest to investigate whether differences observed between *Tritonia* and *Pleurobranchaea* play a role in the differences in the DV motor patterns of those animals, as *Tritonia* swims for longer durations and the flexion cycle periods are less variable than in *Pleurobranchaea* (Jing and Gillette, 1999). It has been shown that differences in intrinsic properties of

neurons involved in rhythmic motor pattern production in crustaceans and leech influence the functional output of their neural circuits (Goaillard et al., 2009; Wright and Calabrese, 2011). The neuron property differences detected here may similarly underlie the DV swim differences in *Tritonia* and *Pleurobranchaea*.

Evolution of behavior due to differences in synaptic connections

Differences in the synaptic connections of homologous neurons could also account for species differences in behavior. As with differences in neuron properties, species differences in synaptic connections may be somewhat constrained as such changes could affect multiple behaviors. However, synaptic differences have been shown to be present in networks underlying behavior within crustacean (Marder, 2011) and leech (Calabrese et al., 2011b; Roffman et al., 2012) species without loss of the motor pattern. The ability to produce stable motor patterns in the face of such synaptic strength variability is presumably due to compensatory mechanisms or redundancy in the systems. Loss of synaptic connections have been reported in two Nudipleura species with no apparent detriment to similar left-right swimming behaviors (Sakurai et al., 2011). Such results suggest that synapses may be able to change, affecting one behavior but not others. Moreover, examples of changes in synaptic connections associated with behavioral differences have been documented in nematodes (Chiang et al., 2006), leech (Baltzley et al., 2010), insects (Wilson et al., 1982; Arbas, 1983b; Shaw and Meinertzhagen, 1986; Shaw and Moore, 1989; Martin et al., 2011), fish (Kawasaki, 2009; Giassi et al., 2011), and birds (Jarvis, 2004). Additionally, it is likely that many of the studies linking increases in neuron number in particular brain regions with behavioral differences (see *Evolution of behavior due to gain or loss of neurons section* above) may involve synaptic changes among homologous neurons or structures as well.

In **Chapter 3**, we investigated whether differences in the synaptic connections between the DSIs, between the C2s, and between DSI and C2 may play a role in behavioral differences in *Tritonia*, *Pleurobranchaea*, and *Hermissenda*. Figure 5.2 summarizes the findings. There

were a number of synaptic differences found. Some differences correlated with phylogeny, and therefore may play a role in *Tritonia* and *Pleurobranchaea* DV swim differences such as swim duration and flexion cycle period. Other differences correlated with behavior, which suggests that they may play a role in the ability to produce a DV swim.

There were several differences that correlated with phylogeny and therefore may play a role in the DV swim differences observed between *Tritonia* and *Pleurobranchaea*. The electrical coupling strengths among the C2s differed between the species. The electrical coupling strengths of the DSIs differ as well (Getting, 1981; Jing and Gillette, 1999; Tian et al., 2006). It has been reported that manipulating electrical coupling relationships of neurons alters rhythmic activity in leech (Wright and Calabrese, 2011) and *Xenopus* (Zhang et al., 2009). Thus, the coupling differences found here could certainly play a role in the differences in the DV swim behaviors of *Tritonia* and *Pleurobranchaea*. Additionally, there were differences in the chemical synapse of the left and right C2. Such a synaptic difference could also play a role in the swim differences of *Tritonia* and *Pleurobranchaea*.

There are also synaptic differences that correlated with behavior and therefore could play a role in the ability to produce a DV swim across species. The electrical coupling relationships of the different classes of DSI homologues differ in *Hermissenda* (Tian et al., 2006) compared to *Tritonia* (Getting, 1981) and *Pleurobranchaea* (Jing and Gillette, 1999) (Figure 5.2A-C). Evidence in rodents has shown that altering electrical coupling relationships of neurons can affect the ability to produce rhythmic output (Blenkinsop and Lang, 2006; Placantonakis et al., 2006). Therefore, such differences in DSI coupling properties could play a role in the ability to produce the rhythmic DV swim motor output across species.

Additionally, there were differences in the C2-to-DSI and DSI-to-C2 synaptic connections that correlated with behavior and may play a role in species differences in DV swim expression (Figure 5.2D-F). Unlike *Tritonia* and *Pleurobranchaea* (Jing and Gillette, 1999), the C2-to-DSI synapse in *Hermissenda* did not consistently produce both the fast excitatory and slow inhibitory

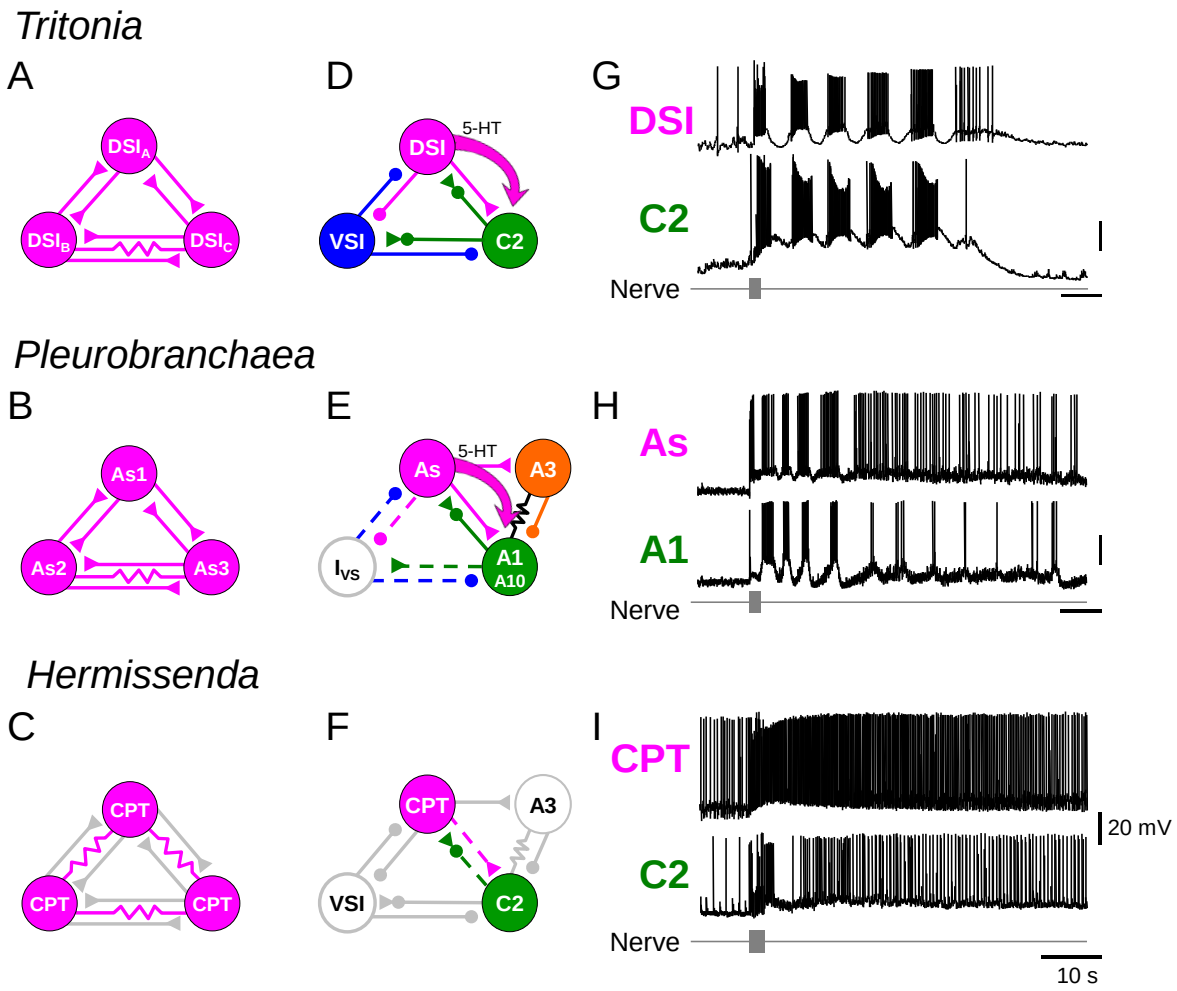

Figure 5.2: Summary of CPG circuitry and swim behaviors in each species. A-C) The DSI homologue electrical coupling relationships and functional synaptic connections in each species. Connections in pink are known, connections in grey have not been tested. The results show differences in the electrical coupling relationships of the DSI homologues in C) *Hermissenda* compared to A) *Tritonia* and B) *Pleurobranchaea*. D-F) Connections and modulation in color are known, grey are untested, dotted synaptic connections indicates possible or variable connections, and things that are missing in one species compared to another have been tested and are absent. The results show differences in synaptic connections and modulation that correlate with behavior. G-I) Stimulating a body wall nerve elicited a dorsal-ventral swim motor pattern as recorded in DSI and C2 homologues in *Tritonia* and *Pleurobranchaea*, but not in *Hermissenda*. Triangles: excitatory synapses, circles: inhibitory synapses, both: multicomponent synapses; resistors: electrical coupling.

synaptic components. Similarly, DSI lacked an excitatory connection with C2 in *Hermissenda* that is present in *Tritonia* and *Pleurobranchaea* (Jing and Gillette, 1999). A *Tritonia* swim CPG modeling study indicates that the inhibitory component of the C2-to-DSI synapse does not play a role in the ability to swim (Calin-Jageman et al., 2007). This is the component that was lacking in some cases in *Hermissenda*. Therefore, the C2-to-DSI synaptic differences may not play a role in the inability of *Hermissenda* to produce a DV swim. Conversely, the *Tritonia* model suggests the lack of a DSI-to-C2 synaptic connection as found in *Hermissenda* would result in an inability of *Tritonia* to produce a DV swim (Calin-Jageman et al., 2007). Thus, the DSI-to-C2 synaptic connection difference found in *Hermissenda* may indeed play a role in the inability of *Hermissenda* to produce a DV swim.

It may be possible that using other neurons aside from or in addition to the currently known CPG circuits could allow *Hermissenda* to produce a DV swim with the existing synaptic connections between DSI and C2. For example, two left-right swimming Nudipleura species, *Melibe* and *Dendronotus iris*, perform similar swim behaviors despite *Dendronotus* lacking synaptic connections that are necessary to produce the behavior in *Melibe* (Sakurai et al., 2011). Overlooking this possibility, our evidence suggests the synaptic connections found in *Hermissenda* would not allow a DV swim using the currently identified neurons.

Thus, our results demonstrate a number of synaptic differences across species. Some of these differences may contribute to the ability of *Tritonia* and *Pleurobranchaea* to produce a swim that most Nudipleura do not express. Moreover, given the amount of synaptic differences we found, our results suggest that changes to synaptic connections may not be highly constrained in these Nudipleura species.

Evolution of behavior due to differences in neuromodulation

The last possibility tested is that differences in neuromodulatory properties can affect species differences in behavior. This scenario would allow neuron properties and synaptic con-

nections to go unchanged across species, but allow for dynamic alterations of those properties to produce different outputs and behaviors. As discussed, constraints on the nervous system may not play as large a role in restricting changes to neuron and synaptic properties as I initially expected. However, neuromodulation could play a significant role in species differences in behavior. I reviewed the evidence for such species differences in neuromodulation underlying behavioral differences in **Chapter 1**. While there was not a diversity of concrete examples, there were some very strong and repeatedly observed examples concerning nonapeptide receptor expression and social behavior differences among vertebrates (e.g., Donaldson and Young, 2008; Young, 2009; Goodson and Thompson, 2010).

In **Chapter 4**, we tested whether species differences in serotonergic modulation of C2 homologues played a role in the ability to produce the DV swim behavior. Our results concerning neuromodulation were much more straightforward than those concerning synaptic connections. We showed that species and individual differences in serotonergic neuromodulation could account for the ability to produce a DV swim. The serotonergic DSIs modulated the C2 synaptic strength in the DV swimming species *Tritonia* and *Pleurobranchaea*. Our *Tritonia* results were similar to those previously published (Katz et al., 1994a; Katz and Frost, 1995b). Additionally, the extent of DSI modulation correlated with the strength of swimming in *Pleurobranchaea*. Moreover, blocking the modulation using the serotonin receptor antagonist methysergide inhibited the ability to swim *in vivo* and inhibited the rhythmic motor pattern in the isolated brain in both species. Again, our *Tritonia* results were similar to previously published studies (McClellan et al., 1994; Katz and Frost, 1995b). This demonstrated that serotonergic neuromodulation was necessary for swimming in both species, as predicted by the *Tritonia* DV swim CPG modeling study (Calin-Jageman et al., 2007). We also showed that neither DSI nor serotonin modulated the strength of C2 synapses in the non-DV swimmer *Hermissenda*. These results strongly suggested that the modulation is necessary for the homologous CPG neurons to produce a functional rhythmic motor pattern, even if this modulation was not the only factor lacking in the non-

DV swimmers. Thus, these results provide an example of a role for species differences in neuromodulation at the cellular level related to the production of similar independently evolved behaviors.

Additionally, our results suggest similarity to studies in voles and other species demonstrating that neuromodulator receptor expression patterns, but not the abundance of the neuromodulator present, were responsible for behavioral differences (Wang et al., 1996; Lim et al., 2004b). This is also similar to species differences in learning among aplysiid molluscs, where it was hypothesized that species differences in serotonin receptor expression, but not the abundance of serotonin in the system, underlie the species differences in learning ability (Marinesco et al., 2003; Hoover et al., 2006). The modulator neuron in our experiments, DSI, is serotonergic in all three species (Katz and Frost, 1995b; Jing and Gillette, 1999; Tian et al., 2006). Moreover, bath applying 5-HT mimicked the results of stimulating DSI, which suggests that a lack of serotonin in the system is not responsible for the species differences in DSI modulation of C2 synaptic strength. Instead, the results suggest that differences in the expression of 5-HT receptors or in the intracellular signaling mechanisms that underlie the modulatory response are responsible for the species differences observed. Therefore, our results indicate that species differences in serotonin receptor expression, possibly at the level of a single neuron, may underlie species differences in DV swim behavior.

Summary of results

Our results and previous published work suggest that neuromodulation of homologous neurons can account for species differences in behavior. Additionally, synaptic differences across species may also play a role in the behavioral differences. We also found that neuron and synaptic properties differed in the two DV swimming species. These differences may underlie the DV swim differences of the two species, but they do not appear to affect the ability to

produce a DV swim. Instead, neuromodulation does appear to influence the ability of individual *Pleurobranchaea* to produce a swim.

Nervous system constraints in the evolution of behavior

Heading into this study, it was clear from the work in vole species that species differences in neuromodulation could underlie behavioral differences across species. However, it had not been shown how such differences would be manifested at the cellular level in circuits underlying behavior. Moreover, despite the strong evidence indicating that modulatory differences are sufficient to produce behavioral differences in voles (e.g., Lim et al., 2004b), it was still possible that differences in neuron or synaptic properties could also play an important role in the species differences in behavior. However, I expected the neuronal and synaptic properties of the homologous CPG neurons to be relatively conserved across species due to nervous system constraints brought upon by neuron multifunctionality. The neuron electrophysiological properties we investigated across species supported that hypothesis, but admittedly we did not test many possible neuron properties. Conversely, the synaptic connections studied did not support the hypothesis, as there were species differences in every synaptic connection measured. This suggests that compensatory mechanisms accounted for whatever detriments such differences had on multiple behaviors or that the differences did not affect multiple behaviors. Alternatively, the differences could affect multiple behaviors that we were not measuring in each species. In any case, it is clear that synaptic connections can differ a great deal across species. Such differences may commonly play a role in species differences in behavior.

Neuromodulation as a substrate for behavioral evolution

The extent of DSI modulation correlated with the strength of DV swimming in *Pleurobranchaea*. This may demonstrate an example of neuromodulatory mechanisms acting as a substrate upon which selection can act. Similar hypotheses have been put forward concerning V1aR expression and social behavior. While the 5’ microsatellite region of the V1aR gene has

since been shown to not influence V1aR expression patterns and behavior in a number of species (Fink et al., 2006; Fink et al., 2007; Phelps, 2010), there are correlations with microsatellite polymorphisms and social behavior in voles and human and non-human primates (see Hammock and Young, 2004; Donaldson and Young, 2008; Hopkins et al., 2012). Because the microsatellite region can influence the expression patterns of V1a receptors, this suggests that differences in neuromodulation play a role in the individual variability in social behaviors. In our studies, we find that similar individual differences in neuromodulation may be responsible for individual variability in *Pleurobranchaea*. Such individual differences in behavior, and thus individual differences in neuromodulation, could be selected for or against. Therefore individual differences in neuromodulation may act as a substrate upon which selection can act, leading to the evolution of behavior within a species.

The evolvability of behavior

The repeated independent evolution of similar behaviors suggests that there may be neural mechanisms that affect the evolvability of behavior. Investigating the neural mechanisms underlying the similar independently evolved behaviors of *Tritonia* and *Pleurobranchaea* allowed us to examine whether certain neural mechanisms appeared to affect the evolvability of the DV swim behavior. Neuromodulation appears to be such a mechanism. Our results suggest that neuromodulation allows homologous neurons to reconfigure to form a stable oscillator in addition to the non-rhythmic functions that DSI and C2 normally serve. Such results mirror the repeated use of nonapeptide receptor expression differences and the independent evolution of similar social behaviors (e.g., Goodson and Thompson, 2010). In that case, a diverse number of vertebrate species appear to be able to acquire, in some cases, similar behaviors independently by differentially modulating conserved circuitry. Our results are similar, but are demonstrated at the individual neuron level, and thus further support such a hypothesis.

Conclusions

The results show that species differences in the neuromodulation of homologous neurons can account for the differences in swim behavior observed. Additional differences in specific synaptic connections also appear to play a role in the behavioral differences. However, other synaptic connections and some electrophysiological properties of homologous neurons do not appear to play a role in species differences in behavior. Thus, the results indicate that the influence of neuronal properties and synaptic connections on neural circuit output depends on the dynamics of the circuit. Additionally, the results further demonstrate the importance of neuromodulation in producing proper circuit output. In terms of evolution, the results show that synaptic connections can vary significantly across related species, which stands in contrast to the expected constraints on such differences. However, a role for neuromodulation in species differences in behavior fits the expected constraints on circuit properties across species. Previous work has suggested such a role for neuromodulation in species differences in behavior, but the work presented here is the first to directly link differences in neuromodulation at the cellular level to species differences in natural behavior. In summary, the dissertation demonstrated a neural mechanism that allows differences in behavior from homologous components while remaining within the apparent evolutionary constraints of nervous systems. However, the results also suggest that the evolutionary constraints of the nervous system may not be as substantial as initially perceived.

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APPENDIX A: ELECTROPHYSIOLOGICAL PROPERTIES OF C2 *MELIBE LEONINA* AND *FLAELLINA IODINEA*

Introduction

In Chapter 2, C2 homologues were identified using anatomical and neurochemical characteristics in *Tritonia diomedea*, *Melibe leonina*, *Flabellina iodinea*, *Hermissenda crassicornis*, and *Pleurobranchaea californica* (Lillvis et al., 2012). In Chapters 3 and 4 the electrophysiological, synaptic, and neuromodulatory properties of the C2 homologues and previously identified DSI homologues (Jing and Gillette, 1999; Tian et al., 2006; Newcomb and Katz, 2007) were investigated in *Tritonia*, *Hermissenda*, and *Pleurobranchaea*. These properties were not reported in *Melibe* and *Flabellina*. However, the response to a body wall nerve stimulus, resting membrane potential, resting activity, input resistance, C2-to-C2 electrical coupling properties, and C2-to-C2 synaptic connection were tested in *Melibe*. The resting activity was also tested in *Flabellina*. The results of those tests are documented here.

Methods

Animal collection, maintenance, and dissection

Melibe leonina and *Flabellina iodinea* individuals were obtained from Living Elements Ltd. (Delta, BC, Canada) or Monterey Abalone Company (Monterey Bay, CA, USA). Animals were maintained in artificial seawater (Instant Ocean) tanks at a fixed 12:12 light/dark cycle at 13 ± 1° C.

Melibe and *Flabellina* individuals were anesthetized by injection of 0.33 M $MgCl_2$ into the body cavity. A cut was made on the dorsal surface of the body wall near the buccal mass. The brain, consisting of the cerebral, pleural, and pedal ganglia, was removed by cutting all nerve

roots. The brain was transferred to a Sylgard-lined dish where it was superfused, at a rate of ~0.5 ml/min, with artificial saline (in mM): 420 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 11 D-glucose, and 10 HEPES, pH 7.5.

Connective tissue surrounding the brain was manually removed with forceps and fine scissors while maintaining a temperature of ~4 °C to reduce neuronal activity. The temperature was raised to 13-14° C for electrophysiological experiments.

Electrophysiology

Intracellular recordings were obtained using 10-80 MΩ glass microelectrodes filled with 3 M KCl connected to Axoclamp 2B amplifiers (Molecular Devices). Extracellular suction electrode recordings were obtained by drawing individual nerves into polyethylene tubing filled with artificial saline connected to an A-M Systems Differential AC Amplifier (model 1700, A-M Systems, Inc.). Both intra- and extracellular recordings were digitized (>1 kHz) with a 1401 Plus A/D converter from Cambridge Electronic Design.

In the isolated brain, body wall nerve stimulations were used to mimic swim inducing stimuli in the whole animal. The stimuli were varied in an effort to thoroughly test whether the DSI and C2 homologues were capable of producing the bursting activity that underlies dorsal-ventral swimming (1-5 s, 5-20 ms pulses, 5-20 Hz). Nerve stimuli were applied to Pedal Nerve 2 or 3 (PdN2/3) (Newcomb et al., 2006) in *Melibe*, which project to the dorsal body wall and reliably elicit swimming in *Tritonia* (Frost and Katz, 1996). This nerve is generically referred to a body wall nerve.

Input resistance tests were conducted in artificial saline. The neuron was monitored using a discontinuous current clamp (DCC) to ensure accurate membrane potential measurements without using two electrodes in the soma. Current steps (3 s, 1 nA to -5 nA) were applied and the change in membrane potential was measured. The current injected was plotted against

the corresponding change in membrane potential. Input resistance was calculated by measuring the slope of the linear regression line fit to the plotted data.

To test electrical coupling and synaptic connections, the bathing medium was switched to high divalent cation (HiDi) saline, which raises the threshold for spiking and reduces spontaneous neural firing. The composition of the HiDi saline was (in mM): 285 NaCl, 10 KCl, 25 $CaCl_2$, 125 $MgCl_2$, 11 D-glucose, and 10 HEPES (pH 7.5). To test electrical coupling, the presynaptic neuron was monitored using DCC to ensure accurate membrane potential measurements without using two electrodes in the soma. Current steps (5 s, 1 nA to -7 nA) were applied to the presynaptic neuron while the membrane potential was monitored in both pre- and postsynaptic neurons. The changes in pre- and corresponding postsynaptic membrane potential were plotted against each other. Coupling coefficients were calculated as the slope of the linear regression line fit to the plotted data. To test synaptic connections, depolarizing current pulses (10 to 20 ms pulses for 0.5 to 3 s at 10 to 20 Hz) were injected into the presynaptic neuron and the voltage response was measured in the postsynaptic neuron. The membrane potential of the postsynaptic neuron was hyperpolarized and depolarized to test for synaptic components that may be hidden at the resting membrane potential.

Neuron Identification

C2 neurons were identified preliminarily using soma position, size, and pigmentation (white). C2 is conspicuous and relatively easy to identify visually across species (Lillvis et al., 2012). In experiments where C2 identity was not certain, the soma was filled with the biotinylated tracer biocytin (2.5% dissolved in 0.75 M KCl) (Invitrogen) or Neurobiotin (2-4% dissolved in 0.75 M KCl) (Vector Laboratories) and processed for FMRFamide and/or Small Cardioactive Peptide B (SCP_B) immunoreactivity (see below) because the axon projection in conjunction with FMRFamide and SCP_B immunoreactivity uniquely identifies C2 across species (Lillvis et al.,

2012). Hereafter, C2 homologues will be referred to by the *Tritonia* name and will distinguish species using the subscripts of $_{Mel}$ and $_{Flab}$.

Biocytin or Neurobiotin processing and immunohistochemistry procedures were identical to those reported in Lillvis et al., 2012. Briefly, after fixation, brains were washed and incubated in one or more of the following primary antiserum: rabbit anti-FMRFamide (Immunostar) or anti-serotonin (Invitrogen) antiserum diluted 1:1000 and/or mouse monoclonal anti-SCP_B (courtesy of Stephen Kempf) diluted in 1:20 antiserum diluent (ASD). Streptavidin-Alexa Fluor 594 conjugate (1:50-1:200, Invitrogen) was also added to visualize the biotinylated tracer. Brains were then washed and incubated in goat anti-rabbit and/or goat anti-mouse antiserum conjugated to Alexa Fluor 488, Alexa Fluor 594 (Invitrogen), or DyLight 405 (Jackson ImmunoResearch) diluted 1:100 in ASD. Brains were then washed, dehydrated, and mounted on a slide to visualize soma immunohistochemistry and axon projection.

Data acquisition, analysis, and statistics

Data acquisition and analysis were performed with Spike2 software (Cambridge Electronic Design) and SigmaPlot (Jandel Scientific). Statistical comparisons were made using a one-way analysis of variance (ANOVA) with post-hoc pair wise multiple comparisons using the Holm-Sidak method for resting membrane potential measurements. The input resistance and electrical coupling data failed the Shapiro-Wilk test of normality. Therefore, the median values were compared and the non-parametric Dunn's post-hoc pairwise multiple comparison test was used. In all cases, $p<0.05$ was considered significant. Results are expressed as the mean ± standard error of the mean (SEM) unless stated otherwise.

Results

Electrophysiological properties of the $C2_{Mel}$ homologue in Melibe leonina

In *Melibe*, the resting membrane potential of $C2_{Mel}$ was -49.9 ± 2.17 (n=16). This value differed from the value of -43.1 ± 1.2 mV in *Hermissenda* ($p<0.05$), but was similar to the value of-45 ± 2.4 mV in *Tritonia* ($p>0.05$) and -45.2 ± 1.1 in *Pleurobranchaea* ($p>0.05$). The spontaneous activity of $C2_{Mel}$ differed in *Melibe* compared to all other species tested. As opposed to being quiescent at rest, $C2_{Mel}$ fired spontaneous action potentials at a rate between 0.5 and 1 Hz (n=15 of 18 preparations) (Figure A.1A). However, like the other species, the left and right $C2_{Mel}$ received common synaptic input and fired action potentials in synchrony (n=6) (Figure A.1A). The median input resistance of $C2_{Mel}$ was 46.33 MΩ (n=5). This value was significantly different from 15.16 MΩ *Pleurobranchaea* ($p<0.05$), but statistically similar to 30.58 MΩ in *Tritonia* ($p>0.05$) and 59.55 MΩ in *Hermissenda* ($p>0.05$).

As in the other species, the left and right $C2_{Mel}$ were electrically coupled. The median coupling coefficient in *Melibe* was 0.059 (n=4) (Figure A.1B). This was significantly different from the median coupling coefficient of 0.159 in *Pleurobranchaea* ($p<0.05$), but statistically similar to the median coefficient of0.026 in *Tritonia* and 0.081 in *Hermissenda*. Like the other species, stimulating one $C2_{Mel}$ excited the contralateral $C2_{Mel}$ (Figure A. 1C).

Melibe can swim using rhythmic left-right flexions, but not dorsal-ventral flexions. Therefore, as expected, $C2_{Mel}$ does not produce rhythmic bursts in response to a body wall nerve stimulus in the isolated *Melibe* brain (Figure A2). In *Tritonia*, *Hermissenda*, and *Pleurobranchaea* a body wall nerve stimulus elicited one or two prominent EPSPs or action potentials followed by a brief period of quiescence in C2 (Figure 3.1D-F). In *Melibe*, such prominent EPSPs or action potentials were generally absent in $C2_{Mel}$ (n=6 of 10 preparations). Instead, the $C2_{Mel}$ response to a body wall nerve stimulus was quiescence that halted the ongoing action potentials being fired at rest (n=8 of 10 preparations) (Figure A2). In the other 2 preparations, $C2_{Mel}$ was excited from the body wall nerve stimulus without the brief period of quiescence. In 5

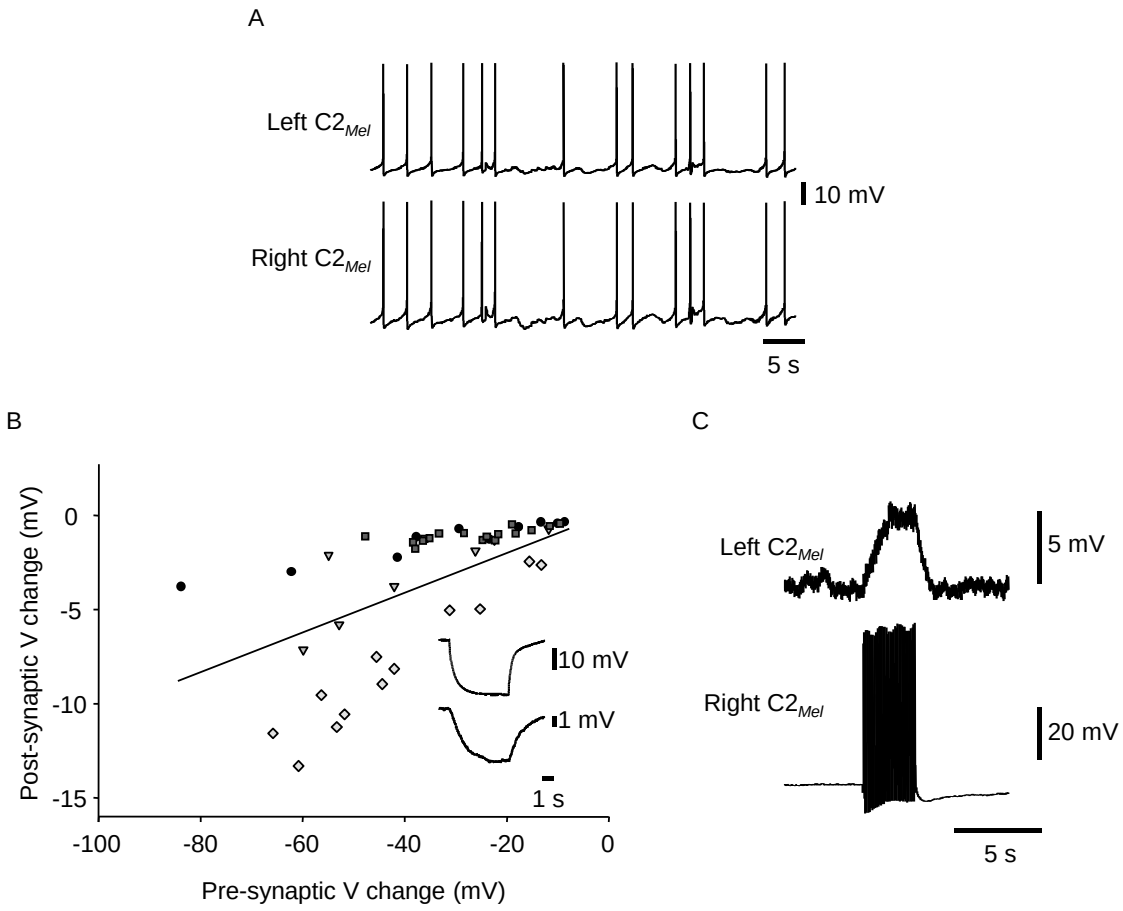

Figure A.1: Electrophysiological properties, electrical coupling, and synaptic connections of C2 in *Melibe*. A) C2 fired spontaneous action potentials in *Melibe*. The left and right receive common excitatory synaptic input and fire action potentials in synchrony. B) Electrical coupling relationships of the left and right C2 in each *Melibe*. Each plot shows the relationship between the membrane potential change of the presynaptic C2 and the corresponding change in the postsynaptic C2. Symbols represent different experiments, and the lines represent the linear regression from all experiments. Inset shows single examples of electrical coupling with similar presynaptic membrane potential changes on the top and the corresponding postsynaptic changes on the bottom. C) Stimulating the right C2 excited the left C2 in *Melibe*. Examples are representative of the population.

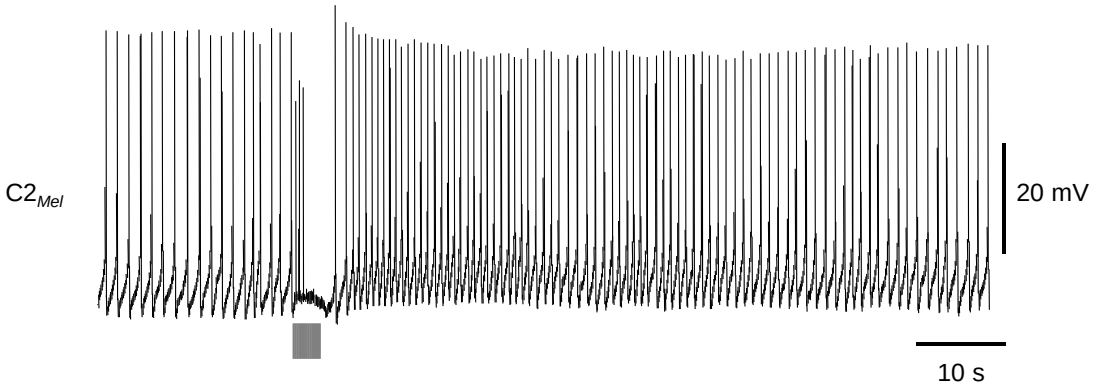

Figure A.2: C2 response to body wall nerve stimulation in *Melibe*. In the isolated brain, stimulating pedal nerve 2 (PdN2; grey bar) in *Melibe* caused a brief period of quiescence followed by higher frequency action potential firing (compared to before the stimulus. The example shown is representative of the population.

of 8 preparations where $C2_{Mel}$ was briefly quiescent, $C2_{Mel}$ was then excited to fire action potentials at a slightly higher rate than before the body wall nerve stimulus. In the remaining 3 preparations, the period of quiescence was followed by action potential firing at the same rate as before the stimulus.

Electrophysiological properties of the $C2_{Flab}$ homologue in Flabellina iodinea

In *Flabellina*, the only property of $C2_{Flab}$ that can be reported is that it was quiescent at rest and received spontaneous EPSPs (Figure A.3) (n=3). This is similar to the resting activity of *Tritonia* and *Hermissenda* (Figure 3.3A).

Discussion

Reasons for not reporting the Melibe and Flabellina data in Chapter 3

The *Melibe* data reported here was not reported in Chapter 3 simply because the data set is incomplete. $C2_{Mel}$ synapses with pedal ganglion neurons or DSI homologues were never found. Efforts to find synaptic connections with DSI homologues were not extensive. However, synaptic connections were sought with motor neurons in the contralateral pedal ganglion in a number of preparations with no success. Moreover, once the data sets with *Tritonia*, *Hermissenda*, and *Pleurobranchaea* became more compelling it seemed wise to focus on those species instead of working on additional experiments with *Melibe* or other species. Therefore, I do not doubt that synaptic connections with $C2_{Mel}$ could be found, but exhaustive efforts were not made. For this reason, it seemed best not to include the data in Chapter 3 which will be submitted for publication because it would open up question from reviewers about the missing synaptic data.

The data presented here in *Flabellina* is extremely limited. Working with *Flabellina* is challenging due mainly to the small size of the brain. Therefore, the experiments conducted in *Flabellina* consisted mainly of attempting to fill the $C2_{Flab}$ homologues and then to quickly fix the

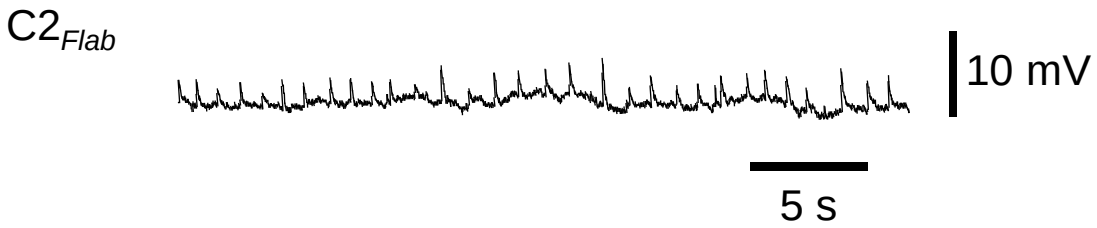

Figure A.3: Electrophysiological properties of C2 in *Flabellina*. A) C2 was quiescent at rest and received spontaneous EPSPs in *Flabellina*.

preparations for immunohistochemistry before the $C2_{Flab}$ membrane was damaged. Thus, the only results that can be reported with confidence are the resting activity results reported here.

Melibe properties compared to other species

Figure A.3 shows the properties of the DSI and C2 homologues in all five species in relation to phylogeny. Generally, *Melibe* and *Flabellina* show C2 and DSI properties that align with phylogeny, rather than swim behavior. Although $C2_{Mel}$ shows difference in its spontaneous activity and response to a body wall nerve stimulus compared to other species, these differences do not correlate with phylogeny or behavior. It is of interest to determine why C2 may be so different in *Melibe* compared to other species. One possible reason is that *Melibe* feeds differently than the other species investigated (Newcomb, 2008); *Melibe* tends to act as a passive feeder as opposed to the more active predatory feeding behaviors of the other species investigated. Given that $C2_{Pleur}$ plays a role in feeding in *Pleurobranchaea* (Jing and Gillette, 1995), it is possible that C2 also plays a role in feeding in other species. If so, it is possible that the properties of $C2_{Mel}$ that differ from other species are better suited for the feeding strategy employed by *Melibe* than the C2 properties found in the other species investigated . Additional experiments are necessary to test this hypothesis.

Summary

In summary, the results demonstrate additional differences in C2 among related species. However, these differences cannot help explain why some species can swim with a dorsal-ventral swim while others cannot. This is because the differences in *Melibe* compared to the dorsal-ventral swimmers are also different than *Hermissenda* and *Flabellina*, two other species that do not express a dorsal-ventral swim. Additional experiments are required to understand why certain properties of C2 may differ in *Melibe* compared to the other species.

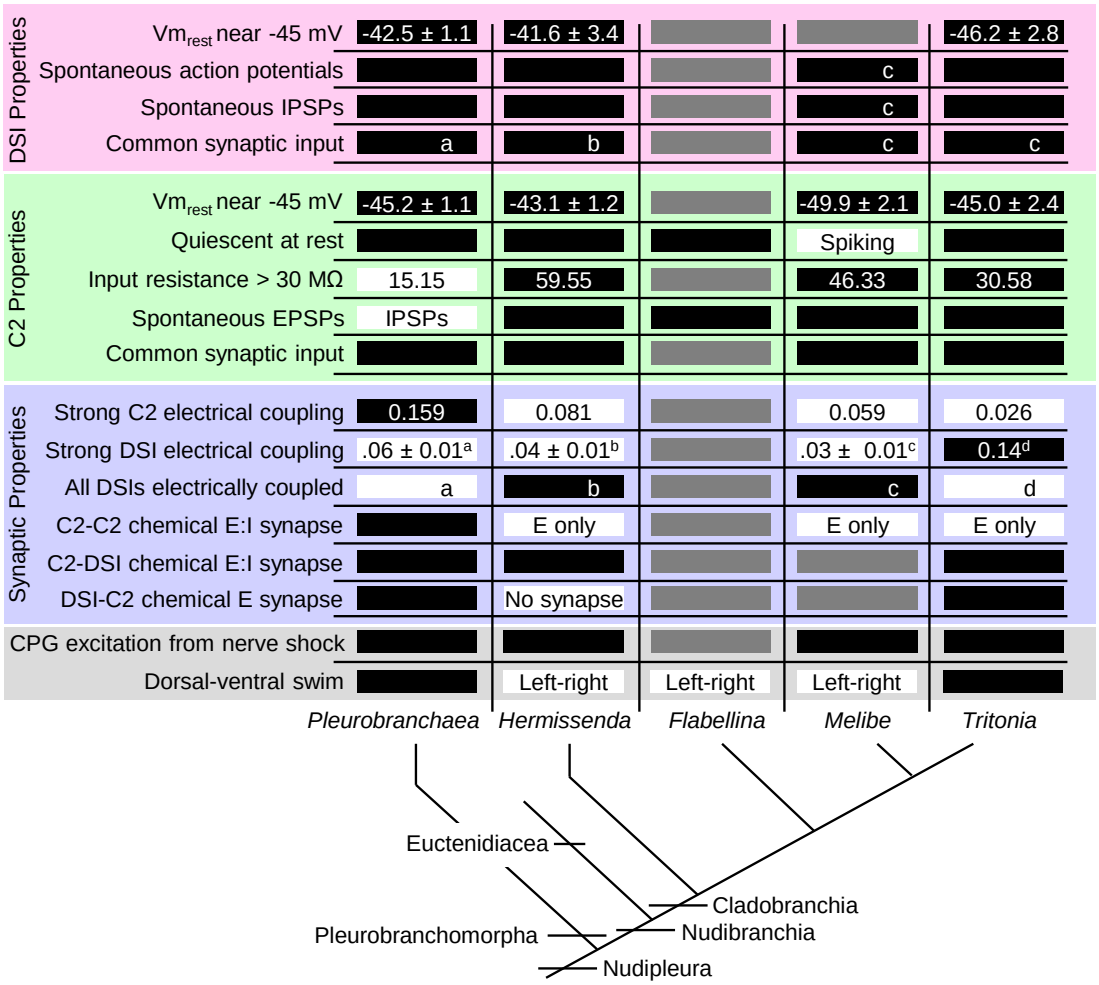

Figure A.4: Summary of results in relation to phylogeny. Black boxes indicate the presence of the characteristic to the left. White boxes indicate absence of the characteristic. Grey boxes indicate the characteristic was untested. Where values were measured, they are indicated in the boxes. The results show C2 electrophysiological properties and synaptic connections that correlated with phylogeny and synaptic connections that correlated with behavior. *Tritonia*, Melibe, Flabellina, and *Hermissenda* are members of the monophyletic clade Cladobranchia. *Pleurobranchaea* is not, but is a member of the monophyletic Nudipleura clade with the other species. References: a, (Jing and Gillette, 1999); b, (Tian et al., 2006); c, (Newcomb and Katz, 2007); d, (Getting, 1981).

APPENDIX B: NEUROPEPTIDE EXPRESSION IN THE *TRITONIA DIOMEDEA* BRAIN

Introduction

The CPG underlying the dorsal-ventral swim of *Tritonia* is composed of three neuron types named DSI, C2, and VSI (Katz, 2009). Previous evidence has demonstrated that DSI is serotonergic (Katz et al., 1994b). This knowledge greatly aided the identification of DSI homologues across species that cannot produce a dorsal-ventral swim (Newcomb and Katz, 2007). Evidence has shown that C2 uses one or more neuropeptide transmitters (Snow, 1982b), but the identity of the neuropeptide content of C2 is unknown. In an effort to determine the neuropeptide content of C2, an expressed sequence tag (EST) library of the *Tritonia* brain was previously constructed (available on NCBI). This EST database was searched for candidate signaling neuropeptide sequences that could be present in C2. Candidate neuropeptide genes were cloned and probes were constructed for in situ hybridization experiments. Ultimately, the experiments did not identify the neuropeptide contents of C2. However, the experiments did provide a glimpse of the expression patterns of several neuropeptides in the *Tritonia* brain. These experiments are described here.

Methods

Methods summary

First, a cDNA library of the *Tritonia* brain was generated. To do this, RNA was isolated from the brain of *Tritonia* and made into a cDNA library representing the brain genome. This cDNA library was the source DNA material used to clone the candidate neuropeptide genes chosen. The neuropeptide clones were converted into an RNA probe. Whole brain *in situ* hybrid-

ization experiments were then run to identify the expression patterns of the chosen neuropeptides.

RNA isolation

An Ambion RNAqueous Isolation Kit (Invitrogen) was used for this procedure. The kit supplied the reagents described and most of the steps detailed followed the kit instructions. The whole brain was removed from *Tritonia* and placed into 350 µl of Lysis Buffer to disrupt the tissue. The solution was mixed to dissolve the connective tissue. Next, 350 µl of 64% EtOH was added to the lysate and mixed. This 700 µl solution was then transferred to a filter cartridge and centrifuged for 1 min at 14,000 rpm. All of the centrifugation steps are for 1 min at 14,000 rpm unless noted otherwise. The flow through was discarded and the filter cartridge was reinserted into the tube. 700 µl of Wash Solution #1 were added to the filter and centrifuged. Flow through was again discarded. 500 µl of Wash Solution #2/3 were added and centrifuged. The flow through was discarded and this step was repeated. The filter cartridge was then placed into a clean collection tube and 40 µl of Elution Solution (preheated to 75°C) was added to the center of the filter and centrifuged. An additional 10 µl of Elution Solution was added and the tube was centrifuged once more yielding isolated RNA in eluate form. The content of the eluate was verified using argarose gel electrophoresis.

cDNA library creation

A SMART PCR cDNA Synthesis Kit (Clontech) was used for the library creation procedure and provided the reagents described. Isolated RNA was reverse transcribed using the reverse transcriptase primer TRsa. 2 µl of this primer was added to 6 µl of RNA, and 2 µl of BD SMART II A oligonucleotides. The tube was mixed and spun in a centrifuge briefly. The tube was then incubated for 2 min at 72 °C and cooled on ice for an additional 2 min. Next, 4 µl of 5x First-Strand Buffer, 2 µl of dithiothreitol (DTT), 2 µl of dNTP Mix, and 2 µl of BD PowerScript

Reverse Transcriptase were added to the cDNA. The tubes were incubated for 1 hour at 42°C to make a single strand cDNA (ss cDNA).

The ss cDNA was then amplified using PCR. The PCR solution consisted of 31 µl of dIH2O, 5 µl of 10x BD Advantage 2 PCR Buffer, 1 µl TRsa, 1 µl 50x dNTP Mix, 1 µl of 5' PCR Primer II A, and 1 µl of 50x BD Advantage 2 Polymerase Mix. This reaction mixture was placed into a thermal cycler with the following parameters: 95 °C for 30 s, and then approximately 18 cycles at: 95 °C for 30 s, 65 °C for 30 s, and 68 °C for 3 min. The content of the reaction was verified via argarose gel electrophoresis.

EST database and analysis

Previously, The University of Florida sequencing core sequenced and annotated a 10,000 EST library. This library was searched for signaling neuropeptide sequences. Several candidate neuropeptides were selected for further investigation. These neuropeptide gene sequences included: *apgwamide*, *clionin*, *fmrfamide*, *myomodulin*, *pleurin*, *small cardioactive peptide* (*scp*), and an unnamed peptide found in the *Aplysia* R3 and R14 neurons that we called *aplysia r3, r14 homologue*. Additionally, *acetylcholine binding protein* (*achbp*) which is not a known signal neuropeptide was investigated.

Primer design

Amino acid sequences from the EST library were used to design degenerate primers used to isolate the sequence of interest for cloning. *Clionin* and *achbp* in *Tritonia* were previously cloned. Here *apgwamide*, *aplysia r3, r14 homologue*, *fmrfamide*, *myomodulin*, *pleurin*, and *scp* were cloned. The following degenerate primers were used for gene isolation.

apgwamide

Forward: 5’-GAGAAACCACAATGCGCCTAACG-3’

Reverse: 5’-GCACAAGACATCCACTTGTCCTCA-3’

aplysia r3/r14 homologue

Forward: 5’-CATCAAGTTCCACACAAAACCCTGC-3’

Reverse: 5’-CATCCATAGACCCTGCGTTC-3’

fmrfamide

Forward: 5’-CTACCGTTGGTGACAT-3’

Reverse: 5’-TCCATGCATAAACCG-3’

myomodulin

Forward: 5’-CATCTTCATCCTCGTCATCATATCAGC-3’

Reverse: 5’-TGCGCCCTAGACACCAAACAAGAG-3’

pleurin

Forward: 5’-ACTCTACCACAATGTACCAAATCC-3’

Reverse: 5’-GCTAGCAGAGAAGATTTTGTTCCA-3’

scp

Forward: 5’-ACCATGGAAATGACAATGCCCCGA-3’

Reverse: 5’-GGCAATAATAATTAGTATTATGGTGCCGGG-3’

Gene Isolation

A reaction mixture consisting of 1 µl of LAtaq to 5 µl of 10x buffer (Takara), 8 µl dNTPs (Takara), 1 µl of our cDNA library, 1 µl of the forward primer, 1 µl of the reverse primer, and 33 µl dIH20 was made. This reaction mixture was then run for 30 PCR cycles at the following temperatures: 94 ºC for 30 s, selected annealing temperature for 30 s, and 72 ºC for 1 min.

The PCR product was then run on a 1% agarose gel and the band of the gene of interest was isolated. Using the QIA quick Gel Extraction Kit (Qiagen), 500 µl of Buffer QG was added into the tube and incubated at 55 ºC for 10 min. The dissolved solution was then transferred to a QIAquick column and centrifuged for 1 min at 13000 rpm. After discarding the flow through, another 500 µl of Buffer QG was added. The tube was then centrifuged for 1 min, and the flow though was discarded. Next, 750 µl of Buffer PE was added, the tube was centrifuged for 1 min, and the flow through was discarded. The tube was then centrifuged for 1 additional min. The column was placed into a clean tube and 30 µl of water were added to the center of the column to elute the product. The column sat for 1 min and was then centrifuged for 1 min. The product was the isolated gene of interest.

Gene Insertion into Plasmid Vector

For the vector creation and insertion processes a TOPO TA Cloning Kit (Invitrogen) was used. A solution consisting of 4 µl of the isolated gene product, 1 µl of Salt Solution, and 1 µl pCR 4-TOPO was made. This was incubated for 5 – 10 min at room temperature before being added competent *E. coli* cells.

Cloning

3 µl of the plasmid vector solution were added to a vial of TOPO One Shot Competent Cells (Invitrogen). The reaction was chilled on ice for approximately 10 min. The cells were then heat shocked in a 42 ºC water bath for exactly 30 s. After heat shocking, which corresponds to

transformation, the tube was immediately placed back on ice. Next, 250 µl of S.O.C. medium (Cellgro) was added to the solution. The tube was then placed into a 37 ºC shaker for 1 hour at 200 rpm. After 1 hour of shaking, the cells were poured onto warmed (37 ºC) Luria Bertani Broth/Carbenicillin (LB/carb) (50 µg carbenicillin per ml of LB broth) plates, spread evenly, and incubated overnight at 37 ºC.

The following day three to four single colonies were removed from the LB/carb plates. Each colony was placed into a glass test tube containing 4 ml of LB/carb (50 ml/50 µl). The tubes were covered and placed into a 37 ºC shaker at 200 rpm overnight.

A QIAprep Spin Miniprep Kit (Qiagen) was used to isolate the clones from the growing medium. The next day, the contents of the test tube were poured into a microcentrifuge tube and centrifuged for 1 min at 13,000 rpm (all future centrifuge steps were at 13,000 rpm unless noted otherwise). The supernatant was discarded and the pellet formed at the bottom of the tube was kept. 250 µl of Buffer P1 was added to resuspend the pellet. After resuspension, 250 µl of Buffer P2 was added and the tube was gently mixed. Then 350 µl of buffer N3 was added and the tube was quickly mixed. This reaction was centrifuged for 10 min. The supernatant left after spinning was transferred to a spin column and centrifuged for 1 min. The flow through was discarded and the column was washed with 500 µl of Buffer PB and centrifuged for 1 min. 750 µl of buffer PE are then added and the column was centrifuged. Flow through is discarded and the tube is centrifuged for 1 additional min. Finally, the cloned plasmid DNA was eluted with 30 – 50 µl of dIH20, depending on size of pellet obtained after previously centrifuging the LB/carb colony. The clones were then sent out for sequencing to confirm identity.

Plasmid Linearization

Once the cloned sequence was confirmed, the plasmid was linearized. If the gene sequence was inserted in the forward direction, Not 1 was used as the restriction enzyme with the associated Buffer 3 (Roche). If inserted in the reverse direction, Pme 1 and Buffer 4 (Roche)

were used. The reaction mixture was as follows: 25 µl plasmid DNA, 0.3 µl bovine serum albumin (BSA) (Sigma), 3 µl of the appropriate buffer, and 2 µl of the appropriate restriction enzyme. The reaction was incubated for 2 hours at 37 ºC. Plasmid linearization was confirmed via agarose gel electrophoresis.

If the plasmid appeared to be linearized, excess plasmid material was cleaned and the sequence was isolated. This was done using the QIAquick PCR Purification Kit (Qiagen). The remaining cut plasmid DNA was added to a tube and a 5:1 volume of Buffer PBI to DNA was added. This mixture was placed into a spin column and centrifuged for 1 min at near 10,000 rcf. The flow through was discarded and the column was washed with 750 µl of Buffer PE. This was then centrifuged for 1 min twice. The linearized sequence was eluted with 30 µl of elution buffer. The column sat for 1 min and then was centrifuged for 1 min. This gave the linearized sequence of the peptide gene of interest.

Probe Creation

Once the plasmid was cut and cleaned, digoxigenin (DIG) labeled RNA antisense probes were constructed. The polymerase added was based on which enzyme was used to cut the plasmid. If cut with Not 1, the T3 RNA polymerase (Roche) was used. If cut with Pme 1, the T7 RNA polymerase (Roche) was used. The whole reaction was as follows: 2 µl of the appropriate polymerase, 2 µl DIG RNA labeling (Roche), 2 µl 10x Buffer, 1 µl RNasin, and 13 µl of DNA. This reaction was incubated for 2 hours at 37 ºC. RNA probe contents and concentration were assessed using a bioanalyzer.

Animal dissection and brain removal

Tritonia individuals were anesthetized by cooling. A cut was made on the dorsal surface of the body wall near the buccal mass. The brain, consisting of the cerebral, pleural, and pedal, and buccal ganglia, was removed by cutting all nerve roots. The brain was either fixed immedi-

ately (see below) or transferred to a Sylgard-lined dish where it was superfused, at a rate of ~0.5 ml/min, with artificial saline (in mM): 420 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 11 D-glucose, and 10 HEPES, pH 7.5.

Connective tissue surrounding the brain was manually removed with forceps and fine scissors while maintaining a temperature of ~4 °C to reduce neuronal activity. The temperature was raised to 10-11° C for electrophysiological experiments.

Lucifer yellow injection

In some cases, the C2 soma was filled with the fluorescent dye Lucifer yellow (LY). In those cases, the C2 soma was impaled with a 10-50 MΩ microelectrode filled with 4% LY in 0.1% LiCl. Hyperpolarizing current pulses (1-10 nA, 50% duty cycle) were used to inject the soma with LY. After filling the preparations were fixed immediately (see below).

In Situ Hybridization

The brain was fixed immediately after LY injection or removal from the animal in 4% formaldehyde (Fisher) in 1x PBS (Fisher) for approximately 24 hours. After fixation, the brain was washed in 1x PBS for 5 min three times. The brain was then desheathed if it had not been desheathed previously. After desheathing, the brain was washed in PTW (0.1% Tween 20 in 1x PBS) (Fisher) for 10 min. Next, the brain was washed for 10 min three times. The first wash was a 3:1 PTW and methanol mixture, followed by a 1:1 mixture, and then a 1:3 mixture. The brain was then placed into 100% methanol for 5 min or stored in 100% methanol for up to 2 weeks at -20ºC.

After methanol treatment, the brain was transferred through three more 10 min PTW and methanol washes: a 1:3 mixture, a 1:1 mixture, and a 3:1 mixture. The brain was then washed in PTW for 10 min, 0.3% Triton X 100 (Fisher) for 10 min, and PTW for 5 more min. The brain was then incubated in 10 µg of proteinase K (New England Biolabs) per 1 ml of PTW for up to

an hour at room temperature. The incubation time varied with brain size. Next, the brain was incubated in 4% formaldehyde in 1x PBS at 4 ºC for 20 min. After fixation the brain was processed through to two quick washes of 2 mg glycine (Sigma) per 1 ml of PTW followed by 3 washes in PTW. The brain was then washed twice quickly in TEA HCl (pH: 8.0) (Sigma) before two 5 min washes in 1 ml of TEA HCl with 2.5 µl of acetic anhydride (Sigma). Next the brain was washed in four quick washes of PTW before incubating in hybridization buffer overnight at -20 ºC. Hybridization buffer was composed of 50% formamide (Sigma), 5 mM EDTA (Invitrogen), 5x SSC (Roche), 1x Denhardt's solution (USB), 0.1% Tween 20, and 0.5 mg/ml yeast tRNA (Invitrogen).

The following day, the brain was placed into hybridization buffer at 50 ºC for 6 – 8 hours on a shaker. Next, the brain was placed into hybridization buffer with the DIG labeled RNA probes (1-4 µl/mL of hybridization buffer) for 12 to 14 hours at 50ºC on a shaker. After the incubation, the brain was washed in a solution composed of 50% formamide, 5x SSC, and 1% SDS (USB) at 60ºC for 30 min on a shaker. Next, it was shaken for another 30 min at 60ºC, this time in a solution composed of 50% formamide, 2x SSC, and 1% SDS. The brain was then washed twice in 0.2x SSC for 30 min at 55ºC on a shaker. This was followed by four quick washes in PBT. The brain was then incubated in 10% normal goat serum (Sigma) in PBT for 60 to 90 min at 4 ºC on a shaker. The brain was then incubated in 0.5 µl of alkaline phosphatase-conjugated DIG antibodies (Roche) for every 1 ml of 1% goat serum in PBT on a shaker at 4 ºC for 12 to 14 hours.

The next day, the brain was washed in PBT for 30 min four to eight times at room temperature. The brain was then washed in detection buffer (100 mM NaCl, 50 mM MgCl2, 0.1% Tween 20, 1 mM Tetramisol HCl (Sigma), and 100 mM Tris HCl at a pH of 9.5) twice for 5 min before development. Finally, the brain was placed into 20 µl of NBT/BICP solution (Roche) to 1 ml of detection buffer in the dark on ice. Once it appeared as though the cells were fully developed and the background staining was rising, the brain was placed into 4% formaldehyde in

methanol at 4 ºC on ice for 1 hour. The brain was then washed in 100% ethanol twice for 10 min. The brain was stored in 100% ethanol at 4 ºC or cleared in methyl salicylate and mounted on a slide in Cytoseal.

In instances where cells were injected with 4% LY, some *in situ* hybridization steps were modified to ensure that the LY fluorescence would be maintained. These alterations did not change the expression patterns observed. The alterations were as follows. 1) The duration of Proteinase K incubation was always 1 hour. In many preparations this time was reduced, but it was always 1 hour here. 2) The post-Proteinase K, 4% formaldehyde fixation was extended from 20 min to 1 hour. 3) The prehybridization period in hybridization buffer at 50 ºC for 6-8 hours was reduced to 2 hours. 4) The hybridization period (DIG-RNA probes in hybridization buffer at 50 ºC for 12-14 hours) was reduced to 3 hours.

Results

We chose to use *in situ* hybridization to screen the *Tritonia* CNS for 7 different peptides found in the EST database. These peptides were chosen because of their documented function as signaling peptides in other systems and because close to full length if not full-length sequences were found in the EST library. The peptides cloned were APGWamide, Clionin, FMRFamide, Myomodulin, Pleurin, SCP, and the *Aplysia R3, R14 homologue*. Additionally, we investigated AChBP expression.

APGWamide

The full-length *apgwamide* sequence in *Tritonia* was cloned successfully and the sequence can be found in the NCBI database (NCBI: EV289979.1). An RNA probe was constructed from the clone and used for *in situ* hybridization (Figure B.1). Five preparations were used for *in situ* hybridization with similar results. There was strong symmetrical staining in the dorsal cer-

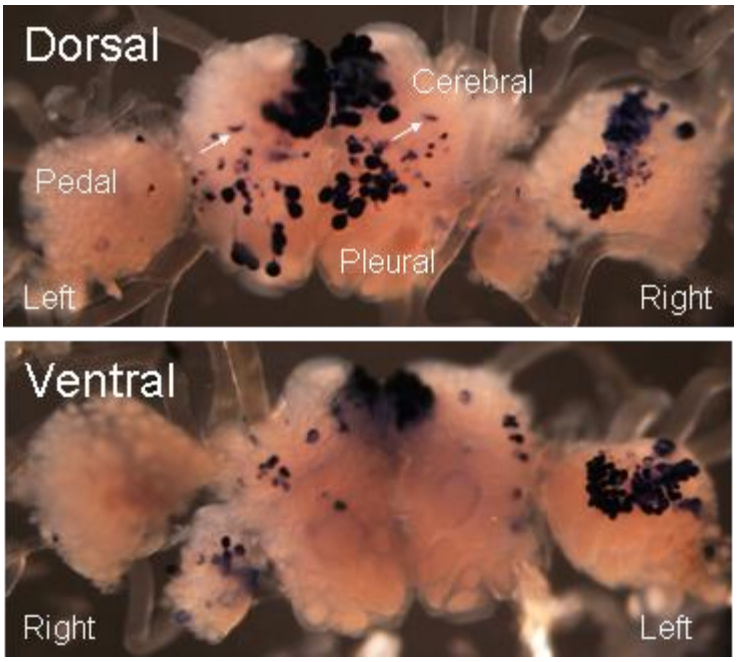

Figure B.1: APGWamide expression in *Tritonia*. Dorsal) APGWamide shows generally symmetrical expression in the cerebral and pleural ganglion. The differences apparent here appear to be due to differences in soma position in the left and right side. There are two asymmetrical clusters of neurons in the right pedal ganglion. There is also some staining near the C2 region in the cerebral ganglion (white arrows). Ventral) Staining is comparatively reduced on the ventral side of the brain compared to the dorsal. There are two asymmetric clusters in the left pedal ganglion.

ebral/pleural ganglia. Although there was no pronounced staining in the C2 region of the cerebral ganglion, there was some staining in the region that could be C2. However, it was not verified whether or not C2 expresses APGWamide. There were also two strongly stained clusters of neurons in the dorsal right pedal ganglion that were absent on the left side of the brain. On the ventral side of the brain, there was roughly symmetrical staining in the cerebral ganglia. There were also two strongly stained clusters of neurons in the ventral left pedal ganglion that were absent on the right side of the brain. Thus, there were asymmetrical clusters of APGWamide positive neurons in the pedal ganglia on the dorsal and ventral side of the brain. This is particularly intriguing in that APGWamide immunoreactivity was found only in the right pedal ganglion in *Aplysia californica* (Fan et al., 1997). Here we see an asymmetry, but that asymmetry is also present in reverse on the ventral side of the brain. There are also one or two neurons that appear to be stained in the buccal ganglion (data not shown).

Clionin

The full-length *clionin* sequence in *Tritonia* was cloned successfully and synthesized into an RNA probe for *in situ* hybridization. The *Tritonia clionin* sequence can be found on the NCBI database (NCBI: EV286941.1). Two preparations were used for *in situ* hybridization with similar results (Figure B.2). The staining for this peptide appeared relatively symmetrical on the dorsal side of the brain. There is sparse staining in the cerebral ganglia but no apparent staining in the pleural ganglia. One stained cluster of cells in the cerebral ganglion is near the C2 region. We did not do any further testing to confirm or deny whether C2 expresses Clionin. A large cluster of cells was also stained in the left and right pedal ganglia. This region of the pedal ganglion has also been shown to stain intensely for serotonin immunoreactivity in *Tritonia* (Fickbohm et al., 2001). The ventral cerebral and pleural ganglia stain for a greater number of cells which are scattered throughout the region. A single large neuron is asymmetrically stained in the ventral right pedal ganglion. Three cells in the buccal are symmetrically stained as well.

Figure B.2: Clionin expression in *Tritonia*. Dorsal) Clionin shows generally symmetrical expression in the cerebral ganglion, including some staining in the C2 soma region (white arrows). There is a symmetrical staining in the pedal ganglia. Ventral) There is comparatively more cerebral ganglia staining on the ventral side of the brain. There is also a prominent asymmetrically stained neuron in the right pedal ganglion.

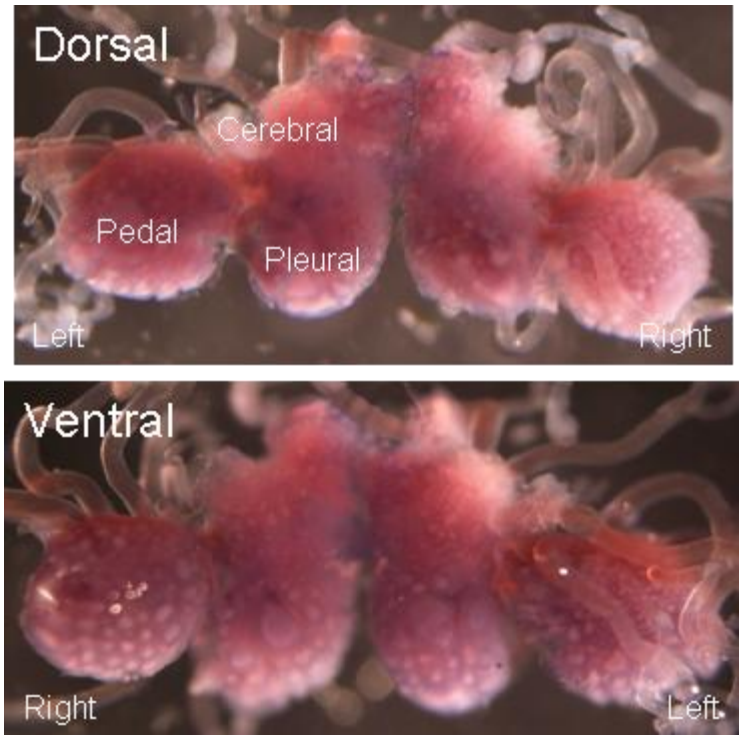

Figure B.3: Myomodulin expression in *Tritonia*. Specific staining was not observed with the Myomodulin probe.

Myomodulin

The full-length *myomodulin* sequence iin *Tritonia* was cloned successfully (NCBI: EV285663.1) and synthesized into an RNA probe for *in situ* hybridization. Three preparations were used for *in situ* hybridization. Unfortunately, the probe was rather weak in concentration. This could be due to a number of factors that may have occurred during the cloning process or during probe synthesis. As a result, we had to incubate the preparations in detection buffer for a longer period which caused high background staining. Making out which cells were stained for myomodulin is difficult (Figure B.3). It appears as though a number of cells were stained around the perimeter of the cerebral and pleural ganglia but any conclusions based on these preparations are far from definitive.

Pleurin

The full-length *pleurin* sequence in *Tritonia* was cloned successfully (NCBI: EV289023.1) and synthesized into a RNA probe for *in situ* hybridization. Three preparations were used for *in situ* hybridization, all with similar results (Figure B.4). The dorsal side of the CNS exhibited 4-5 symmetrically stained cells in the cerebral ganglion. These cells do not appear to be in the region of C2 in the cerebral ganglion. In the right dorsal pedal ganglion, 1-3 cells were stained in the medial portion of the ganglion, while the left pedal ganglion appeared to contain no stained cells. The ventral cerebral ganglion showed symmetrical staining of two cells. The ventral pedal ganglia exhibited somewhat symmetrical staining of a small cluster of approximately 3 cells. The buccal ganglion exhibited symmetrical staining of a cluster of cells on the dorsal side and one or two single cells on the ventral side (data not shown).

Aplysia R3/R14 Peptide Homologue

The R3/R14 cells in the abdominal ganglion of *Aplysia* have been demonstrated to control cardiovascular activity (Nabu et al., 1983). The R3/R14 peptide has been documented only

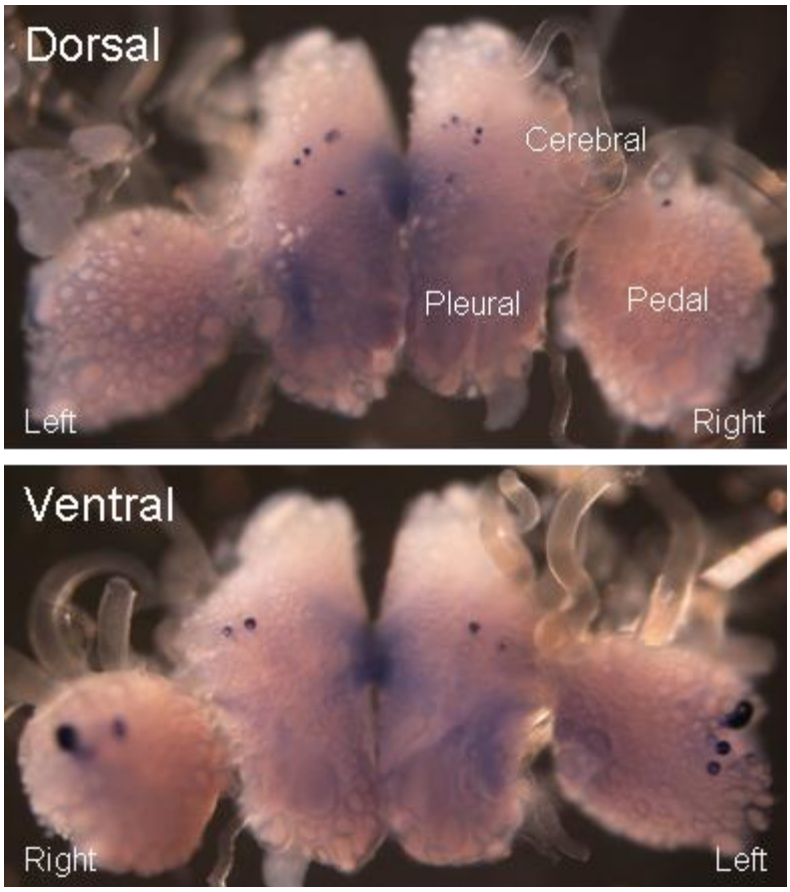

Figure B.4: Pleurin expression in *Tritonia*. Dorsal) There is sparse symmetrical Pleurin staining in the cerebral ganglia. The cell bodies stained are too medial in the ganglia to be C2. Ventral) There is sparse symmetrical staining in the cerebral and pedal ganglia.

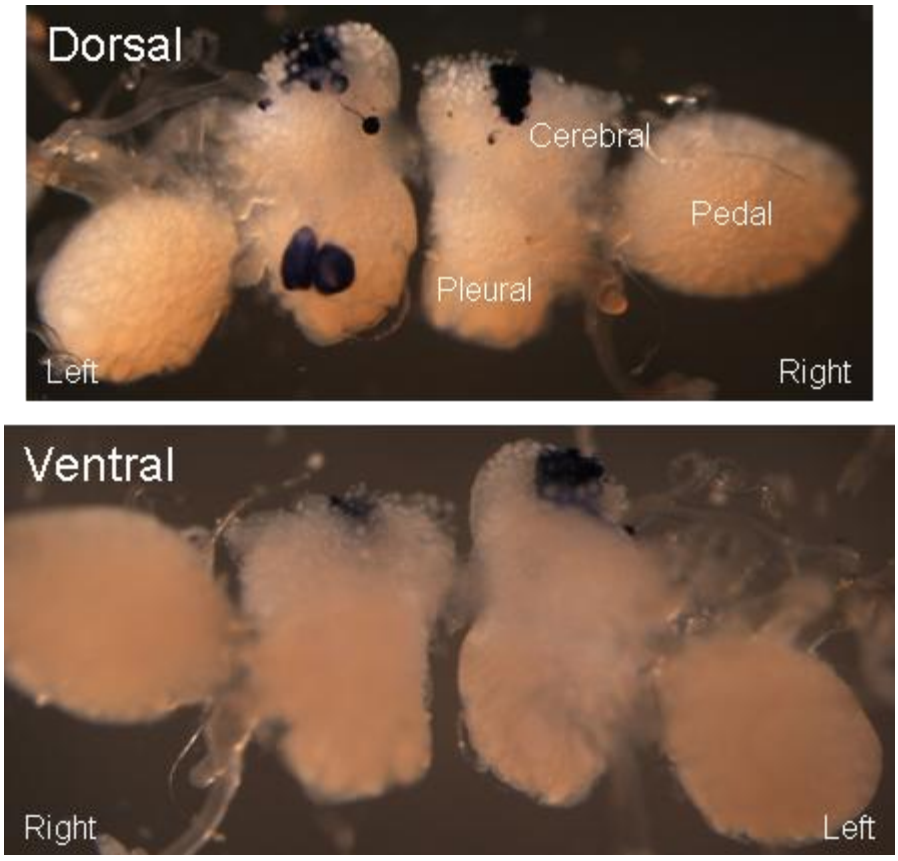

Figure B.5: *Aplysia* R3/R14 homologue expression in *Tritonia*. Dorsal) There is symmetrical staining in the anterior portion of the cerebral ganglia. There are two prominent asymmetrically stained neurons in the left pleural ganglion. Ventral) There is some staining in the cerebral ganglion in the same region as the staining on the dorsal side.

in these cells in *Aplysia* (Kreiner et al., 1984). *Tritonia* does not have an abdominal ganglion, so it was of interest to see where this peptide would be found in the *Tritonia* brain.

The full-length a*plysia r3/314 homologue* sequence in *Tritonia* was cloned successfully (NCBI: EV284612.1) and synthesized into a RNA probe for *in situ* hybridization. Two preparations were used for *in situ* hybridization. The peptide showed distinct expression patterns (Figure B.5). It was found in two large cells in the dorsal left pleural ganglion. It is possible that these cells are homologous to R3 and R14. It also stained symmetrical clusters in the rostral portion of the dorsal cerebral ganglion. There was little or no staining on the ventral side of the brain. There is some staining in the cerebral ganglion on the ventral side of the brain in a similar position to the clusters in the cerebral ganglion on the dorsal side. There was no expression in the buccal ganglion (data not shown).

Acetylcholine Binding Protein

Glial cells make AChBP via release of acetylcholine from nearby neurons. The protein regulates synaptic efficacy of cholinergic cells (Sixma and Smit, 2003). Because the protein should only be expressed in glial cells, in situ hybridization for this transcript promised to show us something that has yet to be viewed: glia in *Tritonia*.

The full-length *achbp* sequence in *Tritonia* was previously cloned (NCBI: EV289393.1). Here the clone was synthesized into a RNA probe for *in situ* hybridization. Five preparations were used for *in situ* hybridization and the results appear similar across all preparations. As expected, AChBP stained heavily in the neuropil, with little to no staining found within cell bodies (Figure B.6).

FMRFamide

Our experiments with FMRFamide immunohistochemistry have shown that C2 is FMRFamide immunoreactive (Chapter 2 of this dissertation; Lillvis et al., 2012). A partial sequence of

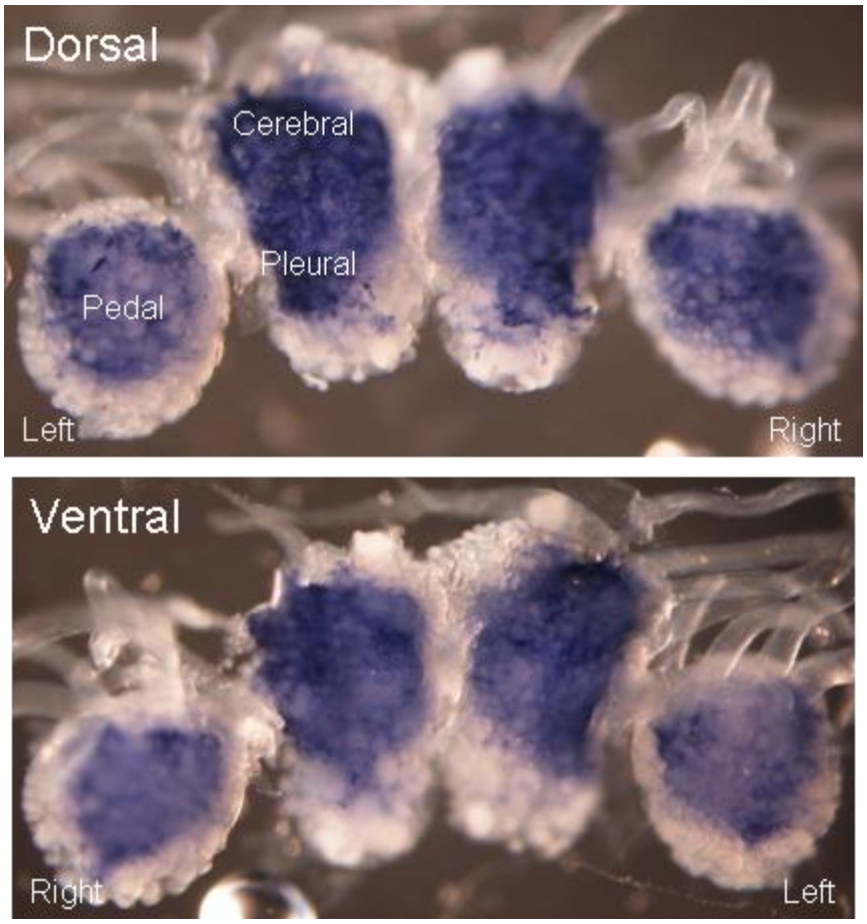

Figure B.6: AChBP expression in *Tritonia*. AChBP appears to be expressed exclusively in the neuropil. This may represent glial staining.

fmrfamide in *Tritonia* was cloned successfully (EV286865.1) and synthesized into a RNA probe for *in situ* hybridization.

Generally, we had difficulty seeing staining from the probes made for FMRFamide (Figure A.7A). This difficulty may be due to the probe not being constructed from a full-length sequence. In nine preparations, we found sparse, apparently symmetrical staining on the dorsal side of the pedal ganglia. There was no apparent staining on the dorsal side of the cerebral or pleural ganglia. On the ventral side of the brain, there were 2-5 cells stained in each pleural ganglion. Furthermore, we filled C2 with LY and tested FMRFamide expression and confirmed that C2 did not express FMRFamide (Figure B.7, inset). There were distinct clusters of symmetrical staining in the buccal ganglia (data not shown).

Small Cardiac Peptide

Our experiments with SCP_B immunohistochemistry have shown that C2 is SCP_B immunoreactive (Chapter 2 of this dissertation; Lillvis et al., 2012). A full-length *scp* sequence in *Tritonia* was cloned (EV290162.1) and a probe was made for *in situ* hybridization of twelve preparations (Figure B.8). It was not clear from the sequence information and annotations whether this *SCP* was SCP_A or SCP_B.

There was one strongly stained symmetrical cluster in the dorsal cerebral ganglion. This staining was near the C2 region of the cerebral ganglion. However, we filled C2 with LY and tested SCP expression and found that C2 did not express SCP (Figure B.8, inset). There were also some large cells in the pleural ganglia that expressed the peptide. The dorsal right pedal expressed some asymmetrical staining as compared to the left pedal. Many axons also showed a great deal of SCP expression. The ventral cerebral and pleural ganglia are similar in expression to the dorsal, containing 2 – 3 symmetrical clusters showing expression. The ventral side of the right cerebral and right pedal ganglia demonstrated two asymmetrical clusters that stained strongly for SCP. The buccal ganglion was heavily stained in a symmetrical fashion as expected

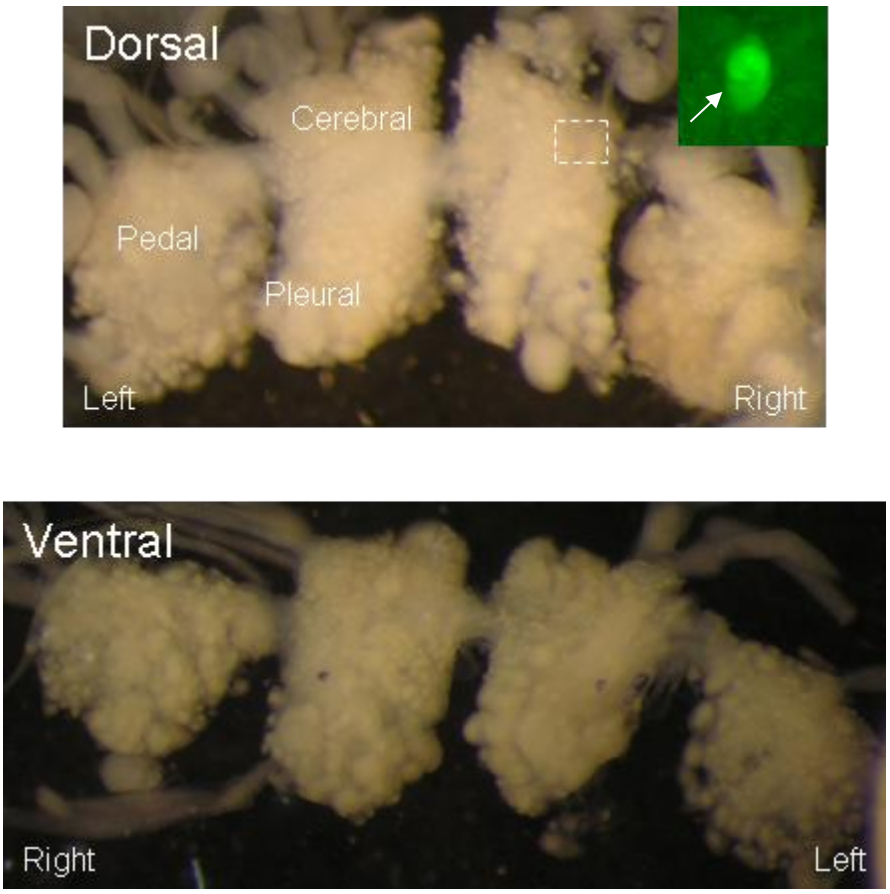

Figure B.7: FMRFamide expression in *Tritonia*. Dorsal) There is some symmetrical staining in the pedal ganglia, though it cannot be seen here. Inset) C2 was filled with LY (white arrow) and does not express FMRFamide. This staining is in neurons just under the surface. Ventral) There is symmetrical staining of 2-3 neurons in the pleural ganglia.

due to the involvement of SCP_B in feeding in other species (e.g., Hurwitz et al., 2000) (data not shown).

Discussion

We were able to successfully clone full-length sequences of six signaling neuropeptides and examine their expression patterns in the *Tritonia* brain. Additionally, we cloned a partial *fmr-famide* sequence and examined the expression of AChBP in *Tritonia*. Unfortunately, we were unable to show that any of the neuropeptide candidates were expressed in C2. However, the results suggested possible C2 neuropeptide candidates that were later tested by other techniques (see below). Additionally, the results allowed the expression patterns of several neuropeptides to be observed. Moreover, the tools used here may be useful for projects in the future.

FMRFamide and SCP

C2 is immunoreactive for FMRFamide and SCP_B in *Tritonia*, but these experiments indicate that neither peptide sequence is expressed in C2. There are three primary possibilities for this discrepancy. 1) The immunohistochemistry antibodies may be non-specific. The commercial FMRFamide antibodies are thought to stain for RFamides in general, and thus could stain a peptide other than FMRFamide. The SCP_B monoclonal antibody may similarly stain a related peptide that is similar to SCP. 2) There are other isoforms of FMRFamide and SCP. Evidence from the EST database suggests that there are several isoforms of FMRFamide in *Tritonia*. The same could be true of SCP. Moreover, the *scp* sequence used may not be SCP_B, as the exact identity of the sequence was unclear. Thus, C2 may contain FMRFamide and SCP, but not the isoforms tested here with *in situ* hybridization. 3) Finally, the FMRFamide probe used here may not have been particularly effective. The general lack of staining and the fact that the *fmrfamide* sequence used was not a full-length suggest that the probe used was not binding to FMRFamide sequences with great efficacy.

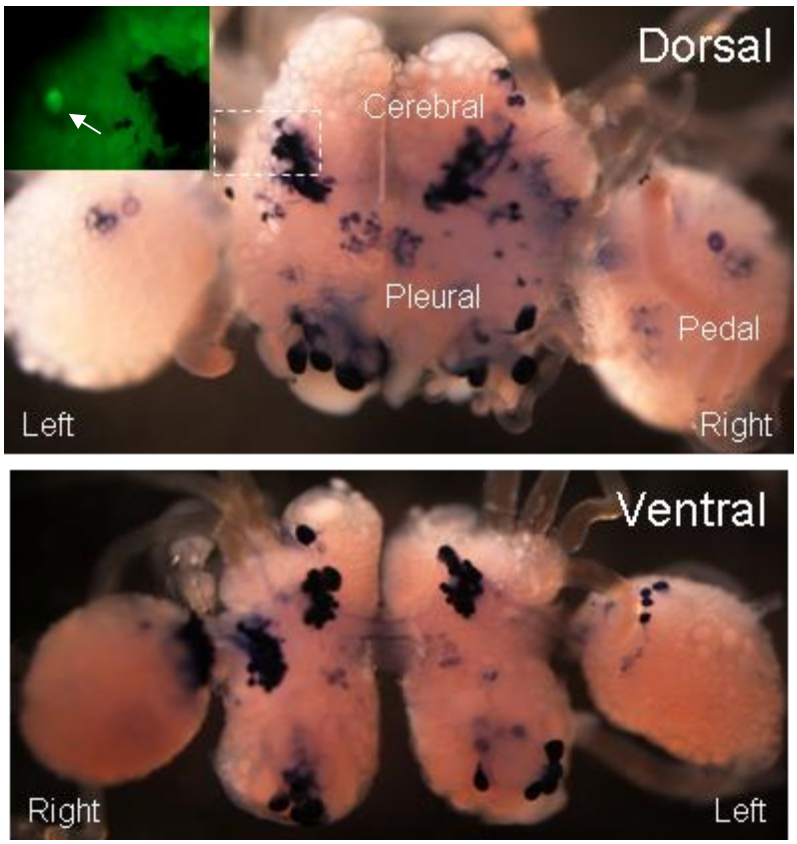

Figure B.8: SCP expression in *Tritonia*. Dorsal) There are prominent symmetrical clusters stained in the cerebral and pleural ganglia. There is comparatively sparse staining in the left and right pedal ganglia. Inset) C2 was filled with LY (white arrow) and does not express SCP. Ventral) There are symmetrical clusters in the cerebral and pleural ganglia stained. There is also an asymmetric cluster of cells stained in the right cerebral and pedal ganglia.

These discrepancies are worth noting, and may be interesting to investigate. However, they do not detract from our use of FMRFamide and SCP_B antibodies in C2 homologue identification across species. In the homologue identification experiments, the antibodies were being used to mark a cell type, but not to make claim of cell contents. In that regard, the antibodies were effective in that they displayed consistent staining across individuals within a species. Additionally, the antibodies showed reliable staining of similar cell clusters across species. The FMRFamide and SCP_B antibodies also consistently stained the C2 homologues in the most distantly related species studied, *Tritonia* and *Pleurobranchaea*, where C2 could be verified physiologically by its involvement in the dorsal-ventral swim CPG (Lillvis et al., 2012). Thus, regardless of what the antibodies were staining, they consistently stained specific neurons and these staining patterns showed similarity across species.

APGWamide, Clionin, Pleurin

APGWamide, Clionin, and Pleurin all showed staining in the vicinity of the C2 region of the cerebral ganglion. The staining patterns of these peptides do not appear to actually be in the correct region to stain C2, but it was never actually verified whether or not C2 expressed these peptides using *in situ* hybridization.

However, it should be noted that all of the peptide sequences discussed here as well as 23 additional peptide sequences were used as the basis of a sequence database that was consulted for matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry experiments conducted on C2. These experiments attempted to identify the neuropeptide contents of C2 by measuring the mass of the peptide fragments in the C2 soma. 30 peptide sequences were consulted as candidate peptides in C2, but the MALDI-TOF results suggested that none of the peptides, including those discussed here, were present in C2. It is possible that extensive post-translational modifications of the peptide sequences caused the peptide masses measured by MALDI-TOF to go unnoticed, but that is unlikely. Instead, the evidence suggests

that none of the peptide sequences identified here or in the EST database in general are present in C2.

Therefore, it appears as though uncovering the peptide contents of C2 will take an extensive amount of work consisting of a combination of genetic and mass spectrometry techniques. Given the fact that we can now identify C2 homologues across species without knowing the exact neuropeptide content of C2, it may not be worth the financial cost and effort necessary for such an endeavor.

Acknowledgments

This work was completed in collaboration with Leonid Moroz's group at the University of Florida's Whitney Lab in St. Augustine, FL. Leonid and Andrea Kohn contributed significantly to this work. Thanks are due to Sammi Jezzini and Yelena Bobokova as well for their help.