Evolution of swimming behaviors in nudibranch molluscs: A comparative analysis of neural circuitry

Charuni Gunaratne

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EVOLUTION OF SWIMMING BEHAVIORS IN NUDIBRANCH MOLLUSCS: A COMPARATIVE ANALYSIS OF NEURAL CIRCUITRY

by

CHARUNI A. GUNARATNE

Under the Direction of Paul S. Katz

ABSTRACT

Behaviors are a product of underlying neural circuits, yet there is a paucity of mechanistic information about how nervous systems contribute to the repeated evolution of similar behaviors. Theoretical studies have predicted that the same behavioral output can be generated by neural circuits with different properties. Here, we test the theory in biological circuits by comparing the central pattern generator (CPG) circuits underlying swimming behaviors in nudibranchs (Mollusca, Gastropoda, Euthyneura, Nudipleura).

In comparative studies of neural circuits, neurotransmitter content can serve as landmarks or molecular markers for neuron types. Here, we created a comprehensive map of GABA-immunoreactive neurons in six Nudipleura species. None of the known swim CPG neurons were
GABA-ir, but they were located next to identifiable GABA-ir neurons/clusters. Despite strong conservation of the GABA-ergic system, there were differences, particularly in the buccal ganglia, which may represent adaptive changes.

We applied our knowledge of neurotransmitter distribution along with morphological traits to identify the neuron type Si1 in *Flabellina*, a species that swims via whole body left-right (LR) flexions and in *Tritonia*, a dorsal-ventral (DV) swimming species. Si1 is a CPG member of the LR species *Melibe*, whereas its homologue in the LR species *Dendronotus* is not. In *Flabellina*, Si1 was part of the LR CPG and despite having similar synaptic connections as *Flabellina* and *Melibe*, Si1 in *Tritonia* was not part of its DV swim CPG.

Side by side circuit comparison of *Flabellina, Melibe* and *Dendronotus* revealed different combinations of circuit architecture and modulation resulting in different circuit configurations for LR swimming. This includes differences in the role and activity pattern of Si1, sensitivity to curare and the effect of homologues of C2, a DV CPG neuron, on the LR motor pattern. These results collectively reveal three different circuit variations for generating the same behavior. It suggests that the neural substrate from which behaviors arise is phylogenetically constrained. While this neural substrate can be configured in multiple different ways to generate the same outcome, the possibilities are finite and, as seen here, similar structural and functional neural motifs are used in the evolution of these circuits.

INDEX WORDS: Evolution, Behavior, Mollusc, Neural Circuit, Homologous neurons
EVOLUTION OF SWIMMING BEHAVIORS IN NUDIBRANCH MOLLUSCS:
A COMPARATIVE ANALYSIS OF NEURAL CIRCUITRY

by

CHARUNI A. GUNARATNE

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CHARUNI A. GUNARATNE

Committee Chair: Paul S. Katz

Committee: Ronald L. Calabrese
Charles D. Derby
Walter Wilczynski

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
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In loving memory of my father
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CHAPTER ONE: GENERAL INTRODUCTION

“When you examine the tapestry of evolution you see the same patterns emerging over and over again.”

-Simon Conway Morris

Despite the extraordinary behavioral diversity observed in the animal kingdom, it is also rife with examples of similar behavioral traits that have re-appeared several times within clades of animals. Behavior is a product of underlying neural circuitry. While there are detailed descriptions of independently evolved, i.e. analogous, behaviors and even of convergent genomic mechanisms related to such behaviors, little is known of how the nervous system contributes to the repeated emergence of similar behaviors. A fundamental question addressed in this dissertation is, does similarity in behavior imply a similarity in underlying neural circuitry?

This is an intriguing question from both an evolutionary neurobiological perspective and a translational neuroscientific point of view where analogies between human and animal behaviors form the basis of many studies that use animal models. Such studies are especially meaningful if the behaviors rely on the same neural circuitry (Gottlieb and Lickliter, 2004; Russell et al., 2005; Shanks and Greek, 2009; Nestler and Hyman, 2010). One approach to addressing the above question is to conduct a systematic study of the neural circuits underlying a similar behavioral phenotype that has appeared several times within a clade of animals. At a very basic level, such a study could reveal if there is a single or if there are multiple neural paths to the same behavior.

At a finer level, such a comparison could illuminate the features of the nervous system that may be important for the appearance of a behavior and also highlight neural circuit properties that may be less integral. In other words, such comparisons will also give us insight into how the nervous system contributes to the evolution of analogous behaviors.
1.1 Repeated evolution of behaviors

There is an ever-expanding list of similar behavioral phenotypes (i.e. analogous behaviors) that have evolved independently. A few examples include myrmecophagy, or ant-eating, in pangolins, numbats, echidnas, anteaters, aardvarks and aardwolves (Schwenk, 2000); pack hunting in New World army ants, African lions, wolves, white pelicans and orcas (McGhee, 2011; Pitman and Durban, 2012); group carrion scavenging in Eurasian black vultures and North American turkey vultures (McGhee, 2011); patagia-assisted gliding in flying squirrels, marsupial sugar gliders and colugos (Jackson, 2012); eusociality in insects, coral shrimp and mole rats (Nowak et al., 2010); bipedal hopping in North American and Old World desert rodents (Mares, 1993); web architecture in Hawaiian nocturnal orb-weaving spiders (Blackledge and Gillespie, 2004); and electrogenesis in six lineages of fishes (Rose, 2004; Gallant et al., 2014). Despite all the descriptions in the literature of behavioral convergence in the animal kingdom, not as much is known about how the nervous systems of these animals contribute to the evolution of these behaviors.

It is possible that analogous behaviors have entirely different underlying neural circuitry. Such is the case with the wave-like undulatory swimming behavior displayed by leeches and lampreys, where despite the behavioral similarity, the underlying CPGs are composed of non-homologous neurons that are wired differently (Mullins et al., 2011). This is an example of convergent evolution, in which similar behaviors have independently evolved from non-homologous neural substrates. In this example, the species belong to two different phyla, Annelida and Chordata, and it is perhaps not surprising that the neural circuits are entirely different.

Alternatively, modification to peripheral structures could also result in analogous behaviors regardless of differences or similarities in underlying circuitry. For example,
temporarily simplified single-click advertisement calls evolved independently at least twice in African clawed tree frogs (Leininger and Kelley, 2013). In *Xenopus borealis*, the fictive motor output from the brain matches the temporal pattern of the call while it does not in *Xenopus boumbaensis*; instead, the simplified call of *X. boumbaensis* is a result of modification to the larynx (Leininger and Kelley, 2013).

Analogous behaviors could also result from similarity in neuromodulation of underlying circuitry. For example, the appearance of swimming by dorsal-ventral flexions in Nudipleura sea slugs correlates with serotonergic neuromodulation of particular synapses in the swim circuit (Lillvis and Katz, 2013). In another example, non-monogamous meadow voles show pair preference behavior like monogamous prairie voles when vasopressin V1 receptor expression is up regulated in the ventral forebrain, similar to what is observed in the brains of monogamous species (Lim et al., 2004).

Analogous behaviors may evolve independently using similar circuitry, with homologous components and organization. For example, manual dexterity has evolved independently in *Cebus apella*, a New World monkey and in Old World macaque monkeys, using similar expansion of proprioceptive cortical areas 2 and 5 (Padberg et al., 2007). Similar to Old World Monkeys, *Cebus* has also independently acquired direct corticospinal projections to ventral horn motor neurons, which in turn project to individual digits of the hand (Bortoff and Strick, 1993; Nakajima et al., 2000; Krubitzer and Seelke, 2012). These similarities are an example of parallel evolution, where similar behaviors have evolved independently using homologous neural structures. Interestingly, such parallelism has been shown many times at the genetic level. For example, evolution of echolocation in bats and toothed whales involves parallel evolution of
genes associated with cochlear amplification and signal transmission (Davies et al., 2012; Shen et al., 2012). Similarly, the independent evolution of the electric organ in different lineages of electric fishes involves similar genetic adaptations, with similar patterns of expression of orthologous genes involved in the construction of functional electrocytes (Zakon et al., 2008; Gallant et al., 2014).

Analogous behaviors could also result from circuits that are configured differently but are still built upon homologous components. For instance, theoretical and experimental studies in the decapod crustacean stomatogastric nervous system (STG) show that CPG neurons with widely varying intrinsic and synaptic properties can still produce the same motor output (Prinz et al., 2004; Marder, 2011). It is possible that this is the case across species and circuits containing differently configured homologous neurons underlie similar behaviors. The last three scenarios suggest that there are evolutionary constraints on the nervous system of related species and neural circuits are drawing upon the same neural building blocks to generate analogous behaviors.

1.2 Dissertation Overview

As described in the previous section, there are numerous examples of similar behaviors that have repeatedly evolved, both within and across clades of animals, and it is not well understood how the nervous system relates to the evolution of many of these behaviors. At a basic level, we can ask if analogous behaviors have similar underlying neural circuitry. In order to address such a question, we need to examine multiple species with comparable behaviors and accessible nervous systems. We need access to information of the phylogenetic relationship of these species and the ability to identify homologues of neural circuit
components across species. As detailed in the following sections, molluse species in the clade Nudipleura are suitable for such a comparative study of neural circuitry.

1.3 Revisions to the Euthyneura phylogeny

The species described in this dissertation are in the monophyletic clade Euthyneura (Mollusca, Gastropoda). Based on morphological and molecular characteristics, major groups in Euthyneura have been recently redefined (Klussmann-Kolb et al., 2008; Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Wägele et al., 2014; Zapata et al., 2014). The traditional taxa Opisthobranchia and Pulmonata were found to be paraphyletic. Instead the new Euthyneura tree has Nudipleura as a basal offshoot and the newly formed Tectipleura as a sister group (Fig 1.1). Tectipleura consists of the newly formed monophyletic clades Euopisthobranchia and Panpulmonata. Among others, Euopisthobranchia contains anaspids such as *Aplysia californica* and *Aplysia brasiliana* and the pteropod *Clione limacina*. Panpulmonata now includes the sacoglossans (e.g., *Cyerce nigricans*) and traditional pulmonates such as *Lymnaea stagnalis*.

1.4 Nudipleura phylogeny

The species used in this dissertation are in the Nudipleura. Nudipleura is a monophyletic clade consisting of the sister clades Nudibranchia and Pleurobranchomorpha. *Pleurobranchaea californica* belongs to the latter clade. Nudibranchia consists of the two major subclades: Cladobranchia and Euctenidiacea. *Tritonia diomedea, Dendronotus iris, Melibe leonina*, as well as the aeolids *Flabellina iodinea* and *Hermisenda crassicornis* belong to Cladobranchia. Recent data based on phylogenetic analyses of whole brain transcriptomes of these six Nudipleura species further resolve Cladobranchia such that *Tritonia* forms a clade with the
aeolids and *Dendronotus* and *Melibe* form a more basal clade (Senatore and Katz, 2014). The species studied in Chapter 2, 3 and 4 include the above species plus *Tochuina tetraquetra*. As seen in the abbreviated phylogeny, these species differ in the swimming behaviors that they express (Fig 1.2). This provides the opportunity to examine how the nervous system of these species relates to phylogeny and function.

### 1.5 Locomotor behavior

The primary form of locomotion in the Nudipleura is crawling via mucociliary beating where the animal glides over a surface of secreted mucus with the use of cilia on the bottom of the foot (Chase, 2002). Some species also crawl via muscular contraction of the foot. In addition to crawling, swimming is another mode of locomotion albeit a rare one. Only 63 species out of the approximately 3000 species in Nudipleura have been reported to exhibit swimming behaviors (Newcomb et al., 2012). The different categories include swimming by left-right (LR) flexions, left-right undulations (LU), dorsal-ventral (DV) flexions, dorsal-ventral undulations (DU), asymmetric undulations (AU), breaststroke (BS) and flapping (F) (Newcomb et al., 2012). While families that exhibit swimming can also contain non-swimming species, the category of swimming typical for that family is usually the same. However, this is not the case in the families Flabellinidae and Pleurobranchidae and this may have implications for the evolution of swimming in these groups. Flabellinidae contains LR, non-swimming and BS species. In BS swimming, the animal moves via coordinated, whip-like strokes of its cerata (Farmer, 1970). BS swimming is also seen in the sacoglossan sea slug *Cyerce nigricans*¹, in the distantly related Panpulmonata (Miller, 1969). Similarly, the family Pleurobranchidae consists of DV, AU and F

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¹ [https://sites.google.com/site/swimmingseaslugs/a-z-index/cyerce-nigricans](https://sites.google.com/site/swimmingseaslugs/a-z-index/cyerce-nigricans)
species (Newcomb et al., 2012). We recently encountered an example of LR swimming in Pleurobranchidae in the species *Pleurobranchaea brockii* \(^2\). AU swimming as displayed by *Pleurobranchaea membranaceus* is a rather peculiar behavior. The animal swims upside with its mantle acting as a keel and the left and right halves of its foot make graceful alternating muscular waves\(^3\) (Thompson and Slinn, 1959). F swimming, as exemplified by *Euselenops luniceps*, involves the left and right halves of the foot making coordinated flapping motions rather like a bird in flight \(^4\) (Farmer, 1970). F swimming via muscular waves of parapodia is seen in Euopisthobranchia, in species such as *Clione limacina* \(^5\) and *Aplysia brasiliana* \(^6\) (Hamilton and Ambrose III, 1975; Satterlie et al., 1985). In this dissertation, I primarily focus on LR and DV swimming. However, these other forms of swimming and where they appear in the phylogeny will be considered when discussing the evolution of LR and DV swimming.

### 1.6 Left-right (LR) swimming behavior

The most common mode of swimming in Nudibranchia is via left-right (LR) flexions. In this mode, the animal flattens its body in the sagittal plane and makes rhythmic, alternating flexions by bending at the midpoint of its body axis with the head and tail moving closer to each other with each lateral flexion (Fig 1.3 A-C, Fig 1.4). This rhythmic motion propels the animal through the water. The LR swimming species in the studies that make up this dissertation include *Flabellina iodinea* or the Spanish shawl, *Melibe leonina* aka the lion’s mane/hooded nudibranch, *Dendronotus iris* and *Hermissenda crassicornis*. LR swimming was thought to only to be present within Nudibranchia, however we recently encountered video records of an LR swimming

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\(^2\) [https://sites.google.com/site/swimmingseaslugs/a-z-index/pleurobranchaea-brockii](https://sites.google.com/site/swimmingseaslugs/a-z-index/pleurobranchaea-brockii)

\(^3\) [https://sites.google.com/site/swimmingseaslugs/a-z-index/pleurobranchus-membranaceus?pli=1](https://sites.google.com/site/swimmingseaslugs/a-z-index/pleurobranchus-membranaceus?pli=1)

\(^4\) [https://sites.google.com/site/swimmingseaslugs/a-z-index/euselenops-luniceps](https://sites.google.com/site/swimmingseaslugs/a-z-index/euselenops-luniceps)

\(^5\) [https://sites.google.com/site/swimmingseaslugs/a-z-index/clione-limacina](https://sites.google.com/site/swimmingseaslugs/a-z-index/clione-limacina)

\(^6\) [https://sites.google.com/site/swimmingseaslugs/a-z-index/aplysia-brasiliana](https://sites.google.com/site/swimmingseaslugs/a-z-index/aplysia-brasiliana)
species in Pleurobranchomorpha called *Pleurobranchaea brockii* (Fig 1.4). Approximately 40 of the 63 species in Nudipleura that have been reported to swim exhibit some form of LR swimming (Newcomb et al., 2012). Of the approximately 47 families in Nudibranchia (Bouchet and Rocroi, 2005), the known LR species represent 9 families (Newcomb et al., 2012). Thus LR swimming, in fact swimming in general, is an extremely rare behavior.

### 1.7 Left-Right swim CPG in Melibe leonina

*Melibe leonina* displays LR swimming (1.3B). The behavior is elicited by loss of contact with the substrate or by noxious stimuli such as the touch of a predatory sea starfish or a high molarity salt solution (Hurst, 1968; Lawrence and Watson, 2002). *Melibe* has also been observed to swim spontaneously; its swim can last up to an hour and the flexion cycle period has been reported to range from 2 to 7 seconds (Watson et al., 2001; Lawrence and Watson, 2002; Sakurai et al., 2011; Sakurai et al., 2014). There appears to be a directional component to the swim with the animal predictably moving in the direction perpendicular to the long axis of its foot (Hurst, 1968; Lawrence and Watson, 2002). It has been suggested that swimming is a means of dispersal for *Melibe*, allowing for mixing of gene pools from spatially isolated populations (Mills, 1994).

### 1.8 Left-Right swim CPG in Melibe leonina

*Melibe leonina* displays LR swimming (Fig 1.3B). The behavior is elicited by loss of contact with the substrate or by noxious stimuli such as the touch of a predatory sea starfish or a high molarity salt solution (Hurst, 1968; Lawrence and Watson, 2002). *Melibe* has also been observed to swim spontaneously; its swim can last up to an hour and the flexion cycle period has been reported to range from 2 to 7 seconds (Watson et al., 2001; Lawrence and Watson, 2002; Sakurai et al., 2011; Sakurai et al., 2014). There appears to be a directional component to the
swim with the animal predictably moving in the direction perpendicular to the long axis of its foot (Hurst, 1968; Lawrence and Watson, 2002). It has been suggested that swimming is a means of dispersal for *Melibe*, allowing for mixing of gene pools from spatially isolated populations (Mills, 1994).

The CPG underlying the *Melibe* LR swim consists of four bilaterally represented cell types called swim Interneuron 1 (Si1), swim interneuron 2 (Si2), swim interneuron 3 (Si3) and swim interneuron 4 (Si4) (Thompson and Watson, 2005; Sakurai et al., 2011; Sakurai et al., 2014). These neurons are arranged into two reciprocally inhibitory kernels, one consisting of Si1, Si2 and the contralateral Si4 and the second kernel consisting of Si3 (Sakurai et al., 2014). These kernels interact with each other to produce a stable rhythmic swim motor pattern. For the sake of simplicity, the version of the *Melibe* CPG represented in this dissertation consists of only Si1 and Si2 (Fig 1.5A). Each Si1 and Si2 makes inhibitory connections across the midline with the Si1 and Si2 on the other hemisphere. The Si1 and Si2 pairs are all electrically coupled to each other, though the coupling between the ipsilateral Si1 and Si2 are 10-30 times stronger than the other connections (Sakurai et al., 2011). Si1 and Si2 synapse onto motor neurons that project to the muscles (Thompson and Watson, 2005). The ipsilateral Si1 and Si2 fire in phase with each other and in antiphase to the contralateral Si1 and Si2, resulting in the alternating left-right bursting activity that drives the rhythmic body flexions. The activity of the CPG cells can be monitored in an isolated brain preparation, in which the fictive swim motor pattern can appear spontaneously or in response to stimulation of a body wall nerve, or a dimming of the lights.
1.9 Left-Right swim CPG in Dendronotus iris

Similar to Melibe, Dendronotus iris swims via LR flexions (1 3A.) (Agersborg, 1922). The Dendronotus swim can be elicited by loss of contact with the substrate, the touch of a predatory sea star or a high-molarity salt solution and has a period similar to that of Melibe (Sakurai et al., 2011; Newcomb et al., 2012). The cells that are homologues of the Melibe Si1 and Si2 have been identified in Dendronotus. However, only Si2 is a member the swim CPG in Dendronotus (Fig 1.5B). The Si2 neurons display mutual inhibition and are electrically coupled to each other and the Si1 pair. The Si1 neurons do not inhibit each other although they are electrically coupled. During a fictive swim motor pattern, the Si2 neurons fire in alternation and in a constant phase with pedal motor neurons and nerves (Sakurai et al., 2011).

Melibe and Dendronotus display similar swimming behaviors but the neural circuitry underlying this behavior has some key differences such as the exclusion of Si1 from the Dendronotus swim CPG. These differences could be due to the two species independently evolving LR swimming or because of circuit divergence from a common LR swimming ancestor in one or both lineages. One way to parse through these possibilities is to examine the swim circuitry of a third LR species and compare it to that of Melibe and Dendronotus.

1.10 Left-Right swimming in Flabellina iodinea

The Spanish shawl, Flabellina iodinea, is an example of another LR swimmer (Fig 1.3C) (MacFarland and MacFarland, 1966; Farmer, 1970). This species typically makes vigorous rhythmic flexions and has been observed to swim spontaneously and for many minutes (personal observation). Flabellina sometimes reacts to the touch of starfish tube feet by swimming. A high concentration salt solution can also induce swimming in vivo (personal observation). Loss of
contact with the substrate does not appear to induce swimming; the animal merely floats to the bottom. In this dissertation, we will compare the neural circuitry underlying LR swimming in *Flabellina*, *Melibe* and *Dendronotus*. In the following section, I will discuss the phylogenetic information concerning these three species, the possible results of comparing these circuits and its implications for the evolution of LR swimming behavior.

1.11 Evolution of LR swimming in Flabellina

The literature on the distribution of LR swimming supports the notion that LR swimming arose independently in at least *Flabellina iodinea*. *Flabellina*, *Melibe* and *Dendronotus* are in the clade Cladobranchia. These three species belong to the families Flabellinidae, Tethydidae and Dendronotidae respectively. Flabellinidae belongs to a sub-clade of Cladobranchia called Aeolidida, which consists of 11 families and whose phylogeny has been examined (Fig 1.6) (Carmona et al., 2013). This means that Flabellinidae is more closely related to these 10 other aeolid families than it is to Tethydidae and Dendronotidae. It is also known that *Flabellina* is more closely related to the DV swimming species *Tritonia diomedea* of the family Tritoniidae than it is to either *Melibe* or *Dendronotus* (Senatore and Katz, 2014). Cladobranchia as a whole contains 31 families (Bouchet and Rocroi, 2005) of which 7 have at least one known LR species (Newcomb et al., 2012). This means that out of the 31 families in Cladobranchia, there are at least 11 non-LR families that Flabellinidae is more closely related to than it is to Tethydidae and Dendronotidae (Fig 1.6). These non-LR families include the non-swimming and breast-stroke (BS) families in the sub-clade Aeolidida and the DV swimming family Tritoniidae. They collectively represent several hundred species. Flabellinidae itself contains just two known LR swimmers, *Flabellina iodinea* and *Flabellina telja* (Newcomb et al., 2012). It also contains three BS swimmers, *Flabellina cynara*, *Cumanotus beaumonti* and *Cumantus cuenoti*, as well non-
swimming species (Newcomb et al., 2012). Thus, when taking into account the distribution of LR behavior in Aeolidida, the known information of the Cladobranchia phylogeny and the presence of BS swimming species in Flabellinidae, it is reasonable to conclude that LR swimming evolved independently in *Flabellina iodinea*.

LR swimming may have evolved independently in Tethyidae and Dendronotidae as well but it is equally possible that these two families cluster together in a completely resolved phylogeny of Cladobranchia and have a last common ancestor that exhibited LR swimming. Interestingly, almost half of the known 40 LR/LU species belong to these two families (Newcomb et al., 2012).

### 1.12 Possible results of comparing the LR circuitry of Flabellina, Melibe and Dendronotus

It is possible that the *Flabellina* circuitry is entirely different from that of *Melibe* or *Dendronotus* and does not employ homologues of any of the known LR CPG neurons (1.7A). This would indicate that LR swimming and the underlying circuitry arose by convergent evolution in *Flabellina*. This would be further supported by the Cladobranchia phylogeny that shows that *Flabellina* is more closely related to several hundred non-swimming species encompassing 11 non-LR families than it is to either *Melibe* or *Dendronotus*.

Another possibility is that the *Flabellina* circuitry is identical or highly similar to either *Melibe* (Fig 1.7B) or *Dendronotus* (Fig 1.7C). This would indicate that LR swimming was present in the last common ancestor of *Flabellina/Melibe* or *Flabellina/Dendronotus* and the circuit diverged in either *Dendronotus* or *Melibe*. This would also entail the loss of the LR behavior in 23 of the 31 families in Cladobranchia, as well as gain of BS swimming in the
families Aeolidiidae and Flabellinidae as well as the gain of DV swimming in the family Tritoniidae (1.6).

The Flabellina circuitry could also contain some circuit components similar to that of Melibe and some to Dendronotus (Fig 1.7D) as well as have its own unique properties (Fig 1.7E). Many studies have shown similarities in the nervous systems of nudibranchs and the above circuit scenario could suggest that Flabellina independently evolved LR behavior using existing homologous structures, and hence the resemblance to the other two species. This would indicate that there are multiple but overall finite number of ways to generate a behavior from a common neural ground plan.

1.13 Dorsal-ventral (DV) swimming behavior

In this dissertation, I will also examine if there is overlap in the neural circuitries underlying LR and DV swimming. The following sections will describe what is known of the DV behavior, its neural control, and its distribution in the Euthyneura phylogeny. After LR swimming, the next most prevalent form of swimming in Nudipleura involves some form of dorsal-ventral flexions (Newcomb et al., 2012). In DV swimming, the animal flattens its body on the horizontal plane and makes repeated alternating flexions where the tail and head coming together above midpoint of the body and then below (1.3D-E). DV swimming has been documented in at least 17 Nudipleura species (Newcomb et al., 2012) and once outside of Nudipleura, in Petalifera ramosa, an aplysiid in the clade Euopisthobranchia7 (Thompson, 1977). The aplysiid Aplysia brasiliana is known to swim via graceful flapping of its parapodia (Hamilton and Ambrose III, 1975). Close observation of videos made of this species swimming in captivity and in their natural environment reveal additional aspects of its swimming behavior.

7 https://sites.google.com/site/swimmingseaslugs/a-z-index/petalifera-ramosa
that make it uncannily close to DV swimming\textsuperscript{8}. There is a dorsal-ventral movement of the body while \textit{A. brasiliana} swims (1. 8A). This movement is not as dramatic as that of \textit{Tritonia} or \textit{Petalífera}. Unlike \textit{Tritonia} and \textit{Pleurobranchae} (Fig 1.3D, E), these species do not bend at the midpoint of their body axis; instead the bend during the dorsal-ventral movement is closer to the head, approximately one fourth of the way down the body axis (Fig 1.8A). Curiously, \textit{Hexabranchus sanguinus}, the Spanish dancer, a DV swimming nudibranch in Doridacea, also makes this bend one fourth of the way down its body axis (Fig1. 8B) (observation from high definition videos of \textit{Hexabranchus} swimming\textsuperscript{9}). In addition to the DV motion during swimming, \textit{Hexabranchus} makes muscular waves with its mantle skirt\textsuperscript{10}, which is reminiscent of the muscular waves of the parapodia in \textit{Aplysia brasiliana}.

\textbf{1.14 Dorsal-ventral swim CPG in \textit{Tritonia diomedea}}

\textit{Tritonia diomedea} swims (Fig 1.3D) in response to the touch of a predatory starfish or contact with a high-molarity salt solution (Willows, 1967; Hume et al., 1982). The CPG underlying the DV swim circuit in \textit{Tritonia} consists of three cell types called the Dorsal Swim interneurons\textsuperscript{11} (DSI), Cerebral Neuron 2\textsuperscript{12} (C2), and Ventral Swim Interneuron-B\textsuperscript{13} (VSI) (Fig 1.9A). The following is a summary of the initiation and production of the swim motor pattern. In reaction to noxious stimuli, sensory neurons excite a gating command neuron called Dorsal Ramp Interneuron (DRI). The DRI excites the DSIs, which in turn excites C2. C2 creates a positive feedback loop by exciting DRI and thus further exciting DSI. C2 provides a delayed

\textsuperscript{8} https://www.youtube.com/watch?v=_A78qeKBfA
\textsuperscript{9} https://sites.google.com/site/swimmingseaslugs/a-z-index/hexabranchus-sanguineus
\textsuperscript{10} https://www.youtube.com/watch?v=ZjNsJIE-bRk&list=PLldzyW6VWRqP1Mk_t0IOftZTySdDz3Lpm&index=67
\textsuperscript{11} http://neuronbank.org/Tri0001043
\textsuperscript{12} http://neuronbank.org/Tri0002380
\textsuperscript{13} http://neuronbank.org/Tri0002436
excitation to VSI, which inhibits DSI and C2, ending each cycle. When C2 ceases to fire, it no longer excites VSI. When VSI falls silent, the C2-DRI-DSI positive feedback loop restarts (Katz, 2009). Unlike the LR swimmers, each CPG cell fires in unison with its respective contralateral partner such that there is synchronized activity across the midline resulting in both sides of the body bending in unison.

1.15 Dorsal-ventral swim CPG in Pleurobranchaea californica

*Pleurobranchaea* displays a swimming behavior that is highly similar to that of *Tritonia* although the threshold for activation of this behavior is much higher in *Pleurobranchaea*. On rare occasions, swimming was observed in the holding tanks in reaction to an attack bite from a conspecific (personal observation). Electric shocks to the dorsal mantle or tail of the animal can sometimes induce the DV swim behavior (Jing and Gillette, 1999; Lillvis and Katz, 2013). Just like in *Tritonia*, a swimming *Pleurobranchaea* bends at the midpoint of its body axis and makes whole body dorsal and ventral flexions (Fig 1.3E).

A comparison of the known CPG elements of *Tritonia* and *Pleurobranchaea* reveals some striking similarities. For instance *Pleurobranchaea* contains DSI and C2 homologues with similar connections to *Tritonia* and there is synaptic evidence of a VSI-like neuron that inhibits the activity of C2 and DSI (Fig 1.9B) (Jing and Gillette, 1999). However, the *Pleurobranchaea* CPG also contains two additional neurons A3 and A10, which are located in the same region as DSI and C2 (Jing and Gillette, 1999). These neurons either do not exist in *Tritonia* or, similar to the case of Si1 in *Melibe* and *Dendronotus*, they are present but are not rhythmically active in *Tritonia*. Both of these scenarios imply a significant difference between the two DV circuits. A third scenario is that A3 and A10 homologues are present in *Tritonia* and are part of the CPG but have just not been found. While plausible, this seems rather unlikely as the *Tritonia* swim circuit
has been intensively studied for almost 50 years and if there were additional CPG cells in the vicinity of C2 and DSI, they would have been found. Another possibility is that the morphology and location of these cells have diverged significantly in *Tritonia*. Another difference between the two DV circuits involves the serotonergic DSIs. It has been previously shown that both *Tritonia* and *Pleurobranchaea* have a set of five serotonin-immunoreactive neurons on the dorsal surface of the cerebral ganglia (Sudlow et al., 1998; Newcomb et al., 2006). In *Tritonia*, at least three of these neurons are the DSIs. However in *Pleurobranchaea*, four of the five serotoninergic neurons are part of the DV CPG (Jing and Gillette, 1999). However, differences in the number of neurons of a particular type across species have been observed in other systems. Given the lack of information of A3 and A10 in *Tritonia*, we can only speculate as to whether the DV swimming circuitry in *Tritonia* and *Pleurobranchaea* are more similar to each other than different.

### 1.16 Identification of homologous neurons

To compare neural circuits underlying swimming across species, it is necessary to be able to identify homologous neurons. Individual neurons can be identified based on a suite of neuroanatomical and neurochemical characteristics. These same characteristics can be used to identify homologous neurons in other species (Weiss and Kupfermann, 1976; Croll, 1987; Bullock, 2000). The homologue of the *Melibe* LR CPG neuron type Si1 has been identified in *Dendronotus* using the criteria that uniquely identify this neuron in *Melibe*. These include its location in the dorsal cerebral ganglion, its axon morphology and projection pattern, its proximity to known serotonergic neurons and its immunoreactivity to the neuropeptide FMRFamide (Thompson and Watson, 2005; Sakurai et al., 2011). Similarly the homologue of the *Melibe* neuron type Si2 has been identified in *Dendronotus* (Sakurai et al., 2011). The
homologues of the serotonergic DSI neuron type of the *Tritonia* DV CPG has been identified in other Nudipleura species including *Melibe, Dendronotus, Flabellina* and *Pleurobranchaea* (Jing and Gillette, 1999; Newcomb et al., 2006; Tian et al., 2006; Newcomb and Katz, 2007) as well as in euopisthobranchs such as *Aplysia californica* and *Clione limacina* (Satterlie and Norekian, 1995; Fickbohm et al., 2001; Jing et al., 2008). Similarly, using criteria such as soma location and white coloration, axon projection and immunoreactivity to the neuropeptides Small Cardioactive Peptide-B (SCP-B) and FMRFamide, the homologues of the *Tritonia* CPG neuron type C2 have been identified in Nudipleura species including *Melibe* and *Flabellina* (Lillvis et al., 2012).

### 1.17 Intersection of LR and DV CPG circuits

LR and DV swimming behaviors are categorically different from each other and the circuit information to date indicates that the CPGs consist of non-overlapping neurons (Newcomb et al., 2012). The DSI homologues have been examined in *Melibe* and *Dendronotus* and were found to be non-rhythmic although in *Melibe*, the DSI homologues act as extrinsic modulators of the swim motor pattern (Newcomb and Katz, 2009; Newcomb et al., 2012). The C2 homologues have been identified in *Melibe* and *Flabellina* (Lillvis et al., 2012) but not in *Dendronotus*. In *Melibe*, the C2 homologue was also found to be non-rhythmic although its involvement in the LR swim was not examined further (Newcomb et al., 2012). The activity and role of the C2 homologue in *Flabellina* has not been examined. In this dissertation, I will identify the C2 homologue in *Dendronotus* using previously established identifying criteria as well as examine the role of C2 homologues in the LR swim in *Flabellina, Melibe* and *Dendronotus*.

To date, homologues of known LR CPG neurons have not been identified in DV species. In this dissertation, I will also identify the Si1 homologue in the DV species *Tritonia* using
previously established identifying criteria and examine its role in the DV CPG. We will also compare the connections made by the Si1 homologues in *Tritonia* to those made by Si1 in *Melibe, Dendronotus* and *Flabellina*. Given that *Tritonia* and *Flabellina* are more closely related to each other than to either *Melibe* or *Dendronotus*, such a comparison would help differentiate between features that are important for the generation of LR swimming versus features that are products of the phylogenetic relationship between the four species.

In summary, there are two main modes of swimming in the Nudipleura, namely LR and DV swimming. *Melibe* and *Dendronotus* are two LR swimmers whose swim CPGs have been examined. A third species, *Flabellina*, has been shown to be a robust LR swimmer as well. The neuroanatomical and neurochemical criteria for identifying homologous neurons such as Si1 have been established. This provides an excellent opportunity to examine and compare the neural circuitry underlying a similar swimming behavior in three species of nudibranchs. Furthermore, the ability to identify certain homologous neurons across species regardless of behavior gives us the opportunity to explore any intersection of LR and DV circuits.

1.18 Dissertation summary

Elucidating the neurotransmitter phenotype of neurons in neural circuits is important for understanding how these neural circuits function. In comparative circuit studies, transmitter distribution maps can also serve valuable tools for establishing neuronal homology across species. **Chapter 2** describes the mapping of GABA-immunoreactive neurons in the central ganglia of four nudibranch molluscs, *Tritonia diomedea, Melibe leonina, Dendronotus iris* and *Hermissenda crassicornis*. I wanted to determine if any of the previously identified neurons in the LR and DV swimming central pattern generator circuits were GABA-ir. While none of the
known swim CPG neurons were immunoreactive for GABA, there was a consistent cluster and distribution pattern of GABA-ir neurons in the brains of these species suggesting that their presence has been strongly conserved across nudibranchs. I also found that certain identified GABA-ir neurons and clusters can serve as landmarks for swim CPG neurons.

**Chapter 3** describes the mapping of GABA-ir neurons in the primarily feeding related buccal ganglia of six nudipleuran molluscs, *Hermisenda crassicornis, Tritonia diomedea, Tochuina tetraquetra, Dendronotus iris, Melibe leonina*, and *Pleurobranchaea californica*. I wanted to know if the distribution pattern varied with the Nudipleura phylogeny. The GABA-ir distribution in *Pleurobranchaea*, which belongs to the clade Pleurobranchomorpha, differed significantly from that of the species in the sister clade Nudibranchia. However, the distribution of GABA-ir in *Pleurobranchaea* is also very different from all other gastropods examined to date including the distantly related euopisthobranchs and panpulmonates, suggesting that the *Pleurobranchaea* GABA distribution is in fact more likely a feature unique to that lineage. Within Nudibranchia, the GABA-ir distribution was consistent except for *Melibe*. This may be an adaptive feature and may be related to the unique feeding behavior of *Melibe*, which is controlled in part by the buccal ganglia.

**Chapter 4** shows that three different neural circuits composed of homologous structures underlie analogous LR swimming in three nudibranchs species. In this chapter, I compare and contrast the swim circuitry of the LR swimming species *Flabellina, Melibe* and *Dendronotus* with the goal of examining if similar behaviors have similar neural underpinnings. The results of this study show that the LR circuits of each species were configured differently, with different combinations of underlying circuit architecture and modulation. I also examined the role of a LR
CPG homologue in the DV swimmer *Tritonia* and vice versa. While the LR and DV swim behaviors are produced by non-overlapping sets of CPG cells, the homologues of the DV swim interneuron C2 acted as an extrinsic modulator of the LR swim and in *Flabellina*, it was also found to be necessary for the swim.

In **Chapter 5**, I review three case studies of independently evolved behaviors within and across phyla, where there has been extensive comparative work on the underlying neural substrates. These include: 1) undulatory swimming behavior in leeches and lampreys; 2) vocal learning in songbirds, parrots and hummingbirds; and 3) electrolocation and the jamming avoidance response in African and South American weakly electric fish. I then review left-right swimming behavior in nudibranch molluscs and its neural bases, which is yet a fourth example, and the focus of this thesis. Finally, I discuss the recurring themes in these examples on how the nervous system contributes to the evolution of analogous behaviors.
Figure 1.1 Abbreviated phylogeny of the reclassified Euthyneura phylogeny. Euthyneura contains two clades: Nudipleura and Tectipleura. Within Nudipleura, there are two clades: Nudibranchia and Pleurobranchomorpha. Tectipleura contains two large clades: Euopisthobranchia and Panpulmonata. Representative species of each major group are shown. The colors represent different types of swimming behaviors. Blue letters indicate dorsal-ventral (DV) swimmers, pink letters indicate left-right (LR) swimmers, orange letters indicate breast-stroke (BS) swimmers and black letters represent non-swimmers (NS). The phylogenetic relationships are based on: (Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Wägele et al., 2014).
Figure 1.2 Abbreviated phylogeny of Euthyneura and swimming behaviors. Pink letters indicate left-right (LR) swimming, blue letters indicate dorsal-ventral (DV) swimming, orange letters indicate breast-stroke (BS) swimming, green letters indicate swimming
via flapping (F), maroon letters indicate swimming by asymmetric undulation (AU) and black letters indicate non-swimmers (NS). Swimming and phylogenetic information are based on: (Miller, 1969; Hamilton and Ambrose III, 1975; Satterlie et al., 1985; Klussmann-Kolb, 2004; Newcomb et al., 2012; Senatore and Katz, 2014; Wägele et al., 2014)

Figure 1.3 Examples of swimming behaviors. Whole body left-right (LR) swimming as exhibited by *Dendronotus iris* (A), *Melibe leonina* (B) and *Flabellina iodinea* (C). Whole body Dorsal-Ventral (DV) flexion swimming as exhibited by *Tritonia diomedea* (D) and *Pleurobranchaea californica* (E). Pink letters and outline indicate LR swimming and blue indicates DV swimming. A; anterior, P; posterior.
Figure 1.4 Whole body left-right (LR) swimming behavior of *Pleurobranchaea brockii*.

The foot is flattened in the sagittal plane as in other LR swimmers and the animal flexes its body from left to right. A; anterior, P; posterior. Images extracted from video 1.
Figure 1.5 Left-right (LR) swim CPG circuits and fictive swim motor pattern recordings in *Melibe* and *Dendronotus*.

A. (i) The *Melibe* LR CPG contains the bilaterally represented neuron types Si1 and Si2 (gray box). (ii) Simultaneous intracellular recordings from both pairs of Si1s and Si2s show the ipsilateral side firing in synchrony and in alternation with the contralateral side.

B. (i) The *Dendronotus* LR CPG consists of the Si2 pair (gray box) and the Si1 pair does not show mutual inhibition. (ii) Simultaneous intracellular recordings show the Si2 pair firing in alternation while the Si1 pair fires irregularly. The resistor symbol represents electrical coupling with thicker lines indicating stronger coupling. The small filled circle and line symbol indicates an inhibitory synapse. Figures from: (Sakurai et al., 2011; Newcomb et al., 2012).
Figure 1.6 Phylogeny showing all families in Cladobranchia. Pink, blue and brown squares indicate families that contain species that exhibit left-right (LR), dorsal-ventral (DV) and breast-stroke (BS) swimming species respectively. All other families have no known swimming species. Shaded box indicates families within Aeolidida, a sub-clade of Cladobranchia. Asterisks indicate families that include species examined in this study.
Swimming and phylogenetic information are based on: (Bouchet and Rocroi, 2005; Pola and Gosliner, 2010; Newcomb et al., 2012; Carmona et al., 2013; Senatore and Katz, 2014)

**Figure 1.7** Possible results of comparing the LR circuitry of *Flabellina, Melibe* and *Dendronotus.*
Schematics of hypothetical circuits where the *Flabellina* LR CPG is: A. different from that of *Melibe/Dendronotus*; B. identical to *Melibe*; C. identical to *Dendronotus*; D. similar to both *Melibe/Dendronotus*; E. similar to both *Melibe/Dendronotus* and has its unique properties. Large filled circles depict neurons. The resistor symbol represents electrical coupling, with thicker lines.
indicating stronger coupling. The line and small filled circle or triangle symbol indicates an inhibitory or excitatory synapse respectively. Green indicates circuit connections unique to *Flabellina*.

Figure 1.8 Swimming behavior of *Aplysia brasiliana* (A) and *Hexabranchus sanguineas* (B). Green letters and outline indicate swimming via flapping and blue indicates dorsal ventral swimming. White arrow indicates the point along the body axis where the animal bends during a dorsal-ventral movement. A; anterior, P; posterior.
Figure 1.9 Dorsal-ventral (DV) swim CPG circuits and intracellular recordings of the fictive swim motor pattern in *Tritonia* and *Pleurobranchaea*.

A. (i) The *Tritonia* DV swim circuit is composed of three neuron types. (ii) Simultaneous intracellular recording from a pair of DSIs and the C2 pair show synchronous firing across the midline. VSI (not shown) fires in alternation with the DSIs and C2.

B. (i) The *Pleurobranchaea* DV swim CPG contains homologues of DSI and C2 (also called As and A1 cells). Ivs has not been found although synaptic input to DSI and C2 suggests the existence of this cell type. A3 and A10 are additional CPG neurons not found in *Tritonia*. (ii) Simultaneous intracellular recording from As, A1, and A3. The fictive motor pattern is elicited by electrical stimulation of a body wall nerve (black bar). The resistor symbol represents electrical coupling. The small filled circle and line symbol indicates inhibitory connections. The small filled triangles and lines indicate excitatory connections. Combined circles and triangles indicate mixed inhibition and excitation. Figures from: (Newcomb et al., 2012).
CHAPTER TWO: COMPARATIVE MAPPING OF GABA-IMMUNOREACTIVE NEURONS IN THE CENTRAL NERVOUS SYSTEMS OF NUDIBRANCH MOLLUSCS

Charuni A. Gunaratne, Akira Sakurai and Paul S. Katz

Neuroscience Institute

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2.1 ABSTRACT

The relative simplicity of certain invertebrate nervous systems, such as those of gastropod molluscs, allows behaviors to be dissected at the level of small neural circuits composed of individually identifiable neurons. Elucidating the neurotransmitter phenotype of neurons in neural circuits is important for understanding how those neural circuits function. In this study, we examined the distribution of GABA-immunoreactive (GABA-ir) neurons in four species of sea slugs (Mollusca, Gastropoda, Opisthobranchia, Nudibranchia): *Tritonia diomedea*, *Melibe leonina*, *Dendronotus iris* and *Hermissenda crassicornis*. We found consistent patterns of GABA immunoreactivity in the pedal and cerebral-pleural ganglia across species. In particular, there were bilateral clusters in the lateral and medial regions of the dorsal surface of the cerebral ganglia as well as a cluster on the ventral surface of the pedal ganglia. There were also individual GABA-ir neurons that were recognizable across species. The invariant presence of these individual neurons and clusters suggests that they are homologous although there were interspecies differences in the numbers of neurons in the clusters. The GABAergic system was largely restricted to the central nervous system with the majority of axons confined to ganglionic connectives and commissures, suggesting a central, integrative role for GABA. Although GABA was a candidate inhibitory neurotransmitter for neurons in central pattern generator (CPG) circuits underlying swimming behaviors in these species, none of the known swim CPG neurons were GABA-ir. Although, the functions of these GABA-ir neurons are not known, it is clear that their presence has been strongly conserved across nudibranchs.
2.2 INTRODUCTION

“The garden of neurology holds out to the investigator captivating spectacles and incomparable artistic emotions. In it, my aesthetic instincts found full satisfaction at last.”

– Santiago Ramón y Cajal, Recollections of My Life

Determining the neurotransmitters present within a neural circuit underlying a behavior can help define the characteristics of the circuit. Gamma-aminobutyric acid (GABA) is a widely distributed and often inhibitory neurotransmitter that has been shown to play important roles in the nervous systems of both vertebrates (Martin and Olsen, 2000; Storm-Mathisen, 1974) and invertebrates (Cline, 1986; Kuffler and Edwards, 1958; McIntire et al., 1993). In this study, we surveyed the overall distribution of GABA immunoreactive (GABA-ir) neurons in the brains of several nudibranch species with the goal of determining if any of the previously identified neurons in the swimming central pattern generator (CPG) circuits are GABA-ir.

We examined GABA-ir in the nudibranch nervous system for several reasons. Nudibranchs, as well as species in the larger class Gastropoda that they belong to, display behaviors that can be dissected with cellular precision. Their nervous systems contain individually identifiable neurons, which form distinct neural circuits that underlie simple behaviors (Carew and Kandel, 1977a; Elliott and Susswein, 2002; Jing and Gillette, 1999; Lillvis et al., 2012; Lillvis and Katz, 2013; Winlow and Syed, 1992). One such behavior, which has been studied in great depth, is swimming. Several species in the order Nudibranchia exhibit one of two different swimming behaviors, which involve either whole body alternating flexions in the dorsal-ventral (DV) directions or alternating left-right (LR) body flexions (Newcomb et al., 2012). These two swimming behaviors are produced by CPG circuits that have fundamentally different circuit architecture with distinct neuronal types, several of which make inhibitory
synapses within their respective circuits (Lillvis and Katz, 2013; Newcomb et al., 2012; Sakurai et al., 2011). It is therefore plausible that GABA, which often functions as an inhibitory neurotransmitter, may mediate some of these connections. It was not known if any of these neurons are GABA-ir; in fact, the GABA distribution in the nudibranch nervous system has never been examined previously.

GABA-mediated neurotransmission and neuromodulation have been shown to be significant for a range of behaviors in gastropods including: feeding (Bravarenko et al., 2001; Diaz-Rios and Miller, 2005; Jing et al., 2003; Norekian and Malyshev, 2005), respiration (Moccia et al., 2009), olfaction (Ito et al., 2004; Kobayashi et al., 2008), graviception (Jin et al., 2009), reproduction (Romanova et al., 1996), and developmental metamorphosis (Morse et al., 1980; Morse et al., 1979). The pattern of GABA distribution in other gastropods has led to the speculation that GABA is involved in coordinating bilateral systems such as those regulating locomotion and feeding (Bravarenko et al., 2001; Diaz-Rios et al., 1999; Ierusalimsky and Balaban, 2001). Swimming in the nudibranchs is such a bilateral system that involves inhibitory connectivity.

Although homologous neurons have been identified across nudibranch species (Baltzley and Lohmann, 2008; Lillvis et al., 2012; Newcomb et al., 2006; Sakurai et al., 2011), little headway has been made in determining which features of the nudibranch nervous system are general and which are species-specific. Comparison of the distribution of GABA-ir neurons in the brains of nudibranch species will help in determining the ground plan for nervous systems in this group of animals.
2.3 MATERIALS AND METHODS

2.3.1 Animal collection and maintenance

The species *Melibe leonina* (30 - 100 mm), *Dendronotus iris* (60 - 200 mm) and *Hermisenda crassicornis* (15 - 40 mm) were collected as adults by Monterey Abalone Company (Monterey, CA, USA). *Tritonia diomedea* (50-200 mm) and additional *Melibe leonina* and *Dendronotus iris* were provided by Living Elements (Vancouver, BC, Canada). All animals were maintained in recirculating artificial seawater (Instant Ocean, Blackburg, VA, USA) tanks on a fixed 12hr:12hr light:dark cycle at 10ºC-12ºC.

2.3.2 Brain nomenclature

We used the same naming conventions for the central ganglia of nudibranchs as established in a previous study by Newcomb et al. (2006). Briefly, the brain is composed of three, bilaterally represented ganglia: the cerebral, pleural and pedal ganglia. The buccal ganglia are the subject of a future study. For naming purposes, each ganglion is divided into quadrants defined as: anterior (A), posterior (P), lateral (L), and medial (M). This applies to both the dorsal (d) and ventral (v) surfaces of the brain (Fig. 2.1A). As the overall brain silhouettes of the species in this study differed from each other, a schematic delineating the quadrants in each species is provided (Fig. 2.1B-E). The symbol γ was used to represent GABA. So, for example, a cluster of GABA-ir neurons located on the dorsal surface of the cerebral ganglia in the posterior medial quadrant would be named dCeγPM (dorsal Cerebral GABAergic Posterior Medial) cluster. This also applies to individually identifiable GABA-ir neurons. For example, an individually identifiable GABA-ir neuron on the lateral half of the ventral pleural ganglion could be named the vPlyL neuron.
2.3.3 Dissection

*Melibe, Dendronotus* and *Hermissenda* were anaesthetized by injecting the body cavity with 0.33 M magnesium chloride solution. *Tritonia* were anaesthetized by chilling. A cut was made on the dorsal body wall near the esophagus. The brain was extracted from the body by cutting all nerve roots. The brain was transferred and pinned to a Sylgard-lined dish superfused by physiological saline or with artificial seawater. Saline composition was (in mM): 20 NaCl, 10 KCl, 10 CaCl$_2$, 50 MgCl$_2$, 10 D-Glucose, and 10 HEPES, pH 7.4. The brain was kept at 4°C while surrounding connective tissue was manually removed using fine forceps and scissors. When electrophysiology was performed before whole-mount immunohistochemistry, the fine sheath immediately encasing the brain was manually removed to allow for penetration of the underlying neurons with sharp electrodes.

2.3.4 Behavioral Whole-mount immunohistochemistry

Brains were fixed for 6-24 hours at 4°C in paraformaldehyde-lysine-periodate fixative: 4% paraformaldehyde, 1.85% lysine monohydrochloride, and 0.22% sodium periodate in cacodylate buffer (0.2 M cacodylic acid in 0.3 M NaCl, pH 7.4 –7.6). The tissue was quickly rinsed several times with phosphate-buffered saline (PBS): 50 mM Na$_2$HPO$_4$ in 140 mM NaCl, pH 7.2. Brains still encased by the fine sheath were desheathed. If the brain is desheathed prior to fixation the cell bodies of neurons could fall off or be mechanically broken during the desheathing and/or the consequent processing, resulting in an undercount of GABA-ir cells. Therefore, to minimize this, the majority of preparations were desheathed after fixation. The desheathed brains were pinned onto small Sylgard blocks. This further minimized cell loss as the brains themselves were not directly handled during consequent processing. Instead, the Sylgard blocks with the brains attached were transferred with forceps into different solutions.
Desheathing was followed by two longer PBS rinses (3 hours each). The tissue was then washed twice with 4.0% Triton X-100 in PBS (3 hours each) and then incubated in antiserum diluent (ASD; 0.5% Triton X-100, 1% normal goat serum, and 1% bovine serum albumin in PBS) for 1-2 hours. The brains were incubated for 3-5 days in the primary antiserum diluted in ASD (see Table 2.1 for primary antibody details). The brains were washed six times (1 hour each) with 0.5% Triton X-100 in PBS and then incubated overnight in goat anti-rabbit antiserum conjugated to Alexa 488 (Invitrogen, Carlsbad, CA) diluted to 1:100 in ASD. Finally, the tissue was washed six times (1 hour each) with 0.5% Triton X-100 in PBS, dehydrated in an ethanol series (70%, 80%, 2x90%, 95%, 3x100%, 20 mins each), cleared in methyl salicylate and mounted on a slide with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). The tissue was kept at 4°C under gentle agitation for the entire immunohistochemistry protocol, except for dehydration and clearing.

2.3.5 Primary antibody characterization and immunohistochemistry controls

GABA immunolabeling was obtained using a polyclonal rabbit antibody (A2052; Sigma-Aldrich) whose specificity was tested with a dot blot assay that showed positive binding with GABA but negative binding with bovine serum albumin (BSA; Sigma datasheet). The activity of the primary antiserum was blocked by incubation with the original antigen. These preabsorption controls were performed on the central ganglia of Tritonia (n=1), Melibe (n=1), Dendronotus (n=1), and Hermissenda (n=1). Additional controls for autofluorescence and secondary specificity were performed by incubating in normal serum and not including the primary antibody: Tritonia (n=1), Melibe (n=1), Dendronotus (n=1), and Hermissenda (n=1). In all the controls, there was no fluorescence observed in the brain.
2.3.6 Nerve backfills

When backfills of the pedal 2 commissure (PP2) were performed, the cut end of the nerve was drawn into a petroleum jelly well created on top of a Sylgard block. Several drops of distilled water (dH2O) were added to the well and the nerve was cut again and left in the dH2O for 30 seconds. The dH2O was then replaced by a 2-2.5% solution of the biotinylated tracer, Biocytin (Invitrogen, Carlsbad, CA) dissolved in 0.75 M KCl. The well was covered by more petroleum jelly to reduce evaporation of the tracer and the preparation was incubated at 4°C for 6-48 hours. During this incubation, the tracer was transported retrogradely to cell bodies of neurons with axons in the PP2 nerve. After incubation, the brain was washed briefly in saline and whole-mount immunohistochemistry for GABA was performed as described earlier. The immunohistochemistry protocol was identical except that the tissue was incubated in primary antiserum mixed with Streptavidin-Alexa Fluor 594 conjugate (Invitrogen, Carlsbad, CA) at 1:200 dilution, to visualize the biotinylated tracer.

2.3.7 Tracer injection and electrophysiology

All intracellular recordings and tracer injections were performed on desheathed brains that were superfused with normal saline at a rate of 1ml/min at 10 °C. In preparations where neurons were filled, the cell body was impaled by a microelectrode filled with a 2% solution of the biotinylated tracer, Biocytin (Invitrogen, Carlsbad, CA) dissolved in 0.75 M KCl. The identities of the swim interneurons of interest were confirmed by a combination of their activity pattern in response to a body wall nerve stimulation, soma location and size, axon projection, and connections to other neurons. Intracellular recordings were made using Axoclamp 2B amplifiers (Molecular Devices, Sunnyvale, CA). The extracellular nerve suction electrodes were connected to an A-M Systems Differential AC Amplifier (Model 1700, A-M Systems, Carlsborg, WA).
After cell identity was confirmed with intracellular recordings, the biotinylated tracer was injected via iontophoresis for 15-30 minutes (bipolar current pulses from -10 nA to +3 nA, 1 Hz, 50% duty cycle). The preparations were maintained in normal saline for 1 - 4 hrs prior to fixation and processing for whole-mount immunohistochemistry with the addition of Streptavidin-Alexa Fluor 594 conjugate at 1:200 dilution to visualize the biotinylated tracer.

2.3.8 Imaging

The tissue was imaged using a Zeiss LSM 700 Axio Examiner D1 confocal microscope (Carl Zeiss, Oberkochen, Germany) with 5-20x objectives. Fluorophores were excited with two lasers (488 and 555 nm) and fluorescent emissions were passed through a 505-550 nm band-pass filter to visualize Alexa Fluro 488 and a 560 nm long-pass filter to visualize Alexa Fluro 594. The LSM 700 software ZEN was used to acquire the images. The thickness of the optical sections within a confocal stack was optimized and kept consistent within a preparation. Maximal projections of confocal stacks were made and exported as TIFF files to Adobe Photoshop CS where montages were assembled. The images were converted to grayscale and the intensity was inverted such that labeled neurons, axons and neuropil appeared dark. For images showing neuron fills combined with GABA-ir, false color was used to differentiate between different neuronal types. Brightness and contrast of all images were adjusted when necessary.

2.3.9 Data Analysis

GABA-ir neuron clusters were defined as a close grouping of GABA-ir neurons that were separated from other GABA-ir neurons by a clear space that lacked any GABA immunoreactivity. In every preparation, the numbers of GABA-ir neurons in each GABA-ir cluster were counted. These counts were made from the original slide at the confocal microscope and not from the confocal stacks. As the data were not always normally distributed, the median
and semi-interquartile range were determined to be the best measure of central tendency with respect to cell counts within individual clusters. In every preparation, individually identifiable GABA-ir neurons that were not part of a cluster were counted from the original slide. As cell body loss during processing can greatly skew the counts of these single neurons, the cell count data for each neuron type was represented as the number of preparations out of the whole, where at least one neuron of that type was observed. In some preparations, a distinct GABA-ir axon without an attached cell body was observed emanating from the characteristic location of a neuron type, suggesting that the cell body was lost during processing. We considered these cases as a positive count for that neuron type. It should be noted that this situation only arose in two preparations.

2.4 RESULTS

The overall organization of GABA-ir neurons was similar across the nudibranch nervous systems. Examination of the dorsal and ventral sides of the brains of Tritonia, Melibe, Dendronotus and Hermissenda revealed several similar GABA-ir clusters and individual neurons (Figs. 2.2 and 2.3). Where possible, we will give these clusters and individual neurons the same name to indicate possible homology.

2.4.1 GABA-ir neurons in the cerebral ganglion

2.4.1.1 dCeyPM Cluster

There was a discrete cluster of GABA-ir neurons on the dorsal surface of the cerebral ganglion in the posterior medial region (dCeyPM; Fig. 2.2, dorsal and Fig. 2.3, red). This cluster consists of a tight grouping of very small neurons (2-12 μm) near the cerebral commissure (Fig. 2.4). In Tritonia (Fig. 4A) and Dendronotus (Fig. 2.4C) the dCeyPM cluster had a similar number
of somata (2.2), while in *Melibe* (Fig. 2.4B) and *Hermissenda* (Fig. 2.4D) the cluster had fewer neurons (Table 2.2).

**2.4.1.2 *CeγL Cluster***

A cluster of GABA-ir neurons spanned the lateral margins on both the dorsal and ventral sides of the cerebral ganglion in each species (*CeγL*; Fig. 2.2 and Fig. 2.3, *orange*). The number of neurons in this cluster varied across species (Table 2.2). The soma size within the *CeγL* cluster was heterogeneous, ranging from 20 to 50 µm (Fig. 2.5A-D). Some of the *CeγL* neurons in *Tritonia, Melibe* and *Dendronotus* appeared to project their axons towards the cerebral commissure. In some *Tritonia, Melibe* and *Dendronotus* preparations, we were able to discern that one or two *CeγL* neurons projected an axon to the cerebral-buccal connective (CBC), which leads to the buccal ganglia.

**2.4.1.3 dCeγAM Cluster**

In *Melibe*, on the dorsal surface of the cerebral ganglion, there was an anterior medial cluster (d*CeγAM*; Fig. 2.2B, *dorsal* and Fig. 2.3B, *brown*), which did not seem to have an equivalent in any of the other species. The d*CeγAM* cluster consisted of 4 GABA-ir neurons that ranged from 10 to 15 µm in diameter (Table 2.2, Fig. 2.5B *black arrow*). These neurons were located close to the *CeγL* cluster and there was a clear space between the two groups (Fig. 2.5B). However, in two preparations, it was difficult to clearly distinguish between one of the d*CeγAM* and CeγL cluster pairs.

**2.4.2 GABA-ir neurons in the pleural ganglion**

There were no clusters of GABA-ir neurons in the pleural ganglia. In one *Tritonia* preparation, there was a single GABA-ir neuron (~200µm) located on the posterior end of the
right pleural ganglion that spanned the dorsal and ventral sides (Fig. 2.2A, black arrows), but this neuron was not found in any other Tritonia preparation or in any other species. However, there were two individually identifiable GABA-ir neurons found in all of the species: vP\(\gamma\)L and dP\(\gamma\)A (Table 2.3).

2.4.2.1 vP\(\gamma\)L neuron

vP\(\gamma\)L was located on the ventral side of the pleural ganglia on the lateral margin (Fig. 2.2, ventral and Fig. 2.3, green) (Table 2.3). This neuron was very distinct and projected its axon ipsilaterally to the pedal ganglion via the posterior cerebral-pedal connective (Fig. 2.6, black arrow). There was extensive neurite arborization near the soma of vP\(\gamma\)L (Fig. 2.6), some of which arose from other neurons. In two Tritonia preparations, there was an additional GABA-ir neuron in the lateral pleural region where vP\(\gamma\)L is located, but this neuron was distinguishable from vP\(\gamma\)L because its dendritic arborization was not as distinct and extensive. In one Hermissenda preparation, there were two additional cells near the right vP\(\gamma\)L but they were located between the dorsal and ventral sides and displayed minimal dendritic arborization close to the soma (Fig. 2.2D, black arrows).

2.4.2.2 dP\(\gamma\)A neuron

dP\(\gamma\)A was located on the dorsal surface of the anterior pleural ganglia (Fig. 2.2, dorsal and Fig. 2.3, dark blue). This neuron was not as strongly GABA-ir as vP\(\gamma\)L; we did not observe any neurite arborization and could not trace its axon projection. While this neuron was reliably present in Tritonia, we observed dP\(\gamma\)A in only 50% or fewer of the preparations in the other three species (Table 2.3). In one Hermissenda preparation there was an additional cell located lateral to the left dP\(\gamma\)A.
2.4.3  *GABA-ir neurons in the pedal ganglion*

2.4.3.1  *vPdγA Cluster*

In each species, there was a cluster of GABA-ir neurons on the ventral surface of the anterior pedal ganglion, close to the cerebral-pedal connective (vPdγA; Fig. 2.2, *ventral* and Fig. 2.3, *cyan*). The median number of neurons in this cluster was consistent across species, ranging from 7 to 11 neurons (Table 2.2). The somata were small, with diameters ranging from 5 to 18 μm in *Tritonia, Melibe* and *Hermissenda* and 10-25 μm in *Dendronotus* (Fig. 2.7A-D). The axons of these neurons projected towards the lateral pedal ganglia in a single highly fasciculated tract in all species except *Hermissenda*, where we were unable to discern the axons of the cluster (Fig. 2.7A-C).

2.4.3.2  *Pdγ*

In each species, the pedal ganglion contained GABA-ir neurons that were dispersed, which we collectively refer to as *Pdγ* (Table 2.2). These neurons were located in positions ranging from the dorsal and ventral surfaces to a few cell layers below the surface (Fig. 2.2 and Fig. 2.3, *purple*). A few of these neurons had characteristics that made them individually recognizable within and across species. These neurons were named *Pdγ* followed by a number (Fig. 2.2, *ventral* and Fig. 2.3, *pink numbers*).

2.4.3.3  *Pdγ1 neuron*

*Pdγ1* in *Tritonia* was ~ 50 μm and located in the lateral region of the pedal ganglion (Table 2.3, Fig. 2.8A). The soma was not on the ganglion surface but is more visible from the ventral surface. The axon that *Pdγ1* sent towards the anterior medial region of the pedal had a characteristic bend (Fig. 2.8A, arrowhead) and traveled towards the *vPdγA* cluster. This neuron
was bipolar and sent a second axon through the pedal commissure 2 (PP2) as confirmed by nerve backfills of PP2 combined with GABA immunohistochemistry (Fig. 2.8B).

Similar to *Tritonia*, there was a 50-60 µm bipolar neuron in *Melibe* that was located in the lateral region of the pedal ganglion, more visible from the ventral surface, and sent an axon through PP2 (Table 2.3, Fig. 2.8C). In *Dendronotus*, there was a single 60-80 µm bipolar neuron in this same position that also had an axon in PP2 (Table 2.3, Fig. 2.8D). It was clearly distinguishable from two or three other bipolar neurons in this region, which did not have axons in PP2 (Fig. 2.8D, white arrows). In *Hermissenda*, there was a ~ 30 µm neuron located in this same position, with the characteristic bend in its axon seen in *Tritonia* (Table 2.3, Fig. 2.8E). As with *Pdy*1 in *Tritonia*, the axon of this neuron in *Hermissenda* projected towards the anterior medial region of the pedal. We do not know if the neuron is bipolar with an axon in PP2 because the axon is not visible in the GABA-immunoreactivity and backfills of PP2 in *Hermissenda* were not feasible due to the short length of this nerve.

In *Melibe* and *Dendronotus*, *Pdy*1 was only ever found on the left side; it was never bilaterally represented. Therefore, in *Melibe* and *Dendronotus*, there may be just one lateralized *Pdy*1, unlike in *Tritonia* and *Hermissenda* where there were always contralateral counterparts.

### 2.4.3.4 Pdy2 neuron

*Pdy*2 in *Tritonia* was ~30 µm and located in the anterior region of the pedal ganglion (Table 2.3, Fig. 2.8A). Like *Pdy*1, its soma was more visible from the ventral side. It was always located lateral to the vPdyA cluster and sent its axon towards the center of the pedal ganglion (Fig. 8A). In *Melibe*, there was also a neuron (~ 50 µm) in the lateral region of the pedal ganglion that sent its axon towards the center of the ganglion (Table 2.3, Fig. 2.8C). We were
unable to distinguish possible equivalents of \(Pd\gamma 2\) in \textit{Dendronotus}. In six out of seven \textit{Hermissenda} preparations, there was a row of three GABA-ir neurons in the anterior region of each pedal ganglion below the \(vPd\gamma A\) cluster (Fig. 2.8F, \textit{black arrows}). These three neurons were \(\sim 25\ \mu m\) in diameter and one soma was located near the ventral surface while the other two somas were more visible from the dorsal surface. The axon projections of these neurons could not be determined. It is possible that one of these neurons is equivalent to \(Pd\gamma 2\).

\textbf{2.4.4 GABA-ir axon tracts}

Almost all of the GABA-ir axons were restricted to the central nervous system, with many axons visible in the ganglionic connectives and commissures (Fig. 2.2). There were axon tracts in the anterior and posterior cerebral commissures (Fig. 2.9A-D). The pedal commissure, PP2 contained GABA-ir axons as did the cerebral-buccal connective in each species. However, nerves projecting towards the periphery were markedly lacking GABA-ir axons with very few exceptions. In three out of seven \textit{Tritonia} preparations, there was a single GABA-ir axon exiting pleural nerve 1, which emanates from the posterior end of the pleural ganglion. In five out of seven \textit{Dendronotus} preparations and two \textit{Tritonia} preparations, we observed a maximum of 2 axons exiting from a large nerve in the anterior cerebral ganglion named cerebral nerve 2. In \textit{Melibe}, we sometimes observed fine GABA-ir processes in a large pedal ganglion body wall nerve (pedal nerve 2). These processes originated from the neuropil in the pedal ganglion and never extended very far down the body wall nerve.
2.4.5 Previously identified swim interneurons are not GABA-ir

2.4.5.1 Swim interneurons in Tritonia are not GABA-ir

The CPG underlying the DV swimming behavior of *Tritonia* contains three identified neuron types: C2 (http://neuronbank.org/Tri0002380), DSI (http://neuronbank.org/Tri0001043) and VSI-B (http://neuronbank.org/Tri0002436) (Fig. 2.10A)(Katz, 2009). These neurons can be unambiguously identified by their pattern of activity during a swim motor pattern (Fig. 2.10B). Each neuron was identified electrophysiologically and then injected with a biotinylated tracer for double-label immunohistochemistry. C2 and DSI are located on the dorsal surface of the cerebral ganglia and project their axons contralaterally through the cerebral commissure. The relative position, size and projection of C2 in particular are comparable to some of the neurons in the CeɣL cluster. However, double labeling experiments combining intracellular tracer fills of C2 (n=4) and DSI (n=4) with GABA immunohistochemistry demonstrated that these swim interneurons are not GABA-ir (Fig. 2.10C).

VSI-B is located in the lateral region of the ventral pleural ganglion and projects its axon ipsilaterally to the pedal ganglion. The vPlɣL neuron was of comparable size and location to VSI-B. Furthermore, the GABA staining revealed that vPlɣL also projected its axon ipsilaterally to the pedal ganglion. However, double labeling experiments combining intracellular tracer fills of VSI-B with GABA immunohistochemistry (n=4) showed that VSI-B is not GABA-ir although its soma is often located immediately adjacent to vPlɣL (Fig. 2.10D). There is another identified neuron, VSI-A, which is rhythmically active and makes an inhibitory connection to DSI (Getting et al., 1980). This neuron was also not GABA-ir (data not shown). Thus, none of the swim CPG neurons in *Tritonia* are GABA-ir, indicating that GABA may not be involved in the swim motor pattern generation.
2.4.5.2 Swim interneurons in Melibe are not GABA-ir

The CPG for LR swimming in Melibe contains two swim interneurons called Si1 (http://neuronbank.org/Mel0002265) and Si2 (http://neuronbank.org/Mel0002582) (Fig. 2.11A) (Thompson and Watson, 2005). These neurons can be identified by their bursting activity during a swim motor pattern with ipsilateral Si1 and Si2 bursting in phase with each other (Fig. 2.11B). Si1 is located on the dorsal medial cerebral ganglion close to the cerebral commissure. Si2 is located on the dorsal surface of the pedal ganglion. Its relative position and size is comparable to some of the Pdɣ neurons. Intracellular tracer fills of Si1 (n=4) and S2 (n=3) combined with GABA immunohistochemistry showed that neither of these swim interneurons were GABA-ir (Fig. 2.11C, D). However, Si2 was located immediately adjacent to two Pdɣ neurons and about 100 µm posterior of the vPdɣA cluster (Fig. 2.11D). The results indicate that GABAergic neurons may not be involved in the swim motor pattern generation in Melibe.

2.4.5.3 Swim interneurons in Dendronotus are not GABA-ir

The neural circuit underlying LR swimming in Dendronotus includes Si2, which is the homologue of the swim interneuron Si2 in Melibe (Fig. 2.11E) (Sakurai et al., 2011). In contrast to the Melibe Si1, in Dendronotus Si1 does not burst during the swim motor pattern (Fig. 2.11F). Nonetheless, Si1 can be identified using the same anatomical criteria used to identify its homologue in Melibe (Sakurai et al., 2011); Si1 is located on the dorsal medial cerebral ganglion close to the cerebral commissure and has a distinctive axon projection pattern. Si2 is located on the dorsal pedal ganglion (Fig. 2.11E). Its relative position and size is comparable to some of the Pdɣ neurons. Double labeling experiments involving intracellular tracer fills of Si1 (n=3) and S2 (n=3) combined with GABA-ir showed that, as in Melibe, neither of these swim interneurons were GABA-ir (Fig. 2.11G,H). As in Melibe, Si2 was located immediately adjacent to two Pdɣ
neurons (Fig. 2.11H).

2.5 DISCUSSION

In this study, we compared the numbers and locations of GABA-ir neurons in the central ganglia of the nudibranch species *Tritonia diomedea, Dendronotus iris, Melibe leonina* and *Hermissenda crassicornis*. There were distinct similarities in the distribution of GABA-ir neurons with recognizable clusters and individual neurons across species. We conclude that these clusters and individual neurons are most likely homologous.

There was large inter-species variation in the total number of GABA immunoreactive neurons in the brain, ranging from $66 \pm 5$ GABA-ir neurons in *Hermissenda* (median ± semi-interquartile range) to $128.5 \pm 5$ in *Dendronotus*. Some clusters varied more than others. In all species, the cerebral ganglion contained the majority of GABA-ir neurons followed by the pedal ganglion. The pleural ganglion contained a maximum of 2 neurons that regularly appeared.

There were GABAergic fiber tracts observed in the cerebral commissure as well as GABA-ir axons in the pedal 2 (PP2) commissure. The GABAergic system appears to be largely restricted to the central nervous system with very few GABA-ir fibers observed in nerves projecting to the periphery. This suggests that GABA serves almost exclusively as a neurotransmitter for interneurons, rather than efferent or afferent neurons.

Examination of the overall distribution revealed GABA-ir neurons that were of similar size and location to certain identified neurons underlying swimming behavior in *Tritonia, Dendronotus* and *Melibe*. However, tracer injections combined with GABA immunostaining determined that these swim interneurons were not GABA-ir. VSI-B is located next to a distinct and recognizable GABA-ir neuron, vPlγL. The Si2 soma is also immediately adjacent to GABA-
ir neurons.

2.5.1 GABA in molluscs

GABA has been shown to be present in the nervous systems of species in all the major classes of Mollusca, including Cephalopoda (Cornwell et al., 1993), Bivalvia (Karhunen et al., 1993; Vitellaro-Zuccarello and De Biasi, 1988) and Gastropoda (Soinila and Mpitsos, 1991). Within Gastropoda, GABA-ir has been described to varying degrees in pulmonate snails and slugs (Mollusca, Gastropoda, Pulmonata). This includes the species Helisoma trivolvis (Richmond et al., 1991), Planorbis corneus (Turner and Cottrell, 1978), Lymnaea stagnalis (Hatakeyama and Ito, 2000), Helix pomatia (Hernadi, 1994), Helix aspersa (Ierusalimsky and Balaban, 2001), Helix lucorum (Bravarenko et al., 2001), Cepaea nemoralis (Dyakonova et al., 1995) and Limax maximus (Cooke and Gelperin, 1988). GABA-ir neurons are located primarily in the cerebral, pedal and buccal ganglia in the above species, although a single pair of neurons was found in the pleural ganglia of Lymnaea.

Among the Opisthobranchia, GABA-ir has been described in only a handful of species, the best-studied being the Sea Hare, Aplysia californica (Diaz-Rios et al., 1999). GABA immunoreactivity has also been described in the Sea Angel, Clione limacina (Arshavsky et al., 1993) and cursorily in the Bubble snail, Bulla gouldiana (Michel et al., 2000). As in the pulmonates, the GABA distribution was restricted primarily to the cerebral, pedal and buccal ganglia.

GABA distribution has not been previously described in the order Nudibranchia, with just a brief description in Pleurobranchaea californica, which is in the sister group Pleurobranchomorpha (Soinila and Mpitsos, 1991). The presence of endogenous GABA was
demonstrated in the nudibranch *Hermissenda crassicornis* by HPLC chromatography using sonicated whole brain samples (Alkon et al., 1993). That study primarily focused on the GABA-ir sensory hair cells located within the statocysts, a fluid filled graviception organ.

### 2.5.2 CeyL cluster

In *Aplysia*, all the GABA-ir neurons in the cerebral ganglion were located in the anterior and lateral regions and ranged from the dorsal to the ventral surface. The neurons were of variable size, shape and staining intensity and some projected their axons towards the cerebral commissure. At least two GABA-ir neurons projected to the cerebral-buccal connective (Diaz-Rios et al., 1999). These features of the *Aplysia* cerebral GABA-ir neurons are comparable to the characteristics of neurons in the CeyL cluster found in this study and may indicate that these clusters are homologous. The *Aplysia* GABA-ir neurons in this region that project to the buccal ganglion via the CBC were identified as the Cerebral Buccal Interneurons, CBI-3 and CBI-11, both of which are involved in feeding behavior (Jing et al., 2003; Wu et al., 2003). A homologous neuron to the GABA-ir CBI-3 neuron was also found in the related Aplysiid, *Aplysia kurodai* (Narusuye et al., 2005). *Clione* also contains an identified feeding neuron (Cr-BM) that is GABA-ir, located in the anterior lateral cerebral ganglion, and projected to the buccal ganglia via the CBC (Norekian and Malyshev, 2005). We observed CeyL neurons that projected through the CBC in the nudibranchs. These neurons may be homologous to the *Aplysia* CBI-3 and CBI-11 and *Clione* Cr-BM neurons.

Another GABA-ir neuron of the prey capture network of *Clione* (Cr-Aint) is located in the anterior lateral cerebral ganglion with an axon that projects towards the cerebral commissure (Norekian, 1999). It is highly likely that GABA-ir neurons in nudibranchs, particularly those in the CeyL cluster, are involved in feeding behavior as well.
2.5.3 dCe\_PM cluster

No clusters in the *Aplysia* or *Clione* cerebral ganglion were overtly similar to the nudibranch dCe\_PM cluster. However, Diaz-Rios et al. (1999) noted that the dorsal cerebral neurons in *Aplysia* ranged in size from 10-80\(\mu\)m and that the smaller neurons were always grouped together. It is plausible that this group is homologous to the dCe\_PM cluster and that the exact location has diverged in the aplysiids and nudibranchs. The cerebral and pleural ganglia in *Aplysia* are separated by long connectives unlike the fused cerebral-pleural ganglia in the nudibranchs. It is possible that this difference in the overall organization of the brain may have resulted in displacement of homologous clusters. The description of the GABA distribution in the Nudipleura mollusc *Pleurobranchaea* by Soinila and Mpiptos (1991) is too brief to make extensive comparisons to the nudibranchs. However, the authors mention the presence of several small GABA-ir neurons on the dorsal cerebral ganglion close to the cerebral commissure. This is similar to the dCe\_PM cluster in the nudibranchs and may indicate homology.

2.5.4 dCe\_AM cluster

*Melibe* exhibited an additional cluster in the cerebral ganglion, dCe\_AM, which was not present in any of the other nudibranchs. There is also no evidence of a similar cluster in the gastropod literature. It is possible that four neurons from the CeyL cluster may have migrated medially to form what appears to be a separate cluster in *Melibe*.

2.5.5 vPd\_A cluster

The *Aplysia* pedal ganglion contains four clusters of small to medium sized GABA-ir neurons located from the anterior medial region to the anterior lateral region of the ganglion (Diaz-Rios et al., 1999). The clusters are located below the surface layer of neurons and are more visible from the ventral surface. Interestingly, these clusters were associated with a single highly
fasciculated fiber tract that projected towards the pedal commissure. This is very similar to the vPdγA cluster in the nudibranchs, which is also associated with a single tight fascicle. The lack of additional clusters in the nudibranch pedal ganglia may represent divergence between the two groups. The anterior pedal ganglion of Clione also contained a cluster of 6-7 small (15-25 µm) GABA-ir neurons that may be homologous to vPdγA (Arshavsky et al., 1993). Interestingly, even the more distantly related pulmonates possess GABA-ir pedal clusters that are highly similar to the vPdγA, with a tight axon bundle projecting towards the pedal commissure (Bravarenko et al., 2001; Richmond et al., 1991). This strong anatomical conservation could indicate an important functional feature.

2.5.6 Species-difference in GABA-immunoreactivity

There are species-differences in the total number of neurons in some of the GABA-ir clusters in the nudibranchs including CeyL and dCeγPM. This is consistent with previous studies that show that while neuronal types are conserved, the exact number of neurons of that type can vary among related species (Baltzley and Lohmann, 2008; Callaway et al., 1987; Katz, 1989; Newcomb et al., 2006; Turrigiano and Selverston, 1991). Pdγ1 is bilaterally represented in Tritonia and Hermissenda, but only appears on the left pedal ganglion in Melibe and Dendronotus. Such instances of asymmetry are not unheard of. The nudibranch Armina californica contains a prominent, bilaterally represented serotonergic neuron in the pedal ganglion (dPdSM) yet only a left dPdSM neuron was observed in the ten other nudibranch species examined (Newcomb et al., 2006). Possible explanations for the variability seen in GABA-ir clusters and individual neurons include changes in the neurotransmitter phenotype of homologous neurons across species (Meyrand et al., 2000), changes in homologous cell number via neuronal deletion or duplication (Paul, 1991) or neuron displacement (Munoz et al., 1983).
2.5.7 **GABA in the pleural ganglion**

No GABA-ir neurons have been previously reported in the pleural ganglion of other Gastropod species except for the pulmonate snail, *Lymnaea*. The presence of the vPlγL and dPlγA neurons in the pleural ganglion across the nudibranchs in this study may represent an aspect of the GABAergic system that is unique to the nudibranch nervous system. Further experiments particularly on vPlγL, are required to understand any functional implications of this unique feature of the nudibranch nervous system.

In rare instances, we observed one to two additional GABA-ir neurons in the pleural ganglion aside from dPlγA or vPlγL in *Tritonia* and *Hermissenda*. While it is possible that we are unable to reliably stain these neurons in every preparation due to an insufficiently sensitive immunohistochemistry protocol, it is also possible that these neurons are products of growth and migratory abnormalities during development in individual animals. Such instances have been noted among identified neurons in *Aplysia* and *Hermissenda* (Hughes, 1967; Newcomb et al., 2006).

2.5.8 **GABA as a central transmitter**

All the previous studies of GABA-immunoreactivity in gastropods indicate that GABA is restricted to the central nervous system. The few exceptions include limited GABA innervation of the peripheral nerve and lips in *Helix pomatia* (Hernadi, 1994), GABA innervation of the *Lymanaea* osphradium, a putative chemosensory organ (Nezlin and Voronezhskaya, 1997), and limited GABA innervation of the *Lymnaea* head retractor muscle (Kononenko and Zhukov, 2005). In this study, we noted that in five out of seven *Dendronotus* preparations, there were a maximum of two GABA-ir fibers that exited the central nervous system via an anterior cerebral nerve. This limited peripheral projection may be a feature that is unique to the *Dendronotus*
lineage. However, the data from this study overwhelmingly indicate that the GABAergic system in the nudibranchs is largely restricted to the central nervous system. This suggests a lack of GABA-ergic involvement in peripheral motor or sensory projections and perhaps more of an involvement in bilateral coordination and integration. While we show in this study that none of identified neurons in the circuits underlying the bilaterally coordinated swimming behavior in nudibranchs are GABAergic, it is likely that GABA-ir neurons are involved in other behaviors that involve bilateral coordination such as approach or avoidance behaviors (Byrne, 1980; 1981; Carew and Kandel, 1977b; Hirayama and Gillette, 2012).

The similarity in the gross GABAergic organization among the nudibranchs, the other opisthobranchs, and the distantly related pulmonates, indicates that the molluscan GABAergic system is ancient and highly conserved. Additional features seen in the nudibranchs, such as the presence of pleural GABA-ir neurons may represent derived features unique to Nudibranchia.

2.6 ACKNOWLEDGEMENTS

The authors thank Joshua Lillvis for helpful comments on the manuscript. This work was supported by National Science Foundation grants IOS-081441, IOS-1120950 and a Sigma Xi GIAR.
Figure 2.1 Nudibranch brain anatomy and nomenclature.
The naming conventions used in this study are based on the nomenclature established by Newcomb et al, 2006. (A) Outlined is a dorsal view of a stereotyped Nudibranch brain. The brain is composed of three, bilaterally symmetric ganglia called the Cerebral (Ce), Pleural (Pl) and
Pedal (Pd) ganglia. Prominent nerves and the pedal commissures (PP1 and PP2) are also pictured on the right. GABA-ir neurons and clusters were named primarily by location on the brain. For this purpose, each ganglion was divided into halves: Anterior (A)/Posterior (P), Lateral (L)/Medial (M). The thick black dashed line delineates the border between the cerebral and pleural ganglia. As the overall brain silhouette, particularly of the pedal ganglia differ across the species in this study, brain schematics of (B) Tritonia diomedea, (C) Melibe leonina, (D) Dendronotus iris and (E) Hermissenda crassicornis, are provided with particular attention to the pedal ganglia. Large black circles on C and E represent the eyes of the respective species.
Figure 2.2  GABA immunoreactivity of nudibranch brains.
A montage of confocal images of the dorsal (left side) and the ventral (right side) surfaces of representative preparations of (A) *Tritonia diomedea*, (B) *Melibe leonina*, (C) *Dendronotus iris* and (D) *Hermissenda crassicornis*. Recognizable clusters and individual neurons are indicated by dashed circles. These include the clusters $\text{Cey}_L$, $\text{dCey}_{PM}$, $\text{dCey}_{AM}$ (*Melibe* only), and $\text{vPdy}_{A}$, and the neurons $\text{dPly}_{A}$ and $\text{vPly}_{L}$, as well as the dispersed group of pedal neurons collectively grouped as the $\text{Pdy}$. Neurons in the $\text{Cey}_L$ cluster are located between the dorsal and...
ventral sides. It should be noted that because of its thinness and optical transparency, some neurons and clusters in the *Melibe* (B) and *Dendronotus* (C) images can be seen from the dorsal and ventral sides. Black arrows on confocal image A and E point to anomalous GABA-ir neurons not observed in other preparations within the respective species.

![Schematic of the GABA immunoreactivity.](image)

**Figure 2.3 Schematic of the GABA immunoreactivity.**
Schematic of the GABA immunoreactivity of nudibranch brains indicating the median number of the left and right GABA-ir neurons and their locations on the dorsal (left side) and ventral (right
side) surfaces in: (A) Tritonia diomedea (n=7), (B) Melibe leonina (n=8), (C) Dendronotus iris (n=8) and (D) Hermissenda crassicornis (n=7). Recognizable clusters and individual neurons are color-coded and indicated by filled circles. The clusters are CeɣL, dCeɣPM, dCeɣAM (Melibe only) and vPdɣA. The recognizable neurons are dPlɣA and vPlɣL and the dispersed group of pedal neurons collectively are grouped as the Pdɣ. Recognizable neurons within the larger Pdɣ group are differentiated with numbers and colors. Neurons in the CeɣL cluster are located between the dorsal and ventral sides. Gray ovals and parallel gray lines indicate areas of extensive GABA-ir neuropil and axon tracts. Bipolar neurons are illustrated by a circle with two short lines extending from either side of the circle.

Figure 2.4 The dCeɣPM cluster. The dCeɣPM cluster on the dorsal surface of the cerebral ganglion in the posterior medial region in (A) Tritonia diomedea, (B) Melibe leonina, (C) Dendronotus iris and (D) Hermissenda crassicornis.
Figure 2.5 The CeγL cluster.
The CeγL cluster in the lateral cerebral ganglion of (A) Tritonia diomedea, (B) Melibe leonina, (C) Dendronotus iris and (D) Hermissenda crassicornis. The unique dCeγAM cluster of Melibe is seen in B (black arrow).
Figure 2.6 The vPlɣL neurons.
The individually identifiable vPlɣL neurons in (A) Tritonia diomedea, (B) Melibe leonina, (C) Dendronotus iris and (D) Hermissenda crassicornis. The black arrow indicates the general direction of the posterior cerebral-pedal connective.
Figure 2.7 The vPdyA cluster.
The vPdyA cluster in (A) Tritonia diomedea, (B) Melibe leonina, (C) Dendronotus iris and (D) Hermissenda crassicornis.
Figure 2.8 The Pdy1 and Pdy2.
A. *Tritonia diomedea* pedal ganglion showing Pdy1, its characteristic axon shape (arrowhead), and Pdy2 in relation to the vPdyA cluster. B. Pdy1 is bipolar and projects through PP2 in *Tritonia*. Bi. Schematic of the left half of a *Tritonia* brain illustrating the location of Pdy1 and a suction pipette on PP2 indicating a backfill of PP2. Bii. Image of Pdy1 whose soma has been retrogradely labeled by a backfill through PP2. Biii. GABA immunolabeling in the same preparation indicating double labeling of Pdy1. C. *Melibe leonina* pedal ganglion showing that Pdy1 is bipolar and sends its axon through PP2. Pdy2 is located nearby. D. *Dendronotus iris* pedal ganglion indicating that Pdy1 is bipolar, sends its axon to PP2, and is located lateral to the vPdyA cluster. Other bipolar neurons that do not project through PP2 are indicated with white arrows. E. *Hermissenda* pedal ganglion showing Pdy1 and its characteristic axon shape.
(arrowhead). F. *Hermisenda* anterior pedal ganglion showing the characteristic row of $Pd_{\gamma}$ neurons (arrows) and their location lateral to the $vPd_{\gamma}A$ cluster.

**Figure 2.9 Axon tracts through the cerebral commissure.**
Extensive axon tracts through the cerebral commissure as viewed from the ventral side in (A) *Tritonia diomedea*, (B) *Melibe leonina*, (C) *Dendronotus iris* and (D) *Hermisenda crassicornis.*

*Ce*, Cerebral ganglion; *Pl*, Pleural ganglion.
**Figure 2.10** Previously identified swim interneurons in *Tritonia* are not GABA-ir.

A. Schematic of the left half of the Tritonia brain illustrating the location of the swim interneurons C2, DSI and VSI-B. Nearby GABA-ir clusters (CeγL and dCeγPM) and individual neurons (vPlγL) are included. B. Simultaneous intracellular electrophysiological recordings of all three *Tritonia* swim interneurons illustrating the rhythmic bursting pattern that corresponds to the rhythmic swimming behavior. The black arrow on the trace signifies an electric shock to a body wall nerve, which initiates the swim motor pattern *in vitro*. C. Double labeling showing that C2 and DSI are not GABA-ir. White indicates intracellular fills of C2 and DSI. In orange are neurons of the nearby GABA-ir CeγL cluster. D. Double labeling showing that VSI-B is not GABA-ir. White indicates intracellular fill of VSI-B. In green is the GABA-ir vPlγL neuron.
Figure 2.11  Previously identified swim interneurons in *Melibe leonina* and their homologues in *Dendronotus iris* are not GABA-ir.

A. Schematic of the left half of the *Melibe* brain illustrating the location of the swim interneurons Si1 and Si2. Nearby GABA-ir groups (dCeyPM, vPdyA and Pdy) are included. B. Simultaneous intracellular electrophysiological recordings of the two *Melibe* swim interneurons illustrating the rhythmic bursting pattern that corresponds to the rhythmic swimming behavior. C. Double labeling showing that Si1 is not GABA-ir.  D. Double labeling showing that Si2 is not GABA-ir.
E. Schematic of the left half of the *Dendronotus* brain illustrating the location of the neurons Si1 and Si2, which are homologues of the *Melibe* Si1 and Si2. Nearby GABA-ir groups (dCeγPM, νPdγA and Pdγ) are included. F. Simultaneous intracellular microelectrode recordings from Si1 and Si2 show that Si2 bursts during the swim motor pattern, but Si1 does not. G. Double labeling showing that Si1 is not GABA-ir. H. Double labeling showing that Si2 is not GABA-ir. White indicates an intracellular fill of Si1 or Si2. In red is the GABA-ir dCeγPM cluster. In lavender are GABA-ir Pdγ neurons. In cyan is the GABA-ir νPdγA cluster.
Table 2.1 Primary antibodies used in this study

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Table 2.2 Median Cell counts (±semi-interquartile range) for GABA-ir neuron clusters

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<th>GABA cluster</th>
<th>Left/Right</th>
<th>Tritonia diomedea (n=7)</th>
<th>Melibe leonina (n=8)</th>
<th>Dendronotus iris (n=8)</th>
<th>Hermisenda crassicornis (n=7)</th>
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Total whole brain $^3$ 127 ± 8 98 ± 4 128.5 ± 5 66 ± 5

1 For CeγL, it was difficult to clearly differentiate dorsal and ventral neurons from those located in between dorsal and ventral in the anterior cerebral region, hence they were grouped together.

2 Pdγ refers to the GABA-ir neurons distributed throughout the pedal ganglia that do not belong to a distinct GABA-ir cluster. They were grouped together as Pedal GABA (Pdγ) neurons. Some of the Pdγ neurons are individually identifiable and are denoted by Pdγ followed by a number.

3 The total includes the individually identified pleural ganglion neurons listed in Table 2.3.
Table 2.3 Number of preparation with at least one unilaterally or bilaterally represented, visually identifiable GABA-immunoreactive neuron

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<th>Dendronotus iris (n=8)</th>
<th>Hermisenda crassicornis (n=7)</th>
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3 CHAPTER THREE: COMPARATIVE MAPPING OF GABA-IMMUNOREACTIVE NEURONS IN THE BUCCAL GANGLIA OF NUDIPLEURA MOLLUSCS

Charuni A. Gunaratne¹ and Paul S. Katz¹

In preparation for Journal of Comparative Neurology

3.1 ABSTRACT

Phylogenetic comparisons of neurotransmitter distribution are important for understanding the ground plan organization of nervous systems. This study describes the GABA-immunoreactive (GABA-ir) neurons in the buccal ganglia of six sea slug species (Mollusca, Gastropoda, Euthyneura, Nudipleura). In the nudibranch species, *Hermisenda crassicornis*, *Tritonia diomedea*, *Tochuina tetraquetra*, and *Dendronotus iris*, the number of GABA-ir neurons was highly consistent. Another nudibranch, *Melibe leonina*, however, contained approximately half the number of GABA-ir neurons. This may relate to its loss of a radula and its unique feeding behavior. The GABA immunoreactivity in a sister group to the nudibranchs, *Pleurobranchaea californica*, differed drastically from that of the nudibranchs. Not only did it have significantly more GABA-ir neurons but it also had a unique GABA-ir distribution pattern. Furthermore, unlike the nudibranchs, the distribution of GABA-ir in *Pleurobranchaea* was also different from that of the more distantly related euopisthobranch and panpulmonate snails and slugs. This suggests that the distribution of GABA-ir in *Pleurobranchaea* may be a derived feature unique to this lineage. The majority of GABA-ir axons and neuropil in the Nudipleura were restricted to the buccal ganglia, commissures and connectives. However, in *Tritonia* and *Pleurobranchaea* a few GABA-ir fibers were detected in buccal nerves that innervate feeding muscles. Although the specific functions of the GABA-ir neurons in the species in this study are
not known, the innervation pattern suggests these neurons may play an integrative or regulatory role in bilaterally coordinated behaviors in the Nudipleura.

3.2 INTRODUCTION

“Inspired by motives of extreme economy, nature delights in repeating itself”

– Santiago Ramón y Cajal, Recollections of My Life

Neurotransmitter phenotype can provide a wealth of information on the functional properties of neurons and can also serve as a neurochemical marker for neuronal types. Comparing neurotransmitter distribution across related species and examining its correlation with phylogeny is particularly useful in comparative studies of neural circuits. Such comparisons can aid in determining neuronal homology, point towards aspects of that transmitter system that may be ancestral and highly conserved, or highlight species differences in transmitter systems that may underlie the evolution of neural circuits and their emergent behaviors. In this study, we examined the distribution of GABA-immunoreactive (GABA-ir) neurons in the buccal ganglia of six species of Nudipleura molluscs with the goal of determining the extent to which the GABA-ir distribution correlated with the Nudipleura phylogeny.

Nudipleura are shell-less sea slugs that belong to the larger molluscan class of Gastropoda. Gastropods have highly accessible nervous systems with individually identifiable neurons that form small neural circuits underlying simple behaviors. Examples of such behaviors include swimming (Getting et al., 1980; Arshavsky et al., 1998; Jing and Gillette, 1999; Sakurai et al., 2014), feeding (Elliott and Susswein, 2002), inking (Carew and Kandel, 1977), learning and memory (Crow, 1988; Kandel, 2001) and respiration (Winlow and Syed, 1992).

Furthermore, GABA transmission and modulation in the buccal ganglion has been implicated in the control of feeding behaviors in *Aplysia* (Jing et al., 2003), *Clione* (Arshavsky et al., 1993), *Helisoma* (Richmond et al., 1986; Murphy, 1993; Richmond et al., 1994), *Helix* (Bravarenko et al., 2001) and *Limax* (Cooke and Gelperin, 1988).

We examined species in the Nudipleura for two reasons. First, there is recent phylogenetic information of many of the Nudipleura species in this study (Senatore and Katz, 2014), allowing us to examine if the pattern of GABA distribution across species correlates with the phylogeny. Comparing the GABA distribution within and beyond the Nudipleura could highlight which features of the gastropod GABA-ergic system are general and which are species-specific. Second, Nudipleura species show distinct differences in feeding behavior and the types of food they consume, which may be reflected in the GABA distribution. For example, *Dendronotus iris* feeds exclusively on burrowing anemones, which it grabs with its radula using a rearing and lunging motion (Shaw, 1991). *Melibe leonina* feeds on small crustaceans by repeatedly casting its large oral hood like a net and swallowing whole prey (Hurst, 1968). *Hermissenda crassicornis* is a generalist carnivore, preying on conspecifics, cnidarians and tunicates (Megina et al., 2007). *Tritonia diomedea* bites off and ingests octocorals such as sea pens and sea whips using its radula and jaws (Willows, 1978). *Tochuina tetraquetra* feeds on soft corals such as sea strawberries (Wicksten and DeMartini, 1973). *Pleurobranchaea* is highly
predatory and hunts other sea slugs including conspecifics and attacks with an explosive projection of its feeding apparatus (Lee et al., 1974). A detailed map of the GABA-ir neurons in the buccal ganglia will provide a ground plan for a comparative study of the role of GABA or GABA-ir neurons in feeding behavior in the Nudipleura.

3.3 MATERIALS AND METHODS

3.3.1 Animal Collection and Maintenance

Adult specimens of *Hermissenda crassicornis* (15 - 40 mm), *Dendronotus iris* (60 - 200 mm), *Melibe leonina* (30 - 100 mm), and *Pleurobranchaea californica* (60 – 400 mm) were collected by Monterey Abalone Company (Monterey, CA, USA). *Tritonia diomedea* (50-200 mm), *Tochuina tetraquetra* (40-150 mm), and additional *Dendronotus* and *Melibe* were provided by Living Elements (Vancouver, BC, Canada). All animals were maintained in tanks with recirculating artificial seawater (Instant Ocean, Blackburg, VA, USA) and on a fixed 12hr:12hr light:dark cycle at 10-12°C.

3.3.2 Dissection

*Hermissenda, Dendronotus, Melibe* and *Pleurobranchaea* were anaesthetized by injecting the body cavity with a solution of 0.33 M magnesium chloride. *Tritonia* and *Tochuina* were anaesthetized by chilling in the refrigerator. A cut was made on the dorsal body surface above the oesophagus. The buccal ganglia were extracted from the body by cutting all nerve roots. In some preparations, the buccal ganglia were left attached to the rest of the brain (cerebral, pleural, and pedal ganglia) via the cerebral-buccal connective (CBC). The ganglia were transferred and pinned to a Sylgard-lined dish superfused by physiological saline or with artificial seawater. Saline composition was (in mM): 20 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 10
D-glucose, and 10 HEPES, pH 7.4. The ganglia were kept at 4°C while surrounding connective tissue was manually removed using fine forceps and scissors.

### 3.3.3 Brain Nomenclature

We adopted a similar naming convention for the buccal ganglia as used for the cerebral, pleural and pedal ganglia of nudibranchs in previous studies (Newcomb et al., 2006; Gunaratne et al., 2014). Briefly, the buccal ganglia are composed of a pair of hemi-ganglia connected by the buccal commissure (BC). For naming purposes, each hemi-ganglion is divided into quadrants defined as anterior (A), posterior (P), lateral (L), and medial (M). This applies to both the dorsal (d) and ventral (v) surfaces of the buccal ganglia. It should be noted that dorsal and ventral correspond to caudal and rostral in the nomenclature of the *Aplysia californica* buccal ganglia (Diaz-Rios et al., 1999). As there were differences in the overall silhouettes of the buccal ganglia and the buccal nerves among the species in this study, a schematic delineating the quadrants in each species is provided (Fig 3.1Ai-Fi). The symbol γ was used to represent GABA. For example, a cluster of GABA-ir neurons located on the ventral surface of the buccal ganglia in the anterior quadrant would be named vBcγA (ventral Buccal GABAergic Anterior) cluster. This also applies to individually identifiable GABA-ir neurons. For example, an individually identifiable GABA-ir neuron on the posterior medial region of the ventral buccal ganglion could be named the vBcγPM (ventral Buccal GABAergic Posterior Medial) neuron.

### 3.3.4 Whole-mount Immunohistochemistry

The GABA immunohistochemistry protocol used is based on Gunaratne et al. (2014). The tissue was fixed for 6-24 hours at 4°C in paraformaldehyde-lysine-periodate fixative: 4% paraformaldehyde, 1.85% lysine monohydrochloride, and 0.22% sodium periodate in cacodylate
buffer (0.2 M cacodylic acid in 0.3 M NaCl, pH 7.4 –7.6). The buccal ganglia were quickly rinsed several times with phosphate-buffered saline (PBS): 50 mM Na$_2$HPO$_4$ in 140 mM NaCl, pH 7.2. The fine sheath encasing the ganglia was removed using fine forceps and scissors. The desheathed ganglia were pinned onto small Sylgard blocks. This minimized cell loss as the ganglia themselves were not handled any further during consequent processing. Instead, the Sylgard blocks with the buccal ganglia attached were transferred with forceps into different solutions.

Desheathing was followed by two longer PBS rinses (3 hours each). The tissue was then washed twice with 4.0% Triton X-100 in PBS (3 hours each) followed by incubation in antiserum diluent (ASD; 0.5% Triton X-100, 1% normal goat serum, and 1% bovine serum albumin in PBS) for 1-2 hours. The ganglia were then incubated for 3-5 days in the primary antiserum diluted in ASD (see Table 3.1 for primary antibody details). The tissue was washed six times (1 hour each) with 0.5% Triton X-100 in PBS and then incubated overnight in goat anti-rabbit antiserum conjugated to Alexa 488 (Invitrogen, Carlsbad, CA) diluted to 1:100 in ASD. Then the tissue was washed six times (1 hour each) with 0.5% Triton X-100 in PBS, dehydrated in an ethanol series (70%, 80%, 2x90%, 95%, 3x100%, 20 min each), cleared in methyl salicylate and mounted on a slide with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). The tissue was kept at 4ºC under gentle agitation for the entire immunohistochemistry protocol (except for dehydration and clearing).

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3.3.5 **Primary Antibody characterization and immunohistochemistry controls**

GABA immunolabeling was obtained using a polyclonal rabbit antibody (A2052; Sigma-Aldrich) whose specificity was tested with a dot blot assay that showed positive binding with GABA but negative binding with bovine serum albumin (BSA; Sigma datasheet). The activity of the primary antiserum was blocked by incubation with the original antigen. These preabsorption controls were performed once each on the buccal ganglia of *Tritonia* and *Pleurobranchaea* as well as nervous tissue from *Melibe*, *Dendronotus*, and *Hermissenda*. Additional controls for autofluorescence and secondary specificity were performed by incubating in normal serum and not including the primary antibody: *Tritonia* (n=1), *Melibe* (n=1), *Dendronotus* (n=1), *Hermissenda* (n=1) and *Pleurobranchaea* (n=1). In all the controls, there was no fluorescence observed in the brain.
3.3.6 Imaging

The tissue was imaged using a Zeiss LSM 700 Axio Examiner D1 confocal microscope (Carl Zeiss, Oberkochen, Germany) with 5-20x objectives. Fluorophores were excited with one laser (488 nm) and fluorescent emissions were passed through a 505-550 nm band-pass filter to visualize Alexa Fluro 488. The LSM 700 software ZEN was used to acquire the images. The thickness of the optical sections within a confocal stack was optimized and kept consistent within a preparation. Maximal projections of confocal stacks were made and exported as TIFF files to Adobe Photoshop CS where montages were assembled. The images were converted to grayscale and the intensity was inverted such that labeled neurons, axons and neuropil appeared dark. Brightness and contrast of all images were adjusted when necessary.

3.3.7 Data Analysis

In every buccal hemi-ganglion, GABA-ir neurons that were not part of a cluster were counted. GABA-ir neuron clusters were defined as a close grouping of GABA-ir neurons that were separated from other GABA-ir neurons by a clear space that lacked any GABA immunoreactivity. When present, the numbers of GABA-ir neurons in each GABA-ir cluster of every preparation were counted. All counts were made from the original slide at the confocal microscope and not from the confocal stacks. The cell counts were reported as the median ± semi-interquartile range.

3.4 RESULTS

In this study, we compared the number and locations of GABA-ir neurons in the buccal ganglia of six species of Nudipleura molluscs. The phylogenetic relationships between the species in this study are summarized in Figure 3.2. The overall organization of the GABA-ergic system was similar within Nudibranchia except for Melibe leonina, which has highly reduced
buccal ganglia (see Fig. 3.1Eii and Fig. 3.3E) (Trimarchi and Watson, 1992). We found significant differences in GABA distribution between the two sister clades of Nudibranchia and Pleurobranchomorpha.

3.4.1 GABA-ir neurons in the Nudibranchia

3.4.1.1 Location

The majority of GABA-ir cells in the nudibranchs were located anterior to a major axon tract that runs through the BC and the central region of each ganglion (Fig. 3.1Ai-Fii; Fig. 3.3). In Dendronotus, the GABA-ir cells were mostly located in the anterior lateral region of each hemi-ganglion (Fig. 3.1Dii; Fig. 3.3D). In Melibe, it was difficult to infer much about soma location since the buccal ganglion was morphologically very different from the other nudibranchs. The axons of some GABA-ir neurons projected contralaterally through the buccal commissure (BC) and to the other half of the buccal ganglion (Fig. 3.3A-E). Unlike other nudibranchs, a long BC separated each Melibe buccal hemi-ganglion and we could not reliably follow an axon all the way through to the other hemi-ganglion.

3.4.1.2 Number and size

The total number of GABA-ir neurons in the buccal ganglia was very consistent between Hermissenda, Tritonia, Tochuina and Dendronotus with the median ranging from 13 to 15 cells (Table 3.2, Fig. 3.1 Aii-Dii and 3.3A-D). Melibe however had nearly half the number of GABA-ir neurons observed in the buccal ganglia of other nudibranchs, with approximately 4 GABA-ir neurons in each hemi-ganglion (Table 3.2, Fig. 3.1Eii, 3.3E). Each Melibe hemi-ganglion is approximately 200µm in diameter and consists of about 30-40 neurons while other nudibranchs have 10 fold more cells in each hemi-ganglion (Trimarchi and Watson, 1992; Watson and
Willows, 1992). Thus, although Melibe has fewer GABA-ir neurons than the other nudibranchs, they represent a larger proportion of the total buccal neuron population. We did not observe any clearly discernable clusters of GABA-ir neurons in the buccal ganglia of the nudibranch species, as we saw in the central ganglia of those species (Gunaratne et al., 2014). The somata sizes of the GABA-ir neurons in the nudibranchs were heterogeneous, with diameters ranging from 25-50 µm in Hermissenda and Melibe to 30-100 µm in Tritonia, Tochuina and Dendronotus (Fig. 3.3A-E).

3.4.2 Bipolar GABA-ir neurons in T

3.4.2.1 vBcyP neuron

In Tritonia, there was a bilaterally represented and brightly stained GABA-ir bipolar neuron (60 – 80 µm diameter) that was consistently seen in all the preparations (n=8) (Fig. 3.1Bii, red; Fig. 3.3B). This neuron was located closer to the ventral surface and posterior to the central axon tract that runs through the BC. It had one process that tapered into a thick axon, which projected to the contralateral hemi-ganglion via the BC, and another process that terminated in a lateral dendritic arborization (Fig. 3.4Ai). The soma and its two main processes made a shallow “V” shape with the tip pointing to the posterior (Fig 3.3B; Fig 3.4Ai). We were unable to follow the medial process in its entirety as it merged with the dense GABA-ir central axon tract in the BC.

3.4.2.2 vBcyAM neuron

Another pair of ventral bipolar neurons was observed in the anterior medial region (n=8). These neurons were smaller (30-50 µm) and sent one process medially through the BC and the other process laterally towards the CBC (Fig 3.1Bii, blue; Fig. 3.3B; Fig 3.4Aii). In preparations
with clearer staining, we were able to discern the lateral process entering the CBC. In three preparations, we observed another pair of bipolar neurons near vBcɣAM.

3.4.3 GABA-ir neurons in Pleurobranchaea californica

The GABA-immunoreactivity in the Pleurobranchomorph *Pleurobranchaea californica*, was very different from that of any of the nudibranchs. *Pleurobranchaea* had a median of 59.5 ± 2 (n=4) GABA-ir neurons in the buccal ganglia, which is four times more than any of the nudibranchs (Table 3.2). The distribution pattern of these cells was also very different in *Pleurobranchaea*. There were at least two bilateral clusters and two individually identifiable bipolar neurons (Fig 3.1Fii; Fig 3.3F).

3.4.3.1 vBcɣL cluster

This cluster consisted of 9-10 GABA-ir neurons and was located on the ventral surface near the lateral margin, close to the buccal-cerebral connective (Table 3.2; Fig. 3.1Fii, pink; Fig. 3.3F). The neurons in this cluster were small, with diameters ranging from 15 to 20 µm (Fig 3.4Bi).

3.4.3.2 vBcɣM cluster

This cluster consisted of 14-17 GABA-ir neurons on the ventral surface of the buccal ganglion (Table 3.2). The cluster was located in the medial region of each hemi-ganglion, near or around the central axon tract that crossed the buccal commissure (Fig. 3.1Fii, purple; Fig. 3.3F). The cells in this cluster were heterogeneous in size, ranging from 10 to 40 µm in diameter (Fig. 3.4Bi).
3.4.4 Bipolar GABA-ir neurons

3.4.4.1 vBcyPM neuron

In every Pleurobranchaea preparation, we observed another pair of bipolar GABA-ir cells close to the ventral surface of the buccal ganglion. This neuron was smaller (40-60 µm) than vBcy1 and was located posterior to the central axon tract and medial to the first buccal root and the vBcyA neuron. (Fig 3.1Fii, green; Fig 3.3F). The two processes emanating from this neuron appear to encircle part of this central axon bundle (Fig 3.4Bii).

3.4.4.2 vBcyA neuron

There was a large, bilaterally represented GABA-ir bipolar neuron in Pleurobranchaea that was observed in all preparations (n=4). The soma was located closer to the ventral surface, lateral to the first buccal root and anterior to the axon tract that ran through the BC (Fig 3.1Fii, orange; Fig 3.3F). This neuron was 100-140 µm in diameter and had one thick process that projected to the contralateral hemi-ganglion via the BC and another process that elaborated into a lateral dendritic arborization (Fig 3.4Biii). We were unable to follow the contralateral process in its entirety as it merged with the dense GABA-ir central axon tract.

3.4.5 GABA-ir axon tracts and fibers in the Nudipleura

In all of the species, the central region of each hemi-ganglion was rich with GABA-ir neuropil. Similarly, the buccal commissure was also dense with GABA-ir axon tracts (Fig 3.5A-E). Every species also contained GABA-ir fibers in the cerebral-buccal connective (CBC) that connects the buccal ganglion to the cerebral ganglion. In Tritonia we observed a maximum of six axons in the CBC, Tochuina and Hermissenda had a maximum of four, Dendronotus had a maximum of three and we observed a maximum of seven GABA-ir axons in Melibe. In
Pleurobranchaea, there was a maximum of five GABA-ir axons in the CBC.

The buccal nerves projecting towards the periphery were markedly lacking GABA-ir axons with few exceptions. In all eight Tritonia preparations, we observed a maximum of two thin GABA-ir axons in buccal nerve, BN5 (Fig 3.5G). In three out of eight Tritonia preparations, we observed a single GABA-ir fiber in buccal nerve 2. In one preparation, we noted a single GABA-ir axon in buccal nerve 4. In three out of four Pleurobranchaea preparations, the first buccal root, r1, contained a maximum of two fine GABA-ir axons and fine fibers were observed close to the base of the nerve (Fig 3.5H). In one Pleurobranchaea preparation, we noted a single thin GABA-ir fiber in the third buccal root, r3. We did not observe any GABA-ir fibers in buccal nerves projecting to the periphery in any other species.

3.5 DISCUSSION

In this study, we compared the numbers and locations of GABA-ir neurons in the buccal ganglia of six Nudipleura molluscs (Mollusca, Gastropoda, Heterobranchia). Five of the species (Hermissenda crassicornis, Tritonia diomedea, Tochuina tetraquetra, Dendronotus iris and Melibe leonina) belong to the subclade Nudibranchia and the sixth (Pleurobranchaea californica) belongs to Pleurobranchomorpha, a sister clade to Nudibranchia. We did not detect any phylogenetic signal in GABA immunoreactivity of the five nudibranchs. Within Nudibranchia the number of GABA immunoreactive neurons was highly consistent. The only exception was Melibe, which had half as many, due to the overall reduction in the number of neurons in the buccal ganglion, which probably relates its lack of a radula and buccal mass.

We found that the GABA immunoreactivity in the out-group species Pleurobranchaea differed significantly from that of the nudibranchs. The GABA-ir axons in Nudipleura were
restricted to the buccal commissures and connectives with very limited peripheral innervation observed in *Tritonia* and *Pleurobranchaea*.

### 3.5.1 GABA distribution in *Melibe*

Gastropod feeding strategies are highly diverse and include rasping, grazing, sucking, suspension feeding, cutting and hunting (Audesirk and Audesirk, 1985; Chase, 2002). While here are differences in choice of prey and feeding habits of the species in this study, feeding in *Melibe* differs drastically in that it is highly specialized and unique in Gastropoda (Hurst, 1968; Watson and Trimarchi, 1992). Given the overall reduction in the feeding system of *Melibe*, it is perhaps not surprising that there are fewer GABA-ir neurons in *Melibe* compared to any other nudibranchs. However, it should be noted that GABA-ir neurons represent a larger proportion of the buccal neuronal population in *Melibe* compared any other nudibranch. This suggests that these GABA-ir neurons serve a highly conserved role. The *Melibe* buccal ganglia have been shown to control the movements of the esophagus, which is required to transport captured food to the stomach (Trimarchi and Watson, 1992). Given the low neuronal population of the *Melibe* buccal ganglia, it is possible that the GABA-ir neurons are also involved with control of the esophagus. If so, it could indicate a conserved function for some of the GABA-ir neurons in the other nudibranchs.

### 3.5.2 GABA distribution in *Pleurobranchaea* differs from other gastropods

*Pleurobranchaea* belongs to the clade Pleurobranchomorpha and is thus the most distantly related to the other species in this study, which belong to the sister clade Nudibranchia. The *Pleurobranchaea* GABA distribution was consistent with the phylogeny in that there were marked differences in the number and distribution of GABA-ir neurons compared to the nudibranchs.
The GABA distribution in the *Pleurobranchaea* buccal ganglia not only differs from that of the nudibranchs, it is also different from other gastropods. *Aplysia californica* has approximately 20 GABA-ir neurons in the buccal ganglia and has no cluster that is comparable in size, location and number to the *Pleurobranchaea* vBcγL and vBcγM (Soinila and Mpitsos, 1991; Diaz-Rios et al., 1999). None of the panpulmonate snails and slugs examined has more than 12 GABA-ir neurons in the buccal ganglia, nor do they have any clusters of GABA-ir neurons. In fact, the number and distribution of GABA-ir neurons in panpulmonates and nudibranchs are very similar to each other. Most of the GABA-ir buccal neurons in *Helisoma trivolis* (Richmond et al., 1991), *Lymnaea stagnalis* (Hatakeyama and Ito, 2000), *Helix pomatia* (Hernadi, 1994), *Helix lucorum* (Bravarenko et al., 2001) and *Limax maximus* (Cooke and Gelperin, 1988) are located anterior or near the central axon tract and at least a few of the buccal neurons project through the CBC and BC. It is therefore possible that the GABA distribution in *Pleurobranchaea* represents derived features that are unique to this lineage.

It is unclear as to why *Pleurobranchaea* has more buccal GABA-ir neurons than all other gastropods examined to date. As mentioned earlier, GABA and the buccal ganglia have been heavily implicated in the control of feeding behavior in gastropods. It is possible that the *Pleurobranchaea* GABA-ir buccal neurons are involved in feeding behavior as well. This species is notable for being a voracious, cannibalistic, predatory hunter (Lee et al., 1974; Willan, 1984). It is known to swallow large prey whole and has numerous modifications to accommodate its feeding habits (Morse, 1984). Some of these modifications include an enlarged buccal mass with well-developed musculature supporting its explosive bite strike, and a large acid gland that secretes acid directly into the buccal cavity. While acid secretion from the skin is used as a defense mechanism in some gastropods, the acid gland in *Pleurobranchaea* is associated with
feeding and immobilization of whole prey in the buccal cavity (Morse, 1984).

3.5.3 Role of identified GABA-ir neurons

The GABA-ergic system in the buccal ganglia of Nudipleura molluscs has not been examined previously barring a brief mention by Soinila and Mpitsos (1991) of a paired GABA-ir neuron and a cluster of small GABA-ir cells in the ventral surface of the *Pleurobranchaea* buccal ganglia. However, individual GABA-ir and their roles have been studied in more detail in distantly related euopisthobranch and panpulmonate species. For example, in the panpulmonate snail *Helisoma trivolis*, there are two bilaterally represented pairs of GABA-ir neurons called the BCN1s that project their axons contralaterally through the BC and CBC and into the cerebral ganglion (Murphy, 2001). Stimulation of these GABA-ir neurons or superfusion of GABA drives the protraction phase of the *Helisoma* feeding motor pattern (Murphy, 1993; Murphy, 2001). In *Aplysia*, B34 and B63 are two bilaterally represented GABA-ir buccal interneurons that are a part of the feeding CPG network and are thought to play a significant role in synchronizing activity in the two buccal hemi-ganglia (Hurwitz et al., 1997). Another buccal neuron, B40 is also a GABA-ir and a member of the feeding CPG in *Aplysia*. All three of these neurons are located in the anterior lateral region of the buccal ganglion and project an axon contralaterally through the BC and into the CBC (Hurwitz et al., 1997; Jing et al., 2003). The axons of each of these neurons follow the central axon tract but each neuron type has a distinct pattern of proximal and distal arborization (Hurwitz et al., 1997; Jing et al., 2003). All the species in this study contained GABA-ir neurons in the anterior lateral region of the buccal ganglion with axons that projected contralaterally through the BC. It is possible that homologues of the *Aplysia* B34, B40 and B63 buccal interneurons or the *Helisoma* BCN1s are present in the Nudipleura as well. While it was difficult to distinguish and follow individual GABA-ir axons from the soma all the
way to the contralateral CBC in this study, it should be noted that every species had GABA-ir axons in the CBC.

3.5.4 Bipolar neurons

Individually identifiable GABA-ir bipolar neurons were found in *Pleurobranchaea* and *Tritonia*. The vBCγAM neuron in *Tritonia* and the vBCγA neuron in *Pleurobranchaea* are superficially similar but are unlikely to be homologous due to differing innervation patterns. Although the *Tritonia* vBCγAM neurons had an axon in the ipsilateral CBC, the process of the vBCγA neuron in *Pleurobranchaea* remained within the ipsilateral hemi-ganglion and ended in a lateral dendritic arborization. Similarly, the bipolar vBCγP neuron in *Tritonia* and vBCγPM neuron in *Pleurobranchaea* are also unlikely to be homologous due to differing axon projection and morphology. The *Tritonia* vBCγP neuron had one thick axon that projected to the contralateral hemi-ganglion via the BC, and another process that terminated in a lateral dendritic arborization within the ipsilateral buccal ganglion. In contrast, the two processes emanating from *Pleurobranchaea* vBCγPM neuron remained within the ipsilateral hemi-ganglion and encircled the central axon bundle. It is possible that there is more to its axon morphology and projection, which we could not observe due to inferior GABA immunoreactivity in *Pleurobranchaea*. We do not know of any GABA-ir bipolar cells in the buccal ganglia of other gastropods except for *Aplysia*, where an unpaired, GABA-ir bipolar cell is present near the midline of the buccal commissure (Diaz-Rios et al., 1999).

The vBCγA neuron in *Pleurobranchaea* is strikingly similar in location, size and morphology to the previously described contralateral corollary discharge (CCD) neurons, which allow for communication between the buccal and cerebral ganglia (Kovac et al., 1986). There are two pairs of CCDs identified by Kovac et al (1986) and this neuron type is bipolar, located in the
anterior region of the ventral side of each buccal ganglion. Similar to vBcγA, the lateral process of the CCD neuron type terminates in an arborization and the thicker medial process projects through the BC to the contralateral hemi-ganglion and into the CBC. We were unable to reliably follow the contralateral process of vBcγA in its entirety as it merged with the dense GABA-ir central axon track in the buccal ganglia. As such, we do not know if the contralateral projecting axon of vBcγA projects into the CBC as in the Pleurobranchaea CCD neurons. Therefore we are not certain if vBcγA neuron and the CCD neurons are one and the same.

3.5.5 GABA as a peripheral transmitter

GABA-ir axons have not been observed in the buccal nerves innervating the periphery in any gastropod examined to date except the panpulmonate snail Helisoma trivolis, where two GABA-ir fibers are present in the esophageal nerve (Richmond et al., 1991). In this study, we consistently found two GABA-ir fibers in buccal nerve 5 of Tritonia diomedea. It has been shown that stimulation of the peripheral cut end of buccal nerve 5 elicits movements in the odonotophore muscles that are responsible for the protraction/retraction of the radula (Willows, 1978). Buccal nerve 5 in Tritonia diomedea is homologous to buccal nerve 3 in the closely related Tritonia homberghi, where it innervates the buccal muscle groups M1-4, which are collectively involved in the protraction of the radula (Bulloch and Dorsett, 1979). A maximum of 2 fine GABA-ir neurons were also found in the Pleurobranchaea buccal root 1. This nerve innervated feeding muscles 2, 4 and 6 (Davis et al., 1973; Lee and Liegeois, 1974) and controls the explosive proboscis eversion phase of feeding. Proboscis eversion refers to the protracted buccal mass and everted oral tube (Lee and Liegeois, 1974; Siegler et al., 1974). It is possible that the peripherally projecting GABA-ir axons in Tritonia and Pleurobranchaea are involved in the protraction phase of the feeding movements in these two species.
3.5.6 GABA as a central transmitter

The vast majority of the GABA-ir fibers and axons in the buccal ganglia of Nudipleura are restricted to the buccal connectives, commissures and the neuropil region of each hemi-ganglion and there is very limited GABA-ergic innervation of the periphery. In nudibranchs, a maximum of two GABA-ir neurons from CeγL cluster of the cerebral ganglia have axons in the CBC (Gunaratne et al., 2014) and GABA-ir cerebral-buccal neurons have been shown to play important roles in initiating and regulating feeding behavior in species such as *Aplysia californica* (Jing et al., 2003; Wu et al., 2003), *Aplysia kurodai* (Narusuye et al., 2005), and *Clione limacina* (Norekian and Malyshev, 2005). We observed two or more GABA-ir fibers in the CBC of all the Nudipleura species and one of the sources of these axons maybe GABA-ir buccal-cerebral neurons. Collectively, this suggests that GABA may play a predominantly central role in the Nudipleura and may be involved in coordinating feeding behavior as in other gastropods.

3.6 ACKNOWLEDGEMENTS

This work was supported by National Science Foundation grants IOS-081441, IOS-1120950 and a Sigma Xi GIAR.
Figure 3.1 Nudipleura buccal ganglia anatomy and nomenclature.

The naming conventions in this study are based on Newcomb et al. (2006). As the overall silhouette of the buccal ganglia differed across species, buccal schematics are provided for: (Ai)
*Hermissenda crassicornis*, (Bi) *Tritonia diomedea*, (Ci) *Tochuina tetraquetra*, (Di) *Dendronotus iris*, (Ei) *Melibe leonina*, and (Fi) *Pleurobranchaea californica*. The buccal ganglia are composed of a pair of bilaterally symmetric hemi-ganglia. The buccal commissure (BC) and the cerebral-buccal connective (CBC) are indicated. The buccal nerve nomenclature in *Tritonia*, *Melibe*, and *Pleurobranchaea* are based on Willows (1978), Trimarchi and Watson (1992), and Lee and Liegeois (1974) respectively. The CBC in *Hermissenda*, *Tochuina* and *Dendronotus* were identified by tracing the nerve from the buccal to the cerebral ganglion. GABA-ir neurons and clusters were named based on location. For this purpose, each hemi-ganglion was divided into halves: Anterior (A) / Posterior (P), Lateral (L) / Medial (M); Gastroesophageal ganglion (G.o.g.), Buccal nerve (BN), Root (R). Aii-Fii. Schematic of the GABA immunoreactivity in the Nudipleura buccal ganglia indicating the median number of the left and right GABA-ir neurons and their locations in (Aii) *Hermissenda crassicornis* (n=8), (Bi) *Tritonia diomedea* (n=8), (Ci) *Tochuina tetraquetra* (n=4), (Di) *Dendronotus iris* (n=7), (Ei) *Melibe leonina* (n=5), and (Fi) *Pleurobranchaea californica* (n=4). Neurons are indicated by filled circles and recognizable GABA-ir neurons and clusters are color-coded. The individually identifiable neurons are vBcγP and vBcγAM (*Tritonia* only), vBcγA and vBcγPM (*Pleurobranchaea* only). The recognizable clusters include vBcγM and vBcL (*Pleurobranchaea* only). Gray ovals and parallel gray lines indicate areas of extensive GABA-ir neuropil and axon tracts. Bipolar neurons are indicated by a circle with two short lines extending from either side of the circle.
Figure 3.2 Abbreviated phylogeny of Euthyneura.
Euthyneura contains two clades: Nudipleura and Tectipleura. Within Nudipleura there are two clades: Nudibranchia and Pleurobranchomorpha. The relationship between Nudipleura species in this study is shown. Tectipleura contains two large clades: Euopisthobranchia and Panpulmonata. Representative species from these two major groups are shown. The phylogenetic relationships are based on: (Senatore and Katz, 2014; Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Wägele et al., 2014).
Figure 3.3 GABA immunoreactivity in the Nudipleura buccal ganglia. Confocal images of representative preparations of (A) *Hermisenda crassicornis*, (B) *Tritonia diomedea*, (C) *Tochuina tetraquetra*, (D) *Dendronotus iris*, (E) *Melibe leonina*, and (F) *Pleurobranchaea californica*. The individually identifiable neurons are vBcγP and vBcγAM (*Tritonia* only) and vBcγA and vBcγPM (*Pleurobranchaea* only). The recognizable clusters in *Pleurobranchaea* are vBcγM and vBcL (circled).
Figure 3.4 Higher magnification images of GABA-ir neurons.
A: *Tritonia* buccal ganglion showing the individually identifiable bipolar GABA-ir neurons vBcγP (i), and vBcγAM (ii). The black arrow in (i) indicates the direction of the buccal commissure. B: *Pleurobranchaea* buccal ganglion showing the GABA-ir clusters vBcγM and vBcγL (i) and the individually identifiable GABA-ir bipolar neurons vBcγPM (ii) and vBcγA (iii).
Figure 3.5 Extensive GABA-ir neuropil and axonal tracts in buccal ganglia. (A) *Hermissenda crassicornis*, (B) *Tritonia diomedea*, (C) *Tochuina tetraquetra*, (D) *Dendrochirus iris*, (E) *Melibe leonina*, and (F) *Pleurobranchaea californica*. (G) GABA-ir neurons in the *Tritonia* CBC and the peripheral buccal nerve 5. (H) GABA-ir axon in the peripheral buccal nerve R1 of *Pleurobranchaea*. Black arrows indicate the direction of the BC. Buccal commissure (BC), cerebral-buccal connective (CBC), Root 1 (R1), buccal nerve (BN), gastroesophageal ganglion (G.o.g).
### Table 3.1 Primary Antibodies used in the study

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### Table 3.2 Median Cell counts (±semi-interquartile range) for GABA immunoreactive neurons in the buccal ganglia

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<tr>
<th>Ganglion</th>
<th>GABA-ir cluster</th>
<th>Left/ Right</th>
<th>Hermissenda crassicornis (n=8)</th>
<th>Tritonia diomedea (n=8)</th>
<th>Tochonia tetraquetra (n=4)</th>
<th>Dendronotus iris (n=7)</th>
<th>Mellea leonina (n=5)</th>
<th>Pleurobranchaea californica (n=4)</th>
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<tr>
<td>Buccal</td>
<td>-</td>
<td>L</td>
<td>7 ± 1</td>
<td>6.5 ± 1</td>
<td>7.5 ± 1</td>
<td>8 ± 1</td>
<td>4</td>
<td>6.5 ± 1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>R</td>
<td>6.5 ± 1</td>
<td>6.5 ± 1</td>
<td>8</td>
<td>4 ± 1</td>
<td>4</td>
<td>9.5 ± 1</td>
</tr>
<tr>
<td>vBcγL</td>
<td>L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14 ± 1</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17 ± 1</td>
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<td>-</td>
<td>-</td>
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<td>13</td>
</tr>
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<td></td>
<td>R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>13.5 ± 1</td>
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<tr>
<td>Total whole buccal</td>
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<td>14.5 ± 1</td>
<td>14 ± 1</td>
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CHAPTER FOUR: A COMPARATIVE ANALYSIS OF BIOLOGICAL NEURAL CIRCUITS REVEALS MULTIPLE SOLUTIONS TO THE SAME PROBLEM

Charuni A. Gunaratne, Akira Sakurai, Adriano Senatore and Paul S. Katz

_in preparation for Current Biology_

4.1 SUMMARY

Theoretical studies suggest that the same behavior could be generated by neural circuits with different properties (Prinz et al., 2004). Here, we explored a real life instantiation of the theory by comparing features of four biological neural circuits underlying analogous and non-analogous behaviors. *Melibe leonina* and *Dendronotus iris* are two nudibranchs (Mollusca, Gastropoda, Euthyneura, Nudipleura) that have analogous swimming behavior consisting of alternating left-right (LR) body flexions. In *Melibe*, swim interneuron 1 (Si1_{Mel}) is part of the central pattern generator (CPG) circuit for swimming, whereas its homologue in *Dendronotus* (Si1_{Dend}) is not (Thompson and Watson, 2005; Sakurai et al., 2011). Here, we identified the Si1 homologue in *Flabellina iodinea* (Si1_{Flb}), a species whose phylogenetic position indicates that it independently evolved LR swimming behavior. We found that its swim circuit shared some features with *Melibe*, some with *Dendronotus*, and also had its own unique properties. 1) Si1_{Flb} was a member of the LR swim CPG and displayed rhythmic bursting activity characteristic of the swim motor pattern (SMP); it also had a mutually inhibitory connection with its contralateral counterpart, collectively making *Flabellina* similar to *Melibe* and unlike *Dendronotus*. We also identified the Si1 homologue in *Tritonia diomedea*, a species more closely related to *Flabellina* that displays dorsal-ventral (DV) swimming, a behavior non-analogous to LR swimming. Si1_{Trit} did not display rhythmic bursting activity nor did it play a role in the DV swim motor pattern. Like Si1_{Flb}, Si1_{Trit} had mutually inhibitory connections with its contralateral partner, suggesting
that mutual inhibition by itself is not sufficient for rhythmic activity and that this may be a property that relates to the phylogeny. 2) In *Flabellina* only, transecting the cerebral commissure blocked the SMP. This was caused by a commissure-crossing neuron, C2, whose homologue is a member of the DV swim CPG in *Tritonia*. C2 activity was found to be sufficient to initiate the SMP in all three LR species. However, its activity was necessary for initiation and maintenance of the SMP only in *Flabellina*, indicating that its swim network is different from that of *Melibe* and *Dendronotus*. 3) Curare blocked the LR swim motor pattern (SMP) in *Flabellina* and *Dendronotus*, but not in *Melibe*, demonstrating another difference between the three LR circuits. Thus, in three species with similar behaviors, there were three distinct solutions to the organization, activation, and pharmacological sensitivity of a CPG.

### 4.2 RESULTS

The *Melibe* circuit underlying LR swimming includes four cell types called Si1, Si2, Si3 and Si4 (Sakurai et al., 2014) (see neuronbank.org/wiki/index.php/Melibe). Although the homologues of the *Melibe* Si1 and Si2 neurons have been identified in *Dendronotus*, Si1 is not a member of the CPG for LR swimming in that species (Sakurai et al., 2011). This difference in roles could represent a derived feature of either lineage or it could be due to independent evolution of LR circuitry in each lineage. *Flabellina iodinea* is another LR swimming nudibranch; it belongs to a sub-clade of Nudibranchia called Aeolidida. The Aeolidida phylogeny (Carmona et al., 2013) along with the distribution of swimming among the 11 aeolid families (Newcomb et al., 2012) suggest that *Flabellina* independently evolved LR swimming. Comparing features of the *Melibe* and *Dendronotus* swim circuitry to that of a species such as *Flabellina* will show if the circuitry for analogous behavior is built differently. Additionally, phylogenetic analysis based on whole brain transcriptomes of six Nudipleura species revealed
that *Tritonia* and *Flabellina* are more closely related to each other than to *Melibe* and *Dendronotus* (Fig. 4.1). Comparing *Flabellina* LR circuit features as well as those of *Melibe* and *Dendronotus* to that of a species that swims differently, such as *Tritonia*, could indicate which circuit features are general to nudibranchs and/or relate to the nudibranch phylogeny.

### 4.2.1 Swimming Behavior

*Flabellina* swims by flattening its whole body in the sagittal plane and bending from side to side, making a semicircle shape with its body with each flexion (Fig. 4.2A, Supplementary video S1). The periodicity of the *Flabellina* swim was 2.3 ± 0.18 seconds (n=14). This LR swimming behavior appeared spontaneously *in vivo* or could be triggered by noxious stimuli such as a tube foot of a sea star or a high molarity salt solution. The *Flabellina* LR swimming behavior is analogous to that of *Melibe* and *Dendronotus* (Lawrence and Watson, 2002; Sakurai et al., 2011) in the movement of the body during swimming and the stimuli that trigger swimming.

### 4.2.2 Identification of the Si1 homologue in *Flabellina*

We identified a single pair of neurons in the cerebral ganglia of *Flabellina* that displayed the same anatomical and neurochemical characteristics that uniquely identify Si1 in *Melibe* (Watson et al., 2001; Thompson and Watson, 2005) and its homologue in *Dendronotus* (Sakurai et al., 2011). We concluded that these neurons are also Si1 homologues. Hereafter, the Si1 homologue in each species will be distinguished by the abbreviated subscript of its respective genus. Similar to Si1<sub>Mel</sub> and Si1<sub>Dend</sub>, Si1<sub>Flb</sub> had a non-pigmented soma located on the dorsal surface of the cerebral ganglion near the cerebral commissure (Fig. 4.2B, C). Intracellular injection of the tracer Biocytin revealed that the axon of Si1<sub>Flb</sub> (N=7) projected ipsilaterally to the pedal ganglion (Fig. 4.2C, D) and had the same characteristic bend near the soma as seen in
Si1\textsubscript{Mel} and Si1\textsubscript{Dend} (Fig. 4.2E). The axon of Si1\textsubscript{Flb} dipped ventrally (Fig. 4.2E) as opposed to posteriorly as in the other species (Watson et al., 2001; Sakurai et al., 2011). Similar to Melibe and Dendronotus, Si1\textsubscript{Flb} neurites spread towards the pedal commissure 2 (PP2) (Fig. 4.2D) and fine neurites also emanated from the length of axon near the soma (Fig. 4.2E).

Serotonin immunoreactivity combined with intracellular fills revealed that just as with Si1\textsubscript{Mel} and Si1\textsubscript{Dend}, the somata of Si1\textsubscript{Flb} (N=3) were always located near a previously identified and highly conserved cluster of five serotonergic neurons, which includes the three dorsal swim interneurons (DSIs, neuronbank.org/wiki/index.php/DSI) (Newcomb et al., 2006) (Fig. 4.2F). Furthermore as in Melibe and Dendronotus (Sakurai et al., 2011), Si1\textsubscript{Flb} was immunoreactive to the neuropeptide FMRFamide (Fig. 4.2G, N=6). This suite of neuroanatomical and neurochemical characteristics identified the Si1 homologues in Flabellina just as it do in Melibe and Dendronotus, and distinguished them from all other neurons in the brain.

### 4.2.3 Role of the Si1 homologue in Flabellina

A fictive motor pattern was reliably evoked in Flabellina by brief electrical stimulation of a body wall nerve (BWN). The motor pattern resembled that of Melibe and Dendronotus (Sakurai et al., 2011) in that it was characterized by alternating bursts of spikes in the left and right BWNs (Fig. 4.3A). The average burst period was $2.5 \pm 0.51$ sec (n=10), which was not statistically significant from the period of the swimming behavior in vivo ($p=0.16$, Student’s unpaired t-test). The left-right alternation was also observed in the spiking activity of the left and right Si1\textsubscript{Flb} (Fig. 4.3A) and in neurons in the pedal ganglia (Fig. 4.3B).

To determine whether Si1\textsubscript{Flb} is a member of the LR swim CPG, brief depolarizing (Fig. 3Bi) or hyperpolarizing (Fig. 4.3Bii) current pulses were injected into Si1\textsubscript{Flb} (N=10). These
manipulations to Si1\textsubscript{Flb} phase delayed (Fig. 4.3Bi) or phase advanced (Fig. 4.3Bii) the swim motor pattern. Thus, Si1\textsubscript{Flb} is a member of the LR CPG, making it similar to Si1\textsubscript{Mel} and unlike Si1\textsubscript{Dend}.

4.2.4 Difference in Duty cycle of Si1 homologues

Despite similarity in their roles in the LR CPG, we observed differences in the duty cycle of Si1\textsubscript{Flb} and Si1\textsubscript{Mel} during the swim motor pattern. The duty cycle of a neuron refers to the percentage of a full cycle that the neuron is active and can reflect some of the internal dynamics of the circuit. For example, Si1\textsubscript{Mel} had a duty cycle of 0.31 ± 0.1 (Sakurai et al., 2014) and its burst is cut short as a direct result of inhibitory input from another neuron in the Melibe CPG called Si3 (Sakurai et al., 2014). Si1\textsubscript{Flb} has a duty cycle of 0.50 ± 0.05 (N=8), which is significantly different from that of Melibe (p<0.001, Student’s unpaired t-test). We do not know what terminates each Si1\textsubscript{Flb} burst but the difference in Si1 homologue duty cycle between Flabellina and Melibe is indicative of differences in the internal dynamics of the CPG.

4.2.5 Difference in duty cycle of Si1 homologues

Despite similarity in their roles in the LR CPG, we observed differences in the duty cycle of Si1\textsubscript{Flb} and Si1\textsubscript{Mel} during the swim motor pattern. The duty cycle of a neuron refers to the fraction of a full cycle that the neuron is active and can reflect some of the internal dynamics of the circuit. Si1\textsubscript{Mel} had a duty cycle of 0.31 ± 0.1 (Sakurai et al., 2014) and its burst was cut short as a direct result of inhibitory input from another neuron in the Melibe CPG called Si3 (Sakurai et al., 2014). Si1\textsubscript{Flb} has a duty cycle of 0.50 ± 0.05 (N=8), which is significantly different from that of Melibe (p<0.001, Student’s unpaired t-test). We do not know what terminates each Si1\textsubscript{Flb} burst but Si1 homologue duty cycles of Flabellina and Melibe is suggestive of differences in the internal dynamics of the CPG.
4.2.6 Identification of the Si1 homologue in Tritonia

In contrast to Melibe, Dendronotus and Flabellina, Tritonia swims by flattening its body in the horizontal plane and making whole body DV flexions (Willows and Hoyle, 1969; Newcomb et al., 2012) (Fig. 4.4A). Based on the same anatomical and neurochemical criteria used to identify Si1_{Flb}, Si1_{Mel} and Si1_{Dend}, we identified a pair of neurons in the cerebral ganglia of Tritonia that we conclude to be Si1 homologues. Briefly, the Si1_{Trit} soma is located on the dorsal surface of the cerebral ganglion (Fig. 4.4B, C). The Si1_{Trit} axon makes a characteristic posterior bend (N=8) near the soma and projects ipsilaterally to the pedal ganglion (Fig. 4.4C, E) and into the pedal commissure 2 as confirmed by simultaneous electrophysiological recordings of Si1_{Trit}, a motor follower neuron and PP2 (Fig. 4.4D). Similar to Melibe, Dendronotus and Flabellina, we observed fine neurites emanating from the length of axon near the soma of Si1_{Trit} (Fig. 4.4E). Si1_{Trit} is always located next to the serotonergic DSIs (N=3) (Fig. 4.4F) and is immunopositive for the neuropeptide FMRFamide (N=6) (Fig. 4.4G). This suite of neuroanatomical and neurochemical characteristics identified the Si1 homologues in Tritonia just as they do in Melibe, Dendronotus and Flabellina, and distinguished them from all other neurons in the brain.

In Tritonia, the DV swim motor pattern is bilaterally symmetric as would be expected for the dorsal and ventral swimming motion (Dorsett et al., 1973; Hoyle and Willows, 1973; Willows et al., 1973). We found that Si1_{Trit} did not fire rhythmic bursts of action potentials during a DV swim motor pattern (Fig. 4.4H). Instead, the neuron decreased its firing, often stopping altogether (N=9). Although it did not participate in the DV swim motor pattern, it received sub threshold synaptic input phase-locked to the DV swim motor pattern.
4.2.7 **Synaptic connectivity of Si1<sub>Flb</sub> and Si1<sub>Trit</sub>**

An important difference between Si1 in *Melibe* and *Dendronotus* is that there is mutual inhibition between the two contralateral Si1s in *Melibe*, which is absent in *Dendronotus* (Sakurai et al., 2011). Si1<sub>Mel</sub> participates in the swim motor pattern through its mutually inhibitory and electrical connections with other members of the CPG (Thompson and Watson, 2005; Sakurai et al., 2014). Here we found that the Si1 homologues in *Flabellina* and *Tritonia* also have mutually inhibitory connections; injection of depolarizing current into either the left or right Si1 inhibited the contralateral counterpart in both *Flabellina* (Fig. 4.5Ai, ii) and *Tritonia* (Fig. 4.5Bi, iii, iv). Si1<sub>Trit</sub> is also electrically coupled to its contralateral counterpart (Supplementary Fig. 4.9 S1ii) and a similar connection is visible in Si1<sub>Flb</sub> in normal saline (Supplementary Fig. 4.9 S1i). Similarly, the paired Si1 neurons in both *Melibe* and *Dendronotus* are coupled (Sakurai et al., 2011).

4.2.8 **Effect of cerebral commissure transection on the swim motor pattern of LR species**

None of the known swim interneuron homologues in *Melibe* (Sakurai et al., 2014), *Dendronotus* (Sakurai et al., 2011) or *Flabellina* have axons that cross the cerebral commissure (Fig. 4.6Ai, Bi, Ci). Therefore, we predicted that transection of the cerebral commissure would not disrupt their swim motor pattern. In *Flabellina*, cutting the cerebral commissure eliminated the swim motor pattern (N=8) (Fig. 4.6Aii, iii) and no recovery was observed up to 24 hrs after transection. In contrast, the motor pattern continued after transection in *Melibe* (N=3) (Fig. 4.6Bii, iii) and *Dendronotus* (N=3) (Fig. 4.6Cii, iii). These results indicate that, unlike the swim circuits of *Melibe* and *Dendronotus*, a commissure-crossing neuron is necessary for the *Flabellina* swim motor pattern.
4.2.9 Sufficiency and necessity of C2 activity for the swim motor pattern of LR species

We identified the commissure-crossing neuron that was necessary for the *Flabellina* swim motor pattern. It was the homologue of Cerebral Neuron 2 (C2, neuronbank.org/wiki/index.php/C2_neuron), which was first identified in *Tritonia* as a member of the DV CPG (Getting, 1977; Taghert and Willows, 1978; Getting et al., 1980). C2 homologues have been identified in other species including *Melibe* and *Flabellina* (Lillvis et al., 2012). We identified the C2 homologue in *Dendronotus* based on similar criteria used to identify C2 in the other species (Supplementary Fig. 4.10 S2). Just as in *Tritonia*, the C2 homologues in *Flabellina* (Fig. 4.7Ai), *Melibe* (Fig. 4.7Bi) and *Dendronotus* (Fig. 4.7Ci) projected their axons through the cerebral commissure and into the contralateral half of the brain.

Although C2 was not rhythmically active during the LR swim motor pattern in *Flabellina*, its spiking activity was both necessary and sufficient. In a quiescent preparation, depolarization of a single C2 (N=12, Fig. 4.7Aii) was sufficient to initiate the LR swim motor pattern. Furthermore, hyperpolarization of a single C2 terminated an ongoing swim motor pattern (N=12, Fig. 4.7Aiii). Fictive swims were typically elicited in *Flabellina* by brief shock to a body wall nerve (Fig. 4.7Aiv). This also resulted in an increase in the basal firing rate of C2. Hyperpolarization of a single C2 in *Flabellina* blocked this nerve-evoked swim motor pattern (N=12, Fig. 4.7Av). In some preparations, Si1f0 fired action potentials in response to the nerve shock but no rhythmic bursting was observed while C2 was hyperpolarized.

C2 spiking activity was sufficient to initiate rhythmic activity in both *Melibe* and *Dendronotus* but unlike C2 in *Flabellina*, its activity was not necessary for the swim motor pattern. In quiescent preparations, depolarization of a single C2 initiated rhythmic bursting
activity in *Melibe* (N=3, Fig. 4.7Bii) and *Dendronotus* (N=7, Fig. 4.7Cii). Hyperpolarization of one or both C2s in *Melibe* (N=3, Fig. 4.7Biii) and *Dendronotus* (N=3, Fig. 4.7Ciii) did not prevent the swim motor pattern from being elicited by nerve shock. It also did not prevent the spontaneous occurrence of motor patterns such as those seen in *Melibe* (Supplementary Fig. 4.11 S3). These results support the model that there is a fundamental difference in the importance of C2 for the production of the swim motor pattern in *Flabellina* compared to *Melibe* and *Dendronotus*.

### 4.2.10 Curare blocks the motor pattern in *Flabellina* and *Dendronotus*

The effect of pharmacological manipulations on the swim motor pattern can provide valuable information on the underlying neural circuit. Superfusion of the acetylcholine receptor blocker, d-tubocurarine (curare, 50 - 100 µM), had different effects on the swim motor pattern of the three LR species. In *Flabellina*, curare eliminated nerve evoked swim motor patterns (N=7, Fig. 4.8Ai,ii). In contrast in *Melibe*, curare never eliminated bursting, but instead increased the period significantly from 4.82 ± 1.27 s in normal saline to 24.30 ± 12.3 s, (N=10, p=0.006, Student’s paired t-test) (Fig. 4.8Bi,ii). The effect of curare in *Dendronotus* was similar to that in *Flabellina*, it halted the motor pattern (N=16, Fig. 4.8Ci,ii). In *Flabellina* and *Dendronotus*, the swim motor pattern did not first slow down before stopping. These results suggest that the neural circuits underlying LR swimming in *Flabellina* and *Dendronotus* are different from that of *Melibe* in that they contain curare-sensitive synapses that are necessary for the swim motor pattern.
4.3 DISCUSSION

4.3.1 Analogous behavior, multiple neural circuit solutions

The swimming behaviors exhibited by *Melibe*, *Dendronotus* and *Flabellina* are analogous, yet there are important differences in the underlying CPG circuits; namely the connectivity, the pharmacological sensitivity, and role of homologous neurons in the initiation, production and maintenance of the swim motor pattern differ. In *Melibe* and *Flabellina* but not in *Dendronotus*, the Si1 homologues are core members of the swim circuit. Furthermore, the difference in duty cycle of the Si1 homologues in *Flabellina* and *Melibe* during a SMP signify differences in the internal dynamics of the two CPGs. In *Flabellina* but not *Melibe* or *Dendronotus*, the commissure-crossing neuron C2 is necessary for the initiation and maintenance of the swim motor pattern. The LR circuitry of *Dendronotus* and *Flabellina* but not *Melibe* is functionally sensitive to curare. Thus, while there were overlapping components, the LR neural circuit of each species differs from each other in ways that are functionally relevant to the expression of the behavior. These results represent a real life instantiation of the theoretical studies that have suggested that circuits with different properties can generate the same behavioral output (Prinz et al., 2004).

4.3.2 Phylogenetically conserved circuit features

In *Tritonia* as well as in *Flabellina*, the Si1 homologue pairs are mutually inhibitory. Mutually inhibitory neurons are the most common circuit building block for network based CPGs. The neurons in such half-center oscillators (HCOs) are not rhythmogenic individually but produce rhythmic outputs when reciprocally inhibited (Marder and Calabrese, 1996; Hooper, 2001). The presence of mutual inhibition between the Si1 homologues in *Flabellina* and *Melibe* and its absence in *Dendronotus* is consistent with the hypothesis that mutual inhibition is
necessary for alternating bursting in Si1. However, the fact that mutual inhibition was observed in *Tritonia* in the absence of rhythmic bursting activity suggests that its presence by itself is neither predictive nor sufficient to cause alternating firing in Si1. This shows that mutual inhibition among the Si1 homologues may be a phylogenetically constrained feature given that *Flabellina* and *Tritonia* are more closely related to each other than they are to *Melibe* or *Dendronotus*. Moreover, the integral role of C2 in the *Flabellina* LR swim and the *Tritonia* DV swim could also be due to the phylogenetic relationship between the two species. In contrast, C2 plays a non-integral role in the LR swim motor pattern in *Melibe* and *Dendronotus*.

### 4.3.3 Interspecies variability in other neural circuits

Electrogenesis and the jamming avoidance response (JAR) in African mormyrid and South American gymnotid electric fish represent a fascinating example of interspecies similarities and differences in circuitry. These fish independently evolved the capacity for active electrosensing, electric organ discharge (EOD) and the JAR (Hopkins, 2009). The EOD in both groups is initiated in pacemaker/command neurons in medullary nuclei (Hopkins, 2009). The signals are then projected via relay nuclei to electromotor neurons that drive the electric organ (Carlson, 2002; Kawasaki, 2009). Both groups also have similar descending thalamic input to pacemaker nuclei (Caputi et al., 2005). Mormyrid and gymnotid wave-type fish alter the frequency of their EODs to avoid jamming when a conspecific using a similar EOD frequency is nearby. However, the brain areas that control the JAR are different in the two groups. In the African mormyrids, timing difference computations occur in a medullary structure called the electrosensory lateral line lobe (ELL) whereas in the South American gymnotids, these computations occur within the midbrain structure called the torus semicircularis (Kawasaki, 2009). Thus, in electric fish, neural circuitry underlying the analogous JAR behavior can involve
similar computations and comparable pathways but also involve non-homologous brain areas. It has been proposed that the preexisting architecture of the nervous system has affected the ability for these groups to evolve behaviors such as electrolocation and the JAR (Rose, 2004).

Vocal learning in birds has evolved independently two or three times - in the oscine songbirds and parrots or their last common ancestor and in the more distantly related hummingbirds (Petkov and Jarvis, 2012). Vocal learning birds possess specialized forebrain circuitry consisting of seven comparable vocal nuclei that are not found in non-vocal learners (Jarvis, 2006). It is proposed that the vocal nuclei found in the three avian groups share a deep homology and are in fact, specializations of a preexisting motor system (Feenders et al., 2008; Jarvis, 2013). While the brain structures and connectivity in the vocal learning pathways are remarkably similar among the vocal learners, there are differences as well. For example, there are some clear differences between oscines and parrots in the connectivity between the anterior and posterior vocal pathways (Jarvis, 2006). Also in parrots, the NLC and ACC vocal nuclei (HVC and RA in oscines) have unique outer shell regions and associated connectivity patterns not found in other vocal learners (Chakraborty et al., 2013). It is thought that these additional outer shell regions in parrots relate to their more advanced vocal learning abilities compared to oscines.

In our example of swimming in nudibranchs, it is notable that of the 5,000-10,000 neurons in the nudibranchs brain, the same neuronal types (Si1 and C2) were incorporated into the neural circuits underlying analogous swimming behavior. It is even more striking that the C2 neuronal type is also a core member of the CPG underlying DV swimming, a behavior that has also evolved independently in a handful of lineages. As has been proposed by others, such similarities in neural substrate underlying independently evolved behaviors might not just be
extraordinary coincidences (Farries, 2001; Katz, 2011; Jarvis, 2013). Parallel evolution (i.e. independent evolution of behavior using homologous neural substrate) is thought to be a reflection of the extent to which the nervous system organization constrains the evolution of the behavior (Katz, 2011). Swim circuitry in nudibranchs represent a single neuron level example of how the structure of the nervous system provides particular avenues for the independent evolution of behavior. Numerous studies identifying homologous neurons within and beyond Nudipleura have shown that nervous system organization tends to be conserved (Weiss and Kupfermann, 1976; Pentreath et al., 1982; Sudlow et al., 1998; Newcomb et al., 2006; Lillvis et al., 2012; Gunaratne et al., 2014); it possible the LR swim circuitry evolved from preexisting neural circuitry that served a different function in the common ancestor. This has been suggested for the DV CPG neuronal type DSI, which is thought to have evolved from ancestral non-rhythmic circuits involved in the response to noxious stimuli such as aversive turning and increased crawling (Jing and Gillette, 2000; Katz, 2011).

4.4 EXPERIMENTAL PROCEDURES

4.4.1 Animal collection and dissection

Flabellina iodinea (2.5 – 6 cm in body length) were obtained as adults from Marinus Scientific (Long Beach, CA). Adult Melibe leonina (3 – 10 cm) and Dendronotus iris (6 - 20 cm) were obtained from Monterey Abalone Company (Monterey, CA). Tritonia diomedea (50-200 mm) were provided by Living Elements (Vancouver, BC, Canada). All animals were maintained in tanks with recirculating artificial seawater (Instant Ocean, Blacksburg, VA, USA) and a fixed 12hr:12hr light:dark cycle at 10-13°C.
Flabellina, Melibe and Dendronotus were anaesthetized by injecting the body cavity with 0.33 M MgCl₂ solution. Tritonia were anaesthetized by chilling in a refrigerator at 4ºC. To remove the brain, a cut was made on the dorsal body wall near the esophagus. The brain, consisting of the paired cerebral, pleural and pedal ganglia, was extracted from the body by cutting all nerve roots. The brain was transferred and pinned to a Sylgard-lined dish and superfused with physiological saline or artificial seawater. Saline composition was (in mM): 420 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 10 D-glucose, and 10 HEPES, pH 7.4. The surrounding connective tissue and the fine sheath immediately encasing the brain were removed using fine forceps and scissors. The brain was kept at 4ºC during the dissection procedure.

4.4.2 Electrophysiology

All intracellular recordings were performed on desheathed brains that were superfused with normal saline at a rate of 1 ml/min at 10-12 ºC. Intracellular recordings were made using 12-30 MΩ glass microelectrodes filled with 3 M KCl and connected to an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA). Extracellular nerve recordings were made by gently drawing nerves of interest into polyethylene tubing filled with normal saline. These suction electrodes were connected to an A-M Systems Differential AC Amplifier (Model 1700, A-M Systems, Carlsborg, WA). Both intra- and extracellular recordings were digitized (>2 kHz) with a 1401Plus or Micro1401 A/D converter from Cambridge Electronic Design (Cambridge, UK).

The effects on the swim motor pattern of current injection to Si₁fib or C2 homologues were examined by injecting positive or negative current (-9 to 5 nA) through a bridge-balanced microelectrode. Data acquisition and analysis were performed with Spike2 software (CED,
Statistical comparisons were made using Student’s t-test. Results were shown as mean ± standard deviation.

For d-tubocurarine bath application experiments, left and/or right body wall nerves in the isolated nervous system were stimulated every 10 min to elicit a fictive motor pattern in the control and experimental conditions; 50 – 100 µM d-tubocurarine (Sigma Aldrich-check) in saline was superfused into the dish, followed by a normal saline washout.

The cerebral commissure was transected by cutting the commissure with fine scissors. To ensure that the results were not a product of direct damage to cell bodies by the scissors, we also transected several preparations with a fine silk thread. The sheath surrounding the cerebral commissure was carefully removed and control motor patterns were elicited by nerve shock. A fine silk thread was then slipped around the commissure and quickly pulled, resulting in a clean transection of the commissure and physical separation of the two cerebral-pleural ganglia.

4.4.3 Tracer injections, immunohistochemistry, and imaging

In preparations where neurons were filled, the cell body was impaled by a microelectrode filled with a 2% solution of the biotinylated tracer, Biocytin (Invitrogen, Carlsbad, CA) dissolved in 0.75 M KCl. Biocytin was injected via iontophoresis for 15-30 min (bipolar current pulses from -10 to +3 nA, 1 Hz, 50% duty cycle). The preparations were maintained in normal saline for 1 - 4 hr prior to fixation overnight in paraformaldehyde-lysine-periodate fixative: 4% paraformaldehyde, 1.85% lysine monohydrochloride, and 0.22% sodium periodate in cacodylate buffer (0.2 M cacodylic acid in 0.3 M NaCl, pH 7.4 –7.6). The tissue was quickly rinsed several times with phosphate-buffered saline (PBS): 50 mM Na₂HPO₄ in 140 mM NaCl, pH 7.2, followed by two longer PBS rinses (3 hr each). The tissue was then washed twice with 4.0%
Triton X-100 in PBS (3 hr each) and then incubated in antiserum diluent (ASD; 0.5% Triton X-100, 1% normal goat serum, and 1% bovine serum albumin in PBS) for 1-2 hr. The brains were incubated for 3-5 days in either primary rabbit polyclonal anti-serotonin (ImmunoStar, Inc., Hudson, WI), anti-FMRFamide (ImmunoStar, Inc., Hudson WI) antiserum diluted 1:1000 in ASD and anti-GABA details. To visualize biocytin, Streptavidin-Alexa Fluor 594 conjugate at 1:200 dilution was added. The brains were washed six times (1 hr each) with 0.5% Triton X-100 in PBS and then incubated overnight in goat anti-rabbit antiserum conjugated to Alexa 488 (Invitrogen, Carlsbad, CA) diluted to 1:100 in ASD. Finally, the tissue was washed six times (1 hr each) with 0.5% Triton X-100 in PBS, dehydrated in an ethanol series (70%, 80%, 2x90%, 95%, 3x100%, 20 min each), cleared in methyl salicylate and mounted on a slide with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). The tissue was kept at 4°C under gentle agitation for the entire immunohistochemistry protocol, except for dehydration and clearing.

The tissue was imaged using a Zeiss LSM 700 Axio Examiner D1 confocal microscope (Carl Zeiss, Oberkochen, Germany) with 5-20x objectives. Fluorophores were excited with two lasers (488 and 555 nm) and fluorescent emissions were passed through a 505-550 nm band-pass filter to visualize Alexa Fluor 488 and a 560 nm long-pass filter to visualize Alexa Fluor 594. The LSM 700 software ZEN was used to acquire the images. Maximal projections of confocal stacks were made and exported as TIFF files to Adobe Photoshop CS where montages were assembled. Brightness and contrast of all images were adjusted when necessary.

4.5 ACKNOWLEDGEMENTS

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Figure 4.1 Phylogenetic tree of the Nudipleura.
Species in this study are marked with an asterisk. The Nudipleura is composed of the monophyletic clades Nudibranchia and Pleurobranchomorpha.
Figure 4.2 Identification of Si1 homologue in *Flabellina iodinea*

A. *Flabellina* exhibits Left-Right (LR) swimming, in which the animal flattens its body in the sagittal plane, bends at the midpoint and makes whole body left and right flexions. B. Schematic of the *Flabellina* brain showing the location of the soma and axon of swim interneuron 1 homologue (Si1, pink) relative to the locations of the serotonergic DSI somata (green). Abbreviations: cerebral ganglion (Ce), pleural ganglion (Pl) and pedal ganglion (Pd), pedal commissures 1 (PP1) and 2 (PP2). C. Biocytin fills of Si1$_{Flb}$ show the location of the soma in the
cerebral ganglia and the projection of the Si1 axon into the ipsilateral pedal ganglia. M: medial, L: lateral, D: dorsal, V: ventral. D. Fine neurites in the pedal ganglia from the axon of Si1
spread towards PP2 (arrow). E. The axon of Si1 showed a characteristic bend in the cerebral
ganglia. In Si1 this bend was in the dorsal-ventral plane. F. Si1 was labeled by intracellular
injections of Biocytin (pink) and was surrounded by the DSI neurons, which are serotonin-
immunoreactive (green). G. Si1 showed double labeling (pink-white) when stained with
antiserum against FMRFamide (green).
Figure 4.3 Role of Si1 homologue in *Flabellina iodinea*
A. Simultaneous intracellular recordings of the left and right Si1<sub>Fib</sub> and extracellular recording of the left and right body wall nerve (BWN). The left and right Si1<sub>Fib</sub> were rhythmically active, firing bursts of action potentials in anti-phase with each other and in phase with the corresponding ipsilateral BWN. The gray bar indicates the duration of one L-Si1 burst. B. Brief depolarizing (i) and hyperpolarizing (ii) current injections into the right Si1<sub>Fib</sub> reset the motor pattern. The red dots indicate the expected times of bursts in a right pedal motor follower neuron (PdN: Pedal Neuron). The phase relationship of the swim motor pattern remained stable before and after the reset (gray bars).
Figure 4.4 Identification of Si1 homologue in *Tritonia diomedea*

A. *Tritonia* exhibits Dorsal-Ventral (DV) swimming, in which the animal flattens in the horizontal plane, bends at the midpoint and makes whole body dorsal and ventral flexions. B.
Schematics of the *Tritonia* brain showing the location of the soma and axon of the swim interneuron 1 homologue (Si1, pink) relative to the locations of the serotonergic DSI somata (green). Abbreviations: cerebral ganglion (Ce), pleural ganglion (Pl) and pedal ganglion (Pd), pedal commissures 1 (PP1) and 2 (PP2). C. Biocytin fills of Si1<sub>Trit</sub> show the location of the soma in the cerebral ganglia and the projection of the Si1 axon into the ipsilateral pedal ganglia. M: medial, L: lateral, D: dorsal, V: ventral. D. Simultaneous electrophysiological recordings of action potentials in the Si1<sub>Trit</sub> soma, EPSPs in a neuron in the ipsilateral pedal ganglion, and Si1<sub>Trit</sub> impulse on extracellular recordings on PP2. E. The axon of Si1<sub>Trit</sub> showed a characteristic bend in the cerebral ganglia. F. Si1<sub>Trit</sub> (Bvi) was labeled by intracellular injections of Biocytin (pink) and was surrounded by the DSI neurons, which are serotonin-immunoreactive (green). G. Si1<sub>Trit</sub> was labeled by intracellular injections of Biocytin (pink) and showed double labeling (pink-white) using antiserum against FMRFamide (green). H. Brief electrical stimulation of a body wall nerve elicited a fictive DV swim motor pattern in *Tritonia*. Si1<sub>Trit</sub> did not show bursting activity, whereas the DV CPG neurons C2 and DSI and the nerve connective PP2 displayed rhythmic bursting activity typical of the DV swim motor pattern.
Figure 4.5 Synaptic connectivity of Si1<sub>Flb</sub> and Si1<sub>Trit</sub>
Si1<sub>Flb</sub> (A) and Si1<sub>Trit</sub> (B) inhibited their contralateral counterparts. Depolarizing current injections into the right Si1 in Flabellina (Ai) and Tritonia (Bi) inhibited the activity of the left Si1 and vice versa (Aii, Bii).
Figure 4.6  Effect of cerebral commissure transection on LR swim motor pattern.
None of the known LR swim interneuron homologues in Flabellina (Ai), Melibe (Bi) and Dendronotus (Ci) project axons through the cerebral commissure. Si1 (pink), Si2 (blue), Si3 (orange) and Si4 (turquoise). A. In Flabellina, the fictive swim was recorded by the bursting activity of a left pedal neuron (ii, top trace) and in the bursting activity of a body wall nerve (ii, bottom trace) under control conditions. Transection of the cerebral commissure (i, red dotted line) halted the swim motor pattern (iii). B. In Melibe, transection of the cerebral commissure (i, red dotted line) did not eliminate the swim motor pattern as shown by the bursting activity of the right Si2 and a left motor follower neuron before (ii) and after (iii) the transection. C. In Dendronotus, the activity of the right Si2 before (ii) and after (iii) the cerebral commissure transection (i, red dotted line) show that the swim motor pattern was not eliminated. Si1 in Dendronotus is not part of the CPG and as expected, did not oscillate.
Figure 4.7 Effect of the homologues of the DV CPG neuron, C2, on the LR swim motor pattern.

Brain schematics showing that the C2 homologues (red) in *Flabellina* (Ai), *Melibe* (Bi) and *Dendronotus* (Ci) send their axons through the cerebral commissure and into the contralateral side of the brain. Aii. In *Flabellina*, C2 activity was sufficient to activate the LR swim. Injecting
depolarizing current (1 to 5 nA) into C2 initiated rhythmic bursting in Si1. Aiii. C2 activity was necessary for maintaining the LR swim in Flabellina. Silencing a single C2 in Flabellina by injecting hyperpolarizing current (-9 nA) was sufficient to halt an ongoing swim motor pattern as recorded by the change in bursting activity of the right Si1 and the left BWN. Releasing C2 and allowing it to fire action potentials restored the swim motor pattern. Aiv and v. C2 activity was necessary for the LR swim. Brief electrical shock to the left BWN initiated a fictive swim as recorded by the bursting activity of the right Si1 and left BWN (iv, middle and bottom trace). Nerve shock also increased the basal firing rate of the left C2 (iv, top trace). Suppression of C2 firing by injection of hyperpolarizing current blocked the nerve evoked swim motor pattern (v). B and C. C2 activity was not necessary for the LR swim in Melibe or Dendronotus. Hyperpolarizing current (-1 to -5 nA) was injected into both C2s to suppress its firing in Melibe (Biii, black arrow) and Dendronotus (Ciii, black arrow) but this did not prevent nerve stimulation (black bar) from evoking a swim motor pattern.
Figure 4.8 Effect of d-tubocurarine (100 μM) on the LR swim motor pattern.
A. In Flabellina, curare blocked the swim motor pattern as recorded from the right Si1, a left motor pedal neuron and the right body wall nerve Si2. Although Si1_{Flb} was silenced in this example, it sometimes fired action potentials in curare. B. In Melibe, superfusion of curare slowed but did not halt the swim motor pattern as recorded from the right Si1 and Si2 before (i) and during (ii) curare application. C. In Dendronotus, curare blocked the swim motor pattern as monitored from the right Si1 and Si2.
Figure 4.9 S1 Electrical coupling in Si1 homologues.
In normal saline, hyperpolarization of Si1 in *Flabellina* (A) and *Tritonia* (B) by negative current injection induced hyperpolarization of the contralateral counterpart.

Figure 4.10 S2 Identification of the C2 homologue in *Dendronotus*.
Just as in the C2 homologues in other Nudipleura molluscs including *Tritonia*, *Melibe* and *Flabellina* [18], C2<sub>Dend</sub> was located on the dorsal surface of the cerebral ganglion by cerebral Nerve 1 with an axon that projected contralaterally through the cerebral commissure and to the contralateral pedal ganglion (i). It was observed to be a whitish cell just as in the other species.
Biocytin fills of $C_{2Dend}$ (red) and immunostaining for FMRFamide (blue) showed that this cell was double labeled ($ii$), which is another identifying criterion for C2 homologues. However, unlike in other species examined to date, $C_{2Dend}$ was not immunopositive for SCP-B (green). $C_{2Dend}$ was not rhythmically active during the nerve-evoked LR swim motor pattern ($iii$), just as in other non-DV swimmers [7]. The $C_{2Dend}$ pairs received common synaptic input ($iv$). The right $C_{2Dend}$ was stimulated with brief current pulses (20 ms, 10 nA) at 15 Hz for 1.5 sec. This caused excitation followed by slower inhibition in the contralateral C2 ($v$). Stimulation of one $C_{2Dend}$ induced an electrotonic spike in the contralateral C2 ($v$). The C2 pairs were electrically coupled ($vi$); negative current injections to one C2 caused hyperpolarization of the contralateral partner.

**Figure 4.11 S3  $C_{2Mel}$ was not necessary for nerve-evoked swim motor pattern.** Hyperpolarization of both C2 homologues in *Melibe* (black arrow) did not prevent nerve shock (black bar) from eliciting the swim motor pattern.
5 CHAPTER FIVE: GENERAL DISCUSSION

“That famous sentence should perhaps be rewritten to read, 'from so simple a beginning limited forms most beautiful and most wonderful have been, and are being, evolved.'”

- George McGhee

In this dissertation, neuroanatomical features such as soma size, location and axon projection and neurochemical features such as immunoreactivity to neurotransmitters such as GABA, FMRF-amide and serotonin, were used to identify homologous neurons and clusters in seven Nudipleura molluscs. The ability to identify homologous neurons provided an opportunity to examine if species with analogous behaviors used similar underlying neural mechanisms. To this end I compared the neural circuits underlying both analogous and non-analogous swimming behaviors in four nudibranch species. A number of conclusions can be drawn from the results of the studies described in this thesis:

1) Analogous swimming behaviors can be generated by neural circuits that are composed of homologous neurons;
2) Those homologous neurons are arranged in different configurations;
3) The CPGs composed of homologous neurons display different internal dynamics.
4) The parallel evolution of swim neural circuits is likely influenced by constraints on the structure of the nudibranch nervous system.

5.1 Nervous system organization tends to be conserved

The basic ground plan for nervous systems tends to be conserved. Despite large divergence times, similarities in brain structure can be recognized in disparate taxa, suggesting that there are constraints on the organization of the nervous system. For example, barring lampreys and hagfish, which appear to lack a cerebellum, all vertebrate brains have the same
major divisions (Fig 5.1A) (Northcutt, 2002). There can however, be significant distortions in brain geometry in some taxa. In arthropods, the protocerebrum, deutocerebrum and tritocerebrum are generally recognized as the three major divisions in the anterior arthropod head (Scholtz and Edgecombe, 2006). In invertebrates and some vertebrates, the components of the nervous system can be further distinguished down to the level of individual neurons allowing for single neuron level analyses of homology. Neurons can be uniquely identified from animal to animal based on multiple characteristics including morphological and neurochemical criteria (Bullock, 2000; Comer and Robertson, 2001). The homologues of these neurons can be identified in other species using the same identifying criteria. Homologous neurons have been identified in this way in phyla such as Annelida, Arthropoda, Nematoda, Chordata and Mollusca. For example, homologues of the serotonergic Retzius (R) cell, and identified mechanosensory (T,P,N) cells have been described in different leech species (Lent, 1973; Keyser and Lent, 1977). Among others, homologues of the Anterior Burster (AB) interneuron have been identified in the stomatogastric ganglion of several decapod crustaceans (For review see: Katz and Tazaki, 1992). The pharyngeal nervous system of the nematode Caenorhabditis elegans consists of 20 identified neurons, each of which has a homologue in the species Pristionchus pacificus (Fig 5.1B) (Bumbarger et al., 2013). Homologues of the escape reflex related Mauthner cell (M cell) have been identified in lampreys, teleost fish and amphibians (For review see: Zottoli and Faber, 2000).

In Nudipleura, which is the clade of focus in this thesis, homologues of the neuronal type C2 have been identified based on both neurochemical and neuroanatomical criteria (Fig 5.1C) (Jing and Gillette, 1995; Lillvis et al., 2012). This includes immunoreactivity to the neuropeptides FMRFamide and Small Cardioactive Peptide-B (SCP-B), axon morphology and
projection pattern. In Chapter 4, these criteria were used to identify the homologue of C2 in the LR swimming species, *Dendronotus iris*. However, the C2 homologue in *Dendronotus* was not immunopositive for SCP-B. It is possible that the neuron identified is not the true C2 homologue. However, it should be noted that there are examples in the literature of homologous neurons with different neurochemical content. For example, in the crustacean stomatogastric nervous system (STNS), the modulatory projection neuron type MPN in the crab, *Cancer borealis* and the neuron type GN1/2 in the European lobster, *Homarus gammarus*, are considered to be homologues of each other based on a suite of criteria including location, axon morphology and GABA-immunoreactivity (Meyrand et al., 2000). No other neuron in that region of the STNS meets these criteria in either species. However, MPN is immunopositive for the neuropeptide proctolin while GN1/2 is not. Moreover, GN1/2 is immunopositive for cholecystokinin (CKK) and FLMRamide while MPN is not (Meyrand et al., 2000).

Another neuronal type that was a focal point in this thesis is Si1. In Chapter 4, neuroanatomical and neurochemical content was used to identify the homologue of the *Melibe leonina* and *Dendronotus iris* Si1 in two additional species, *Flabellina iodinea* and *Tritonia diomedeia*. The neurochemical criteria include immunoreactivity to FMRFamide. The anatomical criteria include location, proximity to the serotonergic DSI homologues, and axon morphology and projection. That said, there are differences as well. For example, the characteristic loop in the proximal axon of the Si1 homologue in *Flabellina* is in the dorsal-ventral plane whereas its counterparts in *Melibe, Dendronotus* and *Tritonia* are in the anterior-posterior plane (Sakurai et al., 2011). This, however, may also be a reflection of the differences in the overall brain shape among these species.
Another example of a molluscan neuronal type that is heavily conserved is the metacerebral giant (MCG) neuron. The MCG is known to be immunoreactive for serotonin (5-HT-ir). In gastropod molluscs, the serotonergic system is particularly well studied, from individual neurons to whole brain. The MCG can be identified based on the fact that it is a large neuron (often the largest) in the anterior cerebral ganglion that is 5-HT-ir. MCG homologues have been identified in species spanning Euthyneura, from nudibranchs such as *Tritonia* (Newcomb et al., 2006) and *Phestilla sebogae* (Willows, 1985) to euopisthobranchs such as *Aplysia californica* and panpulmonates such as *Lymnaea stagnalis* (Weiss and Kupfermann, 1976; Granzow, 1981; Pentreath et al., 1982). Comparative mapping of the serotonergic system in gastropods has revealed several other homologous 5-HT-ir neurons and clusters as well. For example, the homologues of the *Tritonia* dorsal-ventral (DV) swim CPG neuronal type, DSI, have been identified in several Nudipleura molluscs (Sudlow et al., 1998; Newcomb et al., 2006; Newcomb and Katz, 2007) as well as in the distantly related *Aplysia* (Jing et al., 2008) and *Clione limacina* (Satterlie and Norekian, 1995). Some of these euthyneuran species have divergence times of over a hundred million years (Senatore and Katz, 2014) and yet, anatomical and neurochemical traits can uniquely identify a single neuronal type across species.

The work mapping the GABA-ergic system in the central nervous system of Nudipleura molluscs (Chapter 2 and 3) contributes to the very large body of work on homologous neurons in gastropod molluscs. GABA-ir mapping revealed homologous neurons and clusters (e.g., the pleural vPlγL neuron and the pedal vPdγA cluster), highly conserved GABA-ir axonal tracts and innervation patterns as well as similarities in distribution patterns to distantly related species (e.g. buccal GABA-ir pattern in nudibranchs and panpulmonates) (Gunaratne et al., 2014). These
studies collectively show that despite many million years of evolution, nervous systems tend to be highly conserved.

This raises several interesting questions. 1) If the nervous system is so constrained, how is behavioral diversity achieved? For example, how have some Nudipleura species evolved left-right (LR) swimming while others evolved dorsal-ventral (DV) swimming? 2) How do similar behaviors evolve independently in different lineages yet the same behaviors are absent in intermediate lineages that face similar adaptive pressures? For example, *Dendronotus* (family Dendronotidae) and *Flabellina* (family Flabellinidae) exhibit LR swimming behavior while *Dirona albolineata* from the intermediate family Dironidae does not (Newcomb et al., 2012).

All three species of nudibranchs can be found on the west coast of North America and feed on cnidarians including the burrowing anemone and hydroids (McDonald and Nybakken, 1978). They may be preyed on by common predatory sea star species found in the same region such as the sunstar *Pycnopodia helianthoides* and *Pisaster* sea stars (Morris, 1980). *Dendronotus* is known to swim in response to contact with *Pycnopodia* (Morris, 1980). *Dirona* and *Flabellina* both recoil from the touch of sea star tube feet that, in some instances, cause *Flabellina* to swim (personal observations). 3) Conversely, how does a behavior evolve independently in two separate lineages when the nervous system organization is very different (as can be the case between phyla)? Behaviors are a product of underlying neural circuitry, so how is the nervous system contributing to the above evolutionary scenarios? I will attempt to address some of these questions in the next half of this chapter.

5.2 Neural mechanisms underlying analogous behaviors

There are numerous examples of independently evolved behaviors (i.e., analogous behaviors) in nature. Analogous behaviors have been described within relatively restricted
taxonomic groups to across phyla. A few examples include tree-climbing behavior in Indo-Pacific and Atlantic mangrove crabs (Fratini et al., 2005); patagia-assisted gliding in flying squirrels, marsupial sugar gliders and colugos (Jackson, 2012); bipedal hopping in North American and Old World desert rodents (Mares, 1993); highly specialized seed-caching behavior in corvids (de Kort and Clayton, 2006); eusociality in insects, coral shrimp and mole rats (Nowak et al., 2010); and myrmecophagy, or ant-eating, in pangolins, numbats, echidnas, anteaters, aardvarks and aardwolves (Schwenk, 2000).

Despite a laundry list of examples of behavioral convergence in the animal kingdom, not nearly as much is known about how the nervous systems of these animals contribute to the evolution of these behaviors. Comparative work of this kind is usually impeded by the fact that it can be significant challenge to uncover the neural underpinnings of a behavior in a single species, let alone multiple. There are however a few notable exceptions. In the following section, I will review three examples of independently evolved behaviors within and across phyla, where there has been extensive comparative work on the underlying neural substrates. These include: 1) undulatory swimming behavior in leeches and lampreys; 2) vocal learning in songbirds, parrots and hummingbirds; and 3) electrolocation and the jamming avoidance response in African and South American weakly electric fish. I will then discuss left-right swimming behavior in nudibranch molluscs and its neural bases, which is yet a fourth example, and the focus of this thesis.

Each case study will be described at two levels of analysis, the behavior and the underlying neural circuit. Each level can be described in the context of homology (similarity through common descent, i.e., homologous) or homoplasy (similarity through independent evolution, i.e. analogous). For example, undulatory swimming in leeches and lampreys is an
example of an analogous behavior since the last common ancestor of the two groups did not have this trait (Mullins et al., 2011). All four case studies represent analogous behaviors and any speculation on this point shall also be discussed.

Homoplasy at the neural circuit level can be described in terms of convergence (analogous trait built on non-homologous neural substrate) or parallelism (analogous trait built upon homologous neural structures). For instance, the jamming avoidance response (JAR) in African and South American weakly electric fish is an example of convergence at the neural circuit level since the timing-comparison circuitry that is key to the JAR consists of non-homologous brain areas (Kawasaki, 2009). Manual dexterity in *Cebus apella*, a New World monkey, and in Old World macaque monkeys is an analogous behavior that represents an instance of parallelism at the neural level since both groups use similar expansion of homologous brain areas (Padberg et al., 2007). It has been postulated that parallel evolution reflects the extent to which nervous systems bias the direction of evolutionary change (Katz, 2011).

5.3 Swimming behavior in leech and lamprey

5.3.1 Behavior

Locomotion via swimming in leeches and lampreys is similar in that it involves rhythmic axial bending movements of the body, creating undulations that propagate along the body from head to tail (Fig. 5.2A) (Kristan et al., 1974; Wallen and Williams, 1984). In both groups, the undulation amplitude increases with posterior progression of the wave. The swim cycle period ranges from 0.35 – 1.1 sec in leeches (Kristan et al., 1974) and 0.13 - 0.66 sec in lampreys (Wallen and Williams, 1984). Both groups display constant intersegmental phase lags; this ensures coordination of the muscle wave as it travels posteriorly (Mullins et al., 2011). However
there are also differences in the behavior. Leeches make dorsal-ventral undulation in the frontal plane whereas lampreys make snake-like lateral undulations in the sagittal plane. Leeches flatten and lengthen their body during swimming whereas lampreys, with their rigid notochord, maintain their general body shape. Leeches can only swim in the forward direction while lampreys are also capable of backward swimming (Islam et al., 2006).

5.3.2 Phylogenetic relationship

Lampreys are vertebrates belonging to the phylum Chordata and leeches to the phylum Annelida (Fig 5.2B). The phylogenetic distance between the leech and lamprey is very large, with the two lineages diverging from their last common ancestor about 560 million years ago (Kumar and Hedges, 1998). Undulatory swimming behavior in leeches and lampreys is a clear-cut example of an independently evolved or analogous behavior.

5.3.3 Neural Circuit

The leech central nervous system consists of a head ganglion, a segmental nerve cord composed of 21 ganglia and a tail ganglion (Payton, 1981). Each segmental ganglion contains approximately 400 neurons (Macagno, 1980). The lamprey central nervous system consists of the brain (divided into the telencephalon, diencephalon, mesencephalon and rhombencephalon), and the spinal cord. The spinal cord contains 100 segments, each of which contains approximately 1000 neurons (Rovainen, 1979). There are some similarities in the organization of the nervous system between the two groups (Mullins et al., 2011). For example, they both have segmental nervous systems and have many segments/ganglia containing neurons homologous to that of adjoining segments/ganglia. However, leech and lamprey neurons are not homologous to each other.
The swimming behavior of both leech and lamprey is driven by central pattern generator (CPG) circuits. Individual ganglia/segments in both groups have functional CPG units with intra and intersegmental connections (Grillner et al., 1991; Brodfuehrer et al., 1995). However, the neural circuits themselves are completely different from each other. In the leech, there are 13 central pattern generator (CPG) neurons per segment (six pairs and 1 unpaired) per segment. These neurons can be divided into three groups based on their activity phase (cells 208, 115, and 123; 28; 33, 27, and 60) with each group firing 33% out of phase with the previous group (Fig 5.2C). None of these CPG cells have intrinsic bursting properties and the circuit contains mostly inhibitory synapses, which are considered to be necessary for oscillatory activity of the neurons (Brodfuehrer et al., 1995; Mullins et al., 2011). In the lamprey, the CPG units are described at the level of cell class. The circuit is composed of three classes of neurons that have a reciprocal relationship with their contralateral counterparts. These CPG cell classes include excitatory interneurons (EINs), lateral inhibitory interneurons (LINs) and caudally projecting interneurons (CCINs) (Grillner et al., 1991). The circuit is composed of both excitatory and inhibitory synapses. CPG activity is reflected in the motor output to the ventral roots in each segment with the left and right sides showing motor activity with a simple 50% phasing. Unlike in the leech, the lamprey CPG neurons display intrinsic bursting properties (Mullins et al., 2011).

5.3.4 Conclusion

Leech and lamprey display analogous swimming behaviors but the underlying neural circuits are composed of non-homologous neurons that are wired differently. This would be an example of convergent evolution, in which similar behaviors have independently evolved from non-homologous neural substrates.
5.4 Vocal learning in songbirds, parrots and hummingbirds

5.4.1 Behavior

Vocal learning refers to the ability to imitate sounds by learning versus instinct and modify the sounds produced through auditory feedback (Konishi, 1965; Brainard and Doupe, 2000). Vocal learning is adaptive for individual identification, semantic communication, mate attraction and territorial defense (Jarvis, 2009). It depends on but differs from auditory learning, which is the ability to form auditory memories and make associations to sounds heard. For instance, a dog could associate the sound of the word “fetch” with the act of retrieving but cannot vocalize the sound itself. A parrot on the other hand can imitate the sound of the word “fetch”.

Most vocal learners, however, only imitate sounds from their own species. While all examined vertebrates are capable of auditory learning, the ability to learn vocalization is very rare. Vocal learning has been recorded in three groups of birds (songbirds, parrots and hummingbirds) as well as in distantly related groups of mammals (cetaceans, humans, elephants, bats and pinnipeds) (Jarvis, 2009; Petkov and Jarvis, 2012; Reichmuth and Casey, 2014).

Parrots and a few songbirds are capable of vocal learning throughout their lives and can even combine learned sounds (Nottebohm and Nottebohm, 1978; Farabaugh et al., 1994). In most songbirds and hummingbirds, however, vocal learning involves a restricted song repertoire learned during the bird’s critical period (Marler, 1970; Gahr, 2000). For example, during the sensory learning stage of vocal learning, a juvenile songbird will listen to its father’s songs or that of a conspecific and form a song template by memorizing the spatiotemporal properties of the sounds. During what is referred to as the sensorimotor phase, the juvenile bird will begin producing vocalizations that resemble the learned sounds and continue practicing till its vocalization match that of the memorized song (Marler, 1970).
5.4.2 Phylogenetic relationship

Only three (Trochiliformes, Passeriformes, and Psittaciformes) of approximately 30 avian orders have species that display vocal learning. The avian phylogeny suggests that vocal learning behavior has evolved independently two and possible three times in birds (Fig. 5.3A) (Petkov and Jarvis, 2012). Recent phylogenetic analyses show that Passeriformes (which includes songbirds, i.e., oscines and suboscines) and Psittaciformes (parrots) are more closely related to each other than previously thought (Suh et al., 2011; Zhang et al., 2014). They now belong to a clade named Psittacopasserae. Trochiliformes (hummingbirds) are distantly related to the Psittacopasserae, indicating that vocal learning in hummingbirds evolved independently. Vocal learning may have evolved independently in both parrot and songbird lineages as well or it may have been present in the last common ancestor of parrots and songbirds and the trait lost in the suboscines (Fig. 5.3A) (Suh et al., 2011). The latter scenario is supported by recent studies showing the presence of vocal learning in suboscine Procnias bellbirds (Kroodsma et al., 2013) as well as rudimentary vocal learning brain areas in the suboscine Saynoris phoebe (Liu et al., 2013).

5.4.3 Neural Circuit

The vocal learning neural circuitry in songbirds, parrots and hummingbirds is composed of seven comparable and distinct vocal nuclei (Jarvis, 2006). Barring the example of rudimentary vocal neural substrate in the suboscine phoebe, these vocal nuclei are not found in vocal non-learners. The vocal learning brain areas have been given different names in each bird group (Refer to Table 5.1 for abbreviations). There is debate as to whether these brain areas are homologous or analogous (See reviews by: Jarvis, 2006; Petkov and Jarvis, 2012) but regardless, there are some distinct morphological similarities in the vocal nuclei in each bird group. For
example, the nidopallial vocal nucleus in each group (songbird HVC, parrot NLC and hummingbird VLN) all bulge into the overlying ventricle (Nottebohm et al., 1976; Durand et al., 1997; Jarvis and Mello, 2000; Jarvis et al., 2000). In each vocal learning group, three of the vocal nuclei are found in the same relative position in the anterior forebrain while the four posteriorly located vocal nuclei are found within the same brain divisions despite changes in the relative position. The arcopallial vocal nucleus in each group (songbird RA, parrot AAC, and hummingbird VA) is oval in shape with a distinct cytoarchitecture (Nottebohm et al., 1976; Jarvis and Mello, 2000; Jarvis et al., 2000).

The vocal nuclei form two pathways that have been examined to varying degrees in songbirds, parrots and hummingbirds (Fig 5.3B) (Nottebohm et al., 1976; Scharff and Nottebohm, 1991; Jarvis and Mello, 2000; Jarvis et al., 2000; Jarvis, 2006; Jarvis, 2009). Functionally, the four posteriorly located nuclei in each group form the posterior-lateral vocal pathway, which is also referred to as the vocal motor pathway due to its involvement in the production of learned song. This anterior vocal pathway includes projections from a nidopallial vocal nucleus (songbird HVC, parrot NLC, hummingbird VLN) to an arcopallial vocal nucleus (songbird RA, parrot AAC, hummingbird VA) to the midbrain premotor nucleus (DM) and brainstem vocal motor (nXIIIts) neurons. These neurons project to the muscles of the avian vocal organ, the syrinx. Vocal non-learners also possess the DM and nXIIIts for the production of innate calls but lack the connections with the arcopallium seen in vocal learners. In the songbird there are also projections from the posterior vocal nucleus, Nif, to the HVC and from the HVC to the Av, another posterior vocal nucleus. The connectivity between the comparable regions in the parrot and hummingbird are not as well established.
The three anteriorly located nuclei form the anterior-medial vocal pathway or the vocal learning pathway. This circuit connectivity has been examined in the songbird and parrot. It involves projections from a pallial vocal nucleus (songbird MAN, parrot NAOc) to a striatal vocal nucleus (songbird Area X, parrot MMSt) to a dorsal thalamic nucleus (songbird DLM, parrot DMM) and then loops back to the pallial vocal nucleus (songbird MAN, parrot NAOc).

Although there are striking similarities in brain areas and connectivity in the vocal learners, there are some important differences as well. For example, there are some clear differences between songbirds and parrots in the connectivity between the anterior and posterior vocal pathways (Jarvis, 2006). In songbirds, the posterior pathway connects to the anterior pathway via connection from the HVC to Area X. An analogous connection in parrots would entail projections from the NLC to MMSt but instead the posterior pathway connects to the anterior in parrots via projections from the AAC to the NAOc and MO. Another difference is that in parrots, the NLC and AAC vocal nuclei (songbird HVC and RA) have unique outer shell regions and associated connectivity patterns not found in other vocal learners (Chakraborty et al., 2013). It is thought that these additional outer shell regions in parrots relate to their more advanced vocal learning abilities among birds. Among parrots, species with superior vocal learning capabilities such as the African grey parrot were found to have larger outer shell regions (Chakraborty et al., 2013).

It has been proposed that vocal learning nuclei evolved independently as specializations of a pre-existing motor system inherited from a common ancestor of the three lineages and that this explains the similarities in vocal learning brain areas (Feenders et al., 2008; Petkov and Jarvis, 2012; Jarvis, 2013). Feenders et al. (2008) found that motor behaviors such as hopping
and wing flapping activate regions surrounding or immediately adjacent to the seven vocal nuclei in songbirds, parrots and hummingbirds. In vocal non-learners, motor behavior caused activation of seven comparable regions but without the presence of adjacent vocal nuclei (Feenders et al., 2008). Moreover, the connectivity of motor areas around the vocal nuclei resembles that of the vocal motor and learning pathways. It is proposed that the seven vocal nuclei found in vocal learning birds share a deep homology (Feenders et al., 2008; Jarvis, 2013).

5.4.4 Conclusion

Vocal learning behavior has evolved independently two and possibly three times in birds. The three vocal learning avian groups (songbirds, parrots and hummingbirds) contain seven distinct vocal nuclei that are not found in vocal non-learners. There are striking similarities in known circuitry between the three avian groups that exhibit vocal learning. The notion that vocal nuclei may have evolved as specializations of homologous motor structures would make vocal learning an example of parallel evolution. However, there are also notable differences in the vocal learning circuitry and as well as gaps in the knowledge of the parrot and hummingbird circuit connectivity.

Intriguingly, it was recently found that the suboscine phoebe (in the clade Psittacopasserae) possesses rudimentary neural substrates for vocal learning including a songbird RA-like area (Liu et al., 2013). While more examples are needed from multiple vocal non-learning avian groups, the latter study supports a hypothesis that homologues of what are considered to be brain areas unique to vocal learners are present in other bird groups as well but that these areas are particularly well developed in the vocal learning groups (Jarvis, 2006). Since the sub-oscine phoebe is in the clade Psittacopasserae along with songbirds and parrots, vocal learning may have been present in their last common ancestor and the trait lost in the suboscines
In this scenario, vocal learning would be a homologous behavior in parrots and songbirds and similarities in circuitry between songbirds and parrots could be attributed to homology and differences to circuit divergence. Hummingbirds however are evolutionarily distant from Psittacopasserae. The known circuitry of songbirds/parrots and the distantly related hummingbirds suggest that there are a restricted number of neural solutions to generating vocal learning. In other words, the structure of their nervous systems has provided a particular pathway for the evolution of vocal learning.

5.5 Electrocommunication in African mormyroid and South American gymnotiform weakly electric fish

5.5.1 Behavior

Weakly electric fish generate electric fields in order to locate objects in the environment, i.e., electrolocation and for social communication, conveying information such as sex, species, and individual identity. These fish have an electromotor network with a specialized electric organ (EO) for generating the electric fields and an electrosensory network for detecting changes in amplitude and timing of the electric fields due to nearby objects in its environment (Bullock and Heiligenberg, 1986). However, when gymnotid or mormyrid wave-type fish emitting electric organ discharges (EODs) of similar frequencies encounter each other, the resulting interference of the electric fields can adversely affect electrolocation and communication (Bullock et al., 1972; Bullock et al., 1975). Interference of the two EOD signals results in a combined signal whose amplitude and phase fluctuates slowly with time as the two EOD signals go in and out of phase (Rose, 2004). This slow fluctuation can mask the changes in signal caused by objects in the environment and essentially impair electrolocation in the fish. To counter this situation, both mormyrids and gymnotids wave-type fish evolved a behavior referred to as the jamming
avoidance response (JAR). To avoid jamming, the fish alter the frequency of their EODs to increase the total difference in frequency (Bullock et al., 1972; Bullock et al., 1975). For example, in an encounter between a fish with a 600 Hz EOD and another with a 602 Hz EOD frequency, the 602 Hz fish will increase its EOD frequency to 607 Hz and the other will lower its frequency to 595Hz. In the mormyrids, the JAR has been well studied in *Gymnarchus niloticus*; the only African wave type species. Among the gynotids, the JAR and its neural bases have been examined extensively in *Eigenmannia* and *Apteronotus*.

### 5.5.2 Phylogenetic relationships

The African Mormyriformes and South American Gymnotiformes do not share a common ancestor with electrogenic or electrosensory capabilities. The two groups are in fact very distantly related to each other and had their last common ancestor over 200 million years ago (Fig. 5.4A) (Albert and Crampton, 2005; Gallant et al., 2014). Thus, electrogenesis and the jamming avoidance response arose independently in mormyrid and gymnotid weakly electric fish (Bullock et al., 1972; Hopkins, 2009). Mormyriformes include a single basal wave-type species *Gymnarchus niloticus* and one family of pulse-type fishes. The Gymnotiformes include four pulse-type families and two wave-type families with the wave-type being more derived (Kawasaki, 2009)

### 5.5.3 Neural Circuit

Electrolocation and the JAR in Mormyriformes and Gymnotiformes represent a fascinating example of interspecies similarities and differences in circuitry. The EOD in both groups is initiated in a group of electrotonically-coupled neurons in the medulla reticular formation (mormyrid CN, command nucleus; gymnotids PM, pacemaker nucleus) (Hopkins, 2009; Kawasaki, 2009). The signals from the CN/PM are then projected via relay nuclei to
electromotor neurons that activate electrocytes and drive the electric organ (Fig. 5.5A) (Carlson, 2002; Kawasaki, 2009). Pacemaker nuclei in both groups also have similar descending input from midbrain nuclei (mormyrid PCN, precommand nucleus; gymnotid PCM, prepacemaker nucleus) and thalamic nuclei (mormyrid DP, dorsal posterior nucleus; gymnotid DP, dorsal posterior nucleus) (Caputi et al., 2005; Kawasaki, 2009). There are differences in design as well. For example, the command nuclei in mormyrids connect to spinal motor neurons via two relay nuclei while gymnotids use one; in pulse-type mormyrids the command nuclei also sends a copy of EOD command signals to electro sensory areas while gymnotids and the wave-type mormyrid Gymnarchus lack such corollary discharge (Kawasaki, 2009). Thus, the electromotor pathway in mormyrids and gymnotids share many similarities and some differences in design, which is striking given that the two groups do not share a common ancestor with electrogenic ability and independently evolved both electro sensory and electromotor systems.

Both mormyrids and gymnotids wave-type fish converged on the same JAR behavior and use identical computational rules to determine if their EOD frequency needs to be increased or decreased (Kawasaki, 1993). As mentioned earlier, two similar EOD signals result in a combined EOD signal whose amplitude and phase fluctuates slowly with time (Fig 5.5B). A fish can determine the direction of frequency change needed to prevent jamming by comparing concurrent modulations of amplitude and phase on the combined signal to that of signals detected on reference parts of its body that are minimally affected by the EOD from the second fish (Kawasaki, 1993; Rose, 2004). If for example, comparing the combined signal to the fish’s reference signal reveals a phase advance, which also coincides with an increase in the amplitude of the combined signal, the fish’s EOD frequency is determined to be higher than that of the second fish (Fig 5.5B). It is very striking that the both African and South American wave-type
fish use the same complex computations for the JAR behavior. However, the brain areas where these computations occur are different in the two groups. In the African mormyrids, timing difference computations occur in a medullary structure called the electrosensory lateral line lobe (ELL) whereas in the South American gymnotids, these computations occur within the midbrain structure called the torus semicircularis (Heiligenberg, 1991; Kawasaki, 1993; 2009).

There are also some differences in the output path of these timing difference computations. The mormyrid ELL connects to sensorimotor interface nuclei TM (tectum mesencephali) while the torus semicircularis in the gymnotids connects to the sensorimotor nuclei nE (nucleus electro sensorius) (Carlson, 2002). These nuclei connect to the electromotor pathway, which ultimately controls the EOD frequency. Thus the JAR represents an example of convergent evolution, where the same behavior evolved independently using different underlying neural structures.

5.5.4 Conclusion

The electromotor and electrosensory systems evolved independently in African and South American weakly electric fish. EODs in both groups are produced by electromotor circuitries that are very similar in organization. The jamming avoidance response behavior in the two groups is analogous and the algorithms used to compute timing difference are the same but the calculations occur in non-homologous parts of the brain, representing an example of convergent evolution at the level of neural circuitry.

Intriguingly, the wave-type gymnotid Sternopygus, which is basal to Eigenmannia and Apteronotus, is mostly immune to being jammed but is nonetheless capable of determining if an EOD frequency is lower or higher than its own (Rose, 2004). Electric fish track the movements
of their immediate surroundings and Rose and Canfield (1991) showed that *Sternopygus* could be trained to associate foreign EOD signals of frequencies higher or lower than its own with particular movements of the tube it hides within and faithfully tracks. After this conditioning, *Sternopygus* will make appropriate tracking movements in response to the EOD signals alone indicating its ability to discriminate between frequencies (Rose and Canfield, 1991). Such discrimination may be used for social communication such as that observed during courtship and for electrolocation of distant objects, which would require sensitivity to very small modulations of amplitude/timing in feedback signals. (Hopkins, 2009; Kawasaki, 2009).

In terms of neural substrate, *Sternopygus* possesses both the electrosensory circuitry required to make computations on signal amplitude and timing difference as well as the electromotor circuitry to produce EODs but importantly, it lacks the coupling at the sensorimotor interface that leads to appropriate frequency modulations seen in the JAR producing species (Rose, 2004). Collectively, this has led to the proposal that the nervous system is preadapted for the jamming avoidance response in that that coupling preexisting circuits for timing comparisons and EOD production is the most “natural solution” to evolving JAR-like behavior (Rose, 2004). In other words, the organization of the nervous system has constrained the evolution of the JAR neural circuitry in gymnotids.

The mormyrids and gymnotids however do not share a common ancestor with electrosensory or electromotor systems. The JAR may have evolved in each lineage from traits ancestral to the JAR such as electrolocation, which can require the ability to detect very small changes in amplitude/timing of electric signals. Interestingly, the time-comparison computations in the gymnotid JAR-producing *Eigenmannia*, the gymnotid non-JAR *Sternogyrus* and the
mormyrid pulse-type fish *Brienomyrus* all occur in a homologous midbrain structure (torus semicircularis) (Rose et al., 1987; Rose, 2004; Kawasaki, 2009).

### 5.6 Left-Right swimming behavior in Nudipleura molluscs

#### 5.6.1 Behavior

A very small percentage (~3%) of Nudipleura molluscs are capable of producing swimming behaviors. Swimming can be a means of escape from predators as in the species *Dendronotus*, which swims in response to the touch of a sunflower sea star (Morris, 1980). It has also been suggested that swimming is a means of dispersal in species such as *Melibe*, allowing for mixing of gene pools from spatially isolated populations (Mills, 1994). In some species such as *Flabellina* swimming behavior can occur in the apparent absence of any aversive or predatory stimuli and can continue for minutes to hours (personal observation). Left-right (LR) swimming involves the animal flattening its body in the sagittal plane and making rhythmic, alternating flexions by bending at the midpoint of its body axis with the head and tail moving closer to each other with each lateral flexion (Newcomb et al., 2012). This rhythmic motion propels the animal through the water. In some species such as the nudibranch *Bornella anguilla*, this left-right motion appears more as an eel-like undulatory motion. The neural substrate of LR swimming has been examined in *Flabellina iodinea*, *Melibe leonina*, and *Dendronotus iris* and will be reviewed here.

#### 5.6.2 Phylogenetic relationships

Approximately 40 of the 63 species in Nudipleura that have been reported to swim exhibit some form of LR swimming (Newcomb et al., 2012). LR swimming was thought to only
to be present within Nudibranchia; however I recently encountered video records\(^\text{14}\) showing that
LR swimming is present in *Pleurobranchaea brockii*, a species belonging to
Pleurobranchomorpha, the sister clade to Nudibranchia. Of the approximately 48 families in
Nudipleura (Bouchet and Rocroi, 2005), the known LR species now represent 10 families
(Newcomb et al., 2012). However, LR swimming, in fact swimming in general, is an extremely
rare behavior across the phylogeny.

The phylogenetic distance between *Pleurobranchaea brockii* and the nudibranch LR
species as well as the distribution of swimming in Nudipleura suggests that LR swimming
evolved independently in the family Pleurobranchidae. In addition to LR, three other forms of
swimming are seen in Pleurobranchidae including dorsal-ventral (DV), asymmetric undulations
(AU), and flapping (F) (Newcomb et al., 2012). Within Nudibranchia, LR swimming likely
evolved independently in the subclade Euctenidiacea where out of 17 families, LR swimming
has only been observed in a single family called Polyceridae (Newcomb et al., 2012).

Within Cladobranchia, the other sub-clade of Nudibranchia, the literature on the
distribution of LR swimming supports the notion that LR swimming arose independently in at
least *Flabellina iodinea*. *Flabellina, Melibe* and *Dendronotus* belong to the families
Flabellinidae, Tethyidae and Dendronotidae respectively. Flabellinidae belongs to a sub-clade
of Cladobranchia called Aeolidida, which consists of 11 families and whose phylogeny has been
examined (Fig. 5.6) (Carmona et al., 2013). This means that Flabellinidae is more closely related
to these 10 other aeolid families than it is to Tethyidae and Dendronotidae. It is also known

\(^{14}\) https://sites.google.com/site/swimmingseaslugs/a-z-index/pleurobranchaea-brockii
that *Flabellina* is more closely related to the DV swimming species *Tritonia* of the family Tritoniidae than it is to either *Melibe* or *Dendronotus* (Senatore and Katz, 2014). Cladobranchia as a whole contains 31 families (Bouchet and Rocroi, 2005) of which 7 have at least one known LR species (Newcomb et al., 2012). This means that out of the 31 families in Cladobranchia, there are at least 11 non-LR families that Flabellinidae is more closely related to than it is to Tethydidae and Dendronotidae (Fig. 5.6). These non-LR families include the non-swimming and breast-stroke (BS) families in the sub-clade Aeolidida and the DV swimming family Tritoniidae. They collectively represent several hundred species. Flabellinidae itself contains just two known LR swimmers, *Flabellina iodinea* and *Flabellina telja* (Newcomb et al., 2012). It also contains three BS swimmers, *Flabellina cynara*, *Cumanotus beaumonti* and *Cumantus cuenotii*, as well non-swimming species (Newcomb et al., 2012). Thus, when taking into account the distribution of LR behavior in Aeolidida, the known information of the Cladobranchia phylogeny and the presence of BS swimming species in Flabellinidae, it is reasonable to conclude that LR swimming may have evolved independently in *Flabellina iodinea*.

LR swimming may have evolved independently in Tethydidae and Dendronotidae as well but it is equally possible that these two families cluster together in a completely resolved phylogeny of Cladobranchia and have a last common ancestor that exhibited LR swimming. Interestingly, almost half of the known 40 LR/LU species belong to these two families (Newcomb et al., 2012).

**5.6.3 Neural Circuit**

The LR swimming behavior in *Flabellina*, *Melibe* and *Dendronotus* are controlled by CPG circuits. The activity of the CPG cells can be monitored in an isolated brain preparation, in
which the fictive swim motor pattern can be evoked by stimulation of a body wall nerve. The neural circuit underlying the *Melibe* LR swim consists of four bilaterally represented cell types called swim Interneuron 1 (Si1), swim interneuron 2 (Si1), swim interneuron 3 (Si3) and swim interneuron 4 (Si4) (Thompson and Watson, 2005; Sakurai et al., 2011; Sakurai et al., 2014). These neurons are arranged into two reciprocally inhibitory kernels, one consisting of Si1, Si2 and the contralateral Si4 and the second kernel consisting of Si3 (Sakurai et al., 2014). These kernels interact with each other to produce a stable rhythmic swim motor pattern. For the sake of simplicity, in this dissertation a version of the *Melibe* CPG was represented that consists of only Si1 and Si2 (Fig 5.7). Each Si1 and Si2 makes inhibitory connections across the midline with the Si1 and Si2 on the other hemisphere. The Si1 and Si2 pairs are all electrically coupled to each other, though the coupling between the ipsilateral Si1 and Si2 is 10-30 times stronger than the other connections (Sakurai et al., 2011). Si1 and Si2 synapse onto motor neurons that project to the muscles (Thompson and Watson, 2005). The ipsilateral Si1 and Si2 fire in phase with each other and in antiphase to the contralateral Si1 and Si2, resulting in the alternating left-right bursting activity that drives the rhythmic body flexions.

The cells that are homologues of the *Melibe* Si1 and Si2 have been identified in *Dendronotus*. However, only Si2 is a member of the swim CPG in *Dendronotus* (Fig 5.7). The Si2 neurons display mutual inhibition and are electrically coupled to each other and the Si1 pair. The Si1 neurons do not inhibit each other although they are electrically coupled. During a fictive swim motor pattern, the Si2 neurons fire in alternation and in a constant phase with pedal motor neurons and nerves (Sakurai et al., 2011).

In chapter 4, I identified the homologue of Si1 in *Flabellina* and determined that it is a member of the LR CPG. Similar to *Melibe*, the Si1 makes mutually inhibitory connections to its
contralateral partner on the other hemisphere. The Si1 pair also exhibits electrical coupling as seen in the Si1 homologues of both *Melibe* and *Dendronotus* (Fig 5.7). Si1 fires in antiphase to its contralateral partner resulting in the alternating left-right bursting activity that is also observed on pedal motor neurons and drives the rhythmic body flexions.

Comparing different aspects of the three LR CPGs showed that some circuit features were shared by all three species; some were shared by any two of the three species; and some were unique to each species (Fig. 5.8). For example, all three species contain homologues of the neuronal types Si1. The Si1 homologue is also coupled to its contralateral counterpart in all three species. The neuronal type C2 has also been identified in *Melibe, Flabellina* (Lillvis et al., 2012) and *Dendronotus* (Chapter 4). In each species, the C2 homologue acts as an extrinsic modulator and initiates the swim motor pattern (Chapter 4).

Features that are shared by *Melibe* and *Dendronotus* and not *Flabellina* include the presence of the neuronal types Si2/Si3 and their role as CPG members (Sakurai and Katz, 2013; Sakurai et al., 2014). Given the high degree of conservation in the organization of the nervous system in nudibranchs, it is possible, if not likely that the Si2/Si3 homologues are present in *Flabellina* as well but have not been found yet.

Neural circuit feature shared by *Melibe* and *Flabellina* and not *Dendronotus* include the role of Si1 as a CPG member as well as the mutually inhibitory connection seen between the Si1 pair. Mutually inhibitory neurons are the most common circuit building block for network based CPGs (Marder and Calabrese, 1996). The fact that mutual inhibition is present in the Si1 homologues of *Melibe* and *Flabellina* and not *Dendronotus* would support the hypothesis that mutual inhibition is necessary for alternating bursting in Si1. However, we have also identified
the Si1 homologue in *Tritonia*, a species that does not display LR swimming and found that its Si1 pair also displays mutual inhibition (Fig. 5.7). This shows that mutual inhibition among the Si1 homologues may be a phylogenetically constrained feature especially given that *Flabellina* and *Tritonia* are more closely related to each other than they are to *Melibe* or *Dendronotus*.

The mutual inhibition example highlights the importance of also examining the nervous systems of species that either do not display the behavior of interest or display a non-analogous behavior. If we did not examine the Si1 homologue in *Tritonia*, we would have concluded that mutual inhibition is a feature of the nervous system that is required to produce oscillatory bursting in Si1 and thus contributes to its role as a CPG neuron.

A feature shared by *Dendronotus* and *Flabellina* is the susceptibility of the swim motor pattern to curare. Curare blocks the SMP in *Flabellina* and *Dendronotus* but only slows it down in *Melibe* suggesting that there are curare-sensitive synapses in *Flabellina/Dendronotus* that are necessary for the swim motor pattern. The curare sensitive synapse has been identified in *Melibe* and *Dendronotus* as those made by the swim CPG neuronal type Si3 (Sakurai and Katz, 2013).

A swim circuit feature that is unique to *Flabellina* is the necessity of C2 activity for the swim motor pattern. In *Flabellina*, hyperpolarizing a single C2 could block the SMP or halt an ongoing SMP. Lesioning the cerebral commissure via which C2 makes connections to the contralateral half of the brain also eliminated the SMP in *Flabellina* only.

While Si1 is part of the CPG in *Flabellina* as it is in *Melibe*, the Si1 duty cycle is about 50% (i.e. Si1 fires for 50% of the duration of a full cycle) while the *Melibe* Si1 exhibits a 30% duty cycle. In *Melibe*, it is known that the Si1 firing is cut short by direct inhibitory input from
Si3 (Sakurai et al., 2014). The duty cycles of *Flabellina* and *Melibe* indicate that there are differences in the internal dynamics of the two CPGs.

The *Melibe* circuit includes a fourth CPG neuronal type called Si4 (Sakurai et al., 2014). If present, the Si4 homologues have not been identified in *Dendronotus* or *Flabellina*. Also, the synapses made by the CPG neuron Si3 are inhibitory in *Melibe* while the same synapses are excitatory in *Dendronotus* (Sakurai and Katz, 2013; Sakurai et al., 2014). While Si1 is not a CPG member in *Dendronotus*, it nonetheless acts as an extrinsic modulator of the SMP (Sakurai and Katz, 2013).

5.6.4 Conclusion

The swimming behaviors exhibited by *Flabellina* and *Melibe/Dendronotus* are analogous, and yet there are important differences in the underlying CPG circuits; namely the connectivity, pharmacological sensitivity, and role of homologous neurons in the initiation, production and maintenance of the swim motor pattern. LR swimming in nudibranchs represents an example of an analogous behavior that is produced by neural circuits that are configured differently but are still built upon homologous components, i.e. parallel evolution.

What is striking is that of the thousands of neurons in the brains of Nudipleura sea slugs, the same players repeatedly emerge in the context of the very rare swimming trait. For instance, it seems too extraordinary a coincidence that the C2 neuronal type is a member of the swim CPG in two evolutionary distant species (*Tritonia* and *Pleurobranchaea*) that display analogous DV swimming behavior and that it is an extrinsic modulator of LR swim circuits (and a necessary component of one). Similar to the vocal learning example, swim circuits in Nudipleura likely evolved from pre-existing neural circuits that may have served a different function in the common ancestor.
Work on the roles of the serotonergic DSI homologues across Euthyneura has led to the proposal that escape swim networks such as that of the DV swimming *Tritonia* and *Pleurobranchaea* have evolved from ancestral non-rhythmic circuits involved in the response to noxious stimuli such as aversive turning and increased crawling (Katz et al., 2001; Jing et al., 2008). In line with this notion, the activity of the C2 homologue in *Pleurobranchaea* is known to increase with noxious mechanical or chemical stimuli, strongly inhibit any ongoing feeding behavior, and in whole animal preparations, result in the animal making a single dorsal flexion followed by a lateral turn (Jing and Gillette, 1995).

While components of the DV swim circuitry, such as C2 and DSI, are involved in the LR circuitry, we do not have any evidence of LR circuit components affecting the DV CPG. This could indicate that neurons such as Si1-4 were incorporated into circuits that were built upon ancestral networks that already included DSI/C2. Or it could be because we have so far identified only one LR neuronal type (Si1) in a single DV swimming species (*Tritonia*). In *Pleurobranchaea*, there is a neuronal type, A4, that meets many of the anatomical criteria used to identify Si1 homologues (e.g. characteristic axonal loop, projection pattern, location, proximity to 5-HT-ir DSI homologues) and is known to be a command neuron for turning, causing the animal to make a lateral body bend reminiscent of the body movements during LR swimming (Jing and Gillette, 2003). However, it is not known for certain if A4 is the Si1 homologue since it is not known if A4 meets the neurochemical identifying criteria of Si1 homologues.

### 5.7 General conclusions

A basic question addressed in this dissertation is, do analogous behaviors have similar underlying neural circuitry? A deeper question that is tackled is, how does the nervous system
contribute to the evolution of analogous behaviors? The four examples discussed above – undulatory swimming in leeches and lampreys; vocal learning in songbirds, parrots and hummingbirds; electrolocation and jamming avoidance behavior in African and South American weakly electric fish; and left-right swimming behavior in nudibranchs – all provide insight into these questions.

The example of swimming in evolutionary distant leeches and lampreys shows that analogous behaviors can be produced by entirely different neural circuitry assembled from nervous systems that do not share any apparent homologous components (convergent evolution). The jamming avoidance response (JAR) in African and South American weakly electric fish shows that analogous behaviors can be produced by neural circuits that use identical computational rules executed by non-homologous brains areas (convergent evolution). Vocal learning behavior in songbirds, parrots and hummingbirds suggests that analogous behaviors can be produced by neural circuits composed of homologous vocal nuclei (parallel evolution). The case for homology of vocal nuclei is based on the hypothesis that vocal nuclei are specializations or expansions of preexisting motor brain areas that were involved in an ancestral trait. It should be noted, however, that it has also been proposed that vocal nuclei in the three groups are non-homologous. It is currently unclear which hypothesis is more accurate (See reviews by: Jarvis, 2006; Petkov and Jarvis, 2012). In nudibranch molluscs, there is considerably less debate on the identity of homologous brain structures and homology can be established at the level of single neurons. The example of left-right (LR) swimming behavior in nudibranchs molluscs shows that analogous behaviors are produced by neural circuits composed of homologous neurons that have been configured differently (parallel evolution).
5.8 **Do analogous behaviors have similar underlying neural circuitry?**

The simple answer to this question is no. While not an incorrect statement, it can be an oversimplification. As seen in weakly electric fish, vocal learning birds and swimming nudibranchs, there are uncanny similarities in the underlying neural substrate, be it the evolutionary origin, organization or computational rules used in executing the behavior. As has been proposed by others, these similarities might not just be extraordinary coincidences (Farries, 2001; Rose, 2004; Katz, 2011; Jarvis, 2013). There are two main recurring themes in the examples of analogous behavior that were reviewed: 1) nervous system organization tends to be conserved; and 2) new neural networks build upon pre-existing neural systems. This concept is aptly captured by the following quote:

> “Although an organ may not have been originally formed for some special purpose, if it now serves for this end we are justified in saying that it is specially contrived for it. On the same principle, if a man were to make a machine for some special purpose, but were to use old wheels, springs, and pulleys, only slightly altered, the whole machine, with all its parts, might be said to be specially contrived for that purpose. Thus throughout nature almost every part of each living being has probably served, in a slightly modified condition, for diverse purposes, and has acted in the living machinery of many ancient and distinct specific forms.”

> -Charles Darwin, 1862

Given that behaviors are produced by neural circuits, there are two main conclusions that can be drawn from these recurring themes. One is that there is a limited neural palette from which behaviors arise. The second is that the structure of the nervous system not only puts a cap on the number of neural solutions that can be generated, but that it also herds the evolution of behavior down particular paths.
Figure 5.1  Nervous system organization tends to be conserved.
A. Lateral views of the brains of a number of extant vertebrate species (not drawn to the same scale). While there is tremendous variation in size, most vertebrates possess brains that can be divided into the same number of divisions. aob, accessory olfactory bulb (cross-hatched); cb,
cerebellum (stippled); **ch**, cerebral hemispheres (cross-hatched); **m**, medulla oblongata; **ob**, olfactory bulb (cross-hatched); **ot**, optic tectum (black); and **p**, pituitary gland. Figure adapted from: (Northcutt, 2002). **B.** Nomarski microscopy of the pharynx of *C. elegans* and *P. pacificus* with an overlay indicating the position and shape of cell bodies of neurons (shown in red). Neurons are homologous between *P. pacificus* and *C. elegans* based on position and shape. Note that ventral cells occur in pairs, except for I5. For these cells, only the left side is shown. The pharynx contains four major subdivisions: the corpus, median bulb, isthmus, and the terminal bulb. Interneuron, I; Motorneuron, M. Figure adapted from: (Bumbarger et al., 2013). **C.** Brain schematics of Nudipleura molluscs showing the location and axon projection of the Cerebral 2 (C2) neuron homologues (pink) in *Tritonia diomedea, Hermissenda crassicornis, Flabellina iodinea, Melibe leonina*, and *Pleurobranchaea californica*. Figure adapted from: (Lillvis et al., 2012)
Figure 5.2 Undulatory swimming in leeches and lampreys.
A. Illustration of swimming behavior in a leech and lamprey. The leech makes dorsal-ventral undulations in the frontal plane and the lamprey makes lateral undulations in the sagittal plane.
B. Abbreviated phylogenetic tree of Metazoa illustrating the relationships between major phyla including Annelida and Chordata which contain leeches and lampreys respectively. Figure adapted from: (Edgecombe et al., 2011). C. Circuit diagram for swimming in leeches and lampreys. In the leech, the numbers denote individually identified intersegmental interneurons. DI-102 and DI-1 are inhibitory motorneurons. Phase values for the three groups of CPG neurons are indicated at the top. In the lamprey CPG, crossed inhibitory interactions ensure that when one side is active, the other is inhibited. CCIN, contralaterally and caudally projecting interneuron; LIN, lateral interneuron. Lines ending in filled circles denote inhibitory synapses; those terminating with a Y denote excitatory synapses; diode symbols denote electrical coupling. Figure from: (Mullins et al., 2011).
Figure 5.3 Vocal learning in songbirds, parrots and hummingbirds.

A. Avian phylogenetic tree showing the relationships between the three vocal learning avian groups (in red). Filled circles indicate independent acquisitions of the vocal learning trait. Unfilled circles indicate an alternate scenario involving nine independent losses of the trait from a vocal learning common ancestor. Figure from: (Petkov and Jarvis, 2012). B. Brain schematics illustrating the comparable vocal and auditory brain areas among vocal learning birds. Yellow regions and black arrows indicate proposed posterior vocal pathways; red regions and white arrows indicate proposed anterior vocal pathways; dashed lines show connections between the two vocal pathways; blue indicates auditory regions. For simplification, not all connections are shown. Figure from: (Jarvis, 2009).
Figure 5.4 Phylogenetic tree showing the relationship between the major groups of electric fish.
Electrogenesis evolved independently six times in vertebrates including in the African Mormyriformes and South American Gymnotiformes. Figure from: (Gallant et al., 2014).
Figure 5.5 Electromotor pathway and jamming avoidance response (JAR).

A. Central electromotor pathway in the gymnotid *Eigenmannia* and the mormyrid *Gymnarchus*. The pacemaker/command nuclei initiate the timing for each EOD. Neurons in the consequent downstream structures fire one action potential for each EOD. Figure from: (Kawasaki, 2009). B.
Schematic of the JAR of two wave-type electric fish. The similar EOD frequencies (\(S_1\) and \(S_2\)) of the two fish summate to produce a combined frequency (\(S_1+S_2\)), where the amplitude and phasing of the signal fluctuates slowly with time as the two EOD signals go in and out of phase. The amplitude peak of the combined signal corresponds to the point in time when \(S_1\) and \(S_2\) are in phase (reinforcement) and at the minimum when the two signals are in antiphase (interference). When the frequency of \(S_2 > S_1\), the timing of zero-crossings of the combined signal, relative to those of \(S_1\), are delayed as the amplitude rises, and advanced as the amplitude falls. This is reversed when \(S_1 > S_2\). The fish are capable of computing the relationship between amplitude and phase modulations to determine if its own frequency is higher and lower than that of the neighboring fish. Figure from: (Rose, 2004)
Figure 5.6 Phylogeny showing all families in Cladobranchia.
Pink, blue and brown squares indicate families that contain species that exhibit left-right (LR), dorsal -ventral (DV) and breast-stroke (BS) swimming species respectively. All other families have no known swimming species. Shaded box indicated families within Aeolidida, a sub-clade
of Cladobranchia. Asterisks indicate families that include species examined in this study.

Swimming and phylogenetic information are based on: (Bouchet and Rocroi, 2005; Pola and Gosliner, 2010; Newcomb et al., 2012; Carmona et al., 2013; Senatore and Katz, 2014)

Figure 5.7 Swim CPG circuits and homologous neurons in Nudibranch molluscs. A. The *Melibe* LR CPG contains the bilaterally represented neuron types Si1 and Si2 (gray box). The *Dendronotus* LR CPG consists of the Si2 pair (gray box) and the Si1 pair does not show mutual inhibition. The *Flabellina* LR CPG consists of the Si1 pair. The C2 homologues in
the three LR species are sufficient for the swim motor pattern and necessary in *Flabellina* (pink box). The DSI homologue in *Melibe* is sufficient for its LR swim motor pattern. The *Tritonia* DV CPG consists of the C2, DSI and VSI pairs (gray box). The Si1 pair in *Tritonia* has connections similar to *Melibe* and *Flabellina*. The resistor symbol represents electrical coupling with thicker lines indicating stronger coupling. The small filled circle and line symbol indicates an inhibitory synapse. Figure information from: (Newcomb and Katz, 2009; Sakurai et al., 2011; Newcomb et al., 2012).

**Figure 5.8** Summary of the similarities and differences in LR swim networks of *Melibe*, *Dendronotus* and *Flabellina*. Figure information from: (Thompson and Watson, 2005; Sakurai et al., 2011; Sakurai and Katz, 2013; Sakurai et al., 2014).
Table 5.1 Abbreviations of comparable vocal learning related brain areas of songbirds, parrots and hummingbirds.

<table>
<thead>
<tr>
<th>Subdivision</th>
<th>Songbird</th>
<th>Parrot</th>
<th>Hummingbird</th>
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<tr>
<td>Nidopallium</td>
<td>HVC: a letter based name</td>
<td>NLC: Central nucleus of the lateral nidopallium</td>
<td>VLN: Vocal nucleus of the lateral nidopallium</td>
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<tr>
<td></td>
<td>NIF: Interfacial nucleus of the nidopallium</td>
<td>LAN: Lateral nucleus of the anterior nidopallium</td>
<td>VMN: Vocal nucleus of the medial nidopallium</td>
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<tr>
<td></td>
<td>MAN: Magnocellular nucleus of anterior nidopallium</td>
<td>NAO: Oval nucleus of the anterior nidopallium</td>
<td>VAN: Vocal nucleus of the anterior nidopallium</td>
</tr>
<tr>
<td>Mesopallium</td>
<td>MO: Oval nucleus of the mesopallium</td>
<td>MO: Oval nucleus of the mesopallium</td>
<td>VAM: Vocal nucleus of the anterior mesopallium</td>
</tr>
<tr>
<td></td>
<td>Av: Avalanch</td>
<td>LAM: Lateral nucleus of the anterior mesopallium</td>
<td>VMM: Vocal nucleus of the medial mesopallium</td>
</tr>
<tr>
<td>Arcopallium</td>
<td>RA: Robust nucleus of the arcopallium</td>
<td>AAC: Central nucleus of the anterior arcopallium</td>
<td>VA: Vocal nucleus of the arcopallium</td>
</tr>
<tr>
<td>Striatum</td>
<td>Area X: Area X of the striatum</td>
<td>MMS: Magnocellular nucleus of the anterior striatum</td>
<td>VAS: Vocal nucleus of the anterior striatum</td>
</tr>
<tr>
<td>Thalamus</td>
<td>DLM: medial nucleus of dorsolateral thalamus</td>
<td>DMM: Magnocellular nucleus of the dorsomedial thalamus</td>
<td>DLM: Medial nucleus of dorsolateral thalamus</td>
</tr>
<tr>
<td>Midbrain</td>
<td>DM: Dorsal medial nucleus of the midbrain</td>
<td>DM</td>
<td>DM</td>
</tr>
<tr>
<td>Brainstem</td>
<td>nXII: Tracheoesophageal subdivision of the hypoglossal nucleus</td>
<td>nXII</td>
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