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HOMOLOGOUS NEURONS AND THEIR LOCOMOTOR FUNCTIONS IN  
NUDIBRANCH MOLLUSCS

by  
JAMES M. NEWCOMB

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

ABSTRACT

These studies compare neurotransmitter localization and the behavioral functions of homologous neurons in nudibranch molluscs to determine the types of changes that might underlie the evolution of species-specific behaviors. Serotonin (5-HT) immunohistochemistry in eleven nudibranch species indicated that certain groups of 5-HT-immunoreactive neurons, such as the Cerebral Serotonergic Posterior (*CeSP*) cluster, are present in all species. However, the locations and numbers of many other 5-HT-immunoreactive neurons were variable. Thus, particular parts of the serotonergic system have changed during the evolution of nudibranchs.

To test whether the functions of homologous neurons are phylogenetically variable, comparisons were made in species with divergent behaviors. In *Tritonia diomedea*, which crawls and also swims via dorsal-ventral body flexions, the *CeSP* cluster includes the Dorsal Swim Interneurons (DSIs). It was previously shown that the DSIs are members of the swim central pattern generator (CPG); they are rhythmically active during swimming and, along with their neurotransmitter 5-HT, are necessary and

sufficient for swimming. It was also known that the DSIs excite efferent neurons used in crawling. DSI homologues, the *CeSP-A* neurons, were identified in six species that do not exhibit dorsal-ventral swimming. Many physiological characteristics, including excitation of putative crawling neurons were conserved, but the *CeSP-A* neurons were not rhythmically active in any of the six species. In the lateral flexion swimmer, *Melibe leonina*, the *CeSP-A* neurons and 5-HT, were sufficient, but not necessary, for swimming. Thus, homologous neurons, and their neurotransmitter, have functionally diverged in species with different behaviors.

Homologous neurons in species with similar behaviors also exhibited functional divergence. Like *Melibe*, *Dendronotus iris* is a lateral flexion swimmer. Swim interneuron 1 (Si1) is in the *Melibe* swim CPG. However, its putative homologue in *Dendronotus*, the Cerebral Posterior ipsilateral Pedal (CPiP) neuron, was not rhythmically active during swim-like motor patterns, but could initiate such a motor pattern. Together, these studies suggest that neurons have changed their functional relationships to neural circuits during the evolution of species-specific behaviors and have functionally diverged even in species that exhibit similar behaviors.

Index Words: Central Pattern Generator, Convergent Evolution, Divergent Evolution, Evolution, Gastropod, *Hermisenda*, Homologous Neurons, Homology, Homoplasy, Invertebrate, Locomotion, *Melibe*, Mollusc, Nudibranch, Opisthobranch, Parallel Evolution, Phylogeny, Serotonin, Swimming, *Tritonia*

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NUDIBRANCH MOLLUSCS

by

JAMES M. NEWCOMB

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

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2006



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Georgia State University  
December 2006

## DEDICATION

Swimming slugs are fun and interesting, but nothing compares to welcoming a daughter into this world. This dissertation is dedicated to the new shining star in my life, Brynna Grace Newcomb.

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## LIST OF ABBREVIATIONS

5-HT	serotonin
5-HT-ir	serotonin-immunoreactive
A	anterior
ASD	antiserum diluent
C1	cerebral cell 1
C2	cerebral cell 2
Ce	cerebral ganglion
CED	Cambridge Electronic Design
<i>CeN1</i>	cerebral nerve 1
<i>CeN2</i>	cerebral nerve 2
<i>CeN3</i>	cerebral nerve 3
<i>CeSP</i>	cerebral serotonergic posterior
<i>CeSP-A</i>	cerebral serotonergic posterior A
CI	consistency index
CNS	central nervous system
CPG	central pattern generator
CPiP	cerebral posterior ipsilaterally projecting
CPT	cerebropleural ganglion triplet
D	dorsal
d <i>CeSA</i>	dorsal cerebral serotonergic anterior

<i>dCeSL</i>	dorsal cerebral serotonergic lateral
<i>dCeSP</i>	dorsal cerebral serotonergic posterior
<i>dPdSA</i>	dorsal pedal serotonergic anterior
<i>dPdSL</i>	dorsal pedal serotonergic lateral
<i>dPdSM</i>	dorsal pedal serotonergic medial
<i>dPdSP</i>	dorsal pedal serotonergic posterior
<i>dPlSL</i>	dorsal pleural serotonergic lateral
<i>dPlSM</i>	dorsal pleural serotonergic medial
DSI	dorsal swim interneuron
FHL	Friday Harbor Laboratories
L	lateral
M	medial
MCG	metacerebral giant
MS	methysergide
P	posterior
PBS	phosphate buffered saline
Pd	pedal ganglion
Pd5	pedal 5
<i>PdN1</i>	pedal nerve 1
<i>PdN2</i>	pedal nerve 2
Pl	pleural ganglion
<i>PlN1</i>	pleural nerve 1

<i>PP1</i>	pedal-pedal connective 1
<i>PP2</i>	pedal-pedal connective 2
RC	rescaled consistency index
RI	retention index
Si1	swim interneuron 1
Si2	swim interneuron 2
SMP	swim motor pattern
T	tentacular lobe
TPep	<i>Tritonia</i> pedal peptide
V	ventral
vCeSA	ventral cerebral serotonergic anterior
vCeSL	ventral cerebral serotonergic lateral
vCeSP	ventral cerebral serotonergic posterior
vPdSA	ventral pedal serotonergic anterior
vPdSL	ventral pedal serotonergic lateral
vPdSM	ventral pedal serotonergic medial
vPdSP	ventral pedal serotonergic posterior
vPlSL	ventral pleural serotonergic lateral
vPlSM	ventral pleural serotonergic medial
VSI	ventral swim interneuron

## CHAPTER ONE

### GENERAL INTRODUCTION

The central nervous system is generally considered to have been more conserved during the course of evolution than the periphery (Bramble and Wake, 1985; Wainwright and Lauder, 1986; Lauder and Shaffer, 1988; Sanderson, 1988; Goslow et al., 1989; Wainwright, 1989; Wainwright et al., 1989; Kavanau, 1990; Arbas et al., 1991; Edwards and Palka, 1991; Paul, 1991; Katz and Tazaki, 1992; Nishikawa et al., 1992; Weijs and Dantuma, 1994; Tierney, 1995; Kiehn et al., 1997; Katz and Harris-Warrick, 1999; Herrel et al., 2001; Langenbach and Van Eijden, 2001; Wainwright, 2002; though see Smith 1994). It is hypothesized that one of the reasons for this conservation may be the fact that many neural networks are multifunctional, and therefore alterations to one neural element or circuit will have deleterious repercussions on other circuit functions (Nishikawa et al., 1992; Tierney, 1995). Therefore, species-specific behaviors are thought to often arise from changes in the periphery. There are a number of examples of similar neural circuits producing different behaviors as a result of differences in the periphery (Westneat and Wainwright, 1989; Paul, 1991; Katz and Tazaki, 1992) that support this hypothesis.

However, the central nervous system is evolutionarily labile and capable of changing as a result of natural selective pressures, as is obvious from the wide variety of brain morphologies and neuroanatomical organization present throughout the animal kingdom. A number of studies have demonstrated evolutionary changes in the nervous

system (Shaw and Meinertzhagen, 1986; Wilson and Paul, 1987; Shaw and Moore, 1989; Buschbeck and Strausfeld, 1997), including cases where these changes have been correlated with alteration in function (Liem 1978, 1979, 1980; Wilson et al., 1982; Arbas, 1983a,b; Lauder, 1983; Sillar and Heitler, 1985; Gordon and Herring, 1987; Wainwright et al., 1989; Paul, 1991; Wright, 2000; Hale et al., 2002). However, none of these studies have investigated the evolution of central pattern generators (CPGs) producing a rhythmic motor pattern at the level of identified neurons. In the current study, we demonstrate a number of evolutionary changes in the central nervous system of nudibranch molluscs, including alterations in CPG circuits involved in the production of rhythmic locomotor motor patterns and behaviors.

The relative simplicity of invertebrate nervous systems, in conjunction with the ability to identify individual homologous neurons between species (reviewed in Sakharov, 1976; Weiss and Kupfermann, 1976; Pentreath et al., 1982; Croll, 1987a; Katz, 1991; Paul, 1991; Katz and Tazaki, 1992; Bulloch and Ridgway, 1995; Katz et al., 2001; Murphy, 2001) and the functions of these identified neurons (Bullock, 2000; Comer and Robertson, 2001), provides a means of investigating key neural components associated with the evolution of species-specific behaviors.

### **Homology and homologous neurons**

The concept of homology has evolved and expanded since the term was initially coined in the nineteenth century. Richard Owen is generally attributed with defining

homology and delineating it from analogy (Owen, 1843, 1847), although this distinction had been proposed by others (MacLeay, 1821; Strickland, 1846). In fact, the concept of homology can even be traced back to Aristotle and Belon (Panchen, 1994; Rieppel, 1994). Owen defined homology as, "The same organ in different animals under every variety of form and function" (Owen, 1843, p. 379). Over the last 150 years, this original definition has been modified a number of times for the purpose of making comparisons at differing hierarchical levels of biology (e.g. molecular, genetic, cellular, embryological, organismal, population, behavioral, etc.). In this current study, I use the phylogenetic definition of homology, which defines two structures as homologous if they derive evolutionarily from the same structure in a common ancestor (Rieppel, 1980, 1988; Patterson, 1982, 1988; Bock, 1989; de Pinna, 1991; Striedter and Northcutt, 1991; Shubin, 1994).

Comparisons of homologous neural structures have been useful in investigating the evolution of nervous systems. There are many examples of homology in the vertebrate nervous system (Karten, 1991; Bruce and Neary, 1995; Striedter, 1997; Reiner et al., 2004; Pritz, 2005; LaBerge et al., 2006). In invertebrates, the concept of homology has been applied to individually identifiable neurons and has led to a number of potential evolutionary principles of the nervous system: 1) neurons can be highly conserved despite differences in target structures (Dorsett, 1974; Dickinson, 1979); 2) homologous neurons can exhibit differences in number and synaptic connectivity (Arbas, 1983; Shaw and Meinertzhagen, 1986; Shaw and Moore, 1989; Buschbeck and Strausfeld, 1997); 3) multifunctional circuits can be a source for the evolution of species-specific behaviors



(Wilczynski 1984; Arbas et al. 1991; Katz 1991; Tierney 1995; Wainwright 2002); 4) sensory organs for the external environment can evolutionarily derive from internal proprioceptive sensors (Yack and Fullard, 1990; Yack et al., 1999; Yager, 1999); 5) neuromodulation can be a rich source of evolutionary plasticity (Katz, 1991; Katz and Tazaki, 1992); 6) neuronal deletion can accompany the loss of a specific behavior (Faulkes, 2004); and 7) evolutionary alteration of synaptic transmission in homologous neurons can result in species-specific behaviors (Chiang et al., 2006). However, there have been fewer studies demonstrating that the functions of homologous neurons, in the context of specific neural circuits, can be changed during the evolution of species-specific behaviors (Sillar and Heitler, 1985; Dumont and Robertson, 1986; Paul, 1991; Wright, 2000).

The purpose of this current study was to capitalize on the relative ease of identification and physiological characterization of nudibranch neurons to investigate the extent of functional changes of homologous neurons during the evolution of species-specific behaviors. The behaviors that we focused on were various modes of locomotion that have evolved independently in the nudibranch clade.

### **Nudibranchs as a model system**

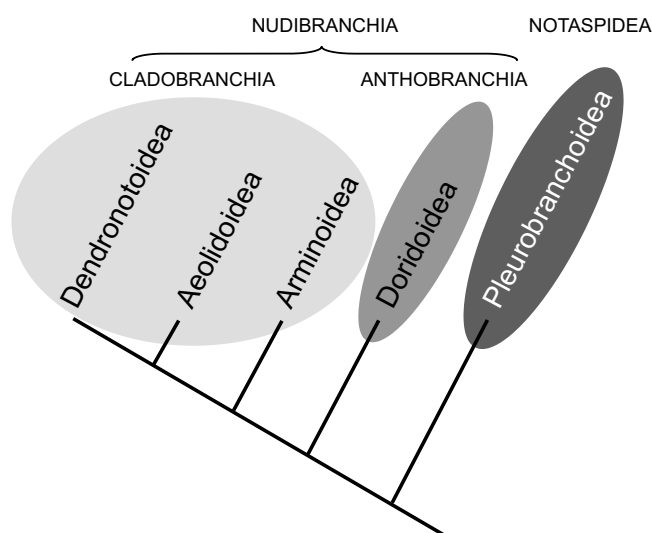
There are a number of criteria which would be useful for comparing homologous neurons and their functional roles in species-specific behaviors. These criteria include: 1) a well-characterized phylogeny; 2) a wide variety of species-specific behaviors; 3)

that are similar enough across the studied phylogenetic range to identify homologous neurons and structures; and 5) large and identifiable neurons. Nudibranchs satisfy all of these criteria and therefore are excellent organisms for investigating evolutionary changes of the nervous system.

### **Phylogeny of nudibranchs**

Nudibranchia is an order of gastropod molluscs in the subclass Opisthobranchia. The phylogeny of the nudibranchs has been analyzed using both morphological and molecular data (Brunckhorst, 1993; Thollessen, 1999, 2000; Wägele and Willan, 2000; Schrodler et al., 2001; Wollscheid-Lengeling et al., 2001; Grande et al., 2002, 2004; Valdes, 2003; Vonnemann et al., 2005). The order Nudibranchia is subdivided into four suborders (Dendronotoidea, Aeolidioidea, Arminoidea, and Doridoidea) that fall within two monophyletic clades: Anthobranchia and Cladobranchia (Fig. 1-1) (Odhner, 1934; Thollessen, 1999; Wägele and Willan, 2000; Wollscheid-Lengeling et al., 2001; Grande et al., 2004; Vonnemann et al., 2005). The nudibranchs form a larger monophyletic clade (Nudipleura) with some members of the order Notaspidea, including *Pleurobranchaea californica* (Wägele and Willan, 2000), providing a closely related sister group for phylogenetic comparisons.

Although the monophyly of the Anthobranchia and Cladobranchia is universally supported, recent data question the monophyly of some of the four suborders. For



**Figure 1-1:** Nudipleura phylogeny. The notaspids and nudibranchs are sister orders. Within the nudibranchs, there are four suborders divided into two monophyletic clades, Cladobranchia and Anthobranchia (Odhner, 1934; Thollessen, 1999; Wägele and Willan, 2000; Wollscheid-Lengeling et al., 2001; Grande et al., 2004; Vonnemann et al., 2005).

example, a comprehensive phylogenetic analysis of morphological characteristics by Wägele and Willan (2000) supported the monophyly of the Doridoidea, Aeolidoidea and Dendronotoidea, but suggested that the Arminoidea may be paraphyletic. Molecular evidence also supports the paraphyly of the Arminoidea (Wollscheid-Lengeling et al., 2001). However, this and other molecular studies to date have only examined a select few genes and do not have enough resolution to determine the specific phylogenetic relationships of the Arminoidea and their relationships to the other suborders. Therefore, it is not yet possible to construct a plausible alternative phylogeny and thus the traditional four suborders are used for interpreting results in this study.

### **Locomotion in nudibranchs**

Nudibranchs exhibit several modes of locomotion. Crawling is the primary form of locomotion for all nudibranchs (Chase, 2002). The majority of nudibranchs crawl via mucociliary locomotion; cilia on the bottom of the foot beat and propel the animal over a surface of secreted mucus (Miller, 1974a,b; Audesirk, 1978a,b; Chase, 2002; Baltzley, 2006). A few species apparently also use muscular crawling; the anterior portion of the foot is stretched and attached to the substrate, whereupon the animal pulls the rest of its foot and body forward (Agersborg, 1922, 1923; Hurst, 1968; Baltzley, 2006).

In addition to crawling, a limited number of species also can swim. Farmer (1970) categorized swimming in opisthobranchs into five types: 1) flapping, 2) undulation, 3) lateral bending, 4) breast stroke, and 5) jet propulsion. All of these modes of swimming,

with the exception of jet propulsion, are exhibited by the nudibranchs (Table 1-1; Farmer, 1970). However, undulation, in the form of dorsal-ventral body flexions (e.g. *Tritonia diomedea* [Willows, 1967; Hume et al., 1982]) and lateral bending (e.g. *Melibe leonina* [Lawrence and Watson, 2002]) are the predominant modes of swimming in the nudibranch clade. Therefore, this study will focus on the underlying neural mechanisms and the evolution of these two types of swimming.

### **Evolution of dorsal-ventral and lateral flexion swimming in nudibranchs**

The phylogenic distribution of dorsal-ventral and lateral flexion swimming in nudibranchs suggests that one or both of these two modes of swimming arose independently multiple times. Three of the four nudibranch suborders have species that exhibit lateral flexion swimming (Table 1-1), Arminoidea being the lone suborder without any swimming species at all. Dorsal-ventral flexion swimmers are also present in Dendronotoidea and Doridoidea (Table 1-1). However, regardless of whether or not they can swim, most nudibranchs exhibit crawling; only three nudibranch species are truly pelagic, *Phylliroë atlantica*, *Phylliroë bucephala*, and *Cephalopyge trematoides* (Lalli and Gilmer, 1989). This is also true for gastropods in general; there are approximately 40,000 marine gastropod species but only about 140 are pelagic (Lalli and Gilmer, 1989). Therefore, crawling is likely to be the basal form of locomotion in the nudibranchs, which means that both modes of swimming arose multiple times (Fig. 1-2A). Even if dorsal-ventral or lateral flexion swimming was the ancestral condition, then parsimony

Table 1-1: Swimming nudibranchs.<sup>1</sup>

	Mode of Swimming	References
<b>Dendronotoidea</b>		
Bornellidae		
<i>Bornella anguilla</i>	L	Johnson, 1984
<i>Bornella calcarata</i>	L	Thompson, 1980
<i>Bornella stellifer</i>	L	Risbec, 1953; Farmer, 1970
Dendronotidae		
<i>Dendronotus albopunctatus</i>	L	Robilliard, 1972
<i>Dendronotus albus</i>	L	Farmer, 1970; Robilliard, 1970
<i>Dendronotus dalli</i>	L	Robilliard, 1970
<i>Dendronotus diversicolor</i>	L	Robilliard, 1970
<i>Dendronotus frondosus</i>	L	Farmer, 1970; Robilliard, 1970
<i>Dendronotus iris</i>	L	Agersborg, 1922; Haefelfinger and Kress, 1967; Farmer, 1970; Robilliard, 1970, 1972; Wobber, 1970
<i>Dendronotus nanus</i>	L	Marcus and Marcus, 1967; Farmer, 1970; Robilliard, 1972
<i>Dendronotus rufus</i>	L	Robilliard, 1970
<i>Dendronotus subramosus</i>	L	Farmer, 1970; Robilliard, 1970
Lomanotidae		
<i>Lomanotus genei</i>	L	Thompson and Brown, 1984
Phylliroidae		
<i>Phylliroë atlantica</i>	L	Lalli and Gilmer, 1989
<i>Phylliroë bucephala</i>	L	Lalli and Gilmer, 1989
<i>Cephalopyge trematoides</i>	L	Lalli and Gilmer, 1989
Scyllaeidae		
<i>Notobryon wardi</i>	L	Thompson and Brown, 1981
<i>Scyllaea pelagica</i>	L	Collingwood, 1879; Pruvot-Fol, 1954; Farmer, 1970
Tethydidae		
<i>Melibe bucephala</i>	L	Schuhmacher, 1973
<i>Melibe engeli</i>	L	Risbec, 1937
<i>Melibe fimbriata</i>	L	Thompson and Crampton, 1984
<i>Melibe leonina</i>	L	Agersborg, 1921; Hurst, 1968; Farmer, 1970; Lawrence and Watson, 2002
<i>Melibe megaceras</i>	L	Gosliner, 1987
<i>Melibe pilosa</i>	L	Pease, 1860; Ostergaard, 1955; Farmer, 1970
<i>Tethys fimbria</i>	L	Pruvot-Fol, 1954; Farmer, 1970
Tritoniidae		
<i>Marionia blainvillea</i>	DV	Pontes, 2002
<i>Marionia tethydes</i>	DV <sup>2</sup>	Haefelfinger and Kress, 1967
<i>Tritonia diomedea</i>	DV	Willows, 1967; Hume et al., 1982
<i>Tritonia festiva</i>	DV	Birkeland, 1974
<i>Tritonia hombergi</i>	DV	Willows and Dorsett, 1975
<b>Aeolidoidea</b>		
Aeolidiidae		
<i>Aeolidiella alba</i>	BS	Pruvot-Fol, 1954; Farmer, 1970
Cumanotidae		
<i>Cumanotus beaumonti</i>	BS	Picton and Morrow, 1994
<i>Cumanotus cuenoti</i>	BS	Tardy and Gantes, 1980
Flabellinidae		
<i>Flabellina cynara</i>	BS	Marcus and Marcus, 1967; Farmer, 1970
<i>Flabellina iodinea</i>	L	MacFarland, 1966; Farmer, 1970
<i>Flabellina telja</i>	L	Marcus and Marcus, 1967; Farmer, 1970; Ferreira and Bertsch, 1972
Glaucidae		
<i>Hermisenda crassicornis</i>	L	unpublished observation

<i>Pteraeolidia ianthina</i>	L	S Kempf, personal communication
<b>Doridoidea</b>		
Discodorididae		
<i>Sebadoris nubilosa</i>	DV/F <sup>3</sup>	Marcus and Marcus, 1967; Farmer, 1970
Dorididae		
<i>Aphelodoris antillensis</i>	DV	Quiroga et al., 2004
<i>Aphelodoris gigas</i>	DV	Wilson, 2003
<i>Aphelodoris karpa</i>	DV	Wilson, 2003
<i>Discodoris evelinae</i>	DV	Marcus, 1955; Marcus and Marcus, 1967
<i>Discodoris pusae</i>	DV	Marcus, 1955
Goniodorididae		
<i>Trapania velox</i>	L	Cockerell, 1901; Farmer, 1970
Hexabranchidae		
<i>Hexabranchus aureomarginatus</i>	DV/F <sup>3</sup>	Neu, 1932; Ostergaard, 1955; Farmer, 1970
<i>Hexabranchus morsomus</i>	DV/F <sup>3</sup>	Risbec, 1928; Marcus and Marcus, 1962
<i>Hexabranchus sanguineus</i>	DV/F <sup>3</sup>	Risbec, 1928; Gohar and Soliman, 1963; Vicente, 1963; Edmunds, 1968; Farmer, 1970
<i>Hexabranchus tinkeri</i>	DV/F <sup>3</sup>	Ostergaard, 1955; Farmer, 1970
Polyceridae		
<i>Nembrotha megalocera</i>	L	Yonow, 1990
<i>Plocamopherus ceylonicus</i>	L	Rudman and Darvell, 1990
<i>Plocamopherus imperialis</i>	L	Ellis, 1999a; Marshall and Willan, 1999
<i>Plocamopherus lucayensis</i>	L	A Valdes, personal communication
<i>Plocamopherus maculatus</i>	L	Pease, 1860
<i>Plocamopherus maderae</i>	L	Lowe, 1842
<i>Plocamopherus pilatecta</i>	L	A Valdes, personal communication
<i>Plocamopherus tilesii</i>	L	Ellis, 1999b
<i>Tambja blackii</i>	L	Pola et al., 2006
<i>Tambja eliora</i>	L	Lance, 1968; Farmer, 1970
<i>Tambja morosa</i>	L	Willan and Coleman, 1984; Marshall and Willan, 1999
<i>Triopha fulgurans</i>	L	Risbec, 1925; Farmer, 1970

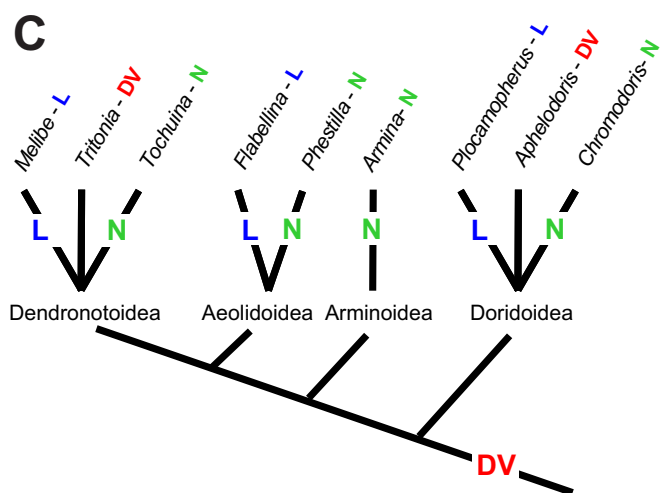
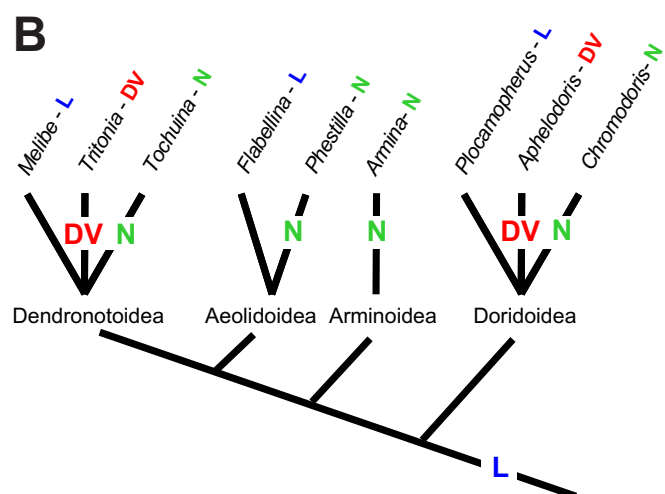
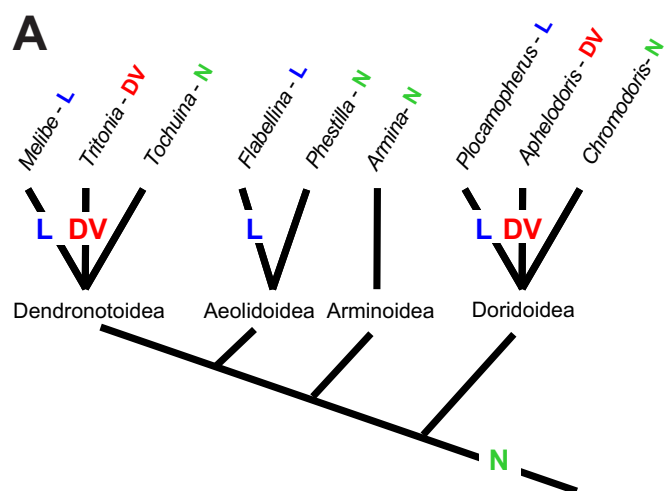
<sup>1</sup> Abbreviations: BS = breast stroke; DV = dorsal-ventral flexion; F = flapping; L = lateral flexion

<sup>2</sup> Farmer (1970) reported that *Marionia* swim via lateral flexions and cited a German reference (Haefelfinger and Kress, 1967). However, a more recent translation of this reference into English, by P Katz, indicates that Haefelfinger and Kress reported dorsal-ventral flexions.

<sup>3</sup> Farmer (1970) categorized swimming in *Sebadoris* and *Hexabranchus* as "flapping". However, swimming in these species appears to include dorsal-ventral flexions of the body, in addition to undulations of the mantle.

**Figure 1-2:** Potential evolutionary histories of locomotion in the nudibranch clade. **A)** If non-swimming (N) was the ancestral condition, then lateral flexion swimming (L) would have evolved independently in three lineages and dorsal-ventral flexion swimming (DV) would have arisen twice. Other evolutionary scenarios are possible in the case of non-swimming being the primitive state, although this is the most parsimonious. The species illustrate representative examples of each mode of locomotion in the corresponding suborders. **B)** If lateral flexion swimming was a synapomorphy of the nudibranchs, then it would have been lost independently four times and dorsal-ventral flexion swimming would have evolved twice. **C)** If dorsal-ventral swimming was the primitive condition, then it would have been lost four lineages and lateral flexion swimming would have arisen three times.





suggests that independent evolution and loss of locomotor behaviors occurred (Fig. 1-2B & C). Therefore, evolution of locomotor behavior in the nudibranch clade has exhibited both divergence and convergence.

### **Convergent and parallel evolution**

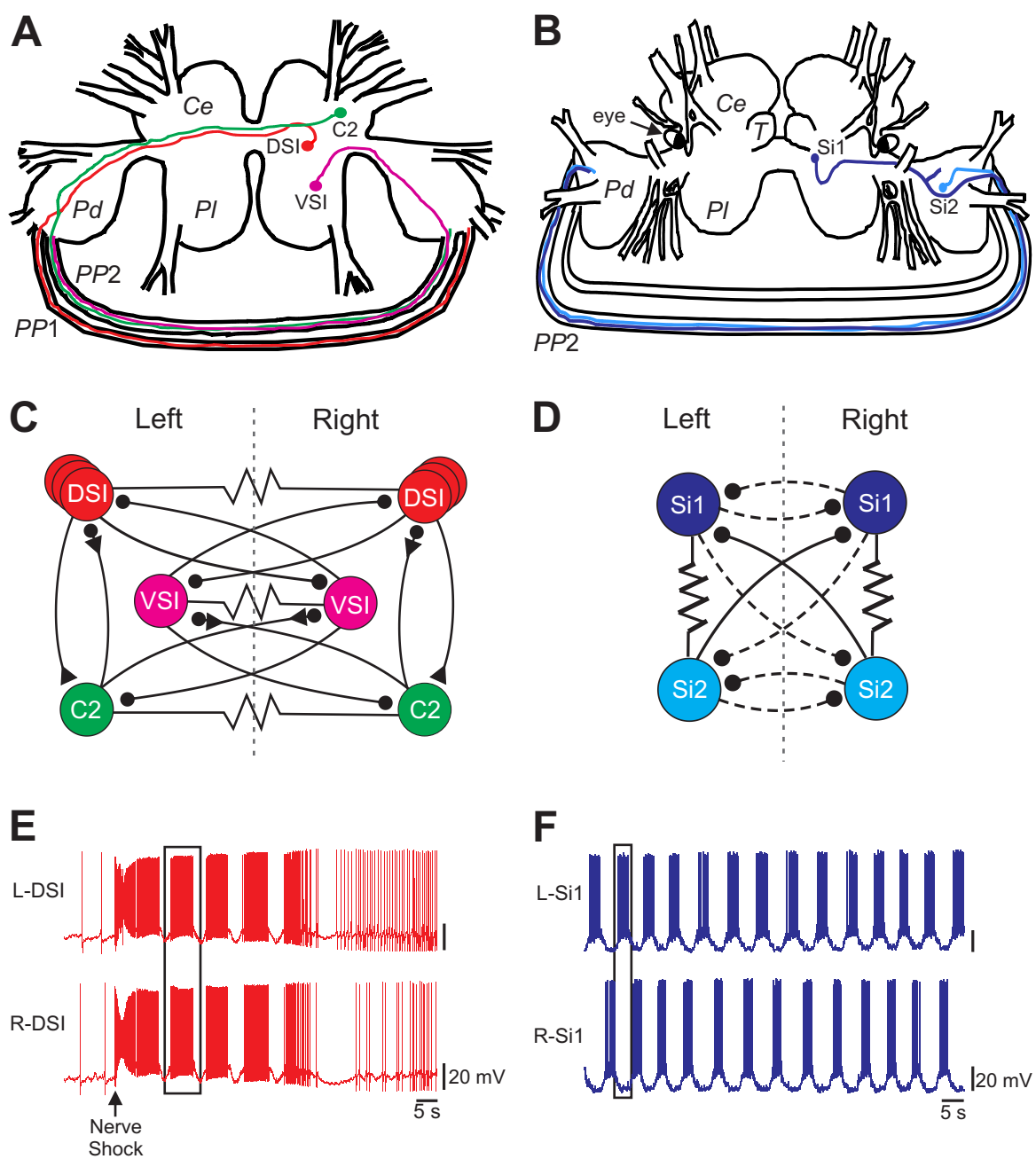
The evolution of similar, independently-derived behaviors, or homoplasy, can be accomplished via convergent or parallel evolution. In convergent evolution, non-homologous traits come to resemble each other, whereas in parallel evolution, homologous traits are used and developed in a similar manner (Futuyma, 1986; Schluter et al., 2004). There have been some anatomical-based studies on parallel evolution of neural circuits (Wullimann et al., 1991; Catania, 2000; Puelles, 2001; Carr and Soares, 2002), but little, if any, neurophysiological work. Parallel evolution of morphological features has been studied in molluscs (Gosliner and Ghiselin, 1984; Gosliner, 1991), and the ability to identify individual neurons within and across molluscan species (Bullock, 2000) suggests that this group of animals is a promising system for investigating the neurophysiological basis of both convergent and parallel evolution of species-specific behaviors.

### **Dorsal-ventral and lateral flexion swimming are not homologous behaviors**

The neural circuits producing dorsal-ventral and lateral flexion swimming have been characterized in two nudibranch species. Willows and colleagues were the first to investigate neural control of dorsal-ventral swimming in *Tritonia diomedea* (Willows, 1967; Dorsett et al., 1969, 1973). Later studies further characterized the swim CPG (Getting et al., 1980; Getting, 1983; Frost and Katz, 1996), which is comprised of at least three types of bilaterally symmetric interneurons: dorsal swim interneurons (DSIs), ventral swim interneurons (VSI-B), and cerebral cell 2 (C2) (Fig. 1-3). There are three DSIs, one VSI-B, and one C2 on each side of the brain. The DSIs and C2 are located on the dorsal surface of the cerebral ganglion and project to the contralateral pedal ganglion, whereas the VSI-B is located on the ventral surface of the pleural ganglion and projects to the ipsilateral pedal ganglion. All of these bilaterally symmetric swim interneurons are electrically coupled to their contralateral counterparts. These electrical connections assist in synchronizing the two sides of the circuit, providing synchronous activity on both sides of the brain such that the two sides of the animal bend in unison.

In contrast, the swim CPG for lateral flexion swimming in *Melibe leonina* is comprised of only two types of bilaterally symmetric interneurons: swim interneuron 1 (Si1) and swim interneuron 2 (Si2) (Fig. 1-3D-F; Thompson and Watson, 2005). Si1 is located in the cerebral ganglion and projects to the ipsilateral pedal ganglion, whereas Si2 is located in the pedal ganglion and projects through one of the pedal-pedal connectives to the contralateral pedal ganglion. Electrical connections exist between ipsilateral swim

**Figure 1-3:** Dorsal-ventral flexion swimming in *Tritonia diomedea* (**A, C & E**) and lateral flexion swimming in *Melibe leonina* (**B, D & F**) are not homologous behaviors, based on fundamental differences in circuit membership, wiring, and output. **A)** In *Tritonia*, the swim central pattern generator (CPG) is comprised of three types of bilaterally symmetric interneurons, the Dorsal Swim Interneurons (DSIs), Cerebral cell 2 (C2), and Ventral Swim Interneuron B (VSI-B) (Getting et al., 1980; Getting, 1983). The approximate somata locations and neurite projections are illustrated for representative examples in the right cerebropleural ganglion. **B)** In *Melibe*, the swim CPG consists of two types of bilaterally symmetric interneurons, Swim interneuron 1 and 2 (Si1 and Si2) (Thompson and Watson, 2005). The somata locations and neurite projections (only the right interneurons are illustrated) are different than the swim interneurons in *Tritonia*, suggesting that these neurons are not homologous. **C)** Contralateral counterparts in the *Tritonia* swim CPG are electrically coupled to each other (Getting, 1981), thereby synchronizing the two sides of the swim circuit. **D)** In contrast, there is reciprocal inhibition between contralateral counterparts in the *Melibe* swim CPG (Thompson and Watson, 2005). This creates a half-center oscillator between the two sides of the swim circuit. **E)** In an isolated *Tritonia* brain, pedal nerve stimulation elicits a transient swim motor pattern, which can be monitored by simultaneous intracellular recordings from swim interneurons. Contralateral DSIs exhibit left-right synchrony (box). **F)** In an isolated *Melibe* brain preparation, a swim motor pattern can occur spontaneously and last for many minutes. Furthermore, contralateral swim interneurons exhibit alternating bursts of activity (box). In the circuit diagrams, circles represent inhibitory connections, triangles represent excitatory connections, and resistors indicate electrical connections. Several of the contralateral inhibitory connections between swim interneurons in the *Melibe* swim circuit are represented with dotted lines because it is unclear whether these connections are monosynaptic or polysynaptic. Abbreviations: Ce = cerebral ganglion; Pd = pedal ganglion; Pl = pleural ganglion; T = tentacular lobe.



interneurons but, in contrast to *Tritonia*, contralateral counterparts are mutually inhibitory. Thus, the two sides of the circuit in *Melibe* operate in antiphase, resulting in a bilateral half-center oscillator that produces the alternating left-right flexions of the body (Agersborg, 1921; Hurst, 1968; Lawrence and Watson, 2002).

Comparison of the swim CPGs between *Tritonia* and *Melibe*, which are in the same suborder, shows that the swim interneurons are located in different areas of the brain of each species, have different projection patterns, are wired differently, and produce different outputs (Fig. 1-3). Therefore it is most parsimonious to assume that these two CPGs, and thus the two modes of swimming, are not homologous behaviors and that they arose independently during evolution.

### **Homologous neurons in nudibranchs**

Although dorsal-ventral and lateral flexion swimming are not homologous behaviors, homologous neurons can be readily identified between nudibranch species (Dorsett, 1974; Dickinson, 1979; Pentreath et al, 1982; Croll, 1987a; Longley and Longley, 1987; Katz et al., 2001). Homologous neurons have usually been identified in nudibranchs based on soma location, size and color, neurite morphology, neurotransmitter, synaptic connections, and electrophysiological properties. Though it is rare for all of these criteria to be met in any particular case for homology, an accumulation of several of these criteria can be supportive of such a claim.

The C1 neuron, also referred to as the metacerebral giant cell, is an excellent example of a homologous neuron in nudibranchs. The C1 neuron is the largest serotonergic neuron in the anterior region of the cerebral ganglion, and it projects to the buccal ganglion where it serves as a neuromodulator of feeding (Weiss and Kupfermann, 1976; Barber, 1983). Based on its morphological criteria, it has been identified in several nudibranchs, including *Hermisenda crassicornis* (Croll, 1987b; Auerbach et al., 1989), *Phestilla sibogae* (Croll et al., 2001), and *Tritonia* (Weinreich et al., 1973; Sudlow et al., 1998; Fickbohm and Katz, 2000; Fickbohm et al., 2001). A similar neuron is present in many other gastropods (Sakharov, 1976; Weiss and Kupfermann, 1976; Pentreath et al., 1982; Barber, 1983; Ono and McCaman, 1984; Murphy et al., 1985; Longley and Longley, 1986; Croll, 1988; Hernádi et al., 1989; Satterlie et al., 1995; Shirahata et al., 2004). Therefore this neuron is considered to be highly conserved and homologous between gastropod species (Sakharov, 1976; Weiss and Kupfermann, 1976; Pentreath et al., 1982; Barber, 1983; Croll, 1987a).

The serotonergic cluster that contains the DSIs in *Tritonia* is present in several other nudibranchs (Land and Crow, 1985; Croll, 1987b; Auerbach et al., 1989; Croll et al., 2001) and even other opisthobranchs (Katz et al., 2001), suggesting that DSI homologues may be present in a number of species. The DSIs are integral members of the dorsal-ventral flexion swim CPG in *Tritonia* (Getting et al., 1980). However, the other nudibranchs that are known to have a similar serotonergic cluster do not exhibit dorsal-ventral swimming. Furthermore, there are other nudibranchs that do exhibit dorsal-ventral swimming (see Table 1-1) but which may also have putative homologues of the DSIs;

however, the distribution of serotonergic neurons is not known for these species. Thus, this may be an excellent opportunity to investigate the functional roles of putative homologues in species with divergent and convergent behaviors.

### **Dissertation outline**

The first three chapters of this dissertation focus on homologous neurons in species with divergent behaviors. I hypothesized that homologues of the serotonergic DSIs would be present in a wide array of nudibranchs. Therefore, chapter one describes serotonin immunoreactivity in the central nervous system of eleven nudibranch species, encompassing all four suborders of the nudibranch clade. Despite a high degree of homoplasy in the presence and number of many serotonergic neurons and clusters, there was a small group of serotonergic neurons, the Cerebral Serotonergic Posterior (*CeSP*) cluster, located in the same vicinity as the *Tritonia* DSIs in all eleven species. This chapter was published in The Journal of Comparative Neurology (Newcomb et al., 2006). David J. Fickbohm is an additional author on this manuscript because he provided the data regarding the distribution of serotonin-immunoreactive neurons in *Tritonia*.

In chapter two, dye fills of neurons in the vicinity of the *CeSP* cluster, combined with serotonin immunohistochemistry, indicated the presence of putative DSI homologues, the *CeSP*-A neurons, in six nudibranch species representing three of the four suborders. None of these six species exhibit dorsal-ventral flexion swimming.



Despite this divergence in locomotion, the *CeSP-A* neurons were remarkably similar to the DSIs in many of their physiological properties, with the exception that they were not rhythmically active in any species other than *Tritonia*. This chapter was published in The Journal of Comparative Physiology A (Newcomb and Katz, in press).

A more thorough comparison of the DSIs in the dorsal-ventral swimming *Tritonia* with their homologues, the *CeSP-A* neurons, in the lateral swimming *Melibe* is described in chapter three. The DSIs are integral members of the swim CPG in *Tritonia* and are necessary and sufficient for swimming (Getting et al., 1980). They also serve as intrinsic neuromodulators of the swim CPG (Katz et al., 1994; Katz and Frost, 1995a,b, 1997; Katz, 1998; Sakurai and Katz, 2003). In *Melibe*, electrophysiological analysis indicated that the *CeSP-A* neurons were not members of the lateral swim CPG. However, they were sufficient to elicit a swim motor pattern and therefore served as extrinsic modulators of the swim CPG.

The last chapter describes homologous neurons in species with similar behaviors. In this case, a putative homologue of Si1, an integral member of the swim CPG in *Melibe* (Thompson and Watson, 2005), was identified in another lateral flexion swimmer, *Dendronotus iris*. Despite the fact that these two species are relatively closely related, the putative Si1 homologue, the Cerebral Posterior ipsilateral Pedal (CPiP) neuron, was not rhythmically active during a putative swim motor pattern and thus may not be a member of the swim CPG in *Dendronotus*. However, it was sufficient to elicit a swim motor pattern and thus may be involved with initiation or modulation of this behavior. These last two chapters will also each be submitted for publication in peer-reviewed journals.

In summary, it appears that neurons tend to be highly conserved but can change their functions during the evolution of species-specific behaviors. This suggests that neural circuits are often sculpted from preexisting networks rather than created by the addition of new neurons.

## CHAPTER TWO

### COMPARATIVE MAPPING OF SEROTONIN-IMMUNOREACTIVE NEURONS IN THE CENTRAL NERVOUS SYSTEMS OF NUDIBRANCH MOLLUSCS

#### **Abstract**

The serotonergic systems in nudibranch molluscs were compared by mapping the locations of serotonin-immunoreactive (5-HT-ir) neurons in eleven species representing all four suborders of the nudibranch clade: Dendronotoidea (*Tritonia diomedea*, *Tochuina tetraquetra*, *Dendronotus iris*, *Dendronotus frondosus*, and *Melibe leonina*), Aeolidioidea (*Hermisenda crassicornis* and *Flabellina trophina*), Arminoidea (*Dirona albolineata*, *Janolus fuscus* and *Armina californica*), and Doridoidea (*Triopha catalinae*). A nomenclature is proposed to standardize reports of cell location in species with differing brain morphologies. Certain patterns of serotonin (5-HT) immunoreactivity were found to be consistent for all species, such as the presence of 5-HT-ir neurons in the pedal and cerebral ganglia. Also, particular clusters of 5-HT-ir neurons in the anterior and posterior regions of the dorsal surface of the cerebral ganglion were always present. However, there were inter-species differences in the number of 5-HT-ir neurons in each cluster and some clusters even exhibited strong intra-species variability that was only weakly correlated with brain size. Phylogenetic analysis suggests that the presence of particular classes of 5-HT-ir neurons exhibits a great deal of homoplasy. The conserved features of the nudibranch serotonergic system presumably represent the shared ancestral structure, whereas the derived characters suggest substantial independent evolutionary changes in

the number and presence of serotonergic neurons. While a number of studies have demonstrated phylogenetic variability of peptidergic systems, this study suggests that serotonergic systems may also exhibit a high degree of homoplasy in some groups of organisms.

## **Introduction**

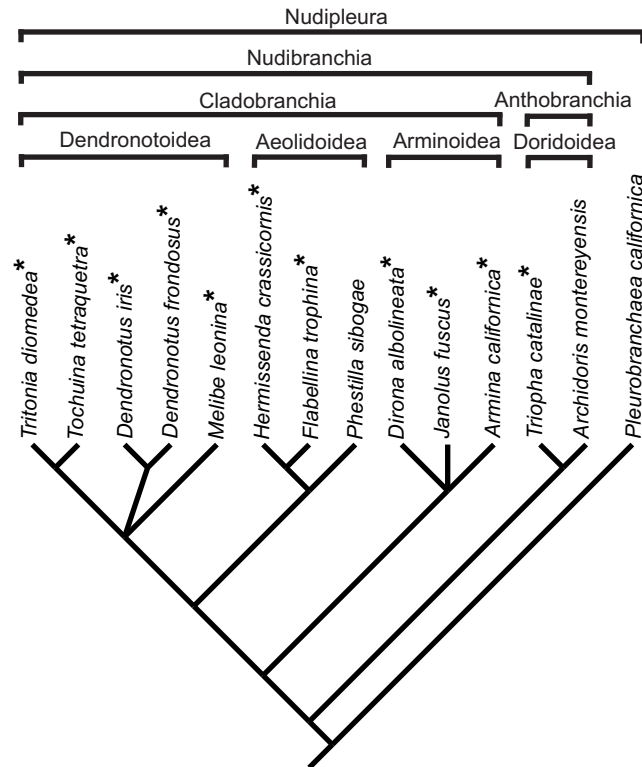
Species differences in the number and presence of neuronal cell types could underlie the evolution of neural circuits. Nudibranch molluscs have relatively simple nervous systems with large identifiable neurons and clusters of neurons, making them amenable to neural circuit analysis. Neuronal cell types can be classified by their neurotransmitter phenotype and their location within the central nervous system. This study mapped the location of serotonin-immunoreactive (5-HT-ir) neurons in the central nervous systems of representative nudibranch species to determine the extent to which the presence, size, location, and number of serotonergic neurons are correlated with the currently understood phylogeny.

Serotonin is known to play important roles in many different behaviors. Some of the key functions for 5-HT in the nervous system of nudibranchs and other opisthobranchs include locomotion (Audesirk et al., 1979; Mackey and Carew, 1983; Parsons and Pinsker, 1989; McClellan et al., 1994; Satterlie and Norekian, 1996), feeding (Palovcik et al., 1982), and learning (Farley and Wu, 1989; Barbas et al., 2003).

Serotonin has been implicated in a number of behaviors in two species of nudibranchs

that have been used extensively as neurophysiological preparations: *Tritonia diomedea* and *Hermisenda crassicornis*. Serotonergic neurons participate in the production of locomotion in *Tritonia* (Gettings et al. 1980, McClellan et al., 1994, Katz et al., 1994, Audesirk et al., 1979, Popescu and Frost, 2002). In *Hermisenda*, serotonergic modulation of photoreceptors plays an important role in associative learning (Farley and Wu, 1989, Crow, 2004). Homologous serotonergic neurons, such as the cerebral serotonergic giant neuron, which is involved in feeding behaviors in a wide variety of gastropod molluscs (Pentreath et al., 1982), have been identified in nudibranchs, including *Tritonia* (Weinreich et al., 1973; Sudlow et al., 1998; Fickbohm and Katz, 2000; Fickbohm et al., 2001) and *Hermisenda* (Land and Crow, 1985; Croll, 1987b; Auerbach et al., 1989). Therefore, it would be of interest to compare the serotonergic systems of nudibranchs to determine the extent to which they are phylogenetically conserved.

The phylogeny and systematics of nudibranchs have been determined using both morphological and molecular criteria. The order Nudibranchia is subdivided into four suborders (Dendronotoidea, Aeolidioidea, Arminoidea, and Doridoidea) that fall within two monophyletic clades: Anthobranchia and Cladobranchia (Fig. 2-1) (Odhner, 1934; Thollessen, 1999; Wägele and Willan, 2000; Wollscheid-Lengeling et al., 2001; Grande et al., 2004; Vonnemann et al., 2005). Morphological and molecular data suggest that each of these suborders is monophyletic (Grande et al., 2004; but see Wägele and Willan, 2000; and Wollscheid-Lengeling et al., 2001). The nudibranchs form a larger monophyletic clade (Nudipleura) with some members of the order Notaspidea, including



**Figure 2-1:** Phylogeny of Nudipleura. There are four suborders of nudibranchs, Dendronotoidea, Aeolidioidea, Arminoidea, and Doridoidea. These suborders form two monophyletic nudibranch clades, Cladobranchia and Anthobranchia. Only nudibranchs marked with an asterisk were used in the current study. The closest sister group to the nudibranchs are the pleurobranchs, such as *Pleurobranchaea californica*. The nudibranchs and pleurobranchs form the monophyletic clade Nudipleura. This phylogeny summarizes morphological and molecular data (Thollessen, 1999; Wägele and Willan, 2000; Wollscheid-Lengeling, et al., 2001; Grande et al., 2004; Vonnemann et al., 2005).

*Pleurobranchaea californica*, providing a closely related sister group for phylogenetic comparisons.

Published examples of 5-HT immunolabeling in adult nudibranchs are limited to four species representing three suborders: Dendronotoidea [*Tritonia diomedea* (Sudlow et al., 1998; Fickbohm and Katz, 2000; Fickbohm et al., 2001)], Aeolidioidea [*Hermisenda crassicornis* (Land and Crow, 1985; Croll, 1987b; Auerbach et al., 1989; Tian et al., 2006) and *Phestilla sibogae* (Croll et al., 2001)], and Doridoidea [*Archidoris montereyensis* (Wiens and Brownell, 1995)]. Serotonin immunohistochemistry also has been published for *Pleurobranchaea californica* (Sudlow et al., 1998), providing an out-group comparison. This study includes additional species in each of these three nudibranch suborders and three species of Arminoidea. Serotonin immunoreactivity observed from both the dorsal and ventral views are included to give a complete description of the location of 5-HT-ir neurons.

The results show that while much of the serotonergic system is conserved in all nudibranchs, there are some derived traits that appear to have evolved independently in separate lineages, i.e. homoplasy. Previous studies have indicated that serotonergic neurons tend to be relatively conserved across large phylogenetic distances (Sakharov, 1976; Weiss and Kupfermann, 1976; Pentreath et al., 1982; Barber, 1983; Croll, 1987a; Stuart et al., 1987; Katz and Tazaki, 1992; Kempf et al., 1997; Beltz, 1999; Hay-Schmidt, 2000; Katz et al., 2001; Santagata and Zimmer, 2002; Marinesco et al., 2003). Therefore, the extent of homoplasy within serotonergic localization in nudibranchs is important

because it indicates that the serotonergic system may actually be an important site of evolutionary change.

## **Methods**

### *Animal collection and maintenance*

All specimens were collected as adults. *Dendronotus iris* (10 – 20 cm in body length), *Hermisenda crassicornis* (1.5 – 4 cm), *Janolus fuscus* (5 – 8 cm in body length), *Melibe leonina* (5 – 10 cm), and *Triopha catalinae* (6 – 12 cm) were collected off of the dock at Friday Harbor Laboratories (FHL), Friday Harbor, WA. Additional *Hermisenda* were provided by Charles Hollahan (Santa Barbara, CA). *Armina californica* (5 – 12 cm), *Dirona albolineata* (5 – 8 cm), *Tochuina tetraquetra* (5 – 20 cm), *Tritonia diomedea* (5 – 20 cm), and additional *Melibe* were provided by Shaun Cain and Jim Murray (FHL), as well as Living Elements (Vancouver, British Columbia). *Dendronotus frondosus* (4 – 8 cm) was provided by Win Watson (University of New Hampshire).

Animals were either kept in seawater tables at FHL at ambient seawater temperatures and light/dark cycle, or in recirculating artificial seawater tanks at Georgia State University at 10° C and a fixed 12:12 light/dark cycle.

### *Dissection*

Animals were anesthetized by chilling and either injection of 0.33 M magnesium chloride into the body cavity or immersion in a 50:50 mixture of 0.33 M magnesium



chloride and sea water for 15 – 30 min. A cut was made in the dorsal integument above the esophagus and buccal mass. The brain, consisting of the cerebral, pedal, and pleural ganglia, was removed by cutting all nerve roots. The buccal ganglion was not included in this study. The brain was transferred to saline in a Sylgard-lined dish. Saline composition was (in mM): 420 NaCl, 10 KCl, 10 CaCl<sub>2</sub>, 50 MgCl<sub>2</sub>, 11 D-glucose, and 10 HEPES, pH. 7.6. Connective tissue surrounding the brain was manually removed with forceps, fine scissors and a tungsten needle.

#### *Whole-mount immunohistochemistry*

Some brains were entirely desheathed prior to 5-HT immunohistochemistry to enable separate electrophysiology experiments that were not part of this study. In these cases, the fine sheath immediately surrounding the neurons was removed to allow access with intracellular glass electrodes and the brains were later fixed for 5-HT immunohistochemistry (as indicated below). Other brains were subjected to protease treatment (0.5% Type IX Protease, Type XIV Protease, or Subtilisin [Sigma]), for 10 – 15 min at room temperature, to permeabilize the sheath and allow penetration of histochemical substances. Desheathing may result in an underestimation of the number of 5-HT-ir cells because some cell bodies might be destroyed during tissue processing. The number of preparations in each species that were not manually desheathed (i.e. had intact sheaths) is noted in Table 2-1.

Brains were fixed 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) (McLean and Nakane, 1974). After fixation, ganglia were washed twice (30 min each) with cacodylate buffer and then overnight with 4% Triton X-100 in phosphate buffered saline (PBS, 50 mM Na<sub>2</sub>HPO<sub>4</sub> in 140 mM NaCl, pH 7.2). The tissue was incubated for 1 hr in antiserum diluent (ASD, 0.5% Triton X-100, 1% normal goat serum and 1% bovine serum albumen in PBS) followed by 48 – 96 hr in primary antiserum (rabbit polyclonal anti-5-HT, #20080, ImmunoStar, Inc., Hudson, WI) diluted 1:1000 in ASD. This rabbit antibody was raised against serotonin coupled to bovine serum albumin with paraformaldehyde. Specificity of the antiserum has been tested with preadsorption controls and it does not cross-react with closely related amines such as 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, and dopamine (manufacturer's technical information). After four washes (1 hr each) with 0.5% Triton X-100 in PBS, ganglia were incubated overnight in goat anti-rabbit antiserum conjugated to Alexa 488 (Molecular Probes, Eugene, OR) diluted 1:50 or 1:100 in ASD. After this step, ganglia were washed four times (1 hr each) with PBS, dehydrated in an ethanol series, cleared in methyl salicylate, and mounted on a depression slide with Cytoseal 60 (Electron Microscopy Sciences, Washington, PA). The ganglia were kept at 4° C for the entire immunohistochemistry protocol (except protease treatment) and all of the steps between fixation and dehydration were done with gentle agitation on a shaker. The shape and size of brains remained relatively constant throughout the immunohistochemistry process.

### *Immunohistochemistry controls*

Previous studies with the same antibody had already confirmed its specificity for 5-HT in nudibranchs via preadsorption controls, as well as a lack of autofluorescence in these animals in our emission range (Fickbohm et al., 2001). In this study, we obtained further confirmation of this by the fact that 5-HT-ir in *Tritonia* was the same as previously indicated with this antibody (Fickbohm et al., 2001). Additional controls for autofluorescence and secondary specificity were done in this study by omitting the primary antibody: *Tritonia* (n = 1), *Tochuina* (n = 1), *Melibe* (n = 3), *Hermisenda* (n = 1), and *Dirona* (n = 1). In all of these control preparations, there was no fluorescence on either the dorsal or ventral sides of the brain (data not shown).

### *Imaging*

Fluorescence images were visualized on an Axiovert 100M microscope (Carl Zeiss, Inc., Thornwood, NY) using confocal microscopy (LSM 510, Carl Zeiss, Inc.) with a 10X- or 20X-objective. Fluorophores were excited with a laser (488 nm) and fluorescent emissions were passed through a band-pass filter (505 – 550 nm) for visualization of Alexa 488. LSM 510 software was used to acquire images. Brains were imaged twice, once from the dorsal surface and once from the ventral surface. Confocal stacks consisted of 12 – 80 optical sections, depending on the thickness of tissue, and included the entire dorsal/ventral span of the brain. The thickness of each optical section, which was optimized and kept consistent within a preparation, ranged from 2 – 14  $\mu\text{m}$ . To compile images of 5-HT immunoreactivity, the first half of a confocal stack was

merged into a single maximal projection and exported as a TIFF file. This provided separate images of immunoreactivity from the dorsal and ventral surfaces that lacked bleed-through from the other side. These projections were then imported into Adobe Photoshop CS (Adobe, San Jose, CA) and assembled into a montage of the entire brain from both dorsal and ventral perspectives. Masking methods were used to minimize visibility of borders between images in the montages (Beck et al., 2000). The montages were then converted to grayscale, color was inverted such that labeled neurons appeared dark, and overall brightness and contrast were adjusted. The ventral image was flipped left to right so that it appears to be viewed from the dorsal surface. Thus, the left half of the brain is on the left side of both the dorsal and ventral images. All images are oriented such that anterior is at the top.

#### *Data analysis*

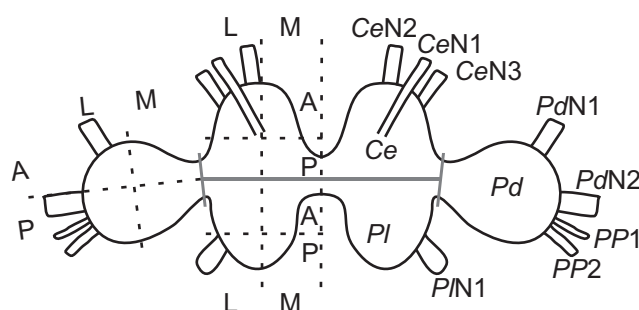
Serotonin-ir neurons that were grouped close together and delineated from other 5-HT-ir neurons by a clear space that lacked 5-HT immunoreactivity were considered a cluster. For each preparation, the number of neurons was counted in each 5-HT-ir cluster. Cell counts were made from the original slides, not the confocal images. The data were not always normally distributed, so the median and interquartile range were determined to be the best measure of central tendency regarding cell counts for individual clusters. SPSS 10.0 for Windows was used to calculate the median and interquartile range. Mean and standard error were used for any other analyses of data. The diameter of the soma was used as a measure of size.

Brain size was calculated as visible surface area, as viewed from the dorsal perspective in Image J (<http://rsb.info.nih.gov/ij/>). The ganglia of nudibranchs are roughly equivalent to flattened ellipsoids with the neuronal somata located on the surface. Linear regressions of brain size vs. number of 5-HT-ir neurons were calculated in SigmaPlot (Systat, Point Richmond, CA).

MacClade 4.08 (Maddison and Maddison, 2005) was used for phylogenetic analysis. Characteristics of 5-HT immunoreactivity that were uniform, or differed for only a single species, were excluded from the analysis because they lacked information for phylogenetic resolution. In addition, characteristics that could not be categorized into discrete variables were excluded from analysis. This left sixteen characteristics identified as informative characters (see Table 2-5 for list of characters used for analysis). Consistency index, retention index, and rescaled consistency index were calculated for each character (scale = 0 to 1, with 1 indicating minimum homoplasy [independent evolution of a similar character]; Farris, 1989). These same statistics, along with tree length, were determined for the entire tree with all sixteen characters. The maximum parsimony method, with random resolution of polytomies, was used for constructing the most parsimonious tree with all sixteen characters.

### *Naming conventions*

A generalized map of a nudibranch brain contains three bilaterally represented ganglia (cerebral, pleural and pedal) that were each further subdivided into anterior-posterior and lateral-medial quadrants (Fig. 2-2). The quadrants are applied to both dorsal



**Figure 2-2:** Brain anatomy and nomenclature. Outline of a stereotypical nudibranch brain, as viewed from the dorsal perspective, with anterior at the top. There are three, bilaterally symmetric ganglia (delineated by gray lines): cerebral (Ce), pleural (Pl) and pedal (Pd). For the purpose of specifying the location of 5-HT-ir neurons/clusters, each ganglion is divided into anterior (A)/posterior (P) and lateral (L)/medial (M) halves, as indicated. Nerves (N) and pedal-pedal connectives (PP) present in all species are also illustrated, with a corresponding name. These names were used for any species without prior publication of nerve nomenclature.

and ventral aspects. The locations of nerves were typically more consistent among species than other anatomical characters. Therefore, large nerves that were present in most species were used as landmarks and provided with names on the generalized map (Fig. 2-2). Names of nerves followed the convention of Willows and colleagues (1973); nerves were numbered in order from medial to lateral and anterior to posterior for each ganglion (Fig. 2-2). The neuroanatomy of four of the nine species in this study had previously been described to some extent (*Tritonia* [Willows et al., 1973], *Melibe* [Hurst, 1968; Newcomb and Watson, 2001], *Hermisenda* [Jerussi and Alkon, 1981], and *Armina* [Dorsett, 1978]), and the original names for specific nerves and neurons are noted in these cases. The names of nerves are not meant to connote homology; they merely indicate the position of that nerve relative to the ganglion.

Naming of 5-HT-ir clusters (> 1 neuron) and individual neurons was based on location and also does not imply homology between species. Our naming convention for newly described 5-HT-ir clusters/neurons consisted of the side of the brain (d = dorsal, v = ventral), the ganglion where the soma was located (*Ce* = cerebral, *Pd* = pedal, *Pl* = pleural), the neurotransmitter (S = serotonin), and location within the ganglion (A = anterior, P = posterior, L = lateral, M = medial). Many clusters spanned more than one quadrant of a ganglion. Therefore, we used the one descriptor (A, P, L, or M) that could be used to clearly distinguish a cluster/neuron from all other clusters/neurons in the same ganglion. For example, the most anterior cluster of 5-HT-ir neurons on the dorsal surface of the cerebral ganglion was referred to as the dorsal cerebral serotonergic anterior

(dCeSA) cluster. Most clusters/neurons were bilaterally represented in the brain but instances with asymmetry were noted by using "left" or "right" preceding the name.

## **Results**

This study compared the number and locations of 5-HT-ir neurons in the central nervous system (CNS) of nudibranchs. As a first approximation, we counted the total number of 5-HT-ir neurons in each species (Table 2-1). There was a large range in the total number of 5-HT-ir neurons across species, from  $126 \pm 29$  (median  $\pm$  interquartile range) in *Dendronotus frondosus* to  $624 \pm 179$  in *Tochuina*. Despite the almost 5-fold difference in the total number of 5-HT-ir neurons, similarities in the organization of the 5-HT-ir neurons were observed and some clear homologies could be established.

Previous studies had indicated that the majority of the serotonergic neurons in the CNS are located in the pedal ganglia in nudibranchs (Land and Crow, 1985; Croll, 1987b; Wiens and Brownell, 1995; Sudlow et al., 1998; Fickbohm and Katz, 2000; Croll et al., 2001; Fickbohm et al., 2001), as well as other, more distantly-related gastropods (Ono and McCaman, 1984; Croll and Lo, 1986; Longley and Longley, 1986; Croll, 1988; Hernádi et al., 1989; Satterlie et al., 1995; Sudlow et al., 1998; Shirahata et al., 2004) and even bivalves (Croll et al., 1995) and chitons (Moroz et al., 1994). We also found that the majority ( $76.3 \pm 0.7$  %) of 5-HT-ir neurons in the CNS were located in the pedal ganglion in all eleven species. Most of the remaining 5-HT-ir neurons were located in the cerebral ganglia ( $23.1 \pm 0.7$  %). There were 5-HT-ir neurons in the pleural ganglia of eight species,



**Table 2-1:** Median cell counts ( $\pm$  interquartile range) for serotonin-immunoreactive (5-HT-IR) clusters

Ganglion	5-HT Cluster <sup>1</sup>	Dendronotoidea					Aeolidioidea		Arminoidea		Doridoidea	
		<i>Tritonia diomedea</i> (11 / 11) <sup>2</sup>	<i>Tochuina tetraquetra</i> (2 / 6)	<i>Dendronotus iris</i> (0 / 10)	<i>Dendronotus frondosus</i> (0 / 8)	<i>Melibe leonina</i> (4 / 31)	<i>Hermisenda crassicornis</i> (5 / 7)	<i>Flabellina trophina</i> (10/10)	<i>Dirona albolineata</i> (10 / 10)	<i>Janolus fuscus</i> (2 / 7)	<i>Armina californica</i> (5 / 11)	<i>Triopha catalinae</i> (0 / 10)
Cerebral	dCeSA	2 ± 0.5	6 ± 3	13 ± 1	9 ± 4	15 ± 3	4 ± 1.5	2 ± 0.5	9 ± 15 <sup>4</sup>	4 ± 1	3 ± 0.5	2 ± 3
	dCeSP	5 ± 0.3	6 ± 4	5 ± 0.4	5 ± 1	5 ± 0.6	5 ± 0.6	5 ± 0	5 ± 0	5 ± 0.5	11 ± 2	5 ± 0.5
	dCeSL	-	24 ± 7	-	-	-	-	-	-	-	-	11 ± 2
	vCeSA	21 ± 5	35 ± 20	-	3 ± 3	-	4 ± 1	14 ± 2	22 ± 9 <sup>5</sup>	18 ± 3	-	23 ± 9
	vCeSP	-	-	-	-	-	-	-	6 ± 1	-	4 ± 2	-
	vCeSL	-	-	-	-	-	-	-	4 ± 3 <sup>6</sup>	-	-	-
Pedal	dPdSA	-	-	15 ± 6	-	32 ± 8	-	-	-	4 ± 3	26 ± 5	8 ± 4
	dPdSP	-	70 ± 24	-	39 ± 9	-	-	-	20 ± 6	22 ± 9	18 ± 4	8 ± 10
	dPdSL	33 ± 9	-	45 ± 12	-	-	30 ± 8	35 ± 9	46 ± 8	-	-	-
	vPdSA	42 ± 15	128 ± 54	18 ± 5	- <sup>3</sup>	9 ± 2	24 ± 9	36 ± 11	53 ± 14	11 ± 9	22 ± 4	-
	vPdSP	41 ± 8	42 ± 24	1 ± 1	6 ± 4	-	8 ± 3	23 ± 4	17 ± 3	22 ± 5	7 ± 4	-
	vPdSL	-	-	38 ± 7	-	27 ± 5	-	-	-	-	-	38 ± 9
Pleural	vPdSM	-	-	-	-	-	-	-	10 ± 11 <sup>7</sup>	-	-	25 ± 4
	dP/SL	-	-	-	-	-	-	2 ± 0.5	-	-	-	- <sup>8</sup>
Median Total in CNS	left vP/ISM	-	-	2 ± 1	2 ± 0.4	-	-	-	-	2 ± 0.5	-	-
	left vP/SL	-	-	-	-	-	-	-	3 ± 0	-	-	-
Median Total in CNS		281 ± 53	624 ± 179	272 ± 44	126 ± 29	174 ± 22	152 ± 18	235 ± 38	445 ± 58	178 ± 31	185 ± 15	261 ± 81

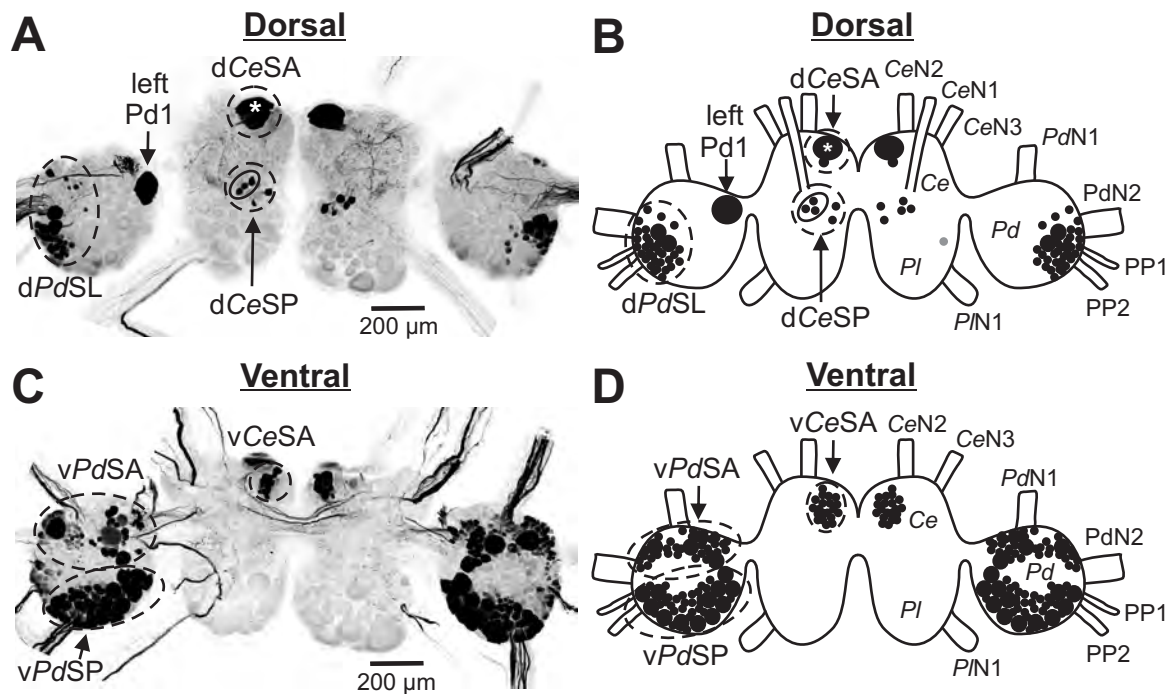
<sup>1</sup>CNS, central nervous system; d, dorsal; v, ventral<sup>2</sup>Number of preparations with intact sheaths / total number of preparations<sup>3</sup>In 2 of 8 preps, there were ~20 particularly small (< 10  $\mu$ m) 5-HT-IR neurons in this area.<sup>4</sup>The left dCe SA cluster had 3  $\pm$  0.1 neurons and the right dCe SA cluster had 31  $\pm$  14 neurons.<sup>5</sup>The left vCe SA cluster had 15  $\pm$  3 neurons and the right vCe SA cluster had 32  $\pm$  7 neurons.<sup>6</sup>The left vCe SL cluster had 1  $\pm$  2 neurons and the right vCe SL cluster had 8  $\pm$  4 neurons.<sup>7</sup>The left vPd SM cluster had 5  $\pm$  3 neurons and the right vPd SM cluster had 24  $\pm$  10 neurons.<sup>8</sup>In 3 of 10 preps, there were two 5-HT-IR neurons in this area.

where they constituted only  $0.7 \pm 0.1$  % of the total number of 5-HT-ir neurons in the brain. Thus, the overall pattern of serotonin localization was similar between species, and was largely centered in the pedal and cerebral ganglia (Fig. 2-3 - 2-13).

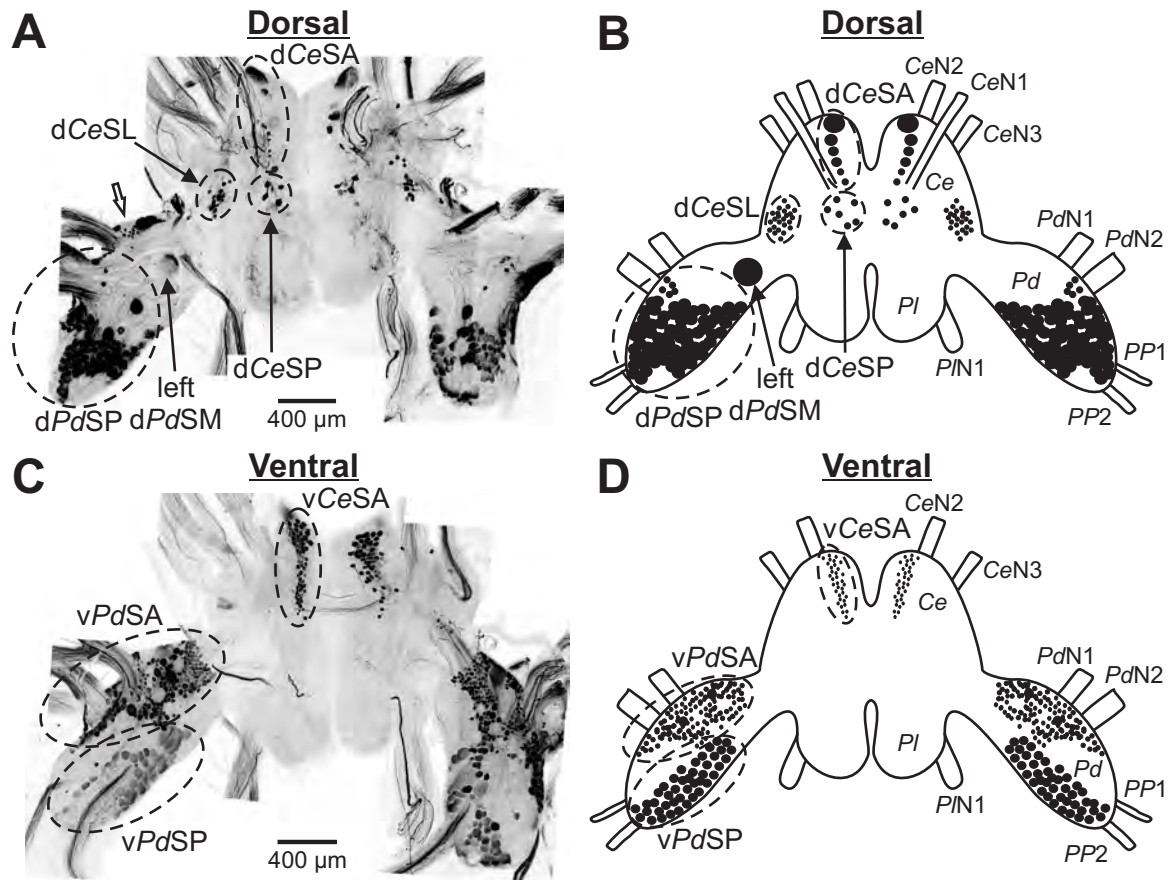
### *Serotonin in the cerebral ganglion*

The pattern of 5-HT immunoreactivity in the cerebral ganglion was the most consistent between species. Serotonin-ir neurons were present in at least two distinct clusters in the cerebral ganglion in all of the species: a posterior cluster on the dorsal surface (dCeSP) and an anterior cluster (dCeSA and vCeSA) (Figs. 2-3 – 2-13, Table 2-1). The consistent presence of these clusters suggests that they are homologous across species. Additional cerebral ganglion 5-HT-ir clusters were present in *Tochuina* (dCeSL, Fig. 2-4A & B), *Dirona* (vCeSP and vCeSL, Fig. 2-10C & D), *Armina* (vCeSP, Fig. 2-12C & D), and *Triopha* (dCeSL, Fig. 2-13A & B). It is not clear if these additional clusters have homologues in other species.

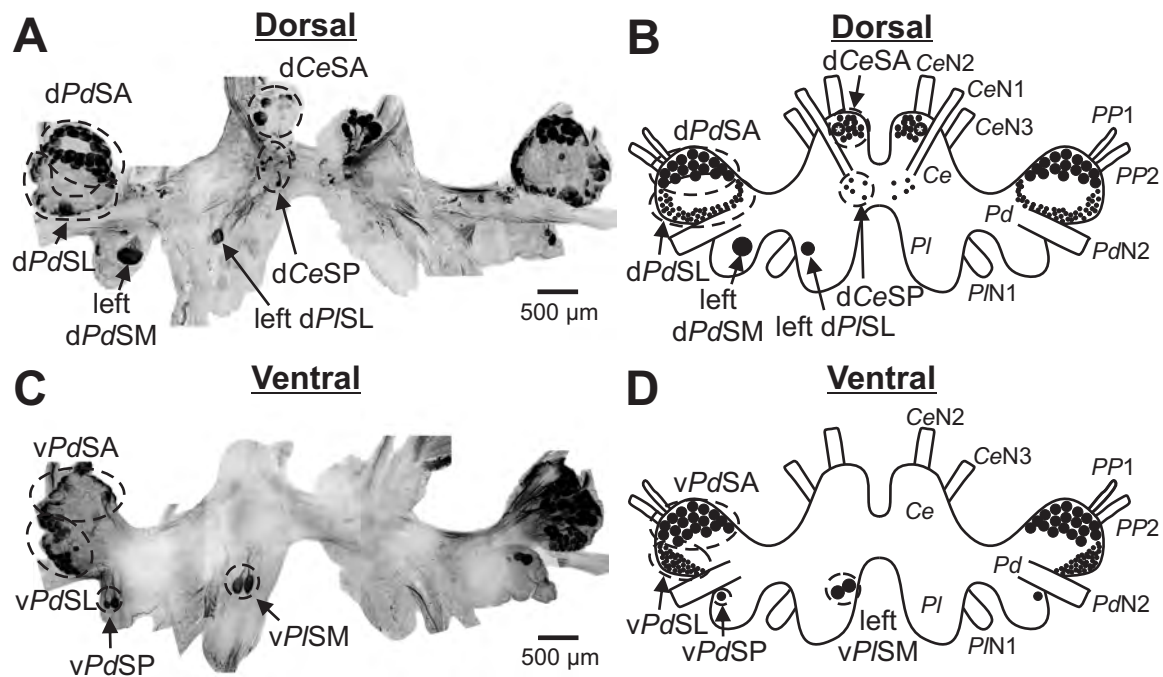
Previous studies indicate that disparate opisthobranchs have a small group of five serotonergic neurons in the posterior region of the cerebral ganglion (Land and Crow, 1985; Croll, 1987b; Auerbach et al., 1989; Wiens and Brownell, 1995; Katz et al., 2001; Tian et al., 2006). As indicated above, we found a similar group of 5-HT-ir neurons, the dCeSP cluster, which was present in all of the species in our study. Generally, the number of neurons in the dCeSP cluster was very consistent: in nine of the eleven species, the median number of dCeSP neurons was five. However, two species, *Tochuina* and *Armina*,



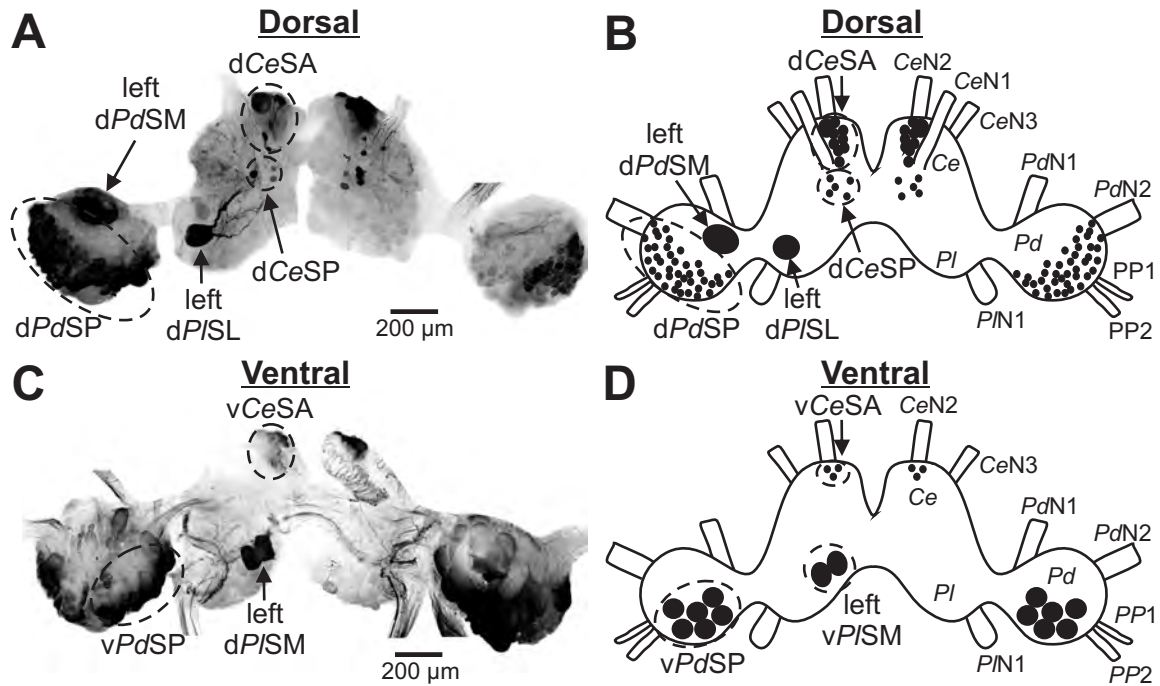
**Figure 2-3:** Serotonin (5-HT) immunoreactivity in *Tritonia diomedea*, illustrated with confocal images of the dorsal (A) and ventral (C) surfaces of a single brain and drawings indicating the median 5-HT localization from eleven brains (B & D). The cerebral ganglion had two clusters of 5-HT-immunoreactive (5-HT-ir) neurons on the dorsal surface, the dCeSA and dCeSP clusters, and one on the ventral surface, the vCeSA cluster. The dCeSA cluster contained the previously identified cerebral neuron 1 (C1, indicated by an asterisk; Willows et al., 1973; Fickbohm et al., 2001). The dCeSP cluster contained the previously identified dorsal swim interneurons (solid circle; Getting et al., 1980; Katz et al., 1994; McClellan et al., 1994). The pedal ganglion had a single group of 5-HT-ir neurons on the dorsal surface, the dPdSL cluster, and two groups on the ventral surface, the vPdSA and vPdSP clusters. There was also a single, asymmetric 5-HT-ir neuron on the dorsal surface of the left pedal ganglion, the previously identified left pedal 1 neuron (left Pd1; Weinreich et al., 1973; Willows et al., 1973; Audesirk et al., 1979; Sudlow et al., 1998). The right pleural ganglion occasionally had a small 5-HT-ir neuron (gray circle) on the lateral edge of the dorsal surface. Nerve nomenclature follows our convention described in Figure 2 for ease in comparing to other species. Previous nomenclature (Willows et al., 1973) differs for PdN2 (originally PdN3), PP1 (PdN5), and PP2 (PdN6).



**Figure 2-4:** Serotonin immunoreactivity in *Tochuina tetraquetra*, illustrated with confocal images of the dorsal (A) and ventral (C) surfaces of a single brain and a drawing of median 5-HT immunoreactivity from six preparations (B & D). The cerebral ganglion contained three 5-HT-ir groups of neurons on the dorsal surface, the dCeSA, dCeSP and dCeSL clusters, and single group of 5-HT-ir neurons on the ventral surface, the vCeSA cluster. There was a single 5-HT-ir group of neurons on the dorsal surface of the pedal ganglion, the dPdSP cluster, and two groups of 5-HT-ir neurons on the ventral surface, the vPdSA and vPdSP clusters. The pedal ganglion also exhibited an asymmetric 5-HT-ir neuron on the dorsal surface, the left dPdSM neuron. In the example illustrated in (A), there were also a few 5-HT-ir neurons along the anterior margin of the pedal ganglion medial to pedal nerve 1 (PdN1; white arrow), although these neurons were not seen in other preparations.

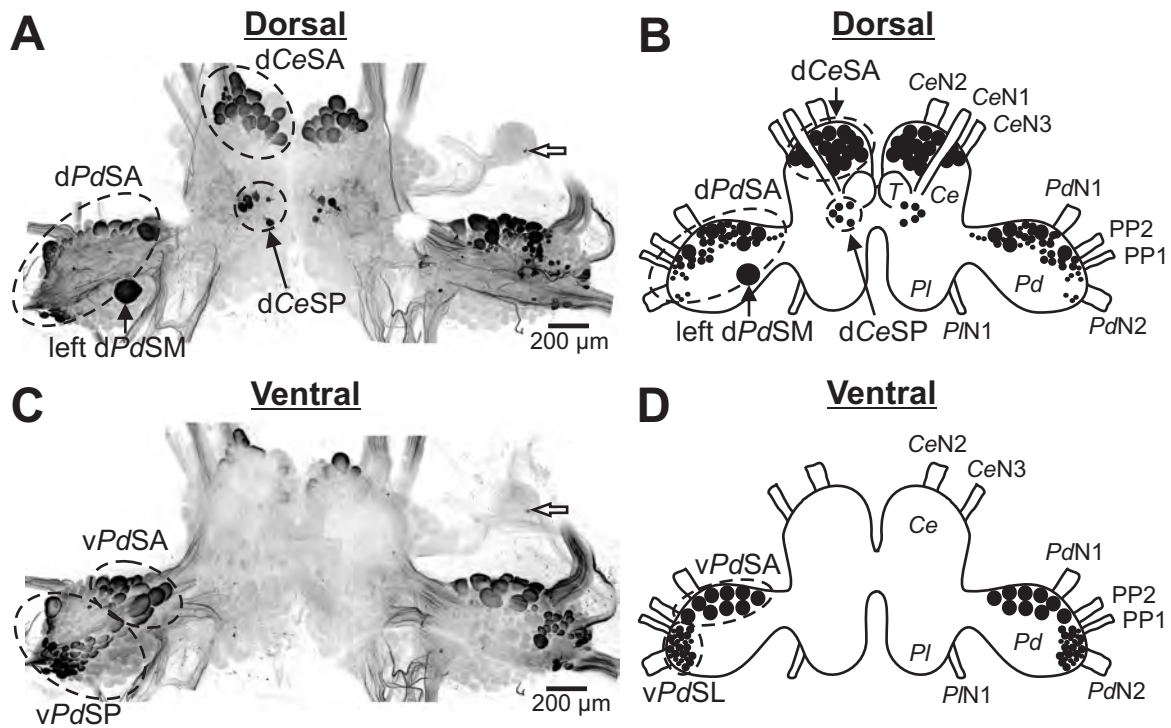


**Figure 2-5:** Serotonin immunoreactivity in *Dendronotus iris*, illustrated by confocal images of the dorsal (A) and ventral (C) surfaces of a single brain, and drawings of median 5-HT localization in ten preparations (B & D). The cerebral ganglion had two groups of 5-HT-ir neurons on the dorsal surface, the dCeSA and dCeSP clusters. There were two groups of 5-HT-ir neurons on the dorsal surface of the pedal ganglion, the dPdSA and dPdSL clusters, and three groups on the ventral surface, the vPdSA, vPdSL and vPdSP clusters. There was also an asymmetric 5-HT-ir neuron on the dorsal surface of the left pedal ganglion, the dPdSM neuron. The left pleural ganglion contained an asymmetric 5-HT-ir neuron on the dorsal surface, the left dP/SL neuron, and an asymmetric group of 5-HT-ir neurons on the ventral surface, the vP/ISM cluster.

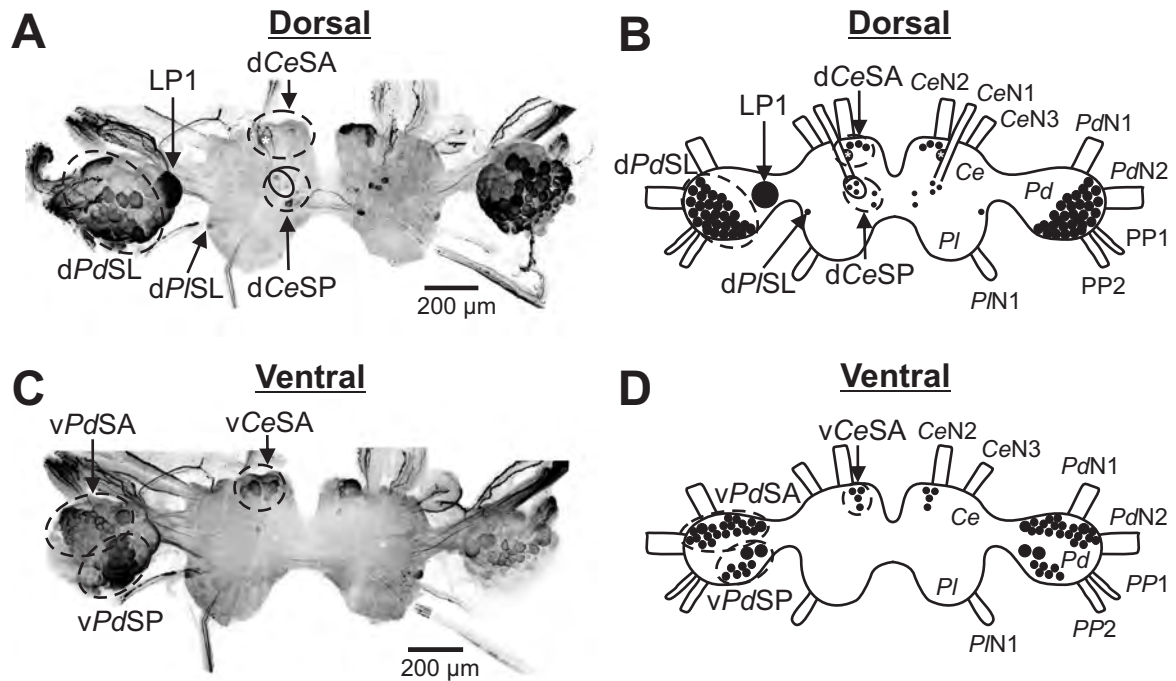


**Figure 2-6:** Serotonin immunoreactivity in *Dendronotus frondosus*, illustrated by confocal images of the dorsal (A) and ventral (C) surfaces of a single brain and drawings of median 5-HT-immunoreactivity in eight preparations (B & D). The cerebral ganglion contained two 5-HT-ir groups of neurons on the dorsal surface, the dCeSA and dCeSP clusters, and a single group on the ventral surface, the vCeSA cluster. The pedal ganglion contained a PdSP cluster of 5-HT-ir neurons on both the dorsal and ventral surfaces. There was also an asymmetric 5-HT-ir neuron on the dorsal surface of the left pedal ganglion, the left dPdSM neuron. The pleural ganglion contained an asymmetric 5-HT-ir neuron on the dorsal surface, the dPISL neuron, and an asymmetric group on the ventral surface, the vPISM cluster.



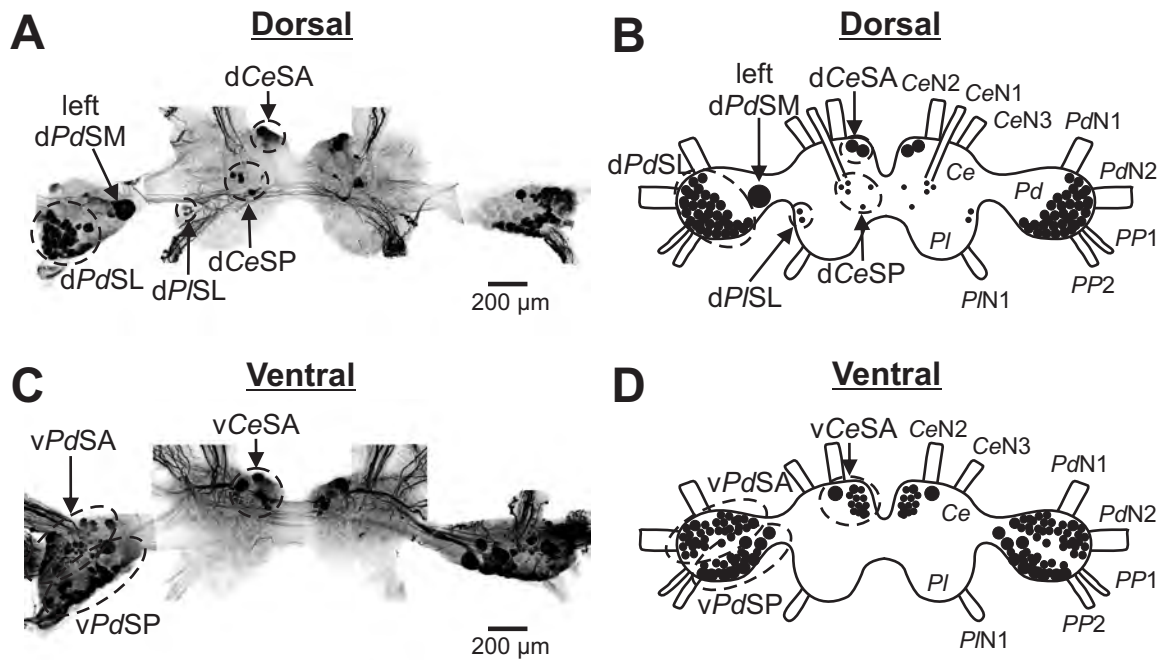


**Figure 2-7:** Serotonin immunoreactivity in *Melibe leonina*, illustrated in confocal images of the dorsal (A) and ventral (C) surfaces of a single brain, and drawings of median 5-HT localization from 31 preparations (B & D). There were two groups of 5-HT-ir neurons on the dorsal surface of the cerebral ganglion, the dCeSA and dCeSP clusters. The pedal ganglion exhibited 5-HT immunoreactivity in a single group of neurons on the dorsal surface, the dPdSA cluster, and two groups on the ventral surface, the vPdSA and vPdSL clusters. There was also an asymmetric 5-HT-ir neuron on the dorsal surface of the left pedal ganglion, the left dPdSM neuron. In (A) and (C), a single 5-HT-ir neuron can also be seen in the right buccal ganglion (white arrow). Nerve nomenclature follows our convention described in Figure 2 for ease in comparison to other species. Previous nomenclature (Hurst, 1968; Newcomb and Watson, 2001) differs for CeN1 (originally C4), CeN2 (C2), CeN3, (C3), PdN1 (PD5), PdN2 (PD4), PP1 (PPC), PP2 (PC), and P/N1 (P1). Abbreviation: T = tentacular lobe.

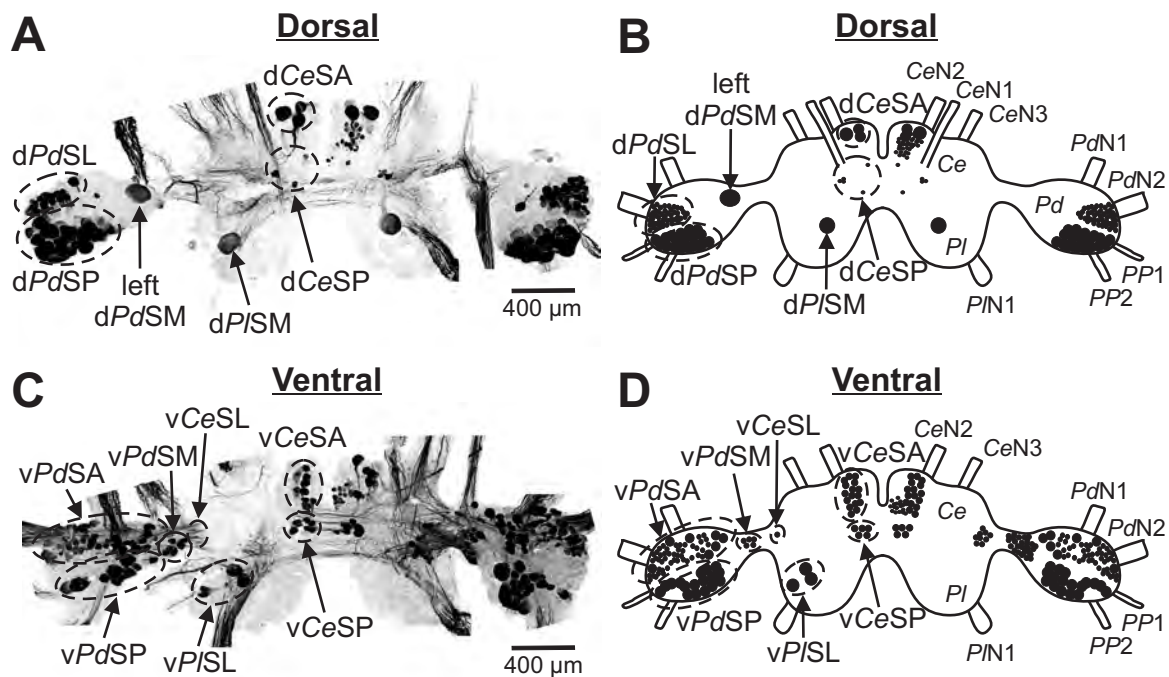


**Figure 2-8:** Serotonin immunoreactivity in *Hermisenda crassicornis*, illustrated by confocal images of the dorsal (A) and ventral (C) surfaces of a single brain, and drawings of median 5-HT localization from seven preparations (B & D). The cerebral ganglion contained two 5-HT-ir groups of neurons on the dorsal surface, the dCeSA and dCeSP clusters, and a single group on the ventral surface, the vCeSA cluster. The dCeSA cluster contained one neuron (asterisk) that was larger than other neurons in the cluster and is the previously identified metacerebral giant cell (MCG; Croll, 1987a). The three lateral neurons in the dCeSP cluster are the recently identified cerebropleural ganglion triplet (CPT) interneurons (solid circle; Tian et al., 2006). The pedal ganglion contained a single 5-HT-ir group of neurons on the dorsal surface, the dPdSL cluster, and two groups on the ventral surface, the vPdSA and vPdSP clusters. There was also an asymmetric 5-HT-ir neuron on the dorsal surface of the left pedal ganglion, the previously identified left pedal neuron 1 (LP1; Jerussi and Alkon, 1981; Land and Crow, 1985; Croll, 1987a). The dorsal surface of the pleural ganglion contained a single, bilaterally represented 5-HT-ir neuron, the dPISL neuron. Nerve nomenclature follows our convention described in Figure 2 for ease of comparison to other species. Previous nomenclature (Jerussi and Alkon, 1981) differs for PdN1 (originally "2"), PdN2 ("1"), and PIN1 ("4").

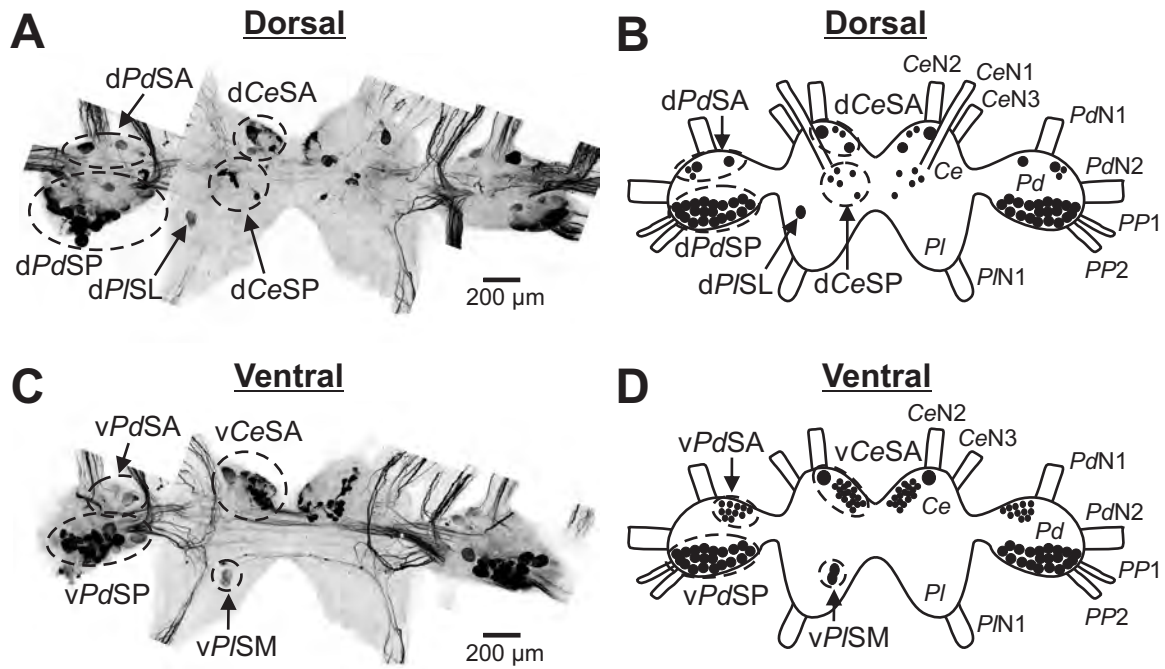




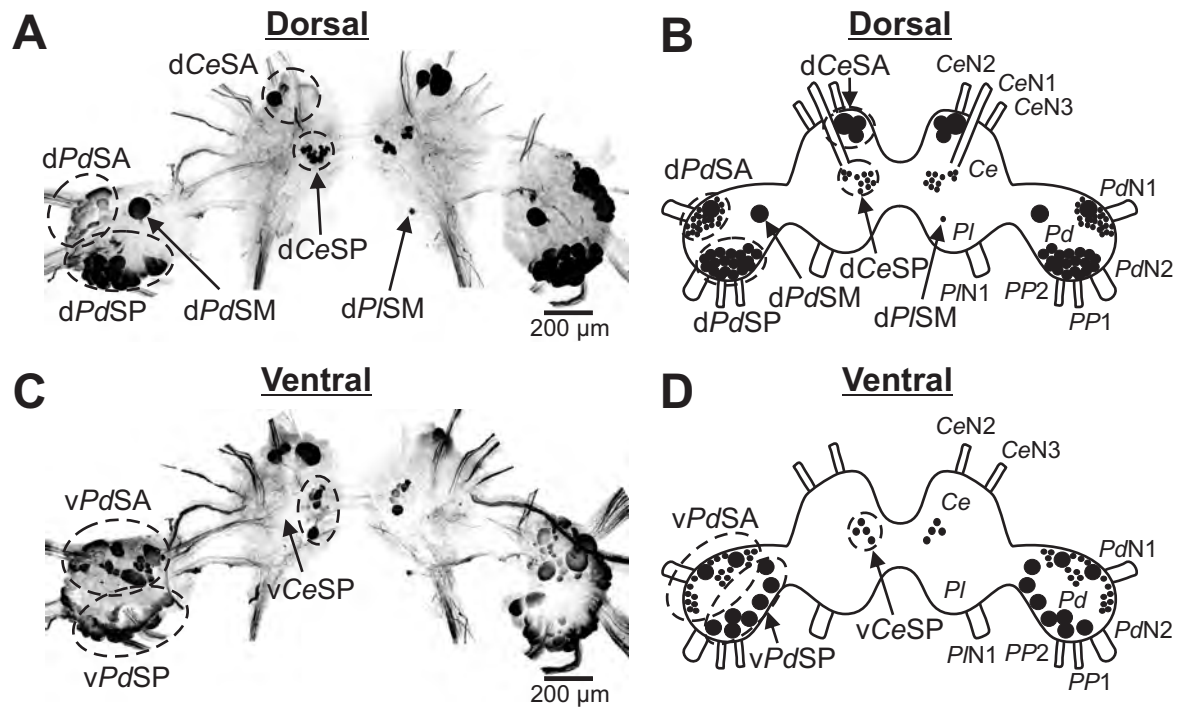
**Figure 2-9:** Serotonin immunoreactivity in *Flabellina trophina*, illustrated by confocal images of the dorsal (A) and ventral (C) surfaces of a single brain, and drawings of median 5-HT localization from ten preparations (B & D). The cerebral ganglion contained two 5-HT-ir groups of neurons on the dorsal surface, the dCeSA and dCeSP clusters, and a single group on the ventral surface, the vCeSA cluster. The pedal ganglion contained a single 5-HT-ir group of neurons on the dorsal surface, the dPdSL cluster, and two groups on the ventral surface, the vPdSA and vPdSP clusters. There was also an asymmetric 5-HT-ir neuron on the dorsal surface of the left pedal ganglion, the left dPdSM neuron. The dorsal surface of the pleural ganglion contained a single, bilaterally represented group of 5-HT-ir neurons, the dP/SL cluster.



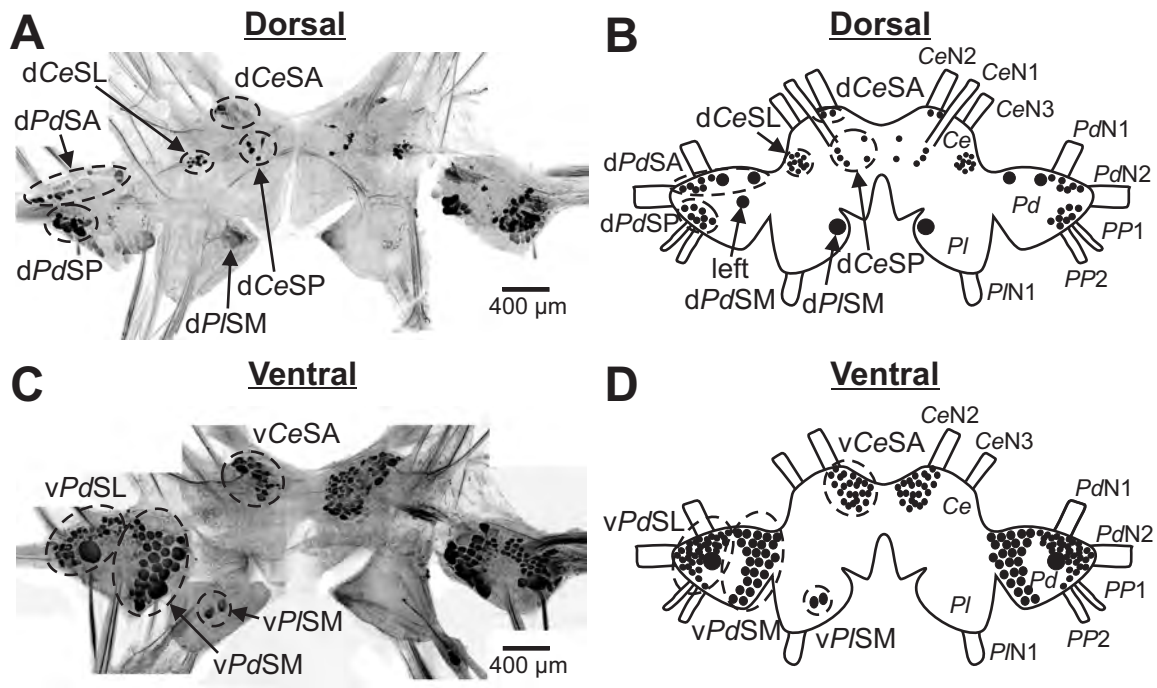
**Figure 2-10:** Serotonin immunoreactivity in *Dirona albolineata*, illustrated by confocal images of the dorsal (A) and ventral (C) surfaces of a single brain, and drawings of the median 5-HT localization from ten preparations (B & D). The cerebral ganglion contained two 5-HT-ir groups of neurons on the dorsal surface, the dCeSA and dCeSP clusters, and two groups on the ventral surface, the vCeSA and vCeSP clusters. The pedal ganglion contained two 5-HT-ir groups of neurons on the dorsal surface, the dPdSL and dPdSP clusters, and three groups on the ventral surface, the vPdSA, vPdSP and vPdSM clusters. There was also an asymmetric 5-HT-ir neuron on the dorsal surface of the left pedal ganglion, the left dPdSM neuron. The pleural ganglion contained a bilaterally represented 5-HT-ir neuron on the dorsal surface, the dPISM neuron, and an asymmetric 5-HT-ir group of neurons on the ventral surface, the vPISL cluster. The dCeSA, vCeSA, vCeSL, and vPdSM clusters on the right side of the brain contained more neurons than their contralateral counterparts.



**Figure 2-11:** Serotonin immunoreactivity in *Janolus fuscus*, illustrated by confocal images of the dorsal (A) and ventral (C) surfaces of a single brain, and drawings of median 5-HT localization from seven preparations (B & D). There were two groups of 5-HT-ir neurons on the dorsal surface of the cerebral ganglion, the dCeSA and dCeSP clusters, and a single group on the ventral surface, the vCeSA cluster. The pedal ganglion contained two groups of 5-HT-ir neurons, the PdSA and PdSP clusters, on both the dorsal and ventral surfaces. The left pleural ganglion contained a single asymmetric 5-HT-ir neuron on the dorsal surface, the left dPISL neuron, and a single asymmetric group of 5-HT-ir neurons on the ventral surface, the vPISM cluster.



**Figure 2-12:** Serotonin immunoreactivity in *Armina californica*, illustrated by confocal images of the dorsal (A) and ventral (C) surfaces of a single brain, and drawings of median 5-HT localization from eleven preparations (B & D). The cerebral ganglion contained two groups of 5-HT-ir neurons on the dorsal surface, the dCeSA and dCeSP clusters, and a single group on the ventral surface, the vCeSP cluster. The pedal ganglion contained two groups of 5-HT-ir neurons, the PdSA and PdSP clusters, on both the dorsal and ventral surfaces. There was also a bilaterally represented, 5-HT-ir neuron on the dorsal surface of the pedal ganglion, the dPdSM neuron. The pleural ganglion contained a single, asymmetric 5-HT-ir neuron on the dorsal surface of the right pedal ganglion, the dPISM neuron [not seen in (A)]. Nerve nomenclature follows our convention described in Figure 2 for ease in comparing to other species. Previous nomenclature (Dorsett, 1978) differs for the three cerebral nerves: CeN1 (originally CN1), CeN2 (CN2), and CeN3 (CN3).



**Figure 2-13:** Serotonin immunoreactivity in *Triopha catalinae*, illustrated by confocal images of the dorsal (A) and ventral (C) surfaces of a single brain, and drawings of median 5-HT localization from ten preparations (B & D). The cerebral ganglion contained three groups of 5-HT-ir neurons on the dorsal surface, the dCeSA, dCeSP and dCeSL clusters, and a single group on the ventral surface, the vCeSA cluster. The pedal ganglion contained two groups of 5-HT-ir neurons on the dorsal surface, the dPdSA and dPdSP clusters, and two groups on the ventral surface, the vPdSM and vPdSL clusters. There was also a single asymmetric, 5-HT-ir neuron on the dorsal surface of the left pedal ganglion, the left dPdSM neuron. The left dPdSM neuron was not present in the brain illustrated in (A) but was present in six of the ten preparations and is therefore shown in (B) in its appropriate location. The pleural ganglion contained a bilaterally represented, 5-HT-ir neuron on the dorsal surface, the dPISM neuron, and an asymmetric 5-HT-ir group of neurons on the ventral surface of the left pleural ganglion, the left vPISM cluster.

contained a larger number of dCeSP neurons (median of six and eleven, respectively [Fig. 2-4A & B and 2-12A & B, Table 2-1]).

With the dCeSP cluster, a subset of neurons could be recognized: three of the five neurons tended to be located more laterally, near the root of cerebral nerve 1. This was the only 5-HT-ir cluster in which we were able to recognize a consistent substructure. In *Tritonia*, the three lateral neurons are the previously identified serotonergic Dorsal Swim Interneurons (DSIs, Fig. 2-3A & B) (Getting et al., 1980; Katz et al., 1994; McClellan et al., 1994). These three lateral neurons have also previously been identified in *Hermisenda*, as the cerebropleural ganglion triplets (CPT, Fig. 2-8A & B) (Tian et al., 2006). The remaining two more medial dCeSP neurons were within a few cell diameters of the lateral dCeSP neurons in the dendronotids (see Figs. 2-3 – 2-7), while in the other suborders, there could be a considerable distance separating the medial and lateral dCeSP neurons (see Figs. 2-8 – 2-13). The consistency of this anatomy suggests that the lateral dCeSP neurons are homologous across species and distinguishable from the medial dCeSP neurons.

In the anterior region of the cerebral ganglion, 5-HT-ir neurons were present on the dorsal surface of all species (dCeSA, Figs. 2-3 – 2-13, Table 2-1) and on the ventral surface in eight of the species (vCeSA, Figs. 2-3, 2-4, 2-6, 2-8 – 2-11, 2-13, & Table 2-1). Although the vCeSA neurons were often smaller than the dCeSA neurons within the same brain (Table 2-2), the two clusters typically converged at the anterior tip of the cerebral ganglion, suggesting that they comprised a single group of 5-HT-ir neurons. This anterior group of 5-HT-ir cerebral neurons ranged in number from 3 in *Armina* (Fig.

Table 2-2: Range of soma diameters (in  $\mu\text{m}$ ) for serotonin-immunoreactive clusters

Ganglion	5-HT Cluster	Dendronotoidea					Aeolidioidea		Arminoidea			Doridoidea
		<i>Tritonia diomedea</i>	<i>Tochuina tetraquetra</i>	<i>Dendronotus iris</i>	<i>Dendronotus frondosus</i>	<i>Melibe leonina</i>	<i>Hermisenda crassicornis</i>	<i>Flabellina trophina</i>	<i>Dirona albolineata</i>	<i>Janolus fuscus</i>	<i>Armina californica</i>	<i>Triopha catalinae</i>
Cerebral	dCeSA	30-50, >150 <sup>2</sup>	50-100, 200-300	75-150, ~200	20-50, 50-100	50-100	30-40, ~50	~50	70-100, 100-150	30-80, 80-100	50-100, 100-150	30-50
	dCeSP	20-30	50-70	50-60	10-30	20-40	10-20	10-30	20-40	20-50	10-30	20-50
	dCeSL	-	40-60	-	-	-	-	-	-	-	-	10-50
	vCeSA	20-30	30-50	-	20-40	-	20-40	10-40, 60-70	10-40	20-50, ~100	-	20-100
	vCeSP	-	-	-	-	-	-	-	30-50	-	10-30	-
Pedal	vCeSL	-	-	-	-	-	-	-	20-40	-	-	-
	dPdSA	-	-	100-175	-	5-100	-	-	-	20-50, 50-100	10-100	20-70, ~100
	dPdSP	-	50-100	-	20-40	-	-	-	75-150	70-100	50-100	20-100
	dPdSL	20-30, 50-70	-	~50	-	-	30-50	10-50	30-60	-	-	-
	vPdSA	10-30, 50-100	20-50, 70-100	100-175	-	50-100	20-30, ~50	10-20, 50-80	20-100	30-50	10-100	-
Pleural	vPdSP	20-30, 50-100	50-100	100-150	50-100	-	40-50, 50-60	20-30, 50-100	30-150	50-100	50-100	-
	vPdSL	-	-	~50	-	20-40	-	-	-	-	-	20-50, 150-200
	vPdSM	-	-	-	-	-	-	-	20-50	-	-	50-100
	dP/SL	-	-	-	-	-	-	~20	-	-	-	-
	left vP/SM	-	-	100-200	~100	-	-	-	-	70-100	-	100-150
	left vP/SL	-	-	-	-	-	-	-	~100	-	-	-

<sup>1</sup>d, dorsal; v, ventral<sup>2</sup>Clusters that contain multiple size classes have ranges separated with commas.

2-12) to 41 in *Tochuina* (Fig. 2-4). The anterior cerebral 5-HT-ir clusters and the dCeSP cluster were the only 5-HT-ir clusters that were consistently present in all of the species in our study.

A notable, highly conserved serotonergic neuron is the metacerebral giant (MCG) neuron of gastropods, located in the anterior region of the cerebral ganglion (Sakharov, 1976; Weiss and Kupfermann, 1976; Pentreath et al., 1982; Barber, 1983; Croll, 1987a). The MCG neuron is substantially larger than other surrounding serotonergic neurons and provides the sole serotonergic innervation to the buccal ganglion. Therefore, we expected to see an MCG candidate in all of the species in our study. In nine of the species in this study, one of the dCeSA or vCeSA neurons was larger than the other neurons in these clusters (Table 2-2) and is likely to be the MCG neuron (Figs. 2-1 – 2-6 & 2-8 – 2-12). However, two species lacked a giant MCG candidate [*Melibe* (Fig. 2-7A & B) and *Triopha* (Fig. 2-13A & B)]; the neurons in the dCeSA cluster in these two species were uniform in size. It is likely that the MCG homologue is present in these species because there was a 5-HT-ir axon in the connective that projects to the buccal ganglion. However, none of the neurons in this cluster had a conspicuously large soma in these two species.

#### *Serotonin in the pedal ganglion*

The locations and number of 5-HT-ir neurons in the pedal ganglion exhibited more inter- and intra-species variation than the cerebral 5-HT-ir clusters. Although the majority of 5-HT-ir neurons in the brain were in the pedal ganglion, there was no specific 5-HT-ir cluster or individual neuron that was present in all species. However, some of the



variability can be attributed to the difficulty in determining a consistent frame of reference for orienting the pedal ganglion because it can vary in shape markedly in different species.

The most consistent similarity across species was the presence of a large 5-HT-ir neuron on the dorsal surface of the left pedal ganglion that was not present in the right pedal ganglion (left dPdSM, Fig. 2-3 – 2-10 & 2-13, Table 2-3). In *Tritonia*, this left 5-HT-ir pedal neuron may be Pd1, which was previously identified by size and location (Weinreich et al., 1973; Willows et al., 1973; Audesirk et al., 1979; Sudlow et al., 1998; or it may be Pd4 as noted by Fickbohm et al., 2001). In *Hermisenda*, this large pedal neuron was named LP1 (Jerussi and Alkon, 1981; Land and Crow, 1985; Croll, 1987b). Interestingly, one of the *Hermisenda* specimens in this study lacked a large, asymmetric 5-HT-ir neuron in the left pedal ganglion, but had a comparable-sized neuron in the left pleural ganglion instead, which may have been a displaced LP1 neuron because it was located near the cerebral-pedal connective (not shown). Of the eleven species in this study, only *Janolus* consistently lacked a large 5-HT-ir neuron in the left pedal ganglion (Fig. 2-11A & B). Although *Armina* had a large 5-HT-ir neuron in the medial region of the left pedal ganglion, it also had a large neuron in a comparable location in the right pedal ganglion (Fig. 2-12A & B). It is not clear if this symmetric pair of neurons is homologous to the asymmetric neurons of other species. Thus, the presence of an asymmetric left dPdSM neuron was consistent in all species except some arminoids.

The locations and sizes of 5-HT-ir clusters in the pedal ganglion were much more variable between species than the presence of the left dPdSM neuron. The most prevalent

**Table 2-3:** Percentage of preparations with visually identifiable, individual, serotonin-immunoreactive neurons

Ganglion	5-HT Neuron <sup>1</sup>	Dendronotoidea					Aeolidioidea		Arminoidea			Doridoidea
		<i>Tritonia diomedea</i> (11 / 11) <sup>2</sup>	<i>Tochuina tetraquetra</i> (2 / 6)	<i>Dendronotus iris</i> (0 / 10)	<i>Dendronotus frondosus</i> (0 / 8)	<i>Melibe leonina</i> (4 / 31)	<i>Hermisenda crassicornis</i> (5 / 7)	<i>Flabellina trophina</i> (10 / 10)	<i>Dirona albolineata</i> (10 / 10)	<i>Janolus fuscus</i> (2 / 7)	<i>Armina californica</i> (5 / 11)	<i>Triopha catalinae</i> (0 / 10)
Pedal	left dPdSM	91% <sup>3</sup>	83%	80%	25%	48%	100% <sup>5</sup>	100%	70%	-	64%	60%
	right dPdSM	-	-	-	-	-	-	-	-	-	36%	-
Pleural	left dP/SL	-	-	70% <sup>4</sup>	88%	-	100%	-	-	71%	-	-
	right dP/SL	-	-	-	-	-	100%	-	-	-	-	-
	left dP/SM	-	-	-	-	-	-	-	100%	-	-	60%
	right dP/SM	-	-	-	-	-	-	-	100%	-	82%	40%

<sup>1</sup>d, dorsal<sup>2</sup>Number of preparations with intact sheaths / total number of preparations<sup>3</sup>Previously identified as left Pd1 (Weinreich *et al.*, 1973; Willows *et al.*, 1973a; Audesirk *et al.*, 1979; Sudlow *et al.*, 1998) and Pd4 (Fickbohm *et al.*, 2001)<sup>4</sup>In 3 of 10 preps, there was an additional, bilaterally symmetric neuron in this area.<sup>5</sup>Previously identified as LP1 (Jerussi and Alkon, 1981; Land and Crow, 1985; Croll, 1987b)

5-HT-ir pedal clusters were the vPdSA cluster (Figs. 2-3 – 2-5 & 2-7 – 2-12), which was present in all species except *Dendronotus frondosus* and *Triopha*, and the vPdSP cluster (Figs. 2-3 – 2-6 & 2-8 – 2-12), which was present in all species except *Melibe* and *Triopha*. When both dorsal and ventral PdSA clusters were present, they were comprised of similarly-sized neurons (Table 2-2) and converged at the anterior tip of the pedal ganglion, suggesting that they comprise a single anterior cluster of 5-HT-ir neurons that spans the dorsal and ventral surfaces (cf: *Dendronotus iris* [Fig. 2-5], *Melibe* [Fig. 2-7], *Janolus* [Fig. 2-11], and *Armina* [Fig. 2-12]). Similarly, there appeared to be a single posterior cluster that includes both dPdSP and dPdSA clusters (cf: *Tochuina* [Fig. 2-4], *Dendronotus frondosus* [Fig. 2-6], *Dirona* [Fig. 2-10], *Janolus* [Fig. 2-11], and *Armina* [Fig. 2-12]).

The presence and number of these pedal 5-HT-ir neurons was variable across species (Table 2-1). In species with 5-HT-ir neurons in the anterior or posterior regions of the pedal ganglion, the number of these neurons ranged from 8 in *Triopha* to 128 in *Tochuina* for the anterior region, and 8 in *Hermisenda* and *Triopha* to 112 in *Tochuina* in the posterior portion of the pedal ganglion. There was a tendency for 5-HT-ir neurons in the anterior region of the pedal ganglion to be smaller than 5-HT-ir neurons in the posterior part of the ganglion, on both the dorsal and ventral surfaces (Table 2-2).

Contributing to the variability of 5-HT-ir localization in the pedal ganglion, 5-HT-ir neurons were not always distributed in just anterior and posterior clusters. Some species had 5-HT-ir neurons that clustered laterally around the roots of the pedal-pedal connectives on the dorsal surface (dPdSL: *Tritonia* [Fig. 2-3A & B], *Hermisenda* [Fig.

2-8A & B], *Flabellina* [Fig. 2-9A & B], and *Dirona* [Fig. 2-10A & B]) or the ventral surface (vPdSL: *Melibe* [Fig. 2-7C & D]). *Dirona* (Fig. 2-10C & D) and *Triopha* (Fig. 2-13C & D) also had a cluster of 5-HT-ir neurons in the medial region of the pedal ganglion (vPdSM). Thus, it is very difficult to assess the congruencies of the pedal 5-HT-ir clusters due to the extreme variability in almost all measures.

#### *Serotonin in the pleural ganglion*

The pleural ganglion contained the lowest number of 5-HT-ir neurons in the brain and exhibited the most asymmetry in 5-HT localization. Three of the dendronotid species generally lacked any 5-HT-ir pleural neurons at all (*Tritonia*, *Tochuina*, and *Melibe*). However, in *Tritonia*, an asymmetric neuron was occasionally observed on the dorsal lateral margin of the right pleural ganglion anterior to pleural nerve 1 (c.f. Fig. 1, Fickbohm and Katz, 2000). In the remaining species in all four suborders, there was generally one large 5-HT-ir neuron on the dorsal surface and two on the ventral surface of just the left pleural ganglion. The large dorsal neuron was located either laterally or medially (dP/SL or dP/SM). The dP/SL/M neuron was present in only the left pleural ganglion in the two *Dendronotus* species (Figs. 2-5 & 2-6) and *Janolus* (Fig. 2-11A & B). However, dorsal 5-HT-ir neurons were bilaterally represented in *Hermisenda* (Fig. 2-8A & B), *Flabellina*, which had two cells instead of one (Fig. 2-9A & B), *Dirona* (Fig. 2-10A & B), and *Triopha* (Fig. 2-13A & B). In *Armina*, there was a dorsal 5-HT-ir neuron in the right pleural ganglion but not the left (Fig. 2-12A & B). Without further

anatomical or physiological characterization, it is not possible to determine if these neurons are homologous.

Serotonin-ir neurons were present on the ventral surface of the pleural ganglion in two of the dendronotids (the two *Dendronotus* species) and two of the arminoids (*Dirona* and *Janolus*). These ventral 5-HT-ir clusters were always asymmetric (left pleural ganglion only) and contained just two or three large neurons. The location of the cluster within the pleural ganglion varied between species, from medial (left *vPLSM*) in the two *Dendronotus* species (Figs. 2-5 & 2-6) and *Janolus* (Fig. 2-11C & D) to lateral (left *vPLSL*) in *Dirona* (Fig. 2-10C & D).

In general, 5-HT-ir neurons in the pleural ganglion were quite large ( $> 70 \mu\text{m}$ ) (Tables 2-2 & 2-4). The only exceptions to this were the aeolids, *Hermisenda* and *Flabellina*, which had small ( $\sim 20 \mu\text{m}$ ) dorsal 5-HT-ir neurons near the pleural-pedal connectives (Figs. 2-8 & 2-9) and *Armina*, which had one small ( $10\text{-}30 \mu\text{m}$ ) 5-HT-ir neuron on the dorsal surface of the right pleural ganglion (Fig. 2-12A & B).

#### *Intra- and inter-species variability in number of 5-HT-ir neurons*

We tested whether the variability in the number of 5-HT-ir neurons within and between species was correlated with the size of the animal. Correlations between the size of animals and the numbers of neurons have been demonstrated previously in juvenile gastropods (Cash and Carew, 1989; Croll and Chiasson, 1989; Nolen and Carew, 1994; Marois and Carew, 1997; Zakharov et al., 1998; Ierusalimsky and Balaban, 2001). Body length and ganglion size are strongly correlated in gastropods (Croll and Chiasson, 1989;

**Table 2-4:** Range of soma diameters (in  $\mu\text{m}$ ) for individual serotonin-immunoreactive neurons

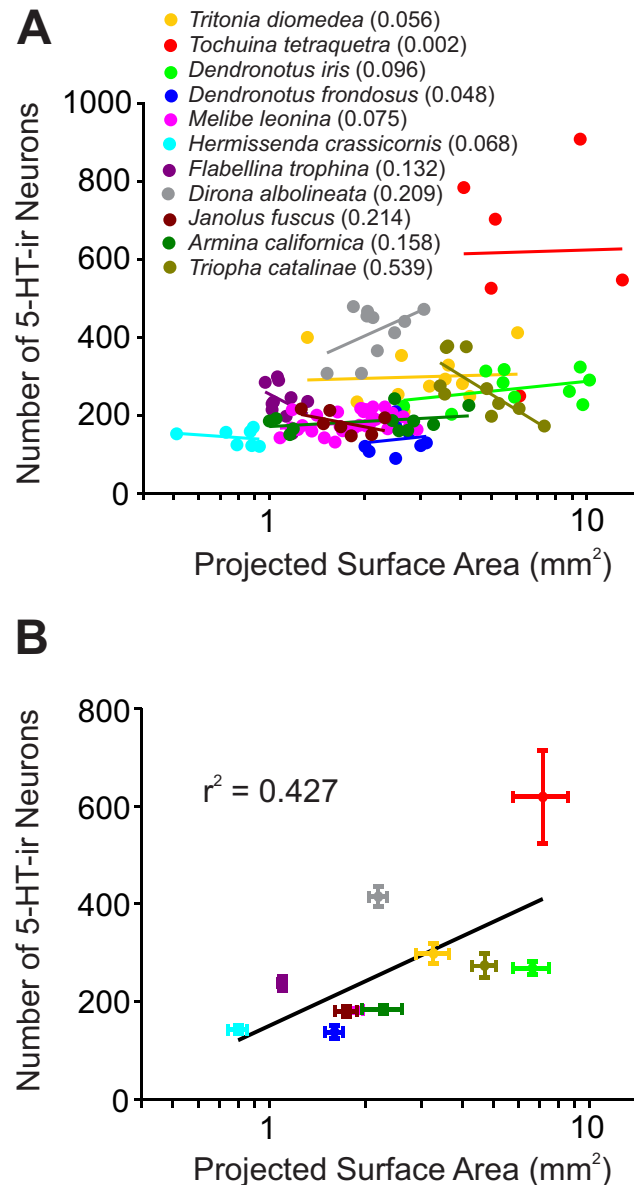
Ganglion	5-HT Cluster <sup>1</sup>	Dendronotoidea				Aeolidioidea		Arminoidea		Doridoidea		
		<i>Tritonia diomedea</i>	<i>Tochuina tetraquetra</i>	<i>Dendronotus iris</i>	<i>Dendronotus frondosus</i>	<i>Melibe leonina</i>	<i>Hermisenda crassicornis</i>	<i>Flabellina trophina</i>	<i>Dirona albolineata</i>	<i>Janolus fuscus</i>	<i>Armina californica</i>	<i>Triopha catalinae</i>
Pedal	left dPd/SM	100-150	200-300	200-300	100-200	~150	~100	~100	~150	-	~100	~100
	right dPd/SM	-	-	-	-	-	-	-	-	-	~100	-
Pleural	left dPl/SL	-	-	~150	~100	-	~20	-	-	70-100	-	-
	right dPl/SL	-	-	-	-	-	~20	-	-	-	-	-
	left dPl/SM	-	-	-	-	-	-	-	150-200	-	-	~200
	right dPl/SM	-	-	-	-	-	-	-	150-200	-	10-30	~200

<sup>1</sup>d, dorsal

Croll et al., 2001), therefore we plotted the total number of 5-HT-ir neurons against the projected surface area of each brain that is visible when the ganglion is mounted on a depression slide.

Regression analysis indicated that brain size was a poor predictor of the total number of 5-HT-ir neurons in the adults of most species (Fig. 2-14A). The only species where brain size accounted for more than 50% of the variability in number of 5-HT-ir neurons was *Triopha* ( $r^2 = 0.539$ ). However, in this case, as well as in *Flabellina* ( $r^2 = 0.132$ ) and *Janolus* ( $r^2 = 0.214$ ), the number of 5-HT-ir neurons actually decreased as brain size increased. There was a slight trend in the other eight species for larger brains to have more 5-HT-ir neurons than smaller brains, although the correlations were very weak ( $r^2$  values: *Tritonia* = 0.056, *Tochuina* = 0.002, *D. iris* = 0.096, *D. frondosus* = 0.048, *Melibe* = 0.075, *Hermisenda* = 0.068, *Dirona* = 0.209, and *Armina* = 0.158.) Thus, brain size does account for much of the intra-species variability in number of 5-HT-ir neurons.

Interspecies variability in the number of 5-HT-ir neurons was also not fully predicted by brain size (Fig. 2-14B). Brain size accounted for less than half of the variability in total number of 5-HT-ir neurons ( $r^2 = 0.427$ , Fig. 2-14B). These results suggest that additional factors, other than brain size, probably contribute to intra- and inter-species variability in the number of 5-HT-ir neurons. However, it is feasible that another measure of brain size might have yielded a clearer correlation.



**Figure 2-14:** The number of 5-HT-ir neurons in adult nudibranchs is not well correlated with brain size, as determined by the projected visible surface area. **A)** Each data point represents an individual specimen. Colors indicate the species. The regression lines and their  $r^2$  values (parentheses) are indicated for each species. **B)** Mean and standard error in the number of 5-HT-ir neurons is plotted versus the mean and standard error in projected brain surface area. The color key for species is the same as in **(A)**.



### *Phylogenetic analysis*

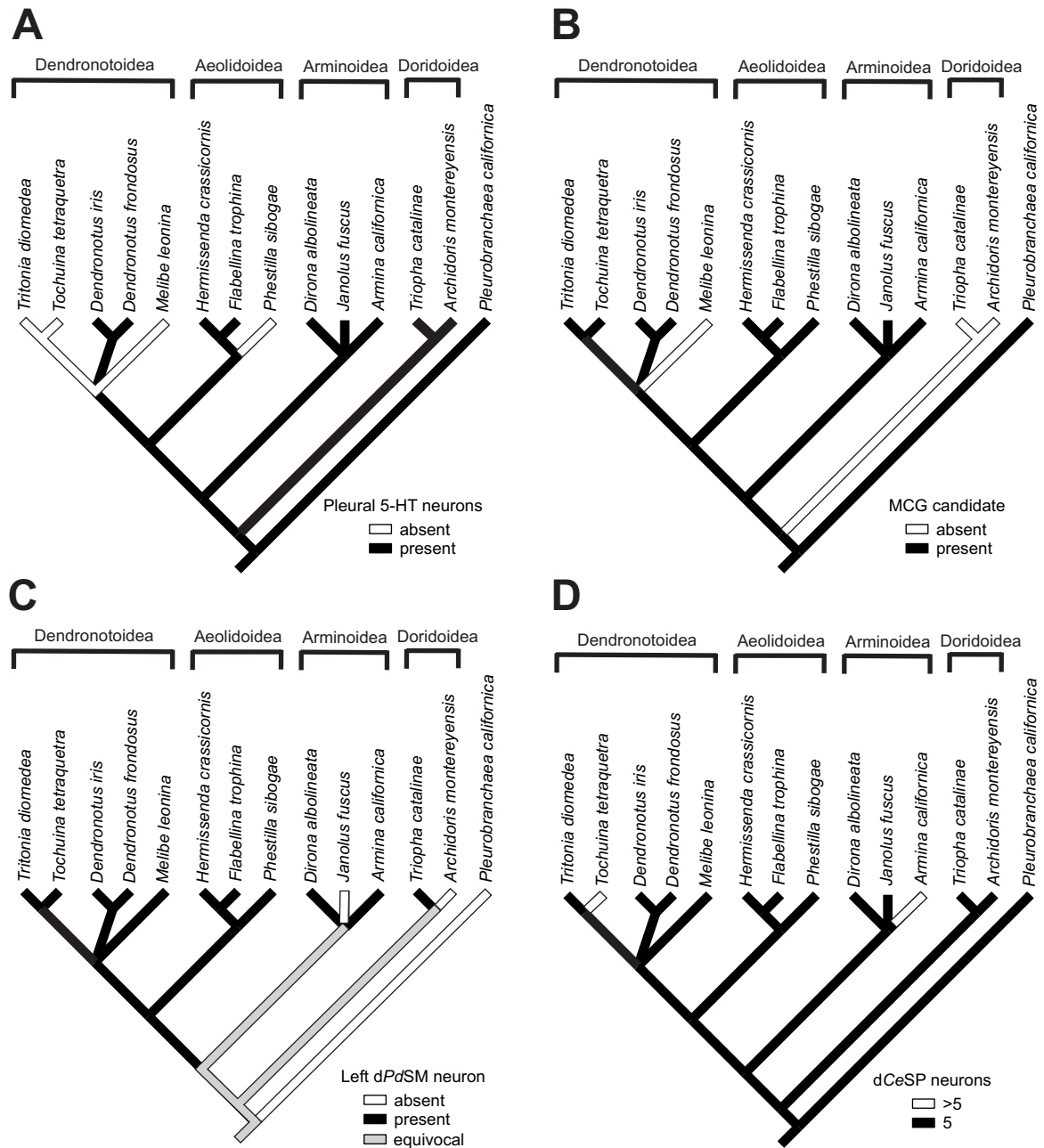
Much of the inter-species variability did not appear to correspond to shared derived features within lineages, but instead seemed to represent independent evolution of similar traits. To determine the extent of homoplasy in the nudibranch serotonergic system, a phylogenetic analysis was performed on sixteen characters measuring aspects of the 5-HT-ir variability (Table 2-5). These sixteen characters were compared among the eleven species included in this study and three additional species with published 5-HT-ir data: two nudibranchs, *Phestilla sibogae* (Croll et al., 2001) and *Archidoris montereyensis* (Wiens and Brownell, 1995), and an out-group, represented by the notaspid *Pleurobranchaea californica* (Sudlow et al., 1998). The analysis was performed with respect to the most widely accepted phylogeny (Fig. 2-1). Statistical measures of fit indicated that the only character that varied between species and exhibited minimum homoplasy was the presence or absence of the vCeSP cluster (rescaled consistency index [RC] = 1; see Table 2-5), which is present only in two arminoids (*Dirona* and *Armina*). All other characters had an RC index of less than 0.5 (Table 2-5), indicating a relatively high degree of homoplasy. Representative examples of such homoplasy are illustrated in Figure 2-15.

Overall, the inter-species variability in 5-HT immunoreactivity was not strongly correlated with the phylogeny of the Nudipleura. When all sixteen characters were analyzed together, the tree length was 44+ (the "+" being a result of unresolved polytomies in the tree) and the RC index was only 0.18, indicating a high degree of homoplasy and a poor fit to the currently understood phylogeny. In addition, a new tree

**Table 2-5:** Characters used for phylogenetic analysis and their statistical indices measuring their fit to the tree in Fig. 2-1

Character	CI	RI	RC
Presence of a <i>vCe</i> SP cluster	1.00	1.00	1.00
Positional relation of medial to lateral <i>dCe</i> SP neurons	0.50	0.80	0.40
Presence of pleural 5-HT-ir neurons (see Fig. 2-15a)	0.50	0.67	0.33
Presence of a right <i>dPl</i> SM neuron	0.50	0.67	0.33
Presence of an MCG candidate (see Fig. 2-15b)	0.50	0.50	0.25
Presence of a left <i>dPl</i> SM neuron	0.50	0.50	0.25
Presence of a <i>vPd</i> SM cluster	0.50	0.50	0.25
Asymmetry of dorsal pleural 5-HT-ir neurons	0.40	0.50	0.20
Presence of a <i>vPd</i> SL cluster	0.33	0.33	0.11
Presence of a left <i>vPl</i> SM cluster	0.33	0.33	0.11
Presence of a <i>dPd</i> SL cluster	0.25	0.40	0.10
Presence of a left <i>dPl</i> SL neuron	0.20	0.20	0.04
Presence of a <i>dCe</i> SL cluster	0.33	0.00	0.00
Presence of a left <i>dPd</i> SM neuron (see Fig. 2-15c)	0.33	0.00	0.00
Number of <i>dCe</i> SP neurons (see Fig. 2-15d)	0.50	0.00	0.00
Number of 5-HT-ir neurons in the brain	0.67	0.00	0.00

<sup>1</sup>5-HT-ir, serotonin-immunoreactive; CI, consistency index; d, dorsal; MCG, metacerebral giant; RC, rescaled consistency index; RI, retention index; v, ventral



**Figure 2-15:** Homoplasy of 5-HT immunoreactivity characteristics in the Nudipleura clade. **A)** Serotonin-ir pleural neurons have been lost in two or three lineages (depending on the resolution of the dendronotid polytomy). **B)** The large MCG neuron has been reduced or lost in two separate lineages. **C)** The dendronotids and aeolids have a left dPdSM neuron, although it is still unclear whether this was the ancestral condition due to the variety of states in the remaining groups. **D)** The number of dCeSP neurons has increased in two separate lineages. Data for species not examined in this study: *Phestilla* (Croll et al., 2001), *Archidoris* (Wiens and Brownell, 1995), and *Pleurobranchaea* (Sudlow et al., 1998).

created with all sixteen characters and using maximum parsimony methods (not shown) did not resemble the current phylogeny. These data suggest that the differences in the presence and location of 5-HT-ir neurons are not strongly correlated to the phylogenetic relationships of these nudibranchs.

## **Discussion**

In this study, we mapped the locations of 5-HT-ir neurons in nudibranch species representing all four nudibranch suborders in order to determine the extent to which the presence, size, location, and number of 5-HT-ir neurons are correlated with the phylogeny of these animals. Some patterns of 5-HT localization were conserved in all species, such as the presence of an anterior and posterior 5-HT-ir cluster on the dorsal surface of the cerebral ganglion. Other features were more variable between species, such as the specific number, location and size of 5-HT-ir clusters in the pedal and pleural ganglia. This variability was not strongly correlated with brain size. Furthermore, a phylogenetic analysis indicated that there was a large degree of homoplasy underlying the inter-species differences in 5-HT localization. The intra- and inter-species variability in a subset of the 5-HT-ir neurons suggests that there is evolutionary plasticity in the serotonergic system in nudibranch molluscs.

### *Dorsal CeSP cluster*

Previous studies have demonstrated that a group of five serotonergic neurons in the posterior region of the cerebral ganglion is conserved in a number of nudibranchs (Land and Crow, 1985; Croll, 1987b; Auerbach et al., 1989; Wiens and Brownell, 1995; Sudlow et al., 1998; Fickbohm and Katz, 2000; Croll et al., 2001; Fickbohm et al., 2001; Tian et al., 2006) and even across other opisthobranchs (Katz et al., 2001). Although we found this group of neurons, the dCeSP cluster, in all eleven species in our study, the number of neurons in this cluster differed in two species. Rather than the typical five neurons, the dCeSP cluster contained six neurons in *Tochuina* and eleven neurons in *Armina*. The increased number of dCeSP neurons arose independently in these two species (Fig. 2-15D).

In *Tritonia*, the three lateral dCeSP neurons are the DSIs (Katz et al., 1994; McClellan et al., 1994), which are involved in swimming (Getting et al., 1980) and crawling (Popescu and Frost, 2002). The three lateral dCeSP neurons have also been recently identified in *Hermisenda*, where they are called the CPT interneurons and are involved with movement of the foot (Tian et al., 2006). Homologues of the DSIs and CPT interneurons in other opisthobranchs are likewise involved in diverse forms of locomotion (Panchin et al., 1995; Satterlie and Norekian, 1995; Jing and Gillette, 1999). However, it remains unclear why *Tochuina* and *Armina* have a greater number of dCeSP neurons than other opisthobranchs and future studies on the function of these neurons in these species may shed some light on this issue.

## MCG

The MCG neuron is a well-established example of an identified neuron with homologues in a wide array of gastropods (Sakharov, 1976; Weiss and Kupfermann, 1976; Pentreath et al., 1982; Barber, 1983; Ono and McCaman, 1984; Murphy et al., 1985; Longley and Longley, 1986; Croll, 1987a,b; Croll, 1988; Auerbach et al., 1989; Hernádi et al., 1989; Satterlie et al., 1995; Sudlow et al., 1998; Croll et al., 2001; Fickbohm et al., 2001; Shirahata et al., 2004). However, in this study, *Melibe* and *Triopha* lacked a clear MCG candidate. This neuron may be present in these species but, based on soma location and size, it was not as conspicuous as it was in the other species. The inability to readily identify a MCG candidate in *Melibe* and *Triopha* may be due to a reduction in the size of this neuron. *Melibe* and *Triopha* are members of different suborders, indicating that such a reduction evolved independently at least twice in the nudibranch clade (Fig. 2-15B). *Triopha* was the only dorid in our study but previous work has indicated that the dorid *Archidoris montereyensis* also does not have an obvious MCG candidate based on soma location and size (Wiens and Brownell, 1995). Thus, it may be that the entire suborder Doridoidea lacks an obvious MCG neuron.

The inability to clearly identify a putative MCG neuron in *Melibe*, *Triopha*, and *Archidoris* may be related to the feeding apparatus in these species. Unlike the other serotonergic neurons in the anterior cerebral ganglion, the MCG neuron projects to the buccal ganglion and then innervates the esophagus, salivary glands, buccal mass and lips (Weiss and Kupfermann, 1976; Pentreath et al., 1982; Barber, 1983). *Melibe* and *Triopha* are the only nudibranchs in our study that lack solid jaws. Most nudibranchs have jaws

and a radula (Wägele and Willan, 2000) controlled by a buccal mass and its associated buccal ganglia (Willows, 1980). In the dorids, such as *Triopha* and *Archidoris*, the jaws consist of multiple structures arising developmentally from different generative grooves (Mikkelsen, 1996) and this is considered to be the plesiomorphic state (Gosliner, 1994; Wägele and Willan, 2000). In the cladobranchs, consisting of the other three nudibranch suborders, the jaws are fused into a "solid jaw" (Wägele and Willan, 2000). This apomorphic trait has been secondarily lost in some species, such as *Melibe*, which not only lacks solid jaws, but also a radula and a buccal mass (Gosliner, 1987). Therefore, the lack of an obvious MCG neuron in *Melibe*, *Triopha*, and *Archidoris* may be related to the absence of solid jaws in these lineages and a reduction in peripheral sites of innervation for the MCG.

#### *Asymmetric pedal and pleural neurons*

Large, identifiable neurons, such as the MCG neuron, are extremely useful for studying serotonergic function because their large size makes them very amenable to neurophysiological recording. Phylogenetic conservation of identified neurons enables comparative and evolutionary analysis of their functions. We have identified another large, 5-HT-ir neuron that is highly conserved in nudibranchs, the left dPdSM neuron. This neuron was present in every species in our study except the arminoid *Janolus*. The other arminoids, *Dirona* and *Armina*, did have a left dPdSM neuron (Fig. 2-15C), suggesting that the loss of this neuron in *Janolus* is not pervasive within the suborder Arminoidea. The lack of an equivalent neuron in *Archidoris* (Wiens and Brownell, 1995)

and *Pleurobranchaea* (Sudlow et al., 1998) suggests that the loss or gain of this neuron has occurred multiple times during the evolution of the Nudipleura.

The left *dPdSM* neuron was asymmetric, lacking a serotonergic counterpart in the right pedal ganglion in all species except for *Armina*. This asymmetry could be due to displacement of the soma, a change in neurotransmitter phenotype, or complete loss of the right counterpart. This left *dPdSM* neuron has been tentatively identified in *Tritonia*, (left Pd1; Weinreich et al., 1973; Willows et al., 1973; Audesirk et al., 1979; Sudlow et al., 1998; but see Fickbohm et al., 2001 for identification as left Pd4). Willows and colleagues (1973) reported that the right Pd1 was frequently absent, although they did not use serotonin as a marker for identification of the left or right Pd1. In these cases, there was often an extra cell of similar size, pigmentation, and physiology in the right pleural ganglion. They concluded that the right Pd1 often becomes misplaced during development and ends up in the right pleural ganglion instead of the right pedal ganglion. In our study, there was a small, unpaired 5-HT-ir neuron present in the right pleural ganglion of *Armina* and occasionally in *Tritonia*. However, this neuron was significantly smaller than the left *dPdSM* neuron. None of the species in our study had an unpaired 5-HT-ir neuron in the right pleural ganglion that was comparable in size to the left *dPdSM* neuron.

The left *dPdSM* neuron has also previously been identified in *Hermissenda* (LP1; Jerussi and Alkon, 1981; Land and Crow, 1985; Croll, 1987b). Although there is no apparent serotonergic counterpart in the right pedal ganglion of *Hermissenda*, there is an unpaired, large, catecholaminergic neuron in the right pedal ganglion (Croll, 1987b). A



similar unpaired, large, dopaminergic neuron is also present in the right pedal ganglion of the pulmonate snail *Lymnaea stagnalis* (Werkman et al., 1991). Further comparative study will be necessary to determine whether the presence of a large, unpaired dopaminergic neuron in the right pedal ganglion is common in gastropods, and whether these left and right aminergic pedal neurons are unrelated or are functional counterparts with different neurotransmitter phenotypes. The fact that *Armina* does have a right 5-HT-ir dPdSM neuron may prove to be valuable in addressing this issue.

#### *Intra-species variability in 5-HT immunoreactivity*

We expected that 5-HT immunoreactivity would be relatively consistent within each species. However, although the presence and location of 5-HT-ir clusters and neurons was consistent between animals of the same species, the number of 5-HT-ir neurons within a given cluster was often quite variable, as indicated by a large interquartile range for many of the clusters (Table 2-1). Although previous studies have demonstrated an increase in the number of neurons in juvenile gastropods as they grow (Cash and Carew, 1989; Croll and Chiasson, 1989; Nolen and Carew, 1994; Marois and Carew, 1997; Zakharov et al., 1998; Ierusalimsky and Balaban, 2001), we found that brain size was not a good predictor of the variability in the total number of 5-HT-ir neurons in the adults of any of the species in our study (Fig. 2-14A). This is consistent with other studies that indicate that increases in the size of gastropod brains during adulthood is correlated with increases in neuron size but not an increase in the number of

neurons (Croll and Chiasson, 1989; LaBerge and Chase, 1992; Sadamoto et al., 2000; Croll et al., 2001; however, see Coggeshall, 1967).

The variability in number of 5-HT-ir neurons within a species was not constant throughout the brain. Certain clusters, especially in the cerebral ganglion, consistently exhibited low intra-species variability, whereas the number of neurons in many of the pedal 5-HT-ir clusters exhibited high intra-species variability. While it is possible that our immunohistochemistry protocol did not always label every serotonergic neuron, the fact that different clusters exhibited different degrees of variability suggests that incomplete labeling is not the source of the differences.

#### *Inter-species variability in 5-HT immunoreactivity*

We had hypothesized that the overall pattern of 5-HT immunoreactivity would be similar between nudibranch species because previous studies suggested that aminergic neurons are often evolutionarily conserved (Sakharov, 1976; Weiss and Kupfermann, 1976; Pentreath et al., 1982; Barber, 1983; Croll, 1987a; Stuart et al., 1987; Katz and Tazaki, 1992; Kempf et al., 1997; Beltz, 1999; Hay-Schmidt, 2000; Katz et al., 2001; Santagata and Zimmer, 2002; Marinesco et al., 2003; Harzsch, 2004). However, we found that the total number of 5-HT-ir neurons was quite variable between species. This variability could not be adequately accounted for by differences in brain size (Fig. 2-14B). In addition to the overall number of 5-HT-ir neurons, there was also high variability in the locations of 5-HT-ir neuron clusters in the pedal and pleural ganglia, whereas the locations of clusters in the cerebral ganglion were more consistent. There are three

potential causes for the inter-species differences in the presence of 5-HT-ir neurons: 1) the neurons are present in all species but differ in neurotransmitter phenotype; 2) the neurons are present but their locations within the CNS have shifted; and 3) there is a real difference in the presence or absence of homologous neurons (change in cell number).

There are some examples of identified neurons whose homologues have a different neurotransmitter phenotype (Katz and Tazaki, 1992; Meyrand et al., 2000). The apparent rarity of this sort of evolutionary change might be due to the fact it would necessitate alterations in both pre- and postsynaptic elements. Therefore, although it is possible that the inter-species differences that we observed in the presence of 5-HT-ir neurons are caused by neurons either gaining or losing the 5-HT neurotransmitter phenotype, it is likely that these differences are more often the result of changes in location, size or number of 5-HT-ir neurons.

Soma location is not highly stereotyped in gastropod brains. Indeed, we observed a large pedal neuron in *Hermisenda* that appeared displaced to the pleural ganglion in one specimen. Kandel (1979) has also reported an example of displacement of the abdominal R2 neuron into the right pleural ganglion in the opisthobranch *Aplysia californica*. Entire clusters of neurons could be displaced by altered growth or migration patterns during development (Karten, 1997). It has been proposed that a change in a single gene is significant enough to alter neuronal position within a ganglion (Sasakura et al., 2005). Therefore, it is conceivable that the 5-HT-ir pleural neurons seen in some species in this study may be displaced homologues of 5-HT-ir pedal neurons in other species.

Differences in the number of neurons in a cluster could be the result of neuronal deletion or duplication. Although rare, there have been some reported examples of neuron deletion and duplication in *Aplysia* (Treisman and Schwartz, 1976; Treisman, 1979), the leech (Kuffler and Muller, 1974), and the locust (Goodman, 1977). This rarity suggests that neuronal deletion and duplication may not explain intra-species differences in the number of 5-HT-ir neurons in a particular cluster but may contribute to the evolution of inter-species differences.

Regardless of the mechanism for the inter-species differences in 5-HT localization, this variability contrasts with previous studies indicating a high degree of similarity amongst related taxa in the distribution of serotonergic neurons in the CNS (Sakharov, 1976; Weiss and Kupfermann, 1976; Pentreath et al., 1982; Barber, 1983; Croll, 1987a; Stuart et al., 1987; Katz and Tazaki, 1992; Kempf et al., 1997; Beltz, 1999; Hay-Schmidt, 2000; Katz et al., 2001; Santagata and Zimmer, 2002; Marinesco et al., 2003). Even the developing nervous system of embryos and larvae of some of the nudibranchs in this study have similar distribution of serotonergic neurons in the apical sensory organ (Kempf et al., 1997). In arthropods, 5-HT-immunoreactivity in the CNS is similar enough at the taxonomic level of class that it can be used to elucidate phylogenetic relationships (Harzsch, 2004). There is some inter-species variability in the number of 5-HT-ir neurons in the developing nervous system of caenogastropod larvae (Page and Parries, 2000), but these differences only constitute a few neurons and it is unknown whether this variability is maintained in the adult nervous system. The lack of a phylogenetic correlation for inter-species variability in 5-HT localization in our study contrasts with these previous

reports and suggests that differences in adult morphology, environmental habitats, and functional needs may underlie the independent evolution of certain derived traits of 5-HT localization in the adult nudibranch CNS.

## CHAPTER THREE

### HOMOLOGUES OF SEROTONERGIC CENTRAL PATTERN GENERATOR NEURONS IN RELATED NUDIBRANCH MOLLUSCS WITH DIVERGENT BEHAVIORS

#### **Abstract**

Homologues of a neuron that contributes to a species-specific behavior were identified and characterized in species lacking that behavior. The nudibranch *Tritonia diomedea* swims by flexing its body dorsally and ventrally. The DSIs are components of the CPG underlying this rhythmic motor pattern and also activate crawling. Homologues of the DSIs were identified in six nudibranchs that do not exhibit dorsal-ventral swimming: *Tochuina tetraquetra*, *Melibe leonina*, *Dendronotus iris*, *Dendronotus frondosus*, *Armina californica*, and *Triopha catalinae*. Homology was based upon shared features that distinguish the DSIs from all other neurons: 1) serotonin immunoreactivity, 2) location in the CeSP cluster, and 3) axon projection to the contralateral pedal ganglion. The DSI homologues, named CeSP-A neurons, share additional features with the DSIs: irregular basal firing, synchronous inputs, electrical coupling, and reciprocal inhibition. Unlike the DSIs, the CeSP-A neurons were not rhythmically active in response to nerve stimulation. The CeSP-A neurons in *Tochuina* and *Triopha* also excited homologues of the *Tritonia* Pd5 neuron, a crawling efferent. Thus, the CeSP-A neurons and the DSIs may be part of a conserved network related to crawling that may have been co-opted into a rhythmic swim CPG in *Tritonia*.

## **Introduction**

The extent to which the evolution of species-specific behavior involved re-specification of the functions of central neural circuits has not been well studied. There is much evidence to suggest that nervous systems often appear more evolutionarily conserved than the musculoskeletal systems that they control (Sanderson, 1988; Goslow et al., 1989; Wainwright, 1989; Kavanau, 1990; Katz, 1991; Katz and Tazaki, 1992; Nishikawa et al., 1992; Weijs and Dantuma, 1994; Tierney, 1995; Kiehn et al., 1997; Katz and Harris-Warrick, 1999; Herrel et al., 2001; Langenbach and Van Eijden, 2001; Wainwright, 2002; though see Smith, 1994). Homologous neural structures can be recognized across wide ranges of species and neural circuits can be multifunctional. Thus, the same organization can produce different outputs (Harris-Warrick and Marder, 1991). However, there have not been many studies that directly test the degree to which central neural circuits diverged to produce species-specific behaviors (Arbas et al., 1991; Smith, 1994). This is partly because there are so few preparations in which the central circuits have been worked out and where the circuit components can be unambiguously identified as homologous if the behaviors are different. Invertebrate nervous systems offer the possibility of identifying homologous neurons across species and assessing the functions of individual neurons in species-specific behaviors (Paul, 1991; Bullock, 2000; Comer and Robertson, 2001).

Identified neurons underlying a species-specific behavior have been extensively studied in the nudibranch mollusc, *Tritonia diomedea* (Family: Tritoniidae; Suborder:

Dendronotoidea) (Bergh, 1894). *Tritonia* produces a swimming behavior by rhythmically flexing its body in the dorsal and ventral directions (Willows, 1967; Hume et al., 1982). This rhythmic motor pattern is controlled by a CPG circuit comprised of identified neurons (Gettings et al., 1980; Gettings, 1983), which include the three DSIs (DSI-A,B,C). The DSIs can be unambiguously distinguished from other neurons in the fused cerebral-pleural ganglion based on neuroanatomical characteristics alone; they are serotonin-immunoreactive, their cell bodies are located in the posterior region of the cerebral portion of the cerebral-pleural ganglion lateral to two other serotonergic neurons, and they each have an axon that projects to the contralateral pedal ganglion (Gettings et al., 1980; Katz et al., 1994; McClellan et al., 1994). The DSIs also can be distinguished from neighboring neurons using purely electrophysiological criteria: they receive common synaptic input, they have a distinctive spike shape, and they are rhythmically active during the swim motor pattern (Gettings et al., 1980; Gettings, 1981). The DSIs make synaptic connections to other CPG neurons, efferent flexion neurons, and efferent crawling neurons (Hume and Gettings, 1982; Popescu and Frost, 2002). DSI-A is electrically coupled to its contralateral counterpart, whereas DSI-B,C are electrically coupled both ipsilaterally and contralaterally (Gettings, 1981). These anatomical and physiological characteristics can be used to identify homologous neurons in other species. Here the term "homologous" is meant to imply that the same cell type was likely present in a common ancestor (Striedter and Northcutt, 1991).

Serotonin immunohistochemistry alone suggests that DSI homologues exist in other nudibranchs (Land and Crow, 1985; Croll, 1987b; Auerbach et al., 1989; Wiens and



Brownell, 1995; Croll et al., 2001; Tian et al., 2006; Newcomb et al., 2006), and even in other non-nudibranch opisthobranchs (Ono and McCaman, 1984; Longley and Longley, 1986; Arshavsky et al., 1992; Panchin et al., 1995; Satterlie and Norekian, 1995; Sudlow et al., 1998; Fickbohm et al., 2001; Katz et al., 2001; McPherson and Katz, 2001; Marinesco et al., 2004a). In all nudibranchs, there is a cluster of serotonin-immunoreactive neurons, the *CeSP* cluster (Newcomb et al., 2006), that contains putative DSI homologues. In one other nudibranch, *Hermisenda crassicornis*, these neurons were individually identified and named the CPT neurons (Tian et al., 2006). Also, in the notaspid *Pleurobranchaea californica*, homologues of the DSIs, the As1-3 neurons, have been physiologically identified and shown to be members of the CPG underlying dorsal-ventral swimming (Jing and Gillette, 1999). In each case, the putative DSI homologues had the same morphological properties that uniquely distinguish the DSIs from other neurons in *Tritonia*.

In this study, we neurophysiologically identified and characterized putative homologues of the DSIs, which we named the *CeSP-A* neurons, in six other nudibranch species. These species represent three of the four suborders: 1. Dendronotoidea (*Tochuina tetraquetra*, Family: Tritoniidae; *Melibe leonina*, Family: Tethydidae; *Dendronotus iris* and *Dendronotus frondosus*, Family: Dendronotoidea), 2. Arminoidea (*Armina californica*, Family: Arminidae), and 3. Doridoidea (*Triopha catalinae*, Family: Polyceridae). The fourth suborder, Aeolidioidea includes *Hermisenda*, in which putative DSI homologues have already been identified (Tian et al., 2006). Three of these species in this study (*Tochuina*, *Triopha*, and *Armina*) do not swim at all. The other three species

(*Melibe* and the two *Dendronotus* species) swim with side-to-side (lateral) body flexions. The *Melibe* lateral-flexion swim CPG has been characterized (Thompson and Watson, 2005) and is comprised of neurons that are distinct from those in the *Tritonia* swim CPG (Katz and Newcomb, in press). Comparisons of the properties of homologous neurons across different species could suggest which properties are important for the production of the species-specific behavior and which are shared regardless of the behavior.

In *Tritonia*, the DSIs are multifunctional neurons; in addition to being members of the dorsal-ventral swim CPG, they also have a neuromodulatory role (Katz et al., 1994) and they accelerate crawling by exciting efferent neurons, including the Pedal 21 neuron and the giant Pd5 neuron (Popescu and Willows, 1999; Popescu and Frost, 2002). Pd5 is distinguishable from surrounding neurons based on morphological characteristics and its immunoreactivity to *Tritonia* pedal peptide (TPep; Willows et al., 1973; Lloyd et al., 1996; Cain et al., 2006). As with the *CeSP* cluster, Pd5 appears to be highly conserved in the nudibranch clade (Baltzley, 2006). Although the other species in this study do not swim in the manner that *Tritonia* does, we hypothesized that the DSI homologues in those species would synapse on the Pd5 homologue and thus potentially have a role in crawling.

## **Methods**

### *Animal collection and maintenance*

All specimens were collected as adults. *Dendronotus iris* (10 – 20 cm in body length) and *Triopha catalinae* (6 – 12 cm) were collected off of the dock at FHL, Friday Harbor, WA. *Tritonia diomedea* (5 – 20 cm), *Tochuina tetraquetra* (5 – 20 cm) and *Armina californica* (5 – 12 cm) were collected via trawling or SCUBA in Bellingham Bay and Dash Point, Puget Sound, WA. Additional *Tritonia*, *Tochuina*, *Dendronotus iris*, and *Armina* were collected similarly by Living Elements (Vancouver, British Columbia, Canada) at Yellow Bank in Clayoquot Sound near Tofino, British Columbia. *Melibe leonina* (5 – 10 cm) was collected from the dock at FHL and from subtidal eelgrass beds at nearby Shaw Island, WA. Additional *Melibe* were collected by Living Elements at the Indian Arm extension of Burrard Inlet in Vancouver Harbor. *Dendronotus frondosus* (4 – 8 cm) was collected from pens at a finfish aquaculture site off the coast of New Hampshire.

Animals were either kept in seawater tables at FHL at ambient seawater temperatures and light/dark cycles, or in recirculating artificial seawater tanks at Georgia State University at 10° C and a fixed 12:12 light/dark cycle.

### *Dissection*

Animals were anesthetized by chilling and either injection of 0.33 M magnesium chloride into the body cavity or immersion in a 50:50 mixture of 0.33 M magnesium

chloride and sea water for 15 – 30 min. A cut was made in the dorsal integument above the esophagus and buccal mass. The brain, consisting of the cerebral, pedal, and pleural ganglia, was removed by cutting all nerve roots. The brain was transferred to a Sylgard-lined dish where it was perfused, at a rate of 0.5 ml/min, with artificial saline (in mM): 420 NaCl, 10 KCl, 10 CaCl<sub>2</sub>, 50 MgCl<sub>2</sub>, 11 D-glucose, and 10 HEPES, pH. 7.6.

Connective tissue surrounding the brain was manually removed with forceps, fine scissors and a tungsten wire while keeping the brain at ~ 4 °C to reduce neuronal firing. The temperature was raised to 10 °C for electrophysiological experiments.

### *Electrophysiology*

Intracellular recordings were obtained using 10-30 M $\Omega$  glass microelectrodes filled with 3 M potassium chloride and connected to Axoclamp 2B (Axon Instruments, Union City, CA) or Dagan IX2-700 (Dagan Corporation, Minneapolis, MN) amplifiers. Electrodes were dipped in ink extruded from a black permanent marker (Sharpie, Sanford Corporation, Oak Brook, IL) to make it easier to see the fine tip. Extracellular suction electrode recordings were obtained by drawing individual nerves into polyethylene tubing filled with artificial saline and connected to an A-M Systems Differential AC Amplifier (model 1700, A-M Systems, Inc., Sequim, WA). Both intra- and extracellular recordings were digitized (>1 kHz) with a 1401 Plus or Micro 1401 A/D converter from Cambridge Electronic Design (Cambridge, UK). Nerve stimulation was applied via the extracellular suction electrodes from an A-M Systems Isolated Pulse Stimulator (model 2100) or a Grass Instruments S48 Square Pulse Stimulator (Grass Telefactor, Warwick, RI). Pedal

nerve 2 (PdN2) was consistently used for nerve stimulation. The stimulation consisted of 10 – 20 V, 2 ms pulses at 2 - 10 Hz for 1 - 5 s. Data acquisition and analysis were performed with Spike2 software (Cambridge Electronic Design).

### *Nerve nomenclature*

The names of nerves follow standardized nomenclature used in Newcomb et al. (2006), which is based upon Willows et al. (1973). Nerves were numbered in order from medial to lateral and anterior to posterior for each ganglion and have the name of the originating ganglion: Pedal nerve 1 would be the most anterior nerve exiting the pedal ganglion. PP1 and PP2 are the two pedal-pedal connectives called Pedal nerves 5 and 6 in *Tritonia* (Willows et al., 1973). In *Melibe* these two connectives were called the parapedal connective (PPC) and pedal connective (PC) respectively (Watson et al., 2002).

### *Neuron identification*

DSIs in *Tritonia* were identified according to a set of characteristics that, together, uniquely delineate them from surrounding neurons (Getting et al., 1980; Getting, 1981; Katz et al., 1994; McClellan et al., 1994; Popescu and Frost, 2002). To identify DSI homologues, the *CeSP-A* neurons, in the other species, candidate neurons were identified based on soma location in relation to the previously described serotonergic *CeSP* cluster (Newcomb et al., 2006). After physiological experiments, *post hoc* confirmation of neuron identity was obtained by injection with 2 - 4% Neurobiotin Tracer (Vector Laboratories, Inc., Burlingame, CA, dissolved in 0.75 M KCl, pH 7.4) and serotonin

immunohistochemistry (see below). Neurobiotin was loaded via iontophoresis for 30 min (1 – 10 nA, 1 Hz, 50% duty cycle) and preparations were fixed (see below) 0.5 – 4 hr after injection.

Pd5 was identified in *Tritonia* based on morphological characteristics, such as its location in the posterior region of the dorsal surface of the pedal ganglion and the fact that it is the largest neuron in this area (Willows et al., 1973). Electrophysiological confirmation was obtained by noting monosynaptic excitatory connections from the DSIs (Popescu and Willows, 1999; Popescu and Frost, 2002) and the presence of a corresponding large unit in pedal nerve 3 (Willows et al., 1973; Popescu and Willows, 1999; Cain et al., 2006). This latter characteristic was very useful because Pd5 is the only giant pedal neuron to project out pedal nerve 3 (Willows et al., 1973). Putative Pd5 homologues in *Tochuina* and *Triopha* were identified based on the criteria of being the largest neuron with an axon in the homologue of pedal nerve 3 and being TPep immunoreactive.

### *Electrical coupling*

To test for electrical coupling between *CeSPs*, the bathing medium was switched to high divalent cation saline, which raises the threshold for spiking and reduces spontaneous neural firing. The composition of the high divalent cation saline was (in mM): 285 NaCl, 10 KCl, 25 CaCl<sub>2</sub>, 125 MgCl<sub>2</sub>, 11 D-glucose, and 10 HEPES (pH 7.6). Brief hyperpolarizing current steps (1 – 5 s, 1 – 15 nA) were applied to one *CeSP* ("presynaptic") while the membrane potential was monitored in other *CeSPs*

("postsynaptic"). The presence of Neurobiotin in the presynaptic electrode often increased the electrode resistance enough to prevent effective balancing of the bridge during current injection. This meant that it was often not possible to obtain an accurate measurement of the presynaptic membrane potential. In those preparations where the bridge could be balanced, coupling coefficients were calculated as the change in membrane potential of the postsynaptic neuron divided by the change in membrane potential in the presynaptic neuron.

#### *Whole-mount immunohistochemistry and Neurobiotin processing*

Brains were fixed 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) (McLean and Nakane, 1974). After fixation, ganglia were washed twice (30 min each) with cacodylate buffer and then twice (30 min each) with 4% Triton X-100 in PBS (50 mM Na<sub>2</sub>HPO<sub>4</sub> in 140 mM NaCl, pH 7.2). The ganglia were then incubated for 1 hr in ASD (0.5% Triton X-100, 1% normal goat serum and 1% bovine serum albumen in PBS). This was followed by 48 – 96 hr in primary antiserum: either rabbit polyclonal anti-serotonin (ImmunoStar, Inc., Hudson, WI), diluted 1:1000 in ASD, or rabbit polyclonal anti-TPep (courtesy of Shaun Cain and A.O. Dennis Willows), diluted 1:500 in ASD. After four washes (1 hr each) with 0.5% Triton X-100 in PBS, ganglia were incubated overnight in goat anti-rabbit antiserum conjugated to Alexa 488 (Molecular Probes) diluted 1:50 or 1:100 in ASD. After this step, ganglia were washed four times (1 hr each) with PBS,

dehydrated in an ethanol series, cleared in methyl salicylate, and mounted on a depression slide with Cytoseal 60 (Electron Microscopy Sciences, Washington, PA). The ganglia were kept at 4° C for the entire immunohistochemistry protocol and all of the steps between fixation and dehydration were done with gentle agitation on a shaker.

In preparations that had Neurobiotin injected into candidate *CeSP-A* neurons, 0.1% Streptavidin-Alexa 594 conjugate (Molecular Probes, Eugene, OR), further diluted 1:50 in ASD, was added during the primary antibody step of the immunohistochemistry protocol above. Thus, neurons double-labeled with both fluorophores (Alexa 488 and 594) would be members of the serotonergic *CeSP-A* cluster.

### *Imaging*

Fluorescence images were visualized using confocal microscopy (LSM 510 mounted on an Axiovert 100M microscope, Carl Zeiss, Inc., Thornwood, NY) with a 10X- or 20X-objective. Fluorophores were excited with two lasers (488 and 543 nm) and fluorescent emissions were passed through a band-pass filter (505 – 550 nm) for visualization of Alexa 488 and a 560 nm long-pass filter to visualize Alexa 594. LSM 510 software was used to acquire images. Confocal stacks consisted of 12 – 80 optical sections, depending on the thickness of tissue, and included the entire dorsal/ventral span of the brain. The thickness of each optical section, which was optimized and kept consistent within a preparation, ranged from 2 – 14  $\mu$ m. Maximal projections of confocal stacks were exported as TIFF files and imported into Adobe Photoshop CS (Adobe, San Jose, CA). In Photoshop, projections were assembled into a montage of the entire CNS



and overall brightness and contrast were adjusted. Outlines of brains and neuron projection patterns were traced in Microsoft PowerPoint 2002.

### *Data analysis*

Spike after-hyperpolarizations were analyzed in Spike2 and measured from the resting membrane potential. The CeSP-A neurons fire at less than 1 Hz (see Table 3-1) but receive much synaptic input (see Fig. 3-4), making it difficult to accurately determine the resting membrane potential from a specific time point. Therefore, we determined the resting membrane potential by averaging the membrane potential over 50 ms, 100 – 200 ms prior to the peak of the action potential. After-hyperpolarization amplitudes were compared in InStat (GraphPad Software, San Diego, CA) using a one-way ANOVA and a Tukey *post-hoc* test.

Analysis of spike frequency, in response to nerve stimulation, was done by binning the spike frequency into 20 s-bins. The three 20 s-bins preceding nerve stimulation were averaged to provide a baseline spike frequency. This was compared to subsequent, post-stimulation 20s-bins in InStat with a repeated-measures ANOVA and a Dunnett *post-hoc* test. Results for all statistical analyses were considered significantly different with a *p*-value less than 0.05. Results are expressed as means  $\pm$  standard error.

## Results

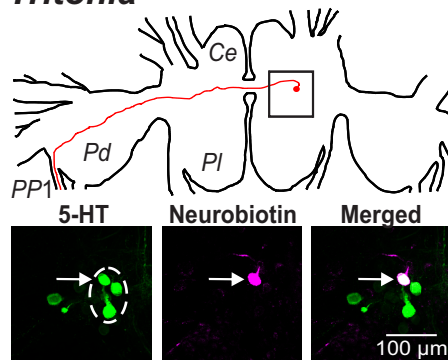
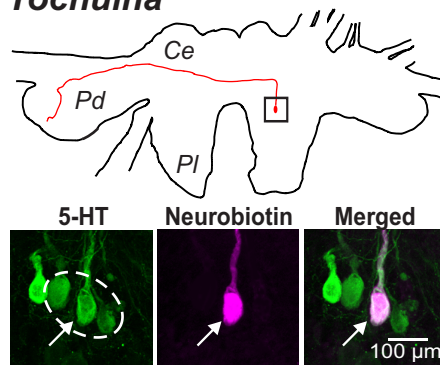
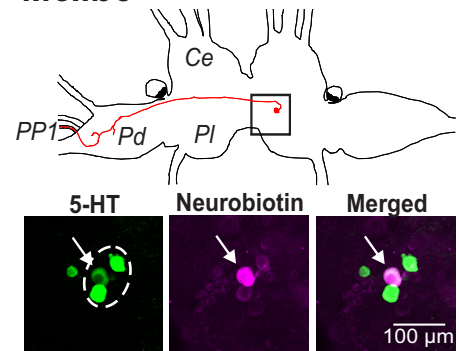
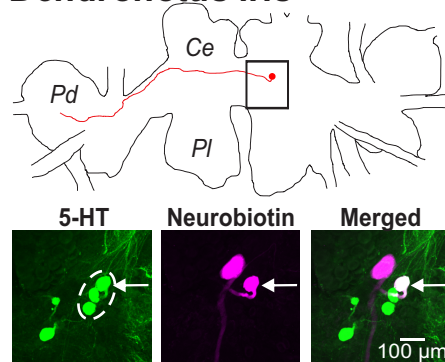
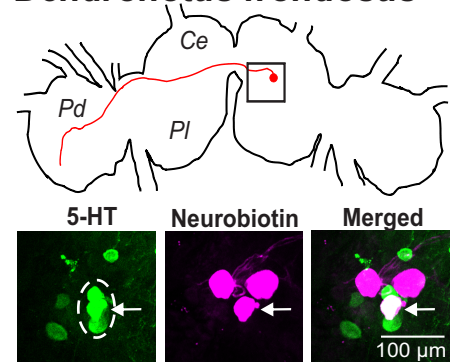
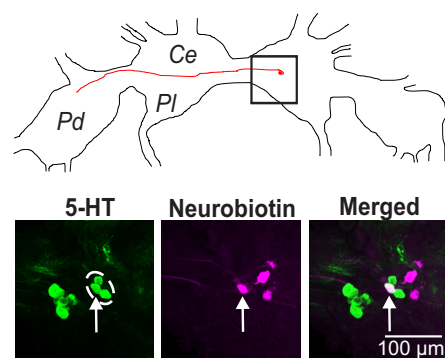
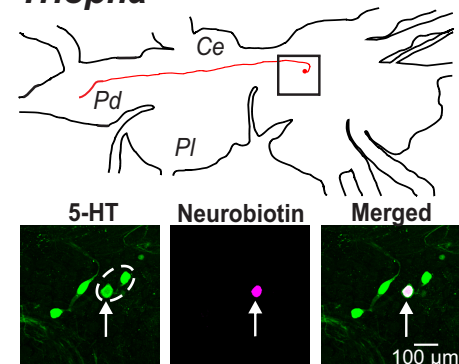
### *The DSI homologues, the CeSP-A neurons, can be identified neuroanatomically*

The DSIs in *Tritonia* can be identified based on neuroanatomical criteria: they are a group of three serotonin-immunoreactive somata located lateral to two additional serotonin-immunoreactive neurons in the posterior region of the cerebral ganglion (Fig. 3-1A) (Katz et al., 1994; McClellan et al., 1994; Fickbohm et al., 2001). The three DSIs are clearly distinguishable from the other two serotonin-immunoreactive neurons in the five-cell cluster based on their soma positions. Furthermore, one of the other two medial cells has a much smaller soma. The same criterion of soma position was used to identify putative homologues of the DSIs in six other nudibranch species.

In naming the DSI homologues, it was important to avoid names that implied function, such as swim interneuron. The somata of the DSI homologues are located in the CeSP cluster (Newcomb et al., 2006). To distinguish the DSI homologues from the other serotonergic neurons in the CeSP cluster, we named them the CeSP-A neurons.

There are three DSIs in *Tritonia* and there appear to be three CeSP-A neurons in some other species. However, the total number of neurons in the CeSP cluster shows some variability, particularly in *Tochuina* and *Armina* (Newcomb et al., 2006). Thus, it is possible that there may be interspecies, or even intraspecies, variability in the number of CeSP-A neurons. Regardless of the number, we have not found any morphological or electrophysiological differences between the CeSP-A neurons within a single animal. Therefore, to distinguish recordings of multiple neurons in the same animal, we simply

**Figure 3-1:** *CeSP-A* neurons in six species of nudibranchs resembled the *Tritonia diomedea* DSIs in soma location, serotonin-immunoreactivity, and axon projection pattern. **A)** The DSIs are the three lateral members (circled) of the *CeSP* cluster of five serotonin-immunoreactive neurons (green) in *Tritonia* (Katz et al., 1994; McClellan et al., 1994; Newcomb et al., 2006). Each DSI projects to the contralateral pedal ganglion (Getting et al., 1980) and through pedal-pedal connective 1 (*PP1*). In this preparation, the anterior-most DSI (arrow) was injected with Neurobiotin (magenta) and the axon was traced. **B-G)** In each of the other six species, *Tochuina tetraquetra*, *Melibe leonina*, *Dendronotus iris*, *Dendronotus frondosus*, *Armina californica*, and *Triopha catalinae*, lateral members (circled) of the *CeSP* cluster (green) had a similar projection pattern as the DSIs (arrows; *CeSP-A* neurons). In *Dendronotus iris*, *Dendronotus frondosus*, and *Armina*, multiple neurons were filled with Neurobiotin but only one was also serotonin immunoreactive.

**A** *Tritonia***B** *Tochuina***C** *Melibe***D** *Dendronotus iris***E** *Dendronotus frondosus***F** *Armina***G** *Triopha*

number them in order of recording *CeSP-A<sub>1</sub>*, *CeSP-A<sub>2</sub>*, and *CeSP-A<sub>3</sub>*. Note that we are not renaming the DSIs in *Tritonia*, nor are we equating the *CeSP-A* neurons with just the *Tritonia* DSI-A.

In *Tritonia*, the three DSIs can be distinguished from the other neurons in the *CeSP* cluster by their axon projection patterns; each DSI has an axon that projects to the contralateral pedal ganglion (Getting et al., 1980). We found, using Neurobiotin injection into the DSI soma, that the DSI axon extends through one of the two pedal-pedal connectives (pedal nerve 5, which is equivalent to PP1 in other species) and presumably continues to the pedal ganglion ipsilateral to the soma (Fig. 3-1A; n = 8). This axon pathway is consistent with extracellular recordings of DSI action potentials on PP1 (Sakurai and Katz, 2003).

Neurobiotin fills were combined with serotonin immunohistochemistry to identify the *CeSP-A* neurons and determine their axon projections in the other six nudibranchs in this study (Fig. 3-1B - G): *Tochuina* (8 total neurons in 4 preparations), *Melibe* (23 neurons in 15 preparations), *Dendronotus iris* (4 neurons in 4 preparations), *Dendronotus frondosus* (2 neurons in 2 preparations), *Armina* (1 neuron in 1 preparation), and *Triopha* (2 neurons in 2 preparations). In each case, the axon projected from the soma through the anterior tract of the cerebral-cerebral connective and the anterior tract of the cerebral-pedal connective to the contralateral pedal ganglion.

As with the *Tritonia* DSIs, the axons of the *CeSP-A* neurons were observed to continue through the contralateral pedal ganglion and enter one of the two pedal-pedal connectives (PP1 or PP2) in three species, *Tochuina*, *Melibe*, and *Dendronotus iris*. In

*Tochuina*, this projection pattern was seen once, where the *CeSP-A* axon projected through the larger-diameter pedal-pedal connective, PP2. In *Dendronotus iris*, two of the four *CeSP-A* neurons that were successfully double-labeled with Neurobiotin and 5-HT immunohistochemistry were seen to project out a pedal-pedal connective (one coursed through PP1 and the other went through PP2). In *Melibe*, 11 of the 13 *CeSP-A* neurons that could be seen to project through a pedal-pedal connective went through the smaller-diameter PP1, the parapedal connective. The length of axon that labeled for Neurobiotin in all of our preparations was variable, indicating that it did not always transport the same length down the axon in each preparation. Thus, the fact that we did not observe a *CeSP-A* neuron project through a pedal-pedal connective in some of our preparations, including the three species with no examples of such a projection pattern, may be due to the fact that the Neurobiotin did not travel far enough in these preparations.

We were unable to establish the number of *CeSP-A* neurons using dye fills because we were never successful at injecting all three potential *CeSP-A* neurons in one cluster with dye. In seven preparations (*Tochuina* [n = 3] and *Melibe* [n = 4]), we were able to inject Neurobiotin into two *CeSP-A* neurons in a single cluster. However, in 27 of the 28 preparations with dye fills and serotonin immunohistochemistry, there were three lateral neurons in the *CeSP* cluster. Thus, although we have not definitively demonstrated the presence of three *CeSP-A* neurons in the cerebral ganglion of each species, it is quite plausible to hypothesize that this is the case.

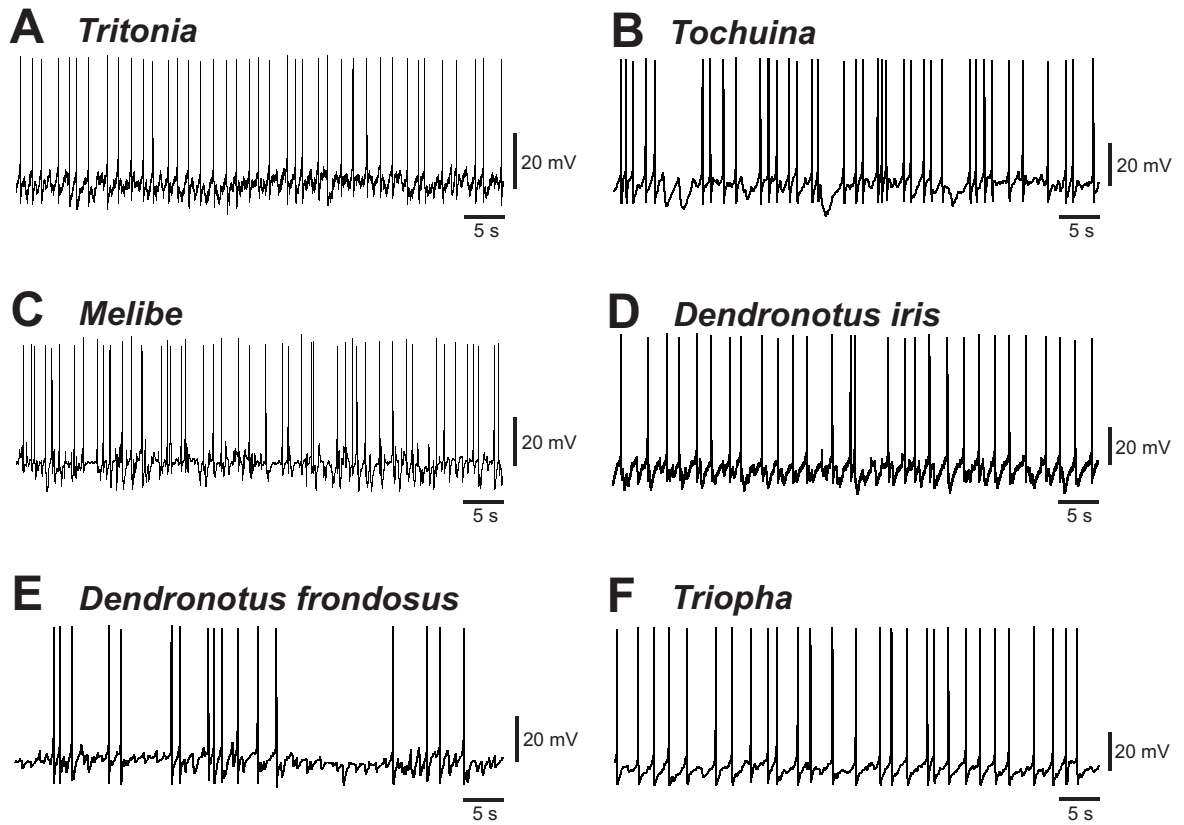
Together, these results show that in six nudibranch species, serotonin-immunoreactive neurons in the same region of the cerebral ganglion as the *Tritonia* DSIs

have a similar axon projection pattern.

#### *Electrophysiological properties of the CeSP-A neurons*

There are a number of physiological characteristics used to identify DSIs in *Tritonia*, including the fact that they are irregularly active at rest (Fig. 3-2A). We found that the CeSP-A neurons were also irregularly active at rest in all five species from which we successfully obtained intracellular recordings (Fig. 3-2B - F; *Tochuina* [9 total neurons in 4 preparations], *Melibe* [48 neurons in 24 preparations], *Dendronotus iris* [5 neurons in 5 preparations], *Dendronotus frondosus* [1 neuron in 1 preparation], and *Triopha* [4 neurons in 3 preparations]). After impalement with an intracellular electrode, the firing frequency of the CeSP-A neurons was determined once the neurons exhibited a constant activity level for at least 1 min. The firing frequency of these neurons ranged from 0.19 Hz in *Dendronotus frondosus* to  $0.73 \pm 0.09$  Hz in *Melibe* (Table 3-1). There were frequent and large spontaneous IPSPs and EPSPs at irregular intervals that likely contributed to the irregular pattern of spiking.

The action potentials of the *Tritonia* DSIs have an after-hyperpolarization that distinguishes them from surrounding neurons (Fig. 3-3). Analysis of spike after-hyperpolarizations indicated that the CeSP-A neurons in *Melibe*, *Dendronotus iris*, and *Triopha* had after-hyperpolarizations that were significantly reduced in comparison to the *Tritonia* DSIs (Fig. 3-3). In each species, regression analysis indicated that there was no correlation between the amplitude of the after-hyperpolarization and the resting membrane potential (not shown). Only the *Tochuina* CeSP-A neurons had an after-



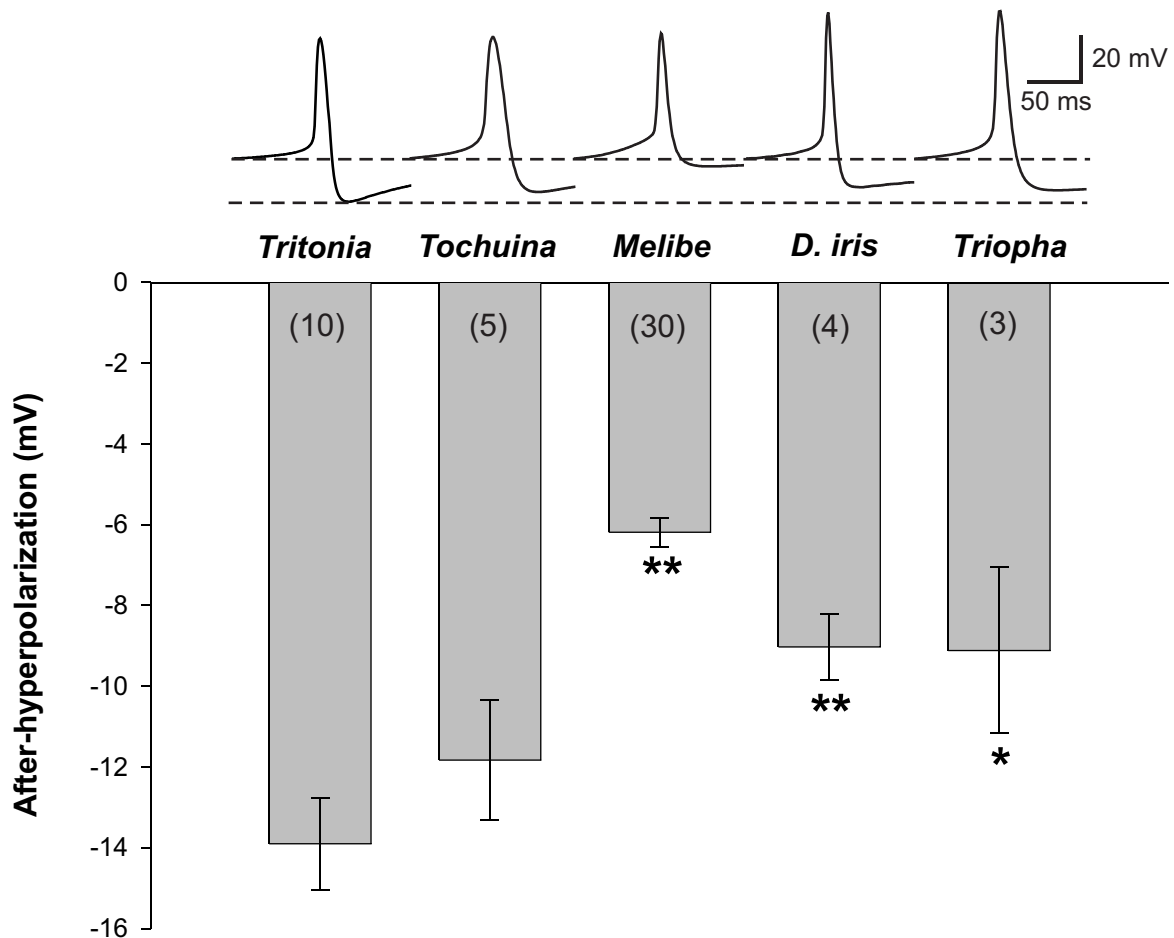
**Figure 3-2:** The spontaneous irregular firing patterns of a DSI (A) and CeSP-A neurons in five species of nudibranchs, *Tochuina* (B), *Melibe* (C), *Dendronotus iris* (D), *Dendronotus frondosus* (E), and *Triopha* (F).



**Table 3-1:** Electrophysiological properties of *Ce* SP-A neurons in five species of nudibranchs

Species	Number of Preparations	Number of Total <i>Ce</i> SP-A Neurons	Resting Membrane Potential (mV)	Spike Frequency (Hz)
<i>Tochuina tetraquetra</i>	4	9	-54 ± 8	0.30 ± 0.12 <sup>1</sup>
<i>Melibe leonina</i>	22	44	-51 ± 3	0.73 ± 0.09
<i>Dendronotus iris</i>	4	4	-44 ± 3	0.31 ± 0.08
<i>Dendronotus frondosus</i>	1	1	-36	0.19
<i>Triopha catalinae</i>	2	3	-50 ± 5	0.55 ± 0.07

<sup>1</sup>Five of the 9 *Ce*SP-A neurons in *Tochuina* were silent. The average firing frequency of the remaining 4 *Ce*SP-A neurons was 0.66 ± 0.08 Hz.



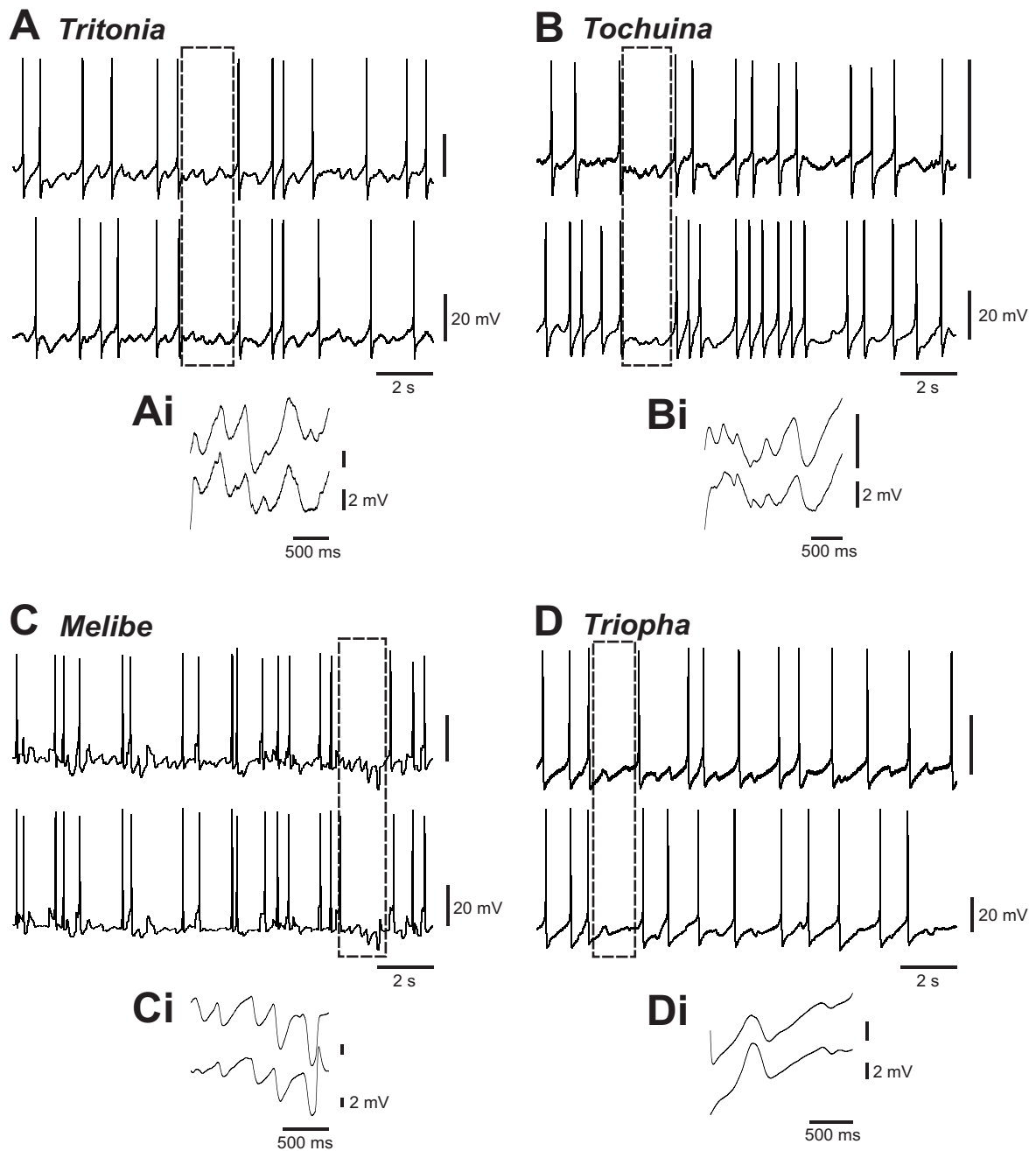
**Figure 3-3:** The CeSP-A neurons in *Melibe*, *D. iris*, and *Triopha* had a significantly reduced after-hyperpolarization compared to the DSIIs. The after-hyperpolarization was measured by subtracting the most negative membrane potential immediately following the action potential (bottom dashed line) from the 50 ms-average of the membrane potential 100–200 ms prior the peak of the action potential (top dashed line). Waveforms are averages of four min of spiking activity in a single neuron. Bars represent mean values  $\pm$  standard error. Numbers in parentheses are the sample sizes of neurons. Asterisks indicate  $p$ -value less than 0.01 (\*\*) or 0.05 (\*).

hyperpolarization that was not significantly different in amplitude than the DSIs (Fig. 3-3).

In *Tritonia*, the spikes from multiple DSIs exhibit near synchrony when recorded simultaneously in the same preparation ( $n = 11$ ) (Fig. 3-4A). This may arise because the DSIs receive synchronous spontaneous synaptic input (Fig. 3-4Ai). Similar near synchrony was observed in the spontaneous action potentials recorded from multiple *CeSP-A* neurons (Fig. 3-4B - D). We successfully obtained simultaneous recordings of multiple *CeSP-A* neurons in three species: *Tochuina* ( $n = 4$ ), *Melibe* ( $n = 15$ ), and *Triopha* ( $n = 1$ ). The neurons always received synchronous, large, and irregularly-timed spontaneous synaptic inputs that probably contributed to the similarity in firing patterns (Fig. 3-4Bi - Di). None of the neurons that we recorded from in the vicinity of the DSIs or *CeSP-A* neurons were ever seen to receive synaptic input or fire action potentials in synchrony with the *CeSP-A* neurons (*Tritonia* [2 total neurons in 2 preparations], *Tochuina* [4 total neurons in 3 preparations], *Melibe* [14 total neurons in 12 preparations], and *Triopha* [6 total neurons in 3 preparations]). The synchronous spontaneous synaptic potentials appear to be an identifying feature that distinguishes the *CeSP-A* neurons from neighboring neurons.

#### *The CeSP-A neurons are electrically coupled*

Electrical coupling between DSIs is another identifying feature of these neurons in *Tritonia*. Based on connectivity, there are two classes of DSIs: DSI<sub>A</sub> and DSI<sub>B/C</sub>. Electrical coupling exists within, but not between, the two classes of DSIs (Getting,



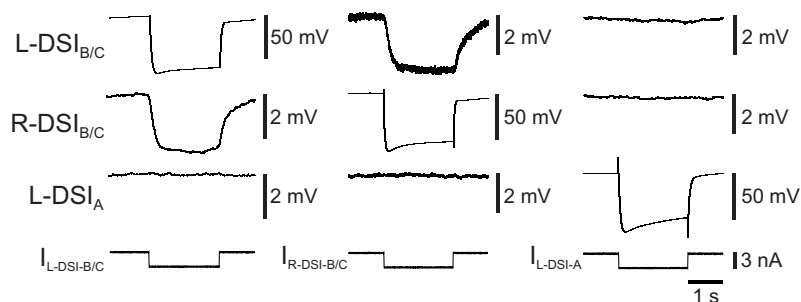
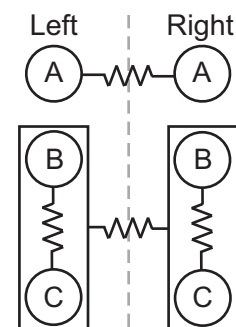
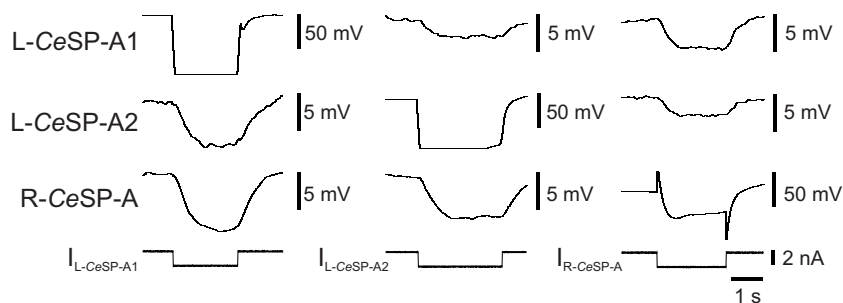
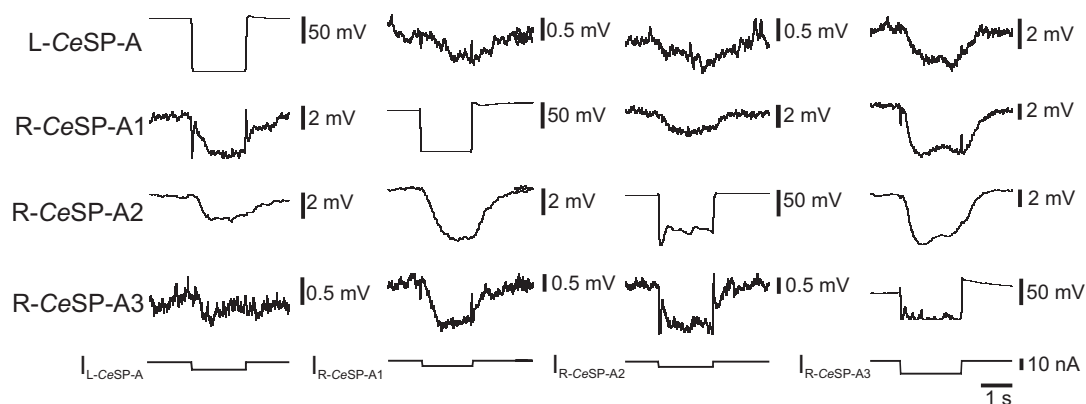
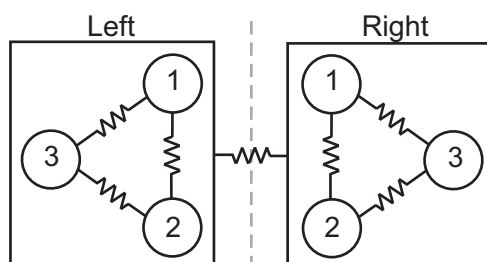
**Figure 4-4:** Simultaneous intracellular recordings from two DSIs in *Tritonia* (A) and two CeSP-A neurons in *Tochuina* (B), *Melibe* (C), and *Triopha* (D). The neurons fire at similar times and receive synchronous spontaneous synaptic inputs (insets). Synaptic input traces (Ai-Di) are expanded from highlighted regions (dashed boxes) of the intracellular recordings.

1981). Thus the contralateral DSI<sub>A</sub> are electrically coupled to each other and not coupled to DSI<sub>B/C</sub>. DSI<sub>B</sub> and DSI<sub>C</sub> are electrically coupled to each other both ipsilaterally and contralaterally (Fig. 3-5A & B).

Using high-divalent cation saline to reduce synaptic transmission, we tested electrical coupling between *CeSP-A* neurons in two species, *Tochuina* (a total of 3 ipsilateral and 2 contralateral neuron pairs in 3 preparations) and *Melibe* (a total of 9 ipsilateral and 8 contralateral pairs of neurons in 10 preparations). In both species, steps of hyperpolarizing current injected into one *CeSP-A* neuron always resulted in concurrent hyperpolarization of the membrane potential in other *CeSP-A* neurons (Fig. 3-5C & D). We could not determine whether the electrical connectivity in *Tochuina* was exactly like the *Tritonia* DSIs because we were not able to successfully record from three ipsilateral *CeSP-A* neurons simultaneously. However, in *Melibe*, simultaneous recordings from three ipsilateral *CeSP-A* neurons indicated that all three were electrically coupled to each other (Fig. 3-5D). Because all of the pairs exhibited electrical connections, it suggests that all of the *CeSP-A* neurons in *Melibe* are electrically coupled to each other, both ipsilaterally and contralaterally (Fig. 3-5E).

The electrical coupling between *CeSP-A* neurons in *Melibe* was weaker than the coupling between *Tritonia* DSIs. Getting (1981) determined that the average coupling coefficient between DSIs in five preparations was 0.19. In our study, the bridge could be sufficiently balanced in the intracellular electrodes in three of the *Melibe* preparations. In these cases, the coupling coefficient between *CeSP-A* neurons was  $0.03 \pm 0.01$  ( $n = 6$  pair-wise combinations).

**Figure 3-5:** The *CeSP-A* neurons were electrically coupled in *Tochuina* and *Melibe*, though the pattern of connectivity in *Melibe* differed from the *Tritonia* DSIs. **A)** Simultaneous intracellular recordings from three DSIs in high divalent cation saline. The top two traces illustrate contralateral DSIs that were electrically coupled. However, another DSI (L-DSI<sub>A</sub>) was not electrically coupled to either of the other two DSIs. **B)** Schematic representing the electrical connectivity of the two classes of DSIs (DSI<sub>A</sub> and DSI<sub>B/C</sub>) on each side of the brain (Getting, 1981). **C)** One right (R) and two left (L) *CeSP-A* neurons in *Tochuina* were electrically coupled to each other. **D)** In *Melibe*, one left and three right *CeSP-A* neurons were electrically coupled to each other. **E)** Unlike in *Tritonia* (**A**), there were no classes of *CeSP-A* neurons in *Melibe* with respect to their electrical connectivity; all *CeSP-A* neurons were electrically coupled to each other, both ipsilaterally and contralaterally.

**A** *Tritonia***B** *Tritonia***C** *Tochuina***D** *Melibe***E** *Melibe*

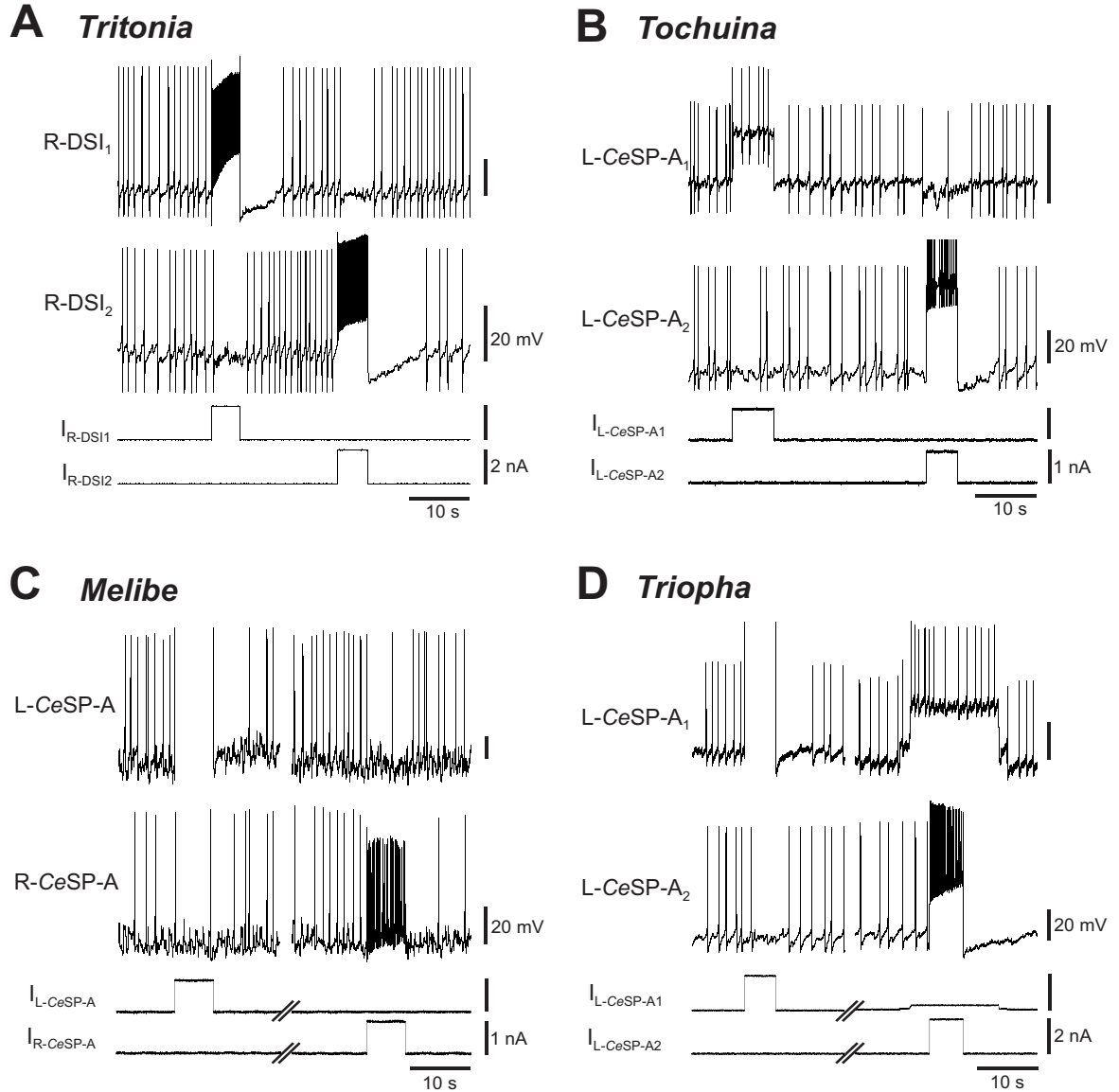
*The CeSP-A neurons are reciprocally inhibitory*

In *Tritonia*, the DSIs recruit inhibition onto each other through a polysynaptic pathway that includes an unidentified inhibitory neuron (Fig. 3-6A; Getting and Dekin, 1985). To determine whether the *CeSP-A* neurons also may recruit inhibition, they were depolarized to increase their firing rate while the responses of other *CeSP-A* neurons were monitored. *CeSP-A* neurons did cause inhibition in the three species examined, although not in every specimen. In *Tochuina*, *CeSP-A* neurons caused inhibition in 2 of 6 pairs (Fig. 3-6B; n = 4). In *Melibe*, 8 of 28 pairs of *CeSP-A* neurons exhibited reciprocal inhibition (Fig. 3-6C; n = 13). In *Triopha*, there was only a single preparation with two simultaneous *CeSP-A* neuron recordings; this pair did exhibit reciprocal inhibition (Fig. 3-6D). The ability of a *CeSP-A* neuron to cause inhibition of another *CeSP-A* neuron was not correlated with whether the neurons were ipsilateral or contralateral to each other. Thus, *CeSP-A* neurons in *Tochuina*, *Melibe*, and *Triopha* do resemble the *Tritonia* DSIs in their ability to reciprocally inhibit each other. However, it is not yet clear whether this is a monosynaptic inhibitory connection or recruitment of inhibition from an intermediary neuron.

*Nerve stimulation increases the firing rate of the CeSP-A neurons without eliciting rhythmic activity*

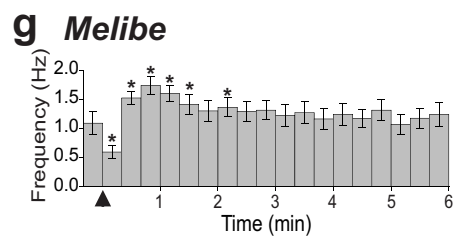
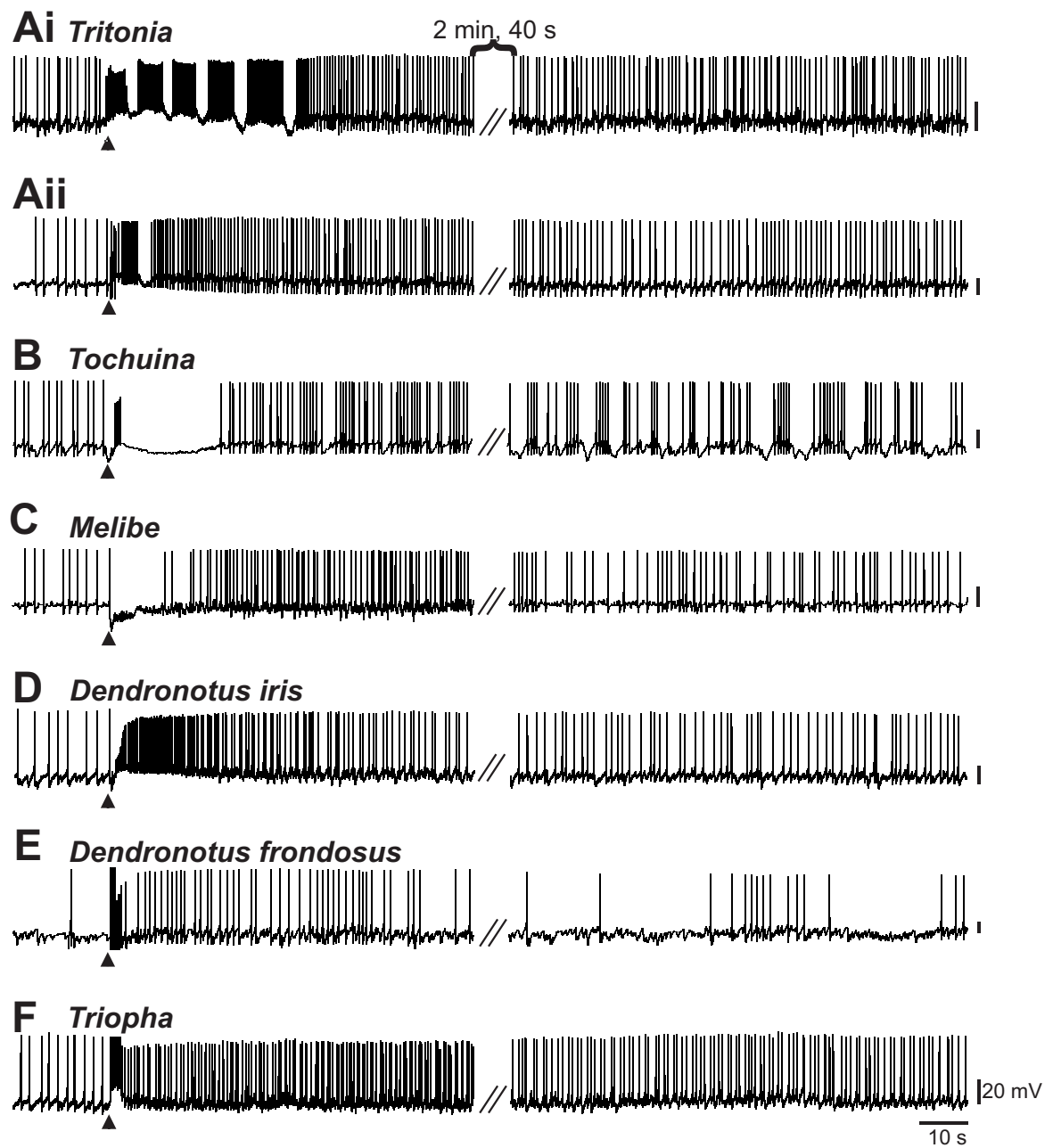
In *Tritonia*, the DSIs are members of the swim CPG and fire bursts of action potentials during a swim motor pattern, which can be elicited by stimulation of cerebral nerves 2 or 3, pleural nerve 1, or pedal nerves 2 or 3 (Fig. 3-7Ai; Dorsett et al., 1969;





**Figure 3-6:** Reciprocal inhibition by the *Tritonia* DSIs (A) and the *CeSP-A* neurons in *Tochuina* (B), *Melibe* (C), and *Triopha* (D). Depolarization of a DSI / *CeSP-A* neuron elicited inhibition of other ipsilateral and contralateral DSI / *CeSP-A* neurons. The DSIs in (A) were not identified as DSI<sub>A</sub> or DSI<sub>B/C</sub> and were therefore numbered for the purpose of this figure. In (D), the firing rate of the *CeSP-A*<sub>1</sub> neuron was increased with a slight depolarizing current pulse (top trace) to more clearly see the inhibition caused by depolarization of the *CeSP-A*<sub>2</sub> neuron.

**Figure 3-7:** Nerve stimulation of pedal nerve 2 (arrowhead) elevated the firing rate of the *CeSP-A* neurons in five species without eliciting rhythmic activity in these neurons. **Ai)** In *Tritonia*, a suprathreshold nerve stimulus elicited a transient swim motor pattern with rhythmic bursting activity in the DSI. This was followed by a prolonged period of elevated firing. In all figures, the diagonal lines separating traces represent 2 min 40 s of intervening time. **Aii)** Nerve stimulation (arrowhead) below the threshold for eliciting a swim motor pattern did not induce rhythmic activity in a DSI but did cause a prolonged increase in the firing rate. In *Tochuina* (**B**), *Melibe* (**C**), *Dendronotus iris* (**D**), *Dendronotus frondosus* (**E**), and *Triopha* (**F**), the response of *CeSP-A* neurons to pedal nerve stimulation (arrowheads) also mimicked the non-rhythmic, elevated firing exhibited by DSIs following a subthreshold stimulus (**Ai**). This particular example from *Tochuina* (**B**) exhibited periodic inhibitory input several minutes after nerve stimulation which made the firing pattern slightly rhythmic. However, this was not seen in any other preparations. **G)** Statistical analysis of the average spike frequency of *Melibe* *CeSP-A* neurons indicated that they were significantly inhibited for the first 20 s after nerve stimulation (arrowhead), followed by a significant increase in activity that eventually returned to baseline levels after 160 s ( $n = 6$ ). The bars represent 20-s bins, with the exception of the first bar (baseline), which is the average of the minute preceding nerve stimulation. Asterisks indicate a significant difference from baseline ( $p < 0.05$ ).



Getting et al., 1980; nerve nomenclature as in Willows et al., 1973). Following the swim motor pattern, the DSIs maintain an elevated level of spontaneous firing. Nerve stimulation that is subthreshold for a swim motor pattern does not elicit rhythmic bursts of action potentials in the DSIs but does still elevate the firing rate of the DSIs for up to an hour (Fig. 3-7Aii; Popescu and Frost, 2002).

Pedal nerve 2 was stimulated in five species: *Tochuina* (4 total neurons in 4 preparations), *Melibe* (6 neurons in 3 preparations), *Dendronotus iris* (4 neurons in 4 preparations), *Dendronotus frondosus* (1 neuron in 1 preparation), and *Triopha* (1 neuron in 1 preparation). The stimulus parameters that typically evoked rhythmic activity in *Tritonia* (10 – 20 V, 20 ms pulses, 2 - 10 Hz for 1 - 5 sec) did not elicit rhythmic bursting in the CeSP-A neurons of other species (Fig. 3-7B - F). However, the firing rate of the CeSP-A neurons was transiently elevated for several minutes. In some cases, the CeSP-A neurons were also initially inhibited for 10 – 20 s before proceeding to fire at an elevated frequency (*Tochuina* [50% of CeSP-A neurons], *Melibe* [100%], *Dendronotus iris* [25%]). Thus, although the CeSP-A neurons did not become rhythmically active in response to nerve stimulation in any species, the transiently elevated firing in CeSP-A neurons somewhat mimicked the response of the DSIs to low-intensity nerve shock.

Our sample size in *Melibe* was sufficiently large enough to statistically analyze this transient elevation of firing rate. Baseline (the 60 s prior to nerve stimulation) and post-stimulation firing rates were averaged into 20-second bins. In the first 20 s following extracellular stimulation of pedal nerve 2, the firing frequency of CeSP-A neurons was significantly lowered (Fig. 3-7G); baseline activity of the CeSP-A neurons was  $1.1 \pm 0.2$

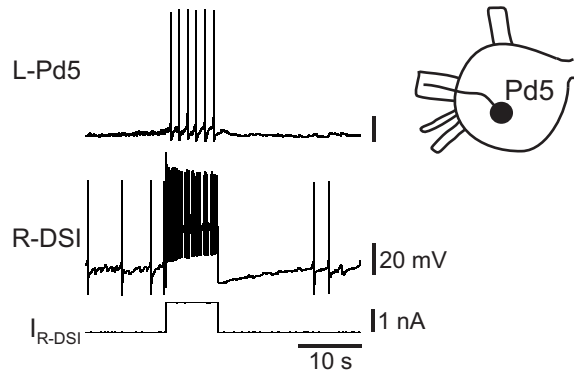
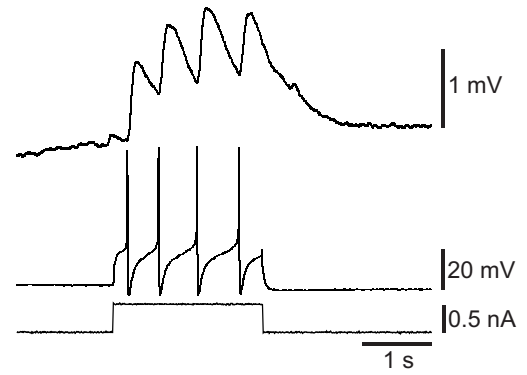
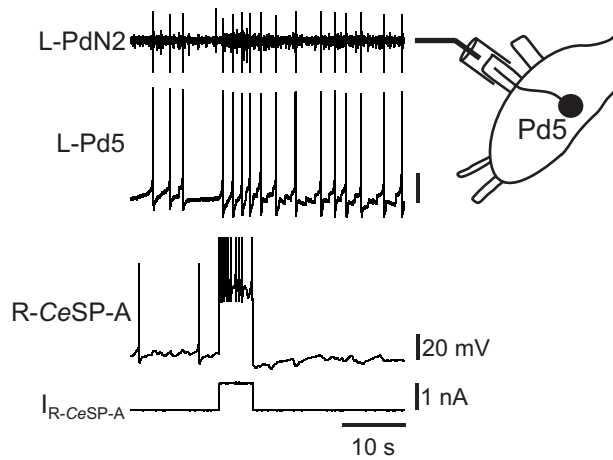
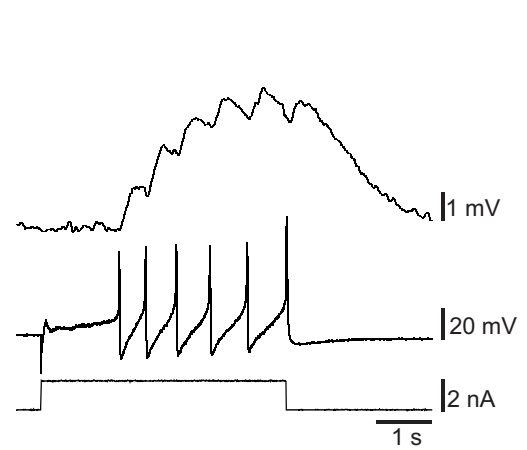
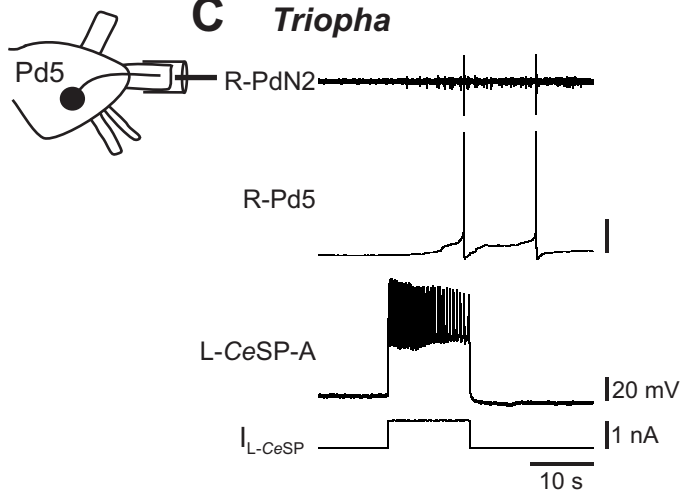
Hz and the firing rate during the 20 s following nerve shock dropped to  $0.6 \pm 0.1$  Hz. However, within 40 s after nerve shock, the firing rate had significantly increased to  $1.5 \pm 0.1$  Hz and reached an eventual maximal level of 150% of baseline by 60 s ( $1.7 \pm 0.2$  Hz). The firing frequency of the *CeSP-A* neurons returned to baseline levels within 160 s after nerve shock. This return to basal firing rate was more rapid than the prolonged elevation of firing rate in the DSIs (Fig. 3-7Ai; Popescu and Frost, 2002).

#### *CeSP-A neurons excite Pd5 homologues*

Pd5 is uniquely identifiable in *Tritonia*, based simply on its soma location and size; it is the largest neuron in the pedal ganglion (Willows et al., 1973). Additional identifying characteristics include an axon that projects out pedal nerve 3 and TPep immunoreactivity (Lloyd et al., 1996; Popescu and Willows, 1999; Cain et al., 2006). We found similar neurons in *Tochuina* ( $n = 2$ ) and *Triopha* ( $n = 5$ ) that satisfied all of these identifying characteristics. In conjunction with other data that suggest that these neurons are highly conserved in the nudibranch clade (Baltzley, 2006), we conclude that these neurons are homologues of Pd5.

The DSIs, in addition to being members of the swim CPG in *Tritonia*, monosynaptically excite efferent crawling neurons in the contralateral pedal ganglion, such as Pd5, as was previously shown (Popescu and Willows, 1999; Popescu and Frost, 2002) and replicated here (Fig. 3-8A, 1 DSI in 1 preparation). Depolarization of a *CeSP-A* neuron to increase its spiking caused an elevation in the firing rate of the contralateral Pd5 homologue in both *Tochuina* (total of 3 *CeSP-A* neurons in 2

**Figure 3-8:** *CeSP-A* neurons had excitatory connections with homologues of the *Tritonia* Pd5 neuron. **Ai)** In *Tritonia*, elevating the firing rate of a DSI with current injection induced action potentials in a silent, contralateral Pd5. The location of Pd5 and its projection out pedal nerve 3 is illustrated at right. **Aii)** This excitatory connection was monosynaptic, as indicated by one-for-one EPSPs in the Pd5 in the presence of high-divalent cation saline. **Bi)** In *Tochuina*, increasing the firing frequency of a *CeSP-A* neuron with current injection also caused an increase in the firing rate of the contralateral Pd5 homologue. The Pd5 homologue was identified based on size and a corresponding large unit in pedal nerve 2, which is the equivalent to the *Tritonia* pedal nerve 3. **Bii)** As with *Tritonia*, this excitatory connection persisted in high-divalent cation saline, indicating it was monosynaptic. **C)** Depolarization of a *CeSP-A* neuron in *Triopha* also elicited spiking activity in the contralateral Pd5 homologue, which was identified as in *Tritonia* and *Tochuina*.

**Ai** *Tritonia***Aii****Bi** *Tochuina***Bii****C** *Triopha*

preparations) and *Triopha* (1 *CeSP-A* neuron in 1 preparation) (Fig. 3-8Bi & C). In *Tochuina*, this excitatory connection remained after perfusion with high-divalent cation saline; action potentials in *CeSP-A* neurons produced one-for-one excitatory post-synaptic potentials in the Pd5 homologue (Fig. 3-8Bii, n = 2). Thus, as with the *Tritonia* DSIs, the *CeSP-A* neurons in *Tochuina* and *Triopha* made excitatory synaptic connections with the contralateral Pd5.

## **Discussion**

In this study, we identified and characterized the *CeSP-A* neurons in six nudibranch species. Morphological characteristics, such as serotonin immunoreactivity, soma location, and axon projection pattern, were remarkably similar between *CeSP-A* neurons in each species (Fig. 3-9). Furthermore, these characteristic also apply to neurons in other opisthobranchs such as *Tritonia* (Getting et al., 1980; Katz et al., 1994; McClellan et al., 1994), *Hermisenda* (Tian et al., 2006), *Pleurobranchaea* (Jing and Gillette, 1999), *Aplysia californica* (McPherson and Katz, 2001), and *Clione limacina* (Panchin et al., 1995; Satterlie and Norekian, 1995) (Fig. 3-9). The most parsimonious explanation for this consistent morphological pattern is that this cell type was present in a common ancestor and, therefore, that these neurons are homologous to each other (Striedter and Northcutt, 1991).

Many electrophysiological characteristics of these homologous neurons have been conserved, including the basal firing pattern, synchronous synaptic input, electrical



**Figure 3-9:** Comparison of the *CeSP-A* neurons and their homologues in other opisthobranch species. "+" indicates the presence of a characteristic, "-" indicates the lack of a characteristic, and a blank space indicates that the characteristic was not determined. Species marked with an asterisk were examined in this study. Data for other species as follows: *Hermisenda* (Tian et al., 2006), *Pleurobranchaea* (Jing and Gillette, 1999, 2000), *Aplysia* (McPherson and Katz, 2001), and *Clione* (Panchin et al., 1995; Satterlie and Norekian, 1995). The phylogeny summarizes morphological and molecular data (Thollessen, 1999; Wägele and Willan, 2000; Wollscheid-Lengeling et al., 2001; Grande et al., 2004; Vonnemann et al., 2005).

Subclass	Opisthobranchia										
Order	Nudibranchia					Notaspidea		Anaspidea		Gymnosomata	
Family	Dendronotoidea					Aeolidioidea	Arminoidea	Doridoidea	Pleurobranchioidea	Aplysioidea	Gymnosomata
Genus/Species	<i>Tritonia diomedea</i> *	<i>Tochuina tetraquetra</i> *	<i>Melibe leonina</i> *	<i>Dendronotus irirs</i> *	<i>Dendronotus frondosus</i> *	<i>Hermisenda crassicornis</i>	<i>Armina californica</i> *	<i>Triopha catalinae</i> *	<i>Pleurobranchaea californica</i>	<i>Aplysia californica</i>	<i>Cione limacina</i>
Neuron Name	DSI	CeSP-A				CPT	CeSP-A		As1-3	CC9,10	Cr-SP
Defining Morphological Characteristics											
Soma in posterior cerebral ganglion	+	+	+	+	+	+	+	+	+	+	+
Projects to contra. pedal ganglion	+	+	+	+	+	+	+	+	+	+	+
Serotonin-immunoreactive	+	+	+	+	+	+	+	+	+	+	+
Physiological Characteristics											
Irregularly active at rest	+	+	+	+	+	+		+	+	+	+
Increased firing after pedal nerve stim.	+	+	+	+	+	+		+	+		
Synchronous firing & synaptic input	+	+	+			+		+		+	
Reciprocal inhibition	+	+	+					+		+	
Electrically coupled	+	+	+			+			+	+	
Excites pedal efferents	+	+				+		+	+	+	+
Large spike after-hyperpolarization	+	+	-	-		-		-		+	
Rhythmic activity	+	-	-	-	-			-	+		

coupling, reciprocal inhibition, and excitatory connections to homologous pedal neurons (Fig. 3-9). However, none of the *CeSP-A* neurons exhibited rhythmic activity in response to nerve stimulation. This contrasts with the *Tritonia* DSIs, which are members of a CPG circuit for a rhythmic swim motor pattern (Getting et al., 1980). These results suggest that the *CeSP-A* neurons are highly conserved and may have a common, non-rhythmic function involved in exciting crawling efferent neurons.

#### *Homologues of the CeSP-A neurons in other species*

Homologues of the *CeSP-A* neurons have been recently identified in another nudibranch, *Hermissenda*, where they are referred to as the cerebropleural triplet neurons (CPT; Tian et al., 2006). As with the DSIs / *CeSP-A* neurons, the CPT neurons respond with elevated firing in response to noxious sensory stimuli. Crawling is the primary form of locomotion for *Hermissenda*, although they will occasionally dislodge from a substrate and exhibit a brief bout of lateral flexions reminiscent of lateral flexion swimming in other nudibranchs (unpublished observation). The CPT neurons were shown to not elicit ciliary activity or excite identified ciliary efferents in *Hermissenda*, but they do excite pedal motoneurons responsible for contractions of the anterior foot (Tian et al., 2006), suggesting a potential role in arousal based on sensory cues.

The As1-3 neurons in the notaspid *Pleurobranchaea* are remarkably similar to the DSIs. As with *Tritonia*, *Pleurobranchaea* swims by alternately flexing its body in the dorsal and ventral directions (Jing and Gillette, 1995). The As1-3 neurons are members

of the dorsal-ventral swim CPG in *Pleurobranchaea* (Jing and Gillette, 1999), playing a role similar to the DSIs in *Tritonia* (Getting et al., 1980). The As1-3 neurons resemble the DSIs in many other ways as well, such as their basal firing properties, elevated levels of activity after a swim, and synaptic connections to crawling neurons (Jing and Gillette, 1999, 2000, 2003). These features also resemble the CeSP-A neurons in this study (Fig. 3-9), suggesting that this group of neurons was present in a common ancestor of the nudibranchs and *Pleurobranchaea*, a group sometimes referred to as the Nudipleura (Wägele and Willan, 2000). Jing and Gillette (1999) concluded that rhythmic activity in the As1-3 neurons and the *Tritonia* DSIs represents the ancestral state for the Nudipleura. However, the absence of rhythmic activity in nudibranchs in each of the nudibranch suborders suggests that rhythmic activity in *Tritonia* and *Pleurobranchaea* may have arisen independently (Katz and Newcomb, in press).

*Aplysia californica* and *Clione limacina* are more distantly-related opisthobranchs with different modes of locomotion than *Tritonia* and *Pleurobranchaea*. *Aplysia californica* cannot swim and crawls via muscular contractions of the foot (Bebbington and Hughes, 1973). *Clione* is purely pelagic and swims via parapodial flapping (Satterlie et al., 1985). Although the DSI homologues in these species, the CC9 & 10 cells in *Aplysia* and the Cr-SP neurons in *Clione*, are not part of the locomotor CPGs in these animals, they can elicit locomotion and have synaptic connections with pedal motoneurons (Fredman and Jahan-Parwar, 1983; Arshavsky et al., 1992; Panchin et al., 1995; Satterlie and Norekian, 1995; McPherson and Katz, 2001), reminiscent of the *Tritonia* DSIs (Popescu and Willows, 1999; Fickbohm and Katz, 2000; Frost et al., 2001;

Popescu and Frost, 2002; Katz et al., 2004) and the *CeSP-A* neurons in the current study. Another *Aplysia* species, *Aplysia brasiliana*, swims by parapodial flapping. The CPG underlying this rhythmic behavior has been localized to the pedal ganglia (von der Porten, et al., 1982). Command neurons in the cerebral ganglion activate the swim motor pattern (Gamkrelidze et al., 1995) and injection of 5-HT into the animal also elicits swimming (Parsons and Pinsker, 1989). The description of these command neurons leaves open the possibility that they are homologous to the *CeSP-A* neurons, but intracellular dye fills with 5-HT staining would be required to make a stronger case for homology. Thus, putative homologues of the *CeSP-A* neurons may be present and exhibit similar functions across distantly related opisthobranchs with different locomotor behaviors.

#### *Basal function of CeSP-A neurons*

The high degree of conservation of the *CeSP-A* neurons suggests that they may serve a common, important function in these animals. Serotonin is known to be important for arousal in mammals (Lucki, 1998) and evidence suggests that the serotonergic system of opisthobranchs constitutes an arousal network (Katz et al., 2001; Marinesco et al., 2004a, b). Our results demonstrate that the *CeSP-A* neurons in six species of nudibranchs exhibit a transient period of elevated firing rate in response to nerve stimulation. The homologues of the *CeSP-A* neurons in *Tritonia*, *Hermisenda*, *Pleurobranchaea*, and *Clione* also respond with heightened activity in response to noxious stimuli (Arshavsky et al., 1992; Jing and Gillette, 2000; Katz et al., 2001; Popescu and Frost, 2002; Tian et al., 2006). In *Aplysia californica*, the majority of serotonergic neurons in the brain fire in

response to tail-nerve shock, including the CB1/CC3 neuron (Mackey et al., 1989; Wright et al., 1995; Marinesco, 2004a), which is a medial member of the *CeSP* cluster (Xin et al., 2001).

One of the potential purposes of this arousal is to initiate or modulate locomotion. As noted above, the homologues of the *CeSP*-A neurons in *Tritonia*, *Pleurobranchaea*, *Aplysia*, and *Clione* all serve locomotor functions. Considering that the *CeSP*-A neurons are serotonergic and serotonin has potent effects on locomotion in opisthobranchs (Audesirk et al., 1979; Palovcik et al., 1982; Mackey and Carew, 1983; Parsons and Pinsker, 1989; Kabotyanskii and Sakharov, 1991; McClellan et al., 1994), it is quite plausible that the basal function of the *CeSP*-A neurons is to elicit or modulate locomotion. With the exception of the pelagic *Clione*, all of the opisthobranchs that are known to have homologues of the *CeSP*-A neurons, including the species in this study, use crawling as their primary form of locomotion and it is most likely the basal locomotor condition in the opisthobranch clade (Thompson, 1976; Chase, 2002). In addition, the *CeSP*-A neurons excite putative crawling motoneurons and other pedal cells in all species examined to date. Thus, the *CeSP*-A neurons may act as initiators or modulators of crawling.

In *Tritonia*, the DSIs play an important neuromodulatory role; altering the synaptic and cellular properties of other members of the swim CPG (Katz et al., 1994; Sakurai and Katz, 2003). It is possible that neuromodulatory actions of the DSIs reconfigure a crawling network into a rhythmically active swim CPG. Thus, differences

in the effects of the DSIs and their homologues, the *CeSP-A* neurons might be crucial for understanding the evolution of the *Tritonia* swimming behavior.

#### *Divergent characteristics of CeSP-A neurons*

There must be species differences in neuronal properties or synaptic connectivity that would account for the fact that the *Tritonia* DSIs are members of a CPG for a rhythmic motor pattern (Getting et al., 1980), even though their homologues, the *CeSP-A* neurons, are not rhythmically active in any of the other nudibranchs so far examined. The only differences that we were able to determine between the DSIs and the *CeSP-A* neurons were the lack of a significant after-hyperpolarization following action potentials in the *CeSP-A* neurons of some species and differences in specific electrical connections in *Melibe* compared to *Tritonia*. An after-hyperpolarization does not necessarily appear to confer rhythmic capability in these neurons because the *CeSP-A* neurons in *Tochuina*, which is a non-swimmer in the same family as *Tritonia*, did have an after-hyperpolarization that was not significantly different from that seen in the DSIs.

The differences in electrical coupling may correspond to the ability to produce rhythmic activity. The *CeSP-A* neurons in *Melibe* are all electrically coupled to each other, both ipsilaterally and contralaterally (Fig. 3-5E). In contrast, the As1-3 neurons in *Pleurobranchaea* have the same pattern of electrical connectivity as the *Tritonia* DSIs (Fig. 3-5B); the As1 neuron is electrically coupled only to the contralateral As1 neuron, and the As2/3 neurons are electrically coupled to each other both ipsilaterally and

contralaterally (Jing and Gillette, 1999). As the *Melibe* CeSP-A neurons are not rhythmically active, whereas the DSIs and the As1-3 neurons are components of CPGs for rhythmic dorsal-ventral swimming; it is possible that this pattern of electrical connectivity is related to the ability of these neurons to be rhythmically active. Additional species will need to be examined to further test this correlation.

The lack of rhythmic activity in the *CeSP-A* neurons may also be caused by different synaptic connectivity than the *Tritonia* DSIs. In addition to the recruitment of inhibition, the DSIs also make direct excitatory chemical synapses with each other (Getting, 1981). We did not test whether this mutual excitation is present in the *CeSP-A* neurons of other nudibranchs. Furthermore, since we have not yet identified homologues of the other *Tritonia* CPG neurons, we were unable to test their interactions with the *CeSP-A* neurons.

The conservation of the *CeSP-A* neurons in a wide array of taxonomic groups with differing locomotor behaviors provides an excellent opportunity to investigate evolutionary changes at the single-neuron and circuit levels. Characteristics of the *CeSP-A* neurons that have been highly conserved, such as morphology, neurotransmitter, and even certain synaptic contacts, must be evolutionarily constrained and thus might be expected to serve essential core functions. In contrast, divergent characteristics, such as spike properties, electrical connectivity, and intrinsic or extrinsic factors contributing to rhythmicity appear to be evolutionarily labile and may have contributed to the evolution of species-specific behaviors.



These results also suggest that the evolution of novel rhythmic motor patterns can involve changes in the nervous system, as opposed to just the periphery. It has already been demonstrated that differences in homologous neural structures can correlate with variation in behavior (Wilson et al., 1982; Shaw and Meinertzhagen, 1986; Shaw and Moore, 1989; Buschbeck and Strausfeld, 1997; Wright, 2000; Chiang et al., 2006) and that the evolutionary loss of specific motor patterns can involve changes in the central nervous system (Arbas, 1983a,b; Paul, 1991; Faulkes, 2004). However, the evolutionary gain of function also can involve changes in the nervous system. Rhythmic motor patterns have evolved from non-rhythmic motor systems many times during the course of evolution. For example, rhythmic vocalization and communication have evolved multiple times in the vertebrate lineage (Bass, 1986; Dye and Meyer, 1986; Grant et al., 1986; Bass and Baker, 1990; Schmidt, 1992; Margoliash et al., 1994; Streidter, 1994; Wild, 1994, 1997; Metzner, 1999; Bass and McKibben, 2003). It has been proposed that the networks producing these rhythmic motor patterns evolved from pre-existing circuitry (Bass and Baker, 1997). Comparisons of homologous neurons in our study support this idea by suggesting that such evolutionary innovations in rhythmic circuitry can be produced by subtle reconfiguration of neurons already present in the central nervous system.

## CHAPTER FOUR

### SEROTONIN AND HOMOLOGOUS SEROTONERGIC INTERNEURONS PARTICIPATE DIFFERENTLY IN DIVERGENT RHYTHMIC BEHAVIORS

#### **Abstract**

The functions of neurotransmitters and central neural circuits are considered to have been relatively conserved during the evolution of species-specific behaviors. Nudibranch molluscs exhibit species-specific swimming behaviors: *Tritonia diomedea* swims by flexing its body in the dorsal and ventral directions, whereas *Melibe leonina* swims by flexing its body from side-to-side. In *Tritonia*, the DSIs are members of the swim CPG. It was previously shown that these neurons and their neurotransmitter 5-HT are necessary and sufficient for production of this rhythmic behavior. In *Melibe*, homologues of the DSIs, the *CeSP-A* neurons, were not rhythmically active during the lateral flexion swim motor pattern (SMP) and therefore are not intrinsic to the swim CPG. However, the *CeSP-A* neurons could influence the swim CPG; depolarization of a *CeSP-A* neuron was sufficient to initiate the SMP in quiescent preparations, and hyperpolarization halted an ongoing SMP. Bath-application of 5-HT also could initiate and regularize the SMP. Neither the *CeSP-A* neurons nor 5-HT was necessary for production of the SMP: 1) SMPs could occur under several conditions in which the *CeSP-A* neurons were inactive; and 2) methysergide blocked the effects of both 5-HT and the *CeSP-A* neurons but did not prevent production of the SMP. In summary, the

functions of these homologous neurons (and their neurotransmitter 5-HT) differ in these two species: in *Tritonia*, the DSIs are intrinsic to the dorsal-ventral swim CPG and necessary for the behavior, whereas in *Melibe*, the *CeSP-A* neurons are extrinsic to the lateral swim CPG and not necessary for the behavior. This suggests that the evolution of species-specific behaviors could have involved alterations of interneuronal circuitry with a corresponding change in the role of a neurotransmitter.

### **Introduction**

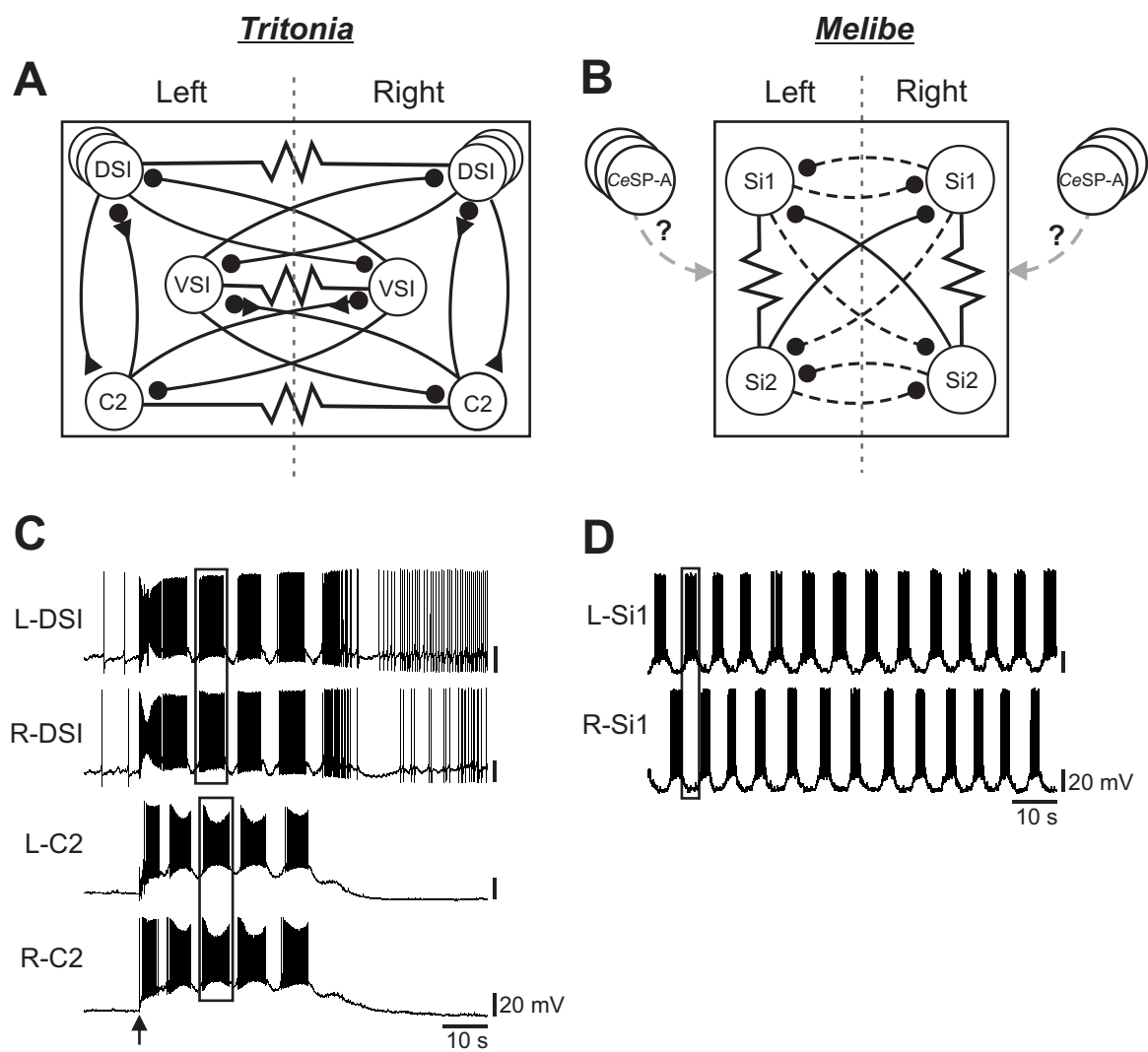
There has been much speculation regarding the extent to which the central nervous system changes during the evolution of species-specific behaviors (Dumont and Roberston, 1986; Kavanau, 1990; Arbas et al., 1991; Smith, 1994; Tierney, 1995; Nishikawa, 1997; Katz and Harris-Warrick, 1999; Wainwright, 2002). Respecification of motor neurons and sensory structures is known from several examples (Sillar and Heitler, 1985; Shaw and Meinertzhagen, 1986; Shaw and Moore, 1989; Paul, 1991; Katz and Tazaki, 1992; Volman, 1994; Buschbeck and Strausfeld, 1997; Wright, 2000; Chiang et al., 2006), but little is known about the degree to which homologous interneurons change their functions to produce species-specific behaviors. Nudibranch molluscs offer the possibility of studying the evolution of neural mechanisms underlying species-specific behavior. Homologous neurons can be identified across species (see Chapter Three) and the neural circuits for some behaviors have already been established in some

key species. In this study, we examine the function of homologous neurons in two closely related nudibranchs that exhibit different locomotor behaviors.

*Tritonia diomedea* and *Melibe leonina* are two nudibranchs in the suborder Dendronotoidea that differ in their swimming behavior: *Tritonia* swims by alternately flexing its body in the dorsal and ventral directions (Willows, 1968; Hume et al., 1982), whereas *Melibe* swims by flexing its body from side-to-side (Agersborg 1919, 1921; Hurst, 1968; Watson et al., 2001; Lawrence and Watson, 2002). These behaviors differ in a number of other fundamental ways. The *Tritonia* swim is an escape response; it is produced in response to only a few types of intense stimuli (Willows, 1968). Although the *Melibe* swim also can function as an escape response, it additionally can occur spontaneously or as a result of the animal being dislodged from the substrate (Lawrence and Watson, 2002). A *Tritonia* swim episode lasts for less than a minute (Hume et al., 1982), which is much shorter than episodes of *Melibe* swimming which can last for up to an hour (Mills, 1994; Lawrence and Watson, 2002; Caldwell and Donovan, 2003).

The neural bases of the two behaviors are also distinct. Neurons comprising the CPGs for these two rhythmic behaviors have been identified (Gettings, 1981, 1989; Thompson and Watson, 2005) (Fig 4-1). The neurons in the *Tritonia* swim CPG are not homologous to the neurons in the *Melibe* swim CPG (Katz and Newcomb, in press). Furthermore, the CPGs are organized in fundamentally different fashions; contralateral counterparts are electrically coupled in *Tritonia* (Fig. 4-1A), whereas they are reciprocally inhibitory in *Melibe* (Fig 4-1B). This leads to a difference in their respective outputs, with left and right sides operating in synchrony in *Tritonia* (Fig. 4-1C) and in

**Figure 4-1:** Neural bases of dorsal-ventral flexion swimming in *Tritonia diomedea* (**A & C**) and lateral flexion swimming in *Melibe leonina* (**B & D**). **A)** The *Tritonia* swim CPG consists of three bilateral interneurons, the serotonergic DSIs, C2, and VSI (Gettings, 1989). Contralateral counterparts are electrically coupled to each other (Gettings, 1981). **B)** The swim CPG in *Melibe* is comprised of two bilateral swim interneurons, Si1 and Si2 (Thompson and Watson, 2005). Neither of these neurons is homologous to any of the *Tritonia* swim interneurons. However, homologues of the *Tritonia* DSIs, the *CeSP-A* neurons, have been identified in *Melibe* (see Chapter Three). It is currently unknown whether the *CeSP-A* neurons are involved in lateral swimming in *Melibe* (gray dotted arrows). **C)** Interneurons in the *Tritonia* swim CPG, including the DSIs, exhibit rhythmic bursting activity during a transient swim motor pattern elicited by pedal nerve stimulation (arrow) in an isolated brain preparation. Contralateral counterparts burst in unison (boxes). **D)** In *Melibe*, contralateral swim interneurons exhibit alternating bursts of activity (box) during a spontaneous swim motor pattern in an isolated brain preparation. In the circuit diagrams, circles represent inhibitory connections, triangles represent excitatory connections, and resistors indicate electrical connections. Several of the contralateral inhibitory connections between swim interneurons in the *Melibe* swim circuit are represented with dotted lines because it is unclear whether these connections are monosynaptic or polysynaptic. Abbreviations: L = left; R = right.



alternation in *Melibe* (Fig. 4-1D).

The DSIs are serotonergic neurons (Katz et al., 1994; McClellan et al., 1994) that are members of the dorsal-ventral swim CPG in *Tritonia* (Gettings et al., 1980) (Fig 4-1). The DSIs are both necessary and sufficient for swimming in *Tritonia*: hyperpolarization of the DSIs can halt the swim motor pattern (Gettings et al., 1980) and activation of the DSIs can initiate the motor pattern (Fickbohm and Katz, 2000; Frost et al., 2001; Katz et al., 2004). We define “sufficiency” as the capability of initiating the behavior and motor pattern when all other components of the circuit are allowed to function normally. Similarly, 5-HT is also necessary and sufficient for swimming behavior: application of 5-HT can initiate swimming in the animal and the swim motor pattern in the isolated nervous system, and the 5-HT antagonist, methysergide, can block swimming and the swim motor pattern (McClellan et al., 1994).

We recently identified putative DSI homologues, which we named the *CeSP-A* neurons, in a number of nudibranch species that do not swim with dorsal-ventral flexions (see Chapter Three). Here, we examine how the *CeSP-A* neurons and 5-HT in *Melibe* interact with the swim CPG for lateral flexion (side-to-side) swimming and how these interactions compare with the functional effects of the *Tritonia* DSIs.

We found that the *Melibe CeSP-A* neurons play a different role from the *Tritonia* DSIs; they are not part of the lateral-flexion swim CPG and are not necessary for production of the swim motor pattern, but their activity is sufficient to elicit the swim motor pattern. This suggests that the evolution of neural circuits underlying swimming

behaviors in these species involved a divergence in the function of homologous neurons and their neurotransmitter, 5-HT.

## **Methods**

### *Animal collection and maintenance*

Adult *Melibe leonina* (5 – 10 cm) were collected from the dock at FHL, Friday Harbor, WA and from subtidal eelgrass beds at nearby Shaw Island, WA. Additional specimens of *Melibe* were collected by Living Elements (Vancouver, British Columbia) at the Indian Arm extension of Burrard Inlet in Vancouver Harbor.

Animals were either kept in seawater tables at FHL at ambient seawater temperatures and light/dark cycles, or in recirculating artificial seawater tanks at Georgia State University at 10° C and a fixed 12:12 light/dark cycle.

### *Isolated brain preparation*

Animals were anesthetized by chilling and then pinned on their side in a Sylgard-lined dish. The integument was cut lateral to the esophagus and the brain, consisting of the cerebral, pedal, and pleural ganglia, was removed by cutting all nerve roots. Care was taken to leave the two pedal-pedal connectives intact, as the brain does not exhibit swim motor pattern activity if these connectives are damaged (Thompson and Watson, 2005). The brain was transferred to a smaller Sylgard-lined dish where it was perfused, at a rate of 0.5 ml/min, with artificial saline (in mM): 420 NaCl, 10 KCl, 10 CaCl<sub>2</sub>, 50 MgCl<sub>2</sub>, 11



D-glucose, and 10 HEPES, pH. 7.6. Connective tissue surrounding the brain was manually removed with forceps, fine scissors, and a tungsten wire while keeping the brain at  $\sim 4^{\circ}\text{C}$  to reduce neuronal firing. The temperature was raised to  $10^{\circ}\text{C}$  for electrophysiological experiments.

### *Electrophysiology*

Intracellular recordings were obtained using 10 - 30 M $\Omega$  glass microelectrodes filled with 3 M potassium chloride and connected to Axoclamp 2B (Axon Instruments, Union City, CA) or Dagan IX2-700 (Dagan Corporation, Minneapolis, MN) amplifiers. Electrodes were dipped in ink extruded from a black permanent marker (Sharpie, Sanford Corporation, Oak Brook, IL) to make it easier to see the fine tip. Extracellular suction electrode recordings were obtained by drawing individual nerves into polyethylene tubing filled with saline and connected to an A-M Systems Differential AC Amplifier (model 1700, A-M Systems, Inc., Sequim, WA). Both intracellular and extracellular recordings were digitized with a 1401 Plus or Micro 1401 A/D converter from Cambridge Electronic Design (CED, Cambridge, UK) and acquired with Spike2 software (CED). Nerve stimulation was performed with Spike2, via the 1401, or applied from an A-M Systems Isolated Pulse Stimulator (model 2100) or a Grass Instruments S48 Square Pulse Stimulator (Grass Telefactor, Warwick, RI). Pedal nerve 2 (PdN2; according to nerve nomenclature in Newcomb et al., 2006) was consistently used for nerve stimulation. The stimulation consisted of 10 – 20 V, 2 ms-pulses at 2 Hz for 5 s. *CeSP-A* neurons, as well as Si1 and Si2, were identified based on soma location and electrophysiological

properties, as previously characterized (Thompson and Watson, 2005; Chapter Three). A fiber-optic light with rheostat-controlled intensity was used for illumination of the preparation.

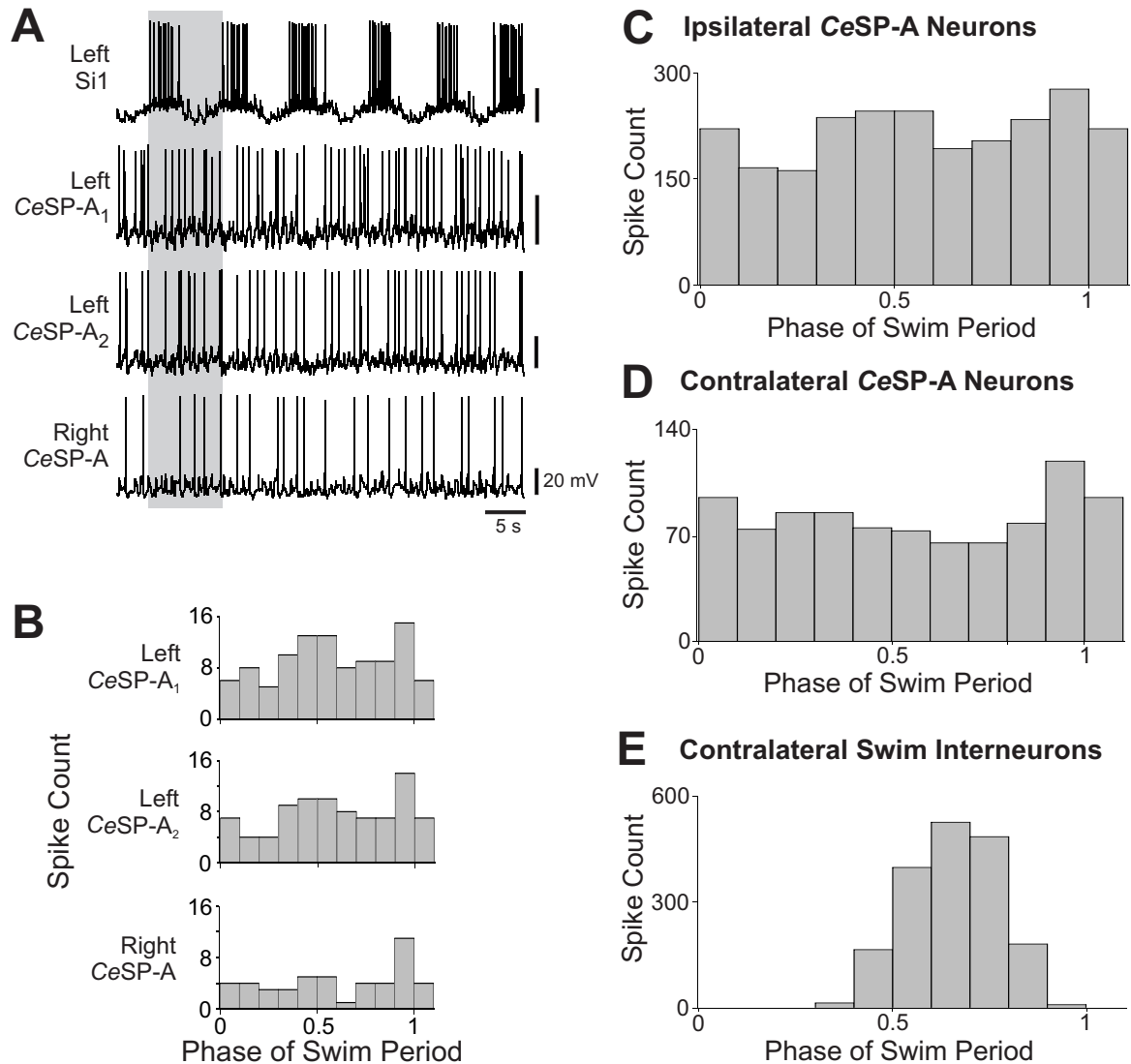
Analysis of electrophysiological data was performed using Spike2 and SigmaPlot (Systat Software, Inc., Point Richmond, CA). Statistical comparisons of means were made in InStat (GraphPad Software, San Diego, CA) with repeated measures, one-way ANOVAs and Dunnett *post hoc* tests. Results were considered significantly different with a *p*-value less than 0.05. Results are expressed as mean  $\pm$  standard error.

Serotonin (5-hydroxytryptamine creatine sulfate) was dissolved in saline at final concentrations (1 – 100  $\mu$ M) just before use. The serotonin receptor antagonist, methysergide, was dissolved in dimethyl sulfoxide at 10 mM and diluted to 1 – 200  $\mu$ M in saline just before use. Drugs were bath applied by switching perfusion paths. All drugs were acquired from Sigma (St. Louis, MO).

## **Results**

### *CeSP-A neurons are not intrinsic to the swim CPG*

The *CeSP-A* neurons in *Melibe* do not display characteristics of swim CPG neurons. Simultaneous intracellular recordings showed that the *CeSP-A* neurons did not fire rhythmic bursts of action potentials during a swim motor pattern typical of a CPG neuron such as Si1 (Fig. 4-2A, *n* = 25). Phase histograms indicated only weak correspondence between firing in *CeSP-A* neurons and swim interneurons during swim



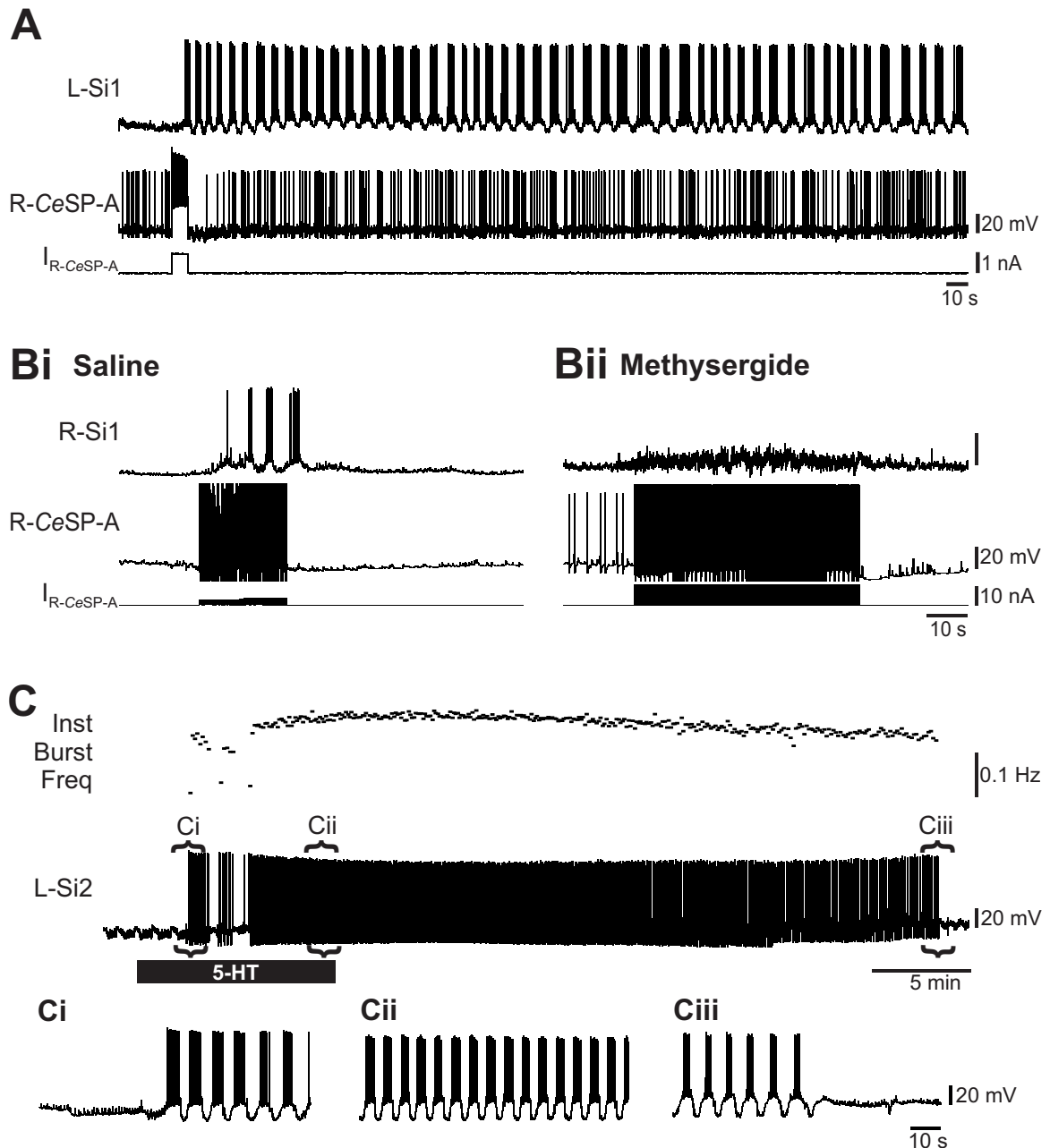
**Figure 4-2:** The *CeSP-A* neurons are not members of the swim CPG. **A)** Simultaneous intracellular recordings indicate that both the left and right *CeSP-A* neurons were not very rhythmically active in relation to the left Si1. **B)** Phase histogram of recording in **(A)**, plotting the total number of spike counts for each *CeSP-A* neuron during ten equivalent portions of the cycle period in Si1. A cycle period was designated as the time between the first action potential of consecutive bursts in Si1 [gray box in **(A)**]. There was a very slight drop in activity of the *CeSP-A* neurons a third of the way through the swim cycle and a slight increase in activity halfway through the swim cycle. This pattern was slightly more pronounced in the *CeSP-A* neurons ipsilateral to Si1. The largest peak of activity in the *CeSP-A* neurons was just prior to the start of a swim cycle in Si1. Across multiple preparations, the *CeSP-A* neurons ( $n = 9$  in **[C]**;  $n = 5$  in **[D]**) exhibited relatively weak rhythmic activity in relation to ipsilateral **(C)** ( $n = 6$ ) and contralateral **(D)** ( $n = 4$ ) swim interneurons, suggesting that they are not members of the swim CPG. **E)** A phase histogram of contralateral swim interneurons ( $n = 2$ ) illustrates strong rhythmic activity in relation to the swim motor pattern.

motor patterns (Fig. 4-2B - D). Because the *Melibe* swim motor pattern alternates between activity in the left and right sides, one might expect that if the *CeSP-A* neurons were members of the swim CPG, then their activity relative to the ipsilateral swim interneurons would be 50% out of phase with the activity relative to the contralateral swim interneurons. This was not the case; the correspondence between *CeSP-A* neurons and either ipsilateral or contralateral swim interneurons did not show complementary relationships (Fig. 4-2C & D). In contrast, the firing of Si1 and Si2 was highly correlated with the swim motor pattern expressed in the contralateral swim interneurons (Fig. 4-2E,  $n = 3$ ), as would be expected for a CPG neuron. These results suggest that the *CeSP-A* neurons are not members of the lateral flexion swim CPG in *Melibe*.

*CeSP-A activity or 5-HT is sufficient to elicit the swim motor pattern*

A depolarizing current pulse or a train of action potentials in one or more *CeSP-A* neurons was sufficient to elicit a swim motor pattern in 16 of 18 preparations at times when the swim motor pattern was not spontaneously expressed (Fig. 4-3A & Bi). The *CeSP-A* elicited swim motor pattern could continue for a long time following the end of the *CeSP-A* stimulus, with durations ranging from 3 s to 82 min (mean duration  $214 \pm 122$  s). Therefore, the *Melibe CeSP-A* neurons can initiate a swim motor pattern that can continue for a substantial period of time after the end of the stimulus.

To determine if 5-HT is responsible for the ability of the *CeSP-A* neurons to elicit a swim motor pattern, we used the 5-HT antagonist methysergide, which is effective in *Tritonia* (McClellan et al., 1994; Katz and Frost, 1995a; Sakurai and Katz, 2003). Bath-



**Figure 4-3:** The *CeSP-A* neurons and 5-HT are sufficient to elicit the swim motor pattern. **A)** During a period of quiescence in the swim motor pattern, depolarization of a single *CeSP-A* neuron elicited a prolonged swim motor pattern, as monitored via Si1. A train stimulus applied to a single *CeSP-A* neuron (20ms pulses at 10 Hz) elicited a brief swim motor pattern (**Bi**) and this was blocked by perfusion with the 5-HT receptor antagonist methysergide (100  $\mu$ M) (**Bii**). **C)** Similar to a *CeSP-A* neuron-induced swim motor pattern, perfusion of a quiescent preparation with 5-HT (10  $\mu$ M) elicited a swim motor pattern, as monitored via Si2 (**Ci** and **Cii**) and its instantaneous burst frequency (Inst Burst Freq). The preparation returned to quiescence after washout with saline (**Ciii**).

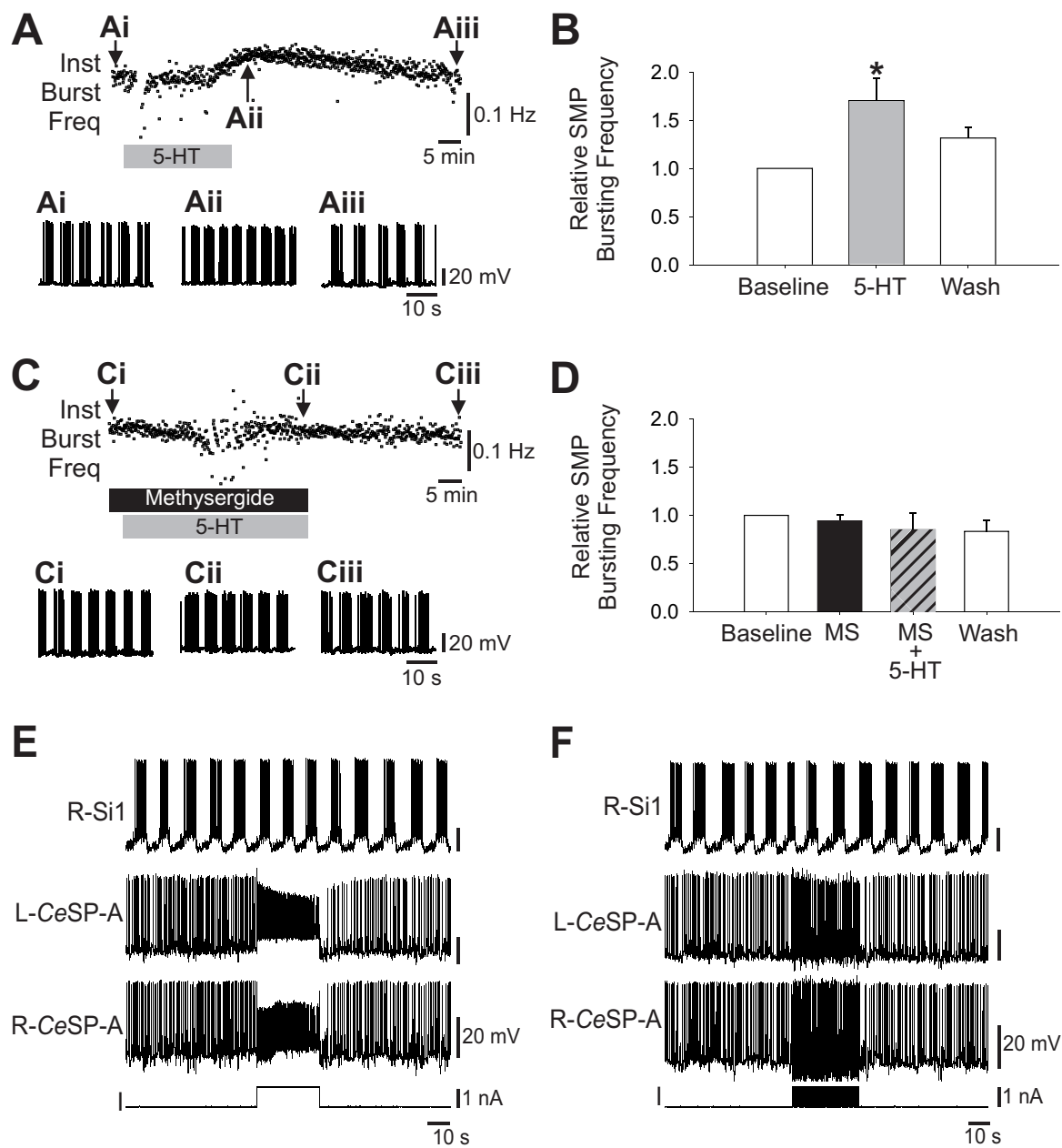
applied methysergide (1 – 100  $\mu$ M) blocked the ability of the CeSP-A neurons to elicit a swim motor pattern (Fig. 4-3B,  $n = 6$ ), suggesting that the effect of the CeSP-A neurons is mediated by 5-HT. The effect did not reverse during a 1-hr wash ( $n = 6$ ) or 2-hr wash ( $n = 3$ ) with saline.

Bath application of 5-HT (5 – 100  $\mu$ M) itself elicited the swim motor pattern in quiescent preparations (Fig. 4-3C,  $n = 6$ ). Swim interneurons displayed robust bursting that became more regular with shorter burst periods as 5-HT continued to be applied to the bath (Fig. 4-3Cii). The swim motor pattern evoked by 5-HT was not transient, but persisted 30 – 60 min past the washout of 5-HT from the bath. The effect of 5-HT reversed in the presence of saline (Fig. 4-3Ciii).

#### *5-HT modulates ongoing swim motor patterns*

Bath-applied 5-HT (1 – 10  $\mu$ M) during an ongoing spontaneous swim motor pattern consistently caused a significant increase in the instantaneous burst frequency of the swim motor pattern (Fig. 4-4A, B,  $n = 8$ ,  $p < 0.01$ ). Associated with the increase in burst frequency were a depolarization of the membrane potential ( $3.8 \pm 1.7$  mV) and a decrease in the amplitudes of the swim interneuron action potentials ( $7.3 \pm 0.6$  mV). Perfusion of the brain with methysergide (50 – 100  $\mu$ M) had no effect on the basal burst frequency, but blocked the increase that would have been caused by bath-application of 5-HT (Fig. 4-4C, D,  $n = 3$ ,  $p > 0.05$ ). However, methysergide did not prevent 5-HT from causing a slight depolarization of the membrane potential ( $6.0 \pm 0.6$  mV) and a decrease in spike amplitude ( $8.0 \pm 0.2$  mV). Thus, 5-HT may act at multiple receptors and only

**Figure 4-4:** Serotonin, but not the *CeSP-A* neurons, could increase the frequency of a swim motor pattern. Bath applied 5-HT (10  $\mu$ M) significantly increased the frequency of bursting in an ongoing swim motor pattern, as seen in a single preparation (**A**) and across eight preparations (**B**) (gray bar;  $p < 0.01$ ). The instantaneous burst frequency of the swim motor pattern was averaged for five minute intervals prior to (baseline), during (5-HT), and after (wash) bath application of 5-HT and then normalized to the baseline frequency for statistical comparison. Methysergide (MS, 50  $\mu$ M) blocked 5-HT from increasing the swim motor pattern burst frequency, as seen in a single preparation (**C**) and the mean normalized values from three preparations (**D**). In (**C**), methysergide was perfused for 20 min prior to bath application of 5-HT and the methysergide did not have any direct effect on burst frequency. **E**) In contrast, depolarization of two *CeSP-A* neurons with a square-pulse current injection had no effect on the swim motor pattern, as indicated by a lack of a change in the rhythmic activity of Si1. **F**) A train stimulus (20 ms-pulses at 10Hz) applied to both *CeSP-A* neurons also had no effect on an ongoing swim motor pattern.





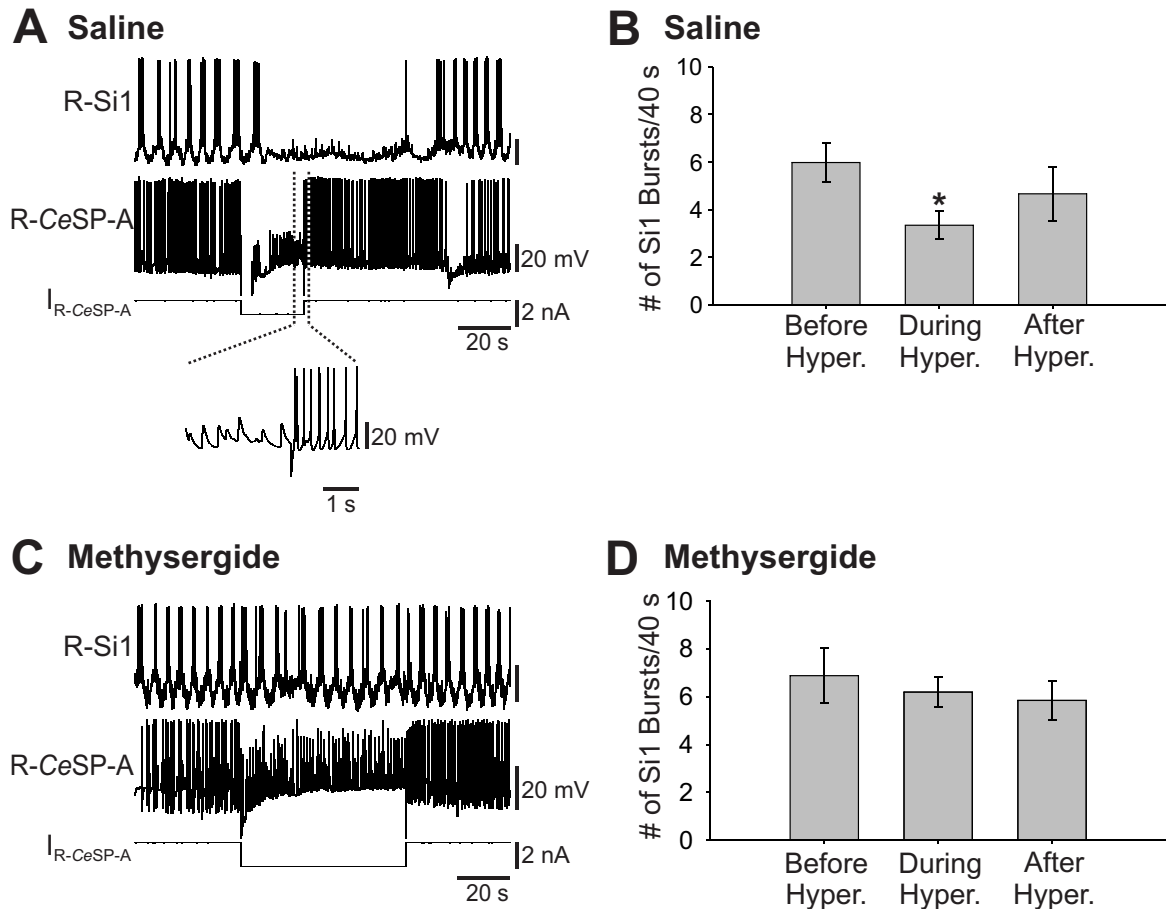
the methysergide-sensitive receptors affect burst frequency.

In contrast to the effect of bath-applied 5-HT, activation of the *CeSP-A* neurons had no apparent effect on the ongoing swim motor pattern (Fig. 4-4E, F). Depolarization of a single, or even multiple, *CeSP-A* neurons with a square pulse current injection had no significant effect on bursting frequency, burst duration, or the number of spikes/burst (Fig. 4-4E,  $n = 6$ ,  $p > 0.05$ ). Additional experiments stimulating one ( $n = 1$ ), two ( $n = 2$ ), or three ( $n = 1$ ) *CeSP-A* neurons with pulse trains (20 ms pulses at 10 Hz) also had no significant effect on these bursting parameters (Fig. 4-4F,  $n = 4$ ,  $p > 0.05$ ). Thus, depolarization of the *CeSP-A* neurons was not sufficient to increase the burst frequency of the ongoing swim motor pattern although it could elicit bursting in quiescent preparations (Fig. 4-3A, B).

#### *Spontaneous CeSP-A firing plays a role in maintaining the swim motor pattern*

Hyperpolarization of a *CeSP-A* neuron, to prevent it from spiking, caused an ongoing swim motor pattern to cease within about 20 s (Fig. 4-5A). The swim motor pattern resumed upon release of the *CeSP-A* neuron from hyperpolarization. This was consistent across preparations, with a significant decrease in the number of swim interneuron bursts in the 40 s following the cessation of spiking in a *CeSP-A* neuron (Fig. 4-5B,  $n = 7$ ,  $p < 0.05$ ). This suggests that the *CeSP-A* neurons may play a role in maintaining the swim motor pattern.

Spontaneously-released 5-HT was not necessary for production of the swim motor pattern. As noted before, the 5-HT antagonist, methysergide, did not block spontaneous



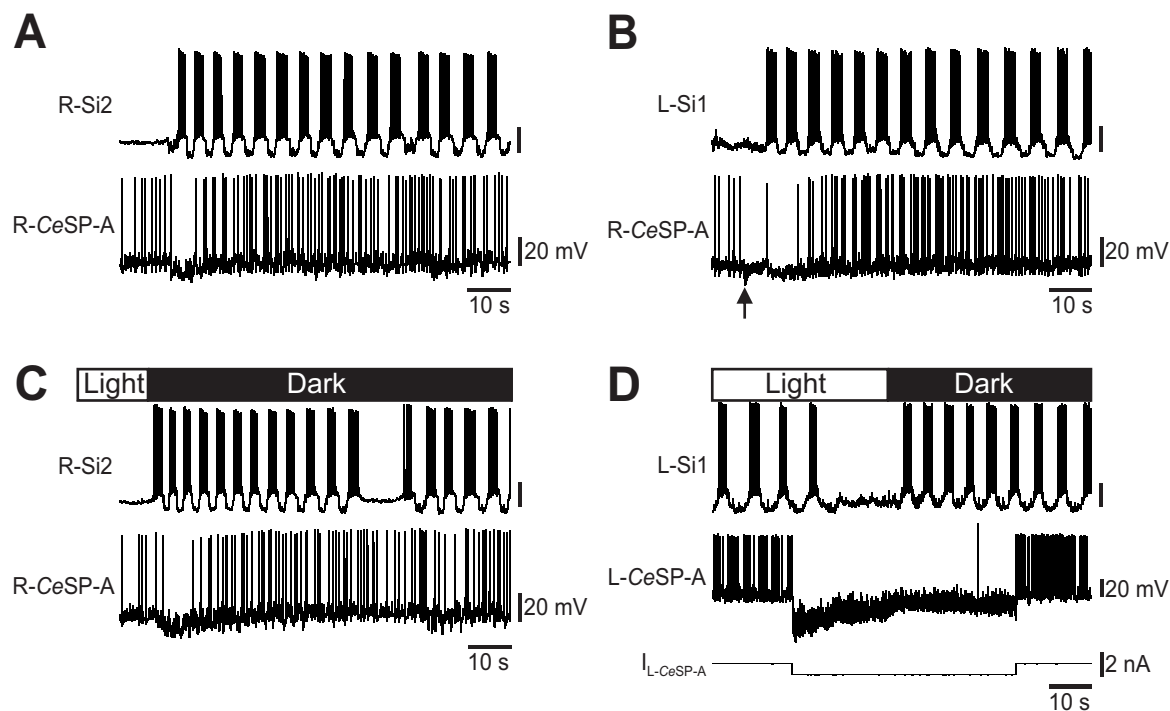
**Figure 4-5:** *CeSP-A* neurons contribute to a serotonergic tone that facilitates the swim motor pattern. **A)** Hyperpolarization of even a single *CeSP-A* neuron, sufficient to prevent action potentials (inset), stopped an ongoing swim motor pattern. **B)** The mean number of bursts in Si1 was significantly reduced during the first 40 s-interval of hyperpolarization of a *CeSP-A* neuron, compared to the 40 s-intervals immediately preceding or following hyperpolarization ( $n = 7$ ;  $p < 0.05$ ). **C)** In the same preparation as **(A)**, hyperpolarization of the *CeSP-A* neuron did not inhibit the swim motor pattern in the presence of methysergide (100  $\mu$ M) and this was consistent across preparations **(D)** ( $n = 3$ ). Note that methysergide did not inhibit the swim motor pattern by itself, suggesting that 5-HT is not necessary for swimming to occur.

swim motor patterns (Fig. 4-4D). Furthermore, in the presence of bath-applied methysergide (50 - 100  $\mu$ M), hyperpolarization of a *CeSP-A* neuron no longer inhibited the ongoing swim motor pattern (Fig. 4-5C, D,  $n = 3$ ,  $p > 0.05$ ).

*CeSP-A neurons are not necessary to elicit a swim motor pattern*

There are three additional conditions for initiating the swim motor pattern that showed that the *CeSP-A* neurons were not necessary for the production of rhythmic activity. First, in 23 of 24 preparations that exhibited spontaneous swim motor patterns, the firing rate of the *CeSP-A* neurons was inhibited at the onset of the swim motor pattern (Fig. 4-6A). Second, stimulation of pedal nerve 2 elicited the swim motor pattern, but consistently caused the *CeSP-A* neurons to stop firing for 10 – 20 s (Fig. 4-6B,  $n = 6$ ; see Chapter Three).

The third set of conditions that show that the *CeSP-A* neurons were not necessary for initiation of the swim motor pattern are related to the effect of ambient light on the swim motor pattern. *Melibe*'s eyes are located directly on the brain, making the isolated brain preparation sensitive to changes in ambient levels of illumination. It was previously shown that offset of light elicits a swim motor pattern (Newcomb et al., 2004). In the present study, decreasing the ambient illumination transiently reduced *CeSP-A* firing frequency in 15 of 19 preparations, while eliciting the swim motor pattern in all 19 preparations (Fig. 4-6C). Furthermore, when the bursting was halted by hyperpolarization of a *CeSP-A* neuron, the swim motor pattern could be recovered by turning off the light (Fig. 4-6D,  $n = 4$ ). These results indicate that the swim motor pattern can be initiated by



**Figure 4-6:** The *CeSP-A* neurons are not necessary to elicit a swim motor pattern. **A)** Isolated brain preparations exhibit alternating periods of quiescence with spontaneous swim motor patterns. This example clearly shows that a *CeSP-A* neuron stopped firing as a spontaneous swim motor pattern was initiated. **B)** Stimulation of pedal nerve 2 (arrow; 2-ms, 5V-pulses for 2 s at 10 Hz) in a quiescent preparation also elicited a swim motor pattern. As noted previously (see Chapter Three), the *CeSP-A* neuron was initially inhibited in response to nerve stimulation, before firing at an elevated rate for several minutes. **C)** The eyes of *Melibe* are located directly on the brain and offset of light also elicits a swim motor pattern (Watson et al., 2001; Newcomb et al., 2004). Similar to spontaneous- and nerve shock-induced swim motor patterns, the *CeSP-A* neurons were initially inhibited in response to offset of light. This inhibition was followed by a period of elevated firing. **D)** As seen in Fig. 4-5, hyperpolarization of a *CeSP-A* neuron stopped the swim motor pattern. However, offset of light was still capable of eliciting a swim motor pattern while the *CeSP-A* neuron was hyperpolarized.

mechanisms that do not involve the *CeSP-A* neurons.

## **Discussion**

In this study, we characterized the functional roles of the *Melibe* *CeSP-A* neurons in lateral flexion swimming. These neurons are homologues of the *Tritonia* DSIs, which are integral members of the dorsal-ventral flexion swim CPG in *Tritonia* and, as such, are necessary and sufficient for production of the swim motor pattern (Getting et al., 1980; Fickbohm and Katz, 2000; Frost et al., 2001; Katz et al., 2004). In *Melibe*, depolarization of a *CeSP-A* neuron was sufficient to elicit a swim motor pattern, and hyperpolarization of a *CeSP-A* neuron inhibited an ongoing swim motor pattern. However, the *CeSP-A* neurons were not members of the swim CPG and were not necessary for initiation of the swim motor pattern. Methysergide, a serotonin receptor antagonist, blocked the effects of the *CeSP-A* neurons, without stopping a swim motor pattern, and swim motor patterns could occur without activity in the *CeSP-A* neurons. These results suggest that unlike their homologues in *Tritonia*, the *CeSP-A* neurons in *Melibe* are extrinsic to the swim CPG and not necessary for the production of the swim motor pattern.

The role of 5-HT also differs between *Tritonia* and *Melibe*. Although bath application of 5-HT could initiate a swim motor pattern in both species, blocking 5-HT receptors with methysergide blocked the swim motor pattern only in *Tritonia* (McClellan et al., 1994). In *Melibe*, the swim motor pattern could continue in the presence of methysergide, although the effects of 5-HT and the *CeSP-A* neurons were blocked. This

suggests that whereas the neurotransmitter 5-HT plays an essential role in the production of the *Tritonia* swim motor pattern, in *Melibe*, 5-HT is not essential.

#### *Multiple pathways for eliciting swimming in Melibe*

In addition to stimulation of the *CeSP-A* neurons, other stimuli can elicit an equivalent swim motor pattern in an isolated *Melibe* brain including offset of light (Watson et al., 2001; Newcomb et al., 2004) and pedal nerve shock. Furthermore, the swim motor pattern occurs spontaneously in the isolated brain, without any externally-applied stimulus. Despite the fact that stimulation of the *CeSP-A* neurons is sufficient to elicit the swim motor pattern, the *CeSP-A* neurons are often silent when the swim motor pattern is initiated through other means. This suggests that the inputs to the CPG may be arranged in parallel such that the *CeSP-A* neurons and other pathways can directly activate the CPG. Alternatively, the *CeSP-A* neurons may not directly activate the CPG but may excite the actual initiating input to the CPG.

The swim behavior in intact *Melibe* also can be elicited by multiple stimuli. The most consistent stimulus for initiating swimming is contact with the predatory seastar *Pycnopodia helianthoides* (Lawrence and Watson, 2002; although, see Page, 1993). This is likely mediated through chemosensory detection of surfactants present on the tube feet of predatory seastars (Mauzey et al., 1968; Mackie, 1970). *Melibe* also will swim in response to concentrated salt solutions (Lawrence and Watson, 2002) and in response to being dislodged from the substrate (unpublished observation). In addition, *Melibe* will

start swimming "spontaneously"; that is, without any obvious external cues. These spontaneous swims occur with significantly greater frequency at night than during the daytime, suggesting that *Melibe* are nocturnal (Newcomb et al., 2004). Thus, a number of internal and external cues elicit swimming in *Melibe*. It is unclear which of these stimuli, if any, involves the *CeSP-A* neurons.

#### *Contribution of CeSP-A neurons to the likelihood of swimming*

There are a total of six *CeSP-A* neurons (see Chapter Three), but prolonged hyperpolarization of just a single *CeSP-A* neuron was sufficient to stop an ongoing swim motor pattern. The robustness of this effect is striking because initiation of the swim motor pattern does not require the *CeSP-A* neurons. It typically took more than 20 s for hyperpolarization of a *CeSP-A* neuron to inhibit the swim motor pattern. This inhibition could potentially be caused by a loss of serotonergic neuromodulatory tone. If this were the case, it may be possible for the circuit to adjust to new serotonin levels, as decreasing the ambient light with a *CeSP-A* neuron hyperpolarized will reinitiate the swim motor pattern.

Homologues of the *CeSP-A* neurons in other opisthobranch species can contribute to the excitability of the serotonergic system. The DSIs are known to excite other serotonergic neurons in *Tritonia*, such as Pd21 (Popescu and Frost, 2002) and the metacerebral giant cell (unpublished observation). The presumed *CeSP-A* homologues in *Pleurobranchaea californica*, the As1-3 neurons, and in *Clione limacina*, the Cr-SP

neurons, also excite other serotonergic neurons (Satterlie and Norekian, 1995, 1996; Jing and Gillette, 2000). Therefore, it has been hypothesized that these neurons comprise part of a general serotonergic arousal system in opisthobranchs (Satterlie and Norekian, 1996; Jing and Gillette, 2000; Katz et al., 2001).

In *Aplysia californica*, evidence suggests that most of the serotonergic neurons in the brain constitute a global arousal network (Marinesco et al., 2004a). A large proportion of the serotonergic neurons in the *Aplysia* brain are interconnected and sensitive to 5-HT. Marinesco and colleagues (2004a) hypothesized that this constituted a distributed serotonergic network that was sensitive to recurrent feedback within the system and/or circulating levels of 5-HT. If this is the case in *Melibe* as well, then it is plausible that the *CeSP-A* neurons have an integral role in this serotonergic network and in contributing to the general serotonergic tone in the brain.

Unlike hyperpolarization, prolonged depolarization of *CeSP-A* neurons during an ongoing swim motor pattern in *Melibe* had no significant effect on burst frequency or other parameters of the swim motor pattern. In contrast, bath-applied 5-HT did increase the burst frequency of the swim motor pattern. However, this effect may not be physiologically relevant, as the duration and amplitude of lateral flexions in freely-swimming *Melibe* do not vary (Lawrence and Watson, 2002). Therefore, the *CeSP-A* neurons may be more important in contributing to a serotonergic tone that influences the likelihood of swimming, as opposed to other parameters of the swim behavior. Serotonin is known to be a gain setter for the initiation of specific behaviors in other animals



(McCall and Aghajanian, 1979; Ma et al., 1992; Yeoman et al., 1994; Pickard and Rea, 1997; Kravitz, 2000; Straub and Benjamin, 2001).

*Phylogenetic alteration of neural networks*

If the evolution of species-specific behaviors involves alteration of existing neural networks, comparison of homologous neurons between species with different behaviors can be informative of this evolutionary process (Sillar and Heitler, 1985; Dumont and Robertson, 1986; Paul, 1991; Buschbeck and Strausfeld, 1997; Wright, 2000; Chiang et al., 2006). Opisthobranch molluscs exhibit several modes of escape behavior and locomotion, such as dorsal-ventral flexions, lateral flexions, and parapodial flapping (Farmer, 1970). The *CeSP-A* neurons and their presumed homologues are involved in all of these modes of locomotion (Getting et al., 1980; Arshavsky et al., 1992; Satterlie and Norekian, 1995; Arshavsky et al., 1998; Jing and Gillette, 1999; Popescu and Frost, 2002; also see Chapter Three). However, homologues of the *CeSP-A* neurons have an intrinsic role in dorsal-ventral flexion swimming and an extrinsic role in other locomotor behaviors. Thus, the functions of homologous neurons have diverged over the course of evolution.

In a phylogenetic context, these functional differences in homologous neurons can be useful in understanding the evolution of species-specific behaviors. Phylogenetic analysis, based on both morphological and molecular data, suggest that nudibranchs, and their sister group the notaspids (e.g. *Pleurobranchaea*) are evolutionarily recent lineages

of opisthobranchs (Salvini-Plawen and Steiner, 1996; Thollessen, 1999; Grande et al., 2004; Vonnemann et al., 2005). Dorsal-ventral flexion swimming is also rare and only exhibited by these derived lineages (Farmer, 1970). This suggests that the intrinsic function of the *CeSP-A* homologues in species that exhibit dorsal-ventral flexion swimming may be an apomorphic trait, whereas the extrinsic function of these neurons in other modes of locomotion may be the ancestral condition for opisthobranchs. Furthermore, the fact that *CeSP-A* neurons are present and influence locomotion in a number of phylogenetically disparate species, suggests that each of these swim circuits is derived from a common, ancestral network that has been phylogenetically altered multiple times.

Comparisons of homologous neurons in other species also have provided a framework for understanding the evolution of neural circuits. For example, many crustaceans exhibit one or more different tail-flipping escape behaviors involving rapid flexion of the abdominal segments (Silvey and Wilson, 1979; Paul, 1989; Edwards et al., 1999). Inter-species comparisons of homologous giant neurons mediating these behaviors indicate that loss (Wilson and Paul, 1987; Paul, 1989, 1990, 1991) or modification (Sillar and Heitler, 1985) of these neurons is probably responsible for the species-specific differences in tail-flip behaviors. Furthermore, this work also has provided a phylogenetic framework for understanding the likely ancestral condition and how it was modified during the evolution of species-specific tail-flip behaviors (Paul, 1989, 1990, 1991; Edwards et al., 1999). Thus, this “neurophylogenetic” approach (Paul, 1989, 1990) is

useful for understanding the evolution of species-specific behaviors in a number of groups of organisms.

## CHAPTER FIVE

DIVERGENT LOCOMOTOR FUNCTIONS OF PUTATIVE HOMOLOGOUS  
INTERNEURONS IN TWO LATERAL-FLEXION SWIMMING NUDIBRANCH  
MOLLUSCS**Abstract**

Neural circuits responsible for locomotion have been characterized in a number of nudibranch molluscs, such as the lateral-flexion swimming *Melibe leonina* (Family: Tethydidae). The swim CPG in *Melibe* is comprised of two types of uniquely identifiable swim interneurons (Si1 and Si2). In this study, a putative homologue of Si1 was identified in another, closely-related, lateral-flexion swimming nudibranch, *Dendronotus iris* (Family: Dendronotidae). The case for homology is based on similarities in a number of morphological features, including location in the posterior region of the cerebral ganglion, soma size, and a characteristic axon projection to the ipsilateral pedal ganglion. Therefore, we have named this neuron in *Dendronotus* the Cerebral Posterior ipsilateral Pedal (CPiP) neuron. In contrast to its homologue in *Melibe*, the CPiP neuron was not rhythmically active during a swim motor pattern, indicating that it is not a member of the swim CPG in *Dendronotus*. However, depolarization of a CPiP neuron could initiate a swim motor pattern. These results suggest that there is a difference in neuronal composition of the swim CPGs for two closely-related, lateral-flexion swimming nudibranchs. This difference in circuit organization is due to either convergent evolution of lateral swimming with novel CPGs or, more likely, a divergence of a basal lateral-

flexion swim circuit. Thus, CPGs may not be as resistant to evolutionary change as previously thought.

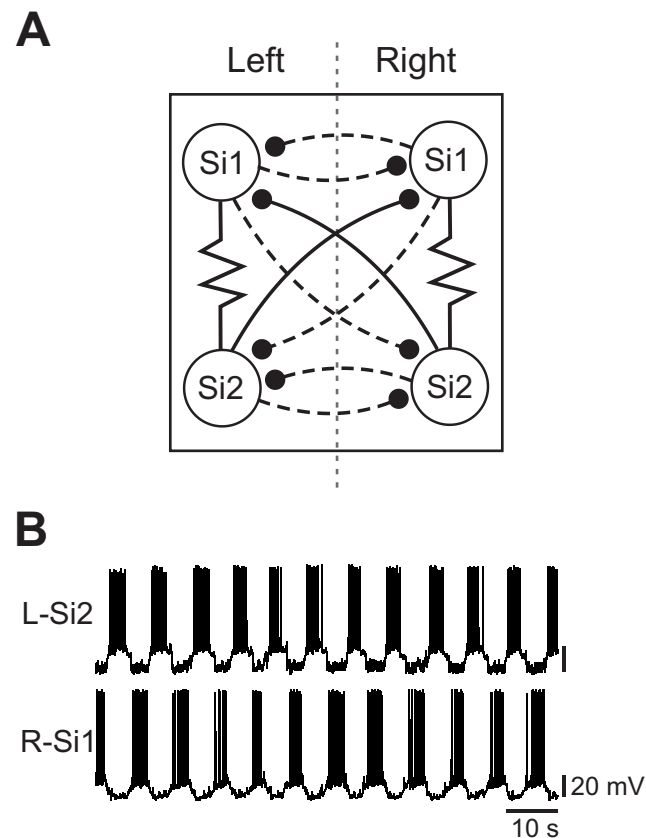
## **Introduction**

There is speculation as to the degree to which the composition of CPGs have been evolutionarily conserved (Wainwright and Lauder, 1986; Sanderson, 1988; Goslow et al., 1989; Wainwright et al., 1989; Kavanau, 1990; Edwards and Palka, 1991; Paul, 1991; Katz and Tazaki, 1992; Nishikawa et al., 1992; Weijs and Dantuma, 1994; Tierney, 1995; Kiehn et al., 1997; Katz and Harris-Warrick, 1999; Herrel et al., 2001; Langenbach and Van Eijden, 2001; Wainwright, 2002). The highly integrated and multifunctional nature of CPGs suggests that alterations in one element might cause disruptions in the whole neural circuit (Nishikawa et al., 1992; Tierney, 1995). However, divergence in neural circuits among related taxa has been reported (Hale et al., 2002). In this study, comparison of homologous neurons between two related nudibranch molluscs suggests that the composition of CPGs producing lateral-flexion swimming in these two species has diverged.

*Melibe leonina* is a nudibranch in the suborder Dendronotoidea that exhibits lateral-flexion swimming (i.e. bending side-to-side) (Agersborg 1921; Hurst, 1968; Watson et al., 2001; Lawrence and Watson, 2002). Briefly, it consists of a sagittal flattening of the body, including the foot, oral hood, and cerata (respiratory projections from the dorsal surface), followed by body flexions from side-to-side. The average

periodicity of swim cycles is  $2.7 \pm 0.2$  s (range = 2 to 4 s; Lawrence and Watson, 2002). Swimming can occur spontaneously and can also be triggered by a pinch of the skin, contact with concentrated salt solutions or the touch of tube feet of the predatory seastar, *Pycnopodia helianthoides* (Lawrence and Watson, 2002). In contrast to the rare and transient escape swim displayed by other swimming nudibranchs (Willows, 1967; Farmer, 1970; Willows et al., 1973; Hume et al., 1982; Wyeth and Willows, 2006), *Melibe* will swim for extended periods of time (Lawrence and Watson, 2002).

The neural correlate of swimming has been characterized (Watson et al., 2001; Watson et al., 2002), including the CPG responsible for the behavior (Fig. 5-1A; Thompson and Watson, 2005). The CPG is comprised of two types of bilaterally represented swim interneurons (Si1 and Si2). Reciprocal inhibition between contralateral interneurons creates a half-center oscillator that produces alternating bursts of activity in the two sides of the circuit (Fig. 5-1B). Si1 is an integral component of this CPG. Si1 exhibits rhythmic bursting activity phase-locked to the swim motor pattern, brief depolarization of Si1 can reset the swim rhythm, and hyperpolarization of Si1 halts an ongoing swim motor pattern. Si1 is also readily identifiable based on a number of morphological criteria that uniquely delineate it from surrounding neurons: 1) Si1 is the largest neuron in the medial, posterior region of the cerebral ganglion; 2) Si1 has a posteriorly directed axon that extends into the ipsilateral pleural ganglion, where it has a very characteristic bend; 3) Si1 projects to the ipsilateral pedal ganglion; and 4) Si1 is an interneuron, based on the fact that its neuronal process does exit through a peripheral nerve, but instead projects out of the pedal ganglion through one of the pedal-pedal



**Figure 5-1:** Swim circuit (**A**) and swim motor pattern (**B**) for lateral swimming in *Melibe*. **A**) The swim CPG is comprised of two types of bilaterally represented interneurons, Si1 and Si2. The circuit consists of ipsilateral electrical and contralateral inhibitory connections, resulting in a half-center oscillator. Circuit diagram adapted from Thompson and Watson (2005). **B**) Simultaneous intracellular recordings from swim interneurons in an isolated brain preparation demonstrate the bursting activity that alternates between the two sides of the circuit.

connectives to the contralateral pedal ganglion.

*Dendronotus iris* is another dendronotid nudibranch that exhibits lateral-flexion swimming (Agersborg, 1922; Robilliard, 1970). Similar to *Melibe*, it is a robust swimmer and can swim for extended periods of time. The neural correlate of swimming in *Dendronotus* has not yet been rigorously examined. However, *Dendronotus* is in the same suborder as *Melibe* (Dendronotoidea) (Wollscheid-Lengeling et al., 2001; Grande et al., 2004) and thus may have similar neural mechanisms underlying swimming. Homologous neurons have already been identified between these two species (see Chapter Three), suggesting that it may be possible to identify homologues of the *Melibe* swim interneurons in *Dendronotus*. We consider neurons to be homologous if the most parsimonious explanation for their similarity is that these neurons were present in a common ancestor.

In this study, we identified a putative homologue of the *Melibe* Si1 in *Dendronotus*, based on similarities in a number of distinguishing morphological characteristics. This Si1 homologue was not rhythmically active during a putative swim motor pattern, suggesting that it is not a necessary component of the swim CPG in *Dendronotus*. However, depolarization of the Si1 homologue in *Dendronotus* elicited a transient swim-like motor pattern, indicating a triggering role for this neuron in lateral swimming. These results suggest that the cellular composition of the CPG for lateral-flexion swimming in *Melibe* and *Dendronotus* has diverged during evolution.



## **Methods**

### *Animal collection and maintenance*

All specimens were collected as adults. *Dendronotus iris* (10 – 20 cm in body length) and *Melibe leonina* (5 – 10 cm) were collected from the dock at FHL, Friday Harbor, WA. Additional animals were collected by Shaun Cain, David Duggins, and the authors from subtidal eelgrass beds at nearby Shaw Island, WA (*Melibe*), and by Living Elements (Vancouver, British Columbia [BC]) at the Indian Arm extension of Burrard Inlet in Vancouver Harbor (*Melibe*) and Yellow Bank in Clayoquot Sound near Tofino, BC (*Dendronotus*).

Animals were kept either in seawater tables at FHL at ambient seawater temperatures and light/dark cycles or in recirculating artificial seawater tanks at Georgia State University at 10° C and a fixed 12:12 light/dark cycle.

### *Video recording*

Animals were induced to swim in a tank by dislodging them from the substrate, squirting them with a concentrated salt solution, or touching them with the tube feet of a predatory seastar, *Pycnopodia helianthoides*. Animals were videotaped with a Sony DCR-HC85 digital video camera recorder. Videos were downloaded to a computer with Adobe Premier (Adobe, San Jose, CA) or Microsoft Windows Movie Maker (Microsoft Corporation, Redmond, WA) for viewing and analysis. Individual frames were exported from Movie Maker. One swim cycle was considered as the time between two complete

flexions to a particular side of the animal. The average swim cycle period for an episode of swimming was calculated by dividing the duration of a swim episode by the number of flexion cycles. All results are presented as mean  $\pm$  standard error.

#### *Isolated brain preparation*

Animals were anesthetized by chilling and then pinned in a Sylgard-lined dish. The integument was cut lateral or dorsal to the esophagus, and the brain, consisting of the cerebral, pedal, and pleural ganglia, was removed by cutting all nerve roots. Care was taken to leave the two pedal-pedal connectives intact in both species, as the *Melibe* brain does not exhibit swim motor pattern activity if these connectives are damaged (Thompson and Watson, 2005). The brain was transferred to a smaller Sylgard-lined dish where it was superfused, at a rate of 0.5 ml/min, with artificial saline (in mM): 420 NaCl, 10 KCl, 10 CaCl<sub>2</sub>, 50 MgCl<sub>2</sub>, 11 D-glucose, and 10 HEPES, pH. 7.6. Connective tissue surrounding the brain was manually removed with forceps, fine scissors and an electrolytically-sharpened tungsten wire. During the dissection, the saline temperature was kept at  $\sim 4$  °C to reduce neuronal firing. For electrophysiological experiments, the temperature was raised to 10 °C, the ambient temperature of the water in which the animals live.

### *Electrophysiology*

Intracellular recordings were obtained using 10-30 M $\Omega$  glass microelectrodes filled with 3 M potassium chloride and connected to Axoclamp 2B (Axon Instruments, Union City, CA) or Dagan IX2-700 (Dagan Corporation, Minneapolis, MN) amplifiers. Electrodes were dipped in ink extruded from a black permanent marker (Sharpie, Sanford Corporation, Oak Brook, IL) to make it easier to see the fine tip. Recordings were digitized with a 1401 Plus or Micro 1401 A/D converter from Cambridge Electronic Design (CED, Cambridge, UK). Data acquisition and analysis were performed with Spike2 software (CED).

Serotonin (5-hydroxytryptamine creatine sulfate; Sigma, St. Louis, MO) was dissolved in saline just before use at final concentrations (10 – 100  $\mu$ M) and bath applied by switching perfusion paths.

### *Dye injections*

The axon projection pattern and soma position of a neuron were determined by injecting with 2 - 4% Neurobiotin Tracer (Vector Laboratories, Inc., Burlingame, CA, dissolved in 0.75 M KCl, pH 7.4). Neurobiotin was loaded via iontophoresis for 30 min (1 – 10 nA, 1 Hz, 50% duty cycle), and preparations were fixed (see below) 0.5 – 4 hrs after injection.

*Whole-mount immunohistochemistry and Neurobiotin processing*

Brains were fixed 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) (McLean and Nakane, 1974). After fixation, ganglia were washed twice (30 min each) with cacodylate buffer and then twice (30 min each) with 4% Triton X-100 in PBS (50 mM Na<sub>2</sub>HPO<sub>4</sub> in 140 mM NaCl, pH 7.2). The ganglia were then incubated for 1 hr in ASD (0.5% Triton X-100, 1% normal goat serum and 1% bovine serum albumen in PBS). This was followed by 48 – 96 hr in 0.1% Streptavidin-Alexa 594 conjugate (Molecular Probes, Eugene, OR), further diluted 1:50 in ASD, and primary 5-HT antiserum (rabbit polyclonal anti-5-HT, #20080, ImmunoStar, Inc., Hudson, WI) diluted 1:1000 in ASD. This rabbit antibody was raised against serotonin coupled to bovine serum albumin with paraformaldehyde. Specificity of the antiserum was tested with preadsorption controls and it does not cross-react with closely related amines such as 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, and dopamine (manufacturer's technical information). After four washes (1 hr each) with 0.5% Triton X-100 in PBS, ganglia were incubated overnight in goat anti-rabbit antiserum conjugated to Alexa 488 (Molecular Probes) diluted 1:50 or 1:100 in ASD. After this step, ganglia were washed four times (1 hr each) with PBS, dehydrated in an ethanol series, cleared in methyl salicylate, and mounted on a depression slide with Cytoseal 60 (Electron Microscopy Sciences, Washington, PA). The ganglia were kept at 4° C for the entire immunohistochemistry protocol and all of the steps between fixation and dehydration were done with gentle agitation on a shaker.

Previous studies with the same primary antibody had already confirmed its specificity for 5-HT in nudibranchs via preadsorption controls, as well as a lack of autofluorescence in these animals in our emission range (Fickbohm et al., 2001). In this study, we obtained further confirmation of this by the fact that 5-HT immunoreactivity in both species was the same as previously indicated with this antibody (Newcomb et al., 2006).

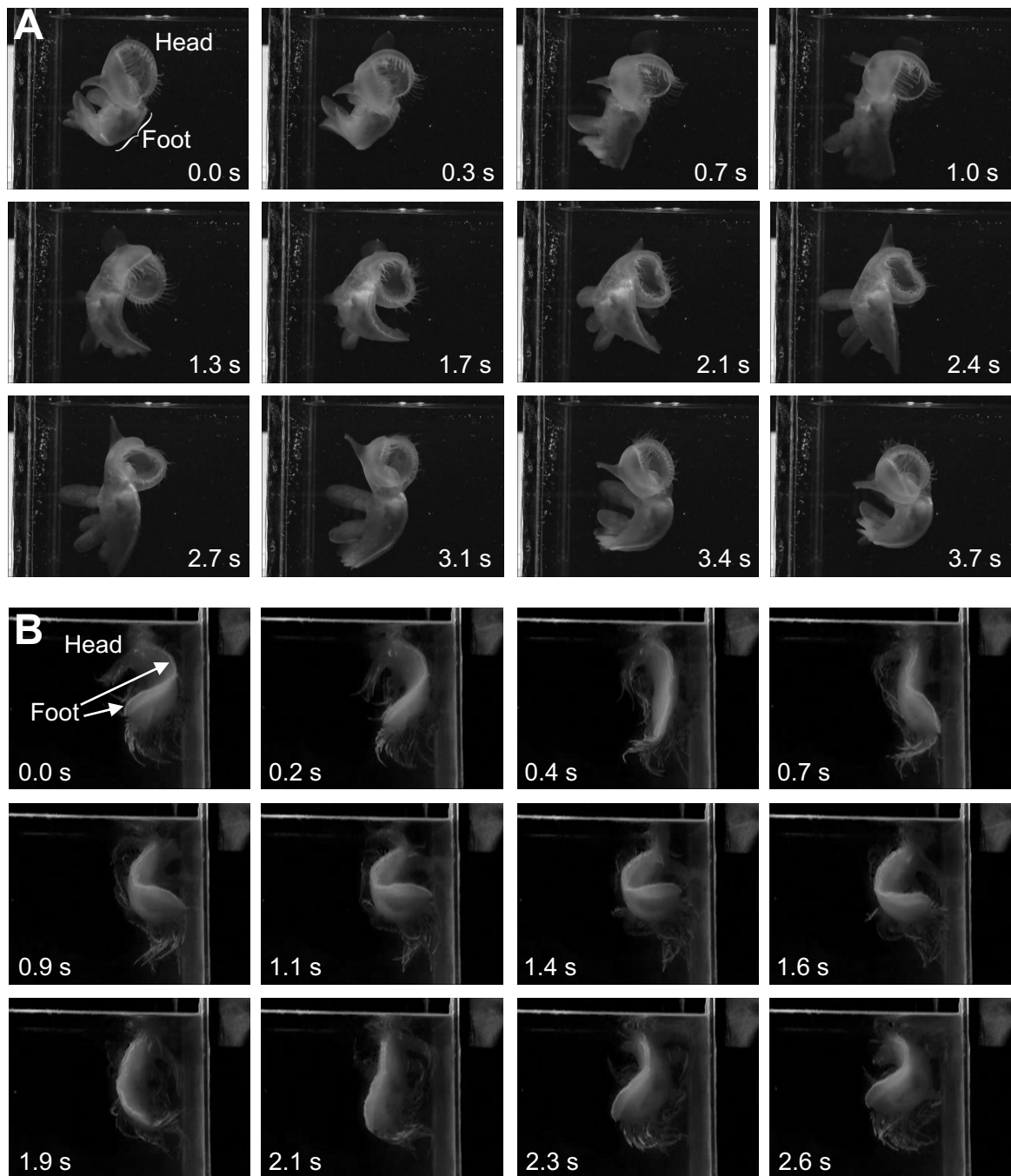
### *Imaging*

Fluorescence images were visualized on an Axiovert 100M microscope (Carl Zeiss, Inc., Thornwood, NY) using confocal microscopy (LSM 510, Carl Zeiss, Inc.) with a 10X-objective. Fluorophores were excited with two lasers (488 and 543 nm) and fluorescent emissions were passed through a band-pass filter (505 – 550 nm) for visualization of Alexa 488 (5-HT immunoreactivity) and a 560 nm long-pass filter to visualize Alexa 594 (Neurobiotin dye fill). LSM 510 software was used to acquire images. Confocal stacks consisted of 12 – 80 optical sections, depending on the thickness of tissue, and included the entire dorsal/ventral span of the brain. The thickness of each optical section, which was optimized and kept consistent within a preparation, ranged from 2 – 14  $\mu\text{m}$ . Maximal projections of confocal stacks were exported as TIFF files and imported into Adobe Photoshop CS (Adobe). In Photoshop, projections were assembled into a montage of the entire CNS and overall brightness and contrast were adjusted. Outlines of brains and neuron projection patterns were traced in Microsoft PowerPoint 2002.

## **Results**

### *Lateral flexion swimming*

Spontaneously swimming *Melibe* were videotaped for comparison to *Dendronotus* (Fig. 5-2A). Videotapes of lateral flexion swimming in *Dendronotus* indicated that this behavior is similar to *Melibe* in many respects. The foot and body flatten in the sagittal plane, followed by body flexions from side-to-side (Fig. 5-2B, n = 3). Swimming episodes were spontaneously exhibited by each of the *Dendronotus*, and could also be consistently elicited by touching the skin with sodium chloride crystals or tube feet of *Pycnopodia*. The average cycle period for lateral flexions in *Dendronotus* was  $3.4 \pm 0.3$  s (n = 3, range = 2.8 to 4.4 s). The cerata on the dorsal surface of *Dendronotus* are substantially more numerous, longer, and more flexible than the cerata in *Melibe*. These cerata passively trail behind the body during swimming flexions in *Dendronotus*. This differs from *Melibe*, which increases its lateral surface area by actively flattening the cerata in the sagittal plane (Fig. 5-2A; Lawrence and Watson, 2002). Analysis of swimming with a larger sample size of *Dendronotus* will be needed to more definitively determine the similarities and differences between lateral swimming in *Dendronotus* and *Melibe*.



**Figure 5-2:** Lateral flexion swimming in *Melibe leonina* (A) and *Dendronotus iris* (B). Time series of swim illustrates one full cycle, determined as the time between two complete flexions to the animal's right side. Animals are viewed from their ventral surface and the top of the figures is "up".

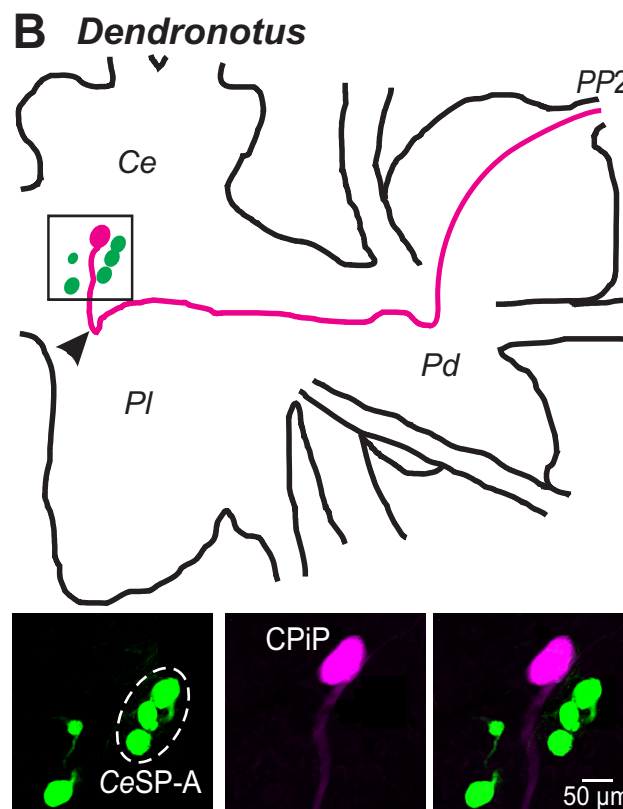
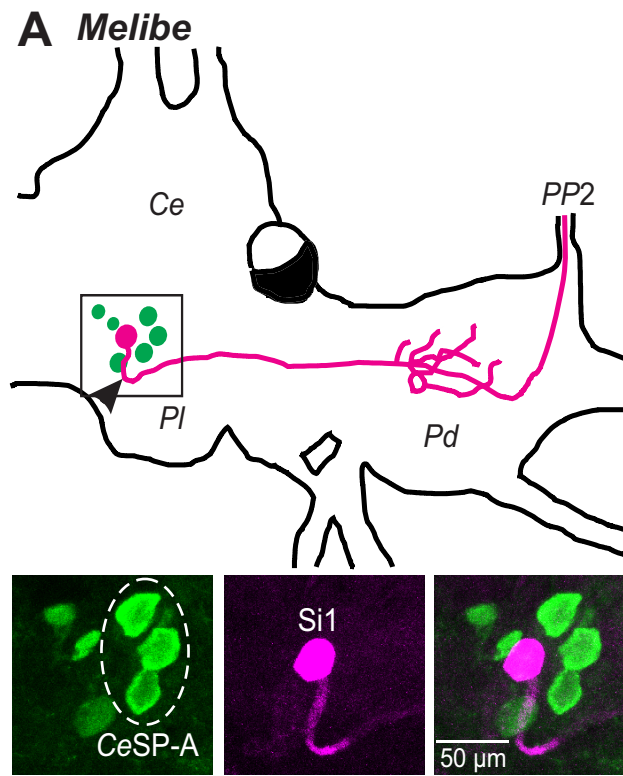
*Putative homologue of Si1*

Si1 is readily identifiable in *Melibe* based on a set of criteria that uniquely delineate it from surrounding neurons, including morphological characteristics such as soma size and location, and projection pattern (Thompson and Watson, 2005). In this study, dye fills of Si1, combined with 5-HT immunohistochemistry, determined that Si1 is not serotonin-immunoreactive but is surrounded by neurons in the serotonin-immunoreactive *CeSP* cluster (Fig. 5-3A, n = 9). Specifically, the soma of Si1 is located just medial to the previously identified serotonergic *CeSP*-A neurons. As previously reported, the axon of Si1 has a posteriorly directed axon that extends into the ipsilateral pleural ganglion, where it has a very characteristic bend. The axon then projects to the ipsilateral pedal ganglion (Thompson and Watson, 2005). However, in 3 of the 9 preparations in this study, the dye fill of the Si1 axon did not end in the ipsilateral pedal ganglion, but continued through the large-diameter pedal-pedal connective, pedal-pedal connective 2 (PP2 using nomenclature of Newcomb et al. [2006]); also referred to as pedal connective [PC] in Watson et al., 2001, 2002) to the contralateral pedal ganglion (Fig. 5-3A).

In *Dendronotus*, a single, bilaterally represented neuron was identified that had morphological characteristics matching those of the *Melibe* Si1 (Fig. 5-3B, total of 10 neurons in 7 preparations). The largest soma in the posterior region of each cerebral ganglion in *Dendronotus* was not serotonergic but was surrounded by the serotonergic neurons of the *CeSP* cluster. This large, non-serotonergic neuron was located just medial to the *CeSP*-A neurons. It had an axon that projected posteriorly into the ipsilateral



**Figure 5-3:** Putative homologue of *Melibe* Si1 in *Dendronotus*. **A)** The *Melibe* Si1 (magenta) is located just medial to the *CeSP-A* neurons, indicated by 5-HT immunohistochemistry (green). The axon of Si1 has a characteristic dip into the ipsilateral pleural ganglion (arrowhead), followed by a projection to the ipsilateral pedal ganglion and through pedal-pedal connective 2 (PP2) to the contralateral pedal ganglion (contralateral projection not shown). Si1 was injected with Neurobiotin (magenta) and the axon was traced. **B)** There is a single neuron, the CPiP neuron, in *Dendronotus* with the same morphological characteristics as the *Melibe* Si1, including the characteristic dip into the pleural ganglion (arrowhead). The drawings in **(A)** and **(B)** represent only the right half of each brain. The confocal micrographs are of the area indicated by the box in each drawing.



pleural ganglion and exhibited a bend reminiscent of Si1 before projecting to the ipsilateral pedal ganglion. Based on these anatomical criteria, we have named this neuron the Cerebral Posterior ipsilateral Pedal (CPiP) neuron. In 5 of the 10 CPiP neurons that were labeled with Neurobiotin, the axon could be traced beyond the ipsilateral pedal ganglion as it projected through the large-diameter pedal-pedal connective (PP2) to the contralateral pedal ganglion. None of the other neurons that we labeled with Neurobiotin in the vicinity of the CPiP neuron had projections to the ipsilateral pleural and then pedal ganglia (26 total neurons in 10 preparations). Thus, based on gross morphology, the CPiP neuron in *Dendronotus* has a projection pattern which uniquely delineates it from surrounding neurons and also resembles the *Melibe* Si1. These similarities lead us to propose that these neurons are putative homologues. Further analysis quantifying the projection patterns of both Si1 and the CPiP neuron will be necessary to confirm this hypothesis.

#### *Electrophysiological properties of the CPiP neuron*

When *Melibe* is not swimming, Si1 is typically silent (Thompson and Watson, 2005). In this study, 7 of the 9 CPiP neurons that we recorded from in *Dendronotus* were silent (data not shown). All 7 of these silent CPiP neurons fired action potentials in response to positive current injection, suggesting that they were not damaged. Thus, resting activity of the CPiP neuron resembled that of the *Melibe* Si1.

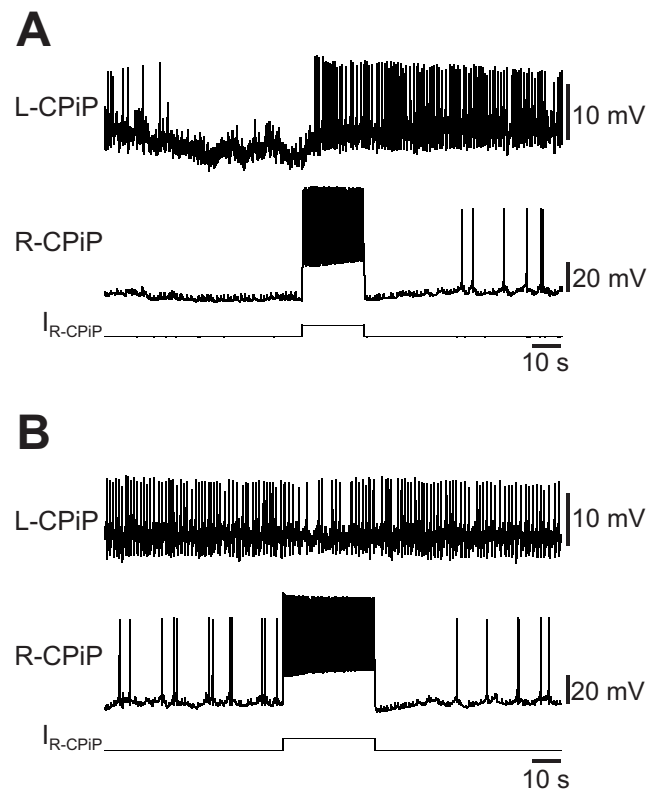
In *Melibe*, contralateral Si1s reciprocally inhibit each other, although it is not known whether these are mono- or polysynaptic connections (Fig. 5-1A; Thompson and

Watson, 2005). In this study, we recorded simultaneously from both the left and right CPiP neurons in three preparations and saw variable effects from intracellular stimulation. In one of these preparations, depolarization of either of the CPiP neurons had no effect on its contralateral counterpart (data not shown). In the other two preparations, depolarization of a CPiP neuron induced action potentials in the contralateral counterpart after a slight delay (Fig. 5-4A). However, in one of the preparations, both of the CPiP neurons were uncharacteristically active without stimulation. In this case, increasing the firing rate of one CPiP neuron inhibited firing in the other CPiP neuron (Fig. 5-4B). Thus, the synaptic effects of the CPiP neurons were variable. Further analysis with additional preparations will be necessary to determine the nature of synaptic connections between CPiP neurons.

*CPiP neuron is not rhythmically active during rhythmic pedal activity*

Si1 is a necessary component of the swim CPG in *Melibe* (Thompson and Watson, 2005). Therefore, we hypothesized that the putative homologue in *Dendronotus*, the CPiP neuron, would also be a member of the swim CPG for lateral-flexion swimming in *Dendronotus*.

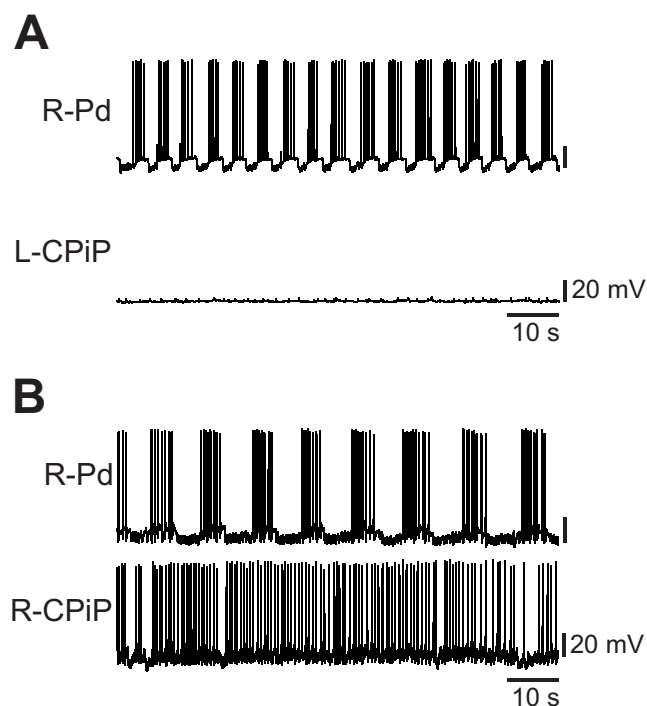
In contrast to *Melibe*, isolated brain preparations of *Dendronotus* did not exhibit spontaneous swim motor pattern activity. Intracellular recordings from pedal neurons (26 total neurons in 7 preparations) and extracellular recordings from pedal nerves (n = 7) did not show any spontaneously rhythmic activity indicative of a swim motor pattern (data not shown). In *Melibe*, swim motor patterns can be elicited in isolated brain preparations



**Figure 5-4:** Synaptic connections between contralateral CPiP neurons. **A)** Depolarization of a right CPiP neuron (R-CPiP) elicited action potentials in the left CPiP neuron (L-CPiP) if the L-CPiP neuron was silent at the time of current injection. **B)** In the same preparation as **(A)**, if the L-CPiP neuron was active, depolarization of the R-CPiP neuron caused inhibition instead of excitation.

by stimulating pedal nerve 2 or with bath application of 5-HT (personal observation). In *Dendronotus*, nerve stimulation of pedal nerve 2 did not elicit rhythmic activity in the CPiP neuron, pedal neurons, or in pedal nerves ( $n = 10$ , data not shown). However, in 2 of 5 preparations, bath application of 10 - 100  $\mu\text{M}$  5-HT did elicit rhythmic activity in pedal neurons and in pedal nerves (Fig. 5-5), that reversed upon washout. The cycle period of this rhythmic activity ranged from 4 to 10 s. This cycle period was slightly longer than the cycle period of the actual swim behavior (Fig. 5-2). However, the cycle period of swim motor patterns in isolated brain preparations of *Melibe* also are longer than the cycle period of the actual behavior (Watson et al., 2002). Furthermore, this motor pattern in *Dendronotus* clearly exhibited alternating activity between the two sides of the brain. Therefore, we conclude that this 5-HT-induced rhythmic pedal activity is a putative swim motor pattern.

The CPiP neuron was never observed to exhibit rhythmic bursting in unstimulated preparations, in response to nerve stimulation, or in the presence of 5-HT ( $n = 7$ ). The CPiP neuron exhibited a varied response to 5-HT that did not include rhythmic activity. In one preparation, a healthy CPiP neuron remained silent during bath application of 100  $\mu\text{M}$  5-HT, while two simultaneously recorded pedal neurons expressed rhythmic activity (Fig. 5-5A). In four other preparations, a total of 6 CPiP neurons fired irregularly in response to 10 – 100  $\mu\text{M}$  5-HT (Fig. 5-5B), despite the fact that in one of these four preparations, rhythmic activity was exhibited by a pedal neuron. The effect of 5-HT was not dependent on concentration, as the CPiP neurons either were silent or fired irregularly, regardless of the concentration of 5-HT. The lack of rhythmic activity in the



**Figure 5-5:** CPiP neuron is not a member of the swim CPG in *Dendronotus*. **A)** Bath application of 100  $\mu$ M 5-HT in an isolated brain preparation elicited a prolonged swim motor pattern, as indicated by a rhythmically active right pedal neuron (R-Pd). However, in this preparation, the left CPiP neuron was silent, demonstrating that it is not a necessary component of the swim CPG. Depolarization of the CPiP neuron indicated that it was not damaged (not shown). **B)** Several hours later in the same animal as **(A)**, bath application of 100  $\mu$ M 5-HT still elicited a swim motor pattern. The frequency of the pattern was less than in **(A)**, as indicated by a recording from the same R-Pd neuron. Although the right CPiP neuron was irregularly active, as opposed to the silence exhibited by the left CPiP neuron, it was also not rhythmically active, indicating that it is not a necessary component of the swim circuit.

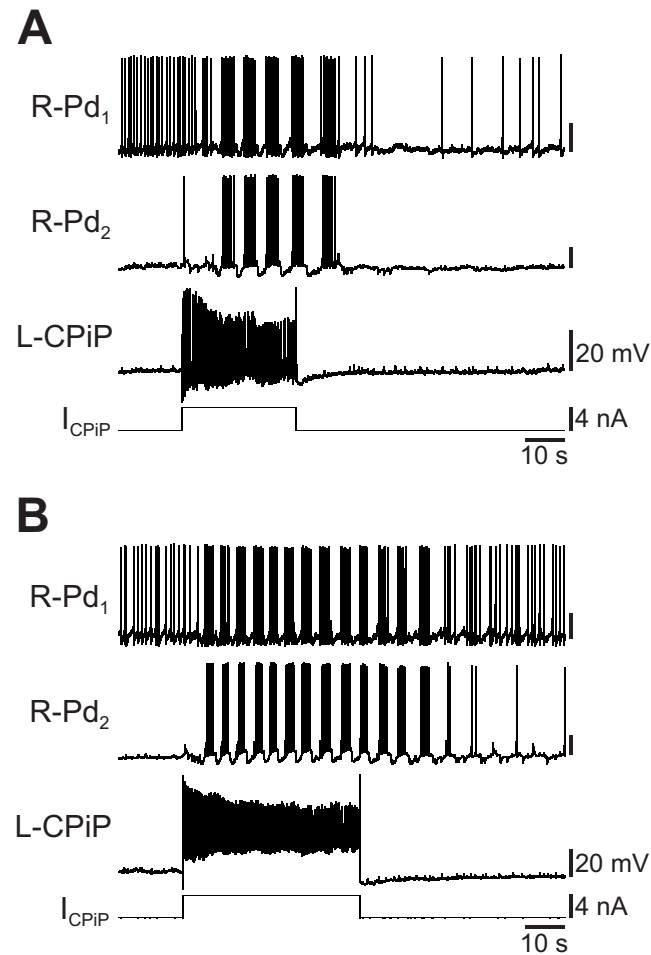
CPiP neuron in the two preparations that exhibited a 5-HT-induced rhythmic motor pattern indicates that it is not an active member of the CPG generating this motor pattern.

*CPiP neuron can elicit rhythmic pedal activity*

Although the CPiP neuron is not rhythmically active, it may still play a role in generating the rhythmic pedal motor pattern. Therefore, a CPiP neuron was stimulated to fire action potentials while pedal neurons were monitored for rhythmic activity. In two preparations with simultaneous intracellular recordings from a CPiP neuron and pedal neurons, a prolonged square-pulse injection of positive current in a CPiP neuron induced high-frequency firing in the CPiP neuron and rhythmic activity in the pedal neurons (Fig. 5-6A). The periodicity of the rhythmic pedal activity ranged from 4 to 6 s. The pedal rhythmic activity was transient, ending within 10 – 20 s after cessation of current injection into the CPiP neuron. Thus, the CPiP neuron was capable of initiating rhythmic pedal activity.

An additional experiment indicated that 5-HT can modulate the CPiP-induced rhythmic activity. In one of the above preparations, bath application of 10  $\mu$ M 5-HT did not elicit rhythmic pedal activity by itself. Depolarization of the CPiP neuron in the presence of 10  $\mu$ M 5-HT elicited rhythmic activity in the pedal neurons similar to the effect of similar stimulation in saline. However, the periodicity of this rhythmic activity was decreased from 6 to 4 s in 5-HT (Fig. 5-6B). Furthermore, although the CPiP-elicited rhythmic pedal activity was still transient in 5-HT, it lasted longer than in saline, not ending until 20 – 30 s after cessation of stimulation of the CPiP neuron (Fig. 5-6B). Thus,





**Figure 5-6:** CIPiP neuron can elicit swim-like motor pattern in *Dendronotus*. **A)** Prolonged stimulation of the CIPiP neuron from a square-pulse injection of positive current elicited a transient swim-like motor pattern in saline, as indicated by recordings from two pedal neurons. The swim-like motor pattern ended shortly after cessation of current injection into the CIPiP neuron. Note that the two pedal neurons were in the same ganglion and therefore fired in phase with each other. **B)** In the same preparation as **(A)**, stimulation of the CIPiP neuron in the presence of 100 μM 5-HT elicited a swim-like motor pattern that had a higher cycle frequency and lasted longer after the cessation of current injection than previously.

5-HT could modulate both the frequency and duration of a CPiP-induced putative swim motor pattern. Additional experiments will be necessary to determine whether or not 5-HT can consistently modulate the frequency and duration of a CPiP-induced putative swim motor pattern.

### **Discussion**

In this study, we identified a putative homologue of the *Melibe* Si1 in another lateral-flexion swimming nudibranch, *Dendronotus*, based on similarities in distinguishing morphological characteristics. We named this putative homologue the CPiP neuron. In contrast to *Melibe*, where the Si1 is an integral component of the swim CPG, the CPiP neuron was not rhythmically active during rhythmic pedal bursting. However, depolarization of a CPiP neuron elicited a transient rhythmic motor pattern in select pedal neurons. The cycle period and left-right alternation of this motor pattern resembled the swim behavior, indicating that it may be a putative swim motor pattern. These results suggest that the CPiP neuron is not a member of the swim CPG for lateral-flexion swimming in *Dendronotus* but that it can activate the swim circuit. Thus, the swim CPGs producing lateral-flexion swimming in two closely-related species have different cellular membership.

*CPiP neuron is a putative homologue of the Melibe Si1*

The CPiP neuron has a number of morphological characteristics that resemble those of the *Melibe* Si1, including soma size and location, and projection pattern. In both species, these characteristics are sufficient to distinguish these neurons from other neurons in the vicinity. Nothing is known about the neurotransmitter or developmental history of Si1, so such features cannot be used as additional tests of homology. While it is possible that a homologue of Si1 is not present or has a different location or morphology in *Dendronotus*, and that the CPiP neuron is a different neuron with similar soma size, location and morphology, this is not the most parsimonious conclusion. It is more likely to assume that these neurons are homologous and represent extant examples of a homologue present in a common ancestor.

Morphological characteristics have formed the sole basis for a number of cases of proposed homology (Wilson et al., 1982; Arbas, 1983a,b; King and Valentino, 1983; Roubos and van de Ven, 1987; Breidbach and Kutsch, 1990; Wiens and Wolf, 1993; Alania, 1995; Faulkes and Paul, 1997). For example, in the optic cartridge of dipteran compound eyes, morphological studies have determined that the photoreceptors and their post-synaptic targets in the first optic neuropil are homologous across a great diversity of dipteran families (Shaw and Meinertzhagen, 1986; Shaw and Moore, 1989). The number of post-synaptic targets has increased from two to four during the evolution of the dipteran lineage and has been accompanied by other structural synaptic changes. As in our study, homology of these neurons and their post-synaptic targets cannot be irrefutably proven by morphological analysis. However, the most parsimonious conclusion regarding

the gross neuroanatomical similarities among these neurons in related species is that they are indeed homologous.

*Neural mechanisms underlying lateral-flexion swimming in the dendronotids*

The results of this study suggest that the swim CPGs responsible for lateral swimming in *Dendronotus* and *Melibe* are different. Si1 is an integral member of the swim CPG in *Melibe* but its homologue, the CPiP neuron, is not a component of the swim circuit in *Dendronotus*. Instead, the CPiP neuron can initiate a swim-like motor pattern.

The phylogeny of the dendronotids has not been sufficiently resolved to determine the exact relationship between *Melibe* and *Dendronotus*. Therefore, there are three possible evolutionary explanations for these differences in swim circuit organization: 1) the swim CPGs in these two species represent convergent evolution and are comprised of different neurons; 2) the *Dendronotus* condition represents the ancestral state and incorporation of Si1 into the CPG is a derived feature in the *Melibe* lineage; or 3) the *Melibe* condition represents the ancestral state and the removal of the CPiP neuron from the CPG is a derived feature in the *Dendronotus* lineage. Regardless of which of these hypotheses is correct, there is a possibility that homologues of the *Melibe* Si2 are also present in *Dendronotus* and that they alone comprise the lateral swim CPG in this animal. Future investigation of the neural mechanisms underlying lateral swimming in *Dendronotus* and other lateral-swimming species are necessary to further elucidate the evolution of the lateral-flexion swim circuits in nudibranchs.

### *Divergence of CPGs*

The functional divergence of homologous neurons in two species that exhibit similar behaviors contrasts with previous work demonstrating that homologous neurons can retain functional similarities in species that exhibit divergent behaviors (see Chapter Three). The DSIs in the nudibranch *Tritonia diomedea* are members of the CPG responsible for rhythmic dorsal-ventral flexion swimming in this animal (Getting et al., 1980), as well as being important in non-rhythmic mucociliary crawling (Popescu and Frost, 2002). Homologues of the DSIs, the *CeSP-A* neurons, are present in a wide array of nudibranchs that do not exhibit dorsal-ventral swimming (see Chapter Three). Despite the fact that the *CeSP-A* neurons are not rhythmically active in any of these other species, these neurons do have excitatory connections with putative crawling neurons just like in *Tritonia*. This suggests that homologous neurons can retain similar functions in species with divergent behaviors. Therefore, it is somewhat surprising in this study to find that homologous neurons have divergent functions in two of these nudibranch species that exhibit similar behaviors.

The divergence of swim CPG organization in two related nudibranch species also contrasts with the evolutionary conservation typically attributed to neural circuits (Wainwright and Lauder, 1986; Sanderson, 1988; Goslow et al., 1989; Wainwright et al., 1989; Edwards and Palka, 1991; Nishikawa et al., 1992; Weijs and Dantuma, 1994; Tierney, 1995; Kiehn et al., 1997; Katz and Harris-Warrick, 1999; Herrel et al., 2001; Langenbach and Van Eijden, 2001; Wainwright, 2002). Diversification in CPG output

has more often been attributed to peripherally-related components, such as neuromodulatory input (Kavanau, 1990; Katz and Tazaki, 1992), sensory input (Heiligenburg et al., 1996; Juranek and Metzner, 1996; Nishikawa, 1997), or motor and muscular components (Westneat and Wainwright, 1989; Paul, 1991), rather than changes within the neural circuit itself. It is possible that there have been peripheral changes in *Melibe* or *Dendronotus* that have contributed to the alteration of the swim CPG, despite the outward similarity of the lateral-flexion behaviors in the two species. However, this does not change the fact that the swim circuits have likely diverged in their neuronal composition.

Divergence of motor pattern circuitry has been previously demonstrated in fish, where many species exhibit a fast-start startle escape response (Domenici and Blake, 1997). Direct comparison of kinematic and electromyogram features of the fast-start startle response in four fish species indicates that the evolution of this behavior, and specifically the underlying neural circuitry, has not been conservative (Hale et al., 2002). When these results are mapped onto a larger vertebrate phylogeny, it becomes apparent that there have been major changes in the circuit for startle behavior at numerous levels of the vertebrate lineage (Hale et al., 2002).

The functional divergence of homologous neurons reported in the current study provides another example of likely divergence of neural circuitry among related taxa. While peripheral components of behavior may often be targets for natural selection, these results suggest that the underlying neural circuits themselves may also contribute to evolutionary changes.

## CHAPTER SIX

### GENERAL DISCUSSION

In these studies, we used morphological features, such as soma size and location, neurite projection pattern, and neurotransmitter to identify homologous neurons across a wide array of nudibranch molluscs that exhibit a variety of locomotor behaviors. Electrophysiological characterization of these homologous neurons indicates that many of the intrinsic physiological features, synaptic connections, and even putative basal functions of these homologues have been conserved across large phylogenetic distances. Despite these similarities, certain locomotor roles for these neurons have diverged, with homologous neurons sometimes adopting additional functions, even in closely-related species with similar behaviors. A number of conclusions can be drawn from these results, including: 1) homologous neurons can be identified between distantly-related species; 2) the central nervous system (CNS) is evolutionarily labile; and 3) the evolution of species-specific behaviors can involve novel configurations of pre-existing neurons.

#### *What defines neurons as homologous?*

Many characteristics can potentially be used to support homology between neurons or other structures, but how much evidence, and of which type, is necessary before making a claim of homology is unclear. Characteristics that can be used to determine homology between identified neurons include location, size, color, projection pattern, neurotransmitter phenotype, intrinsic physiological properties, synaptic

connections, molecular markers, genes, developmental history, and function. Historically, morphological characteristics were used to describe homology (Lauder, 1994). However, with the advent of evolutionary developmental biology and the concept of "biological homology", developmental history has become increasingly popular as a hallmark of homology (Roth, 1984; Wagner, 1989a,b).

One of the problems with attempting to define homology based on a single "optimal" characteristic is that homologous structures often lack similarity in this particular feature. For instance, despite the growing emphasis on the need for common developmental history in homologous structures, many examples exist of well-supported homology that lack similar developmental processes (de Beer, 1958, 1971; Sander, 1983; Hanken, 1986; Thorogood, 1987; Henry and Raff, 1990; Wray and Raff, 1990; Striedter and Northcutt, 1991; Harzsch, 2001).

To complicate matters further, similarity itself is neither necessary nor sufficient to claim homology (Striedter and Northcutt, 1991). For example, homologous elements can be quite dissimilar because of evolutionary divergence (e.g. human hands and bat wings) and others that are remarkably similar may actually be the result of convergent evolution (e.g. shark and dolphin dorsal fins). However, similarities are often the first indicator of homology and are still very important in identifying homologous elements.

The current lack of a single, accurate criterion for homology means that neurons cannot be definitively demonstrated to be homologous. Rather, an accumulation of evidence, based on similarities in a number of characteristics, can only support the parsimonious interpretation of homology. The more similarities that are exhibited by



neurons in different species, then the more likely it is that these cells are evolutionarily derived from the same ancestral neuron (Simpson, 1967; Lauder, 1994; Kutsch and Heckmann, 1995).

In these studies, we use a number of morphological criteria to hypothesize homology between neurons. In the case of the *Tritonia* DSIs and the *CeSP-A* neurons in other nudibranchs, we found inter-species similarities in soma size and location, neurite projection pattern, and neurotransmitter. The same criteria were used in comparing the *Melibe* Si1 and *Dendronotus* CPiP neuron, with the exception of neurotransmitter phenotype, which is unknown for these neurons. In all cases, these morphological criteria were sufficient to distinguish these neurons from all other neurons in the brain of each species, although additional work in *Dendronotus* is necessary to quantify these comparisons. While it is plausible that these neurons exhibit these similarities due to convergent evolution, it is most parsimonious at this time to conclude that they are homologous. As our knowledge of the physiological, synaptic, molecular and genetic characteristics of these neurons increases over the years, additional evidence will accumulate to either support or undermine this conclusion.

#### *Evolution of neural circuits producing rhythmic output*

Dorsal-ventral and lateral flexion swimming in nudibranchs are rhythmic behaviors that have likely evolved independently multiple times. This evolution appears to have involved novel configuration of pre-existing neurons, as our results demonstrate that homologous neurons involved in these behaviors are conserved across species,

regardless of whether they exhibit rhythmic swimming behavior or not. For example, the DSIs are integral members of the dorsal-ventral swim CPG in *Tritonia* (Getting et al., 1980) but their homologues, the *CeSP-A* neurons, are not rhythmically active in species that do not exhibit dorsal-ventral flexion swimming (see Chapter 3, Fig. 3-7). Likewise, *Si1* is a necessary component of the lateral flexion swim CPG in *Melibe* (Thompson and Watson, 2005) but its homologue in the closely related *Dendronotus iris*, the CPiP neuron, is not rhythmically active during a swim-like motor pattern (see Chapter 5, Fig. 5-5). At this time, what enables a particular neuron to be rhythmically active in one species and not in another species is not clear. There are several possible mechanisms for the evolution of rhythmicity in these neurons, including changes in intrinsic cellular properties, alterations of synaptic connections, and differences in neuromodulatory input.

*Evolution of rhythmicity due to changes in intrinsic cellular properties*

Evolutionary changes in the intrinsic cellular properties of neurons have been correlated to functional differences (Wright, 2000; Chiang et al., 2006) and could contribute to the capacity for rhythmicity. Circuits can exhibit rhythmic activity as a result of endogenously bursting neurons (Strumwasser, 1968; Kandel, 1976; Miller and Selverston, 1982a; Peterson and Calabrese, 1982; Egelhaaf and Benjamin, 1983; Hartline and Russell, 1984; Arshavsky et al., 1985, 1988; Dekin et al., 1985; Andrew, 1987; Wallen and Grillner, 1987; Ramirez and Pearson, 1991; Johnson et al., 1994; Thoby-Brisson and Ramirez, 2001; Cymbalyuk et al., 2002) or synaptic connections that form a network oscillator (Stent et al., 1978; Miller and Selverston, 1982b; Getting, 1989;

Calabrese et al., 1995; Cymbalyuk et al., 2002; Straub et al., 2002; Fischer, 2004).

Rhythmic activity in the *Tritonia* DSIs and *Melibe* Si1 is a result of network properties of their respective circuits. Therefore, if changes in intrinsic properties contributed to the evolution of this rhythmicity, these alterations would have taken place in the context of a network oscillator.

A number of intrinsic neuronal properties are considered important for contributing to the capacity for rhythmicity, including adaptation (Benjamin and Rose, 1979; Berman et al., 1989; Guckenheimer et al., 1997; Lansner et al., 1998; van Vreeswijk and Hansel, 2001), depolarizing spike after-potentials (Thompson and Smith, 1976; Wong and Prince, 1978), plateau potentials (Miller and Selverston, 1982b; Arbas and Calabrese, 1987; Katz and Harris-Warrick, 1989; Hartline and Graubard, 1992; Dicaprio, 1997; Schwindt and Crill, 1999), post-inhibitory rebound (Benjamin and Rose, 1979; Mulloney et al., 1981; Miller and Selverston, 1982b; Elliott and Benjamin, 1985; Satterlie, 1985; Hartline and Graubard, 1992; Mangan et al., 1994; Bertrand and Cazalets, 1998), and after-hyperpolarization (Hellgren et al., 1992; Wallen et al., 1992; Chang et al., 1993; Mangan et al., 1994). The after-hyperpolarization exhibited by the *Tritonia* DSIs is the only one of these cellular properties that we have systematically examined in the homologous neurons in this study. Although an after-hyperpolarization is absent in the *CeSP-A* neurons of most of the species that we investigated, the non-rhythmic *CeSP-A* neurons in *Tochuina* have an after-hyperpolarization that is not significantly different from that exhibited by the *Tritonia* DSIs. Therefore, although a role for this after-hyperpolarization in DSI bursting cannot be ruled out, it is apparent that it is not the only

factor that contributes to the capacity for rhythmic activity in the DSIs. Future studies will be necessary to investigate the role of the after-hyperpolarization and other intrinsic neuronal properties in the evolution of rhythmic activity in these neurons.

*Evolution of rhythmicity due to changes in synaptic connectivity*

Alterations in synaptic connectivity could also contribute to the capacity for a neuron to be rhythmically active. During development, a neuron's axonal and dendritic path-finding and connectivity are strictly guided by growth factors, adhesion molecules, and post-synaptic targets (Waites et al., 2005; Wen and Zheng, 2006). Perturbations of this developmental pattern can result in regressive changes (Cowan et al., 1984) or novel connections (Shaw and Meinertzhagen, 1986) with functional consequences. For example, Arbas (1983b) determined that homologues of the locust DCMD visual interneuron in secondarily flightless grasshoppers either lacked particular branches or had aberrant projections to different neuropil areas. In dipteran flies, there are changes in synaptic connectivity of homologous interneurons in the first visual neuropil (Shaw and Meinertzhagen, 1986; Shaw and Moore, 1989) and in deeper visual neuropils (Buschbeck and Strausfeld, 1997) that may relate to inter-species differences in visual resolution.

Examination of synaptic connectivity requires identification of both pre- and post-synaptic neurons. In this study, we identified one post-synaptic target of the *CeSP-A* neurons, a homologue of the *Tritonia* Pd5 neuron in *Tochuina* and *Triopha*. In *Tritonia*, the DSIs make monosynaptic, excitatory connections with Pd5 (Popescu and Willows, 1999; Popescu and Frost, 2002), an efferent that contributes to pedal ciliary

beating and crawling (Willows et al., 1997; Popescu and Willows, 1999; Popescu and Frost, 2002). This synapse was conserved in both *Tochuina* and *Triopha* (see Chapter 3, Fig. 3-8). However, crawling in these species is not a rhythmic behavior (Baltzley, 2006). Therefore, the fact that there is conservation of synaptic contacts between disparate species does not elucidate potential mechanisms for the evolution of rhythmic activity in the *CeSP-A* neurons.

Electrical synapses may also affect the capacity for a network to exhibit rhythmicity. Surprisingly, electrical coupling between neurons that are otherwise non-oscillatory can produce rhythmic network activity (Marder, 1998). Modeling studies of inferior olivary cells (Manor et al., 1997) and pancreatic  $\beta$ -cells (Smolen et al., 1993) support this hypothesis, and it has been demonstrated experimentally with viral (Placantonakis et al., 2006) and pharmacological (Blenkinsop and Lang, 2006) blockade of electrical synapses in the inferior olive. In molluscs, dynamic clamp has been used to artificially create simultaneous reciprocal inhibition and electrical coupling between snail neurons (Tiaza et al., 2005). These studies demonstrate that such a synapse can generate a bistable network switching between synchronous firing and antiphase firing of the two network partners. This suggests that electrical coupling can contribute to an immediate reconfiguration of activity patterns without resorting to alterations of modulatory processes.

Interestingly, we saw differences in electrical coupling connectivity between homologous neurons in our study that correlated with whether or not a neuron or circuit was rhythmically active. In *Tritonia*, electrical coupling exists within, but not between,

the two classes of DSIs (DSI<sub>A</sub> and DSI<sub>B/C</sub>; Getting, 1981; see Chapter 3, Fig. 3-5B). In contrast, the *CeSP-A* neurons in *Melibe* are all electrically coupled to each other, both ipsilaterally and contralaterally, with no distinction between any possible classes of *CeSP-A* neurons (see Chapter 3, Fig. 3-5E). However, it still remains to be determined whether this difference in electrical connectivity between DSIs/*CeSP-A* neurons contributes to differences in rhythmicity between these homologous neurons.

Electrical coupling may also be important for rhythmicity of Si1 and its homologues. In *Melibe*, Si1 and Si2 are electrically coupled ipsilaterally (Thompson and Watson, 2005), although the function of this electrical connection is not yet fully understood. The Si1 neuron in *Dendronotus iris*, the CPiP neuron, is not rhythmically active, even during a swim motor pattern. The CPiP neuron may not be electrically coupled to the homologue of Si2 in *Dendronotus*. Thus, even if all other synaptic connectivity is similar between the two species, this lack of an electrical connection could contribute to the fact that the CPiP neuron is not rhythmically active or a member of the lateral swim CPG in *Dendronotus*. Identification of the Si2 homologue in *Dendronotus* will be necessary to definitively test this hypothesis.

The strength of electrical coupling can also switch a rhythmic network between stimulus-driven and spontaneous oscillator states (Manor et al., 1997). One of the interesting findings from this study is that in contrast to *Melibe*, where the isolated brain spontaneously expresses swim motor patterns, swim motor patterns are only elicited in *Dendronotus* preparations in response to bath perfusion of serotonin or depolarization of the CPiP neuron (see Chapter 5, Figs. 5-5 & 5-6). Thus, even if electrical coupling occurs

between the *Dendronotus* CPiP neuron and a putative homologue of the *Melibe* Si2, it could be weak enough to effectually remove the CPiP neuron from the swim circuit and also make the swim circuit more of a stimulus-driven and less of a spontaneous oscillator.

*Evolution of rhythmicity due to changes in neuromodulation*

Many neural circuits are multifunctional (McClellan, 1982; Ayers et al., 1983; Hooper and Moulins, 1989; Mortin and Stein, 1989; Dickinson et al., 1990; Harris-Warrick and Marder, 1991; Katz and Harris-Warrick, 1991; Meyrand et al., 1991; Dickinson and Moulins, 1992; Dickinson, 1995; Rozsa, 1995; Kittmann et al., 1996; Quinlan and Murphy, 1996; Lieske et al., 2000; Tazaki and Tazaki, 2000; Popescu and Frost, 2002; Berkowitz, 2005; Norris et al., 2006), and neuromodulation is known to be important in some of these networks for determining which motor pattern is expressed at a given time (Katz and Harris-Warrick, 1990; Harris-Warrick et al., 1992; Marder and Weimann, 1992; Nusbaum et al., 2001). This real-time neuromodulation could also occur over an evolutionary timescale, with the addition or subtraction of particular neuromodulatory effects resulting in the production of novel, species specific behaviors or the suppression of pre-existing motor patterns (Kavanau, 1990; Katz and Tazaki, 1992; Antonsen and Paul, 1997, 2001). The escape swim circuit in *Tritonia* is one of the first examples of a multifunctional circuit and was originally termed "polymorphic" (Getting and Dekin, 1985). This network mediates both withdrawal and swimming, and subtle evolutionary changes in the modulation of this circuit could bias its function and suppress one of these behaviors (Kavanau, 1990; Arbas et al., 1991).

In the case of the DSIs, driving these neurons to fire at an elevated rate will elicit a rhythmic swim motor pattern in the absence of extrinsic neuromodulatory input to the circuit (Fickbohm and Katz, 2000; Frost et al., 2001; Katz et al., 2004). However, the DSIs are serotonergic (Katz et al., 1994; McClellan et al., 1994) and are intrinsic neuromodulators of the swim CPG (Katz et al., 1994; Katz and Frost, 1995a,b, 1997; Katz, 1998; Sakurai and Katz, 2003). Serotonin is necessary for swimming in *Tritonia* (McClellan et al., 1994), and release of serotonin by the DSIs increases the excitability of another member of the swim CPG, C2. This modulation is likely to be important in enabling the circuit to function in a rhythmic mode and maintaining it in that state (Katz and Frost, 1997).

The *CeSP-A* neurons in other nudibranch species are also serotonergic (see Chapter 2), and both depolarization of the *CeSP-A* neurons (see Chapter 4, Fig. 4-3; Chapter 5, Fig. 5-6) and serotonin (see Chapter 4, Fig. 4-3; Chapter 5, Fig. 5-5) can elicit lateral-flexion swimming in *Melibe* and *Dendronotus iris*. However, serotonin does not elicit rhythmic activity in the *CeSP-A* neurons in these species. This suggests that these neurons are not part of the lateral-flexion swim CPGs and also that there is not a latent dorsal-ventral swim CPG in these species. This lack of rhythmic activity could be related to differences in the neuromodulatory functions of the *CeSP-A* neurons. Due to the similarity of nudibranch nervous systems, homologues of the remaining neurons in the *Tritonia* swim CPG, VSI and C2, probably occur in the other species. Identification of these homologues will be necessary to investigate the hypothesis that the neuromodulatory functions of the *CeSP-A* neurons are different than the *Tritonia* DSIs.



Differences in extrinsic modulation of this network of neurons in these species may also exist, such that the *CeSP-A* neurons are involved in withdrawal but not rhythmic dorsal-ventral flexions in *Melibe* and *Dendronotus*. Semi-intact preparations involving stimulation of the *CeSP-A* neurons while monitoring the behavioral response of the animal would potentially elucidate whether the circuitry for withdrawal is conserved in these species.

### *Evolution of swimming*

To fully understand the evolution of swimming in nudibranchs, it is necessary to determine the basal mode of locomotion for this clade. However, it is not clear whether dorsal-ventral flexion swimming, lateral flexion swimming, or non-swimming was the ancestral state (see Chapter 1, Fig. 1-2). Based on the similarities between the CPGs underlying dorsal-ventral flexion swimming in *Tritonia* and the notaspid *Pleurobranchaea*, Jing and Gillette (1999) proposed that dorsal-ventral swimming was exhibited by their common ancestor. Assuming monophyly of Nudibranchia, this would make dorsal-ventral swimming the ancestral condition for nudibranchs. However, since *Tritonia* and *Pleurobranchaea* may be phylogenetically disparate, with intervening suborders that do not contain any dorsal-ventral swimming species, it is also possible that this is a case of parallel evolution (Katz and Newcomb, in press).

Undoubtedly, the nudibranch ancestor crawled, as all but a few nudibranchs exhibit this form of locomotion (Lalli and Gilmer, 1989), as well as all of the pleurobranchids (order: Notaspidea), which are the closest sister group to the nudibranchs

(Wägele and Willan, 2000). The conservation of synaptic connections between DSIs/CeSP-A neurons and putative efferents involved in crawling (see Chapter 3, Fig. 3-8) further supports this conclusion. However, the comparison of homologous neurons in this study does not elucidate whether dorsal-ventral flexion or lateral flexion swimming was also an ancestral trait for nudibranchs. Finer resolution of the nudibranch phylogeny is necessary to refine this hypothesis regarding the basal mode of locomotion in this clade.

Although it is not possible at this time to definitively determine the ancestral locomotor state for nudibranchs, it is still feasible to make some conclusions about the evolution of swimming within this clade. One of these conclusions is that divergent modes of swimming have evolved independently multiple times. For example, in three of the four nudibranch suborders, there are multiple modes of swimming represented in extant species (see Chapter 1, Table 1-1), indicating divergent evolution of swimming behaviors. The suborders Dendrodonotoidea and Doridoidea have dorsal-ventral flexion swimmers (e.g. *Tritonia* and *Aphelodoris*) and lateral flexion swimmers (e.g. *Melibe* and *Plocamopherus*). The suborder Aeolidioidea has lateral flexion swimmers (e.g. *Flabellina*) and species that swim by beating their cerata ("breast-stroke swimmers"; e.g. *Cumanotus*). Arminoidea is the only suborder without any species that are known to exhibit swimming, although the capacity for swimming has not been systematically investigated in any of the nudibranch clades so it is still possible that some arminoid species can swim.

In the case of the dorsal-ventral swimming *Tritonia* and the lateral swimming

*Melibe*, the neural circuits underlying these behaviors provide additional evidence that these behaviors are divergent. Neurons comprising these circuits are entirely different and thus the circuits and behaviors are not homologous (see Chapter 1, Fig. 1-3). However, there are neurons in the brain of *Melibe* that are homologous to neurons in the *Tritonia* swim circuit (i.e. the DSI homologues and the *CeSP-A* neurons). This suggests that these species share a common neural organization and that these two non-homologous swim CPGs have evolved within the confines of a similar set of neurons.

Lateral swimming in the dendronotids may be divergent at the neural level. Both *Melibe* (Agersborg, 1921; Hurst, 1968; Farmer, 1970; Lawrence and Watson, 2002) and *Dendronotus iris* (Agersborg, 1922; Haefelfinger and Kress, 1967; Farmer, 1970; Robilliard, 1970, 1972; Wobber, 1970) exhibit lateral flexion swimming but the CPGs producing these behaviors are different. Si1 is a member of the swim CPG in *Melibe* (Thompson and Watson, 2005) but its homologue in *Dendronotus*, the CPiP neuron, is not part of the putative swim circuit in *Dendronotus* (see Chapter 5, Fig. 5-5). The phylogeny of the dendronotids is not defined with enough resolution to determine what is the ancestral state for the lateral flexion swim circuit in the dendronotids. In fact, these differences in the swim CPGs might even indicate convergent evolution, as lateral swimming could have evolved twice in the dendronotid lineage. However, it is more parsimonious to conclude that it evolved only once, or was already present in the dendronotid ancestor, and that the circuits have merely diverged over time. Identification and characterization of the Si2 homologue in *Dendronotus* would be useful in resolving this issue. If *Dendronotus* has a homologue of Si2 and this neuron is a member of the

lateral-flexion swim CPG, then the most parsimonious conclusion would be that the difference in Si1/CPiP function is the result of divergence. However, if the Si2 homologue in *Dendronotus* is not part of the swim CPG in this species, similar to the CPiP neuron, this would suggest that lateral-flexion swimming evolved convergently in *Melibe* and *Dendronotus*.

#### *Selective pressures for the evolution of swimming*

The multiple occurrences of independent evolution of swimming in the nudibranchs suggest that strong selective pressures exist for the evolution of this behavior. Swimming in many nudibranchs has been proposed to be a defensive strategy for escape from predators (Farmer, 1970; Thompson, 1976). Support for this hypothesis is the fact that the touch of a predatory seastar will elicit swimming in *Dendronotus* (Robilliard, 1972), *Melibe* (Lawrence and Watson, 2002), and *Tritonia* (Mauzey et al., 1968; Willows, 1968). Many nudibranchs are carnivorous and will eat other nudibranchs, including conspecifics (Megina and Cervera, 2003). *Tambja eliora* swims in response to the predatory nudibranch *Roboastra* (Farmer, 1970), *Hermisenda crassicornis* exhibits lateral flexions in response to bites from conspecifics (G Clark, personal communication), and *Flabellina iodinea* will swim in response to an attack from the carnivorous notaspid *Pleurobranchaea* (R Gillette, personal communication). However, other nudibranchs are likely to be under similar predatory pressures and do not exhibit swimming. For example, *Armina californica* is a common prey item of *Pleurobranchaea* but does not swim (Battle and Nybakken, 1998). *Armina* burrows in

the sand (Bertsch, 1968; Ricketts et al., 1985) and this behavior may be used as an escape response in this animal instead of swimming.

A number of other defensive strategies are used by nudibranchs, including autotomy of cerata (Stasek, 1967; Kress, 1968), crypsis (Edmunds, 1987; Gosliner and Behrens, 1990; Marin et al., 1997), dermal spicules (Harris, 1973; Cattaneo-Vietti et al., 1993), incorporation of nematocysts from cnidarian prey into cerata (Grosvenor, 1903; Kepner, 1943; Conklin and Mariscal, 1977; Day and Harris, 1978; Greenwood and Mariscal, 1984; Lalli and Gilmer, 1989; Östman, 1997; Martin, 2003; Frick, 2005), and secretion of noxious chemicals (Thompson, 1960; Edmunds, 1966; Cimino et al., 1983; Faulkner and Ghiselin, 1983; Gustafson and Andersen, 1985; Karuso, 1987; Williams and Andersen, 1987; Faulkner et al., 1990; Avila et al., 1991a,b; Faulkner, 1992; Gavagnin et al., 1992; Fontana et al., 1993; McClintock et al., 1994; Avila, 1995; Graziani et al., 1996; Graziani and Andersen, 1996; Dumdei et al., 1997; Kubanek et al., 1997; Cimino and Ghiselin, 1999; Cimino et al., 1999; Avila et al., 2000). An alternative explanation for the variety of locomotor modes in the nudibranch clade is that the capacity for swimming was lost multiple times over the course of evolution. The existence of alternative defensive strategies might mean that swimming is a redundant defensive strategy for some species and therefore subject to being lost with minimal cost to the survival of the species. Interestingly, some of the species that swim also utilize some of these alternative defensive strategies (Ayer and Anderson, 1983; Pawlik et al., 1988; Bickell-Page, 1989, 1991; Miller and Byrne, 2000; Barsby, 2002), suggesting that these behaviors may not necessarily be functionally redundant for some species.

Other hypotheses have been proposed to explain why certain nudibranchs exhibit swimming behavior. The pelagic nudibranchs (i.e. *Phylliroë* and *Cephalopyge*) have likely evolved swimming as a means to occupy an ecological niche beyond the benthos (Lalli and Gilmer, 1989). *Melibe*, which readily swims for extended periods of time (Mills, 1994; Lawrence and Watson, 2002; Caldwell and Donovan, 2003), may swim as a means of reproductive dispersal or to search for food (Agersborg, 1921; Mills, 1994; Willows, 2001). Other nudibranchs, such as *Dendronotus iris* (Agersborg, 1922; Mills, 1994), *Flabellina cynara* (Farmer, 1970) and *Hexabranchus sanguineus* (Gohar and Soliman, 1963; Edmunds, 1968), will swim for prolonged periods and therefore also may use this mode of locomotion to find food or mates.

Select modes of swimming also have different degrees of directionality. Dorsal-ventral flexion swimming is quite non-directional, resulting in the animal tumbling through the water column (Willows, 2001). In contrast, although lateral-flexion swimming has been described as non-directional (Farmer, 1970; Daniel, 1984) more recent evidence suggests that lateral-flexion swimming does have some directionality to it, with the animal traveling perpendicular to the plane of the foot (Lawrence and Watson, 2002). Likewise, swimming involving flapping tends to be directional and purposeful (Farmer, 1970; though see Gohar and Soliman, 1963).

This variety of directionality and potential functions for swimming suggests that a single selective pressure probably cannot explain the evolution of swimming throughout this clade. Unfortunately, very little is known about the ecology of most nudibranchs and therefore determining the selective forces contributing to the evolution of swimming (or

loss of swimming) in any of these species is difficult.

### *Evolution of neural circuits*

This study provides one of the first examples of neurons that are members of a CPG in one species being non-rhythmic in another species. In fact, this result was demonstrated not just once, but in two different CPGs. These results suggest that the neuronal composition of CPGs may be more flexible than previously thought.

Other studies have indicated that CPGs may be evolutionarily altered, but they have not demonstrated this at the level of individual neurons. For example, fish exhibit a fast-start escape response initiated by the Mauthner cell (Eaton et al., 1981). Phylogenetic comparison of the kinematics and muscle activity patterns of the startle response in a wide range of aquatic vertebrates suggests that the startle neural circuit itself is not evolutionarily conservative, despite the fact that the Mauthner cell is a synapomorphy of vertebrates (Hale et al., 2002). Thus, the specific neural changes responsible for the evolution of the startle neural circuit in the vertebrate lineage still remain to be determined.

In the case of nudibranchs, the presence of homologous interneurons in phylogenetically disparate species suggests that the interneuronal composition of the central nervous system may be relatively conserved. Therefore, different behaviors are produced from a similar set of neurons. The differences between the resulting circuits could be subtle, involving slight differences in connectivity, intrinsic properties, and neuromodulation, as mentioned above.

Although these modifications have not been demonstrated in other systems at the level of individual neurons, it is reasonable to hypothesize that evolution of neural circuits in other animals could happen in a similar fashion. For example, the evolution of a “hopping” gait from an alternating gait in tetrapods, or the evolutionary changes in the startle response in aquatic vertebrates, may merely be the result of a slight modification of pre-existing neurons. With recent studies honing in on the interneuronal circuitry responsible for mammalian locomotion (Kiehn, 2006), escape responses in fish (Ritter et al., 2001), and other CPGs, the evolution of these behaviors may soon be able to be examined at the neuronal level.

#### *What next?*

The data from these studies have produced some interesting findings but also additional questions. What is the role of the DSI homologues, the *CeSP-A* neurons, in species that exhibit dorsal-ventral flexion swimming like *Tritonia*? Are there homologues in other species of the remaining swim interneurons in the *Tritonia* dorsal-ventral swim CPG and the *Melibe* lateral swim CPG? What is the neurotransmitter of the *Melibe* Si1? What is the swim CPG in *Dendronotus*? What is the role of the periphery in the evolution of swimming in nudibranchs? Are there other nudibranchs that swim that we are not aware of yet, and how did the circuits controlling their swimming evolve? Some of these questions and others are outlined below.



*What is the role of the DSI homologues, the CeSP-A neurons, in species that exhibit dorsal-ventral flexion swimming like Tritonia?*

In this study, we identified and characterized homologues of the *Tritonia* DSIs, the CeSP-A neurons, in species that do not exhibit dorsal-ventral flexion swimming. In these species, the CeSP-A neurons were very similar to the DSIs, including a conserved synaptic connection with putative crawling efferents (see Chapter 3, Fig. 3-8). However, the CeSP-A neurons were not rhythmically active in response to nerve stimulation in any species. This raises the interesting possibility that these neurons may be part of a rhythmic circuit only in dorsal-ventral flexion swimmers. Therefore, a next logical step is to identify and characterize the CeSP-A neurons in nudibranchs that do exhibit this mode of swimming, such as *Aphelodoris* (Wilson, 2003, Quiroga et al., 2004) and even *Hexabranchus*, which has a dorsal-ventral flexion component to its swimming behavior (Gohar and Soliman, 1963; Vicente, 1963; Edmunds, 1968; Farmer, 1970). If the CeSP-A neurons are not rhythmically active in these species under similar stimulation parameters, this would suggest that the incorporation of these neurons into the dorsal-ventral swim CPG in *Tritonia* is a derived condition. However, if the CeSP-A neurons are rhythmically active in these other dorsal-ventral swimming species in response to nerve stimulation, this would suggest that the participation of these neurons in the dorsal-ventral swim CPG is an ancestral condition (Jing and Gillette, 1999). By default, this latter conclusion would also mean that dorsal-ventral swimming was evolutionarily lost multiple times and that lateral swimming evolved multiple times (see Chapter 1, Fig. 1-2C).

*Are there homologues in other species of the remaining swim interneurons in the Tritonia dorsal-ventral swim CPG and the Melibe lateral swim CPG?*

In this study, we identified and characterized homologues of the *Tritonia* DSIs and the *Melibe* Si1, both integral members of the respective swim CPGs for these animals (Gettting et al., 1980; Thompson and Watson, 2005). However, other components of these swim CPGs exist: VSI and C2 in *Tritonia* (Gettting, 1977; Taghert and Willows, 1978; Gettting et al., 1980; Gettting, 1983) and Si2 in *Melibe* (Thompson and Watson, 2005). Considering the similarities in nudibranch brains and the existence of homologous neurons between species (Dorsett, 1974; Dickinson, 1979; Pentreath et al, 1982; Croll, 1987a; Katz et al., 2001), homologues of VSI, C2 and Si2 may be present in many other nudibranch species. It would be interesting to investigate whether homologues of these neurons are rhythmically active in species with divergent forms of behavior. This would help explain whether just the DSIs/*CeSP-A* neurons and the Si1/*CPiP* neuron have diverged in their capacity for rhythmicity or if the entire homologous swim circuits no longer express rhythmic activity in species with different modes of locomotion. In other words, identification and characterization of additional swim interneuron homologues might clarify the extent of circuit modification that has occurred during the evolution of these swim behaviors.

C2 is the best candidate from the remaining swim interneurons for identifying putative homologues in other species. While the neurotransmitters for C2, VSI, and Si2 are currently unknown, evidence suggests that C2 may be peptidergic (Snow, 1982) and

the peptide may be related to FMRFamide (Longley and Longley, 1987). As with the DSIs/*CeSP-A* neurons in the current study, knowledge of the neurotransmitter of C2 would be invaluable in identification of homologues in other species. Upon identification of C2 homologues, a plethora of experimental options would be available for investigating synaptic connectivity and neuromodulation in homologous circuits.

*What are the roles of CPiP neurons in species that do not exhibit lateral swimming?*

Similar to the investigation in this study of the functional role of *CeSP-A* neurons in species that do not exhibit dorsal-ventral flexion swimming, it would be interesting to determine whether homologues of the *Melibe* Si1, the CPiP neurons, are present and can elicit locomotor motor patterns in species that do not exhibit lateral swimming. The obvious species to first examine the function of the CPiP neuron is *Tritonia* because the dorsal-ventral flexion swim CPG is already characterized in this species (Getting et al., 1980). Thus, if *Tritonia* has a CPiP neuron, it would be easy to monitor the rhythmic motor pattern during stimulation of the CPiP neuron. Furthermore, because the Si1/CPiP neuron is located just medial to the *CeSP-A* neurons (see Chapter 5, Fig. 5-3) and the homologues of the *CeSP-A* neurons in *Tritonia*, the DSIs, are readily identifiable in this species, it may be easier to identify a putative CPiP neuron in *Tritonia* than in some other nudibranchs. Characterization of the functional role of the CPiP neuron in species that do not exhibit lateral flexion swimming may further elucidate how circuits are modified during the evolution of species-specific behaviors.

*What is the swim CPG in Dendronotus iris?*

One of the surprising findings from this study was that the swim CPGs in two, relatively closely-related lateral-flexion swimmers, *Melibe leonina* and *Dendronotus iris*, are different. However, it is not clear yet whether these two swim CPGs are homologous and only slightly divergent or if they are completely different and are an example of convergent evolution. The identification of the swim CPG in *Dendronotus* would help to answer this question. The most parsimonious hypothesis is that there are homologues of the *Melibe* Si2 and that these comprise the circuit in *Dendronotus*. If this is the case, then Si1 was co-opted during evolution to be part of the swim circuit in *Melibe*, or its homologue, the CPiP neuron, was excluded from the circuit in *Dendronotus*. Unfortunately, the phylogeny of the dendronotids is not sufficiently resolved to determine which of these scenarios describes the evolution of the Si1/CPiP neuron and the lateral swim CPGs in these animals. However, either case would indicate divergent evolution of the circuits producing lateral swimming in the dendronotids.

*What is the role of the periphery in the evolution of swimming in nudibranchs?*

As indicated previously, the CNS is generally considered to be relatively conserved during evolution in relation to the periphery (Bramble and Wake, 1985; Wainwright and Lauder, 1986; Lauder and Shaffer, 1988; Sanderson, 1988; Goslow et al., 1989; Wainwright, 1989, 2002; Wainwright et al., 1989; Kavanau, 1990; Arbas et al., 1991; Edwards and Palka, 1991; Paul, 1991; Katz and Tazaki, 1992; Nishikawa et al., 1992; Weijs and Dantuma, 1994; Tierney, 1995; Kiehn et al., 1997; Katz and Harris-

Warrick, 1999; Herrel et al., 2001; Langenbach and Van Eijden, 2001; though see Smith 1994). Our results from this study indicate that the presence of specific neurons across a diverse array of species does indeed remain conserved, although the features and functional roles of these neurons are subject to evolutionary change. These evolutionary changes can include alterations of CPG output, in contrast to previous suggestions of circuit conservation during evolution. However, these evolutionary changes in the CNS do not preclude alterations in the periphery that may also be responsible for the evolution of species-specific behaviors.

Nudibranchs exhibit diverse body forms (Willows, 2001) and it is possible that morphological changes in the periphery may have contributed to driving alterations of the CNS. Wilczynski (1984) has suggested that the interplay between changes in the periphery and the development of the nervous system may be important in reconfiguring neural circuitry to deal with an altered periphery. Peripherally-induced changes of the CNS have been demonstrated in other invertebrates, such as annelids (Baptista et al., 1990) and arthropods (Schneiderman et al., 1982), although it still remains to be determined whether peripheral changes can alter the CNS in molluscs.

*Which nudibranchs swim and what mode of swimming do they use?*

Of the more than one thousand species of nudibranchs, only 61 have been documented in the scientific literature to swim (see Chapter 1, Table 1-1). Since most of the 61 swimmers do so very rarely, many of the other nudibranchs may also be swimmers but just not yet observed doing so. Swimming nudibranchs usually respond to a number

of noxious stimuli, such as concentrated salt solutions, removal from the substrate, and predators (Mauzey et al., 1968; Willows, 1968; Farmer, 1970; Robilliard, 1972; Willows et al., 1973; Lawrence and Watson, 2002), and these can be used to systematically analyze the capacity for swimming in other species. Systematic investigations of the capacity and mode of swimming throughout this clade are necessary for a thorough understanding of the evolution of these behaviors in the nudibranch lineage.

*What are the phylogenetic relationships in the nudibranch clade?*

Although nudibranchs have traditionally been subdivided into four suborders based on morphological features (Odhner, 1934; Schmekel, 1985; Schrödl et al., 2001), the advent of molecular cladistics has begun to cast doubt on the monophyly of these suborders or even of nudibranchs in general (Thollessen, 1999; Wollscheid-Lengeling et al., 2001; Grande et al., 2004). However, in light of these recent molecular data, no consensus yet exists on the overall phylogeny of the nudibranchs. Furthermore, the current resolution of the phylogenetic relationships within individual suborders is still quite low. Therefore, additional cladistic analysis is necessary to clarify these issues. Studies that combine new molecular data with traditional morphological data, such as those by Wägele and Willan (2000), will likely prove most useful in this endeavor. Such clarifications and increased resolution will enable more thorough and accurate interpretation of results from comparative studies and, therefore, a better understanding of the evolution of species-specific behaviors in this clade.

*Summary and conclusions*

In summary, we compared neurotransmitter localization and homologous neurons in a wide array of nudibranch molluscs to investigate the role of the CNS during the evolution of species-specific locomotor behaviors. Using morphological criteria, we identified and characterized a number of homologous neurons between species, suggesting some degree of conservation of the CNS. However, in contrast to many previous studies, we found that the CNS was evolutionarily labile in this group of animals. Substantial homoplasy exists in the location and number of serotonergic neurons between species, and, despite the conservation of certain neurons in a wide array of species with divergent locomotor behaviors, the properties and functions of homologous neurons varies. This functional divergence means that, even in closely related species, similar behaviors are produced by different neural circuits. The existence of homologous neurons differentially involved in a variety of locomotor behaviors also suggests that neural circuits responsible for controlling species-specific behavior evolved from pre-existing circuitry. This is yet another example of the fact that evolution often involves subtle modification of an existing template, rather than building from scratch.

## REFERENCES

- Agersborg HPK (1919) Notes on *Melibe leonina* (Gould). Publ Puget Sound Biol Sta 2: 269-277, pls. 49-50.
- Agersborg HPK (1921) Contributions to the knowledge of the nudibranchiate mollusk, *Melibe leonina* (Gould). Amer Naturalist 55: 222-253.
- Agersborg HPK (1922) Notes on the locomotion of the nudibranchiate mollusk *Dendronotus giganteus* (O'Donoghue). Biol Bull 42: 257-266.
- Agersborg HPK (1923) The morphology of the nudibranchiate mollusc *Melibe* (syn. *Chioraera*) *leonina* (Gould). Q J Microsc Sci 67: 507-592.
- Alania M (1995) Pleuro-buccal projections in pulmonate molluscs. Acta Biol Hung 46: 267-270.
- Andrew RD (1987) Endogenous bursting by rat supraoptic neuroendocrine cells is calcium dependent. J Physiol 384: 451-465.
- Antonsen BL, Paul DH (1997) Serotonin and octopamine elicit stereotypical agonistic behaviors in the squat lobster *Munida quadrispina*. J Comp Physiol A 181: 501-510.
- Antonsen BL, Paul DH (2001) Serotonergic and octopaminergic systems in the squat lobster *Munida quadrispina* (Anomura, Galatheidae). J Comp Neurol 439: 450-468.
- Arbas EA (1983a) Neural correlates of flight loss in a Mexican grasshopper, *Barytettix psolus*. I. Motor and sensory cells. J Comp Neurol 216:369-380.
- Arbas EA (1983b) Neural correlates of flight loss in a Mexican grasshopper, *Barytettix psolus*. II. DCMD and TCG interneurons. J Comp Neurol 216: 381-389.
- Arbas EA, Calabrese RL (1987) Slow oscillations of membrane potential in interneurons that control heartbeat in the medicinal leech. J Neurosci 7: 3953-3960.
- Arbas EA, Meinertzhagen IA, Shaw SR (1991) Evolution in nervous systems. Annu Rev Neurosci 14: 9-38.
- Arshavsky YI, Beloozerova IN, Orlovsky GN, Panchin YV, Pavlova GA (1985) Control of locomotion in marine mollusc *Clione limacina*. III. On the origin of



- locomotory rhythm. *Exp Brain Res* 58: 273-284.
- Arshavsky YI, Deliagina TG, Orlovsky GN, Panchin YV (1988) Control of feeding movements in the freshwater snail *Planorbis corneus*. II. Activity of isolated neurons of buccal ganglia. *Exp Brain Res* 70: 323-331.
- Arshavsky YI, Deliagina TG, Orlovsky GN, Panchin YV, Popova LB (1992) Interneurons mediating the escape reaction of the marine mollusc *Clione limacina*. *J Exp Biol* 164: 307-314.
- Arshavsky YI, Deliagina TG, Orlovsky GN, Panchin YV, Popova LB, Sadreyev RI (1998) Analysis of the central pattern generator for swimming in the mollusk *Clione*. *Ann NY Acad Sci* 860: 51-69.
- Audesirk G (1978a) Central neuronal control of cilia in *Tritonia diomedea*. *Nature* 272: 541-543.
- Audesirk G (1978b) Properties of central motor neurons exciting locomotory cilia in *Tritonia diomedea*. *J Comp Physiol* 128: 259-267.
- Audesirk G, McCaman RE, Willows AOD (1979) The role of serotonin in the control of pedal ciliary activity by identified neurons in *Tritonia diomedea*. *Comp Biochem Physiol C* 62: 87-91.
- Auerbach SB, Grover LM, Farley J (1989) Neurochemical and immunocytochemical studies of serotonin in the *Hermisenda* central nervous system. *Brain Res Bull* 22: 353-361.
- Avila C (1995) Natural products of opisthobranch molluscs: a biological review. *Oceanogr Mar Biol Ann Rev* 33: 487-559.
- Avila C, Cimino G, Crispino A, Spinella A (1991a) Drimane sesquiterpenoids in Mediterranean *Dendrodoris* nudibranchs: anatomical distribution and biological role. *Experientia* 47: 306-310.
- Avila C, Cimino G, Fontana A, Gavagnin M, Ortea J, Trivellone E (1991b) Defensive strategy of two *Hypselodoris* nudibranchs from Italian and Spanish coasts. *J Chem Ecol* 17: 625-636.
- Avila C, Iken K, Fontana A, Cimino G (2000) Chemical ecology of the Antarctic nudibranch *Bathydoris hodgsoni* Eliot, 1907: defensive role and origin of its natural products. *J Exp Mar Biol Ecol* 252: 27-44.
- Ayer SW, Andersen RJ (1983) Degraded monoterpenes from the opisthobranch mollusc

- Melibe leonina*. *Experientia* 39: 255.
- Ayers JA, Carpenter GA, Currie S, Kinch J (1983) Which behavior does the lamprey central motor program mediate? *Science* 221: 1312-1314.
- Baltzley MJ (2006) Evolution and neurobiology of the neural circuitry underlying crawling in nudibranch molluscs. Ph.D. Dissertation. University of North Carolina, Chapel Hill, NC.
- Baptista CA, Gershon TR, Macagno ER (1990) Peripheral organs control central neurogenesis in the leech. *Nature* 346: 855-858.
- Barbas D, DesGroseillers L, Castellucci VF, Carew TJ, Marinesco S (2003) Multiple serotonergic mechanisms contributing to sensitization in *Aplysia*: evidence of diverse serotonin receptor subtypes. *Learn Mem* 10: 373-386.
- Barber A (1983) Properties of the serotonergic cerebral ganglion neurons of the gastropod mollusc, *Philine aperta*. *Comp Biochem Physiol C* 76: 135-149.
- Barsby T, Linington RG, Andersen J (2002) *De novo* terpenoid biosynthesis by the dendronotid nudibranch *Melibe leonina*. *Chemoecology* 12: 199-202.
- Bass AH (1986) Evolution of a vertebrate communication and orientation organ. In: Bullock TH (ed) *Electric organs revisited*. John Wiley & Sons, Inc. New York, NY.
- Bass AH, Baker R (1997) Phenotypic specification of hindbrain rhombomeres and the origins of rhythmic circuits in vertebrates. *Brain Behav Evol* 50: 3-16.
- Bass AH, McKibben JR (2003) Neural mechanisms and behaviors for acoustic communication in teleost fish. *Prog Neurobiol* 69: 1-26.
- Battle K, Nybakken J (1998) The feeding habits of *Pleurobranchaea californica* MacFarland, 1966 (Opisthobranchia: Notaspidea) in Monterey Bay, California. *Veliger* 41: 213-226.
- Bebbington A, Hughes GM (1973) Locomotion in *Aplysia* (Gastropoda, Opisthobranchia). *Proc Malacological Soc Lond* 40: 399-405.
- Beck JC, Murray JA, Willows AOD, Cooper MS (2000) Computer-assisted visualizations of neural networks: expanding the field of view using seamless confocal montaging. *J Neurosci Methods* 98: 155-163.

- Beltz BS (1999) Distribution and functional anatomy of amine-containing neurons in decapod crustaceans. *Microsc Res Tech* 44: 105-120.
- Benjamin PR, Rose RM (1979) Central generation of bursting in the feeding system of the snail, *Lymnaea stagnalis*. *J Exp Biol* 80: 93-118.
- Bergh R (1894) Reports on the dredging operations off the west coast of Central America to the Galapagos, to the west coast of Mexico, and in the Gulf of California, in charge of Alexander Agassiz, carried on by the U.S. Fish Commission Steamer "Albatross", during 1891, Lieut. Commander Z.L. Tanner, U.S.N., Commanding. XIII. Die Opisthobranchien. *Bull Museum Comp Zool* 25: 125-233.
- Berkowitz A (2005) Physiology and morphology indicate that individual spinal interneurons contribute to diverse limb movements. *J Neurophysiol* 94: 4455-4470.
- Berman NJ, Bush PC, Douglas RJ (1989) Adaptation and bursting in neocortical neurones may be controlled by a single fast potassium conductance. *Q J Exp Physiol* 74: 223-226.
- Bertrand S, Cazalets JR (1998) Postinhibitory rebound during locomotor-like activity in neonatal rat motoneurons *in vitro*. *J Neurophysiol* 79: 342-351.
- Bertsch H (1968) Effect of feeding by *Armina californica* on the bioluminescence of *Renilla kollikeri*. *Veliger* 10: 440-441.
- Bickell-Page LR (1989) Autotomy of cerata by the nudibranch *Melibe leonina* (Mollusca): ultrastructure of the autotomy plane and neural correlate of the behaviour. *Phil Trans R Soc Lond B* 324: 149-172.
- Bickell-Page LR (1991) Repugnatorial glands and associated striated muscle and sensory cells in *Melibe leonina* (Mollusca, Nudibranchia). *Zoomorphology* 110: 281-291.
- Birkeland C (1974) Interactions between a sea pen and seven of its predators. *Ecol Monographs* 44: 211-232.
- Blenkinsop TA, Lang EJ (2006) Block of inferior olive gap junctional coupling decreases Purkinje cell complex spike synchrony and rhythmicity. *J Neurosci* 26: 1739-1748.
- Bock W (1989) The homology concept: its philosophical foundation and practical methodology. *Zool Beitr* 32: 327-353.

- Bramble DM, Wake DB (1985) Feeding mechanisms of lower vertebrates. In: Hildebrand M, Bramble DM, Wake DB, Liem KF (eds). Functional vertebrate morphology. Belknap Press. Cambridge, MA.
- Breidbach O, Kutsch W (1990) Structural homology of identified motoneurons in larval and adult stages of hemi- and holometabolous insects. *J Comp Neurol* 297: 392-409.
- Bruce LL, Neary TJ (1995) The limbic system of tetrapods: a comparative analysis of cortical and amygdalar populations. *Brain Behav Evol* 46: 224-234.
- Brunckhorst DJ (1993) The systematics and phylogeny of phyllidiid nudibranchs (Doridoidea). *Records Australian Museum Supp* 0: 1-107.
- Bullock AGM, Ridgway RL (1995) Comparative aspects of gastropod neurobiology. In: Breidbach O, Kutsch W (eds). The nervous systems of invertebrates: an evolutionary and comparative approach. Birkhäuser. Boston, MA.
- Bullock TH (2000) Revisiting the concept of identifiable neurons. *Brain Behav Evol* 55: 236-240.
- Buschbeck EK, Strausfeld NJ (1997) The relevance of neural architecture to visual performance: phylogenetic conservation and variation in dipteran visual systems. *J Comp Neurol* 383: 282-304.
- Cain SD, Wang JH, Lohmann KJ (2006) Immunochemical and electrophysiological analyses of magnetically responsive neurons in the mollusc *Tritonia diomedea*. *J Comp Physiol A* 192: 235-245.
- Calabrese RL, Nadim F, Olsen OH (1995) Heartbeat control in the medicinal leech: a model system for understanding the origin, coordination, and modulation of rhythmic motor patterns. *J Neurobiol* 27: 390-402.
- Caldwell SL, Donovan DA (2003) Energetics of swimming and crawling in the lion nudibranch, *Melibe leonina*. *Veliger* 46: 355-361.
- Carr CE, Soares D (2002) Evolutionary convergence and shared computational principles in the auditory system. *Brain Behav Evol* 59: 294-311.
- Cash D, Carew TJ (1989) A quantitative analysis of the development of the central nervous system in juvenile *Aplysia californica*. *J Neurobiol* 20: 25-47.
- Catania KC (2000) Cortical organization in Insectivora: the parallel evolution of the sensory periphery and the brain. *Brain Behav Evol* 55: 311-321.

- Cattaneo-Vietti R, Angelini S, Bavestrello G (1993) Skin and gut spicules in *Discodoris atromaculata* (Bergh, 1880) (Mollusca: Nudibranchia). *Bull Malacol* 29: 173-180.
- Chang W, Strahlendorf JC, Strahlendorf HK (1993) Ionic contributions to the oscillatory firing activity of rat Purkinje cells *in vitro*. *Brain Res* 614: 335-341.
- Chase R (2002) Behavior and its neural control in gastropod molluscs. Oxford University Press, Inc. New York, NY.
- Chiang J-TA, Steciuk M, Shtonda B, Avery L (2006) Evolution of pharyngeal behaviors and neuronal functions in free-living soil nematodes. *J Exp Biol* 209: 1859-1873.
- Cimino G, Ghiselin MT (1999) Chemical defense and evolutionary trends in biosynthetic capacity among dorid nudibranchs (Mollusca: Gastropoda: Opisthobranchia). *Chemoecology* 9: 187-207.
- Cimino G, De Rosa S, De Stephano S, Sodano G, Villani G (1983) Dorid nudibranch elaborates its own chemical defense. *Science* 219: 1237-1238.
- Cimino G, Fontana A, Gavagnin M (1999) Marine Opisthobranchia Mollusca: chemistry and ecology in sacoglossans and dorids. *Curr Org Chem* 3: 327-372.
- Cockerell TDA (1901) Three new nudibranchs from California. *J Malacol* 8: 85-87.
- Coggeshall RE (1967) A light and electron microscope study of the abdominal ganglion of *Aplysia californica*. *J Neurophysiol* 30: 1263-1287.
- Collingwood G (1879) *Scyllea pelagica*, specimens from China Sea, and spawn described. *Trans Linn Soc Lond* 2: 137-138.
- Comer CM, Robertson RM (2001) Identified nerve cells and insect behavior. *Prog Neurobiol* 63: 409-439.
- Conklin EJ, Mariscal RN (1977) Feeding behavior, ceras structure, and nematocyst storage in the aeolid nudibranch, *Spurilla neapolitana* (Mollusca). *Bull Mar Sci* 27: 658-667.
- Cowan WM, Fawcett JW, O'Leary DDM, Stanfield BB (1984) Regressive events in neurogenesis. *Science* 225: 1258-1265.
- Croll RP (1987a) Identified neurons and cellular homologies. In: Ali MA (ed). *Nervous systems in invertebrates*. Plenum. New York, NY.

- Croll RP (1987b) Distribution of monoamines in the central nervous system of the nudibranch gastropod, *Hermisenda crassicornis*. Brain Res 405: 337-347.
- Croll RP (1988) Distribution of monoamines within the central nervous system of the juvenile pulmonate snail, *Achatina fulica*. Brain Res 460: 29-49.
- Croll RP, Chaisson BJ (1989) Postembryonic development of serotonin-like immunoreactivity in the central nervous system of the snail, *Lymnaea stagnalis*. J Comp Neurol 280: 122-142.
- Croll RP, Lo RYS (1986) Distribution of serotonin-like immunoreactivity in the central nervous system of the periwinkle *Littorina littorea* (Gastropoda, Prosobranchia, Mesogastropoda). Biol Bull 171: 426-440.
- Croll RP, Too CKL, Pani AK, Nason J (1995) Distribution of serotonin in the sea scallop *Placopecten magellanicus*. Invert Reprod Dev 28: 125-135.
- Croll RP, Boudko DY, Hadfield MG (2001) Histochemical survey of transmitters in the central ganglia of the gastropod mollusc *Phestilla sibogae*. Cell Tissue Res 305: 417-432.
- Crow T (2004) Pavlovian conditioning of *Hermisenda*: current cellular, molecular, and circuit perspectives. Learn Mem 11: 229-238.
- Cymbalyuk GS, Gaudry Q, Masino MA, Calabrese RL (2002) Bursting in leech heart interneurons: cell-autonomous and network-based mechanisms. J Neurosci 22: 10580-10592.
- Daniel TL (1984) Unsteady aspects of aquatic locomotion. Amer Zool 24: 121-134.
- Day RM, Harris LG (1978) Selection and turnover of coelenterate nematocysts in some aeolid nudibranchs. Veliger 21: 104-109.
- de Beer GR (1958) Embryos and ancestors, 3<sup>rd</sup> ed. Oxford University Press. London.
- de Beer GR (1971) Homology, an unsolved problem. Oxford University Press. London.
- Dekin MS, Richerson GB, Getting PA (1985) Thyrotropin-releasing hormone induces rhythmic bursting in neurons of the nucleus tractus solitarius. Science 229: 67-69.
- de Pinna M (1991) Concepts and tests of homology in the Cladistic paradigm. Cladistics 7: 367-394.
- Dicaprio R (1997) Plateau potentials in motor neurons in the ventilatory system of the

- crab. *J Exp Biol* 200: 1725-1736.
- Dickinson PS (1979) Homologous neurons control movements of diverse gill types in nudibranch molluscs. *J Comp Physiol* 131: 277-283.
- Dickinson PS (1995) Interactions among neural networks for behavior. *Curr Opin Neurobiol* 5: 792-798.
- Dickinson PS, Moulins M (1992) Interactions and combinations between different networks in the stomatogastric nervous system. In: Harris-Warrick RM, Marder E, Selverston AI, Moulins M (eds) *Dynamic biological networks: the stomatogastric nervous system*. MIT Press. Cambridge, MA.
- Dickinson PS, Mecsas C, Marder E (1990) Neuropeptide fusion of two motor pattern generator circuits. *Nature* 344: 155-158.
- Domenici P, Blake RW (1997) The kinematics and performance of fish fast-start swimming. *J Exp Biol* 200: 1165-1178.
- Dorsett DA (1974) Neuronal homologies and the control of branchial tuft movements in two species of *Tritonia*. *J Exp Biol* 61: 639-654.
- Dorsett DA (1978) Neurons controlling foot and mantle movements in *Armina californica*. *Mar Behav Physiol* 5: 307-324.
- Dorsett DA, Willows AOD, Hoyle G (1969) Centrally generated nerve impulse sequences determining swimming behaviour in *Tritonia*. *Nature* 224: 711-712.
- Dorsett DA, Willows AOD, Hoyle G (1973) The neuronal basis of behavior in *Tritonia*. IV. The central origin of a fixed action pattern demonstrated in the isolated brain. *J Neurobiol* 4: 287-300.
- Dumdei EJ, Kubanek J, Coleman JE, Pika J, Andersen RJ, Steiner J, Clardy J (1997) New terpenoid metabolites from the skin extracts, an egg mass, and dietary sponges of the northeastern pacific dorid nudibranch *Cadlina luteomarginata*. *Can J Chem* 75: 773-789.
- Dumont JPC, Robertson RM (1986) Neuronal circuits: an evolutionary perspective. *Science* 233: 849-853.
- Dye JC, Meyer JH (1986) Central control of the electric organ discharge in weakly electric fish. In: Bullock TH, Heiligenberg W (eds) *Electroreception*. John Wiley & Sons, Inc. New York, NY.

- Eaton RC, Lavender WA, Weiland CM (1981) Identification of Mauthner-initiated response patterns in goldfish: evidence from simultaneous cinematography and electrophysiology. *J Comp Physiol A* 145: 485-496.
- Edmunds M (1966) Protective mechanisms in the Eolidacea (Mollusca Nudibranchia). *J Linn Soc* 46: 27-71.
- Edmunds M (1968) On the swimming and defensive response of *Hexabranhus marginatus* (Mollusca, Nudibranchia). *J Linnean Soc* 47: 425-429.
- Edmunds M (1987) Color in opisthobranchs. *Amer Malacological Bull* 5: 185-196.
- Edwards DH, Heitler WJ, Krasne FB (1999) Fifty years of a command neuron: the neurobiology of escape behavior in the crayfish. *Trends Neurosci* 22: 153-161.
- Edwards JS, Palka J (1991) Insect neural evolution a fugue or an opera? *Seminars Neurosci* 3: 391-398.
- Egelhaaf M, Benjamin PR (1983) Coupled neuronal oscillators in the snail *Lymnaea stagnalis*: endogenous cellular properties and network interactions. *J Exp Biol* 102: 93-114.
- Elliott CJ, Benjamin PR (1985) Interactions of pattern-generating interneurons controlling feeding in *Lymnaea stagnalis*. *J Neurophysiol* 54: 1396-1411.
- Ellis W (1999a) *Plocamopherus imperialis* Angas, 1864. *Australian Nudibranch News* 11: 1-4.
- Ellis W (1999b) *Plocamopherus tilesii* Bergh, 1877. *Australian Nudibranch News* 2: 1-4.
- Farley J, Wu R (1989) Serotonin modulation of *Hermisenda* type B photoreceptor light responses and ionic currents: implications for mechanisms underlying associative learning. *Brain Res Bull* 22: 335-351.
- Farmer WM (1970) Swimming gastropods (Opisthobranchia and Prosobranchia). *The Veliger* 13: 73-89.
- Farris JS (1989) The retention index and rescaled consistency index. *Cladistics* 5: 417-419.
- Faulkes Z (2004) Loss of escape responses and giant neurons in the tailflipping circuits of slipper lobsters, *Ibacus spp.* (Decapoda, Palinura, Scyllaridae). *Arthropod Struct Dev* 33: 113-123.



- Faulkes Z, Paul DH (1997) A map of distal leg motor neurons in the thoracic ganglia of four decapod crustacean species. *Brain Behav Evol* 49: 162-178.
- Faulkner DJ (1992) Chemical defenses of marine molluscs. In: Paul VJ (ed) *Ecological roles of marine natural products*. Constock Publishing. Ithaca, NY.
- Faulkner DJ, Ghiselin MT (1983) Chemical defense and the evolutionary ecology of dorid nudibranchs and some other opisthobranch gastropods. *Mar Ecol Prog Ser* 13: 295-301.
- Faulkner DJ, Molinski TF, Andersen RJ, Dumdei EJ, de Silva ED (1990) Geographical variation in defensive chemicals from Pacific coast dorid nudibranchs and some related marine mollusks. *Comp Biochem Physiol C* 97: 233-240.
- Ferreira AJ, Bertsch H (1972) Additional data on *Flabellina telja* (Gastropoda: Opisthobranchia). *Veliger* 14: 414-415.
- Fickbohm DJ, Katz PS (2000) Paradoxical actions of the serotonin precursor 5-hydroxytryptophan on the activity of identified serotonergic neurons in a simple motor circuit. *J Neurosci* 20: 1622-1634.
- Fickbohm DJ, Lynn-Bullock CP, Spitzer N, Caldwell HK, Katz PS (2001) Localization and quantification of 5-hydroxytryptophan and serotonin in the central nervous systems of *Tritonia* and *Aplysia*. *J Comp Neurol* 437: 91-105.
- Fischer Y (2004) The hippocampal intrinsic network oscillator. *J Physiol* 554: 156-174.
- Fontana A, Avila C, Martinez E, Ortea J, Trivellone E, Cimino G (1993) Defensive allomones in three species of *Hypselodoris* from the Cantabrian Sea. *J Chem Ecol* 19: 339-356.
- Fredman SM, Jahan-Parwar B (1983) Command neurons for locomotion in *Aplysia*. *J Neurophysiol* 49: 1092-1117.
- Frick KE (2005) Nematocyst complements of nudibranchs in the genus *Flabellina* in the Gulf of Maine and the effect of diet manipulations on the cnidom of *Flabellina verrucosa*. *Mar Biol* 147: 1313-1321.
- Frost WN, Katz PS (1996) Single neuron control over a complex motor program. *Proc Natl Acad Sci USA* 93: 422-426.
- Frost WN, Hoppe TA, Wang J, Tian LM (2001) Swim initiation neurons in *Tritonia diomedea*. *Amer Zool* 41: 952-961.

- Futuyma DJ (1986) Evolutionary biology, 2<sup>nd</sup> ed. Sinauer. Sunderland, MA.
- Gamkrelidze GN, Laurienti PJ, Blankenship JE (1995) Identification and characterization of cerebral ganglion neurons that induce swimming and modulate swim-related pedal ganglion neurons in *Aplysia brasiliana*. J Neurophysiol 74: 1444-1462.
- Gavagnin M, Vardaro RR, Avila C, Cimino G, Ortea J (1992) Ichthyotoxic diterpenoids from the Cantabrian nudibranch *Chromodoris luteorosea*. J Nat Prod 55: 368-371.
- Getting PA (1977) Neuronal organization of escape swimming in *Tritonia*. J Comp Physiol 121: 325-342.
- Getting PA (1981) Mechanisms of pattern generation underlying swimming in *Tritonia*. I. Neuronal network formed by monosynaptic connections. J Neurophysiol 46: 65-79.
- Getting PA (1983) Mechanisms of pattern generation underlying swimming in *Tritonia*. III. Intrinsic and synaptic mechanisms for delayed excitation. J Neurophysiol 49: 1036-1050.
- Getting PA (1989) A network oscillator underlying swimming in *Tritonia*. In: Jacklet JW (ed) Neuronal and cellular oscillators. Marcel Dekker, Inc. New York, NY.
- Getting PA, Dekin MS (1985) Mechanisms of pattern generation underlying swimming in *Tritonia*. IV. Gating of central pattern generator. J Neurophysiol 53: 466-480.
- Getting PA, Lennard PR, Hume RI (1980) Central pattern generator mediating swimming in *Tritonia*. I. Identification and synaptic interactions. J Neurophysiol 44: 151-164.
- Gohar HAF, Soliman GN (1963) The biology and development of *Hexabranchus sanguineus* (Rüpp and Leuck) (Gastropoda, Nudibranchiata). Publ Mar Biol Station, Ghardaqa (Red Sea) 12: 219-247.
- Goodman CS (1977) Neuron duplications and deletions in locust clones and clutches. Science 197: 1384-1386.
- Gordon KR, Herring SW (1987) Activity patterns within the genioglossus during suckling in domestic dogs and pigs: interspecific and intraspecific plasticity. Brain Behav Evol 30: 249-262.
- Gosliner TM (1987) Review of the nudibranch genus *Melibe* (Opisthobranchia: Dendronotacea) with descriptions of two new species. Veliger 29: 400-414.

- Gosliner TM (1991) Morphological parallelism in opisthobranch gastropods. *Malacologia* 32: 313-327.
- Gosliner TM (1994) Gastropoda: Opisthobranchia. In: Harrison FW, Gardiner SL (eds) *Microscopic anatomy of invertebrates*, vol. 5: Mollusca I. Wiley-Liss, Inc. New York, NY.
- Gosliner TM, Behrens DW (1990) Special resemblance, aposematic coloration and mimicry in opisthobranch gastropods. *Proc Symp Amer Soc Zool*, Galveston, TX.
- Gosliner TM, Ghiselin MT (1984) Parallel evolution in opisthobranch gastropods and its implications for phylogenetic methodology. *Systematic Zool* 33: 255-274.
- Goslow GE, Dial KP, Jenkins FA (1989) The avian shoulder: an experimental approach. *Amer Zool* 29: 287-301.
- Grande C, Templado J, Cervera JL, Zardoya R (2002) The complete mitochondrial genome of the nudibranch *Roboastra europaea* (Mollusca: Gastropoda) supports the monophyly of opisthobranchs. *Mol Biol Evol* 19: 1672-1685.
- Grande C, Templado J, Cervera JL, Zardoya R (2004) Phylogenetic relationships among Opisthobranchia (Mollusca: Gastropoda) based on mitochondrial *cox 1*, *trn V*, and *rrnL* genes. *Mol Phylogen Evol* 33: 378-388.
- Grant K, Bell CC, Clausse S, Ravaille M (1986) Morphology and physiology of the brainstem nuclei controlling the electric organ discharge in mormyrid fish. *J Comp Neurol* 245: 514-530.
- Graziani EI, Andersen RJ (1996) Investigations of sesquiterpenoid biosynthesis by the dorid nudibranch *Acanthodoris nanaimoensis*. *J Am Chem Soc* 118: 4701-4702.
- Graziani EI, Andersen RJ, Krug PJ, Faulkner DJ (1996) Stable isotope incorporation evidence for the *de novo* biosynthesis of terpenoid acid glycerides by dorid nudibranchs. *Tetrahedron* 52: 6869-6878.
- Greenwood PG, Mariscal RN (1984) The utilization of cnidarian nematocysts by aeolid nudibranchs: nematocyst maintenance and release in *Spurilla*. *Tissue Cell* 16: 719-730.
- Grosvenor GH (1903) On the nematocysts of aeolids. *Proc R Soc Lond* 72: 462-486.
- Guckenheimer J, Harris-Warrick R, Peck J, Willms A (1997) Bifurcation, bursting, and spike frequency adaptation. *J Comput Neurosci* 4: 257-277.

- Gustafson K, Andersen RJ (1985) Chemical studies of British Columbia nudibranchs. *Tetrahedron* 41: 1101-1108.
- Haefelfinger H-R, Kress A (1967) Der schwimmvorgang bei *Gasteropteron rubrum* (Rafinesque, 1814) (Gastropoda, Opisthobranchiata). *Rev Suisse Zool* 74: 547-554.
- Hale ME, Long JH, McHenry MJ, Westneat MW (2002) Evolution of behavior and neural control of the fast-start escape response. *Evolution* 56: 993-1007.
- Hanken J (1986) Developmental evidence for amphibian origins. In Hecht MK, Wallace B, Prance GT (eds) *Evolutionary biology*, vol. 20. Plenum. New York.
- Harris LG (1973) Nudibranch association. *Curr Top Comp Pathobiol* 2: 213-315.
- Harris-Warrick, RM and Marder, E (1991) Modulation of neural networks for behavior. *Annu Rev Neurosci* 14: 39-57.
- Harris-Warrick RM, Nagy F, Nusbaum MP (1992) Neuromodulation of stomatogastric networks by identified neurons and transmitters. In: Harris-Warrick RM, Marder E, Selverston AI, Moulins M (eds) *Dynamic biological networks: the stomatogastric nervous system*. MIT Press. Cambridge, MA.
- Hartline DK, Graubard K (1992) Cellular and synaptic properties in the crustacean stomatogastric nervous system. In: Harris-Warrick RM, Marder E, Selverston AI, Moulins M (eds) *Dynamic biological networks: the stomatogastric nervous system*. MIT Press. Cambridge, MA.
- Hartline DK, Russell DF (1984) Endogenous burst capability in a neuron of the gastric mill pattern generator of the spiny lobster *Panulirus interruptus*. *J Neurobiol* 15: 345-364.
- Harzsch S (2001) Neurogenesis in the crustacean ventral nerve cord: homology of neuronal stem cells in Malacostraca and Branchiopoda? *Evol Dev* 3: 154-169.
- Harzsch S (2004) Phylogenetic comparison of serotonin-immunoreactive neurons in representatives of the Chilopoda, Diplopoda, and Chelicerata: implications for arthropod relationships. *J Morphol* 259: 198-213.
- Hay-Schmidt A (2000) The evolution of the serotonergic nervous system. *Proc R Soc Lond B* 267: 1071-1079.

- Heiligenburg W, Metzner W, Wong CJH, Keller CH (1996) Motor control of the jamming avoidance response in *Apteronotus leptorhynchus*: evolutionary changes of a behavior and its neuronal substrates. *J Comp Physiol A* 179: 653-674.
- Hellgren J, Grillner S, Lansner A (1992) Computer simulation of the segmental neural network generating locomotion in lamprey by using populations of network interneurons. *Biol Cybern* 68: 1-13.
- Henry JJ, Raff RA (1990) Evolutionary change in the process of dorsoventral axis determination in the direct developing sea urchin, *Heliocidaris erhythrogramma*. *Dev Biol* 141: 55-69.
- Hernádi L, Elekes K, S-Rózsa K (1989) Distribution of serotonin-containing neurons in the central nervous system of the snail *Helix pomatia*: comparison of immunocytochemical 5-6-dihydroxytryptamine labelling. *Cell Tissue Res* 257: 313-324.
- Herrel A, Meyers JJ, Nishikawa KC, De Vree F (2001) The evolution of feeding motor patterns in lizards: modulatory complexity and possible constraints. *Amer Zool* 41: 1311-1320.
- Hooper SL, Moulins M (1989) Switching of a neuron from one network to another by sensory-induced changes in membrane properties. *Science* 244: 1587-1589.
- Hume RI, Getting PA (1982) Motor organization of Tritonia swimming. II. Synaptic drive to flexion neurons from premotor interneurons. *J Neurophysiol* 47: 75-90.
- Hume RI, Getting PA, Del Beccaro MA (1982) Motor organization of *Tritonia* swimming. I. Quantitative analysis of swim behavior and flexion neuron firing patterns. *J Neurophysiol* 47: 60-74.
- Hurst A (1968) The feeding mechanism and behavior of the opisthobranch *Melibe leonina*. *Symp Zool Soc Lond* 22: 151-166.
- Ierusalimsky VN, Balaban PM (2001) Ontogenesis of the snail, *Helix aspersa*: embryogenesis timetable and ontogenesis of GABA-like immunoreactive neurons in the central nervous system. *J Neurocytol* 30: 73-91.
- Jerussi TP, Alkon DL (1981) Ocular and extraocular responses of identified neurons in pedal ganglia of *Hermisenda crassicornis*. *J Neurophysiol* 46: 659-671.

- Jing J, Gillette R (1995) Neuronal elements that mediate escape swimming and suppress feeding behavior in the predatory sea slug *Pleurobranchaea*. *J Neurophysiol* 74: 1900-1910.
- Jing J, Gillette R (1999) Central pattern generator for escape swimming in the notaspid sea slug *Pleurobranchaea californica*. *J Neurophysiol* 81: 654-667.
- Jing J, Gillette R (2000) Escape swim network interneurons have diverse roles in behavioral switching and putative arousal in *Pleurobranchaea*. *J Neurophysiol* 83: 1346-1355.
- Jing J, Gillette R (2003) Directional avoidance turns encoded by single interneurons and sustained by multifunctional serotonergic cells. *J Neurosci* 23: 3039-3051.
- Johnson S (1984) A new Indo-West pacific species of the dendronotacean nudibranch *Bornella* (Mollusca: Opisthobranchia) with anguilliform swimming behaviour. *Micronesica* 19: 17-26.
- Johnson SM, Smith JC, Funk GD, Feldman JL (1994) Pacemaker behavior of respiratory neurons in medullary slices from neonatal rat. *J Neurophysiol* 72: 2598-2608.
- Juranek J, Metzner W (1997) Cellular characterization of synaptic modulations of a neuronal oscillator in electric fish. *J Comp Physiol A* 181: 393-414.
- Kabotyanskii EA, Sakharov DA (1991) Neuronal correlates of the serotonin-dependent behavior of the pteropod mollusc *Clione limacina*. *Neurosci Behav Physiol* 21: 422-435.
- Kandel ER (1976) Cellular basis of behavior: an introduction to behavioral neurobiology. WH Freeman and Company. San Francisco, CA.
- Kandel ER (1979) Behavioral biology of *Aplysia*. WH Freeman and Company. San Francisco, CA.
- Karten HJ (1991) Homology and evolutionary origins of the 'neocortex'. *Brain Behav Evol* 38: 264-272.
- Karten HJ (1997) Evolutionary developmental biology meets the brain: the origins of mammalian cortex. *Proc Natl Acad Sci USA* 94: 2800-2804.
- Karuso P (1987) Chemical ecology of the nudibranchs. In: Scheuer P (ed) Bioorganic marine chemistry. Springer-Verlag. New York, NY.

- Katz PS (1991) Neuromodulation and the evolution of a simple motor system. *Semin Neurosci* 3: 379-389.
- Katz PS (1998) Neuromodulation intrinsic to the central pattern generator for escape swimming in *Tritonia*. *Ann NY Acad Sci* 860: 181-188.
- Katz PS, Frost WN (1995a) Intrinsic neuromodulation in the *Tritonia* swim CPG: serotonin mediates both neuromodulation and neurotransmission by the dorsal swim interneurons. *J Neurophysiol* 74: 2281-2294.
- Katz PS, Frost WN (1995b) Intrinsic neuromodulation in the *Tritonia* swim CPG: the serotonergic dorsal swim interneurons act presynaptically to enhance transmitter release from interneuron C2. *J Neurosci* 15: 6035-6045.
- Katz PS, Frost WN (1997) Removal of spike frequency adaptation via neuromodulation intrinsic to the *Tritonia* escape swim central pattern generator. *J Neurosci* 17: 7703-7713.
- Katz PS, Harris-Warrick RM (1989) Serotonergic/cholinergic muscle receptor cells in the crab stomatogastric nervous system. II. Rapid nicotinic and prolonged modulatory effects on neurons in the stomatogastric ganglion. *J Neurophysiol* 62: 571-581.
- Katz PS, Harris-Warrick RM (1990) Actions of identified neuromodulatory neurons in a simple motor system. *Trends Neurosci* 13: 367-373.
- Katz PS, Harris-Warrick RM (1991) Recruitment of crab gastric mill neurons into the pyloric motor pattern by mechanosensory afferent stimulation. *J Neurophysiol* 65: 1442-1451.
- Katz PS, Harris-Warrick RM (1999) The evolution of neuronal circuits underlying species-specific behavior. *Curr Opin Neurobiol* 9: 628-633.
- Katz PS, Newcomb JM (in press) A tale of two CPGs: phylogenetically polymorphic networks. To appear in: Bullock TH, Kaas J (eds) *Evolution of nervous systems, Volume 2 - Evolution of nervous systems in invertebrates*. Elsevier. Cambridge, UK.
- Katz PS, Tazaki K (1992) Comparative and evolutionary aspects of the crustacean stomatogastric system. In: Harris-Warrick RM, Marder E, Selverston AI, Moulins M (eds). *Dynamic biological networks*. MIT Press. Cambridge, MA.
- Katz PS, Getting PA, Frost WN (1994) Dynamic neuromodulation of synaptic strength intrinsic to a central pattern generator circuit. *Nature* 367: 729-731.

- Katz PS, Fickbohm DJ, Lynn-Bullock CP (2001) Evidence that the central pattern generator for swimming in *Tritonia* arose from a non-rhythmic neuromodulatory arousal system: implications for the evolution of specialized behavior. *Amer Zool* 41: 962-975.
- Katz PS, Sakurai A, Clemens S, Davis D (2004) Cycle period of a network oscillator is independent of membrane potential and spiking activity in individual central pattern generator neurons. *J Neurophysiol* 92: 1904-1917.
- Kavanau JL (1990) Conservative behavioral evolution, the neural substrate. *Animal Behav* 39: 758-767.
- Kempf SC, Page LR, Pires A (1997) Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch mollusks with emphasis on the structure and possible function of the apical sensory organ. *J Comp Neurol* 386: 507-528.
- Kepner WA (1943) The manipulation of the nematocysts of *Pennaria tiarella* by *Aeolis pilata*. *J Morphol* 73: 297-311.
- Kiehn O (2006) Locomotor circuits in the mammalian spinal cord. *Annu Rev Neurosci* 29: 279-306.
- Kiehn O, Hounsgaard J, Sillar KT (1997) Basic building blocks of vertebrate spinal central pattern generators. In: Stein PSG, Grillner S, Selverston A (eds). *Neurons, networks and motor behavior*. MIT Press. Cambridge, MA.
- King DG, Valentino KL (1983) On neuronal homology: a comparison of similar axons in *Musca*, *Sarcophaga*, and *Drosophila* (Diptera: Schizophora). *J Comp Neurol* 219: 1-9.
- Kittmann R, Schmitz J, Buschges A (1996) Premotor interneurons in generation of adaptive leg reflexes and voluntary movements in stick insects. *J Neurobiol* 31: 512-532.
- Kravitz EA (2000) Serotonin and aggression: insights gained from a lobster model system and speculations on the role of amine neurons in a complex behavior. *J Comp Physiol A* 186: 221-238.
- Kress A (1968) Untersuchungen zur histology, autotomie und regeneration dreier *Doto* – Arten *Doto coronata*, *D. pinnatifida*, *D. fragilis* (Gastropoda, Opisthobranchiata). *Revue Suisse Zool* 75: 225-303.
- Kubaneck J, Graziani EI, Andersen RJ (1997) Investigations of terpenoid biosynthesis by the dorid nudibranch *Cadlina luteomarginata*. *J Org Chem* 62: 7239-7246.



- Kuffler DP, Muller KJ (1974) The properties and connections of supernumerary sensory and motor nerve cells in the central nervous system of an abnormal leech. *J Neurobiol* 5: 331-348.
- Kutsch W, Heckmann R (1995) Homologous structures, exemplified by motoneurons of Mandibulata. In: Breidbach O, Kutsch W (eds) *The nervous systems of invertebrates: an evolutionary and comparative approach*. Birkhäuser Verlag. Basel, Switzerland.
- LaBerge F, Muhlenbrock-Lenter S, Grunwald W, Roth G (2006) Evolution of the amygdala: new insights from studies in amphibians. *Brain Behav Evol* 67: 177-187.
- Lalli CM, Gilmer RW (1989) *Pelagic snails*. Stanford University Press. Stanford, CA.
- Lance JR (1968) New panamic nudibranchs (Gastropoda: Opisthobranchia) from the Gulf of California. *Trans San Diego Soc Nat Hist* 15: 3-13, pls 1-2.
- Land PW, Crow T (1985) Serotonin immunoreactivity in the circumesophageal nervous system of *Hermisenda crassicornis*. *Neurosci Lett* 62: 199-205.
- Langenbach GEJ, Van Eijden TMGJ (2001) Mammalian feeding motor patterns. *Amer Zool* 41: 1338-1351.
- Lansner A, Kotaleski JH, Grillner S (1998) Modeling of the spinal neuronal circuitry underlying locomotion in a lower vertebrate. *Ann NY Acad Sci* 860: 239-249.
- Lauder GV (1983) Functional and morphological bases of trophic specializations in sunfishes (Teleosti, Centrarchidae). *J Morphol* 178: 1-21.
- Lauder GV (1994) Homology, form, and function. In: Hall BK (ed) *Homology: the hierarchical basis of comparative biology*. Academic Press. San Diego, CA.
- Lauder GV, Shaffer HB (1988) Ontogeny of functional design in tiger salamanders (*Ambystoma tigrinum*): are motor patterns conserved during major morphological transformations? *J Morphol* 197: 249-268.
- Lawrence KA, Watson WH, III (2002) Swimming behavior of the nudibranch *Melibe leonina*. *Biol Bull* 203: 144-151.
- Liem KF (1978) Modulatory multiplicity in the functional repertoire of the feeding mechanism in cichlids. I. Piscivores. *J Morphol* 158: 323-360.

- Liem KF (1979) Modulatory multiplicity in the feeding mechanism of the cichlids, as exemplified by the invertebrate pickers of Lake Tanganyika. *J Zool* 189: 93-125.
- Liem KF (1980) Adaptive significance of intra- and interspecific differences in the feeding repertoires of cichlid fishes. *Amer Zool* 20: 295-314.
- Lieske SP, Thoby-Brisson M, Telgkamp P, Ramirez JM (2000) Reconfiguration of the neural network controlling multiple breathing patterns: eupnea, sighs and gasps. *Nat Neurosci* 3: 600-607.
- Lloyd PE, Phares GE, Phillips NE, Willows AOD (1996) Purification and sequencing of neuropeptides from identified neurons in the marine mollusc, *Tritonia*. *Peptides* 17: 17-23.
- Longley RD, Longley AJ (1986) Serotonin immunoreactivity of neurons in the gastropod *Aplysia californica*. *J Neurobiol* 17: 339-358.
- Longley RD, Longley AJ (1987) Evolution of behavior: an homologous peptidergic escape swim interneuron in swimming gastropod nudibranchs (*Tritonia*, *Hermisenda*) and a related non-swimming species (*Aeolidia*). Program No. 231.7. Washington, DC: Society for Neuroscience.
- Lowe RT (1842) Description of a new dorsibranchiate gastropod discovered at Madeira. *Proc Zool Soc London* 10: 51-53.
- Lucki I (1998) The spectrum of behaviors influenced by serotonin. *Biol Psychiatry* 44: 151-162.
- Ma PM, Beltz BS, Kravitz EA (1992) Serotonin-containing neurons in lobsters: their role as gain-setters in postural control mechanisms. *J Neurophysiol* 68: 36-54.
- MacFarland FM (1966) Studies of opisthobranchiate mollusks of the Pacific Coast of North America. *Mem Calif Acad Sci* 6: 1-546.
- Mackey SL, Carew TJ (1983) Locomotion in *Aplysia*: triggering by serotonin and modulation by bag cell extract. *J Neurosci* 3: 1469-1477.
- Mackey SL, Kandel ER, Hawkins RD (1989) Identified serotonergic neurons LCB1 and RCB1 in the cerebral ganglia of *Aplysia* produce presynaptic facilitation of siphon sensory neurons. *J Neurosci* 9: 4227-4235.

- Mackie AM (1970) Avoidance reactions of marine invertebrates to either steroid glycosides of starfish or synthetic surface-active agents. *J Exp Mar Biol Ecol* 5: 63-69.
- MacLeay WS (1821) *Horae Entomologicae*: or essays on the annulose animals, vol. I, part II. Containing an attempt to ascertain the rank and situation which the celebrated insect, *Scarabaeus sacer*, holds among organised beings. S Bagster. London.
- Maddison DR, Maddison WP (2005) MacClade 4: Analysis of phylogeny and character evolution. Version 4.08. Sunderland: Sinauer Associates.
- Mangan PS, Curran GA, Hurney CA, Friesen WO (1994) Modulation of swimming behavior in the medicinal leech. III. Control of cellular properties in motor neurons by serotonin. *J Comp Physiol A* 175: 709-722.
- Manor Y, Rinzel J, Segev I, Yarom Y (1997) Low-amplitude oscillations in the inferior olive: a model based on electrical coupling of neurons with heterogeneous channel densities. *J Neurophysiol* 77: 2736-2752.
- Marcus E (1955) Opisthobranchia from Brazil. *Boletim da Faculdade de Filosofia, Ciencias e Letras. Universidade de Sao Paulo, Zoologia*. 20: 89-261. pls 1-30.
- Marcus E, Marcus E (1962) Opisthobranchs from Florida and the Virgin Islands. *Bull Marine Sci Gulf Caribbean* 12: 450-488.
- Marcus E, Marcus E (1967) American opisthobranch mollusks. In: Bayer FM, Owre HB, Smith RA (eds) *Studies in tropical oceanography* No. 6. University of Miami. Miami, FL.
- Marder E (1998) Electrical synapses: beyond speed and synchrony to computation. *Curr Biol* 8: R795-R797.
- Marder E, Weimann JM (1992) Modulatory control of multiple task processing in the stomatogastric nervous system. In: Kien J, McCrohan CR, Winlow W (eds) *Neurobiology of motor program selection*. Pergamon Press. Oxford.
- Margoliash D, Fortune ES, Sutter ML, Yu AC, Wren-Hardin BD, Dave A (1994) Distributed representation in the song system of oscines: evolutionary implications and functional consequences. *Brain Behav Evol* 44: 247-264.
- Marin A, Belluga MDL, Scognamiglio G, Cimino G (1997) Morphological and chemical camouflage of the Mediterranean nudibranch *Discodoris indecora* on the sponges *Ircinia variabilis* and *Ircinia fasciculata*. *J Molluscan Stud* 63: 431-439.

- Marinesco S, Duran KL, Wright WG (2003) Evolution of learning in three aplysiid species: differences in heterosynaptic plasticity contrast with conservation in serotonergic pathways. *J Physiol* 550: 241-253.
- Marinesco S, Kolkman KE, Carew TJ (2004a) Serotonergic modulation in *Aplysia*. I. Distributed serotonergic network persistently activated by sensitizing stimuli. *J Neurophysiol* 92: 2468-2486.
- Marinesco S, Wickremasinghe N, Kolkman KE, Carew TJ (2004b) Serotonergic modulation in *Aplysia*. II. Cellular and behavioral consequences of increased serotonergic tone. *J Neurophysiol* 92: 2487-2496.
- Marois R, Carew TJ (1997) Ontogeny of serotonergic neurons in *Aplysia californica*. *J Comp Neurol* 386: 477-490.
- Marshall JG, Willan RC (1999) Nudibranchs of Heron Island, Great Barrier Reef - a survey of the Opisthobranchia (sea slugs) of Heron and Wistari Reefs. Backhuys Publishers. Leiden, Netherlands.
- Martin R (2003) Management of nematocysts in the alimentary tract and in cnidosacs of the aeolid nudibranch gastropod *Cratena peregrine*. *Mar Biol* 143: 533-541.
- Mauzey KP, Birkeland C, Dayton PK (1968) Feeding behavior of asteroids and escape responses of their prey in the Puget Sound Region. *Ecology* 49: 603-619.
- McCall RB, Aghajanian GK (1979) Serotonergic facilitation of facial motoneuron excitation. *Brain Res* 169: 11-27.
- McClellan AD (1982) Movements and motor patterns of the buccal mass of *Pleurobranchaea* during feeding, regurgitation and rejection. *J Exp Biol* 98: 195-211.
- McClellan AD, Brown GD, Getting PA (1994) Modulation of swimming in *Tritonia*: excitatory and inhibitory effects of serotonin. *J Comp Physiol A* 174: 257-266.
- McClintock JB, Baker BJ, Slattery M, Heine JN, Bryan PJ, Yoshida W, Davies-Coleman MT, Faulkner DJ (1994) Chemical defense of the common Antarctic shallow water nudibranch *Tritoniella belli* Eliot (Mollusca: Tritonidae) and its prey, *Clavularia frankliniana* Rouel (Cnidaria: Octocorallia). *J Chem Ecol* 20: 3361-3372.
- McLean IW, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative. a new fixation for immunoelectron microscopy. *J Histochem Cytochem* 22: 1077-1083.

- McPherson D, Katz PS (2001) Identification of serotonergic cerebral neurons that project to the pedal ganglia of *Aplysia californica*. Program No. 943.10. Washington, DC: Society for Neuroscience.
- Megina C, Cervera JL (2003) Diet, prey selection and cannibalism in the hunter opisthobranch *Roboastra europaea*. J Mar Biol Assoc UK 83: 489-495.
- Metzner W (1999) Neural circuitry for communication and jamming avoidance in gymnotiform electric fish. J Exp Biol 202: 1365-1375.
- Meyrand P, Simmers J, Moulins M (1991) Construction of a pattern-generating circuit with neurons of different networks. Nature 351: 60-63.
- Meyrand P, Faumont S, Simmers J, Christie AE, Nusbaum MP (2000) Species-specific modulation of pattern-generating circuits. Eur J Neurosci 12: 2585-2596.
- Mikkelsen PM (1996) The evolutionary relationships of Cephalaspidea (Gastropoda; Opisthobranchia): a phylogenetic analysis. Malacologia 37: 375-442.
- Miller JA, Byrne M (2000) Ceratal autotomy and regeneration in the aeolid nudibranch *Phidiana crassicornis* and the role of predators. Invert Biol 119: 167-176.
- Miller JP, Selverston AI (1982a) Mechanisms underlying pattern generation in lobster stomatogastric ganglion as determined by selective inactivation of identified neurons. II. Oscillatory properties of pyloric neurons. J Neurophysiol 48: 1378-1391.
- Miller JP, Selverston AI (1982b) Mechanisms underlying pattern generation in lobster stomatogastric ganglion as determined by selective inactivation of identified neurons. IV. Network properties of pyloric system. J Neurophysiol 48: 1416-1432.
- Miller SL (1974a) The classification, taxonomic distribution, and evolution of locomotor types among prosobranch gastropods. Proc Malacological Soc London 41: 233-273.
- Miller SL (1974b) Adaptive design of locomotion and foot form in prosobranch gastropods. J Exp Mar Biol Ecol 14: 99-156.
- Mills CE (1994) Season swimming of sexually mature benthic opisthobranch molluscs (*Melibe leonina* and *Gastropteron pacificum*) may augment population dispersal.

- In: Wilson WH Jr, Stricker SA, Shinn GL (eds) Reproduction and development of marine invertebrates. Johns Hopkins University Press. Baltimore, MD.
- Moroz L, Nezlin L, Elofsson R, Sakharov D (1994) Serotonin- and FMRFamide-immunoreactive nerve elements in the chiton *Lepidopleurus asellus* (Mollusca, Polyplacophora). *Cell Tissue Res* 275: 277-282.
- Mortin LI, Stein PSG (1989) Spinal cord segments containing key elements of the central pattern generators for three forms of scratch reflex in the turtle. *J Neurosci* 9: 2285-2296.
- Mulloney B, Perkel DH, Budelli RW (1981) Motor-pattern production: interaction of chemical and electrical synapses. *Brain Res* 229: 25-33.
- Murphy AD (2001) The neuronal basis of feeding in the snail, *Helisoma*, with comparisons to selected gastropods. *Prog Neurobiol* 63: 383-408.
- Murphy AD, Barker DL, Loring JF, Kater SB (1985) Sprouting and functional regeneration of an identified serotonergic neuron following axotomy. *J Neurobiol* 16: 137-151.
- Neu W (1932) Wie schwimmt *Aplysia depilans* L. *Zeitschr Vergl Physiol* 18: 244-254.
- Newcomb JM, Katz PS (in press) Homologues of serotonergic central pattern generator neurons in related nudibranch molluscs with divergent behaviors. *J Comp Physiol A*.
- Newcomb JM, Watson WH III (2001) Identifiable nitrergic neurons in the central nervous system of the nudibranch *Melibe leonina* localized with NADPH-diaphorase histochemistry and nitric oxide synthase immunoreactivity. *J Comp Neurol* 437: 70-78.
- Newcomb JM, Lawrence KA, Watson WH III (2004) The influence of light on locomotion in the gastropod *Melibe leonina*. *Mar Fresh Behav Physiol* 37: 253-269.
- Newcomb JM, Fickbohm DJ, Katz PS (2006) Comparative mapping of serotonin-immunoreactive neurons in the central nervous systems of nudibranch molluscs. *J Comp Neurol* 499: 485-505.
- Nishikawa KC (1997) Emergence of novel functions during brain evolution. *Bioscience* 47: 341-354.

- Nishikawa KC, Anderson CW, Deban SM, O'Reilly JC (1992) The evolution of neural circuits controlling feeding behavior in frogs. *Brain Behav Evol* 40:125-140.
- Nolen TG, Carew TJ (1994) Ontogeny of serotonin-immunoreactive neurons in juvenile *Aplysia californica* – implications for the development of learning. *Behav Neural Biol* 61: 282-295.
- Norris BJ, Weaver AL, Morris LG, Wenning A, Garcia PA, Calabrese RL (2006) A central pattern generator producing alternative outputs: temporal pattern of premotor activity. *J Neurophysiol* 96: 309-326.
- Nusbaum MP, Blitz DM, Swensen AM, Wood D, Marder E (2001) The roles of cotransmission in neural network modulation. *Trends Neurosci* 24: 146-154.
- Odhner NHJ (1934) The Nudibranchiata. *Nat Hist Rep Br Terra Nova Exped Zool* 7: 229-310.
- Ono JK, McCaman RE (1984) Immunocytochemical localization and direct assays of serotonin-containing neurons in *Aplysia*. *Neurosci* 11: 549-560.
- Ostergaard JM (1955) Some opisthobranch mollusca from Hawaii. *Pacific Science* 9: 110-136.
- Östman C (1997) Abundance, feeding behavior and nematocysts of scyphopolyps (Cnidaria) and nematocysts in their predator, the nudibranch *Coryphella verrucosa* (Mollusca). *Hydrobiologia* 355: 21-28.
- Owen R (1843) Lectures on comparative anatomy and physiology of the invertebrate animals, delivered at the Royal College of Surgeons in 1843. Longman, Brown, Green, and Longman. London.
- Owen R (1847) Report on the archetype and homologies of the vertebrate skeleton. *Meet Br Assoc Adv Sci Rep* 16: 169-340.
- Page LR (1993) Development of behavior in juveniles of *Melibe leonina* (Gastropoda: Nudibranchia). *Mar Behav Physiol* 22: 141-161.
- Page LR, Parries SC (2000) Comparative study of the apical ganglion in planktotrophic caenogastropod larvae: ultrastructure and immunoreactivity to serotonin. *J Comp Neurol* 418: 383-401.
- Palovcik RA, Basberg BA, Ram JL (1982) Behavioral state changes induced in *Pleurobranchaea* and *Aplysia* by serotonin. *Behav Neural Biol* 35: 383-394.

- Panchen AL (1994) Richard Owen and the concept of homology. In: Hall BK (ed). Homology: the hierarchical basis of comparative biology. Academic Press. San Diego, CA.
- Panchin YV, Popova LB, Deliagina TG, Orlovsky GN, Arshavsky YI (1995) Control of locomotion in marine mollusk *Clione limacina*. VIII. Cerebropedal neurons. J Neurophysiol 73: 1912-1923.
- Parsons DW, Pinsker HM (1989) Swimming in *Aplysia brasiliana*: behavioral and cellular effects of serotonin. J Neurophysiol 62: 1163-1176.
- Paul DH (1989) A neurophylogenist's view of decapod Crustacea. Bull Marine Science 45: 487-504.
- Paul DH (1990) Neural phylogeny – it's use in studying neural circuits. In: Frontiers in crustacean neurobiology. Wiese K, Krenze W-D, Tautz J, Reichert H, Mulloney B (eds). Birkhäuser Verlag. Basel.
- Paul DH (1991) Pedigrees of neurobehavioral circuits: tracing the evolution of novel behaviors by comparing motor patterns, muscles, and neurons in members of related taxa. Brain Behav Evol 38: 226-239.
- Pawlik JR, Kernan MR, Molinski TF, Harper MK, Faulkner DJ (1988) Defensive chemicals of the Spanish dancer nudibranch *Hexabranchus sanguineus* and its egg ribbons: macrolides derived from a sponge diet. J Exp Mar Biol Ecol 119: 99-109.
- Pease W (1860) Descriptions of new species of Mollusca from the Sandwich Islands. Proc Zool Soc London 28: 18-36.
- Pentreath VW, Berry MS, Osborne NN (1982) The serotonergic cerebral cells in gastropods. In: Osborne NN (ed). Biology of serotonergic transmission. Wiley. Chichester.
- Peterson EL, Calabrese RL (1982) Dynamic analysis of a rhythmic neural circuit in the leech *Hirudo medicinalis*. J Neurophysiol 47: 256-271.
- Pickard GE, Rea MA (1997) Serotonergic innervation of the hypothalamic suprachiasmatic nucleus and photic regulation of circadian rhythms. Biol Cell 89: 513-523.
- Picton BE, Morrow CC (1994) A field guide to the nudibranchs of the British Isles. Immel Publishing Ltd. London.



- Placantonakis DG, Bukovsky AA, Aicher SA, Kiem HP, Welsh JP (2006) Continuous electrical oscillations emerge from a coupled network: a study of the inferior olive using lentiviral knockdown of connexin36. *J Neurosci* 26: 5008-5016.
- Pola M, Cervera JL, Gosliner TM (2006) Description of two new phanerobranch nembrothid species (Nudibranchia: Polyceridae: Doridacea). *J Mar Biol Ass UK* 86: 403-409.
- Pontes M (2002) Mediterranean nudibranchs: *Marionia blainvillea*. *Nudibranch News* 4: 45-52.
- Popescu IR, Frost WN (2002) Highly dissimilar behaviors mediated by a multifunctional network in the marine mollusk *Tritonia diomedea*. *J Neurosci* 22: 1985-1993.
- Popescu IR, Willows AOD (1999) Sources of magnetic sensory input to identified neurons active during crawling in the marine mollusc *Tritonia diomedea*. *J Exp Biol* 202: 3029-3036.
- Pritz MB (2005) Comparisons and homology in adult and developing vertebrate central nervous systems. *Brain Behav Evol* 66: 222-233.
- Pruvot-Fol A (1954) Mollusques opisthobranchs. *Faune de France*. Lechevalier. Paris.
- Puelles L (2001) Thoughts on the development, structure and evolution of the mammalian and avian telencephalic pallium. *Phil Trans R Soc Lond B Biol Sci* 356: 1583-1598.
- Quinlan EM, Murphy AD (1996) Plasticity in the multifunctional buccal central pattern generator of *Helisoma* illuminated by the identification of phase 3 interneurons. *J Neurophysiol* 75: 561-574.
- Quiroga S, Ardila NE, Bolaños M (2004) *Aphelodoris antillensis* Berg, 1897 (Opisthobranchia: Nudibranchia: Dorididae), first record from the Colombian Caribbean. *Bol Invest Mar Cost* 33: 229-231.
- Ramirez JM, Pearson KG (1991) Octopaminergic modulation of interneurons in the flight system of the locust. *J Neurophysiol* 66: 1522-1537.
- Reiner A, Perkel DJ, Mello CV, Jarvis ED (2004) Songbirds and the revised avian brain nomenclature. *Ann NY Acad Sci* 1016: 77-108.
- Ricketts EF, Calvin J, Hedgpeth JW (1985) *Between Pacific tides*. Stanford University Press. Stanford, CA.

- Rieppel O (1980) Homology, a deductive concept? *Z Zool Syst Evolutionsforsch* 18: 315-319.
- Rieppel O (1988) *Fundamentals of comparative biology*. Birkhäuser. Basel.
- Rieppel O (1994) Homology, topology, and typology: the history of modern debates. In: Hall BK (ed). *Homology: the hierarchical basis of comparative biology*. Academic Press. San Diego, CA.
- Risbec J (1925) Production de lumière par un mollusque nudibranche de la Nouvelle Calédonie. *Compt Rend Acad Sci Paris* 181: 472-473.
- Risbec J (1928) Contribution a l'etude des nudibranches Neo-Caledoniens. *Faune Colon Franç* 2: 11-328, pls1-12.
- Risbec J (1937) Note préliminaire au sujet de nudibranches Néo-Calédoniens. *Bull Mus Natl d'Histoire Naturelle Paris*, series 2,9: 159-164.
- Risbec J (1953) Mollusques nudibranchs de la Nouvelle Calédonie. *Faune l'union Franç* 15: 1-189.
- Ritter DA, Bhatt DH, Fetcho JR (2001) *In vivo* imaging of zebrafish reveals differences in the spinal networks for escape and swimming movements. *J Neurosci* 21: 8956-8965.
- Robilliard GA (1970) The systematics and some aspects of the ecology of the genus *Dendronotus* (Gastropoda: Nudibranchia). *Veliger* 12: 433-479.
- Robilliard GA (1972) A new species of *Dendronotus* from the northeastern Pacific with notes on *Dendronotus nanus* and *Dendronotus robustus* (Mollusca: Opisthobranchia). *Canadian J Zool* 50: 421-432.
- Roth VL (1984) On homology. *Biol J Linn Soc* 22: 13-29.
- Roubos EW, van de Ven AM (1987) Morphology of neurosecretory cells in basommatophoran snails homologous with egg-laying and growth hormone-producing cells of *Lymnaea stagnalis*. *Gen Comp Endocrinol* 67: 7-23.
- Rozsa KS (1995) Two types of reorganization in multifunctional neural networks of *Helix pomatia* L. *Acta Biol Hung* 46: 247-262.

- Rudman WB, Darvell BW (1990) Opisthobranch molluscs of Hong Kong. Part 1. Goniodorididae, Onchidorididae, Triophidae, Gymnodorididae, Chromodorididae, (Nudibranchia). *Asian Mar Biol* 7: 31-79.
- Sadamoto H, Yamanaka M, Hatakeyama D, Nakamura H, Kojima S, Yamashita M, Ito E (2000) Developmental study of anatomical substrate for conditioned taste aversion in *Lymnaea stagnalis*. *Zool Sci* 17: 141-148.
- Sakharov DA (1976) Nerve cell homologues in gastropods. In: Salanki J (ed). *Neurobiology of invertebrates: gastropod brain*. Akadémiai Kiadó. Budapest.
- Sakurai A, Katz PS (2003) Spike timing-dependent serotonergic neuromodulation of synaptic strength intrinsic to a central pattern generator circuit. *J Neurosci* 23: 10745-10755.
- Salvini-Plawen LV, Steiner G (1996) Synapomorphies and plesiomorphies in higher classification of Mollusca. In: Taylor J (ed) *Origin and evolutionary radiation of the Mollusca*. Oxford University Press. Oxford.
- Sander K (1983) The evolution of patterning mechanisms: gleanings from insect embryogenesis and spermatogenesis. In Goodwin BC, Holder N, Wylie CC (eds) *Development and evolution*. Cambridge University Press. Cambridge.
- Sanderson SL (1988) Variation in neuromuscular activity during prey capture by trophic specialists and generalists (Pisces: Labridae). *Brain Behav Evol* 32: 257-268.
- Santagata S, Zimmer RL (2002) Comparison of the neuromuscular systems among actinotroch larvae: systematic and evolutionary implications. *Evol Dev* 4: 43-54.
- Sasakura H, Inada H, Kuhara A, Fusaoka E, Takemoto D, Takeuchi K, Mori I (2005) Maintenance of neuronal position in organized ganglia by SAX-7, a *Caenorhabditis elegans* homologue of L1. *EMBO J* 24: 1477-1488.
- Satterlie RA (1985) Reciprocal inhibition and postinhibitory rebound produce reverberation in locomotor pattern generator. *Science* 229: 402-404.
- Satterlie RA, Norekian TP (1995) Serotonergic modulation of swimming speed in the pteropod mollusc *Clione limacina* III. Cerebral neurons. *J Exp Biol* 198: 917-930.
- Satterlie RA, Norekian TP (1996) Modulation of swimming speed in the pteropod mollusc, *Clione limacina*: role of a compartmental serotonergic system. *Invert Neurosci* 2: 157-165.

- Satterlie RA, LaBarbera M, Spencer AN (1985) Swimming in the pteropod mollusc, *Clione limacina* I. Behaviour and morphology. *J Exp Biol* 116: 189-204.
- Satterlie RA, Norekian TP, Jordan S, Kazilek CJ (1995) Serotonergic modulation of swimming speed in the pteropod mollusc *Clione limacina*. I. Serotonin immunoreactivity in the central nervous system and wings. *J Exp Biol* 198: 895-904.
- Schluter D, Clifford EA, Nemethy M, McKinnon JS (2004) Parallel evolution and inheritance of quantitative traits. *Amer Nat* 163: 809-822.
- Schmekel L (1985) Aspects of evolution within the opisthobranchs. In: Trueman ER, Clarke MR (eds) *The Mollusca*, Vol. 10: Evolution. Academic Press. Orlando, FL.
- Schmidt RS (1992) Neural correlates of frog calling: production by two semi-independent generators. *Behav Brain Res* 50: 17-30.
- Schneiderman AM, Matsumoto SG, Hildebrand JG (1982) Trans-sexually grafted antennae influence development of sexually dimorphic neurons in moth brain. *Nature* 298: 844-846.
- Schrödl M, Wägele H, Willan RC (2001) Taxonomic redescription of the doridoxidae (Gastropoda: Opisthobranchia), an enigmatic family of deep water nudibranchs, with discussion of basal nudibranch phylogeny. *Zoologischer Anzeiger* 240: 83-97.
- Schuhmacher H (1973) Notes on occurrence, feeding and swimming behaviour of *Notarchus indicus* and *Melibe bucephala* at Elat, Red Sea (Mollusca: Opisthobranchia). *Israel J Zool* 22: 13-25.
- Schwindt P, Crill W (1999) Mechanisms underlying burst and regular spiking evoked by dendritic depolarization in layer 5 cortical pyramidal neurons. *J Neurophysiol* 81: 1341-1354.
- Shaw SR, Meinertzhagen IA (1986) Evolutionary progression at synaptic connections made by identified neurones. *Proc Natl Acad Sci USA* 83: 7961-7965.
- Shaw SR, Moore D (1989) Evolutionary remodeling in a visual-system through extensive changes in the synaptic connectivity of homologous neurons. *Visual Neurosci* 3: 405-410.
- Shirahata T, Watanabe S, Kirino Y (2004) Distribution of serotonin-like immunoreactive neurons in the slug *Limax valentianus*. *Cell Tissue Res* 315: 285-290.

- Shubin NH (1994) History, ontogeny, and evolution of the archetype. In: Hall BK (ed). Homology: the hierarchical basis of comparative biology. Academic Press. San Diego, CA.
- Sillar KT, Heitler WJ (1985) The neural basis of escape swimming behaviour in the squat lobster *Galathea strigosa* I. Absence of cord giant axons and anatomy of motor neurons involved in swimming. J Exp Biol 117: 251-269.
- Silvey GE, Wilson IS (1979) Structure and function of the lateral giant neurone of the primitive crustacean *Anaspides tasmaniae*. J Exp Biol 78: 121-136.
- Simpson GG (1967) Principles of animal taxonomy. Columbia University Press. New York, NY.
- Smith KK (1994) Are neuromotor systems conserved in evolution? Brain Behav Evol 43: 293-305.
- Smolen P, Rinzel J, Sherman A (1993) Why pancreatic islets burst but single beta cells do not. The heterogeneity hypothesis. Biophys J 64: 1668-1680.
- Snow RW (1982) Evidence for peptide-mediated neurotransmission in a molluscan brain. J Neurobiol 13: 267-277.
- Stasek CR (1967) Autotomy in the Mollusca. Occ Pap Calif Acad Sci 61: 1-44.
- Stent GS, Kristan WB Jr, Friesen WO, Ort CA, Poon M, Calabrese RL (1978) Neuronal generation of the leech swimming movement. Science 200: 1348-1357.
- Straub VA, Benjamin PR (2001) Extrinsic modulation and motor pattern generation in a feeding network: a cellular study. J Neurosci 21: 1767-1778.
- Straub VA, Staras K, Kemenes G, Benjamin PR (2002) Endogenous and network properties of *Lymnaea* feeding central pattern generator interneurons. J Neurophysiol 88: 1569-1583.
- Strickland HE (1846) On the structural relations of organized beings. Philos Mag J Sci 28: 354-364.
- Striedter GF (1994) The vocal control pathways in budgerigars differ from those in songbirds. J Comp Neurol 343: 35-56.
- Striedter GF (1997) The telencephalon of tetrapods in evolution. Brain Behav Evol 49: 179-213.

- Striedter GF, Northcutt RG (1991) Biological hierarchies and the concept of homology. *Brain Behav Evol* 38: 177-189.
- Strumwasser F (1968) Membrane and intracellular mechanism governing endogenous activity in neurons. In: Carlson FD (ed) *Physiological and biochemical aspects of nervous integration*. Prentice-Hall. Englewood Cliffs, NJ.
- Stuart DK, Blair SS, Weisblat DA (1987) Cell lineage, cell death, and the developmental origin of identified serotonin- and dopamine-containing neurons in the leech. *J Neurosci* 7: 1107-1122.
- Sudlow LC, Jing J, Moroz LL, Gillette R (1998) Serotonin immunoreactivity in the central nervous system of the marine molluscs *Pleurobranchaea californica* and *Tritonia diomedea*. *J Comp Neurol* 395: 466-480.
- Taghert PH, Willows AOD (1978) Control of a fixed action pattern by single, central neurons in the marine mollusk, *Tritonia diomedea*. *J Comp Physiol* 123: 253-259.
- Tardy J, Gantes H (1980) Un Mollusque Nudibranche peu connu: *Cumanotus cuenoti* A. Pruvot-Fol, (1948); redescription, biologie. *Bull Soc Zool France* 105: 199-207.
- Tazaki K, Tazaki Y (2000) Multiple motor patterns in the stomatogastric ganglion of the shrimp *Penaeus japonicus*. *J Comp Physiol A* 186: 105-118.
- Thoby-Brisson M, Ramirez JM (2001) Identification of two types of inspiratory pacemaker neurons in the isolated respiratory neural network of mice. *J Neurophysiol* 86: 104-112.
- Tholleson M (1999) Phylogenetic analysis of dorid nudibranchs (Gastropoda: Doridacea) using the mitochondrial 16s rRNA gene. *J Mollus Stud* 65: 335-353.
- Tholleson M (2000) Increasing fidelity in parsimony analysis of dorid nudibranchs by differential weighting, or a tale of two genes. *Mol Phylogenetics Evol* 16: 161-172.
- Thompson SH, Smith SJ (1976) Depolarizing after potentials and burst production in molluscan pacemaker neurons. *J Neurophysiol* 39: 153-161.
- Thompson SH, Watson WH, III (2005) Central pattern generator for swimming in *Melibe*. *J Exp Biol* 208: 1347-1361.
- Thompson TE (1960) Defensive adaptations in opisthobranchs. *J Mar Biol Assoc UK* 39: 123-134.

- Thompson TE (1976) Biology of Opisthobranch Molluscs, Vol. 1. Ray Society. London.
- Thompson TE (1980) Jamaican opisthobranch molluscs II. J Molluscan Studies 46: 74-99.
- Thompson TE, Brown GH (1981) Biology and relationships of the nudibranch mollusc *Notobryon wardi* in South Africa, with a review of the Scyllaeidae. J Zool 194: 437-444.
- Thompson TE, Brown GH (1984) Biology of Opisthobranch Molluscs, Vol II. The Ray Society. London.
- Thompson TE, Crampton DM (1984) Biology of *Melibe fimbriata*, a conspicuous opisthobranch mollusc of the Indian Ocean, which has now invaded the Mediterranean Sea. J Molluscan Studies 50: 113-121.
- Thorogood P (1987) Mechanisms of morphogenetic specification in skull development. In Wolff JR, Sievers J, Berry M (eds) Mesenchymal-epithelial interactions in neural development. Springer Verlag. Berlin.
- Tian L-M, Kawai R, Crow T (2006) Serotonin-immunoreactive CPT interneurons in *Hermisenda*: identification of sensory input and motor projections. J Neurophysiol 96: 327-335.
- Tiaza B, Le Feuvre Y, Rinzel J, Meyrand P (2005) Electrical coupling induces bistability of rhythms in networks of inhibitory spiking neurons. Eur J Neurosci 22: 2661-2668.
- Tierney AJ (1995) Evolutionary implications of neural circuit structure and function. Behav Processes 35: 173-182.
- Treistman SN (1979) Duplication of a spontaneously active neuron in *Aplysia*: electrical coupling and effects of a phosphodiesterase inhibitor. J Neurobiol 10: 325-330.
- Treistman SN, Schwartz JH (1976) Functional constancy in *Aplysia* nervous systems with anomalously duplicated identified neurons. Brain Res 109: 607-614.
- Valdes A (2003) Preliminary molecular phylogeny of the radula-less dorids (Gastropoda: Opisthobranchia), based on 16S mtDNA sequence data. J Mollus Stud 69: 75-80.
- van Vreeswijk C, Hansel D (2001) Patterns of synchrony in neural networks with spike adaptation. Neural Comput 13: 959-992.

- Vicente N (1963) Une des plus belles espèces de nudibranchs, *Hexabranhus marginatus*. Rec Trav Stat Mar End Bull 28: 99-106.
- Volman SF (1994) Directional hearing in owls: neurobiology, behaviour and evolution. In: Davies MNO, Green PR (eds) Perception and motor control in birds. Springer-Verlag. Berlin.
- von der Porten K, Parsons, DW, Rothman, BS, Pinsker H (1982) Swimming in *Aplysia brasiliana*: analysis of behavior and neuronal pathways. Behav Neural Biol 36: 1-23.
- Vonnemann V, Schrödl M, Klusmann-Kolb A, Wägele H (2005) Reconstruction of the phylogeny of the Opisthobranchia (Mollusca: Gastropoda) by means of 18S and 28S rRNA gene sequences. J Molluscan Stud 71: 113-125.
- Wägele H, Willan RC (2000) Phylogeny of the Nudibranchia. Zool J Linnean Soc 130: 83-181.
- Wagner GP (1989a) The biological homology concept. Annu Rev Evol Syst 20: 51-69.
- Wagner GP (1989b) The origin of morphological characters and the biological meaning of homology. Evolution 43: 1157-1171.
- Wainwright PC (1989) Prey processing in haemulid fishes: patterns of variation in pharyngeal jaw muscle activity. J Exp Biol 141: 359-375.
- Wainwright PC (2002) The evolution of feeding motor patterns in vertebrates. Curr Opin Neurobiol 12: 691-695.
- Wainwright PC, Lauder GV (1986) Feeding biology of sunfishes: patterns of variation in the feeding mechanism. Zool J Linn Soc 88: 217-228.
- Wainwright PC, Sanford CJ, Reilly SM, Lauder GV (1989) Evolution of motor patterns: aquatic feeding in salamanders and ray-finned fishes. Brain Behav Evol 34: 329-341.
- Waites CL, Craig AM, Garner CC (2005) Mechanisms of vertebrate synaptogenesis. Annu Rev Neurosci 28: 251-274.
- Wallen P, Grillner S (1987) N-methyl-D-aspartate receptor-induced, inherent oscillatory activity in neurons active during fictive locomotion in the lamprey. J Neurosci 7: 2745-2755.
- Wallen P, Ekeberg O, Lansner A, Brodin L, Traven H, Grillner S (1992) A computer-



- based model for realistic simulations of neural networks. II. The segmental network generating locomotor rhythmicity in the lamprey. *J Neurophysiol* 68: 1939-1950.
- Watson WH III, Lawrence KA, Newcomb JM (2001) Neuroethology of *Melibe leonina* swimming behavior. *Amer Zool* 41: 1026-1035.
- Watson WH, III, Newcomb JM, Thompson S (2002) Neural correlates of swimming behavior in *Melibe leonina*. *Biol Bull* 203: 152-160.
- Weijjs WA, Dantuma R (1994) Evolutionary approach to masticatory motor patterns in mammals. *Adv Comp Environ Physiol* 18: 281-320.
- Weinreich D, McCaman MW, McCaman RE, Vaughn JE (1973) Chemical, enzymatic and ultrastructural characterization of 5-hydroxytryptamine-containing neurons from the ganglia of *Aplysia californica* and *Tritonia diomedea*. *J Neurochem* 20: 969-976.
- Weiss KR, Kupfermann I (1976) Homology of the giant serotonergic neurons metacerebral cells in *Aplysia* and pulmonate molluscs. *Brain Res* 117: 33-49.
- Wen Z, Zheng JQ (2006) Directional guidance of nerve growth cones. *Curr Opin Neurobiol* 16: 52-58.
- Werkman TR, van Minnen J, Voorn P, Steinbusch HW, Westerink BH, De Vlieger TA, Stoof JC (1991) Localization of dopamine and its relation to the growth hormone producing cells in the central nervous system of the snail *Lymnaea stagnalis*. *Exp Brain Res* 85: 1-9.
- Westneat MW, Wainwright PC (1989) Feeding mechanism of *Epibulus insidiator* (Labridae; Teleostei): evolution of a novel functional system. *J Morphol* 202: 129-150.
- Wiens BL, Brownell PH (1995) Neurotransmitter regulation of the heart in the nudibranch *Archidoris montereyensis*. *J Neurophysiol* 74: 1639-1651.
- Wiens TJ, Wolf H (1993) The inhibitory motoneurons of crayfish thoracic limbs: identification, structures, and homology with insect common inhibitors. *J Comp Neurol* 336: 261-278.
- Wilczynski W (1984) Central neural systems subserving a homoplasous periphery. *Amer Zool* 24: 755-763.

- Wild JM (1994) The auditory-vocal-respiratory axis in birds. *Brain Behav Evol* 44: 192-209.
- Wild JM (1997) Neural pathways for the control of birdsong production. *J Neurobiol* 33: 653-670.
- Willan RC, Coleman N (1984) Nudibranchs of Australia. *Australasian Marine Photographic Index*. Sydney.
- Williams DE, Andersen RJ (1987) Terpenoid metabolites from skin extracts of the dendronotid nudibranch *Tochuina tetraquetra*. *Can J Chem* 65: 2244-2247.
- Willows AOD (1967) Behavioral acts elicited by stimulation of single, identifiable brain cells. *Science* 157: 570-574.
- Willows AOD (1968) Behavioral acts elicited by stimulation of single identifiable nerve cells. In: Carlso FD (ed) *Physiological and biochemical aspects of nervous integration*. Prentice Hall. Englewood Cliffs, NJ.
- Willows AOD (1980) Physiological basis of feeding behavior in *Tritonia diomedea*. II. Neuronal mechanisms. *J Neurophysiol* 44: 849-861.
- Willows AOD (2001) Costs and benefits of opisthobranch swimming and neurobehavioral mechanisms. *Amer Zool* 41: 943-951.
- Willows AOD, Dorsett DA (1975) Evolution of swimming behavior in *Tritonia* and its neurophysiological correlates. *J Comp Physiol* 100: 117-133.
- Willows AOD, Dorsett DA, Hoyle G (1973) The neuronal basis of behavior in *Tritonia*. I. Functional organization of the central nervous system. *J Neurobiol* 4: 207-237.
- Willows AOD, Pavlova GA, Phillips NE (1997) Modulation of ciliary beat frequency by neuropeptides from identified molluscan neurons. *J Exp Biol* 200: 1433-1439.
- Wilson JA, Phillips CE, Adams ME, Huber F (1982) Structural comparison of a homologous neuron in gryllid and acridid insects. *J Neurobiol* 13:459-467.
- Wilson LJ, Paul DH (1987) Tailflipping of *Munida quadrispina* (Galatheidæ): conservation of behavior and underlying musculature with loss of anterior contralateral flexor motoneurons and motor giant. *J Comp Physiol A* 161: 881-890.

- Wilson NG (2003) Australian *Aphelodoris* (Mollusca: Nudibranchia): two new species, sperm ultrastructure and a redescription of *Aphelodoris greeni* Burn. In: Wells FE, Walker DI (eds). The marine flora and fauna of Dampier, Western Australia, Volume 2. Western Australian Museum. Perth, Australia.
- Wobber DR (1970) A report on the feeding of *Dendronotus iris* on the anthozoan *Cerianthus* sp. from Monterey Bay, California. Veliger 12: 383-387, pls. 55-57.
- Wollscheid-Lengeling E, Boore J, Brown W, Wägele H (2001) The phylogeny of Nudibranchia (Opisthobranchia, Gastropoda, Mollusca) reconstructed by three molecular markers. Organisms Diversity Evol 1: 241-256.
- Wong RKS, Prince DA (1978) Participation of calcium spikes during intrinsic burst firing in hippocampal neurons. Brain Res 159: 385-390.
- Wray GA, Raff RA (1990) Novel origins of lineage founder cells in the direct-developing sea urchin *Heliocidaris erythrogramma*. Dev Biol 141: 41-54.
- Wright WG (2000) Neuronal and behavioral plasticity in evolution: experiments in a model lineage. Bioscience 50: 883-894.
- Wright WG, Jones K, Sharp P, Maynard B (1995) Widespread anatomical projections of the serotonergic modulatory neuron, CB1, in *Aplysia*. Invert Neurosci 1: 173-183.
- Wullimann M, Hofmann MH, Meyer DL (1991) Histochemical connectional and cytoarchitectonic evidence for a secondary reduction of the pretectum in the European eel *Anguilla anguilla*: a case of parallel evolution. Brain Behav Evol 38: 290-301.
- Wyeth RC, Willows AOD (2006) Field behavior of the nudibranch mollusc *Tritonia diomedea*. Biol Bull 210: 81-96.
- Xin Y, Koester J, Jing J, Weiss KR, Kupfermann I (2001) Cerebral-abdominal interganglionic coordinating neurons in *Aplysia*. J Neurophysiol 85: 174-186.
- Yack JE, Fullard JH (1990) The mechanoreceptive origin of insect tympanal organs: a comparative study of similar nerves in tympanate and atympanate moths. J Comp Neurol 300: 523-534.
- Yack JE, Scudder GGE, Fullard JH (1999) Evolution of the metathoracic tympanal ear and its mesothoracic homologue in the Macrolepidoptera (Insecta). Zoomorphol 119: 93-103.

- Yager DD (1999) Structure, development, and evolution of insect auditory systems. *Microsc Res Tech* 47: 380-400.
- Yeoman MS, Pieneman AW, Ferguson GP, Ter Maat A, Benjamin PR (1994) Modulatory role for the serotonergic cerebral giant cells in the feeding system of the snail, *Lymnaea*. I. Fine wire recording in the intact animal and pharmacology. *J Neurophysiol* 72: 1357-1371.
- Yonow N (1990) Red Sea Opisthobranchia 3: The orders Sacoglossa, Cephalaspidea, and Nudibranchia: Doridacea (Mollusca, Opisthobranchia). *Fauna of Saudi Arabia* 11: 286-299.
- Zakharov IS, Hayes NL, Ierusalimsky VN, Nowakowski RS, Balaban PM (1998) Postembryonic neurogenesis in the procerebrum of the terrestrial snail, *Helix lucorum* L. *J Neurobiol* 35: 271-276.