Insights from Sponge Transcriptomes & Physiology about the Early Evolution of Nervous Systems

by

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Abstract

The origin of neurons and neural systems is a research area that has begun to experience increased progress with the growing availability of genomic data from a range of basal metazoans and closely related outgroups. This has allowed a reevaluation of previous models of neural evolution. Consequently, the aim of this thesis was to use new genetic and physiological tools to determine what sponges can tell us about the early evolution of nerves. This thesis reports the finding of near-complete sets of post-synaptic density genes across the sponge classes, as well as selected enzymes involved in the synthesis of classical neurotransmitters. Building on the identification of $GABA_B$ receptors I attempted to produce an antibody against the $GABA_B$ receptor from the demosponge *Spongilla lacustris*. However, the polyclonal antibody generated was unable to identify the receptor through Western Blot analysis. Lastly, further elucidation the physiological mechanism behind the demosponge inflation and contraction behavior by demonstrating the presence of a Ca^{2+} wave acting as a coordination signal was attempted. The results I obtained are consistent, though not definitive, with the spread of a calcium wave as a factor in coordinating this response. Collectively I interpret the results to mean that while sponges have molecules and use processes which are important building blocks of conventional nervous systems, sponges ought not to be perceived as animals with a 'near nervous system.' Rather, their genetic components and physiological processes are adaptations to the specific environmental circumstances in which they function.

Chapter Prefaces

Chapter 2 Preface

This extended introduction is planned for submission to *Invertebrate Biology* as a review paper. The paper to be submitted will be substantially as it appears here, though further additions and/or figures are likely. At the time of submission of this thesis, no formal submission to the journal had been made.

Chapter 3 Preface

The work reported in this chapter formed a major portion of my thesis research. It was published with two co-first authors Ana Riesgo and Pam Windsor (a former PhD student in Dr. Leys' laboratory):

*Riesgo, A., *Farrar, N., *Windsor, P.J., Giribet, G., Leys, S.P. (2014). The analysis of eight transcriptomes from all poriferan classes reveals surprising genetic complexity in sponges. *Mol Biol Evol*. **31**(5): 1102-1120. (*co-first authors)

I performed all gene searching, phylogeny and protein modeling for the neural (PSD and neurotransmitter) genes. I also did the gene searches for the Hedgehog signaling pathway and assisted with Wnt pathway and immune-related genes. I also undertook searches of protist genomes for these gene sets. In collaboration with my co-first authors and senior author Dr. Leys, I helped frame the paper conceptually and wrote the sections on the neural genes in each section of the paper.

Chapter 4 Preface

This chapter is original, unpublished work.

Acknowledgements

I have been privileged over the past several years to work in what is unquestionably one of the top sponge biology research laboratories in the world headed by Dr. Sally Leys. I wish to thank Dr. Leys for her scientific mentorship and patience with me throughout my studies, and especially for her assistance in helping me to prepare this thesis.

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The cameraderie of friends is important in any endeavor and so I thank the fellow Leys lab members for their support: Dr. Pam Reid, the future doctors Amanda Kahn and Rachel Brown, and Danielle Ludeman. Recently Nhu Trieu has assisted me, and I thank you for your efforts.

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Chapter 1

General Introduction

Nervous systems are a recognized feature of animals (*i.e.*, metazoans). However, sponges, likely the most basal group of animals, along with placozoans, are the only two animal phyla to lack nervous tissues. In the case of placozoans, the absence of a nervous system may be an example of a secondary loss (Srivastava *et al.*, 2010; Liebeskind *et al.*, 2011), though recent discussion points to these animals having never possessed a conventional nervous system (Smith *et al*., 2014; Jorgensen, 2014). Regarding sponges, the most parsimonious view holds that these animals have never possessed nervous tissue. This places key innovations leading to the evolution of nervous systems in the branch leading to the metazoans, both before the divergence of sponges and in the lineages giving rise to ctenophores and cnidarians. One challenge in reconstructing the origins of neural systems lies in the fact that only extant organisms can be studied and inferences drawn from them. This suggests that the most informative phyla for understanding the evolution of nerves will lie at the base of the metazoan tree. Thus, sponges, ctenophores and cnidarians are of special significance (see, chapter 2, figure 2-2).

Over time zoologists have offered a number of models for conceptualizing the emergence of both neural cells and systems. However, evaluation of them in any systematic and robust manner on the basis of anatomy and basic physiology alone has been challenging. With the increase in genomic data, especially from basal metazoans and closely related outgroups (*e.g.*, choanoflagellates, the sister group to the metazoans (Carr *et al.*, 2008) and other protists) a somewhat clearer picture is beginning to emerge. It has been found that the building blocks of nervous systems – neurotransmitters and their metabolic and/or catabolic enzymes, and synaptic structural proteins – significantly pre-date the origin of neural tissues (Emes & Grant, 2012). 'Neural' elements are found in bacteria, fungi, protists and aneural metazoans such as sponges. This demands a reconsideration of what are so often considered 'neural' genes for clearly their evolutionary history speaks of their

functional plasticity outside of conventional nervous tissues. The study of the manner in which these components function in ancestral, non-neural contexts is an area of research currently in its infancy, nevertheless, studies are beginning to confirm alternate roles for 'neural' genes. For example, the synaptic scaffolding gene Homer was recently shown to interact with Flotillin in the nucleoplasm of the protist *Salpingoeca rosetta* pointing to an ancestral, nuclear function (Burkhardt *et al.*, 2014). Secretory functions have also been suggested as non-neural roles for 'neural' proteins in placozoans (Smith *et al.*, 2014). Similar studies have yet to be conducted in any sponge where numerous 'neural' genes have now been identified. The overarching aim of this thesis is to further our understanding of sponge genetic diversity, physiology and cell structure with specific reference to what these novel discoveries can tell us about the origin of the early nervous systems. I devote one chapter to a discussion of each of these aims and offer a brief rationale.

Given the apparent simplicity of sponges, a high level of genetic complexity would not be expected. The presence of 'neural' genes would be particularly unexpected given sponges' aneural physiology. The first sponge genome to be fully sequenced, annotated and published was the demosponge *Amphimedon queenslandica* (Srivastava *et al.*, 2010). Analysis of this genome revealed the presence of neural genes including a near-complete set of post-synaptic density (PSD) genes (Sakarya *et al.*, 2007). These studies quickly became the predominant basis for discussing sponge genetic complexity. Yet, this was only one species from the class demospongiae. One goal of my thesis was to evaluate the 'neural' gene diversity present across the four classes of sponge. Using a set of 8 transcriptomes I performed extensive searches for genes involved in neurotransmitter synthesis, synaptic structure and neurodevelopment. In chapter 3 I show that a rich array of neural genes is present across the sponge phylum, and that this is not an artifact of genomic research on a single species.

Genomes and transcriptomes tell us which genes are present in an organism but they offer more limited insights into the physiology of the genes identified.

Since sponges are aneural, it is the function of these genes that is of special significance. To date only commercial antibodies, those raised against non-sponge protein epitopes, have been used to investigate the expression pattern of 'neural' genes in sponges. One recent attempt to effectively visualize the GABAergic system in demosponges was done in *Chondrilla nucula*. However, serious questions about the specificity of the antibodies used leaves the work in doubt owing to non-specific staining seen in their images (Ramoino *et al*., 2007). While immunohistochemistry does not directly address physiology, an understanding of where 'neural' genes are expressed in sponges is essential for obtaining an integrated understanding of their function. Previous attempts to localize sponge genes with fluorescent *in situ* hybridization (FISH) have encountered technical challenges yet to be fully resolved. Furthermore, FISH does not directly inform the question of the protein's expression in the sponge cells -- that was my primary interest. Consequently, I adopted a differing approach by creating a fusion protein with a metabotropic GABA receptor from the demosponge *Spongilla lacustris*. The purified recombinant peptide was used as an immunogen to generate polyclonal antibodies against the receptor's intercellular domain. Chapter 4 therefore reports on my attempt to obtain a clear label for these receptors and integrate this knowledge with what is currently known about GABA signaling in demosponges.

The amino acid neurotransmitter glutamate has been shown to induce a stereotypical inflation-contraction (I-C) of the canal system in some demosponges. This I-C response is inhibited by the presence of GABA (Elliott & Leys, 2010). Furthermore, the behavior itself is dependent upon extracellular calcium. Since the I-C response is a coordinated behavior which spreads across the animal's body it requires a coordinating signal. Given the important role calcium is known to play in the physiology of contractility, and its requirement in the I-C response, Dr. Leys and I have hypothesed that a slow moving calcium wave is the coordination signal. If correct, this wave could be visualized using a calcium indicator showing a rise and fall in calcium as the wave passes through a region of the sponge's tissue. Using the

ratiometric calcium indicator fura-2 I attempted to show that changes in calcium do accompany an I-C response (see Appendix 1 for preliminary findings).

In my final summary (chapter 5) I review the key findings from each chapter and offer a brief explanation of how I view these findings in light of the understanding I develop in my extended introduction (see, chapter 2). I also suggest the subsequent directions I think each of these research problems should be taken.

While sponges definitely have and presumably express 'neural' molecules and appear to use physiological signaling pathways known to operate in neural and neuromuscular tissues I will contend that sponges should not be viewed as having a 'near nervous system' but rather they simply use common molecules and pathways in a manner fitting for their ecological niches. To more fully introduce the background relevant to this interpretation of my data I begin with an extended review of the literature covering the sponge 'neural' toolkit and how we might best think of this in light of our understanding of early animal evolution.

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Chapter Two
Sponges and the Question of Neural Origins

2.1 Introduction

Our modern understanding of the origin of nervous tissue and centralized nervous systems comes largely from comparative anatomy, especially of basally branching metazoans. The increasing availability of genomic data promises many new characters, in which gene presence/absence can be used to define what may have comprised early nervous systems and perhaps even point to how they functioned. But fundamental questions still remain. How and why did nerve tissue evolve in the first place? Did it evolve once in a common ancestor of the metazoans, or has it arisen independently among different metazoan taxa?

Genomic data has begun revealing which neural components are present among the various basal metazoan groups and in closely related outgroups. From this increase in genomic data has come a recent and controversial hypothesis that ctenophores are may be situated at the base of the metazoan tree (Dunn *et al.*, 2008; Ryan *et al*., 2013). This finding implies that while functional nervous systems would have been present in the earliest animals, they have been later lost in sponges. However alternative hypotheses, such as multiple independent origins of neural systems have also been proposed to explain the differences and the curious patterns of gain and loss of neural components (Moroz, 2009). But would the earliest nervous system even be recognizable as such? Though nerves are histologically identifiable, defining their essence proves more difficult. One working definition of a nerve is a polarized cell adapted for the unidirectional transmission of information using electrical potentials coupled to paracrine signaling. However, this definition is not beyond dispute (*e.g.*, electrically coupled neurons). This ambiguity raises the question of what a proto-neural system might look like if conventional nerves, as we know them morphologically, were still absent? Furthermore, what evolutionary pressures might drive the formation of neural or neural-like systems and their subsequent increases in complexity?

Sponges are a focal point of this investigation because these animals possess many genes associated with neural signaling, yet have neither nerves nor muscle. The traditional, basal phylogenetic position of these animals (Dohrmann & Worheide, 2013), their physiology (Leys & Hill, 2012), ecology (Wulff, 2012) and genomic make up (Srivastava et al., 2010; Nichols *et al.*, 2011) help us situate classical 'neural' genes in both a sponge-centric and early animal evolutionary context. Sponges provide an extant system in which to study how neural molecules can be used outside conventional nervous systems, and how they might have been coopted into later evolving nervous systems.

2.2 The Sponge Enigma: No Nerves, but a big Neural Toolkit

Although to the naked eye sponges appear to do little, their morphology, physiology, and cell biology are finely adapted to filter feeding, a process that requires regulation (Leys & Hill, 2012). Sponges generate a current to feed, oxygenate tissues and remove waste. This requires monitoring of water flow through their canal system and generation of responses to adjust the flow rate. In cellular sponges a threshold level of irritation, mechanical agitation or clogging of the canal system triggers contractions, which safeguard the filtering system. The response prevents entry of other particles, and flushes out debris and wastes that otherwise left could cause mechanical damage to the more delicate, choanocyte chambers (flagellated feeding tissues). This reaction is not rapid but happens slowly over several minutes (Elliott & Leys, 2007). Other sponges such as *Tethya wilhelma* appear to generate rhythmic contractions of unclear functionality (Nickel, 2004). In syncytial sponges (Hexactinellida) similar irritations trigger an arrest of the feeding current through the sponge (Leys *et al.*, 1999). The result is the same, but the mechanism is dissimilar, involving electrical signalling via syncytial pathways. Nevertheless, all sponges are able to trigger regulated, coordinated behaviour in response to a range of stimuli (Leys & Meech, 2006) without nerves or conventional muscle.

In other animals the functions of sensing the external and internal environment, synthesizing and interpreting information impinging on the sensors, and triggering regulated, coordinated responses are typically carried out by neurosensory and neuromuscular systems. The presence of interconnected sensory, neural and muscular systems allows responses to be generated extremely quickly. In contrast sponge behavior is protracted with, no identifiable neural or muscular tissue involvement. How then does the sponge sense and respond to its environment?

Physiological, histological, genomic and transcriptomic studies now show that sponges (across the phylum) possess genes that in other animals are associated with (a) synaptic signaling (*i.e.*, neurotransmitters), (b) synapse construction, (c) propagated potentials, and (d) neurodevelopment. Additionally, a surprising number of neural protein components classically associated with nervous tissues are found in sponges (see, Table 2-1). Below we examine the size and scope of the sponge's 'neural toolkit'.

2.2.1 Neurotransmitters

Cholinergic Signaling: A surprising number of neural protein components classically associated with nervous tissues are found in sponges. Half a century ago the hydrolytic enzyme *Acetylcholinesterase* (AChE) was reported in *Sycon* (Lentz 1966; 1968). AChE has also been reported in the demosponges *Tethya* and *Hippospongia* (Thiney, 1972; Nickel, 2001). In each case AChE activity was shown. Acetylcholine has physiological effects in at least some sponges: in *Spongia officinalis* it causes the osculum to contract (Pavans de Ceccatty, 1971), and in *Tethya wilhelma* the frequency of whole animal contractions is modulated; (Ellwanger and Nickel, 2006). AChE-like proteins were identified in 8 different sponge transcriptomes (unpublished), though no muscarinic, nicotinic, or ancestral-type acetylcholine receptors (AChR) could be identified.

Adrenergic Signaling: Epinephrine appears to influence pumping rate and contractile activity (Emson, 1966; Pavans de Cecatty, 1971; Ellwanger and Nickel, 2006). Interestingly, *Phenylethanolamine N-methyltransferase* (PNMT), the enzyme that synthesizes epinephrine from norepinephrine, was not identified in a recent study of 8 sponge transcriptomes (Riesgo *et al.*, 2013; chapter 3).

Serotonergic Signaling: Serotonin has been more elusive to find in sponges. Lentz (1966) showed staining for serotonin in *Sycon* using a silver (argenteffin) stain and Weyrer *et al.* (1999) showed alleged staining of six clusters of cells in the larva of *Tedania ignis* using a commercial rabbit anti-serotonin antibody. No physiological role for serotonin was found in *Cliona celata* (Emson, 1966), but in *Tethya wilhelma* serotonin was found to induce contractions (Ellwanger and Nickel, 2006). Interestingly no unequivocal hits for serotonin receptors were found in any of 8 transcriptomes recently described (Riesgo *et al.*, 2014; chapter 3), but *tryptophan hydroxylase* (required for conversion of tryptophan to serotonin) was identified in *Corticium candelabrum*, *Petrosia ficiformis* and *Eunapius fragilis.* *Aromatic L-amino acid decarboxylase* (also known as *DOPA decarboxylase* (required for the conversion of 5-HTP to serotonin, and L-DOPA to dopamine) was also identified in *Corticium candelabrum* and *Sycon coactum* (Riesgo *et al.*, 2014; chapter 3). The presence of serotonin synthesis enzymes without receptors might seem odd, but may be explained by a report on the demosponges *Hyrtios erectus* and *Hyrtios reticulatus* that have numerous serotonin-derived alkaloids (Salmoun *et al.*, 2002). The function of these alkaloids remains unknown, but they may shed light on early reports of the presence of serotonin and the lack of clear physiological effect. Perhaps sponges use serotonin only as a biosynthetic intermediate, rather than a signaling molecule, serotonin being later coopted into neural circuits as a signaling molecule. Alternatively, it may be synthesized by bacterial symbionts in the sponge (Lakshminarayan *et al.*, 2004).

Table 2-1. Summary of literature evidence for neuroactive molecules in sponges.

Neuroactive molecule	Sponges in which Identified	Reference
Acetylcholine	*Cliona celata, Euspongia officinalis, Tethya wilhelma*	Emson, 1966; Pavans de Caccatty, 1971; Ellwanger & Nickel, 2006
Epinephrine	*Sycon ciliatum, Sycon sp., Euspongia officinalis, Hippospongia communis, Tethya wilhelma*	Lentz, 1966; Lentz 1968; Pavans de Caccatty, 1971; Thiney, 1972; Ellwanger & Nickel, 2006
Norepinephrine	*Sycon ciliatum*	Lentz, 1966
Serotonin	*Sycon ciliatum, Cliona celata, Tedania ignis, Tethya wilhelma*	Lentz, 1966; Emson, 1966; Weyrer *et al.*, 1999; Ellwanger & Nickel, 2006
Dopamine	*Neopetrosia exigua*	Liu *et al.*, 2004
Nitric oxide	*Tethya wilhelma, Chondrilla nucula, Ephydatia muelleri*	Ellwanger & Nickel, 2006; Zocchi *et al.*, 2001; Elliott & Leys, 2010
Glutamate	*Clathrina clathrus, Oscarella lobularis, Tethya wilhelma*	Nickel, 2010; Ellwanger *et al.*, 2007; Elliott & Leys, 2010
GABA	*Tethya wilhelma, Cliona celata, Chondrilla nucula, Leucandra aspera, Ephydatia muelleri*	Ellwanger *et al.*, 2007; Emson, 1966; Ramoino *et al.*, 2007; Ramoino *et al.*, 2010; Elliott & Leys, 2010
Glycine	*Tethya wilhelma*	Ellwanger & Nickel, 2006
Adenosine/caffeine	*Tethya wilhelma*	Ellwanger & Nickel, 2006
cAMP	*Spongilla lacustris, Clathrina cerebrum, Tethya wilhelma*	Simpson & Rodan, 1976; Gaino & Magnino, 1996; Ellwanger & Nickel, 2006
Receptors & Enzymes		
Acetylcholinesterase	*Spongilla lacustris, Leucandra aspera, Siphonochalina crassa, Sycon ciliatum, Scypha sp., Hippospongia communis, Tethya wilhelma*	Mitropolitanskaya, 1941; Bacq, 1947; Lentz 1966; Bullocks & Horridge, 1969; Thiney, 1972; Nickel, 2001
Glutamate/GABAergic	*Geodia cydonium*	Perovic *et al.*, 1999

Dopaminergic Signaling: The evidence for a functional role of dopamine is even scarcer. My attempt to identify a dopamine receptor from eight sponge transcriptomes and the *Amphimedon queenslandica* and *Oscarella carmella* genomes were unsuccessful. Dopamine has been isolated from *Neopetrosia exigua* (Liu *et al.*, 2004), but the authors did no functional experiments. Since enzymes for synthesizing dopamine (dopamine-β-hydroxylase and DOPA decarboxylase) were found in some sponge transcriptomes (Riesgo *et al.*, 2014; chapter 3) dopamine may also be a metabolic intermediate (*e.g.*, part of the ephinephrine synthesis pathway), rather than a functional signaling molecule in sponges.

Glutamatergic & GABAergic Signaling: In contrast to other molecules discussed above, the excitatory amino acid glutamate is clearly physiologically active in several sponges. Glutamate induces a contraction response in *Tethya wilhelma* (Ellwanger and Nickel, 2006), and Elliott and Leys (2010) have shown that bath-applied L-glutamate triggers a stereotypical 'inflation-and-contraction' of the canal system in *Ephydatia muelleri* (also inducible in *Spongilla lacustris* (Ludeman et al., 2014)). The behavior is also triggered at a threshold level of mechanical agitation or irritation, and *may* involve glutamatergic signaling as the reponse is generated. The inhibitory signaling molecule γ-aminobutyric acid (GABA) has also been found to have a number of effects on sponge behaviour. In *Tethya* 25□mol L^{-1} triggered a contraction while in *Ephydatia* contractions were caused by initial bath addition of GABA (3/18 sponges at concentrations of 250 and 500μmol L^{-1}), but pre-incubation in GABA (1mmol L^{-1}) prevented *Ephydatia* from carrying out the inflation-contraction behaviour, whether triggered by L-glutamate or by mechanical agitation. Therefore, both excitatory and inhibitory roles for GABA may exist depending on the sponge species, with these experiments suggesting there is considerable variability in the physiological responses among sponges.

Given the opposite responses *Tethya* and *Ephydatia* showed to GABA, one should probably not assume conventional expression or use of these molecules in sponges. The discovery of a peculiar hybrid receptor exhibiting similarities to both

GABA and glutamate receptors (mGlu/GABA-like receptor) in *Geodia cydonium* by Perovic et al. (1999) suggests this is so. The N-terminal region of the receptor is structurally similar to vertebrate MGluR4 and MGluR5 receptors, the latter known to activate the IP_3-Ca^{2+} pathway (Perovic *et al.*, 1999). Since then, metabotropic GABA and glutamate receptors and *glutamate decarboxylase* (which catalyzes the reaction of glutamate to GABA) have been found in the *Amphimedon queenslandica* genome (Sakaraya *et al.*, 2007) and in all sponge classes (Riesgo *et al.*, 2014; chapter 3). Metabotropic receptors are to be expected since they are slower acting and fit the nature of the more gradual contraction responses exhibited by sponges. While reported absent from the genome of *Amphimedon queenslandica* (Srivastava *et al.*, 2010), ionotropic glutamate receptor (iGluR) sequences were identified in each sponge class except for hexactinellida (Riesgo *et al.*, 2014; chapter 3). In addition, Glycine, another inhibitory amino acid neurotransmitter, has been shown to modulate endogenous contractile rhythm in *Tehtya wilhelma* (Ellwanger and Nickel, 2006).

Commercial antibodies to mammalian GABA receptors have been used to attempt labeling of GABAergic signaling components in *Chondrilla nucula* (Ramoino et al., 2007). GABA is also suggested to regulate 'feeding' in *Leucandra aspera* (Ramoino et al., 2010). Experiments using dissociated cells showed enhanced uptake of dextrans when GABA was added to the medium. Furthermore, immunocytochemistry revealed that choanocytes label positive for $GABA_B$ receptors (Ramoino *et al*., 2010). The difficulty with interpreting antibody work is the general lack of specificity of commercial antibodies with sponge epitopes. Ramoino *et al*. (2007) used Western blots to confirm that each of these antibodies recognizes sponge proteins of the correct molecular weight, though only a small piece of the blot was shown. However, the broad range of cells and regions within cells that are labeled still raises questions about specificity that only antibodies raised against sponge-specific epitopes can address (also see chapter 4 of this thesis).

Nitric oxide & Neuropeptide Signaling: Nitric oxide (NO), synthesized by Nitric oxide synthase (NOS), is best known for modulatory roles in vertebrate and invertebrate nervous systems. For example, NO modulates peristalsis in the cnidarian *Renilla koellikeri* (Anctil *et al.*, 2005) and can both trigger and modulate contractions in *Tethya wilhelma* (Ellwanger and Nickels, 2006). In *Ephydatia muelleri* cells lining the excurrent canal system and in particular the osculum showed NOS activity as indicated by positive NADPH-diaphorase staining that appeared to form a base-to-tip gradient (Elliott and Leys, 2010). NO also has different roles in other sponges; in *Axinella polypoides* and *Petrosia ficiformis* it appears to signal in a heat stress pathway which contains a temperature sensitive NOS (Giovine *et al.*, 2001). NOS is found in species from each class of sponges (Sakarya *et al.*, 2007; Riesgo *et al.*, 2014; chapter 3).

Curiously, whereas in both cnidarians and ctenophores neuropeptides have been shown to have a large role in neurotransmission, to date they have not been reported in the poriferans. Ctenophores, notably *Pleurobrachia bachei*, appear to utilize neuropeptides extensively for neurotransmission (Moroz *et al.*, 2014). Neuropeptides are also important modulators of neuronal signaling across the metazoans, and have a deep evolutionary history (Grimmelikhuijzen & Hauser, 2012; Jekely, 2013). At present, whether neuropeptides are used for signaling or modulation in sponges is unknown.

In sum, a diverse set of studies in sponges shows the presence of many signaling pathways that are classically associated with chemical neurotransmission in neural animals. The presence of neural genes becomes increasingly curious with the finding of synaptic structural proteins in the aneural sponges.

2.2.2 Synaptic Construction

Neurons, though difficult to define, are frequently identified on the basis of morphological features including a defined cell body, dendritic processes exhibiting varying degrees of branching and an axon with its terminal processes. Synapses, the

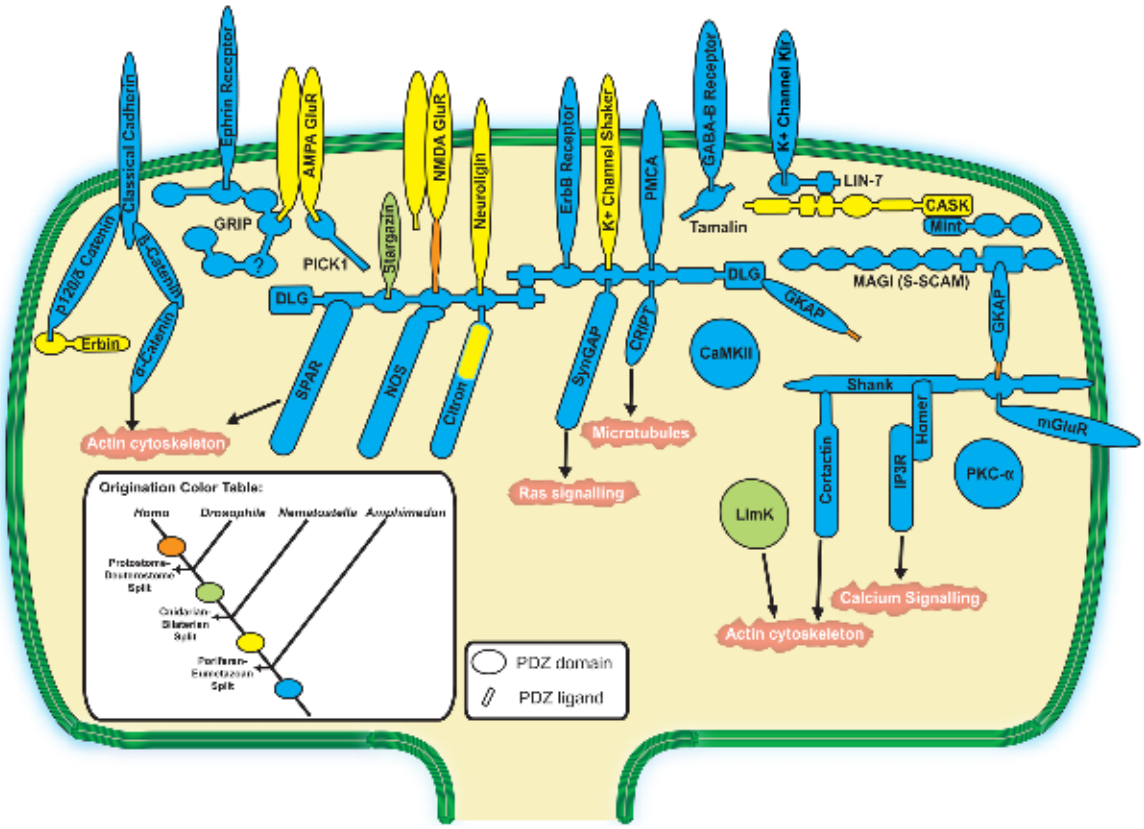
hallmark of nervous systems, are identified by the presence of a post-synaptic density (PSD) on the target nerve cell (Verpelli *et al.*, 2012). This structure, clearly visible under the electron microscope, is composed of scaffolding, as well as receptor and signaling components required for chemical neurotransmission. The PSD is therefore evidence of a synapse. Despite lacking conventional nerves as described above, *Amphimedon queenslandica* (the first sponge to yield a sequenced genome) was found to encode a set of PSD genes (Sakarya *et al.*, 2007; Srivastava *et al.*, 2010). Sakarya *et al*. (2007) reported that of 36 gene families known from vertebrate PSDs many homologs "are present in the sponge and nearly the entire gene set is present in Nematostella." Five PSD genes – DLG, GKAP, GRIP, HOMER and CRIPT – were identified with *in-situ* hybridization in the flask cells of *Amphimedon* larvae. The interactions and functional relationship between these components in sponges has not yet been studied. The lack of clear histological evidence for classical synaptic/PSD-like structures such as those seen in vertebrate synapses in sponges implies that PSD genes are utilized in other ways. A recent study of Homer in the protist *Salpingoeca rosetta* supports the existence of non-neural roles for PSD gene components (Burkhardt *et al.*, 2014) suggesting PSD genes were later coopted into neural pathways during the evolution of synapses.

A subsequent study searched for PSD genes in a taxonomically broader manner. The presence of PSD genes outside the metazoans confirmed these genes evolved in a non-synaptic context and were present in the ancestor of choanoflagellates and metazoans (Alie & Manuel, 2010). Possible roles for some of the earliest evolving PSD genes may have been linking Ca^{2+} influx to dynamic changes in the cytoskeleton (Alie & Manuel, 2010). That these proteins would therefore have been coopted into the evolving synapse is plausible given the role of Ca^{2+} in regulating signaling at the synapse (Llinas *et al.*, 1981). Therefore, while the last common ancestor (LCA) to all metazoans was nerveless, the 'neural' components present were likely functional in other non-neural contexts.

PSD Gene Regulatory Networks: Why do sponges not form a PSD, or similar cell-cell communication platform, when they encode a near-complete set of these genes? After all, sponge PSD genes do contain the domains (*e.g.*, PDZ) required to build such a complex, and it is thought one of the ways such structures evolve is by adding components to an existing structure through domain accretion (Koonin *et al.*, 2000; Kosik, 2009). The answer might lie in the fact that the presence of synaptic components and interaction domains alone is insufficient to build a synapse. The genes must also be expressed together spatially and temporally through a gene expression network. In neural animals a coordinated program of gene expression exists for each component ultimately leading to the formation of a PSD complex. In *Amphimedon* this global co-regulation of PSD gene elements is not observed (Conaco *et al.*, 2012a). While the components for building a conventional PSD are present, the genetic network needed is absent. Neural evolution therefore depends not only on the prerequisite gene set, but also on the evolution of a gene regulatory network required to facilitate the assembly of the structure. Whether *some* PSD components in the sponge function together to support cellular communication is unresolved. One approach to investigate this would be to perform immuno-coprecipitations of sponge PSD components, to determine which interact together *in vivo*. This, however, presupposes specific antibodies are made against sponge PSD components.

Vesicle-based Signaling: Functioning synapses utilize and require machinery (*i.e.*, SNARE proteins) for neurochemical vesicular exocytosis. ESTs from the homoscleromorph *Oscarella carmella* were used to identify a set of SNARE proteins including synaptogamin, syntaxin, N-ethylmaleimide-sensitive fusion protein attachment protein-α and N-ethylmaleimide-sensitive factor. Neurocalin, involved in regulating actin, tubulin and clathrin interactions was also identified in O. carmella (Jacobs *et al.*, 2007). In the 1970s a series of histological studies on *Hippospongia*, *Euspongia* and *Haliclona* species documented a rich diversity of cell-cell contacts, both transient and permanent (Pavans de Ceccatty *et al.*, 1970; Pavans de Ceccatty, 1974a). An EM image from *Hippospongia communis* shows two

Figure 2-1 Schematic diagram of the post-synaptic density (PSD) showing the progressive acquisition of elements across the animal phyla. Reprinted from: Sakarya *et al.*, (2007). *PLoS One*. **2**: e506.



adjoining cells with vesicles clustered near the membrane, an observation consistent with paracrine signaling *via* exocytosis (Pavans de Ceccatty, 1974b). Histological and molecular evidence are consistent with a vesicular signaling system essential for sponge communication and behavior. Such components could easily be coopted into later evolving synapses.

2.2.3 Propagated Action Potentials & Waves

Action Potentials: Sponge vitality is dependent upon an intact, functioning feeding apparatus. Threat of damage to the feeding system therefore demands a rapid response. This fact is likely the biological rationale for the action potential (AP) in the thin-walled, syncytial glass sponge, *Rhabdocalyptus dawsoni* (Leys and Mackie, 1997). It is the only sponge from which an action potential has been recorded to date. The ionic basis for the AP was shown to be Ca^{2+} and K^{+} (Leys *et al*. 1999). Substitution of ¾ choline chloride for NaCl showed minimal reduction in AP amplitude revealing the current is not Na^{+}-dependent. Co^{2+}, Mn^{2+} or nimodipine, each a Ca^{2+} channel blocker, blocked the AP amplitude demonstrating Ca^{2+}-dependence. The AP in *R. dawsoni* triggers the arrest of the beating flagella rapidly halting the sponge's feeding current. This behavior likely serves a protective role by guarding the feeding tissue from potentially damaging particulate matter entering the sponge (Leys *et al*., 1999).

Ca^{2+} Waves: Recording electrical activity from cellular sponges (*e.g.*, demosponges) has encountered unresolved technical challenges (though one non-replicable study reported electrical communication between between cells, Loewenstein, 1967). However, electrical signaling, essential for rapid communication and behavior modification, is less important for many sponges where responses are slower (*e.g.*, rates of contraction between 4 and 400 μm s^{-1}). Spreading contractions have been observed in both the sponge oscula and body of the demosponge *Ephydatia fluviatilis* (McNair, 1923; De Vos & Van de Vyver, 1981) raising the question of what propagating signal coordinates these waves of contraction? Recent work on body contractions in *Ephydatia muelleri* has shown

that in the absence of Ca^{2+} an I/C response is stunted (Elliott & Leys, 2010). Ca^{2+} appears to be the coordinating signal for this behavior. This hypothesis predicts a propagating calcium wave present during an I/C response, which could be visualized with a ratiometric calcium indicator. Since Ca^{2+} waves and oscillations are evolutionarily ancient and important in coordination physiology (*e.g.*, muscle contraction), it seems likely that Ca^{2+} would have a similar role in sponge contractile behavior (Shemarov & Nesterov, 2005a; 2005b; 2007).

2.2.4 Neurodevelopmental Genes

Sponges across the classes possess a many, but not every, developmental toolkit gene possessed by vertebrates (Nichols *et al.*, 2006; Srivastava *et al.*, 2010; Riesgo *et al.*, 2013). In 2008 Richards *et al*. found a small subset of *Amphimedon* larval cells that express delta-notch and the bHLH gene AmqbHLH1. The latter gene promotes neurogenesis when expressed in both *Xenopus* and *Drosophila*. Some interpreted this to mean 'pro-neural' fates exist in the *Amphimedon* larva where they may be involved in external sensing (Richards *et al*., 2008), as in the larval behaviors described and Leys and Degnan, 2001. However, despite these factors being neurogenic in some organisms, this term seems misplaced in an animal that is nerveless!

Axon guidance molecules (AGM) have been identified among sponge genomes and transcriptomes. The AGM *Slit* was identified in *Oscarella* (Jacobs *et al.*, 2007), and *PlexinA1*, *semaphorin3B* and *EphB1* were recently reported in *Amphimedon* (Conaco *et al.*, 2012b). *In vivo* functions for AGMs have not yet been reported. Other classical AGMs including *Slit*, *Robo*, *Netrin* and *UNC5* were not identified in *Amphimedon* (though a DCC receptor has been identified by annotation, GI340383253) (Srivastava *et al*., 2010). Potentially just as AGMs pattern growing neurites (Kolodkin & Tessier-Lavigne, 2011) and shape the developing vascular system (Adams and Eichmann, 2010), in sponges they may shape the branching canal system during development. AGMs may therefore have a more widely

conserved role in morphogenesis outside the nervous system than previous appreciated.

Sponges clearly possess and utilize molecules and receptors classically associated with neurotransmission. They contain a near complete set of PSD genes and machinery associated with SNARE complexes. They express transcription factors involved in neural specification and encode several axon guidance genes. Uniquely, glass sponges are capable of signaling *via* action potentials. These components enable many neural animals to sense their external environment, process information, and generate coordinated behaviors. Yet, sponges lack conventional nerves! How ought we to think rightly about the sponge's 'neural toolkit?' Central to this question are the phylogenetic relationships between the basal metazoans with the correct positioning of the ctenophores relative to poriferans being the central issue. In light of current controversy surrounding this relationship a brief assessment of the current state of basal metazoan and sponge phylogeny is needed.

2.3 Basal Phylogeny & its Implications for Neural Evolution

Correct phylogenetic reconstruction is essential for interpreting molecular and physiological data in the context of neural evolution. This is true with respect to the sponge phylum itself and the four basal metazoan phyla – Porifera, Ctenophora, Cnidaria and Placazoa. For patterns of gain, loss or independent acquisition of neural elements to be mapped the phylogenetic backbone must be correct and this is problematic because this very backbone has been subject to recent debate.

Sponge phylogeny: During the late 1990s phylogenetic reconstructions based on primarily rDNA, and later a set of nuclear housekeeping genes gave rise to polyphyletic models for sponge evolution (Zrzavy *et al*., 1998; Kruse *et al.*, 1998; Borchiellini *et al.*, 2001). A more recent study using seven nuclear housekeeping proteins also recovered polyphyletic relationships with homoscleromorphs in the sister position to the eumetazoans (Sperling *et al.*, 2007). However, wider taxon

sampling across the sponges weakened, but did not wholly eliminate support for this initial reconstruction (Sperling *et al.*, 2009). Subsequent analysis combining nuclear housekeeping and rDNA genes again led to a polyphyletic reconstruction in which both homoscleromorphs and calcarea showed greater relatedness to eumetazoans than the demosponges. Plaguing these studies however was imperfect taxon sampling and the limited gene set utilization. Philippe *et al*. (2009), utilizing a much larger set of 128 nuclear genes, recovered the traditional phylogeny with sponges forming a monophyletic clade sister to the other metazoan phyla. Pick *et al*. (2010)'s reconstruction, based on a previously published set of 150 genes enlarged with broader taxon sampling, also recovered sponges as a monophyletic clade. How are these differing reconstructions to be reconciled? Using 122 nuclear genes Nosenko *et al*. (2013) showed that whether a mono- or polyphyletic tree was generated was dependent on the subset of data analyzed. Due to their essential cellular functions, proteins involved with translation are slowly evolving genes and when used to construct a phylogeny yield a monophyletic reconstruction. In contrast, when genes under less evolutionary constraint are used polyphyletic outcomes are seen. These authors argued that care must be taken in selecting slow evolving genes since these are most likely to give a sound phylogenetic signal. Therefore, present consensus holds that regardless of the correct position of the sponges on the metazoan tree, sponges should be depicted as a monophyletic clade.

Basal metazoan phylogeny: The second issue concerns the placement of sponges on the metazoan tree. Traditionally, sponges have been the first branching group on the tree. However, using EST from animals representing 21 phyla, Dunn *et al*. (2008) placed the ctenophores as the sister group to all other metazoans. This was remarkable, the authors themselves noting this "has not been postulated before" and "should be viewed as provisional until more data are considered from placazoans and additional sponges." The study included only two sponges, *Oscarella carmela* (homoscleromorph) and *Suberites domuncula* (demosponge). Pick *et al*. (2010) who reanalyzed that data set and added 6 demosponges, 3 hexactinellids, 2 calcareans 2 homoscleromorphs and 1 placazoan recovered the traditional

topology. (Phillipe *et al.*, 2009 also recovered the traditional phylogeny.) This controversial 'ctenophore-first' hypothesis has recently gained new life with the publication of both the *Mnemiopsis leidi* and *Pleurobrachia bachei* genomes (Ryan *et al.*, 2013; Moroz et al., 2014).

What implications do these phylogenies have for neural evolution? While sponges lack conventional nerves, ctenophores, which lack any centralized nervous organization, do have a fine nerve net seen around the mouth, pharynx and tentacles (Jager *et al.*, 2011). If sponges are the most basal group of animals then progressive acquisition of neural complexity in subsequently branching taxa is the probable evolutionary scenario.

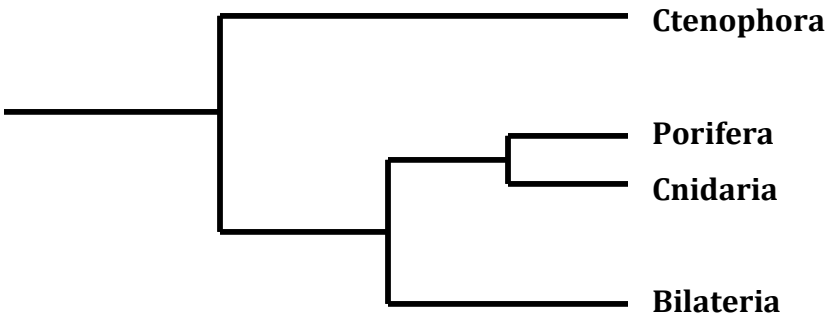
Alternatively, if the reconstruction of Dunn *et al.*, 2008 and recent genomic interpretation by Ryan *et al.*, 2013 is correct then a functioning nervous system existed at the base of the metazoan tree with subsequent loss of nervous tissue (*e.g.*, Poriferans). This scenario could also be consistent with multiple origins for neural tissue making the ctenophore and common ancestor of cnidarian + bilaterian nervous systems examples of convergent evolution. While data from the sponges discussed in this paper can be interpreted within either of these phylogenetic scenarios, I adopt the traditional phylogeny. As Philippe *et al.*, (2011) shows, adjustment to factors such as taxon sampling or the model parameters used in tree construction can lead to biologically significant alterations in the phylogenetic results (also see, Dohrmann & Worheide, 2013). I am therefore hesitant to overturn current understanding on the basis of a few new phylogenetic analyses.

2.4 Comparing Ctenophore and Sponge Neural Components

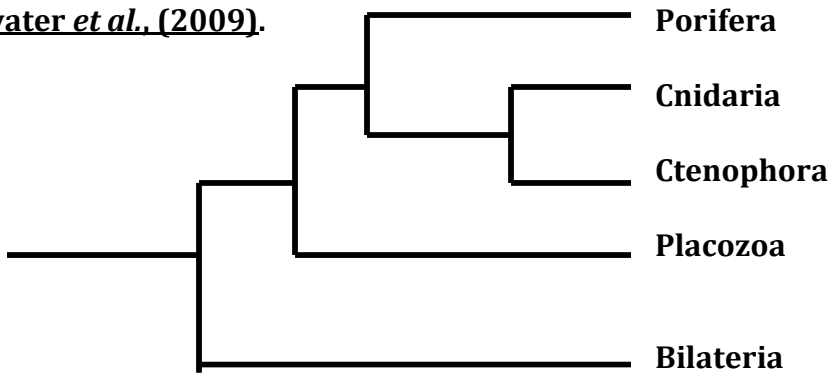
The new *Mnemiopsis* genome allows us to directly compare the neural repertoire of these animals with sponges and other metazoans. While Ryan *et al*. (2014) reported on a limited set of neurotransmitter genes, preliminary comparisons can be made: *Quinoid dihydropteridine reductase*, an enzyme involved phenylalanine metabolism through the recycling of tetrahydrobiopterin and

Figure 2-2 Recent phylogenetic reconstructions have presented several scenarios for the relationships between the basal metazoan taxa. Throughout the thesis I retain the traditional phylogeny which situate sponges as the first branching group of metazoans (as recovered by Philippe *et al.*, 2009). (Modified from Philippe *et al.*, 2011.)

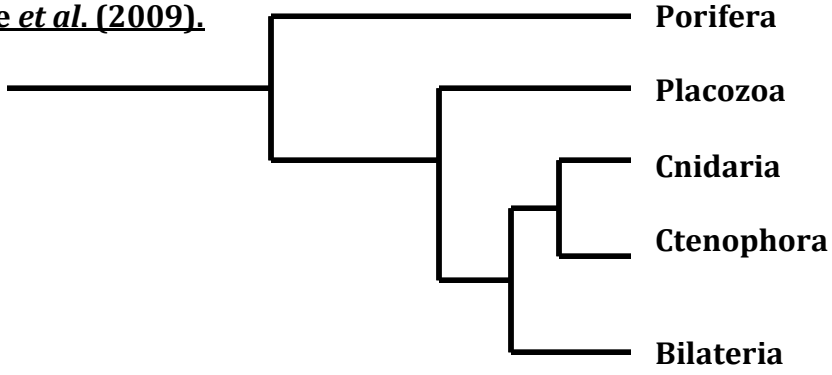
- **Dunn *et al.*, (2008).**



- **Schierwater *et al.*, (2009).**



- **Philippe *et al.* (2009).**



important for some neurotransmitter synthesis, has been reported present in both *Mnemiopsis* and *Amphimedon*. A subsequent search of the previous reported 8 sponge transcriptomes (Riesgo *et al.*, 2014; chapter 3) confirmed the enzyme was present in all sponges. Similarly *Glutamate decarboxylase* (GABA signaling) and A*cetylcholinesterase* (acetylcholine signaling) are present in both sponges and ctenophores. In contrast to *Mnemiopsis* some sponges appear to possess *DOPA decarboxylase* and *Dopamine β-hydroxylase*. The ctenophore genome was unsuccessfully searched for *Homo sapien solute carrier family 18 member 2* (Slc18A2), a vesicular monoamine transporter. It could not be identified in any sponge transcriptome either.

Both ctenophores and sponges have metabotropic glutamate and GABA receptors (Sakarya *et al.*, 2007; Ryan *et al.*, 2013; Riesgo *et al.*, 2014; chapter 3). Interestingly, neither serotonin nor dopamine receptors could be identified in the *Mnemiopsis* genome, though receptors with 7-TM topology and potential homology was detected. Searches for serotonin and dopamine receptors in sponge transcriptomes also did not identify these receptors though mixed Blast hits occasionally weakly identified putative serotonin receptors. Ryan *et al*. (2013) suggests these uncharacterized receptors in *Mnemiopsis* may bind novel neuropeptides, a situation that could also be true in sponges.

Ionotropic glutamate receptors (iGluRs) from *M. leidi* and 7 other ctenophores were not found to be orthologs of AMPA, NMDA, Kainate or Delta-type iGluRs. This suggests the ancestral iGluR found in ctenophores predates the subsequent diversification into the iGluR sub-types. Though iGluRs appear absent in most sponges (*e.g.*, *A. queenslandica*), partial sequences have been identified in species from each class except Hexactinellida (Riesgo *et al.*, 2014; chapter 3).

Core elements of the SNARE complex that facilitate exocytosis of cellular vesicles (*i.e.*, SNAP, syntaxin, synaptotagmin, synaptobrevin) were also identified in *Mnemiopsis*, and as noted above, are encoded by sponges as well. Similarly, the set

of PSD genes reported in *Mnemiopsis* differs little from those reported in *Amphimedon* (Sakarya *et al.*, 2007) and *Oscarella's* genome, as well as the 8 transciptomes (Riesgo *et al.*, 2014; chapter 3). Since core PSD components are widely distributed throughout the protists (Richter & King, 2013; Riesgo *et al.*, 2014) and metazoans (though with increasing complexity as synaptic structure diversified, Grant & Emes, 2012), they are not especially informative for addressing the question of nervous system gain or loss in sponges. There appears to be similarity in the neural components held by ctenophores and sponges. The importance of neuropeptide signaling in ctenophores (Moroz *et al.*, 2014) points to the importance of determining their importance for signaling in sponges. These findings themselves however can be fitted to the traditional or alternate views of neural evolution discussed above.

2.5 The Sponge Enigma in a Paleoecological Context

Nervous and sensory tissues are metabolically expensive to maintain (Laughlin, 2001). The ecological context in which early animals evolved must therefore have produced strong selective pressures favoring emergence of these tissues. What type of world greeted early animals?

The Proterozoic was characterized by changing oceanic chemistry driven by gradual oxygenation of the seas. But, it is thought that only some regions were hospitable to animal life. In fact, the deep ocean water appears to have remained fully anoxic as late as 580 million years ago (Canfield *et al.*, 2007). It is possible that deep waters were also rich in sulfer making these waters non-ideal for the flourishing of early animals (Gaidos, 2011). Early animals would therefore have been restricted to oxygenated areas and limited by sulfidic or anoxic waters. Gaidos (2011) notes that this scenario makes specific predictions about the type of life likely present. He suggests life "adapted to brackish or freshwater, and tolerant of large fluctuations in oxygen concentration brought about by diurnal cycles of primary production and respiration by phototrophs, variable input of freshwater, and tidal- or storm-driven surges of sulfidic seawater." He further notes that

metazoans capable of feeding on bacteria could thrive in such environments. Sponges fit this kind of description – environmentally hardy, capable of surviving on limited resources, tolerant of low O_2 and bacteria consuming. The uncontested fossil record of sponges reaches back to at least the Cambrian period and shows both their presence and early diversification and fits the environmental pictures we have from this early period (Pisera, 2006) (pre-Cambrian sponge fossils may also exist, Maloof *et al.*, 2010; Lowe *et al.*, 2009). During the Cambrian period continued change in oceanic chemistry made the seas more favorable to early animals enabling exploration of new environments.

The restriction of early animals to a small geographical region makes sense of the seemingly prevalent role horizontal gene (and protein motif) transfer (HGT) played in the evolution of animals (Aravind *et al.*, 2003). For example, choanoflagellates, the sister group to metazoans appear to have acquired about 1000 genes through HGT, some from bacteria, other from algae. Some of these genes are also found in metazoans suggesting HGT events in the ancestor of both groups (Tucker, 2013). One could speculate that in the context of neural evolution, HGT from prokaryotes may be the source of a considerable number of enzymes used to synthesize neurotransmitters. These include Glutamate decarboxylase, Phenylalanine hydroxylase and a Nitric oxide synthase domain (Lakshminarayan *et al.*, 2004). Thus, the restrictive environments in which early animals thrived may have promoted HGT that in turn served to increase genetic diversity and latent potential that under different environmental circumstances enabled significant evolutionary change in animals.

As changes in the oceans enabled organisms to extend beyond secluded estuaries, exploring and adapting to novel environments, the need to hunt for prey would be a direct consequence. In contrast to a bacteria-consuming sponge that generates a constant feeding current, the opportunity to feed on larger prey swimming in the water column would necessitate systems to identify, track and capture prey. Searching out new niches would also be aided by more complex

sensory-motor systems. Sensory, nervous and muscle tissues would therefore come under strong selective pressures. Some intriguing work from the Cambrian era highlights the important changes during the period. During the period leading up to, and including the Cambrian explosion a marked increase in defensive morphological diversity is observed, a feature thought to be driven by predation (Butterfield, 2011). This suggests an "evolutionary arms race" was a major driving force for evolutionary innovation during the period.

The origin of carnivory itself appears to date to the Proterozoic-Cambrian transition. A recent study of carnivory among polychaetes in oxygen minimum zones revealed a strong correlation between oxygen levels and the presence of carnivory (Sperling *et al.*, 2013). This observation makes sense in terms of physiology since the anatomical (nerve, muscle, digestive system) and metabolic needs (ATP) for a hunter *may* exceed those of a suspension feeder. In neoproterzoic seas low oxygen levels, among other factors, constrained both animal size and niche. However rising sea oxygen levels opened up novel ecological conditions creating new niches, predation and consequently novel selective pressures. In addition to these factors, there is now evidence that rates of evolution were accelerated in the Cambrian period relative to later periods (Lee *et al.*, 2013). But, if rates of molecular and morphological evolution were elevated enabling novel structures to be built (including the beginnings of complex nervous systems) why did sponges, which have so many neural components not build neural tissues?

To ensure sufficient food capture a sponge regulates its feeding current by sensing of flow rates. Recent evidence suggests this is done through primary cilia in the canal system and osculum (Ludeman *et al*., 2014). Furthermore, the sponge must eliminate any clogging of the canal system, an ability that would be important for an animal living in a shallow, brackish environment. This function is carried out through contraction of the sponge body, in both cellular and syncytial sponges (Leys and Hill, 2012 for review).

Like hunters, sponges must be able to sense their environment, integrate external information, and generate a response. However, unlike hunters, *the speed with which these processes must occur can be slow*. They have no need to actively hunt prey. Acquisition of complex, metabolically expensive neural tissues would not be evolutionarily advantageous for sponges. Rather, they used 'neural' components and signaling pathways needed for their physiology but experienced no selected pressures favoring further development.

2.6 Non-neural Roles for 'Neural' Genes

It should be noted that finding of 'neural' genes in aneural organisms is not new. Acetylcholine, dopamine, norepinephrine, serotonin and histamine have all been identified in bacteria, fungi and plants. Roles played by these molecules in bacteria include regulation of growth and motility (acetylcholine), while in plants they regulate growth and development (reviewed in Roshchina, 2010). Many major enzymes required to synthesize classical neurotransmitters are also found in bacteria (Lakshminarayan *et al.*, 2004). Neurotransmitter homologue GrlE, a G-protein coupled receptor showing sequence similarity to both the metabotropic glutamate receptor and GABA-B receptor, has been described in *Dictyostelium discoideum*. This receptor plays a role in the suppression of cell growth among the amoeba (Fountain, 2010). Additional proteins associated with synaptic structure are also found in the protest *Monosiga brevicollis;* these include SRC kinase, RAF kinase, Calpain, Spectrin, Dlg, and Shank (Alie and Manuel, 2010). These findings highlight an important point: The presence of neurotransmitters, or receptor molecules should not lead us to immediately speak of a 'proto-neural' structure or pathway without having some additional justification for doing so. Like sponges, other organisms utilize 'neural' proteins and molecules to address their physiological needs. However, in no way does this make them 'pre-' or 'proto-neural.'

2.7 Neural Evolution: On Hypotheses, Old and New

Historically, models for neural evolution were monophyletic (*i.e.*, single origin) due to their parsimonious nature. Recently polyphyletic (*i.e.*, multiple origin) models have reemerged. Yet the monophyletic models remain attractive in their simplicity: descent with modification from an ancestor in which a primitive neural system arose. But the anatomy, physiology and especially molecular biology of metazoan nervous systems present challenges to simplistic hypotheses. Leonid Moroz has recently observed that "multiple origins better explains the extant diversity of nervous systems and enormous plasticity in establishment of complex cell phenotypes, development and differentiation programs, transdifferentiation, and redundancy of molecular components in signal transduction pathways (Moroz, 2009, p177-8)." In other words, it is not only behavioral or structural features of nervous systems that result from convergence (Nishikawa, 2002), but nervous systems themselves.

Common to all hypotheses, recent or older, is the view that "protoneurons" arise out of multicellularity and acquire the capacity to transduce an external stimulus into an electrical or chemical signal (Lichtneckert and Reichert, 2007). In 1872 Nicolaus Kleinenberg proposed epithelia-muscular cells in *Hydra* may have been an early neural-like cell and precursor to a neuromuscular system (Kleinenberg, 1872). This idea came from his observation that these polarized cells possessed both sensory and motor properties. This observation suggested a latent potential with both sensory and motor capacities in one cell. Similarly, the study of medusas led Oscar and Richard Hertwig to hypothesize that elementary cell types (*e.g.*, affectors and effectors) evolved from epithelial-type cells and assembled into a primitive neural system (Hertwig & Hertwig, 1878).

Parker (1919) proposed that effectors, cells giving the organism the capacity to respond to a stimulus, could be seen in basal animals like sponges. Affectors, that give an organism the capacity to sense its environment, were discernable in later evolving cnidarians and ctenophores. These less primitive organisms were able to

assemble affector-effector circuits with these cell types. Such circuits increased in complexity in higher animals. Thus, Parker's model proposed a nervous system that steadily increased in complexity and functionality over time.

Subsequent hypotheses began to take seriously the capacity of nervous systems to conduct electrical impulses, since it is the integration of electro-chemical signaling that distinguishes neural communication. The major hypothesis of this type was advanced by George Mackie when he suggested a primitive nervous system may have emerged from myoepithelial cells joined to each other electrically through cell-cell junctions in a tissue that had properties of both affectors and effectors and was capable of contracting (Mackie, 1970). From this primal myoepithelial cell sheet, a small population of cells separated and moved into the interior of the animal. Some cells in the myoepithelial sheet later evolved into 'protoneurons' capable of transmitting signals from the external myoepithelial sheet to the interior muscle-like cells. From this primitive, electrically coupled neural circuit chemical synapses may have evolved establishing the electrochemical neural circuit (Mackie, 1970).

In contrast to a single origin model, several lines of evidence consistent with multiple origins of nerves and/or nervous systems have been described (Moroz, 2009). For example, distinct neurological architecture and unique development (*e.g.*, the specification of neurons in *Nematostella vectensis* is controlled through different genetic pathways than in the cnidarian *Hydra*) is consistent with multiple independent origins (Marlow et al., 2009). Other lines of evidence include absence of Hox genes in neural specification in hemichordates and ctenophores, in sharp contrast to other metazoans (Lowe *et al.*, 2003; Pang & Martindale, 2008). PRD class genes are expressed in neurons in ctenophores but not in the neurons of bilaterians (Pang & Martindale, 2008). Neurons in cnidarians derive from the endoderm while in echinoderms nerves have both mesodermal and endodermal origins rather than an ectodermal origin (Westfall & Elliott, 2002). Different anatomical, genetic and developmental pathways in neural specification in animals today can be interpreted

as evidence of independent neural origins. The recent demonstration of neuropeptide signaling being the major mechanism for communication in the ctenophore *Pleurobrachia bachei* adds further, compelling evidence to the independent origins hypothesis (Moroz *et al.*, 2014).

Is the origin of the nervous system mono- or polyphyletic? This remains an open question, and recent developments in ctenophore genomics make this hypothesis all the more intriguing. However, in surveying the major ideas concerning nervous system evolution I note the importance of coupling both the histological and morphological features of animals along with the molecular evidence. Therefore, the building and testing of credible hypotheses needs to keep the whole animal, its environment and its history firmly in mind, especially now that much research and thinking is heavily influenced by genomics. In light of all this, how best might we think about sponges and their neural molecules? Do they possess an'almost' nervous system, remnants of a lost one, or should we think about this whole question in a less rigid, and conventional way?

2.8 A Different Kind of Integrative System

While there is simply no evidence of neural tissue in sponges, there is nevertheless a large 'neural toolkit'. Sponges sense and respond to both external and internal stimuli and have a significant complement of 'neural' molecules that may be described as an *unconventional neural-like sensory-motor system*. That is to say, sponges sense the environment, generate behaviors in response and do so using molecules associated with neural tissue. Yet, because sponges have no nerves their system is indeed unconventional and does not truly fit into any current or historical model of neural evolution. Sponges are not "on the verge" of acquiring neurons or a nervous system nor do they reflect some primitive stage of neural evolution frozen in time. Rather they employ precisely the neural components they require for the environments and challenges they face—no more, no less.

Historically, hypotheses on neural origins had increasingly focused on the need to couple electrical and chemical signaling. This makes intuitive sense – electrical signaling brings speed, while chemical signaling brings greater diversity and control (*via* signaling molecule, receptor and downstream effector diversity, and combinatorial signaling). The view of neural origins also aligns with ecological changes that faced early animals as they explored new niches, hunted, and avoided predation themselves. Nonetheless, I hypothesize that because the sponge has no special need for speed there developed a predominantly chemical signaling system, with some signal diversity that shares considerable similarity with the chemical signaling we see in the nervous system of other metazoans of common ancestry. However, the coupling of an electrical system into this chemical system was never subject to significant evolutionary pressures, as would be the case with an organism whose survival was dependent upon hunting for food in the water column. Nevertheless, as the glass sponge illustrates, the capacity for electrical signaling in at least some sponges is present should it become evolutionarily advantageous. Yet, since the ionic basis for the glass sponge action potential differs from that of conventional nerves, we are dealing with a unique adaptation in these animals rather than a remnant from a lost system.

What differentiates the sponge's system from the nervous system of other animals is the lack of integration of the chemical and electrical systems. Since morphology is driven by function (*i.e.*, directional signaling in neural circuits), this too explains why neural morphology is not present in sponges. Further insight may be provided by experimental investigation of the function of 'neural' components and signaling partners in sponges versus animals with more developed nervous systems. If the components are in fact being used in decidedly different ways in sponges then this *could* suggest an independent origin. However, if they function in similar ways, supporting effector and affector systems, then such observations would suggest sponges have simply utilized an ancestral set of genes in a unique, non-neural way.

Regardless of whether the nervous system arose once in a common ancestor to 'neural' animals, or has arisen multiple times independently, the key question remains, "What drives the formation of a complex electrochemical signaling system?" Most likely the integration of chemical and electrical systems was selected for because it gives the capacity for rapid acquisition and processing of information with the associated response generation. In the context of early animal evolution where active hunting and novel niche exploration were important for reproductive success (*e.g.*, during the Cambrian), the selective pressure on the emergence of neural systems was very likely strongly positive. This also goes a long way towards helping us understand why sponges never evolved a conventional nervous system.

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Chapter Three

***Riesgo, A., *Farrar, N., *Windsor, P.J. *et al.* (2014). The analysis of eight transcriptomes from all Porifera classes reveals surprising genetic complexity in sponges. *Mol Biol Evol*. 31: 1102-1120 (*, co-first authors)**

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3.1 Abstract

Sponges (Porifera) are among the earliest evolving metazoans. Their filter-feeding body plan based on choanocyte chambers organized into a complex aquiferous system is so unique among metazoans that it either reflects an early divergence from other animals prior to the evolution of features such as muscles and nerves, or that sponges lost these characters. Analyses of the *Amphimedon* and *Oscarella* genomes support this view of uniqueness – many key metazoan genes are absent in these sponges – but whether this is generally true of other sponges remains unknown. We studied the transcriptomes of eight sponge species in four classes (Hexactinellida, Demospongiae, Homoscleromorpha and Calcarea) specifically seeking genes and pathways considered to be involved in animal complexity. For reference, we also sought these genes in transcriptomes and genomes of three unicellular opisthokonts and two bilaterian taxa. Our analyses showed that all sponge classes share an unexpectedly large complement of genes with other metazoans. Interestingly, hexactinellid, calcareous and homoscleromorph sponges share more genes with bilaterians than with non-bilaterian metazoans. We were surprised to find representatives of most molecules involved in cell-cell communication, signaling, complex epithelia, immune recognition and germ-lineage/sex, with only a few, but potentially key, absences. A noteworthy finding was that some important genes were absent from all demosponges (transcriptomes and the *Amphimedon* genome), which might reflect divergence from main-stem lineages including hexactinellids, calcareous sponges, and homoscleromorphs. Our results suggest that genetic complexity arose early in evolution as shown by the presence of these genes in most of the animal lineages, which suggests sponges either possess cryptic physiological and morphological complexity and/or have lost ancestral cell types or physiological processes.

3.2 Introduction

Despite a plethora of genomic data now available current metazoan phylogeny is still in flux, especially with respect to the basal-most branching phyla Porifera, Ctenophora, Cnidaria and Placozoa. Their branching order is however, fundamental for understanding the early evolution of animal features such as tissues and epithelia, nerves and coordination, immune recognition, and propagation of the germ lineage. Traditional markers such as 18S rRNA tend to place sponges as the sister lineage to the rest of metazoans (*e.g.*, Medina *et al*. 2001; Zrzavý *et al*. 2005), and while recent hypotheses using transcriptomic and genomic data from ctenophores have challenged this view (*e.g.,* Dunn *et al*. 2008; Hejnol *et al.* 2009; Ryan *et al.* 2013), the outcome depends on the type of model and parameters used in analysis of these data sets. Other analyses of the same data either confirm that sponges are the sister lineage to the rest of animals (Pick *et al.* 2010; Philippe *et al.* 2011), or fail to resolve this dichotomy (Nosenko *et al.* 2013).

It is commonly considered that morphological complexity in animals is acquired over evolutionary time (McShea 1996). Sponges are morphologically simple in comparison to ctenophores, which possess complex structures such as gonads, nerves and muscles, structures that are not known at all in sponges. The absence of certain homeodomains in both ctenophores and sponges led Ryan *et al*. (2010) to suggest an early branching of ctenophores and sponges prior to placozoans, cnidarians, and bilaterians. In this way perhaps complex structures may have appeared in two branches of early evolving animals almost simultaneously, one in ctenophores and the other giving rise to the rest of animals. Alternatively, sponges might be derived, and having specialized for a filter-feeding lifestyle, become morphologically simplified by losing ancestral cell types. Therefore, the molecular basis to create complex structures might be still present in sponges even though in structure they appear simple.

While sponges might appear morphologically 'simple', analysis of two sponge genomes and transcriptomes, *Amphimedon queenslandica* (Class Demospongiae)

and *Oscarella carmela* (Class Homoscleromorpha) revealed a remarkable molecular complexity (Nichols *et al.* 2006, 2012; Srivastava *et al.* 2010; Conaco *et al.* 2012). The morphology of *Amphimedon queenslandica* corresponds perfectly well to the textbook view of a sponge: a massive body with branching aquiferous canals lined by a single cell layer – the pinacoderm – and enclosing very few cell types many of which are pluripotent (Simpson, 1984). There are however, over 8,500 species of sponge currently recognized with an estimated 12-18,000 more to be described (Appeltans *et al.* 2012). These live in diverse habitats – from the abyssal deep sea to freshwater lakes and rivers – and have contended with changes caused by uptake of symbionts, infection, changing temperature, salinity and food abundance over at least 600 million years (Conway Morris *et al.* 2000; Jackson *et al.* 2007). Some are carnivorous (Vacelet and Boury-Esnault, 1995) and many different ways of forming a skeleton, from calcium carbonate, aragonite, to spongin and silica, exist (Simpson 1984; Jackson *et al.* 2007). Within the context of this extraordinary sponge diversity, the genomes and now transcriptomes of *Amphimedon* and *Oscarella* are informative, but by no means conclusive in terms of providing the absolute gene complement of sponges.

To provide a wider framework for understanding the molecular complexity of sponges, we sequenced the transcriptomes eight sponge species covering all four currently recognized poriferan classes (Fig.1): *Aphrocallistes vastus* (Class Hexactinellida), *Chondrilla nucula, Ircinia fasciculata, Petrosia ficiformis, Spongilla lacustris,* and *Pseudospongosorites suberitoides* (Class Demospongiae). *Sycon coactum* (Class Calcarea), and *Corticium candelabrum* (Class Homoscleromorpha). These species represent for the most part sponges that have been well-studied in other contexts (cell biology, ecology, physiology), and for which quality starting material could be obtained. We analyzed protein families and Gene Ontologies, and specifically screened each transcriptome for the presence of genes involved in signaling, neuronal and ionic conduction, epithelia, immunity, and reproduction.

3.3 Methods

3.3.1 Sample collection

We collected tissue samples from 8 sponge species, belonging to the four currently recognized classes (Fig. 1): *Aphrocallistes vastus* (Hexactinellida), *Sycon coactum* (Calcarea), *Ircinia fasciculata, Chondrilla nucula, Petrosia ficiformis, Spongilla lacustris, Pseudospongosorites suberitoides* (Demospongiae), and *Corticium candelabrum* (Homoscleromorpha). Collecting information is provided in Supplementary File 1. Hereafter we refer to each animal by its genus to ease readability.

3.3.2 Sample preparation

In order to avoid contamination from epibionts, prior to fixation tissues were cleaned carefully using a stereomicroscope. A piece of sponge tissue was removed with razor blades that were rinsed in RNAseZap® (Ambion, Texas, US). All procedures were carried out on ice and quickly to avoid RNA degeneration. Tissues were either flash-frozen in liquid nitrogen and stored at -80oC or they were immersed in at least 10 volumes of RNAlater® at 4oC for 1 hour, incubated overnight at -20 oC, and subsequently stored in the same buffer at -80oC until RNA was extracted (sometimes samples placed in RNAlater were transported back to the laboratory at room temperature, where they were stored at - 80oC). See Supplementary File 1 for details. The amount of tissue used depended on the extent of the spicule skeleton: in most cases 20 to 80 mg of tissue was used but for *Petrosia* and *Aphrocallistes,* 200 mg was needed due to the large silica skeleton (see Riesgo *et al.* 2012a).

3.3.3 mRNA extractions

Two different methods of RNA extraction were used: 1) total RNA extraction followed by mRNA purification for *Corticium* and 2) direct mRNA extraction for all other species. Protocols used for both extraction types are available elsewhere

(Riesgo *et al*. 2012a, b). Total RNA from *Aphrocallistes* was extracted using the Norgen Biotek Animal Tissue RNA Purification Kit (Norgen Biotek, Thorold, ON, Canada). Quantity and quality (purity and integrity) of mRNA were assessed by three different methods, reported in Riesgo *et al*. (2012a) and shown in Supplementary Table 1.

3.3.4 Next-Generation Sequencing

For all sponges except *Aphrocallistes,* next-generation sequencing was performed using Illumina GAII and HiSeq2000 (Illumina, Inc., San Diego, California, USA) platforms at the FAS Center for Systems Biology at Harvard University. mRNA concentrations between 20 and 79.9 ng/µL (Table 1) were used for cDNA synthesis with the TruSeq RNA sample preparation kit (Illumina, Inc.), as described previously (Riesgo *et al.* 2012a,b). cDNA was ligated to homemade adapters in one sample of *Petrosia* (5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GGT T-3'), whereas ds cDNA was ligated to Illumina adapters in the rest of species. Size-selected cDNA fragments of around 300 bp excised from a 2% agarose gel were amplified using Illumina PCR Primers for Paired-End reads (Illumina, Inc.) and 18 cycles of the PCR program 98 oC-30 s, 98 oC-10 s, 65 oC-30 s, 72 oC-30 s, followed by an extension step of 5 min at 72 oC.

The concentration of the cDNA libraries was measured with the QubiT® dsDNA High Sensitivity (HS) Assay Kit using the QubiT® Fluorometer (Invitrogen, Carlsbad, California, USA). The quality of the library and of size selection were checked using the "HS DNA assay" in a DNA chip for Agilent Bioanalyzer 2100 (Agilent Technologies, California, USA). cDNA libraries were considered successful when the final concentration was higher than 1 ng/µL and the Bioanalyzer profile was optimal (see Riesgo *et al*. 2012a). Successful libraries were brought to 10 nM or 7nM depending on the initial concentration prior to sequencing. The paired-end reads had lengths of 100 or 150 bp, depending on availability of sequencers (Illumina GAIIx or HiSeq).

The *Aphrocallistes* transcriptome was prepared by LC Sciences (http://www.lcsciences.com/) using 1 μg of total RNA for polyA tail selection of the mRNA (Supplementary File 1). Library preparation was performed using also the TruSeq RNA sample preparation kit (Illumina,, Inc.) following the manufacturer's instructions and the sequencing of a 9 nM library performed on HiSeq2000 with paired-end 100 nt reads also by LC Sciences (Texas).

3.3.5 Sequence assembly

Thinning and trimming for the raw reads was done with CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark). Thinning refers to discarding of nucleotides and/or entire reads based on quality parameters. It was performed using either 0.05 or 0.005 as the limit, based on *Phred* quality scores, and resulting quality of the thinned reads was visualized in FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). After thinning, only those terminal bases with a *Phred* quality score under 30 were trimmed (where a *Phred* score of 30 corresponds to a probability of 10-3 of incorrect base calling), producing sequences of unequal size. Reads were re-screened to check for presence of adapter or primer sequences using FastQC, and if present, adapters or primers were removed using with CLC Genomics Workbench 5.1.

De novo assemblies with all datasets thinned and/or trimmed were performed with CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark), or Trinity [http://trinityrnaseq.sourceforge.net/]. Global alignments for the *de novo* assemblies were used with the following parameters: mismatch cost=2; insertion cost=3; deletion cost=3; length fraction=0.5; similarity=0.8; and randomly assigning the non-specific matches. Best *k-mer* length was estimated by the software.

3.3.6 Sequence Annotation

For each species, two methods were used for annotation: a more global assignment of gene ontology using BLAST and a more specific assignment of domain by HMMer. For Blast, contigs shorter than 300 bp were removed, as very few of

these short contigs retrieved results for Gene Ontology assignments (see Riesgo *et al*. 2012b). The remaining contigs were independently mapped against three different selections of the non- redundant (nr) NCBI database (all Metazoan proteins in *nr,* all Bacterial proteins in *nr,* and all Protozoan proteins in *nr)* using the blastx program of the BLAST suite. All searches were conducted with BLAST+ (Atschul *et al.* 1990; Camacho *et al*. 2009) using an e-value cut-off of 1e-5. We used the output file from the blast against Metazoa which contained the best hits and Blast2GO v2.5.0 (Conesa *et al*. 2005) to retrieve the Gene Ontology (GO) terms and their parents associated with the top BLAST hit for each sequence. Searches for specific genes were carried out using HMMer hidden Markov models using Interproscan tools (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and either HMM profiles present in the PFAM Protein families database or HMMerbuilds generated specifically using sequences downloaded from NCBI.

To estimate the complexity of the complements of genes involved in different pathways, independently from the general BLAST results, we selected gene targets from conserved developmental signaling pathways, and genes associated with post-synaptic signaling, germ lineage and reproduction, adhesion, and innate immune regulation. We retrieved at least three different sequences of the selected protein targets from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) from a range of metazoan groups to use them for searches in our transcriptome datasets. These sequences were aligned using either T- COFFEE (Notredame *et al*. 2000), MAFFT (Katoh *et al*. 2005), or MUSCLE (Edgar 2004) depending on the level of conservation of the protein, and the alignments used to create HMM profiles for each protein of interest. HMMER searches were performed against all 8 transcriptomes, translated into all 6 reading frames. We selected only the hits with the maximum similarity (cutoff of 1e-05; which varied greatly between groups), and checked each open reading frame with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). A similar approach was performed using the software CLC Genomics Workbench 5.0, selecting three protein sequences from other metazoans and blasting them using the local BLAST suite plug-in with each contig list as the targeted database. Each

predicted protein sequence was reverse blasted against the database *nr* in NCBI using the blastp and DELTA-BLAST programs (http://blast.ncbi.nlm.nih.gov/) and the domain structure checked with SMART (http://smart.embl-heidelberg.de/) using HMMER, PFAM domain, and internal repeats searching. To avoid the bias in the detection of genes derived from the use of cnidarian or bilaterian protein queries, each time we found a target gene in the transcriptomes, we added the sequence to the list of protein queries to improve the searches.

We also confirmed the presence/absence of the same set of conserved developmental signaling pathways, and neuronal signaling, germ lineage and reproduction, adhesion, and innate immune regulation in three different unicellular eukaryote species (Capsaspora *owzarczaki, Monosiga brevicollis,* and *Salpingoeca rosetta)* to determine whether the appearance of the genes in sponges was a novel acquisition or a feature shared with these unicellular organisms. We used the same gene targets and searched in the genomes with the blastp engine implemented in the Broad institute website using default setttings (http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html). We also used searched the *Amphimedon queenslandica* genome and *Oscarella carmela* draft genome (Nichols *et al.* 2006, 2012; Srivastava *et al*. 2010) to confirm the presence/absence of each genes at the genomic level.

We performed 3D reconstructions of the translated sequences of the targeted genes using PHYRE2 for protein fold recognition (Kelley and Sternberg, 2009).

Sequences obtained in this study are available in Supplementary File 5 and also deposited under the Bioproject accession numbers in Genbank: *Aphrocallistes vastus* (PRJNA225584), *Ircinia fasciculata* (PRJNA225586), *Chondrilla nucula* (PRJNA225590), *Petrosia ficiformis* (PRJNA162901), *Spongilla lacustris* (PRJNA225591), *Pseudospongosorites suberitoides* (PRJNA225580), *Sycon coactum* (PRJNA162899), and *Corticium candelabrum* (PRJNA162903).

3.3.7 Phylogenetic analyses

For each of the selected genes, independent protein alignments were built using MUSCLE implemented in SEAVIEW 4.3.0 (Gouy *et al*. 2010) and MAFFT (Katoh *et al*. 2002) with default parameters. For the maximum likelihood phylogenetic analysis of the protein sequences we used RAxML (Stamatakis 2006) with the LG model and an estimated gamma shape parameter and 500 independent searches. Nodal support was estimated via the rapid bootstrap algorithm (1000 replicates) using the WAG-CAT model (Stamatakis *et al*. 2008). Bootstrap resampling frequencies were then mapped onto the optimal tree from the independent searches.

3.4 Results

3.4.1 Sequence assembly and annotation

The cDNA libraries rendered between 38,866,233 reads for *Pseudospongosorites* and 234,585,429 for *Spongilla* of which, between 67% in *Spongilla* and 87% in *Aphrocallistes* of the reads after the thinning process were used for the assemblies (Supplementary Table 1). The assembly of each species produced between 10 Mb and 65 Mb of assembled contigs in all species (Supplementary Table 1).

The average length of the contigs was close to 500 bp in all datasets (Supplementary Table 1), with the transcriptomes of *Aphrocallistes, Sycon, Petrosia* and *Chondrilla* showing the greatest N50 values (N50 is a weighted median statistic such that 50% of the entire assembly is contained in contigs equal to or larger than this value in bp) . The *Corticium* transcriptome had a large number of short contigs that resulted in low N50 values (Supplementary Table 1). The average coverage per contig was 190 reads for the transcriptomes of all species in the study (Supplementary Table 1).

Figure 3-1 A. Phylogenetic relationships in the phylum Porifera. The monophyly of sponges and their sister-group relationship to Placozoa and "Eumetazoa" are shown. "Eumetazoa" in this tree contains Cnidaria, Ctenophora, and Bilateria. **B.** Images of each sponge used in this study: *Aphrocallistes vastus, Spongilla lacustris, Petrosia ficiformis, Pseudospongosorites suberitoides, Ircinia fasciculata, Chondrilla nucula, Sycon coactum,* and *Corticium candelabrum.* (Image authors are provided in the acknowledgements). Colored names indicate their phylogenetic affiliation in Figure 1A. The species *Amphimedon queenslandica* and *Oscarella carmela,* used for gene comparisons, belong to Demopospongiae and Homoscleromorpha respectively.

Sponges host a great number of symbionts (mainly bacteria) within their tissues that are impossible to remove prior to cDNA construction. In order to assess the percentage of sequences that can be assigned to metazoans bacteria and/or protozoans, we compared the results of each independent BLAST analysis against separate databases containing metazoan, bacterial and protozoan proteins (Supplementary File 2A), and found that most contigs in all datasets returned hits from Metazoa. For the BLAST against the Bacteria database, *Corticium* had the highest number of contigs with hits not found in Metazoa and Protozoa (Supplementary File 2A). Only *Sycon* showed unique hits against Protozoa, while the other species produced both protozoan and metazoan hits (Supplementary File 2A).

Among those sequences that blasted to metazoans (more than 60% of the contigs assembled for *Aphrocallistes;* between 40-50% in the demosponges; and around 20-30% in *Sycon* and *Corticium),* most hits were assigned to sponges (mostly *Amphimedon)* for the demosponges, and to bilaterians in the case of *Aphrocallistes, Sycon,* and *Corticium* (see Supplementary File 2B).

Regardless of the potential different physiological states of the sponges when collected, the percentage of sequences with assigned gene ontology (GO) terms was similar for the ontology categories 'biological function', 'molecular function', and 'cellular component' for all datasets (Supplementary File 2C), allowing comparisons at that level. It is important to note however, that the total number of GO terms retrieved for each dataset was very different, with 32,604 in *Corticium* and only 6,501 in *Petrosia.* For all datasets, in the GO category 'biological process', the primary metabolic process was the most abundant term, in the 'molecular function' category, catalytic activity was most common, and in 'cellular component', macromolecular complex was most abundant.

3.4.2 Protein families in Porifera

We analyzed the number of protein families (Pfams) in each sponge dataset and found the highest number of Pfams in *Corticium* (50,798) and the lowest in

Pseudospongosorites (10,137). The number of Pfams in *Corticium* could be either due to the high number of symbionts reported in the transcriptomic dataset (Supplementary File 2D-E) or an enrichment of certain domains in this particular species. We found very similar abundances for all protein families in *Aphrocallistes, Chondrilla, Ircinia, Spongilla, Petrosia,* and *Pseudospongosorites,* and a different profile in *Sycon* and *Corticium* (Supplementary File 2D-E). We obtained the functions for all the most abundant protein families and grouped them under the following categories: 'signaling', 'cell adhesion', 'immune system and metabolism' and 'structural/cytoskeletal', and those which showed more than one main function were grouped under 'multiple functions' (Supplementary File 2D-E). Again, in most cases, the number of protein families was higher in *Corticium* and *Sycon* than in the other species. The families showing the larger differences in the 'cell adhesion' category were I-set and Laminin_EGF, where both *Sycon* and *Corticium* had higher values. For example, the family MAM was only in *Sycon* and *Corticium.* For the category 'metabolism and differentiation', *Corticium* had a much larger complement of protein kinases (Pkinase) and sulfatases.

3.4.3 Targeted Gene Study

We used the transcriptome datasets to search for specific genes in pathways related to the acquisition of morphological complexity in metazoans. Absences of genes in any of the species studied here should be interpreted with caution given that transcriptomes convey expressed transcripts. To confirm the presence/absence of all the genes at the genomic level, we carried out the same surveys of the *Amphimedon queenslandica* genome and *Oscarella carmela* draft genome (Nichols *et al.* 2006, 2012; Srivastava *et al*. 2010)

3.4.4 Developmental Toolkit Genes

Developmental signaling pathways have been considered a hallmark of metazoan complexity. While most components of the major metazoan signaling pathways are present in sponges, some key absences have been noted (Nichols *et al*.

2006). We specifically examined the Hedgehog, Wnt, TGF-β and Notch-Delta pathways. Most components of the Hedgehog (Hh) signaling pathway were present in all sponge classes (Fig. 2A). Since Hh proteins are composed of an N-terminal signaling domain and a C- terminal Hint domain, similarity to Hh may have simply reflected the presence of one of these domains. We therefore included one row for sequences containing the hedge domain and a row for Hint domains. We found no instances in which both a hedge and a hint domain were in the same sequence, and no true hedgehog proteins were found in any sponge. Lastly, Hedgling (Hling) proteins were identified by the whole or partial presence of the domain architecture identified in Adamska *et al*. (2007b), as well as simple blast similarity. Importantly, in *Aphrocallistes, Corticium* and *Sycon* we also found *smoothened,* a component of the Hedgehog pathway that was thought to be absent from sponges (Nichols et al. 2006; Adamska *et al.* 2007b; Srivastava *et al.* 2010; Ingham *et al.* 2011). Phylogenetic analysis indicates that the sponge sequences lie within the smoothened family, and do not cluster together with closely related *frizzled* genes, and predicted 3D structure supports this finding (Fig. 2B, C).

Wnts and other Wnt pathway components identified in *Amphimedon queenslandica* (Adamska *et al*. 2007a, 2010), *Suberites domuncula* (Adell *et al*. 2003, 2007), *Lubomirskia baikalensis* (Harcet *et al*. 2010), *Ephydatia muelleri* (Windsor and Leys 2010) and *Oscarella carmela* (Nichols *et al*. 2006; Lapebie *et al*. 2009) were also found in our transcriptomes (Fig. 2). Of note is the possible absence of *Wnt* in *Aphrocallistes,* despite the presence of other *Wnt* signaling components in that sponge.

Our findings confirm and expand the presence of TGF signaling components in all four sponge classes. We found TGF family ligands as well as TGF family receptors and/or activin receptors in all 8 sponge transcriptomes. Whereas the TGF-β ligand antagonist *noggin* and the downstream effectors *SMADs* were found in all species, *nodal* was not identified in any of the transcriptomes (Fig. 2).

Homologs of *notch* and *delta* were found in all four sponge classes (Fig. 2) as

had been reported previously for *Oscarella carmela* and *Amphimedon queenslandica* (Nichols *et al*. 2006; Richards and Degnan 2012). Some of the sponge sequences showed characteristics aligning them more closely with *jagged* than *delta,* but phylogenetic analysis confirmed that they cluster with *delta* of other sponges (not shown).

3.4.5 Neuronal signaling: Post Synaptic Densities (PSD) and neurotransmission

Genes associated with postsynaptic densities and signaling via neurotransmitters are shown in Figure 3. Perhaps unsurprisingly we found the same general set of genes in all 8 transcriptomes as shown previously for *Amphimedon queenslandica* (Sakarya *et al*. 2007). Several genes however, were not found in some sponges, and importantly we were unable to identify some genes previously described from *Amphimedon* in any of the 8 transcriptomes. For example, *citron* was not found in *Pseudospongosorites* and *cortactin* was not found in *Petrosia* or *Ircinia,* while *homer* was absent from *Petrosia.* Given the presence of these genes in most of the demosponges we studied, it is likely that the variability reflects the fact that these are transcriptomes (only expressed genes are detected) rather than genomes (where all genes are detected).

Our findings are also broadly consistent with gene absences reported from the *Amphimedon* genome (Sakarya *et al*. 2007; in supplementary material), although in contrast to Alié and Manuel (2010) we did not find a true *Shaker-type K+* channel in any sponge nor could we find *neurolignin* or *stargazin* in any of the eight transcriptomes (as reported for *Amphimedon* by Sakaraya et al. 2007). We did find a sequence that blasted to a Kv subfamily-A type, which is characterized as 'shaker-like' in *Corticium,* and genes with sequence similarity to shaker-like Kv channel were also found in the three unicellular eukaryote genomes, but it does not necessarily imply Kv channels are present and further characterization is required.

One significant finding that differs from both previous reports on sponge PSD genes was the presence of an *ionotropic glutamate receptor* (iGluR) in *Corticium,*

Sycon, and *Iricinia.* The iGluRs present in *Corticium* and *Ircinia* appear AMPA-like possessing a Q/R site and all three sponges appear to possess most of the pore motif, SYTANLAAF. Phylogenetic analysis confirmed these channels group with other metazoan iGluRs (Fig. 3 and Supplementary File 3).

We attempted to identify core components of the catecholamine signaling pathway (adrenaline, noradrenaline, epinephrine, etc.; Fig. 3). Curiously, while we found pieces of the biosynthesis pathway in the transcriptomes we were unable to show the full pathway in any single sponge species, and some components were missing from all species. For example dopamine-β-hydroxylase, which catalyzes the reaction of dopamine to norepinephrine, was identified in all transcriptomes, yet *DOPA decarboxylase,* which produces dopamine from L-DOPA could only be identified in *Sycon* and *Corticium.* Furthermore, while gene prediction suggests that the *Amphimedon* genome encodes a *tyrosine hydroxylase* (GI:340369773) an enzyme that catalyzes the reaction of tyrosine to L-DOPA, this gene was not identified in any of the eight sponge transcriptomes studied here, yet *phenylalanine hydroxylase,* the gene that encodes for a protein that catalyzes the synthesis of tyrosine from phenylalanine was identified in all eight transcriptomes. Finally, adrenergic receptors were identified, but subtypes including a *dopamine receptor* were not. Therefore, while components of the catecholamine signaling pathway are present among the four classes of sponges, a more complete picture of this pathway could not be constructed even from data from all of the new transcriptomes. Nevertheless, we did find *glutamate decarboxylase* (which carries out synthesis of GABA from glutamate), which supports previous reports that show both glutamate and GABA are physiologically active in demosponges (Elliott and Leys 2010).

3.4.6 Adhesion and epithelia

Genes involved in maintenance of epithelial polarity, in adhesion to other cells and to a basal matrix, and genes involved in secretion of a basement membrane have previously been considered indicative of evidence of tissue-level

Figure 3-2 Signaling molecules identified in Porifera. **A.** Presence of the genes belonging to the signaling pathways for Hedgehog, Wnt, TGF-β, and Notch-Delta. **B.** Evolutionary relationships of sponge Smoothened proteins determined with Maximum Likelihood analysis. Bootstrap support is shown for nodes greater than 50% of 500 pseudoreplicates. Accession numbers of sequences used to construct the phylogenetic tree are available in Supplementary Table 4. **C.** Three-dimensional reconstructions of the Smoothened proteins in two distant sponges (Aphrocallistes *vastus* and *Sycon coactum)* and the vertebrate *Xenopus laevis.*

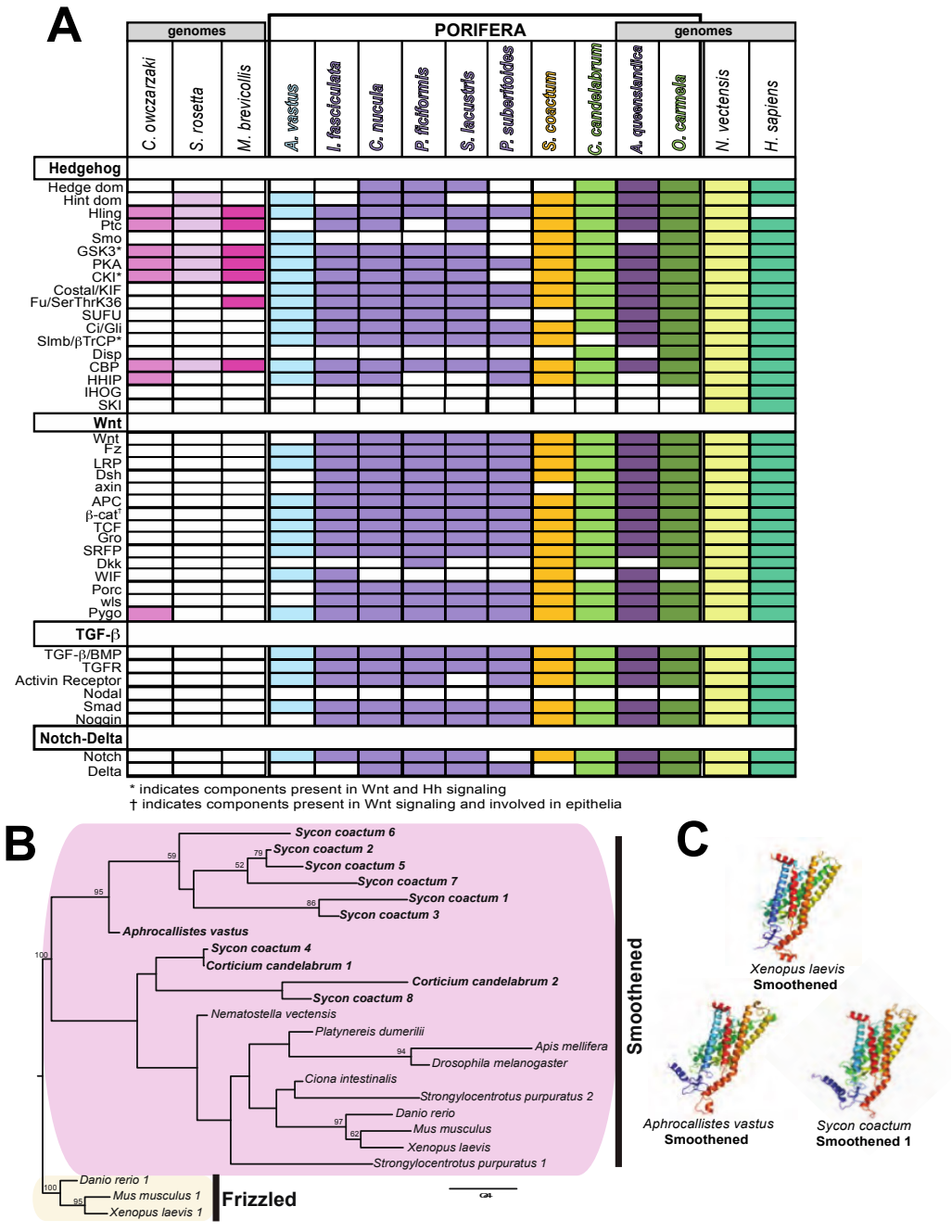
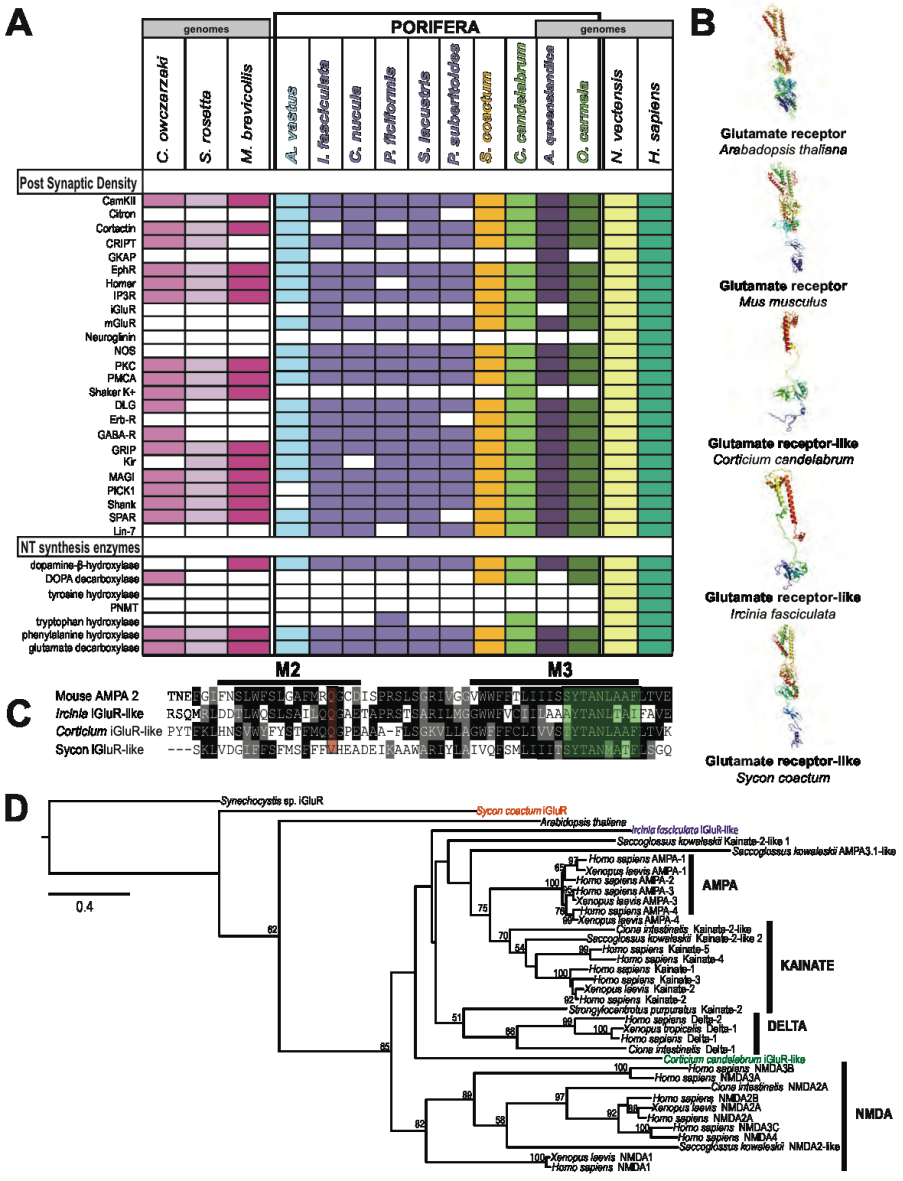


Figure 3-3 Post-synaptic density molecules identified in Porifera. **A.** Presence of genes comprising the PSD and those involved in synaptic neurotransmission in metazoans. **B.** Three-dimensional reconstructions of the ionotropic glutamate receptors in the plant *Arabidopsis thaliana,* the vertebrate *Mus musculus,* and three sponges, *Ircinia fasciculata, Corticium candelabrum,* and *Sycon coactum.* **C.** Alignment of the ionotropic glutamate receptor proteins from the mouse (Mus *musculus;* AMPA type) and the three sponges *Ircinia fasciculata, Corticium candelabrum,* and *Sycon coactum.* **D.** Evolutionary relationships between ionotropic glutamate receptors found in bacteria, plants, and metazoans determined with Maximum Likelihood analysis. Bootstrap support is shown for nodes greater than 50% of 500 pseudoreplicates. Accession numbers of sequences used to construct the phylogenetic tree are available in Supplementary Table 4. Colored sequences are matched to the sponges in Figure 1A.

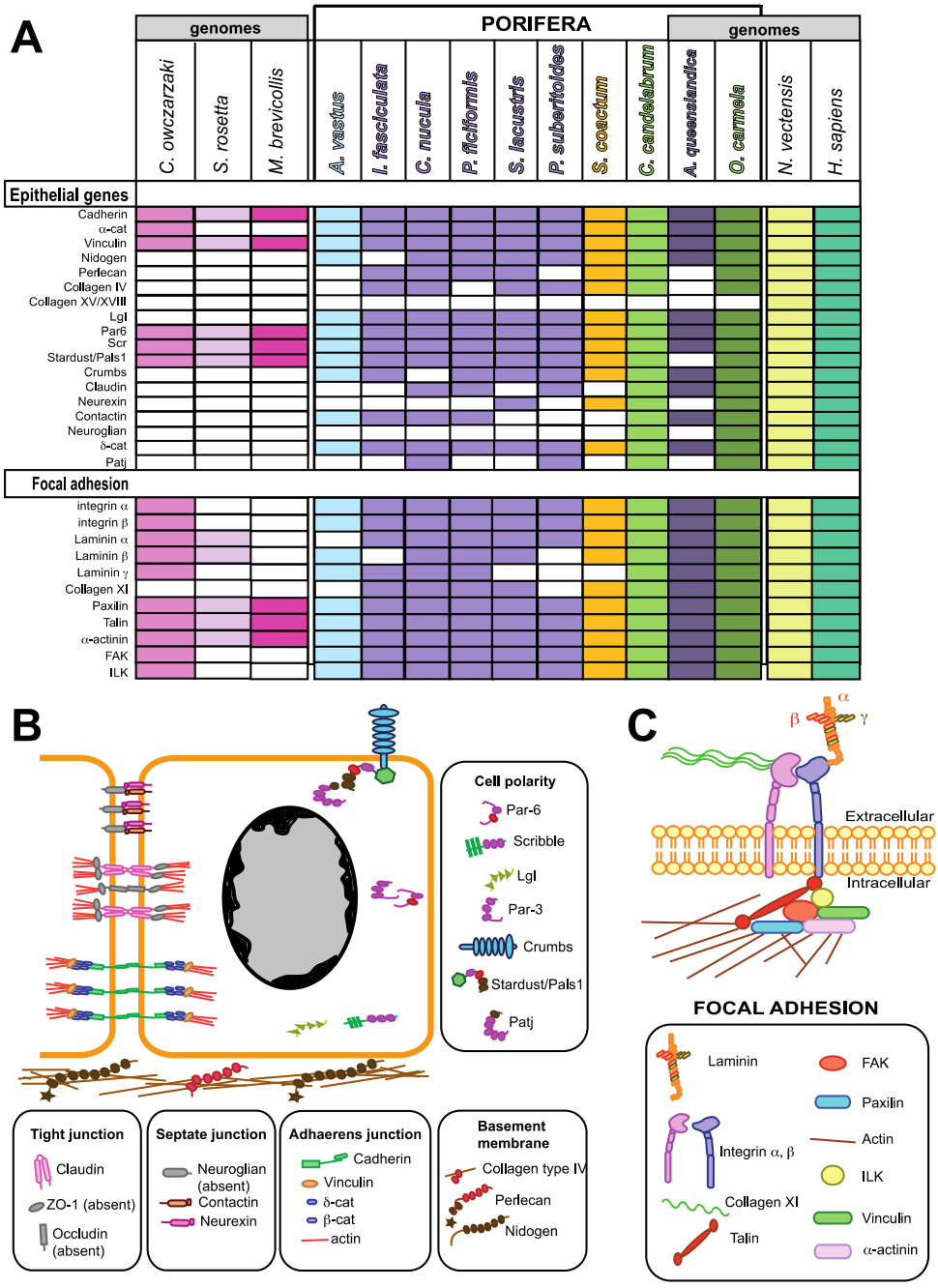


differentiation in some sponges and not others. We found this an ideal opportunity to survey the transcriptomes for the same genes studied previously (Nichols *et al*. 2007; Fahey and Degnan 2010). Our findings are summarized in Figure 5. Unsurprisingly, we found most of the polarity genes *Par3, Par6, Lgl, scribble,* and *disks large* (Fig. 4). We had difficulty identifying strict homologs of *Patj,* previously identified in *Amphimedon* (Fahey and Degnan 2010), but in contrast to previous work, we found good evidence for *stardust/Pals,* and the ligand of *stardust, crumbs,* in all sponge transcriptomes. In terms of adhesion, we found protocadherin in all sponges, and the components typically associated with *cadherin* adhesion (b-catenin, *alpha-catenin, p120 catenin,* and *vinculin)* were all present. What was unusual in comparison with earlier surveys of the *Amphimedon* genome was the presence of homologs of *claudin* in the three demosponges (Spongilla, *Pseudospongosorites,* and *Chondrilla)* as well as in the homoscleromorph *Corticium* and in the calcareous sponge *Sycon* (Fig. 4).

Also in contrast to previous work, we found homologs of important components of basement membrane genes, including *type IV collagen* in *Spongilla, Ircinia, Chondrilla, Sycon and Corticium* (the latter two were shown previously by Leys and Riesgo 2011). *Perlecan* and *nidogen* – molecules that connect the cell membrane to the protein type IV collagen – were also found in all except two of the demosponge transcriptomes (nidogen was not identified in *Ircinia,* and *perlecan* was not found in *Pseudospongosorites).* The genes for Laminins, which play a fundamental role in basement membrane assemblage as well as focal adhesion to the extracellular matrix, are composed of three non-identical chains, alpha, beta, and gamma, whose specific functions depend on the tissue in which they are present (Fig. 4). The three chains (alpha, beta, and gamma) were only found in *Corticium* and *Chondrilla* (Fig. 4), whereas in the other sponge transcriptomes we found only two of the chains, or just one in the case of *Pseudospongosorites* (Fig. 4).

Adhesion of cells to the surrounding extracellular matrix together with their stimulation by growth factors are key features that help cells to survive, proliferate,

Figure 3-4 Cell adhesion, focal adhesion and epithelial development molecules identified in Porifera. **A.** Presence of the genes involved in cell adhesion, epithelia formation, and focal adhesion. **B.** Schematic depicting the cell adhesion, polarity signaling, and basement membrane proteins found in metazoans. **C.** Schematic depicting focal adhesion molecules found in the cell membranes of a metazoan.

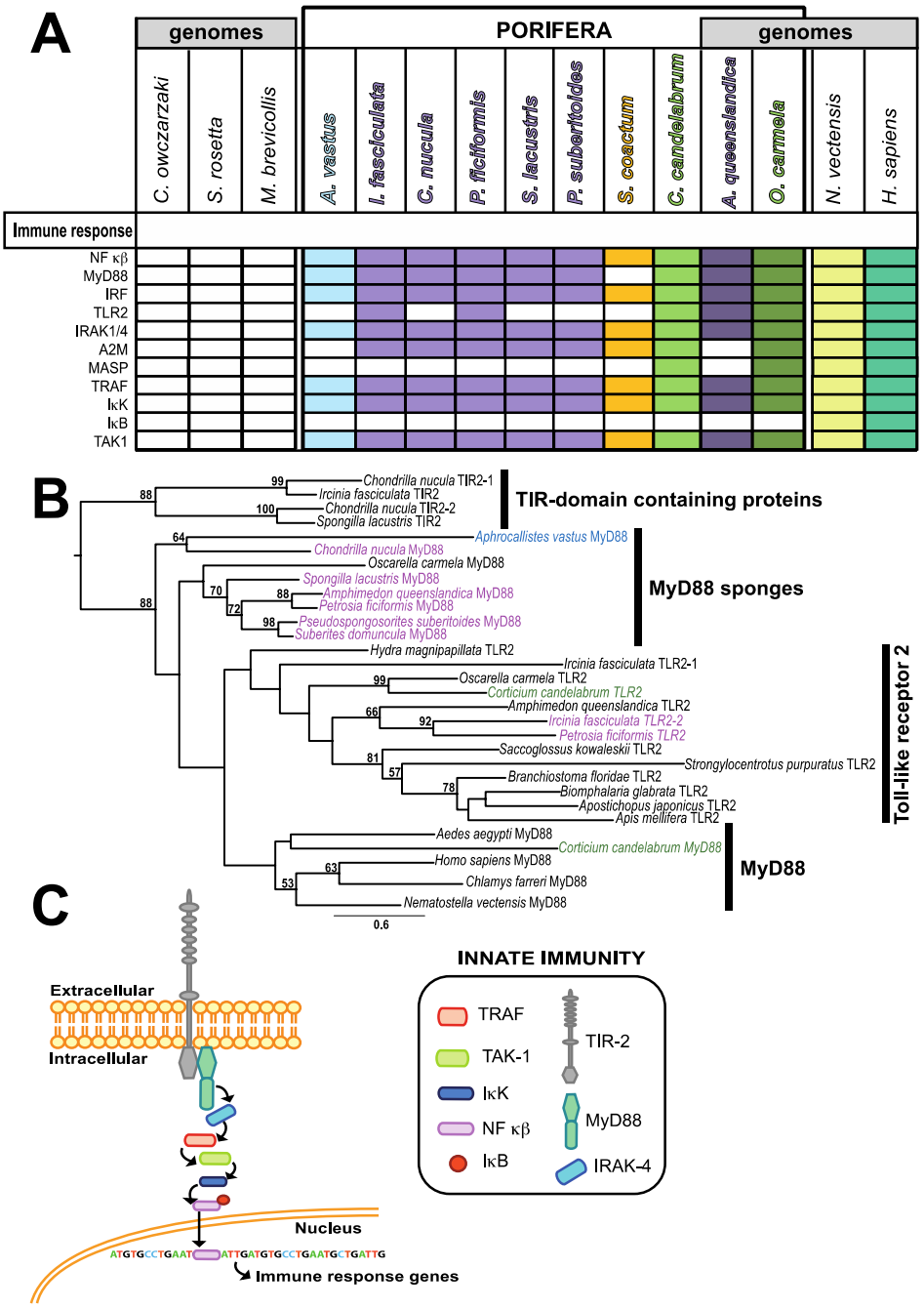


differentiate, or migrate in all animals (Turner 2000; Labouesse and Georges-Labouesse 2003). Cell adhesion is enabled via transmembrane *integrins* and their coupling with extracellular components such as *collagen* and *laminins* as well as their anchoring to *actin* through several protein components such as *focal adhesion kinase, paxillin, talin, integrin- linked kinase,* and *vinculin.* We found all basic components of this mechanism (focal *adhesion kinase, paxilin, talin, integrin alpha and beta, filamin, alpha-actinin,* and *vinculin)* in all eight transcriptomes (Fig. 4). In addition, we found the fibrillar *collagen XI,* known for giving support to connective tissues in mammals, in all species except for *Pseudospongosorites* (Fig. 4).

3.4.7 Innate immunity

While in vertebrates the immune system has a two-tier system consisting of either phagocytic activity or the opsonization and direct lysis of pathogens via the 'complement cascade', basally branching invertebrate phyla typically lack phagocytic activity and only have the ability to detect, contain, and kill pathogens (Miller *et al*. 2007). The complement cascade has been fully described in three cnidarian species (Miller *et al*. 2006), but the *Amphimedon* genome has important absences (Srivastava *et al*. 2010). We focused on the 11 major gene families involved in immunity, as shown in Miller *et al*. (2007) and Srivastava *et al*. (2010). We found all of them in almost all eight sponge transcriptomes, with a few exceptions (Fig. 5), and significantly, there was only one sequence in the unicellular eukaryote genomes (Fig. 5). The *nuclear factor kappa-light-chain-enhancer of activated B cells* (NF kB), the *interleukin receptor-associated kinase 1/4* (IRAK 1/4), *TGF-b activated kinase* (TAK-1), the *TNF receptor-associated factors* (TRAF), and the *interferon regulatory factor* (IRF), were found in all species of sponges and the latter was also found in *Capsaspora* (Fig. 5). The *Toll/interleukin 1 receptor 2* (TLR2) was found in *Ircinia, Petrosia,* and *Corticium* (Fig. 5). In contrast, the *myeloid differentiation primary response 8* (MyD88) gene was found in all sponges except *Sycon* (Fig. 5). α2-macroglobulin (A2M) is an evolutionarily

Figure 3-5 Innate immunity molecules identified in Porifera. **A.** Presence of the genes involved in the innate immune response in metazoans. **B.** Evolutionary relationships of sponge *MyD88* and *Toll-like receptors* determined with Maximum Likelihood analysis. Bootstrap support is shown for nodes greater than 50% of 500 pseudoreplicates. Colored sequences are matched to the sponges in Figure 1A. Accession numbers of sequences used to construct the phylogenetic tree are available in Supplementary Table 4 **C.** Schematic depicting innate immunity molecules in the cell membrane of a metazoan.



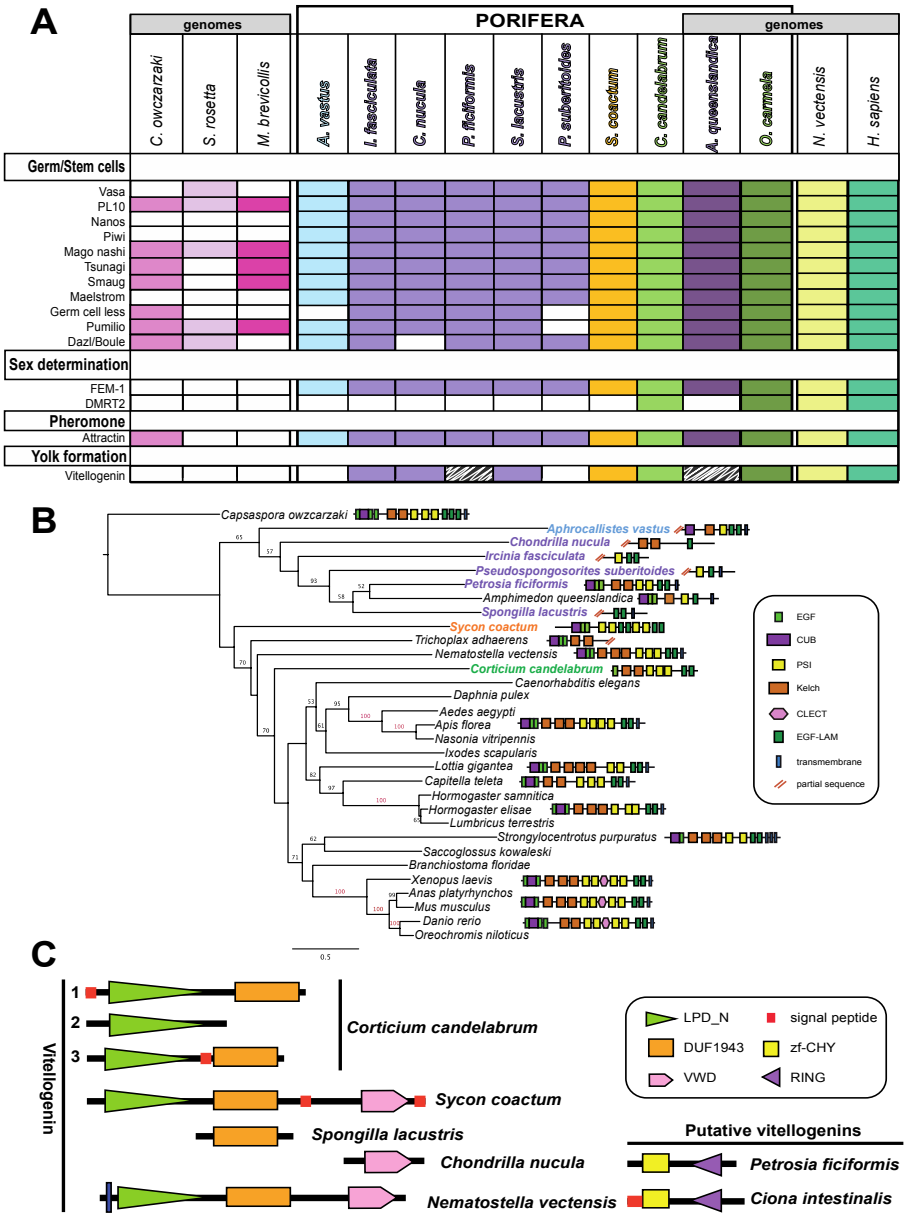
conserved element of the innate immune system whose best- characterized function is the clearance of active proteases from the tissue in many animals (Armstrong and Quigley 1999); it is thought to be absent in *Amphimedon* (Srivastava *et al*. 2010), but we found *A2M* in all the transcriptomes. Similarly, the *mannose-binding lectin associated serine protease* (MASP), which is responsible for activation of the *lectin* complement pathway (Iwaki *et al*. 2011) was not found in *Amphimedon* (Srivastava *et al*. 2010) but was found in the *Corticium* transcriptome (Fig. 5).

3.4.8 Reproductive machinery: germ line, sex determination, pheromones, and vitellogenesis

The ability of differentiated cells to dedifferentiate into dedicated reproductive cell populations (gametes) is exclusive to multicellular animals. Whether these cells are segregated early in the development of the individual, or are continually transformed from undifferentiated cells varies among animal phyla. In sponges, it appears that the mechanism of gamete determination is triggered by environmental cues and involves somatic cell differentiation into gametes (see Riesgo and Maldonado 2008). A well-known germ line machinery exists in metazoans (Ewen-Campen et al. 2010), even though some of the genes may be involved in maintaining totipotency and not specifically in germ line determination (Juliano and Wessel, 2010). Knowing what genetic machinery used for germ line (and eventually gamete) specification and sex determination (Miller *et al*. 2003) exists in sponges can shed light in the evolution of reproduction in metazoans. Of the 20 genes known to be involved in determination of the germ line, we found eleven (with some exceptions) in sponges (Fig. 6). The genes *germ cell-less* and *pumilio* were not present in *Aphrocallistes* and *Pseudospongosorites,* and *boule* was not found in *Chondrilla* (Fig. 6).

For sex determination, all metazoans investigated use *Dmrt* genes, which work as tissue-specific developmental regulators that integrate information about sex, position, and time to direct narrow populations of cells toward male or female fates (Kopp 2012). The sex determination gene *DMRT1* was found exclusively in

Figure 3-6 Reproductive, sex determination, pheromone, and vitellogenesis genes identified in Porifera. **A.** Presence of the genes involved in germ line and sex determination, pheromone communication, and vitellogenesis in metazoans. Hashmarks denote the presence of a sequence with similarity to vitellogenin-like proteins that are highly divergent from vitellogenin. **B.** Evolutionary relationships of the sponge pheromone precursor protein attractin as determined with Maximum Likelihood analysis. Bootstrap support is shown for nodes greater than 50% of 500 pseudoreplicates. Colored sequences are matched to the sponges in Figure 1A. Accession numbers of sequences used to construct the phylogenetic tree are available in Supplementary Table 4 **C.** Protein schematic illustrating the domain structure of vitellogenin and putative-vitellogenin proteins in sponges, cnidarians, and ascidians.



Corticium, while *FEM-1* (a gene involved in gamete specification that appears broadly in metazoans; e.g., Mckeown and Madigan 1992) was found in all sponge transcriptomes (Fig. 6). Another important event genetically and environmentally regulated after gametogenesis is gamete release, which is usually synchronized using pheromones in marine invertebrates (Hardege and Bentley 1997; Painter *et al*. 1998; Counihan *et al*. 2001). Even though it has been suggested that pheromones may synchronize gamete release in *Neofibularia nolitangere* (Hoppe and Reichert 1987), it is only very recently that the presence of a pheromone precursor was shown in a demosponge (Novo *et al*. 2013). In our study, the transcriptomes from all sponge species contained the precursor of the pheromone *attractin* (Fig. 6) and there was a high degree of conservation of amino acid sequence in all sponges except *Sycon.*

Vitellogenesis is also a fundamental reproductive process that occurs during gametogenesis not only in sponges but in all metazoans; it allows embryos and lecithotrophic larvae to survive until they develop feeding structures. The variety of processes converging in the formation of a yolk platelet is remarkable in sponges, as are the various morphologies of yolk (e.g., Riesgo and Maldonado 2009), but the genetic regulation of the yolk formation has been investigated only in bilaterians (e.g., Bownes 1986; Wiley and Wallace 1981). In our datasets, one or several *vitellogenin* genes were also found in all species except for *Aphrocallistes* (Fig. 6), even though the sequences were very divergent among species (not shown).

3.5 Discussion

We searched the transcriptomes of eight sponges for genes that have been considered important for metazoan body organization and function. We also checked for the presence of these genes in three well-referenced unicellular eukaryotic genomes, *Capsaspora owzcarzaki, Monosiga brevicollis, and Salpingoeca rosetta,* and confirmed their presence in two other metazoan genomes (the cnidarian *Nematostella vectensis* and the vertebrate *Homo sapiens).* We found that few genes in the sponge transcriptomes were sponge- specific – sponges shared

between 20 and 50% of genes with other metazoans, between 6.5 and 32% with other eukaryotes (protozoans), and a moderate number were bacterial (between 6 and 24%) – either from bacterial symbionts in the sponge, or in the water they filter; it is unlikely they arise from horizontal gene transfer, which can be the case in pre-metazoans (Tucker 2012). With respect to Pfam domains, *Corticium* contained the highest number, which is similar to that found in the transcriptomes of annelids and molluscs (45,000 to 59,000) and higher than arthropods (around 35,000) (Riesgo *et al*., 2013). However, the transcriptome of *Corticium* may also contain high numbers of bacterial Pfams due to the abundance of symbionts in its mesohyl. From the genes shared with metazoans, in most of the sponges studied more than 50% were most similar to bilaterian genes. In fact, *Aphrocallistes, Sycon,* and *Corticium* showed less than 25% of similarity between their genes and the sponge genes in the NCBI databases; 75% were more similar to bilaterian genes. The number of annotated genes in the sponge transcriptomes was very similar to that of other non-model organisms that also have very few genetic resources (e.g., Riesgo *et al*. 2012b; Pérez-Porro *et al*. 2013). Over 30% of the transcriptomic contigs were not assigned any annotation, which highlights the necessity for a greater effort in sequencing and annotation of sponge genomes and/or transcriptomes.

The complement of genes present in sponges appears far greater than previously understood from the single and now well-studied demosponge species *Amphimedon queenslandica* (Srivastava *et al*. 2010). The publication of the transcriptome of *Oscarella carmela* suggested that homoscleromorph sponges have a far greater complexity than demosponges (Nichols *et al*. 2008). Yet the demonstration here of the broad presence of genes in so many functional categories – development, signaling, adhesion, epithelia, immune recognition, and germ-lineage/specification – across Demospongiae, Homoscleromorpha, Calcarea and Hexactinellida shows that sponges are universally much more complex at the molecular level than previously appreciated.

What then defines metazoan complexity? We found a number of genes which

previously had been associated with complex structures of metazoans and thought to be absent in sponges. For example we found homologs of *smoothened, type IV collagen,* and *iontropic glutamate* receptors in several of the sponges. We found also quite clear differences across the sponge classes – almost all metazoan genes were found in the homoscleromorph *Corticium; Sycon* shared many of these, but the sequences in *Sycon* though blasting with high e-value to the same genes, and folding to the same proposed 3D structure, always had highly divergent amino acid sequences. Interestingly we also found many of these homologs in the glass sponge *Aphrocallistes.* Some of these differences could be due to variation in transcriptome quality (coverage and length of contigs – the most complete transcriptomes were those of *Petrosia, Corticium, Sycon,* and *Aphrocallistes),* but they may also reflect differences in the length of time that the sponge classes have been separated, and in their level of tissue/functional complexity. The fact that sequences found in *Corticium, Sycon,* and *Aphrocallistes* were more similar to bilaterian sequences than to sponge sequences in the databases supports the notion that compared to other sponge groups Demospongiae, broadly speaking, have diverged significantly from other metazoans.

3.5.1 Gene searches

We looked for a total of 127 genes involved in development, neuronal and epithelial signaling, immunity and reproduction. Out of those 127, 100 (78% of the genes) were already identified in the *Amphimedon* genome (Srivastava et al. 2010). In our study, we found 119 genes (18 more than in the *Amphimedon* genome) that were thought to be absent in sponges, mainly in *Corticium* and *Sycon* (see below), which brings the percentage of genes shared by sponges and other metazoans in the pathways studied here to 93%.

3.5.2 Developmental Toolkit Genes

In unicellular opisthokonts such as *Monosiga* and *Salpingoeca* components of the conserved metazoan toolkit signaling pathways are mostly absent (King *et al*.

2008; Fairclough *et al*. 2013); our survey of the genome of *Capsaspora* largely agreed with that finding and confirmed the apparent absence of signaling pathway components outside Metazoa. Notably, however, in *Dictyostelium,* the signaling system involved in forming fruiting bodies contains several elements considered to be critical for Wnt signaling: *Fz receptors, GSK3β,* and β-catenin homologs (called *GSKA* and *aardvark* in this organism) and a *dkk* (Guder *et al*. 2006; Harwood, 2008). Interactions between some members of the Wnt pathway therefore may be predate the origin of metazoans. We found that sponges possess many metazoan toolkit signaling genes in the Wnt, Hedgehog, TGF-β and Notch- Delta pathways, and our study of TGF-β and Notch-Delta signaling gave results generally consistent with what is already know from *Amphimedon* (Richards *et al*. 2008; Richards and Degnan 2009, 2012; Srivastava *et al*. 2010).

The lack of a Hedgehog ligand is unsurprising as it is missing from the genome of *Amphimedon* (Nichols *et al*. 2006; Adamska *et al.* 2007b; Srivastava *et al.* 2010). It was found in expressed sequence tags (ESTs) from *Oscarella carmela* (Nichols *et al*. 2006), but the fragment did not contain all diagnostic domains and the characteristic domain structure of a full-length *hedgehog,* therefore these data should be considered cautiously (see Matus *et al.* 2008 and Ingham *et al*. 2011 for further discussion). Interestingly homologs of the *smoothened* gene in *Aphrocallistes, Sycon* and *Corticium* were found and these new findings push the origin of Smoothened genes further back in metazoan evolution. The lack of *smoothened* in demosponge transcriptomes further supports the idea that the demosponges may have lost certain genes. Furthermore, this trend highlights the need for genomic data from a wider variety of basal branching metazoans – especially sponges – to allow a more complete assessment of the origins of signaling pathways and other characteristic metazoan genes.

3.5.3 Neuronal signaling

Sponges lack conventional neuronal signaling systems and so it is intriguing that molecules of the protein-rich post-synaptic density (PSD) have been

characterized in *Amphimedon* (Sakarya *et al.* 2007; Alié and Manuel 2010; Srivastava *et al.* 2010). We found that PSD genes are present in all 4 classes of poriferans and there is little variation among species, demonstrating that PSD genes were present in the poriferan ancestor. Other genes known to be involved in the development of nervous systems in metazoans (neuralians sensu Nielsen 2010) have been identified in the *Amphimedon* genome, such as *elav- mushashi-like* RNA binding genes, neural transcription factors like *Notch, Delta,* and *BHLH* (Richards *et al*. 2008; Gazave *et al*. 2009; Richards and Degnan 2012). These genes are also widely expressed in the sponge transcriptomes we studied. Molecules involved in signaling (e.g., *G-coupled receptors* (GPCRs)), and neuroendocrine secretion are known in part from the *Amphimedon* genome (Srivastava *et al.* 2010), but clearly this is where sponges vary in complexity.

We found the first evidence of a rapid, ionic-based receptor in sponges, the *ionotropic glutamate receptors* (iGluRs). Both *Corticium* and *Ircinia* transcriptomes have sequences with good similarity to vertebrate *iGluRs,* and we found a similar although more divergent sequence in the calcareous sponge *Sycon.* This pattern of divergence was noted in many genes identified in *Sycon,* an observation that may simply indicate an accelerated rate of evolutionary change in that lineage. In contrast to sponges, in the unicellular eukaryote *Capsaspora* only slightly more than half the PSD genes were present. In general, a small number of structural elements of the PSD that in other metazoans lie deeper within the cell can be found in unicellular eukaryotes, while sponges seem to possess a larger set of PSD genes, notably with an increase in the presence of receptor and signaling molecules. Our results are consistent with the scenario summarized by Ryan and Grant (2009), in which the PSD evolves by adding complexity through the addition of channels and receptors while leaving the underlying scaffolding largely intact. The post-synaptic density – as a structure - therefore appears to be a characteristic of neuralians.

In contrast, components of classical neurotransmitter synthesis pathways do not seem to form a coherent group, but rather are scattered throughout the different

sponge transcriptomes. The presence of genes encoding for enzymes known to be important components of neurotransmitter synthesis, yet lacking the full synthesis pathway could be viewed as evidence of gene loss. An alternate explanation however, is that some enzymes associated with neurotransmitter synthesis are involved in the production of secondary metabolites in the sponge, rather than classical neurotransmitters to be used for signalling. For example, the gene *DOPA decarboxylase,* which codes for an enzyme involved in the synthesis of both dopamine and serotonin, was found in both *Corticium* and *Sycon* yet neither type of receptor was unambiguously identified in any transcriptome. However, a number of serotonin-derived alkaloids have been identified from the demosponges *Hyrtios erectus* and *Hyrtios reticulatus* (Salmoun *et al.,* 2002). This may explain why serotonin has been visualized in sponge tissue and why some synthetic enzymes are present, yet why no clear functional role for the neurotransmitter has been demonstrated (Lentz 1966; Emson, 1966; Weyrer *et al.,* 1999; *cf.* Ellwanger and Nickel, 2006); we were also unable to find sequences for a serotonin receptor in the genomes of *Amphimedon* and *Oscarella.* However, the ubiquitous presence of other enzymes such as *glutamate decarboxylase* among all the sponges is consistent with the demonstrated physiological roles for GABA in sponge physiology (Elliott and Leys 2010). Full genomes showing the concrete absence of any molecules, and careful physiological assessments of neurotransmitter effects on sponges, are needed to fully appreciate the roles these enzymes and molecules might play in sponge physiology and behavior.

It is somewhat surprising not to find sequences for voltage gated potassium channels (Kv) in most of the sponges. Until the genomes of these animals can be surveyed it is difficult to draw conclusions about these absences; however such a consistent absence in most of the sponge transcriptomes could also be an indication of the lack of the need in sponges for rapid changes in membrane potential, typically mediated by Kv channels. The next obvious step will be to experimentally characterize the *shaker- like* sequences we found in the unicellular eukaryotes, and in *Corticium.*

3.5.4 Adhesion and epithelia

Epithelia are complex and highly versatile structures and are one of the unifying characters of multicellular organisms. Even aggregates of unicells form epithelial-like characters such as adherens junctions, for example when *Dictyostelium* amoebae congress to form fruiting bodies (Dickinson *et al.* 2011, 2012). Adherens-junctions probably provide support in the raised structure formed of clones of cells. But the full complement of epithelial characters requires proteins that allow adhesion, sealing, polarity and stability. When these features were assessed for *Amphimedon* (Fahey and Degnan 2010), it was determined that sponges possessed genes allowing polarized epithelia, but lacked the essential conventional molecules typically thought to seal the epithelium from the environment or to stabilize it by attachment to a basement membrane. These conclusions were thought to support the absence of morphological structures for occlusion or a basement membrane in most sponges. In contrast, where these genes were found in *Oscarella,* their presence was justified by ultrastructure showing a typical basement membrane, although it was not considered what this structure would be needed for in a homoscleromorph sponge and not in a demosponge or calcareous sponge.

We found transcripts for genes with homology to *claudin* – involved in sealing the spaces between cells in deuterostomes – and surprisingly *type IV collagens* and other basement membrane genes such as *nidogen* and *perlecan,* which attach *type IV collagen* to the cell's plasma membrane, were present in nearly all sponges. Overall there was no pattern of presence/absence of these genes across the sponge classes that might provide a hint as to the lineage of evolution. The collagen type IV amino acid sequence from *Spongilla* was the most divergent, which might reflect the recent radiation of sponges into freshwater and the challenges of that environment. Indeed the changes involved in the marine-freshwater transition would be exciting to revisit with a survey of ion channels and transport molecules in addition to sealing of epithelia.

3.5.5 Innate Immunity

Immune genes evolve at an extraordinarily rapid pace, which makes it difficult to draw up hypotheses about their evolution (Hughes 1997; Hibino *et al.* 2006). Presumably the pace of mutation is driven by intense selection in the interplay between host and pathogen. As a consequence, finding immune gene homologues with standard molecular strategies and inferring primitive states is a difficult task. The *Amphimedon* genome encodes several molecules involved in innate immunity including *Nod-like* and *Toll-like receptors, IRAK, MyD88, IRF,* and *IKK* (Srivastava *et al.* 2010); many 'immune' molecules however, seem to be largely "eumetazoan" acquisitions (i.e., found in Cnidaria, Ctenophora, and Bilateria). In contrast, we found the most complete molecular machinery involved in sponge immune response to date, finding all genes involved in the innate immune response pathway described in basal invertebrates (Miller *et al.* 2007). Also, two genes previously described as "eumetazoan" acquisitions, *alpha-2 macroglobulin* and *mannose-binding lectin associated serine protease,* were found in sponges, even though they were absent in *Amphimedon* (Srivastava *et al.* 2010). While *A2M* was found in all sponges classes, *MASP* was only in *Corticium.* None of the components from the selected innate immunity response toolkit was present in any of the three unicellular eukaryotes, as also known from the study of Song *et al.* (2012). Therefore, our results indicate an ancient origin of the innate immune response in metazoan evolution, which predated the separation of sponges and other metazoans.

3.5.6 Reproductive machinery

In sexually reproducing animals, germ cells are the source of gametes in the adult (Lin 1997). Germ cells carry the hereditary information for the next generation, thus their segregation and protection from a somatic cell fate is essential for animal development and evolution (Buss 1988; Saffman and Lasko 1999; Wylie 1999; Raz 2000). Modern studies identify primordial germ cells more often by the localization of the products of germ-specific genes (Extavour and Akam 2003; Ewen-

Campen *et al.* 2010). Very recently *vasa, nanos, piwi, and PL10* genes were isolated from sponges (Mochizuki *et al.* 2000, 2001; Funayama *et al*. 2010; Srivastava *et al.* 2010), but whether the complete germ line machinery is present in all sponge classes was not investigated. We found that all classes of sponges possess all the genes reported necessary for germ cell determination (note that those specific to *Drosophila* (e.g., *oskar)* were not found). Interestingly, even though sex has not been reported for the three unicellular eukaryotes surveyed here, we found germ line markers in their genomes (PL10, *mago nashi, smaug, pumilio, germ cell-less,* and *boule).* The presence of germ line genes in unicellular eukaryotes seems to support the alternative suggestion for the function of these genes: it may be that germ line markers originated in multipotent cells, where they maintain multipotency, and were subsequently co-opted by more-specialized, embryonic germ cells to determine their germ fate (Juliano and Wessel 2010). Whether or not these genes are used by sponge cells to maintain multipotency or determine their germ fate remains unknown, but the fact that they possess the complete molecular program for the germ line specification could indicate its potential role in germ line determination, but should be evaluated more closely for their role in determining a germ cell lineage in metazoans.

Sex determination in metazoans involves a wide array of solutions, from splicing- based mechanisms in insects to endocrine regulation in mammals (Kopp 2012). However, the occurrence of the sex determining factors *Dmrt* in all metazoans investigated has emerged as a common theme in sexual dimorphism. The main function of *Dmrt* genes in the gonads of metazoans is to promote male-specific and repress female-specific differentiation (Kopp 2012). Interestingly, we only found an ortholog of *Dmrt1* in *Corticium,* which is a hermaphroditic organism with a remarkable similarity in the gametogenic process with that of other metazoans, for instance in the continuous and asynchronous production of sperm in the cysts (Riesgo *et al.* 2007). Likewise, a *Dmrt1* ortholog is expressed in the hermaphroditic *Acropora millepora* during sexual reproduction (Miller *et al.* 2003). Therefore, our results could suggest that the sex determination mechanisms involving *Dmrt* genes

evolved prior the divergence of Porifera from the rest of metazoans.

Communication via semiochemicals such as pheromones occurs in water by either "sniffing" or by contact chemoreception (Wyatt 2003). The peptide Attractin from *Aplysia* was the first water-borne sex pheromone characterized in invertebrates (Painter *et al.* 1998); and the full length protein Attractin, which has been found expressed in gonads of mammals (Li *et al.* 2009) was recently reported in several metazoans including a demosponge (Novo *et al.* 2013); although the pheromone features were not corroborated. We found that the gene *attractin* was expressed in all classes of sponges, even though it was not originally found in the *Amphimedon* genome. In *Amphimedon,* the gene characterized as *Fanconi anemia group I protein-like* exhibited the highest similarity to *attractin.* Although the potential role of *attractin* on the synchronization of gamete release in sponges remains uninvestigated, it opens the possibility of further research in this novel area.

Vitellogenesis in sponges produces two types of yolk platelets, homogeneous (mainly proteinaceous) and heterogeneous (lipidic and proteinaceous) (see Simpson 1984; Riesgo and Maldonado 2009 for reviews). The participation of autosynthetic and heterosynthetic (through nurse cells) mechanisms has been described for several species, but the protein precursor has never been characterized in sponges. We found two types of yolk precursors in our sponge transcriptomes, one *vitellogenin* gene in *Ircinia, Chondrilla, Spongilla, Sycon,* and *Corticium,* and a *vitellogenin-like* gene in *Petrosia.* Whether the different genes are involved in the formation of multiple yolk platelets as in *Xenopus* (Wiley and Wallace 1981) needs further study.

3.6 Conclusions

This is the first study to survey a wide set of metazoan-specific genes in-depth across all four sponge classes. It provides texture to the question of which molecules might have been present in early animal groups, and more importantly

provides the framework for posing new hypotheses on the evolution of multicellularity and animal complexity.

One of the remarkable outcomes of this work is the understanding that most metazoan genes, or the greater complement of genes involved in complex gene pathways, are present in all sponge groups, including genes supposedly absent in the genome of the demosponge *Amphimedon.* Importantly, there are very few key absences (mainly these concern rapid signaling molecules), but overall transcriptomic datasets proved useful to detect complex molecular machineries, even though they are known to contain genes expressed only at a given time.

Determining gene function in sponges is the next challenge. Usually function of genes in an organism is inferred by comparing to gene function known from other animals. For example, *occludins* are known to seal the epithelium at its apical surface in mice, therefore the presence of *occludins* is taken to imply that sponge epithelia seal. Only one functional study has shown that sponge epithelia are sealed against the outside milieu (Adams *et al*. 2010), but the species in which this work was carried out, *Spongilla lacustris,* does not appear to possess *occludins* in our transcriptome. In *Spongilla,* therefore, sealing function could be the work of other as yet unspecified molecules.

Here we show that the great majority of metazoan genes are present in all sponge groups, but sponges do not have conventional structures, behavior, or even mechanisms of development. So either the genes we know from other animals have a different function in sponges, and were co-opted later in the evolution of metazoans for the function we are familiar with, as suggested for adhesion molecules in unicellular eukaryotes (Sebé-Pedrós *et al.* 2010), or the structure has been lost in sponges. In this sense, the recent publication of the genome of *Mnemiopsis leydi* (Ryan *et al.* 2013), which places ctenophores as the sister group to all other animals, suggests that structures such as nerves might have been present in the metazoan ancestor and were secondarily lost in placozoans and sponges. Another alternative is that sponge genes might carry out a similar function as they

do in other animals, but we do not understand that function because we do not recognize the morphology of such a different structure yet.

Our comparative transcriptomic analysis strengthens the view that sponge complexity as revealed by their molecular toolkit is poorly reflected in their morphology, as it has also been shown in placozoans (Srivastava *et al.* 2008). Quite interestingly, our data provides an indication that demosponges have diverged substantially from other classes of Porifera, and highlights the strong similarity of genes in calcareous, homoscleromorph and even hexactinellid sponges with those in other metazoans. Our data also shows that a number of genes are present in calcareous and homoscleromorph sponges but absent in the Silicea (hexactinellids and demosponges). Both the greater number of genes in Calcarea and Homoscleromorpha and the similarity of those sequences in those two species lend support to the suggested sister relationship of these two groups shown in recent phylogenetic studies (e.g., Nosenko *et al.* 2013; Ryan *et al*. 2013). Taken together the overall view given by patterns shown in gene presence and absence across the 4 sponge classes supports the idea that sponges are monophyletic with Demospongiae + Hexactinellida and Calcarea + Homoscleromorpha forming sister groups, a hypothesis that is in agreement with the latest phylogenomic analysis using several sponge taxa (Nosenko *et al.* 2013). One noteworthy observation provided by the data presented by the publication of the ctenophore genome is the remarkable similarity in the gene absences in the post-synaptic density and signaling pathways in the ctenophore genome (Ryan *et al.* 2013) and our study. These absences (and their significance) deserve more attention given that they may support the placement of ctenophores at the base of the metazoan tree.

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Chapter Four

Generation of a polyclonal metabotropic GABA receptor from *Spongilla lacustris*

4.1 Introduction

A number of 'classical neurotransmitters' have been identified in sponges using different of histochemical techniques including the use of commercially available antibodies (raised against mammalian epitopes). For example, serotonin was reported to be present in the larvae of the demosponge *Tedania ignis* (Weyrer *et al*., 1999). Commerical antibodies were also used to visualize GABA in two demosponges, *Chondrilla nucula* and *Leucandra aspera*, and putatively $GABA_B$ receptors as well (Ramoino *et al.*, 2007; Ramoino *et al*., 2011). However, to date no attempt has been made to generate sponge-specific poly- or monoclonal antibodies against any receptors for the classical neurotransmitters. I am aware of only one study in which an antibody was raised against a sponge protein, a lectin, ACL-1 (Dresch *et al.*, 2011). The use of commercial antibodies to identify sponge proteins is highly problematic in that amino acid sequence identities often differ markedly across the sponge classes, as well as across phyla. Since most commercial antibodies are raised against mammalian epitopes it is unlikely that a clean immunostain will be seen in sponge tissue sections, and the probability of non-specific staining is very high. For example, the 2007 paper by Ramoino and colleagues shows unusual staining of the *Chondrilla* mesohyl, a region made up predominantly of a collagenous matrix. Expression of the receptors in this region seems improbable. In contrast, methodological concerns regarding immunostaining for GABA (an amino acid) are limited and the resulting stain showing expression in the pinacocytes surrounding the canal system seems reasonable. But, even here, some unexpected (*i.e.*, non-cellular) staining in the mesohyl is present. Therefore, it is difficult to be confident that staining patterns seen with commercial antibodies in sponge truly reflect the microanatomy of the tissues. While Ramoino performed Western blots to show the specificity of the commercial antibodies, only a fraction of the gel is shown hiding potential non-specific banding patterns at higher or lower molecular weights.

To address this problem, I identified a $GABA_B$ receptor from the transcriptome of *Spongilla lacustris* with which to construct a fusion protein enabling me to generate the first sponge-specific $GABA_B$ receptor antibody. The $GABA_B$ receptor was selected for three reasons. First, no $GABA_A$ sequences – the ionotropic GABA channels – were identified in the *Spongilla* transcriptomes. Second, the previously characterized inflation-contraction response time course implies the use of metabotropic receptors, and so I believed its selection could further inform our understanding of this unique behavioral response seen in the demosponges. Finally, the successful generation of an antibody to lectin ACL-1 in *Axinella corrugata* resulted in highly specific staining patterns further confirming this approach is necessary for robust labeling in sponges (Dresch *et al.*, 2011).

4.2 Methods

A partial $GABA_B$ receptor protein was identified by BLAST searching the *Spongilla lacustris* transcriptome (same as described in chapter 3, section 3.3.4 – 3.3.6). This work plan was used to construct a fusion protein that upon purification was injected into rabbits to generate polyclonal antibodies. The antibodies were evaluated with Western blots.

4.2.1 Sequence assessment

The *Spongilla lacustris* translated transcriptome (Trinity assembly) was queried with a mouse $GABA_B$ receptor (Genbank, AAG29338.1). The sponge's sequence identity (Sla_contig21594-2) was confirmed with a reciprocal Blast search (e-value top hits: *A. queenslandica*, 0.0; *S. kowalevskii*, 3e-74). Transmembrane (TM) domains were identified using the prediction software, TMMPred (http://www.cbs.dtu.dk/services/TMHMM/). The TM domains were removed as candidate regions leaving the large extracellular (N-terminal) domain and shorter (C-terminal) intercellular domain. These regions were then mapped to the corresponding nucleotide sequences (see Figure 4-1 & 4-2). Synthesizing the

Figure 4-1 Schematic of a $GABA_B$ receptor showing the regions from which the sequence used to generate the Spongilla $GABA_B$ receptor was selected. These identified regions correspond to the given sequences in the methods of the same name.

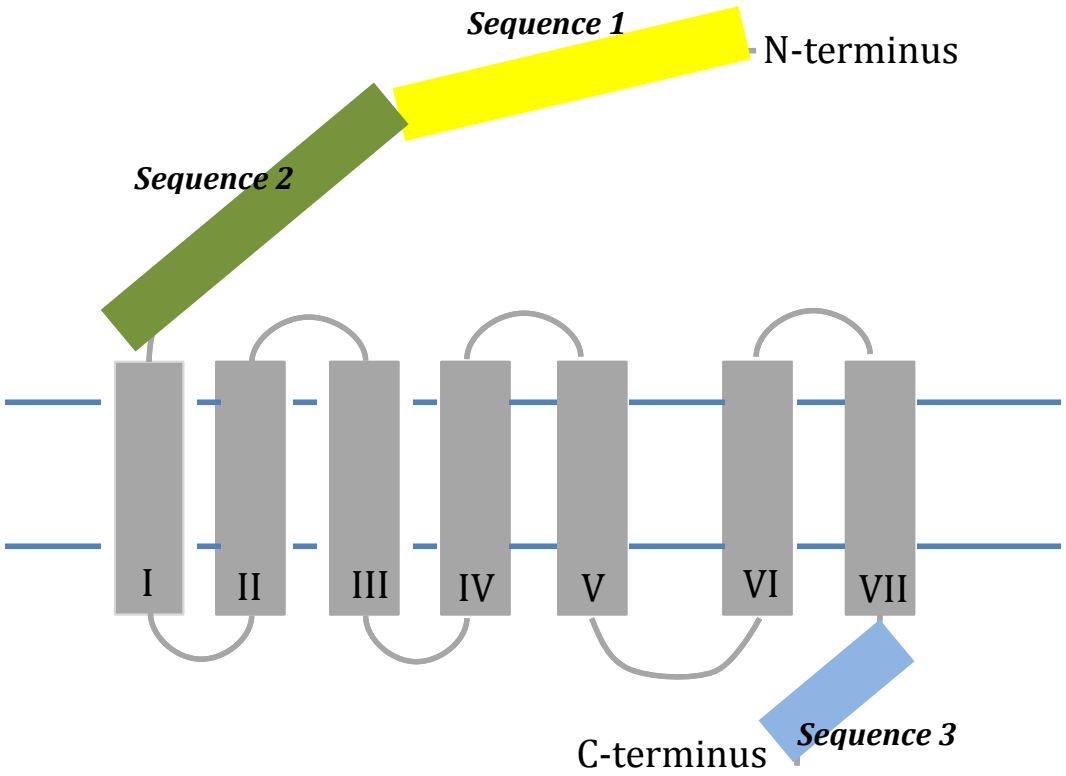
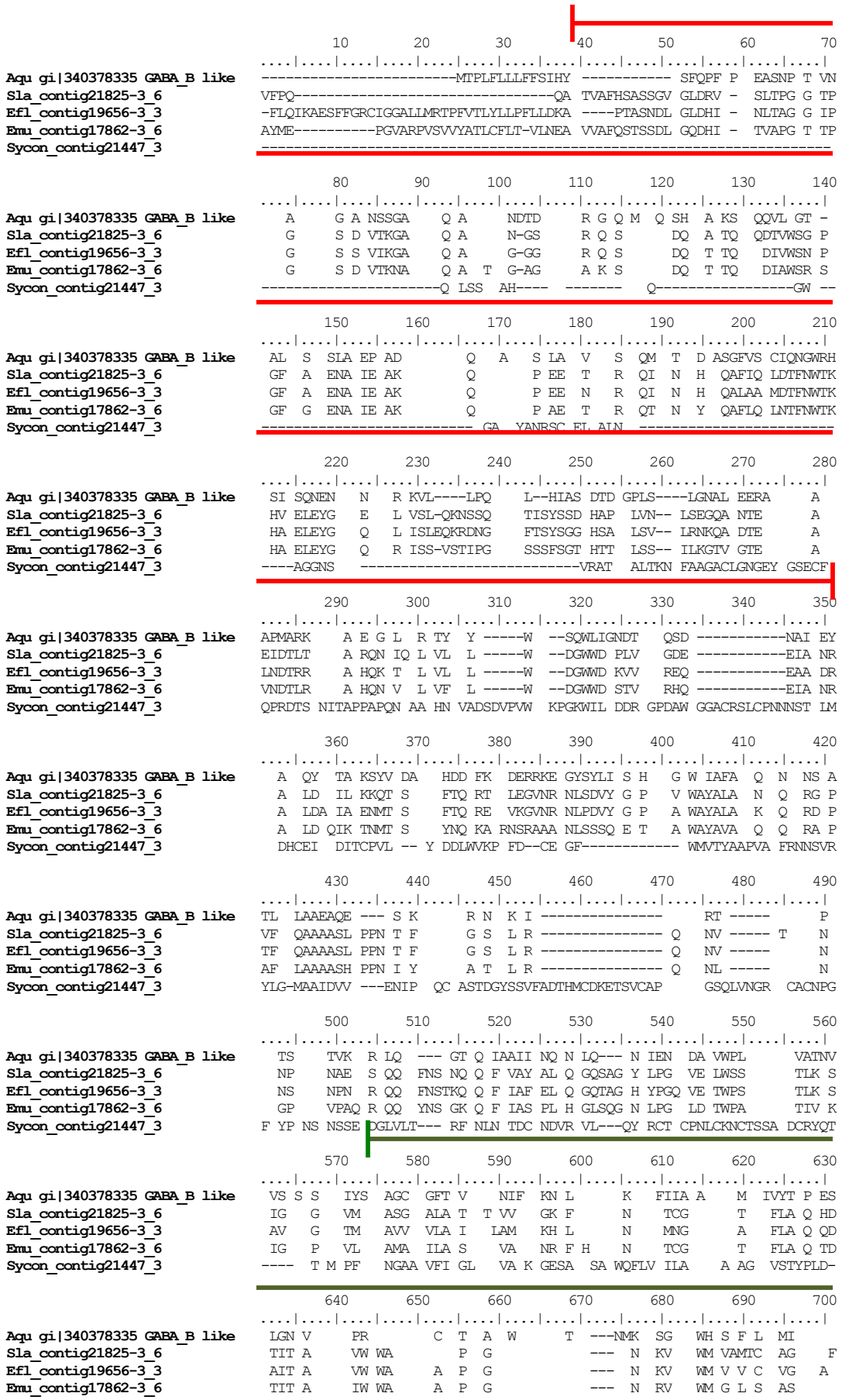
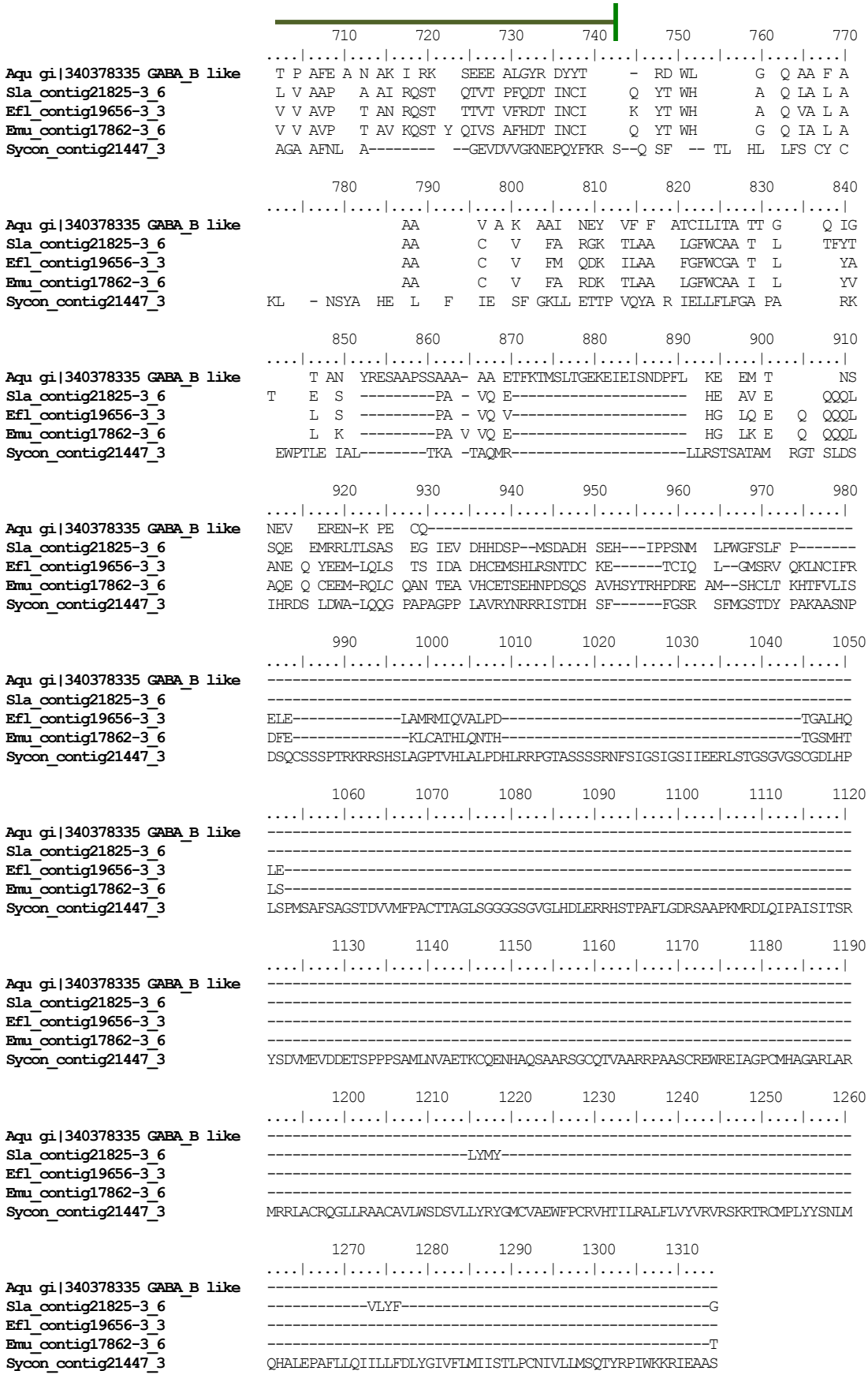


Figure 4-2 MAAFT alignment comparing sponge $GABA_B$ receptors from the *Amphimedon queenslandica* genome, and several $GABA_B$ receptors identified from the transcriptomes reported in this thesis (*Spongilla lacustris, Eunapius fluviatilis, Ephydatia muelleri* and *Sycon coactum.* The alignment shows considerable sequence identity among the demosponges, though the Calcarean Sycon is more divergent. (**Red** line indicates the transmembrane domains (pfam00003); **Green** indicates GABAb receptor binding domain (cd06366). Domains identified with NCBI Conserved Domain Search tool.)


Aqu gi|340378335 GABA_B like
Sla_contig21825-3_6
Efl_contig19656-3_3
Emu_contig17862-3_6
Sycon_contig21447_3


710 720 730 740 750 760 770
Aqu gi|340378335 GABA_B like T P AFE A N AK I RK SEEE ALGYR DYYT - RD WL G Q AA F A
Sla_contig21825-3_6 L V AAP A AI RQST QTVT PFQDT INCI Q YT WH A Q LA L A
Efl_contig19656-3_3 V V AVP T AN RQST TTVT VFRDT INCI K YT WH A Q VA L A
Emu_contig17862-3_6 V V AVP T AV KQST Y QIVS AFHDT INCI Q YT WH G Q IA L A
Sycon_contig21447_3 AGA AFNL A-------- --GEVDVVGKNEPQYFKR S--Q SF -- TL HL LFS CY C
780 790 800 810 820 830 840
Aqu gi|340378335 GABA_B like AA V A K AAI NEY VF F ATCILITA TT G Q IG
Sla_contig21825-3_6 AA C V FA RGK TLAA LGFWCAA T L TFYT
Efl_contig19656-3_3 AA C V FM QDK ILAA FGFWCGA T L YA
Emu_contig17862-3_6 AA C V FA RDK TLAA LGFWCAA I L YV
Sycon_contig21447_3 KL - NSYA HE L F IE SF GKLL ETTP VQYA R IELLFLFGA PA RK
850 860 870 880 890 900 910
Aqu gi|340378335 GABA_B like T AN YRESAAPSSAAA- AA ETFKTMSLTGEKEIEISNDPFL KE EM T NS
Sla_contig21825-3_6 T E S ---------PA - VQ E---------- HE AV E QQQL
Efl_contig19656-3_3 L S ---------PA - VQ V---------- HG LQ E Q QQQL
Emu_contig17862-3_6 L K ---------PA V VQ E---------- HG LK E Q QQQL
Sycon_contig21447_3 EWPTLE IAL--------TKA -TAQMR----------LLRSTSATAM RGT SLDS
920 930 940 950 960 970 980
Aqu gi|340378335 GABA_B like NEV EREN-K PE CQ----------
Sla_contig21825-3_6 SQE EMRRLTLSAS EG IEV DHHDSP--MSDADH SEH---IPPSNM LPWGFSLF P-------
Efl_contig19656-3_3 ANE Q YEEM-LQLS TS IDA DHCEMSHLRSNTDC KE------TCIQ L--GMSRV QKLNCIFR
Emu_contig17862-3_6 AQE Q CEEM-RQLC QAN TEA VHCETSEHNPDSQS AVHSYTRHPDRE AM--SHCLT KHTFVLIS
Sycon_contig21447_3 IHRDS LDWA-LQQG PAPAGPP LAVRYNRRRISTDH SF------FGSR SFMGSTDY PAKAASNP
990 1000 1010 1020 1030 1040 1050
Aqu gi|340378335 GABA_B like ----------
Sla_contig21825-3_6 ----------
Efl_contig19656-3_3 ELE----------LAMRMIQVALPD----------TGALHQ
Emu_contig17862-3_6 DFE----------KLCATHLQNTH----------TGSMHT
Sycon_contig21447_3 DSQCSSSPTRKRRSHSLAGPTVHLALPDHLRRPGTASSSSRNFSIGSIGSIIEERLSTGSGVGSCGDLHP
1060 1070 1080 1090 1100 1110 1120
Aqu gi|340378335 GABA_B like ----------
Sla_contig21825-3_6 ----------
Efl_contig19656-3_3 LE----------
Emu_contig17862-3_6 LS----------
Sycon_contig21447_3 LSPMSAFSAGSTDVVMFPACTTAGLSGGGGSGVGLHDLERRHSTPAFLGDRSAAPKMRDLQIPAISITSR
1130 1140 1150 1160 1170 1180 1190
Aqu gi|340378335 GABA_B like ----------
Sla_contig21825-3_6 ----------
Efl_contig19656-3_3 ----------
Emu_contig17862-3_6 ----------
Sycon_contig21447_3 YSDVMEVDDETSPPPSAMLNVAETKCQENHAQSAARSGCQTVAARRPAASCREWREIAGPCMHAGARLAR
1200 1210 1220 1230 1240 1250 1260
Aqu gi|340378335 GABA_B like ----------
Sla_contig21825-3_6 ----------LYMY----------
Efl_contig19656-3_3 ----------
Emu_contig17862-3_6 ----------
Sycon_contig21447_3 MRRLACRQGLLRAACAVLWSDSVLLYRYGMCVAEWFPCRVHTILRALFLVYVRVRSKRTRCMPLYYSNLM
1270 1280 1290 1300 1310
Aqu gi|340378335 GABA_B like ----------
Sla_contig21825-3_6 ----------VLYF----------G
Efl_contig19656-3_3 ----------
Emu_contig17862-3_6 ----------T
Sycon_contig21447_3 QHALEPAFLLQIILLFDLYGIVFLMIISTLPCNIVLLMSQTYRPIWKKRIEAAS

polynucleotide sequences was done by Integrated DNA Technologies (IDT). Since the maximum synthesizable length that can be generated by IDT is 750 nucleotides the longer N-terminal domain was synthesized as two regions. In total, threesequences were synthesized (two from the N-terminal region and one from the C-terminal region). These sequences were:

N-terminal Domain, sequence 1:
5' -- NdeI RE site – $GABA_B$ receptor sequence -- SapI RE site -- 3'

5'(CATATG***)**GTTTTCCCACAACAAGCATCTACGGTAGCCTTCCATTCGGCTAGCAGTGGT
GTGGAGGGGTTGGATCGCGTCTACCCTAGCAGCCTTACGCCGGGAGACGGTCGAACACCC
CTCACCTTGGGTCTGATGCTAAGCTTTTCGGGGGACTACGTTACGAAAGGAGCTATCCCG
GGGATCCAACTAGCGGTGGACATTATTAACAACGGGAGTATGTTGCCTGGATACCGACT
GCAGTACAGCTTGACTGATTCACGGTGTGACCAGACCGTGGCGCTGACGCAGTTTTTCCA
AGACACCGTGTGGAGCGGCCCTCCTAAGGTGGGCTTCATAGGAGCAGGCTGCGAAAATGC
CACCATCGAAACTGCCAAGATCAGTTACTTCTACAACCTTACCCAGATATCATGTGACTC
ATCGTCCCCTGAAGAGGAGGATAGAACCAGGTTCAAAAGGTACTTCCAAATACTGCCCAA
TGACATCCACCTGTCACAAGCCTTCATTCAAATGCTGGACACCTTTAATTGGACAAAACT
TCATGTTATTGAGCTGGAGTATGGCCTTTTTGTGGAGACAGTGGACCTTTTGGTCTCATT
ACAAAAAAATAGCTCTCAACACAACATCACCACCATCTCATACTCCAGTGACTACCATGC
ACCCAACACCCTCGTGAATATTCTCAGTGAGGGACAAGCATTTAACACAGAATCAAGAAT
ATTCTACCTTG**(**GCTCTTCC****)3'**
*, restriction site for NdeI; **restriction site for SapI

N-terminal Domain, sequence 2:
5' -- AseI RE site – $GABA_B$ receptor sequence -- SapI RE site – 3'

5'(ATTAAT****)**ATGACATTAACAGTTCTGTGTGAGGCATACCGTCAGAACAAGATA
CAACCCCTCTATGTTCTAATCACCCTGGGATGGTACTCTGATGGCTGGTGGGACAGCCCC
CTTGTGAGCTGCACAGGGGATGAGATGGAGATAGCTCTGAACAGATCACTGGCCATCACC
CTAGACCCCGAGATTTTGAACAAGAAGCAGACAACAAGTTCAGGATTGAGCTTCACTCA
GTACAGAACATTGTACCTGGAGGGGGTGAACAGGCTGAACCTGTCTGACGTCTACGATG
GAGAGCCATGTTTTGATGTGGTGTGGGCATATGCCTTAGCCTTGAACAACACCATACAAG
ACCTGAGAGGTGACCCGGTCTTCAACAGACAGGCAGCTGCTGCCTCTCTCCTTCCTCCCA
ACTCCACTTTCTTCATGGAAAACTTCACCTATGGCAACAGTGTCATTTTGGAGAGAATGT
ACCAACACCTGTCCAACGTCACCTTCACTGGGATCACGGGAAATGTTTCCTTCAACCCAA
ATGGTATACGGAATGCTGAACGTATAAGTATTCAGCAGTACAGATTCAACAGCAGTAAT
CAGCTGCAGAAGTTTGACGTTGCTTATGTGGCCTTGGATCAGAATGGACAGTCTGCAGGG
TTTTATTTCCTCCCTGGGGAGTCAGTGGAGACATTGTGGTCATCTGGCATTCCACACGAT
GGGACATTGAAAC**(**GCTCTTCC****)3'**
**restriction site for SapI; ***restriction site for AseI

C-terminal Domain, sequence 3:
5' -- NdeI RE site – $GABA_B$ receptor sequence -- SapI RE site – 3'

5'(CATATG***)**TTCATGCCAACGTTCTATACAACATACAAAGATCCTGAGGGCAAGTCGGT
GTTTCCAGCTACAGTTGTCCAAACGGAGTCAGAGCATGAAAAGATTGCTGTGTTGGAGA
AACGTGTACGAGAACTTCAGCAGCAGCTTTCTCAAGAGCATAAAGAGATGAGGAGGTTG
ACACTATCAGCCAGTTCAAAGGAGGGGTCTATTGAGGTTGTGGACCACCATGACAGTCCC
ATGTCAGACGCAGATCACAGTTCTGAGCACATACCACCATCAAATATGACATCTC**(**GCTC
TTCC****)3'**
*restriction site for NdeI; **restriction site for SapI

Each sequence was tested for internal cut sites for NdeI, SapI and AseI with RestrictionMapper (www.restrictionmapper.org) and found to have none.

4.2.2 Vectors and Cloning

Cloning and expression of each sequence was performed with the New England BioLabs IMPACT Kit™ (https://www.neb.com/products/e6901-impact-kit). I elected to use the pTXB1 vector that generates a C-terminal fusion protein. When the sequence is inserted into the vector, the product is structured as:

T7 promotor – N-$GABA_B$ sequence-C – Intein tag

DNA sequences from IDT (200ng) were dissolved in 20µL of DNase-free waster. The pTXB1 vector was digested according to standard protocols (incubation for 2 hours at 37°C; 20µL total volume: 11µL distilled water, 5µL vector, 2µL digestion buffer, 1µL NdeI and 1µL of AseI or SapI). Ligation of target sequences into the multiple cloning site (MCS) of the vector was also performed overnight at 37°C (20µL total volume: 14µL distilled water, 2µL 10x T4 ligase buffer, 1µL digested pTXB1 vector, 1µL dissolved DNA, and 0.5µL T4 ligase). pTXB1, pTXB1 + insert, and digested pTXB1 + insert for each sequence was analyzed by gel electrophoresis to confirm successful insertion into the vector. Of the three sequences only sequence 3 (C-terminal fragment) was successfully cloned into the vector.

4.2.3 Transformation

Chemically competent *E. coli* cells (One Shot TOP10, Life Technologies) were transformed with the pTXB1 + insert. One vial of *E. coli* cells was placed on ice for 10 minutes after which 10μL of pTXB1 plasmid with insert was added to the cells. Following incubation on ice for 30 minutes the cells were heat shocked at 42°C for exactly 30 seconds after which 950μL of SOC media was added to the cells. After an additional 30 minute incubation on ice the cells were spread on LB-Ampicillin agar plates and allowed to grow overnight at 37°C. The following day (16-18 hours), single transformed colonies were selected and their plasmid isolated using the Qiagen QIAprep Spin Miniprep kit. Plasmid DNA was digested with NdeI and SapI as above and visualized using gel electrophoresis (2% agarose gel). The plasmid (pTXB1 + insert) was sequenced using the Big Dye reaction protocol and was performed by the Molecular Biology Services Unit (MBSU) staff. Following confirmation of successful transformation and proper insertion of the GABA sequence into the pTXB1 vector I established overnight cultures to create a stock of transformed cells. Cultures were made in 500mL flasks with 250mL of Terrific Broth (TB) plus ampicillin (100μg/mL). Each culture was inoculated with a single plate colony and grown overnight in the shaker at 37°C. After 16-18 hours, several freezing vials were filled with 850μL of the overnight culture and 150μL of glycerol and frozen at -80°C as stocks.

4.2.4 Fusion protein induction

Transformed cells from the glycerol stocks were plated on LB-Ampicillin plates overnight at 37°C. Single colonies were used to inoculate cultures as described above. Bacterial growth was monitored with a spectrophotometer that measured the optical density (OD) of the culture at 1-hour intervals. When the OD reached 0.4-0.5 transmissivity the culture was induced with IPTG (40mM). 40μL samples were taken from each culture at 0, 1, 2, 3 and 4 hours and run on SDS-PAGE gel to determine the level at which the induced protein production level plateaued. The remaining culture was spun in the centrifuge (5000 RPM for 15 minutes), the supernatant discarded and the remaining pellet frozen at -80°C.

Fusion protein purification: The frozen cells were resuspended in lysis buffer and sonicated for 20 minutes (cycles of 5 minutes on, 2 minutes off to cool). The lysate was poured over a chitin-lined column and allowed to incubate at room temperature for 48 hours (chitin resin from New England Biolabs, #S66515; chromatography columns from BioRad, #731-1550). Cleavage of the fusion protein from the column was induced by overnight incubation with the reducing agent β-mercaptoethanol (50mM; 2mL total volume in column), the free protein being captured through sequential washes with elution buffer. A 50μL sample was taken to the University of Alberta's Institute for Biomolecular Design for mass determination by mass spectrometry. A protein of approximately 12kDa was expected. However, the sample was found to have degraded and so a second sequencing mass spec was run which showed that the fragments matched the expected sequence. Simone Kerswell of the University of Alberta animal care facility therefore proceeded to inject the *Spongilla* $GABA_B$ receptor C-terminal fragments into two New Zealand white rabbits.

4.2.5 Antibody production and testing

Ethics approval (AUP00000840) was granted for use of rabbits to generate antibodies according to the SOPs used by the department's animal facility. Each injection was 1.5mL total (Injection 1 (day 1): 0.5mL antigen, 1mL Freund's complete adjuvant; Injection 2-4 (days 28, 56, 84): 0.5mL antigen, 1mL Freund's incomplete adjuvant). Test bleeds (5mL per rabbit) were obtained at 15 day intervals following the second injection (days 42, 70 and 98). Test bleeds were kept at 4°C for 5-6 hours the day of drawing before being spun (3000 RPM for 15 minutes), and the plasma was aliquotted and frozen at -80°C. Western blots were used to test the specificity of obtained antibodies. *Spongilla lacustris* gemmules were collected from fresh water lakes in British Columbia and stored at 4°C in the laboratory. Individual gemmules were plated in and hatched in Petri dishes containing M-medium and grown at room temperature as described elsewhere (Elliott & Leys, 2007). Approximately 5 days elapsed between plating of the

gemmules and their collection for Western blot analysis. The fresh *Spongilla* tissue was mechanically ground and suspended in REPA buffer before being frozen at -80°C. This tissue was used for the Western blots (as was frozen trout brain obtained from Dr. Greg Goss' laboratory). Sigma mouse monoclonal anti-tubulin (T9026) was used as a control (1:1000). Fluorescent (Life Technologies Goat anti-Rabbit Alexa Fluor® 488; 1:500 dilution) secondary antibodies were used to visualize the blots (FujiFilm FLA-5000).

Western blot analysis:

Tissues used for the Western blot included *Spongilla* lysate (undiluted, 1:10 and 1:100 dilutions), trout brain lysate (undiluted and 1:10 dilution), and the undiluted fusion protein product (post-cleavage). Proteins were separated by SDS-PAGE (12% gel) and transferred onto nitrocellulose membrane (BioRad, #162-0112). The membrane was blocked overnight (approximately 15 hours) in 5% skim milk. Incubation with the primary antibody (1:500 dilution in 5% skim milk) was for 1 hour. Washes (3 times, 5 minutes each) were done with TTBX (tris buffered saline with 0.025% Tween-20). Secondary antibodies were diluted (1:500) in TTBX and incubated with the membranes for 1 hour. Following three washes, the membranes were visualized using either fluorescence (all test bleed samples) or film (commercial antibodies only).

4.2.6 Commercial antibodies

Two $GABA_B$ receptor antibodies purchased from Santa Cruz Biotechnology (sc-7338 and sc-28792) were also tested for specificity to *Spongilla* $GABA_B$ receptors. HRP-conjugated secondary antibodies (BioRad, Goat anti-Rabbit IgG(H+L)-HRP, #172-1019) were used to visualize protein bands on X-ray film (GenHunter Perfect Film, #B581). A dilution of 1:500 was used for each primary and secondary antibody. *Spongilla* tissue lysate and trout brain lysate were prepared and Western blot analysis performed in the same manner as described above.

Figure 4-3 Induction of the *Spongilla* $GABA_B$-Intein Fusion Protein Construct with IPTG. A basal level of fusion protein is detected at the time of induction (t0), and has plateaued by the 3-hour mark. Samples were taken from the culture at 1-hour time intervals and frozen before being run on 7.5% SDS gel and stained with Coomassie blue.

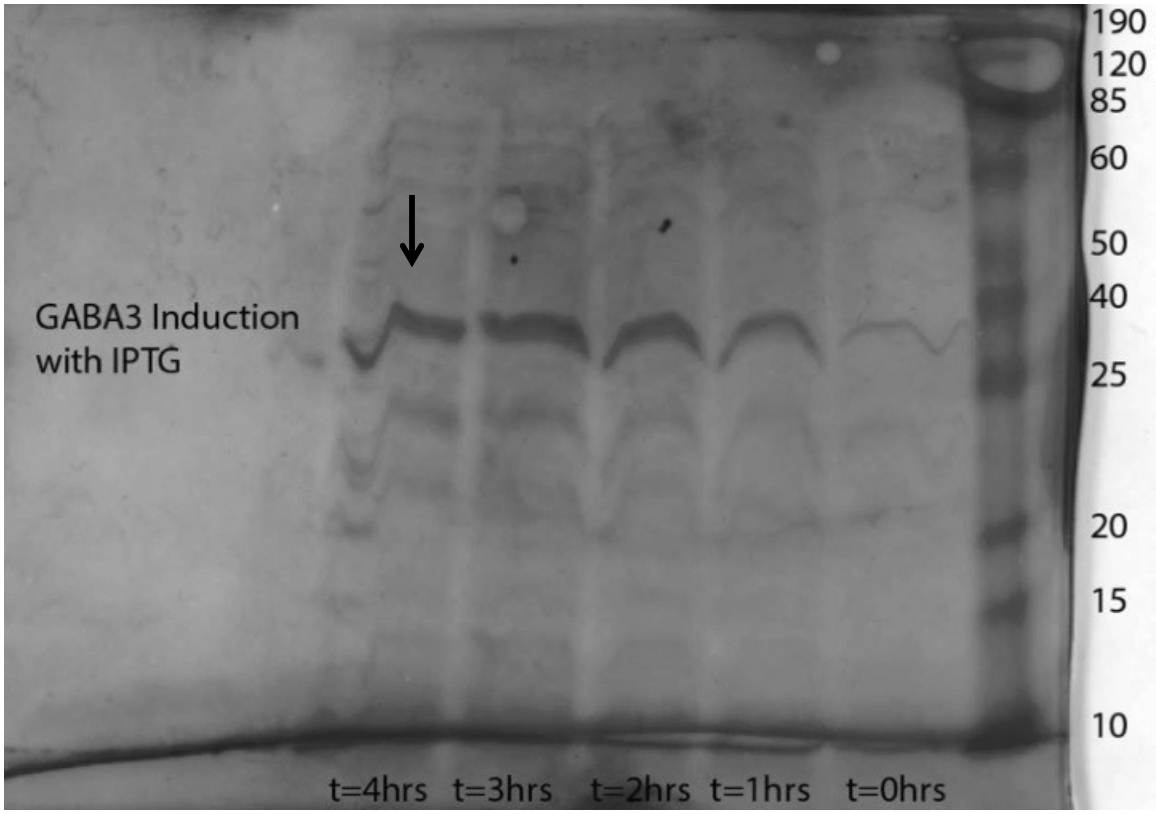


Figure 4-4 Cleavage of the $GABA_B$-intein fusion protein construct from the chitin column. The fusion protein construct is clearly seen in the 3- and 4-hour cultures (left, black arrows) and is identifiable in the protein lysate following sonication. Cleavage of the $GABA_B$-intein protein was by the reducing agent β-mercaptoethanol leaving the smaller $GABA_B$ protein fragment present in the elution (*i.e.*, column) buffer (right, black arrows).

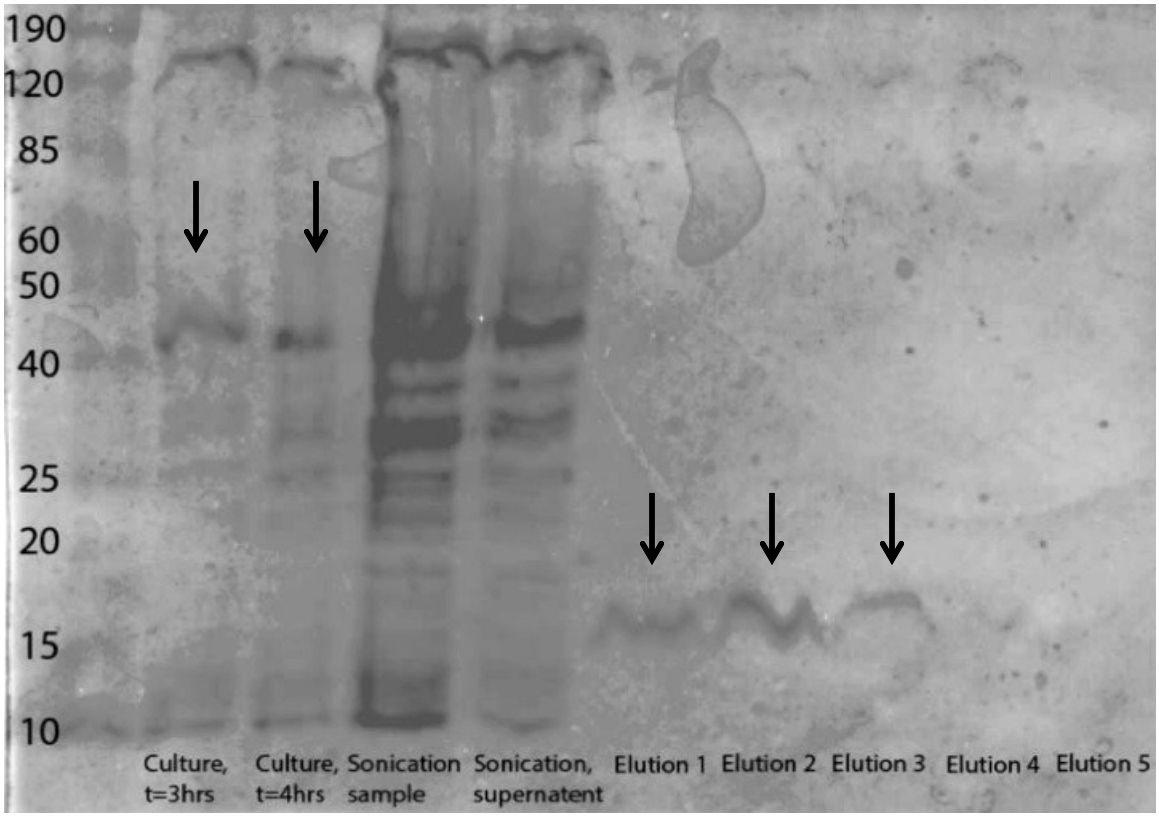


Figure 4-5 $GABA_B$ receptor post-cleavage protein product mass spectrometry. Multiple peaks corresponding to smaller peptides are noted, however, no single peak corresponding to the expected mass (12kDa) is observed.

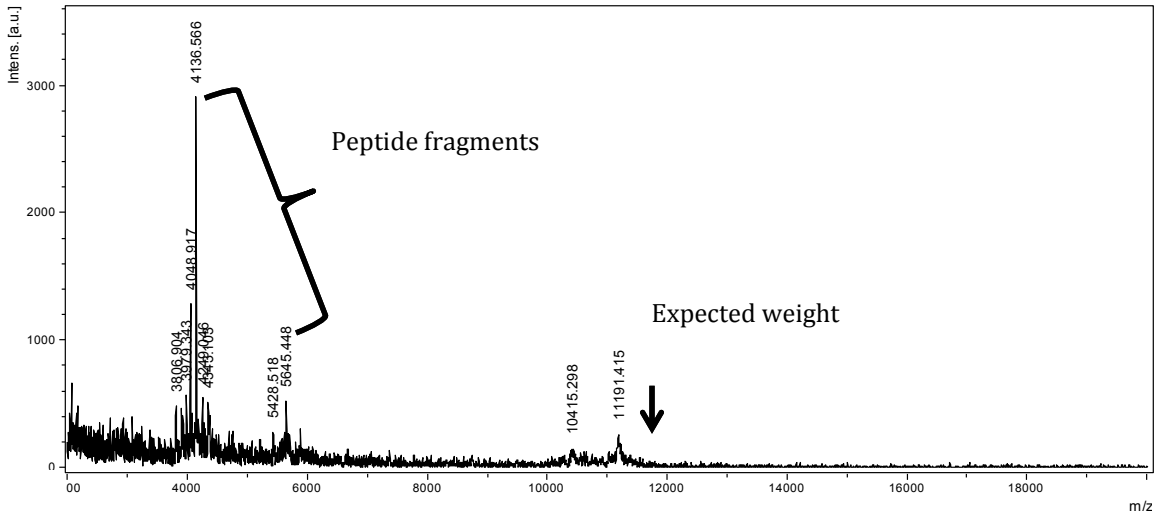


Table 4-1 Amino acid fragments identified by mass spectrometry correspond to the sequence of the $GABA_B$ protein being synthesized. Three fragments are highlighted (pink, yellow & green) in the original sequence for illustrative purposes.

***Spongilla* $GABA_B$ Receptor Sequence**:

LLMRTLFASLYLLHVFPQQASTVAFHSASSGVEGLDRVYPSSLTPGDGRTPLTLGLMLSF
SGDYVTKGAIPGIQLAVDIINNGSMLPGYRLQYSLTDSRCDQTVALTQFFQDTVWSGPPK
VGFIGAGCENATIETAKISYFYNLTQISCDSSSPEEEDRTRFKRYFQILPNDIHLSQAFI
QMLDTFNWTKLHVIELEYGLFVETVDLLVSLQKNSSQHNITTISYSSDYHAPNTLVNILS
EGQAFNTESRIFYLAMYEIDTLTVLCEAYRQNKIQPLYVLITLGWYSDGWWDSPLVSCTG
DEMEIALNRSLAITLDPEILNKKQTTSSGLSFTQYRTLYLEGVNRLNLSDVYDGEPCFDV
VWAYALALNNTIQDLRGDPVFNRQAAAASLLPPNSTFFMENFTYGNSVILERMYQHLSN
VTFTGITGNVSFNPNGIRNAERISIQQYRFNSSNQLQKFDVAYVALDQNGQSAGFYFLPGE
SVETLWSSGIPHDGTLKRSTIGIDVGITVVMYILASGGIALAITCLTFVVIYRGKKFIRL
TSPNLNYITCGGCVVLYTSIFFLAIQSHDETITTAICNVRVWLWAIGYSLSFGPVLGKMY
RVYYIFNNPKPNKKKVVKDWMLVAMTCVLAGLDVVFLLIVTAAPELRATAILRQSTENPQ
TVTGPFQDTTINCIFVCDSQKGYTIWHGILFGYKALIQVLAILLAFGTRKVKVKGLNDSK
YIAAIIYVTSICLVVVIISFATLRGKVNTLAAIYSLGFWCAATTILLLVFMPTFYTTYKD
PEGKSVFPATVVQTESEHEKIAVLEKRVRELQQQLSQEHKEMRRLTLSASSKEGSIEVVD
HHDSPMSDADHSSEHIPPSNMTSL*PWGFSLFVPLYMYVLYCX

Fragments:

VRELQQQLSQEHK	VRELQQQLSQEHKEMR
ELQQQLSQEHKEmR	VRELQQQLSQEHKEmR
ELQQQLSQEHK	ELQQQLSQEHKEMR
SVFPATVVQTESEHEK	SVFPATVVQTESEHEKIAVLEK
FYTTYKDPEGK	IAVLEKR
ELQQQLSqEHKEmR	SVFPATVVQTESEHEKIAVLEKR
RLTLSASSK	DPEGKSVFPATVVQTESEHEK
ELQQqLSQEHK	ELQQQLSqEHKEMR
LTLSASSK	ELQQQLSQEHKEmRR
FYTTYK	SVFPATVVqTESEHEK

Figure 4-6 Plasma from test bleeds 1, 2 and 3 used to probe for the $GABA_B$ protein fragment used in the immunization, and the native protein in *Spongilla* lysate. Each test bleed identifies a distinct band of that corresponds to the $GABA_B$ fragment that was produced and used for immunization, though no bands are identified in the undiluted *Spongilla* tissue lysate.

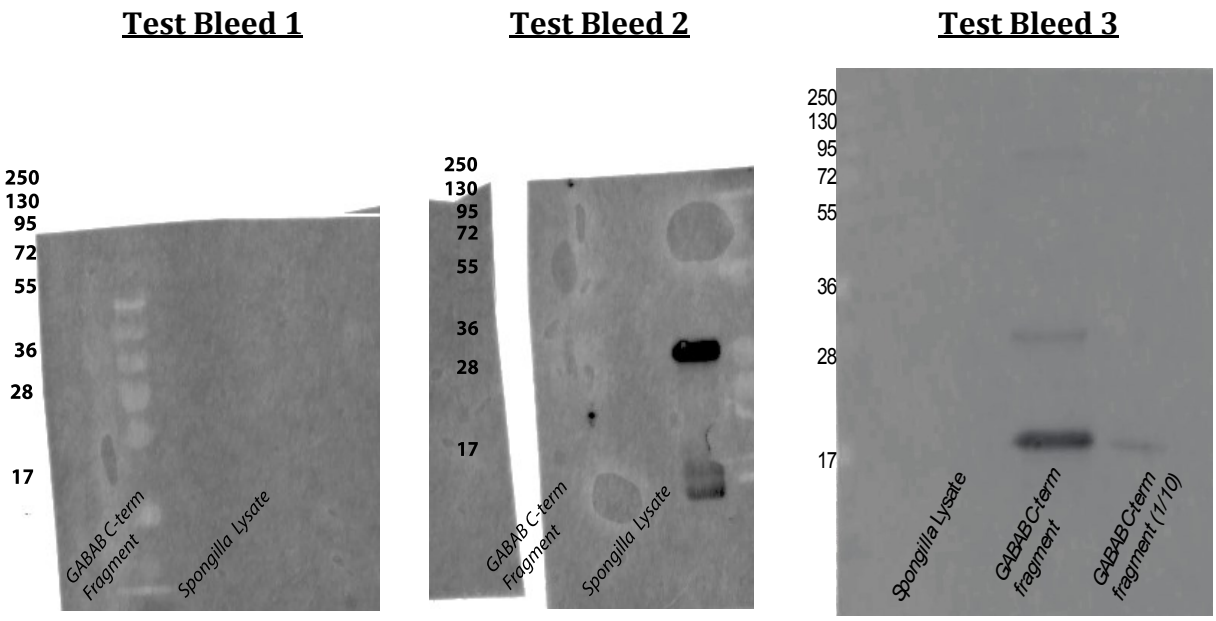
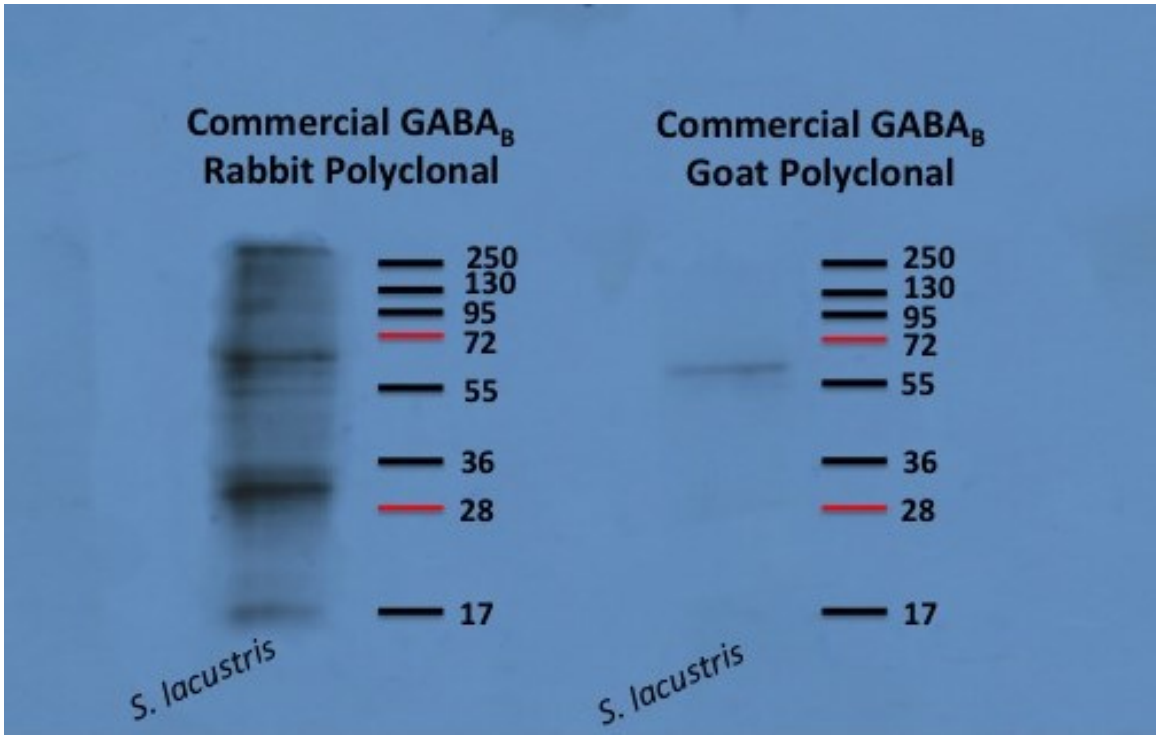


Figure 4-7 Western blot with *Spongilla* tissue lysate probed with rabbit (left) and goat (right) polyclonal $GABA_B$ receptor antibodies. Bands fail to correspond to the predicted molecular mass of the *Spongilla* $GABA_B$ receptor (left: 130kDa; right: 142kDa), and the rabbit polyclonal antibody exhibits non-specific binding affinities in sponge tissue.



4.3 Results

Three vector constructs were made but only one successfully transformed *E. coli*, hereafter called 'GABA3.' This segment corresponded to the intracellular portion of the receptor. The inability to transform with the remaining two vectors was a technical matter, but was not resolved at the time of this writing. Induction with IPTG of the transformed *E. coli* led to the successful synthesis of the fusion protein, with the protein level plateauing approximately 3 hours post-induction (Figure 4-3). Lysate obtained from the transformed bacterial cells was incubated on a chitin-binding column before the final cleavage of the fusion protein from the column (bound *via* the intein tag) was initiated, and the GABA receptor protein segment collected. Samples from each were run to ensure the GABA protein had been cleaved from the intein tag and collected in the wash buffer from the column (Figure 4-4). Analysis of the final protein product was performed with mass spectrometry to confirm the weight of the protein product. However, the mass spectrometry result showed a set of smaller peptides, but not the target peptide weight (Figure 4-5). This suggested the possibility of degradation. To confirm this, a sequencing mass spectrometry reaction was performed. The results confirmed that the protein sequence matched the expected protein sequence (Table 4-1). Although the protein was degraded, the peptide would be cleaved by the animals immune system during the immune response regardless and so the protein sample was given to the animal facility to proceed with the injection of 2 New Zealand white rabbits for antibody production.

Pre-immune, first, second and third test bleed plasma were evaluated for specificity by western blot analysis on *Spongilla* tissue lysate and the GABA3 peptide C-terminal fragment. Pre-immune serum detected no *Spongilla* lysate protein bands. However, test bleeds 1, 2 and 3 also identified no clear bands that correspond to the predicted molecular weight of the *Spongilla* $GABA_B$ receptor. (I do not have the full sequence for the receptor having obtained only a partial sequence from the transcriptome, however, the predicted weight is approximately 130kDa.). When plasma from test bleeds 1, 2 and 3 were used to probe for the

fusion protein a strong band at ~17kDa was identified which matches the weight of the synthetic GABA3 receptor peptide fragment visualized on the gel after purification on the chitin column, and approximates the predicted protein mass (12kDa). However, a faint band is also seen about 28kDa, the identity of which is unknown (Figure 4-6). Therefore the rabbits have successfully generated an immune response against the *Spongilla* protein fragment and have been generating polyclonal antibodies against it though detection of the $GABA_B$ receptor protein in *Spongilla* lysate has been unsuccessful.

Finally, to follow-up on the Ramoino *et al*. paper of 2007, two *Santa Cruz Biotechnology* commercial $GABA_B$ antibodies were tested with *Spongilla* tissue lysate (see Methods for details). Both failed to yield the expected bands and pointed to non-specific binding to other proteins (Expected: $GABA_B$ R1 (sc-7340), 142kDa; $GABA_B$R2 (sc-28972), 130kDa). Control experiment with trout brain also failed to yield single, distinct bands for the antibodies. This result confirms the general inadequacy of commercial antibodies for detecting sponge epitopes and shows the need to reproduce the full result from the Western blot as opposed to simply a small region (Figure 4-7).

4.4 Discussion

The re-analysis of commercial (Santa Cruz Biotechnology) $GABA_B$ antibodies on whole *Spongilla* tissue lysate points towards the need to generate antibodies with specific reactivity to sponge epitopes. In neither case were bands corresponding to the predicted molecular weight identified, and in the case of rabbit polyclonal multiple bands, with differing non-target weights, were identified. Therefore, despite the claim that the GABAergic system has been visualized in a demosponge has been made, the findings are suspect (Ramoino *et al.*, 2007). With the possible exception of antibodies made against chemical neurotransmitters (*e.g.*, GABA, 5-HT) for which no obvious problem would be predicted, vertebrate-derived commercial antibodies against proteins with variation in sequence when compared

to sponges should be considered invalid when used for protein localization in any sponge tissue (*cf*. Appendix 3, Figure A3-1).

The results from the *Spongilla* $GABA_B$ receptor antibody do not show a positive result through Western blot analysis of *Spongilla* tissue lysate, but a robust signal is seen when the test plasma is incubated with the synthesized $GABA_B$ receptor fragment used to inoculate the rabbits. This shows that a robust immune response was generated in the rabbits against the target protein. The region used to make the fusion protein was the short intracellular domain of the $GABA_B$ receptor. The absence of a signal in sponge tissue lysate may be due to very low expression levels of the receptor putting it below the detection limit of the Western. Alternatively, this antibody may not recognize the receptor owing to some remaining secondary or tertiary structure. Future attempts at generating GABA receptor antibodies should target larger segments of the receptor (*e.g.*, extracellular domain), or highly specific regions of the protein of interest (*e.g.*, ligand binding domain). Alternatively, for other proteins of interest, such as a cytosolic NOS, the entire peptide should be synthesized and used to inoculate rabbits. In short, it is possible that the poor result for the $GABA_B$ receptor was due to the short region of the receptor I selected.

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Chapter 5
Implications for the Evolution of Nerves

With the increasing availability of genomes and transcriptomes from basal metazoans, there has been renewed interest in the early emergence of neural systems. A recent symposium supported by the National Science Foundation (NSF, USA) on the theme, *Evolution of the First Nervous Systems*, bears out this resurgence in interest. Furthermore, the recent completion of two ctenophore genomes (Ryan et al. 2013; Moroz et al., 2014), coupled to recent phylogenetic analysis has raised questions about how nervous systems evolved – whether they are best explained by a single origin, or multiple independent origins - and what the exact nature and function of the sponge 'neural repertoire' is given current debates about the phylum's correct positioning. While the data presented in this thesis do not resolve any of these active questions, the findings remain relevant to the broader issues.

This thesis has had three main research aims: The first was to evaluate the 'neural' gene diversity across the sponge classes. It is clear from the pairing of this work with reports in the literature that sponges, though aneural, possess a rich array of 'neural' genes. The second aim was to generate an antibody against one of these neural genes, the $GABA_B$ receptor, in *Spongilla lacustris* allowing subsequent characterization of the expression of this receptor protein. The third aim (Appendix 1) generated only preliminary data suggesting a role for calcium waves in physiology of the inflation-contraction response, a behavior known to be under the regulation of glutamatergic and GABAergic signaling. However, the data provided from each aim points to new questions:

5.1 Future directions – Genes

The gene searches I have performed for this thesis demonstrate that the 'neural' gene toolkit is present across the sponge classes, both in terms of number of genes and types of processes the genes are associated with. However, the restriction of this work to only transcriptomes is problematic, as to date only two

sponge genomes – *Amphimedon queenslandica* (demosponge) and *Oscarella carmella* (homoscleromorph) – are available. While a large number of sponge transcriptomes are now available, there is a pressing need to expand the number of available genomes. As Conaco *et al*. 2012(a) has shown by studying sequential transcriptomes from the *Amphimedon* developmental span, sponges regulate gene expression throughout their development. Having only a single transcriptome per species, as was the case in my own study, places limits on what conclusions are drawn especially regarding genes that are not found, as their absence may simply be an artifact of temporal expression. Genomes from each of the four classes, preferably with multiple representatives will give a much more definitive set of sponge 'neural' genes. However, at present sponge genome assembly has proved difficult, a problem exacerbated by the absence of a well-assembled reference genome. While the *Amphimedon* genome is available, there is some question as to its assembly quality making it less ideal as a reference for assembling additional sponges genomes (L. Moroz, private communication). Once additional, high quality sponges genomes are available it will also allow us to address some curious questions raised by findings with transcriptomes. For example, in the *Spongilla lacustris* transcriptome we find the axon guidance receptor Roundabout (ROBO), but not its conventional ligand Slit (see, Appendix 2). Is this absence an artifact? If not, is this a case of gene loss, or is ROBO functioning in a non-canonical way in sponges? A genome will allow us to answer these questions.

While transcriptomes are valuable for showing us what set(s) of genes are expressed by sponges at given time points, and while genomes show us the full genetic repertoire of an animal, they are less helpful at showing how the individual genes contribute to the behavior and physiology of the animal. Patterns of expression and co-regulation can be determined using transcriptomes as Conaco *et al*. (2012b) has shown. This technique was useful for showing that unlike neural animals which co-express PSD genes to make a functional PSD complex, sponges lack this co-regulation suggesting that the PSD components function in other ways in sponge cells. Nevertheless, such findings are limited if one truly wants

understand how these 'neural' genes function in sponges and how they have been utilized and co-opted throughout the course of evolution. For this level of understanding the *in vivo* function must be studied. Gene hunting is therefore not an end in itself, but rather is a pointer towards the most interesting genes in need of characterization (*e.g.*, fast iGluRs in sponges like *Corticium* which give the appearance of being relatively inactive).

5.2 Future directions – Immunocyto- & histochemistry

Prior to the increased availability of sponge genomic data, use of commercial antibodies on sponge tissue, when done with proper controls, was reasonable because alignments of the relevant genes could not easily be made to assess sequence similarity. Furthermore, antibodies raised against small molecule transmitters (*e.g.,* 5-HT, GABA) would not be thought to pose any substantial concern. However, with the public availability of transcriptomes from all four classes of sponges and genomes from two classes, gene alignments for receptors and other signaling proteins are easily performed and show average-to-poor sequence identity when compared to other animals including vertebrates. Coupled to evidence of the non-specific binding of commercial antibodies in sponge tissue, the use commercial antibodies in sponge tissue should now be considered a sub-par methodology. The challenge here is clear: Large numbers of neural genes have been identified in transcriptomes and genomes (PSD genes, neurodevelopmental genes, *etc.*) however very little is known about the expression pattern of these molecules, especially in the adult sponge. Immunohistochemistry is the bridge that connects genes to physiology by showing how the organization of the tissue enables the physiological processes to be conducted. Unfortunately, save *in situ* hybridizations on several PSD genes in *Amphimedon* larvae there is no histological characterization of any 'neural' genes in sponges. Given the previous and ongoing characterization of glutamatergic and GABAergic signaling systems in *Ephydatia*, *Spongilla* and *Tethya* I am of the opinion that immunolabeling the key components of these pathways (metabotropic glutamate and GABA receptors, GAD, v-GAT, *etc*.) is an excellent starting place as it would provide a detailed picture through the

uniting of the genomics, protein expression and physiology of this sponge system. Additional proteins of interest would be NOS as it has been implicated in the inflation-contraction behavior in *Ephydatia* (Eliott & Leys, 2010) and the iGluR in *Sycon coactum* (Riesgo *et al.*, 2014; chapter 3) as this would provide insights into the types of cells or regions that use fast ionic signaling. Finally, an antibody made a to major PSD scaffolding protein (*e.g.*, Shank or DLG/PSD95, *cf*. Fig. 2-1) could be useful for immune coprecipitation experiments which would enable determination of which components of the 'PSD' gene set actually physically associate within sponge cells *in vivo*. This would be a helpful way to evaluate biochemically whether, and what sort, of 'PSD-like' complex may be utilized in the sponge.

5.3 Future directions – Physiology (Appendix 1)

Aim 3 of my thesis produced a small amount of data as is found in the appendix. It is clear from past work that the inflation-contraction response is dependent on calcium (Elliott & Leys, 2010). The data I acquired appears to show that as a wave of inflation, and its subsequent contraction passes through a canal it is accompanied by a rise, and subsequent fall in calcium. This is consistent with a calcium wave, and is what I expected to see. However, this dynamic is not caught with the imaging system every time. Perhaps this is because some regions of the sponge are better for capturing the change in calcium, or perhaps the wave does not occur throughout the whole sponge body, though this seems unlikely if the wave acts as the coordinating signaling for the inflation-contraction response. Nevertheless, one challenge is to determine how and where to consistently visualize the sponge's calcium dynamics. Once this technical challenge is overcome, the calcium wave's dynamics can be measured (*i.e.*, wave speeds). The source of calcium – intracellular stores vs. extracellular inflow – can also be teased apart using a suite of blockers (*e.g.*, thapsigargin, which depletes the calcium stores of the endoplasmic reticulum). Gap junction blockers such as halothane or octanol would also be informative in determing how a calcium wave may spead in the sponge's tissues. Once the technical challenges surrounding the visualization of the wave are resolved, its subsequent characterization should be rapid and informative.

Nevertheless, the data suggests a calcium wave is an important element in the inflation-contraction behavior.

5.4 Conclusion

With a large repertoire of 'neural' genes, and evidence of neural-like signaling (*e.g.*, excitation by glutamate and inhibition by GABA) it is tempting to speak of sponges being on the verge of becoming neural. Or, if recent phylogenies that place ctenophora in the basal-most position are correct, the loss of a sponge nervous system becomes intriguing. While the data in this thesis does not directly resolve these questions I discern no evidence from the literature or through experimentation that sponges are functioning at sub-optimal levels on account of their lack of nervous tissue. This leads me back to the idea I advanced in chapter 2, namely that the environment to which sponges adapted early in evolution – one requiring an efficient filtering system for feeding and the capacity to regulate and protect it – is the driving force for the physiological adaptations they possess. Certain discoveries such as the presence of many neural 'pseudogenes' in sponge genomes would suggest otherwise, being more consistent with neural system loss, but in the absence of such compelling evidence, sponges have simply used common 'neural' genes to build a non-neural sensory-motor system adapted to the demands of their filter feeding lifestyle. That these molecules can be adapted into neural systems proper ought not to cause us to read properly neural function back onto sponges. While it is true that the function of many of these genes remains unknown, I suspect they will be shown to support a sensory-motor system that has no need of greater speed, integration or processing capacity, and therefore has been under no strong evolutionary pressure favoring the emergence of a nervous system proper in these animals. I therefore contend that sponges have been consistently aneural throughout their history. Subsequent genomic and physiological investigations will determine if my intuition is correct.

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Appendix 1

A non-neural coordination mechanism in the demosponge *Ephydatia muelleri*

5.1 Introduction

Though sponges appear to exhibit low levels of activity, they are frequently seen in varying states of contraction, be this a cyclical contractility or an inducible, dramatic contraction of its body and canal system. In demosponges, the dynamics of contractile behavior has been most thoroughly studied in *Tethya wilhelma* (Nickel et al., 2011) and *Ephydatia muelleri* (Elliott & Leys, 2007). In *Ephydatia*, a particular contractile response, variously described as an inflation-contraction (I-C) response, or 'sneeze' has been investigated in greater detail. This behavior begins with a downward contraction of the sponge osculum, leading to a central-to-peripheral inflating of the canals, followed by their subsequent contraction, the force of which serves to flush the water from the system (Elliott & Leys, 2007). This behavior was found to be inducible by chemical irritation (*i.e.*, dyes in the water) and by mechanical agitation. Later it was shown that this behavior could be induced by bath application of L-glutamate, and antagonized by the presence of GABA in the medium (Elliott & Leys, 2010). The ability of L-glutamate to trigger an inflation-contraction response was dose dependent. Concentrations below 70μM were incapable of generating the behavior, however, high doses led to mechanical tissue damage by creating contractions of excessive force (Elliott & Leys, 2010). The inflation-contraction response was also shown to depend upon the presence of calcium ions in the media. Absence of calcium effectively abolished the inflation-contraction behavior (Elliott & Leys, 2010). These studies on this behavior in *Ephydatia* demonstrated the presence of a highly stereotypical, repeatable behavior in these animals that appeared to be under the control of a glutamatergic (agonist) and GABAergic (antagonist) signaling system. However, the question of what coordinating signal was used to orchestrate this behavior remained. Given the slow speed at which the response occurs (approximately 15 minutes from start to finish), and the essential requirement for calcium, Elliott and Leys had hypothesized that a

slow moving calcium wave, propagating across the sponge body was essential for the coordination of the inflationary and contractile waves.

Calcium signaling is known to play roles in cellular contractility, and has deep evolutionary origins making it unsurprising that signaling with this ion would be an integral part of this sponge system (Shemarova & Nesterov, 2005; 2007). [Several reports show the widespread use of calcium to support other dimensions of sponge physiology including: a sponge stress response signaling cascade (Zocchi *et al.*, 2001; Zocchi *et al.*, 2003), aggregation (Muller *et al.,* 1987; Philip, 2001), calmodulin signaling (Wimmer *et al.*, 1999), and action potential signaling in glass sponges (Leys *et al.,* 1999).]

To evaluate the hypothesis that a calcium wave is a coordination mechanism for the inflation-contraction response I attempted to visualize a rise and fall in calcium levels during an L-glutamate induced response that would be consistent with a wave passing through the region.

5.2 Methods

Ephydatia muelleri gemmules were collected in 2009 from Frederick Lake in British Columbia and stored at 4°C in the laboratory. Plating and hatching of gemmules was done as previously reported by Elliott and Leys (2007). The sponges were loaded with Fura2AM by bath application (final bath concentration of 5μM), and incubated at room temperature for 1 hour to allow sufficient uptake of the calcium indicator. (Fura2 is dissolved in DMSO, final concentration 1mM.) All imaging was performed using a Zeiss inverted (Axiovert) microscope, and associated imaging equipment and software (Eclipse; *Empix Imaging Inc*. provided additional software for ratiometric imaging and analysis, and scripting to set the parameters for each experiment). Prior to beginning any experiment, fura2-loaded sponges were recorded for 30 minutes to establish a stable baseline calcium reading. L-glutamate was added to the sponge media during the recording on the

microscope (Bath concentration, 70μM). Recording of calcium levels were taken for 1 to 1½ hours following the addition of L-glutamate (images were collected every 5 seconds during recording).

5.3 Results

The central objective of these experiments was to determine if a change in calcium accompanies an inflation-contraction cycle in a region of peripheral canal being visualized. Since calcium homeostasis in tissues is dynamic, a perfectly stable baseline was not established (Fig. 5-1), however, when compared to the calcium response recorded during I-C response, the difference is marked. Upon stimulation with L-glutamate, the I-C response is triggered. As can be seen (Fig. 5-2) a rise in calcium accompanies the inflation and contraction of the tissues in the peripheral canal. In several recordings, the subsequent decline in calcium levels is also observed (Fig. 5-2A). In some cases, more than one canal was visible within the imaging field (Fig. 5-2B). Presumably the calcium wave moving through the region is the coordinating signal for each canal, however as can be seen, the magnitude of the I-C response is not identical in each canal suggested some degree of autonomy (differing % change values for the canals) or physical constraint on individual canals. Despite these differences, the recordings show that during the time the I-C response is occurring within a canal segment, a detectable change in the level of calcium in the tissues is recorded. This finding is consistent with the requirement for calcium in generating the I-C response (Elliott & Leys, 2010), and is consistent with a calcium wave-like phenomenon playing a key role in the coordination and timing of the spreading I-C wave.

5.4 Discussion

These findings open up clear trajectories of inquiry. For example, by what mechanism does the wave propagate? At what rate does the wave spread? Is the source of calcium from internal stores or the extracellular environment? One

Figure A1-1 Recording from an unstimulated control fura-2 loaded sponge. The 340/380 ratio reflects the change in calcium bound to fura.

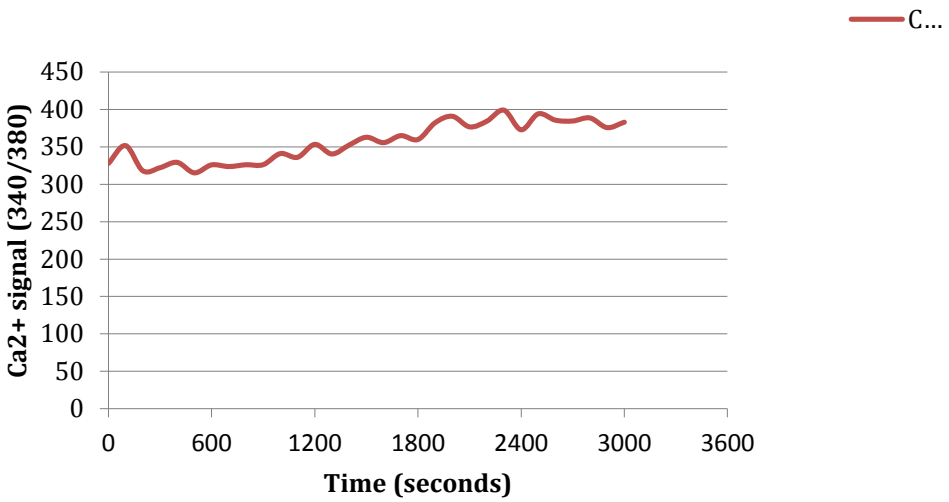
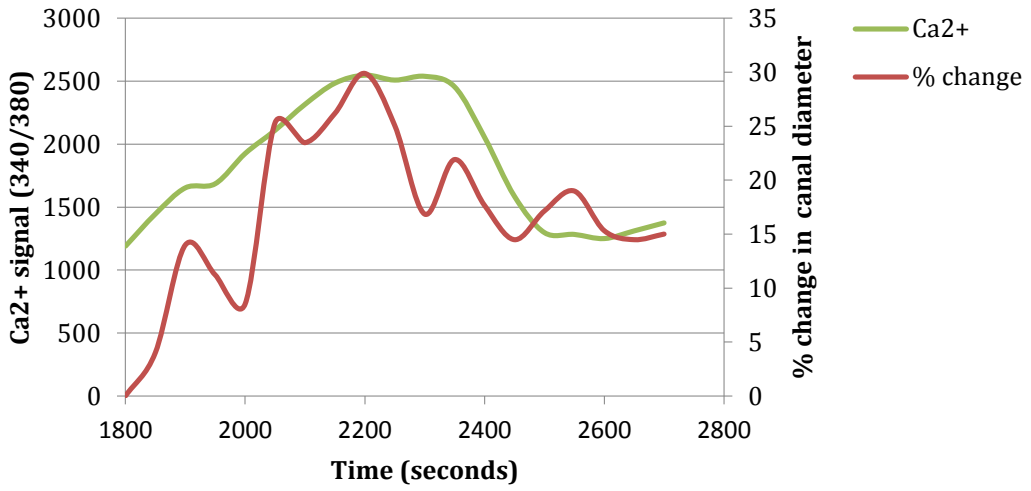
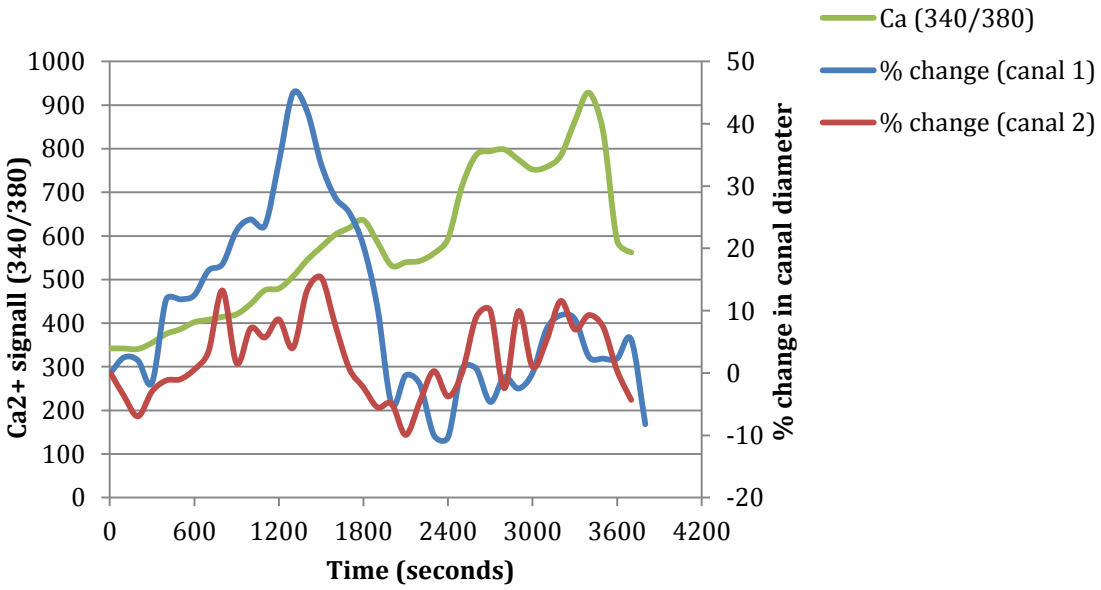


Figure A1-2 Ratiometric calcium imaging on individual, fura-2 loaded juvenile *Ephydatia muelleri*. **(A)** Recording from an individual sponges, focusing on a single canal in the peripheral region of the body following stimulation with 70µM L-glutamate. (Percent [%] change refers the change in the diameter of the individual canal throughout the recording.) **(B)** Recording from a single, stimulated (70 µM L-glutamate) individual where two peripheral canals were within the imaging field of view.

(A)



(B)



difficulty appears to be the unpredictability of the response, both in terms of consistently generating an I-C response and capturing a clear calcium signal (*i.e.*, imaging location in the tissue, robust loading of fura2 throughout the tissues). There are therefore technical issues to overcome. If one cannot consistently generate an I-C response, and see a consistent calcium signal, how could one be sure for example, that a particular blocker is effective at abolishing or perturbing the I-C response? Perhaps genetic approaches that incorporate genes with a calcium indicator tag into the animal's genome, and are subsequently expressed would provide a more reliable method for measuring calcium changes (in development at University of Alberta's Department of Chemistry). Once a stable method is determined for the visualization of calcium changes, assessing the wave dynamics, including its cellular mechanism, will be in reach. However, the evidence to date does clearly point to the importance of calcium signaling in the propagation of the sponge's I-C behavior.

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Appendix 2

Axon guidance molecules in sponges [supplement to Chapter 3]

This brief appendix outlines my findings for axon guidance molecules that were not included in the MBE paper (Chapter 3) as the searches had not been completed at that point in time. They can be viewed as a supplement especially to Figure 3-3.

A2.1 Introduction

Axon guidance molecules (AGMs) play an important role in establishing attractive and repulsive growth zones for elongating neural processes during the development of nervous tissues. While a range of molecules have been found to contribute to the developmental guidance of axons and dendrites (*e.g.*, classical morphogens such as Wnt; Zou & Lyuksyutova, 2007), several major families are best known. These include: Netrin/DCC, Slit/Robo, Ephrin/Eph and Semaphorin/Neuropilin/Plexin (Chilton, 2006). Interestingly, several AGMs have been identified in transcriptomes of *Amphimedon queenslandica* including PlexinA1, Semaphorin3B and EphB1 (Conaco *et al.,* 2012). In one respect, this is unsurprising given the large number of other 'neural' genes sponges have been found to encode. However, it does raise the question of how extensive a set of neurodevelopmental molecules are present in sponges, and what physiological role they may play in sponge development, if any. To address this I searched a narrow set of transcriptomes, plus the *Oscarella* genome, for the classical axon guidance molecules as well as several downstream effectors.

A2.2 Methods

The method used to search for, identify and confirm the identity for each axon guidance genes was identical to the method described in chapter 3. The only difference was the use of a smaller set of transcriptomes and the inclusion of an additional demosponge transcriptome, *Ephydatia muelleri*.

A2.3 Results

Of the AGMs, Netrin and Semaphorin were identified in each transcriptome. However, their receptors were not uniformly identified. Interestingly, while

Table A2.1 Classical axon guidance molecules (AGMs) identified in a set of newly produced (2013) transcriptomes in the lab of Dr. Sally Leys. (*Note*, this set of transcriptomes partially overlaps with those in the MBE paper, however *Ephydatia muelleri* is a new addition here.)

✔, present; ?, likely present; ✗, absent

(A) AXON GUIDANCE FAMILIES

	Hexactinellida	**Calcarea**	**Homoscleromorpha**	**Demospongia**	
	Aphrocallistes	*Sycon*	*Oscarella (genome)*	*Spongilla*	*Ephydatia*
Netrins:					
Netrin	✔	✔	✔	✔	✔
DCC	✗	✔	✗	✗	✗
Unc5	✔	✗	✔	✗	✗
Neogenin	✗	✗	✗	✗	✗
Slit/Robo:					
Slit	✗	?	✗	✗	✗
ROBO	✗	✗	✗	✔	✔
Semas:					
Semaphorin	✔	✔	✔	✔	✔
Plexin	✔	✔	✗	✔	✔
Neuropilin	✗	✗	✗	✗	✗
DSCAM	✔	✔	✔	✗	✗

(B) MAJOR DOWNSTREAM SIGNALING EFFECTORS FOR AGMs

	Hexactinellida	**Calcarea**	**Homoscleromorpha**	**Demospongia**	
	Aphrocallistes	*Sycon*	*Oscarella (genome)*	*Spongilla*	*Ephydatia*
CDC42	✔	✔	✔	✔	✔
RhoA	✔	✔	✔	✔	✔
ROCK	✔	✔	✔	✔	✔
Ephexin	?	✔	✔	✔	✔
srGAP	✔	✔	✔	✔	✔
PAK4	✔	✔	✔	✔	✔

Semaphorin was identified in *Oscarella*, neither Plexin nor Neuropilin was found which may indicate a novel signaling mechanism for Semaphorin in homoscleromorphs. The Slit/Robo signaling set appears to be largely absent from the sponge classes, though Robo appears present in at least some demosponges (Table A2.1A). Downstream signaling effectors involved in cytoskeletal dynamics are present throughout the sponge classes (Table A2.1B).

A2.4 Discussion

In neural animals AGMs signal through the growth cone of the elongating process. AGMs signal to the cytoskeleton where remodeling either drives further growth or retraction. Since the molecules involved in this signaling are involved more broadly in cytoskeletal dynamics it was expected that they should be ubiquitous across the sponge classes. What possible role might AGMs play in sponges? It is also known that AGMs are important in the shaping of the vasculature in higher animals (Adams & Eichmann, 2010). Since a vascular system is essentially an extensive, interconnected set of canals through an animal, I hypothesize that AGMs in sponges may play a role in shaping a set of interconnected canals in their body known as the aquiferous system. The canal system is highly ordered, directional and branching, all features that require developmental guidance and control. The possible role of AGMs in the process warrants investigation and could be examined with RNAi during early development since the resultant canal phenotype may be quite dramatic when compared to sponges with unperturbed development of the aquiferous system.

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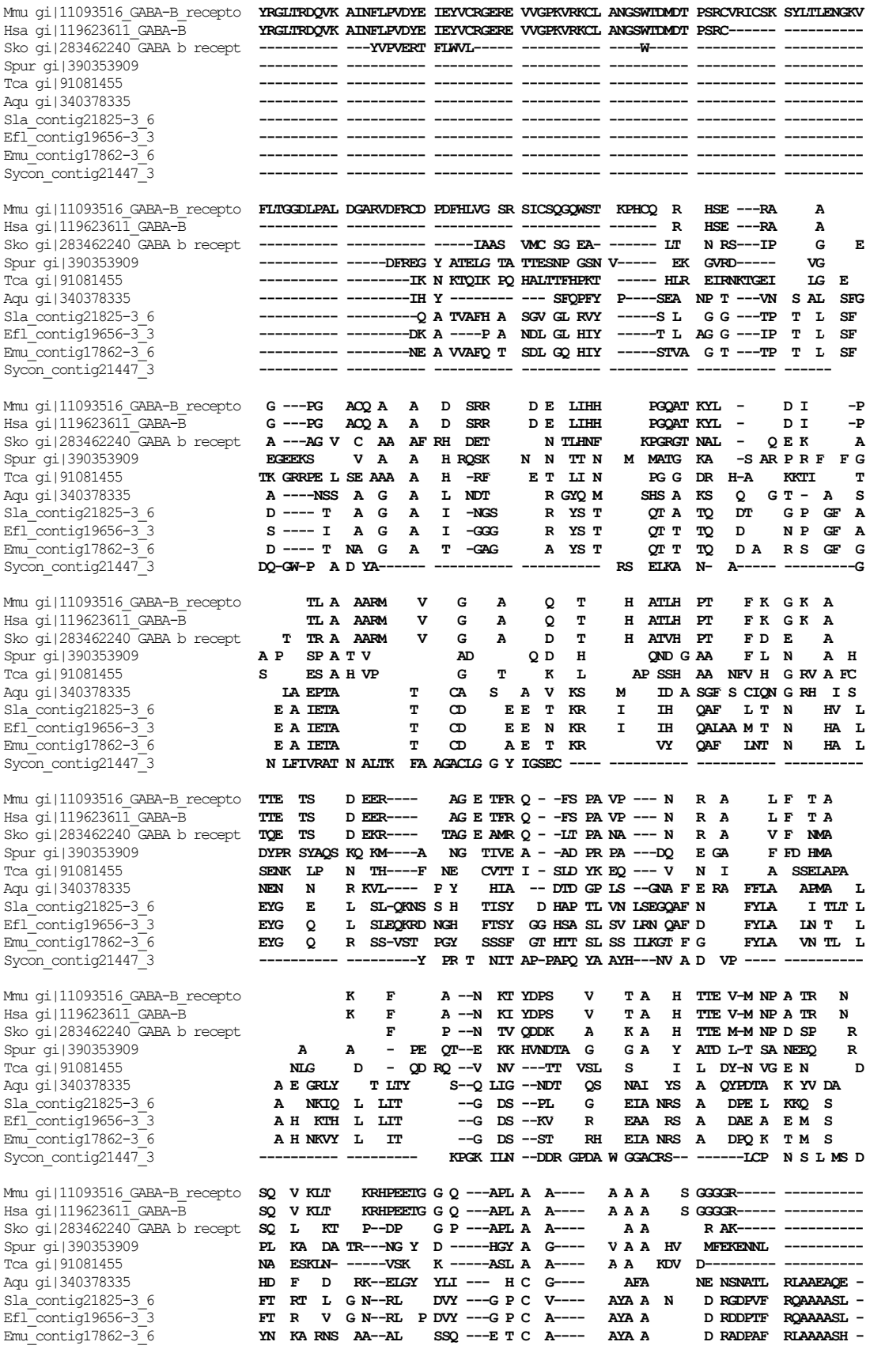
Appendix 3

Additional alignment of $GABA_B$ receptors showing broader taxon sampling

Figure A3.1 MAAFT alignment of $GABA_B$ with sequences from vertebrates and invertebrates, including sequences identified from sponge transcriptome searches. NCBI accession numbers are given for each sequence in the alignment. Broader taxonomic sampling decreases the sequence identity as the resultant alignment demonstrates. Mmu, *Mus muscularis*; Has, *Homo sapien*; Sko, *Saccoglossus kowalevskii*; Spur, *Strongylocentrotus purpuratus*; Tca, *Tribolium castaneum*; Aqu, *Amphimedon queenslandica*; Sla, *Spongilla lacustris*; Efl, *Eunapius fluviatilis*; Emu, *Ephydatia muelleri*; Sycon, *Sycon coactum*.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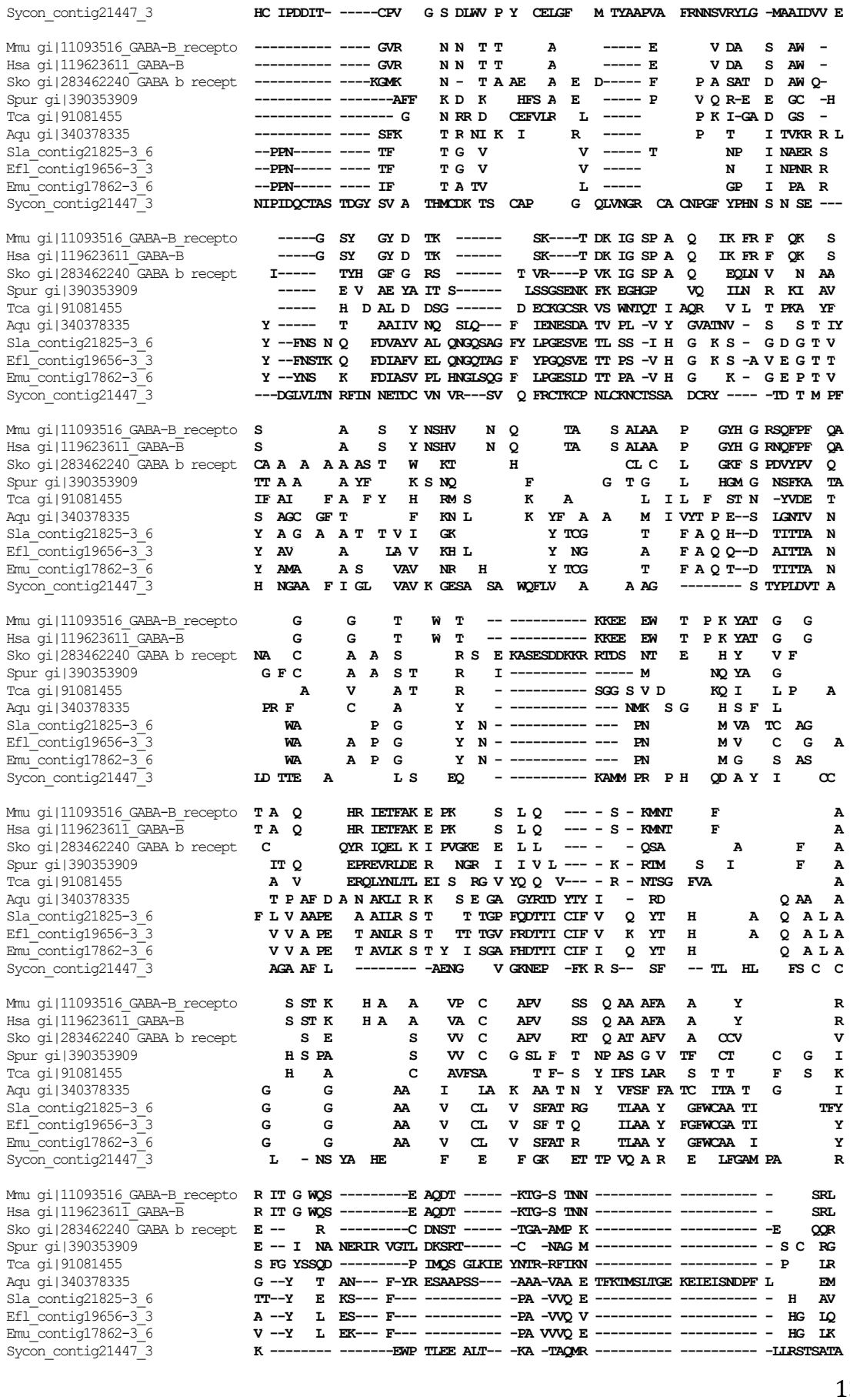
Hsa gi|119623611_GABA-B YRGLTRDQVK AINFLPVDYE IEYVCRGERE VVGPKVRKCL ANGSWTDMDT PSRC------ ----------
Sko gi|283462240_GABA_b_recept ---YVPVERT FLWVL----- ---------- ----W----- ---------- ----------
Spur gi|390353909 ---------- ---------- ---------- ---------- ---------- ---------- ----------
Tca gi|91081455 ---------- ---------- ---------- ---------- ---------- ---------- ----------
Aqu gi|340378335 ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sla_contig21825-3_6 ---------- ---------- ---------- ---------- ---------- ---------- ----------
Efl_contig19656-3_3 ---------- ---------- ---------- ---------- ---------- ---------- ----------
Emu_contig17862-3_6 ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sycon_contig21447_3 ---------- ---------- ---------- ---------- ---------- ---------- ----------
Mmu gi|11093516_GABA-B_recepto FLTGGDLPAL DGARVDFRCD PDFHLVG SR SICSQGQWST KPHCQ R HSE ---RA A
Hsa gi|119623611_GABA-B ---------- ---------- ---------- ---------- ------ R HSE ---RA A
Sko gi|283462240_GABA_b_recept ---------- ---------- -----IAAS VMC SG EA- ------ LT N RS---IP G E
Spur gi|390353909 ---------- -----DFREG Y ATELG TA TTESNP GSN V----- EK GVRD----- VG
Tca gi|91081455 ---------- --------IK N KTQIK PQ HALTTFHPKT ----- HLR EIRNKTGEI LG E
Aqu gi|340378335 ---------- --------IH Y -------- --- SFQPFY P----SEA NP T ---VN S AL SFG
Sla_contig21825-3_6 ---------- ---------Q A TVAFH A SGV GL RVY -----S L G G ---TP T L SF
Efl_contig19656-3_3 ---------- --------DK A ----P A NDL GL HIY -----T L AG G ---IP T L SF
Emu_contig17862-3_6 ---------- --------NE A VVAFQ T SDL GQ HIY -----STVA G T ---TP T L SF
Sycon_contig21447_3 ---------- ---------- ---------- ---------- ---------- ---------- ------
Mmu gi|11093516_GABA-B_recepto G ---PG ACQ A A D SRR D E LIHH PGQAT KYL - D I -P
Hsa gi|119623611_GABA-B G ---PG ACQ A A D SRR D E LIHH PGQAT KYL - D I -P
Sko gi|283462240_GABA_b_recept A ---AG V C AA AF RH DET N TLHNF KPGRGT NAL - Q E K A
Spur gi|390353909 EGEEKS V A A H RQSK N N TT N M MATG KA -S AR P R F F G
Tca gi|91081455 TK GRRPE L SE AAA A H -RF E T LI N PG G DR H-A KKTI T
Aqu gi|340378335 A ----NSS A G A L NDT R GYQ M SHS A KS Q G T - A S
Sla_contig21825-3_6 D ---- T A G A I -NGS R YS T QT A TQ DT G P GF A
Efl_contig19656-3_3 S ---- I A G A I -GGG R YS T QT T TQ D N P GF A
Emu_contig17862-3_6 D ---- T NA G A T -GAG A YS T QT T TQ D A R S GF G
Sycon_contig21447_3 DQ-GW-P A D YA------ ---------- ---------- RS ELKA N- A----- ---------G
Mmu gi|11093516_GABA-B_recepto TL A AARM V G A Q T H ATLH PT F K G K A
Hsa gi|119623611_GABA-B TL A AARM V G A Q T H ATLH PT F K G K A
Sko gi|283462240_GABA_b_recept T TR A AARM V G A D T H ATVH PT F D E A
Spur gi|390353909 A P SP A T V AD Q D H QND G AA F L N A H
Tca gi|91081455 S ES A H VP G T K L AP SSH AA NFV H G RV A FC
Aqu gi|340378335 LA EPTA T CA S A V KS M ID A SGF S CIQN G RH I S
Sla_contig21825-3_6 E A IETA T CD E E T KR I IH QAF L T N HV L
Efl_contig19656-3_3 E A IETA T CD E E N KR I IH QALAA M T N HA L
Emu_contig17862-3_6 E A IETA T CD A E T KR VY QAF LNT N HA L
Sycon_contig21447_3 N LFIVRAT N ALTK FA AGACLG G Y IGSEC ---- ---------- ---------- ----------
Mmu gi|11093516_GABA-B_recepto TTE TS D EER---- AG E TFR Q - -FS PA VP --- N R A L F T A
Hsa gi|119623611_GABA-B TTE TS D EER---- AG E TFR Q - -FS PA VP --- N R A L F T A
Sko gi|283462240_GABA_b_recept TQE TS D EKR---- TAG E AMR Q - -LT PA NA --- N R A V F NMA
Spur gi|390353909 DYPR SYAQS KQ KM----A NG TIVE A - -AD PR PA ---DQ E GA F FD HMA
Tca gi|91081455 SENK LP N TH----F NE CVIT I - SLD YK EQ --- V N I A SSELAPA
Aqu gi|340378335 NEN N R KVL---- P Y HIA -- DTD GP LS --GNA F E RA FFLA APMA L
Sla_contig21825-3_6 EYG E L SL-QKNS S H TISY D HAP TL VN LSEGQAF N FYLA I TLT L
Efl_contig19656-3_3 EYG Q L SLEQKRD NGH FTSY GG HSA SL SV LRN QAF D FYLA LN T L
Emu_contig17862-3_6 EYG Q R SS-VST PGY SSSF GT HTT SL SS ILKGT F G FYLA VN TL L
Sycon_contig21447_3 ---------- ---------Y PR T NIT AP-PAPQ YA AYH---NV A D VP ---- ----------
Mmu gi|11093516_GABA-B_recepto K F A --N KT YDPS V T A H TTE V-M NP A TR N
Hsa gi|119623611_GABA-B K F A --N KI YDPS V T A H TTE V-M NP A TR N
Sko gi|283462240_GABA_b_recept F P --N TV QDDK A K A H TTE M-M NP D SP R
Spur gi|390353909 A A - PE QT--E KK HVNDTA G G A Y ATD L-T SA NEEQ R
Tca gi|91081455 NLG D - QD RQ --V NV ---TT VSL S I L DY-N VG E N D
Aqu gi|340378335 A E GRLY T LTY S--Q LIG --NDT QS NAI YS A QYPDTA K YV DA
Sla_contig21825-3_6 A NKIQ L LIT --G DS --PL G EIA NRS A DPE L KKQ S
Efl_contig19656-3_3 A H KTH L LIT --G DS --KV R EAA RS A DAE A E M S
Emu_contig17862-3_6 A H NKVY L IT --G DS --ST RH EIA NRS A DPQ K T M S
Sycon_contig21447_3 ---------- ---------- KPGK ILN --DDR GPDA W GGACRS-- ------LCP N S L MS D
Mmu gi|11093516_GABA-B_recepto SQ V KLT KRHPEETG G Q ---APL A A---- A A A S GGGGR----- ----------
Hsa gi|119623611_GABA-B SQ V KLT KRHPEETG G Q ---APL A A---- A A A S GGGGR----- ----------
Sko gi|283462240_GABA_b_recept SQ L KT P--DP G P ---APL A A---- A A R AK----- ----------
Spur gi|390353909 PL KA DA TR---NG Y D -----HGY A G---- V A A HV MFEKENNL ----------
Tca gi|91081455 NA ESKLN- -----VSK K -----ASL A A---- A A KDV D--------- ----------
Aqu gi|340378335 HD F D RK--ELGY YLI --- H C G---- AFA NE NSNATL RLAAEAQE -
Sla_contig21825-3_6 FT RT L G N--RL DVY ---G P C V---- AYA A N D RGDPVF RQAAAASL -
Efl_contig19656-3_3 FT R V G N--RL P DVY ---G P C A---- AYA A D RDDPTF RQAAAASL -
Emu_contig17862-3_6 YN KA RNS AA--AL SSQ ---E T C A---- AYA A D RADPAF RLAAAASH -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Hsa gi|119623611_GABA-B
Sko gi|283462240 GABA b recept
Spur gi|390353909
Tca gi|91081455
Aqu gi|340378335
Sla_contig21825-3_6
Efl_contig19656-3_3
Emu_contig17862-3_6
Sycon_contig21447_3

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