The Anatomical Distribution Patterns, Physiological Effects, and Quantification of Biogenic Amines in the Central Nervous Systems of Araneae and Scorpiones (Arthropoda: Chelicerata)

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DEDICATION

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ABSTRACT

The arthropod subphylum Chelicerata is one of the most diverse groups of organisms on the planet, and yet relatively little is known about the structural and functional organization of chelicerate central nervous systems (CNSs). To address this knowledge gap, I conducted a comparative study of biogenic amines in the CNSs of three representative chelicerates: the wolf spider *Hogna lenta* (Araneae: Lycosidae), the jumping spider *Phidippus regius* (Araneae: Salticidae), and the bark scorpion *Centruroides sculpturatus* (Scorpiones: Buthidae). In *H. lenta* and *P. regius*, I mapped the anatomical distribution of catecholaminergic neurons (i.e., those that produce dopamine [DA] or norepineprhine [NE]) in the CNS, using an antiserum against tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis. TH immunoreactivity was detected throughout the spider CNS, including in the visual system, the arcuate body (a site of sensorimotor integration), and the neuromeres of the appendages and opisthosoma, thus suggesting that catecholamines play vital roles in many different behaviors and other physiological processes in spiders. Using similar immunocytochemical methods, I also described the distribution of catecholaminergic neurons in the ventral nerve cord (VNC) of *C. sculpturatus*, as well as neurons that contain octopamine (OA) and serotonin (5-hydroxytryptamine, 5-HT). Of particular note in the scorpion were clusters of large efferent TH-ir neurons, which exited the CNS to directly innervate the

tissues of the book lungs, implying a role for catecholaminergic modulation of respiratory functions. These studies include the first description of catecholamines in any chelicerate taxon, and provide a much-needed foundation upon which future functional studies of biogenic amines in chelicerates can be based. Additionally, I utilized a combination of immunocytochemistry, quantitative chemistry, electrophysiology, and bioinformatics techniques to examine the possibility that NE is an endogenous signaling molecule in chelicerates, despite the widespread notion that invertebrates lack NE. Using ultra-performance liquid chromatography and mass spectrometry, I detected non-trace amounts of NE in the CNSs of both *C. sculpturatus* and *H. lenta*. Endogenous NE was localized to cells of the supraneural lymphoid glands in the scorpion, which implies a previously unrecognized secretory role for these structures. NE was also shown to elicit robust patterned electrophysiological activity in the terminal nerves of the scorpion, which was distinct from the patterns produced by other amines. Finally, I identified genes for distinct NE, OA, and DA receptors in the *C. sculpturatus* genome. Taken together, my results support the idea that NE is an endogenous and physiologically active modulator in scorpions, and possibly in the Chelicerata more broadly, thus challenging the idea that adgrenergic signaling is exclusive to the vertebrates. The implications of these findings are discussed in relation to the evolution of aminergic systems within the Arthropoda and the Bilateria as a whole.

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CHAPTER 1

Introduction: Biogenic amines and the Chelicerata

1.1 I Overview

The arthropod subphylum Chelicerata (arachnids and their relatives) contains over 130,000 currently described species, making it second only to the Insecta as the most diverse group of animals on Earth (Garb et al., 2018; Lozano-Fernandez et al., 2019; Misof et al., 2014; Shultz, 2007). This astonishing diversity encompasses such ubiquitous and recognizable organisms as the spiders (Araneae), scorpions (Scorpiones), and mites/ticks (Acari), as well as several smaller, more obscure groups. There are many compelling reasons to study the Chelicerata— for instance, chelicerates include numerous species of medical significance (Calderón et al., 2015; Ortiz et al., 2015; Rodríguez et al., 2018; Santos et al., 2016; Vetter, 2018), economic value (Attia et al., 2013; King & Hardy, 2013; Nyffeler & Sunderland, 2003; Rangel & Ward, 2018), and ecological importance (Goldschmidt, 2016; Hodkinson et al., 2001; Nyffeler & Birkhofer, 2017), while other species are useful model systems for scientific research and biomimetic engineering (Han et al., 2017; Walter & Proctor, 2013; Yanagida et al., 2017; Yang et al., 2016; Zhao et al., 2016). Additionally, chelicerates are known to exhibit a wide array of fascinating behaviors, including complex courtship and reproductive strategies (Elias et al., 2012; Girard et al., 2015; Gordon & Uetz, 2011; Hrušková-Martišová et al., 2010; Olivero et al.,

2017; Wilgers & Hebets, 2012), varying levels of sociality (Del-Claro & Tizo-Pedroso, 2009; Machado, 2002; Rayor & Taylor, 2006; Viera & Agnarsson, 2017; Walter & Bilde, 2015), spatial navigation (Gaffin & Brayfield, 2017; Jackson & Nelson, 2011; Ortega-Escobar & Ruiz, 2017; Wiegmann et al., 2016), and a myriad of predatory and anti-predator behaviors (Carlson et al., 2014; Hara & Gnaspini, 2003; Li & Lim, 2005; Lohrey et al., 2009; Pékar & Toft, 2015).

All of these reasons make the Chelicerata very attractive study organisms across a wide array of disciplines. Yet surprisingly, investigations into the neurobiology of these intriguing animals remain somewhat limited relative to the wealth of research conducted on the nervous systems of other arthropods (e.g., insects and crustaceans). My dissertation addresses this knowledge gap by presenting a comparative analysis of biogenic amines in the Chelicerata. I utilized histological techniques to characterize the distribution of several key biogenic amines in the central nervous systems (CNSs) of three representative chelicerates—the wolf spider *Hogna lenta* (Araneae: Lycosidae), the jumping spider *Phidippus regius* (Araneae: Salticidae), and the scorpion *Centruroides sculpturatus* (Scorpiones: Buthidae). To complement my histological data, I also integrated physiological techniques, analytical chemistry, and bioinformatics to explore functional aspects of these amines in *C. sculpturatus*, with a particular focus on the possibility that norepinephrine, a compound long thought to be exclusive to vertebrates, could function as an endogenous signaling molecule in chelicerates.

In this chapter, I briefly summarize pertinent background information about the Chelicerata and biogenic amines in order to provide a comprehensive context for the remainder of the dissertation. I also identify current open questions about aminergic systems in chelicerates and the evolution of amine signaling in bilaterian animals, which are addressed in more detail in subsequent chapters. My analyses of *H. lenta* and *P. regius* are presented in Chapter 2, and Chapter 3 details my analyses of *C. sculpturatus*. A brief conclusion, including a discussion of the broader impacts of my research, is presented in Chapter 4.

1.2 I Taxonomy of the Chelicerata

The Chelicerata are one of the four traditional subdivisions of the extant Arthropoda, along with the Hexapoda (insects and allies), Crustacea (crustaceans), and Myriapoda (centipedes, millipedes, and relatives). A multitude of morphological and molecular analyses support the monophyly of these subphyla with the exception of Crustacea, which is now widely considered to be paraphyletic with respect to Hexapoda (Giribet, 2018; Legg et al., 2013; Lozano-Fernandez et al., 2019; Meusemann et al., 2010; Misof et al., 2014; Regier et al., 2005; Regier et al., 2010; Rehm et al., 2014; Rota-Stabelli et al., 2011; Sharma et al., 2014; Shultz, 2007; von Reumont et al., 2011; Wheeler & Hayashi, 1998). These same analyses (along with many others) have also resolved the phylogenetic relationships of these arthropod groups, which I have summarized graphically in Figure 1.1 A. There is a broad consensus that the Chelicerata are

the basal-most lineage of the Arthropoda, and that they are an exceptionally ancient taxon— fossil chelicerates have been dated to the mid-Cambrian (approximately 500 million years ago), and molecular estimates suggest that the Chelicerata could have diverged from other arthropods as early as the Ediacaran-Cambrian boundary (540-550 million years ago) (Dunlop, 2010; Legg, 2014; Misof et al., 2014; Rota-Stabelli et al., 2013). These aspects of the Chelicerata make them a crucial component of comparative studies on arthropod biology and evolution, including those presented in this dissertation.

Sister to the Chelicerata are all remaining extant arthropods, which are collectively known as the Mandibulata on the basis of their mandibulate mouthparts. Within the Mandibulata, there is strong support for a monophyletic taxon uniting crustaceans and hexapods (Pancrustacea, also known as Tetraconata), which is sister to the Myriapoda (Legg et al., 2013; Meusemann et al., 2010; Misof et al., 2014; Regier et al., 2005; Regier et al., 2010; Rehm et al., 2014; Rota-Stabelli et al., 2011; von Reumont et al., 2011). Although mandibulate arthropod groups were not directly studied as part of this dissertation, my findings in the Chelicerata are discussed extensively in relation to previous work in the Mandibulata.

The internal relationships of taxa within the Chelicerata have also been studied extensively using morphological and molecular datasets (Giribet, 2018; Lozano-Fernandez et al., 2019; Sharma et al., 2014; Shultz, 2007; Wheeler & Hayashi, 1998). The majority of chelicerates belong to the class Arachnida,

which contains twelve extant orders: the Araneae (true spiders), Scorpiones (scorpions), Acariformes (acariform mites), Parasitiformes (ticks and parasitiform mites), Opiliones (harvestmen), Pseudoscorpiones (pseudoscorpions), Solifugae (camel spiders), Amblypygi (whip spiders), Thelyphonida (whip scorpions), Schizomida (short-tailed whip scorpions), Palpigradi (micro whip scorpions), and Ricinulei (hooded tick-spiders). The Chelicerata also include two non-arachnid taxa, the Xiphosura (horseshoe crabs) and Pycnogonida (sea spiders, also known as the Pantopoda). Figure 1.1 B summarizes the current view of chelicerate phylogeny, which is referenced extensively throughout the dissertation as I place my findings in a broader evolutionary context. Of particular note is the clade Arachnopulmonata, first proposed by Sharma et al. (2014) and consistently supported in subsequent analyses (Giribet, 2018; Klußmann-Fricke & Wirkner, 2016; Lehman & Melzner, 2019; Leite et al., 2018; Lozano-Fernandez et al., 2019). This clade, which is defined by shared respiratory structures known as book lungs, includes the two orders (Araneae and Scorpiones) that serve as the foci of this dissertation.

1.3 I The Biogenic Amines

The biogenic amines are a class of neuroactive compounds that are derived from the decarboxylation of amino acid precursors (Evans, 1980; Libersat & Pflueger, 2004; Walker et al., 1996). Seven biogenic amines in particular have been shown to exhibit widespread physiological and behavioral

functions as neurotransmitters, neuromodulators, and neurohormones across animal taxa; these compounds are dopamine (DA), norepinephrine (NE), epinephrine (E), serotonin (5-hydroxytryptamine, 5-HT), histamine (HA), octopamine (OA), and tyramine (TA) (Barron et al., 2010; Evans, 1980; Farooqui, 2012; Gallo et al., 2016; Libersat & Pflueger, 2004; Pflüger & Stevenson, 2005; Roeder 2005; Schneider et al., 2014; Torrealba et al., 2012; Verlinden et al., 2010; Vleugels et al., 2014; Walker et al., 1996; Weiger, 1997). Several additional biogenic amines have been identified in animal tissues, but are only present in trace quantities, and their physiological roles appear to be limited and poorly understood; such compounds include tryptamine, β-phenylethylamine, and synephrine (among others) (Berry, 2004; Borowsky et al., 2001; Farooqui, 2016; Grandy, 2007; Zucchi et al., 2006). Aside from the biogenic amines, a variety of other neuroactive substances have been identified as important modulators of animal behavior, including the organic ester acetylcholine, the amino acids glutamate, glycine, and γ-aminobutyric acid (GABA), and an array of different neuropeptides (Grimmelikhuijzen & Hauser, 2012; Walker et al., 1996). My dissertation centers exclusively on the distribution and functions of the behaviorally important “classical” biogenic amines (particularly DA, NE, OA, and 5-HT), which are described in more detail below. When appropriate, comparisons are drawn to other signaling molecules, but these compounds are not major foci of my research.

1.3.1 I Catecholamines

DA, NE, and E are derived from the amino acid tyrosine, and are collectively known as the catecholamines on the basis of a shared catechol moiety (Gallo et al., 2016; Molinoff & Axelrod, 1971; Roberts & Fitzpatrick, 2013). The metabolic pathway by which these catecholamines are synthesized is shown in Figure 1.2 A. Tyrosine is initially converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH, also known as tyrosine 3-monooxygenase), which is the rate limiting enzyme in catecholamine synthesis (Molinoff & Axelrod, 1971; Roberts & Fitzpatrick, 2013) and is utilized extensively as a proxy for catecholamines in this dissertation. L-DOPA itself is not released from neurons as a signaling molecule, but is rather converted to DA via the enzyme dopamine decarboxylase (DDC). NE is synthesized directly from DA via dopamine β-hydroxylase (DβH), and can be converted into E via phenylethanolamine N-methyltransferase (PNMT). NE and E are alternatively known as noradrenaline and adrenaline, respectively; as such, neurons that produce NE or E as their end products are often termed “adrenergic”.

DA has been shown to be an important modulator of sensory and motor processing, and influences an array of behaviors and other phenomena across diverse animal taxa (Gallo et al., 2016; Libersat & Pflueger, 2004; Walker et al., 1996). For example, DA plays a major role in vision and visually-guided behaviors (Kulkarni & Fingerman, 1986; Pfeiffer & Homberg, 2014; Vieira et al., 2018), chemosensation and olfactory memory (Aso et al., 2012; Mercer &

Menzel, 1982; Mizunami & Matsumoto, 2017; Schwaerzel et al., 2003; Unoki et al., 2005; Vergoz et al., 2007), courtship and reproductive behaviors (Akasaka et al., 2010; Creighton et al., 2013; Neckameyer, 1998; Rauceo et al., 2008), reward-seeking and punishment avoidance (Adamantidis et al., 2010; Barron et al., 2010; Kelley & Berridge. 2002; Unoki et al., 2006), aggression (Alekseyenko et al., 2013; Rillich & Stevenson, 2014; Schwartzer & Melloni, 2010), feeding and appetite (Inagaki et al., 2012; Riemensperger et al., 2011; Tsao et al., 2018), locomotion and motor control (Mesce & Pierce-Shimomura, 2010; Mustard et al., 2010; Puhl & Mesce, 2008; Sharples et al., 2014), sleep and arousal (Eban-Rothschild et al., 2016; Kume et al., 2005; Liu et al. 2012; Ueno et al., 2012; van Swinderen & Andretic, 2011), and circadian rhythms (Hirsh et al., 2010; Korshunov et al., 2017). Unlike DA, which appears to be universally present as a signaling molecule in the nervous systems of all animals, NE and E are widely reported to be physiologically active only in vertebrates (Adamo, 2008; Blenau & Baumann, 2001; Farooqui, 2012; Gallo et al., 2016; Pflüger & Stevenson, 2005; Roeder, 1999, 2005; Verlinden, 2010; Walker et al., 1996). NE and E are perhaps best known for playing major roles in the acute “fight or flight” stress response, in which they function to heighten alertness and prime the body for increased motor activity (Adamo, 2008; Berridge et al., 2012; Cecchi et al., 2002; Keller et al., 2006; Singh et al. 2015; Wirz et al., 2017; Wong et al., 2012; Zhang et al., 2003). In addition, NE and E have been shown to be key modulators of other phenomena, including learning and memory, social interactions, and

courtship behaviors (Chen & Williams, 2012; Griffin & Taylor, 1995; Marino et al., 2005; Wade, 2013). In invertebrates, many of these functions are thought to be modulated by OA (and, to a lesser extent, TA), as described below (Adamo & Baker, 2011; Farooqui, 2012; Pflüger & Stevenson, 2005; Roeder, 1999, 2005; Verlinden, 2010).

The physiological effects of the catecholamines are mediated by a suite of highly specific G protein-coupled receptors (GPCRs). At least five subtypes of dopaminergic receptors have been identified, designated as D_1-D_5 in both vertebrates and invertebrates. These receptors are classified into two broad families, based on the identities of their associated G proteins: the D_1-like receptor family, which includes subtypes D_1 and D_5, and the D_2-like family, which contains the D_2, D_3, and D_4 subtypes (Beaulieu & Gainetdinov, 2011; Vallone et al., 2000). Two families of adrenergic receptors, which bind both NE and E with varying affinities, have also been identified on the basis of their pharmacological properties. The α adrenergic family contains two known receptor subtypes (α_1 and α_2), whereas the β adrenergic family contains three (β_1, β_2, β_3) (Bylund, 2007; Perez, 2006).

1.3.2 I Phenolamines

TA and OA are structurally similar to the catecholamines, with the exception of a phenol moiety in place of the catechol; for this reason, they are classified as phenolamines (Farooqui, 2012; Gallo et al., 2016; Roeder, 1999,

2005). Like catecholamines, the phenolamines are derived from tyrosine, but via a completely separate metabolic pathway (Figure 1.2 A). The enzyme tyrosine decarboxylase (TDC) catalyzes the conversion of tyrosine to TA, which can be further converted to OA via tyramine β-hydroxylase (TβH). OA and TA are synthesized in trace quantities in the Vertebrata (Berry, 2004; Borowsky et al., 2001; Farooqui, 2016; Grandy, 2007; Zucchi et al., 2006), but physiologically relevant quantities of these phenolamines are only known from invertebrate taxa (Adamo, 2008; Blenau & Baumann, 2001; Farooqui, 2012; Gallo et al., 2016; Pflüger & Stevenson, 2005; Roeder, 1999, 2005; Verlinden, 2010; Walker et al., 1996).

OA has a multitude of modulatory functions in arthropods and other invertebrates, many of which mirror the roles of NE in the vertebrates; as such, OA is sometimes referred to as the phenol analogue of NE. Hormonal release of OA occurs during the acute stress response of many invertebrates, and can modulate aspects of metabolism, locomotion, aggression, and escape behaviors in stressed animals (Adamo & Baker, 2011; Adamo et al., 1995; Davenport & Evans, 1984; Dierick, 2008; Li et al., 2016; Mentel et al., 2003; Orchard et al., 1993). Beyond the stress response, OA directly modulates sensory processing across multiple modalities (e.g., by adjusting the sensitivity of sensory receptors) (de Haan et al., 2012; Erber et al., 1993; Farooqui et al., 2013; Longden & Krapp, 2010; Spivak et al., 2003; Suver et al., 2012; Widmer et al., 2005), and is involved in the modulation of visual and olfactory learning and memory, often

acting in an antagonistic manner to DA (Hammer & Menzel, 1998; Mizunami & Matsumoto, 2017; Schwaerzel et al., 2003; Unoki et al., 2005, 2006; Zhukovskaya & Polyanovsky, 2017). OA is also known to be involved in the initiation and modulation of rhythmic motor patterns such as insect flight and crawling (Duch & Pflüger, 1999; Martínez-Rubio et al., 2010; Orchard et al., 1993; Selcho et al., 2012; Wong & Lange, 2014). These functions of OA, and many others, have been reviewed in great detail by Farooqui (2012), Pflüger & Stevenson (2005), and Verlinden et al. (2010). In contrast, relatively little is known about the functional roles of TA, as TA has long been considered to be a physiologically irrelevant intermediate product of OA synthesis. It is only relatively recently that TA has begun to be examined as a potent neuromodulator in its own right (Roeder, 2005; Roeder et al., 2003). Several lines of research indicate that TA can modulate locomotory patterns and behaviors in insects, often acting antagonistically to the modulatory effects of OA (Ma et al., 2015; Pflüger & Duch, 2011; Roeder, 2005; Roeder et al., 2003; Saraswati et al., 2004; Selcho et al., 2012). In the nematode *Caenorhabditis elegans*, TA appears to play important roles in modulating motor programs independently of OA, including several key escape responses (Alkema et al., 2005; Donnelly et al., 2013). These studies underscore the potentially widespread physiological actions of TA in invertebrates, which warrant future investigation.

Currently, there are three major families of octopaminergic receptors, following the revised classification framework of Evans and Maqueira (2005).

Two of these families, the α-adrenergic-like OA receptors (OA-αRs) and β-adrenergic-like OA receptors (OA-βRs), are named for their structural similarities to the adrenergic receptors of vertebrates. The third family, the so-called octopamine/tyramine (Oct/Tyr Rs) receptors, is capable of binding both OA and TA, and these receptors are also structurally similar to α-adrenergic receptors (Bayliss et al., 2013; Evans & Maqueira, 2005; Ohta & Ozoe, 2014). When they bind to TA, the Oct/Tyr Rs are sometimes called tyramine-1 receptors. In addition, two other families of invertebrate tyraminergic receptors are known (tyramine-2 and tyramine-3), both of which are much more highly selective for TA over OA (Bayliss et al., 2013; Cazzamalli et al., 2005; Ohta & Ozoe, 2014). Notably, these tyraminergic receptor families do not appear to be homologous with the trace amine-associated receptors (TAARs) that can bind trace TA in vertebrates (Bayliss et al., 2013; Zucchi et al., 2006). As is true of other aminergic receptors, all of the known OA and TA receptors are GPCRs.

1.3.3 I Serotonin (5-Hydroxytryptamine)

The indoleamine 5-HT is derived from the amino acid tryptophan, via the metabolic pathway shown in Figure 1.2 B (Roberts & Fitzpatrick, 2013; Vleugels et al., 2014). Tryptophan is initially converted to the intermediate product 5-hydroxytryptophan (5-HTP) via the enzyme tryptophan hydroxylase (TPH), and 5-HTP is subsequently converted to 5-HT by 5-HTP decarboxylase. Like DA, 5-HT is a ubiquitous signaling molecule, and is thought to be present in all animal

groups that possess a CNS (Vleugels et al., 2014; Walker et al., 1996; Weiger, 1997).

The myriad functions of 5-HT have been extensively documented, and include modulation of locomotion (Crisp & Mesce, 2003; Evans & Myers, 1986; Hardaker et al., 2001; McClellan et al., 1994; Schmidt & Jordan, 2000), learning and memory (Cassel, 2010; Shomrat et al., 2010; Sitaraman et al., 2012), appetite and feeding behaviors (Campanella et al., 2009; Dacks et al., 2003; French et al., 2014; Lent et al., 1989; Novak & Rowley, 1994), sexual behaviors (Dias and Crews, 2006; Hull et al., 2004; Lee et al., 2001; Olivier et al., 2010), aggression (Johnson et al., 2009; Kravitz, 2000; Zubizarreta et al., 2012), gregarious and social behaviors (Anstey et al., 2009; Bubak et al., 2016; Edsinger & Dölen, 2018), chemosensation (Chao et al., 2004; Dacks et al., 2008; Petzold et al., 2009), and sleep (Iwasaki et al., 2018; Yuan et al., 2006). Underlying these effects are at least eight distinct families of serotonergic receptors, which are designated as 5-HT_1-5-HT_8 (Hoyer et al., 2002; Tierney, 2018; Vleugels et al., 2014). Within these families are many different receptor subtypes. Serotonergic receptors, like those for other biogenic amines, are GPCRs, and their structures are highly conserved across animal taxa.

1.3.4 I Histamine

HA is synthesized directly from the amino acid histidine via the enzyme histidine decarboxylase (HDC), as shown in Figure 1.2 C (Elias & Evans, 1983;

Leurs et al., 2012). Like DA and 5-HT, HA appears to be present in the CNS of both vertebrates and invertebrates (Elias & Evans, 1983; Haas et al., 2008; Nässel, 1999; Walker et al., 1996). HA acts as a key signaling molecule in many sensory systems, particularly in the Arthropoda. It is the primary neurotransmitter in arthropod photoreceptors, and thus plays important roles in visually-guided behaviors (Melzig et al., 1996; Nässel, 1999; Orona & Ache, 1992; Stewart et al., 1997; Stuart et al., 2007). HA is also involved in aspects of chemosensation (Del Rio et al., 2008; Sachse et al., 2006; Wachowiak, 2002), mechanoreception (Fabian & Seyfarth, 1997; Ikoma et al., 2006; Melzig et al., 1996), and auditory processing (Skiebe et al., 1990) across a diverse array of taxa. In addition, HA is an important modulator of various homeostatic processes, including wakefulness (Haas et al., 2008; Oh et al., 2013; Panula & Nuutinen, 2013; Parmentier et al., 2002), appetite (Morimoto et al, 2001), thermoregulation (Hong et al., 2006; Lundius et al., 2010), and metamorphosis (Jin et al., 2014; Sutherby et al., 2012), among others. In vertebrates, the physiological effects of HA are mediated by four families of GPCRs (H_1-H_4), the structure and pharmacology of which are well-characterized (Hill et al., 1997; Hough, 2001). Much less is known about the histaminergic receptors of invertebrates— multiple HA-gated chloride ion channels have been identified in insect photoreceptors (Gisselmann et al., 2002; Stuart et al., 2007), but metabotropic HA receptors (e.g., GPCRs) have yet to be definitively identified in invertebrates (Roeder, 2003).

1.4 I Unanswered Questions

Detailed morphological studies of the CNS of chelicerates are relatively rare, especially in relation to the corpus of neuroanatomical research on other arthropod groups. In the Araneae, for example, much of what we know about the structural organization of the CNS is derived from early neuroanatomical treatments of just two taxa— the tarantula *Poecilotheria* (Theraphosidae) (Babu 1965, 1969) and the wandering spider *Cupiennius salei* (Trechaleidae) (Babu & Barth, 1984; Strausfeld et al., 1993). In recent years, these seminal works have been supplemented by a small number of additional investigations, including the comparative study of Long (2016) and the microCT analyses of Steinhoff et al. (2017), but there still remains a need for further structural studies of the CNS in additional spider species. In the Scorpiones, our knowledge of CNS structure stems largely from a single anatomical study on the forest scorpion *Heterometrus* (Scorpionidae) (Babu, 1965). Descriptions of the CNS in the other chelicerate orders are equally scarce or, in the case of several smaller taxa, completely lacking. For a comprehensive synthesis of current knowledge about the structural organization of chelicerate CNSs, the reader is referred to a series of excellent recent reviews by Brenneis (2016) on the Pycnogonida, Battelle et al. (2016) on the Xiphosura, Wolf (2016) on the Scorpiones, and Lehmann et al. (2016) on the Araneae and other non-scorpion arachnids. Additionally, pertinent morphological details of the CNS in spiders and scorpions are discussed later in this dissertation, in Chapters 2 and 3, respectively.

Published accounts of biogenic amines in the Chelicerata are similarly sparse and are limited to a very small subset of chelicerate taxa. Indeed, the majority of these studies have been conducted in just two species—the horseshoe crab *Limulus polyphemus* (Xiphosura: Limulidae) and the spider *Cu. salei*, whose serotonergic systems (Batelle et al., 1999; Chamberlain et al., 1986; Harzsch, 2004; Harzsch et al., 2005; Seyfarth et al., 1990; Washington et al., 1994), octopaminergic systems (Batelle et al., 1999; Lee & Wyse, 1991; Seyfarth et al., 1993), and histaminergic systems (Batelle et al., 1991; Batelle et al., 1999; Harzsch et al., 2005; Schmid & Becherer, 1999; Schmid & Duncker, 1993) have been revealed via immunocytochemistry. Immunolabeling studies of biogenic amines in other chelicerates are restricted to isolated reports of 5-HT in the Pycnogonida (Brenneis & Scholtz, 2015), Scorpiones (Harzsch, 2004; Wolf & Harzsch, 2012), Opiliones (Breidbach & Wegerhoff, 1993), and Parasitiformes (Hummel et al., 2007; Lees & Bowman, 2007). This relative dearth of chelicerate research stands in stark contrast to the abundant volumes of immunolabeling studies performed in other arthropod groups, such as the Pancrustacea, whose aminergic systems have been much more thoroughly described across a substantially broader array of representative species (e.g., in insects: Blenau et al., 1999; Hamanaka et al., 2012; Haselton et al., 2006; Hörner et al., 1996; Kononenko et al., 2009; Mesce et al., 2001; Monastirioti et al., 1995; Nässel, 1988; Nässel & Elekes, 1992; Seid et al., 2008; Sinakevitch et al., 2005; Stemme et al., 2017; Thamm et al., 2017; in crustaceans: Antonsen & Paul, 2001; Beltz,

1999; Cournil et al., 1994; Fingerman et al., 1994; Hartline & Christie, 2010; Kress et al., 2016; Sayre & Strausfeld, 2019; Semmler et al., 2008; Stegner & Richter, 2011; Stemme et al., 2013; Thompson et al., 1994; Tinikul et al., 2016).

Thus, there is a pressing need for additional studies of aminergic systems in the Chelicerata. The catecholamines, in particular, warrant special attention, as there are currently no published reports of the distribution of catecholaminergic neurons in any chelicerate species. Given the multitude of important functions that DA is known to have in other arthropods, it is a likely candidate to modulate many of the fascinating and important behaviors that chelicerates exhibit. It is therefore rather surprising that their catecholaminergic system remains uncharacterized. The distribution of tyraminergic neurons is also not known in any chelicerate taxon, which is perhaps less surprising because TA has only recently been recognized as a major signaling molecule. Nevertheless, the emerging evidence that TA is an important modulator of multiple behaviors calls for detailed studies of tyraminergic systems in the Chelicerata (and other invertebrates). Furthermore, although the distributions of the other major amines (OA, 5-HT, and HA) have previously been described for several chelicerate species, those species represent a very small fraction of the astounding diversity within the Chelicerata. Presently, it is not feasible to conclude whether the distribution patterns of biogenic amines observed in that small handful of taxa are truly representative of the Chelicerata as a whole. As such, there is a broad need

for comparative studies to characterize the distributions of multiple biogenic amines across a greater diversity of chelicerate taxa.

It is also noteworthy that recent research has begun to question the long-standing notion that adrenergic signaling systems are unique to the vertebrates. Bauknecht & Jékely (2017) have identified distinct adrenergic receptors in the genomes of multiple marine invertebrates, including members of the Xenocoelomorpha, Priapulida, Annelida, Mollusca, Hemichordata, and Tunicata. Notably, these taxa are not closely related to one another, but are instead widely distributed throughout the phylogenetic tree of bilaterian animals, as shown in Figure 1.3 (tree topology adapted from Cannon et al., 2016; Giribet & Edgecombe, 2017; Marlétaz et al., 2019; Simakov et al., 2015). This finding raises a very interesting possibility— i.e., that adrenergic signaling is not an autapomorphy of the Vertebrata (as previously suggested), but is rather an evolutionarily ancient phenomenon that originated in the common ancestor of the Bilateria, and which may have been subsequently lost in a subset of invertebrate lineages. In addition, Sukumar et al. (2018) recently identified adrenergic receptors in the spider *Cu. salei*, which suggests that adrenergic signaling may be retained within the Chelicerata as well. However, endogenous NE has not yet been detected in any chelicerate species, and the potential physiological effects of NE on the functioning of chelicerate CNSs have not yet been examined. Such studies are necessary to test the hypothesis that adrenergic signaling systems are, indeed, present in the Chelicerata.

1.5 I Chapter Summaries

My dissertation directly addresses many of these open questions about aminergic systems in the Chelicerata. In Chapter 2, I present a detailed anatomical characterization of catecholaminergic neurons in the CNS of the wolf spider *H. lenta*, which I achieved by using a highly specific antiserum against the rate-limiting enzyme in catecholamine synthesis (TH). For comparative purposes, I also examined several aspects of the catecholaminergic system in a second species, the jumping spider *P. regius*. This chapter constitutes the first study of catecholamine-synthesizing neurons in any chelicerate taxon, thus filling a conspicuous gap in our knowledge of arthropod biology. I describe the dense innervation by catecholaminergic fibers in several key regions of the spider visual system, including sites of early visual processing and higher-order integration centers, as well as an extensive collection of catecholaminergic arborizations in the neuromeres of the appendages. Furthermore, I identify multiple populations of catecholaminergic neurons throughout the CNS, including cells in the opisthosomal neuromeres that give rise to striking plurisegmental fiber tracts. Based on these findings, I propose several hypotheses regarding the functions of catecholamines in spiders, including the possible modulation of visually-guided behaviors, sensorimotor integration, and motor patterning.

My studies of catecholaminergic neurons in *H. lenta* and *P. regius* complement previously published reports of OA, HA, and 5-HT in *Cu. salei*, and help paint a more complete picture of aminergic systems in spiders. In Chapter 3,

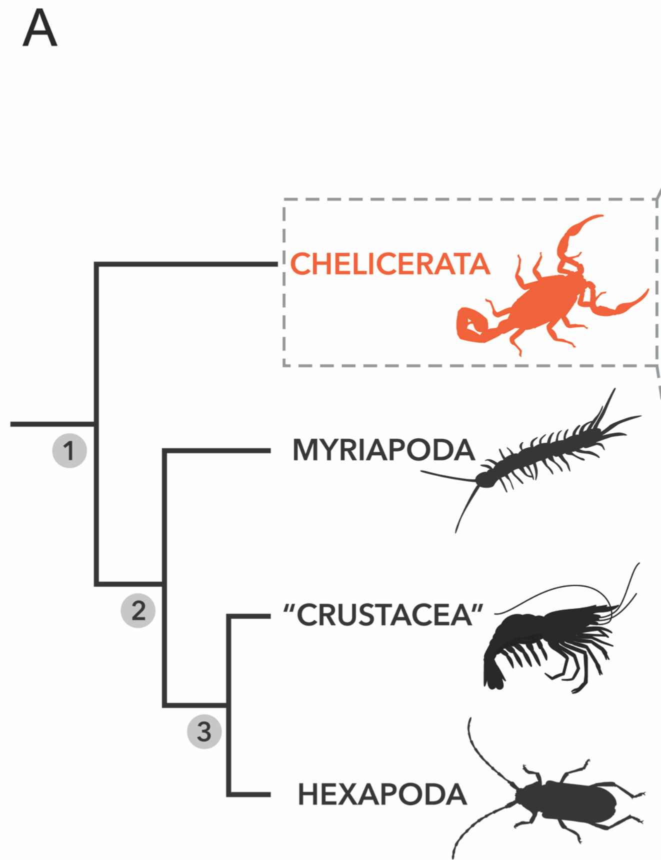
I build upon these studies even further, by conducting a comparative analysis of multiple biogenic amines in another chelicerate lineage— the Scorpiones. In particular, I report the anatomical distributions and projection patterns of catecholaminergic, octopaminergic, and serotonergic neurons in the ventral nerve cord (VNC) of the bark scorpion *C. sculpturatus*. These studies are the first to localize catecholamines and OA in any scorpion species, and greatly expand upon the limited accounts of 5-HT in scorpions. Among the structures highlighted in this chapter are conspicuous clusters of large, efferent catecholaminergic neurons that directly innervate peripheral respiratory structures. Potential functions of these neurons, as well as the others that I have identified, are discussed in relation to the known roles of catecholamines, OA, and 5-HT in other organisms.

In Chapter 3, I also test the hypothesis that NE is present and physiologically active in addition to DA and OA in the scorpion, using a comprehensive approach that integrates analytical chemistry, histology, bioinformatics, and electrophysiology. I report appreciable amounts of all three amines from the CNS of *C. sculpturatus* (as well as *H. lenta*) via ultra-performance liquid chromatography and mass spectrometry. Using NE immunolabeling, I found that adrenergic neurons are absent in the VNC of *C. sculpturatus*, indicating that the catecholaminergic neurons observed are truly dopaminergic. However, NE-synthesizing cells were observed in the supraneural lymphoid glands attached to the VNC, and thus I propose a new function for

these structures as secretory organs, which may be involved in paracrine and/or hormonal release of NE in scorpions. I also report the presence of distinct adrenergic, dopaminergic, and octopaminergic receptors in the genome of *C. sculpturatus*, and present evidence that DA, NE, and OA can elicit distinct patterns of electrical activity in the telsonic nerve of the scorpion. Based on these multiple lines of evidence, I conclude that NE is an endogenous signaling molecule in *C. sculpturatus*. The broad implications of this discovery are discussed in relation to previously reported notions about the evolution of aminergic signaling in the Bilateria.

Finally, in Chapter 4, I briefly summarize the major findings of my research, and discuss how they may inform future investigations into the functional roles of biogenic amines in the Chelicerata.

1.6 | Figures



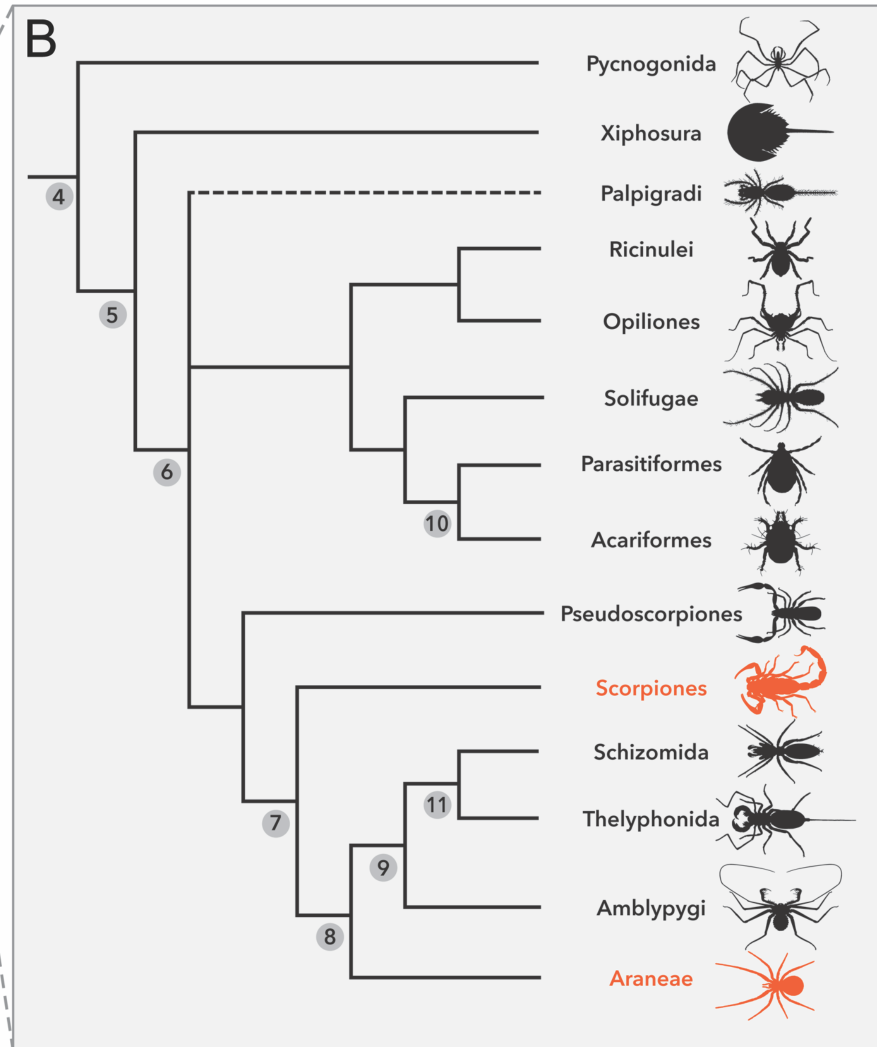
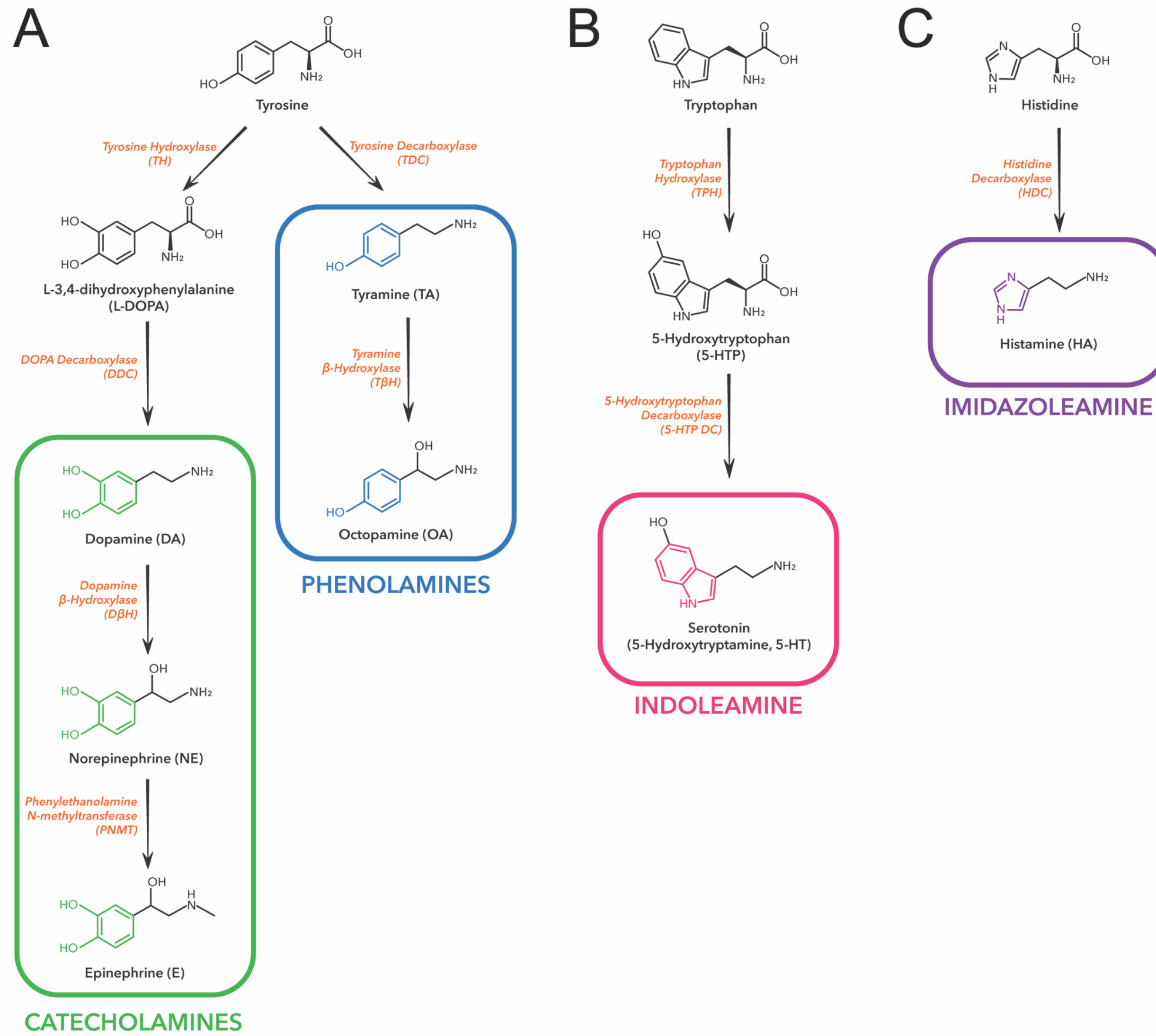


Figure 1.1. Phylogenetic placement of the Chelicerata within the Arthropoda and the relationships among chelicerate orders. **A**. Phylogenetic tree highlighting the evolutionary relationships of the major extant subdivisions of the Arthropoda, modeled after Rota-Stabelli et al. (2011) and supported by many other analyses. Numbered nodes are as follows: 1= Arthropoda, 2= Mandibulata, 3= Pancrustacea. The Chelicerata are consistently recovered as the most basal group of arthropods, sister to the Mandibulata, in numerous studies. The Pancrustacea (= crustaceans + Hexapoda) was once a controversial taxon, but is now widely accepted as a monophyletic group; the "Crustacea" (excluding hexapods) is paraphyletic. **B.** Phylogenetic relationships among the extant chelicerate orders, as revealed by the analysis of genome-scale datasets by Lozano-Fernandez et al. (2019). Numbered nodes are as follows: 4= Chelicerata, 5= Euchelicerata, 6= Arachnida, 7= Arachnopulmonata, 8= Tetrapulmonata, 9= Pedipalpi, 10= Acari, 11= Uropygi. The two orders examined in the present study (Araneae and Scorpiones) are highlighted; both belong to the taxon Arachnopulmonata, which is well-supported by numerous analyses. The Tetrapulmonata, Pedipalpi, and Uropygi are also well-supported; most other supraordinal relationships remain controversial. The position of the Palpigradi (dashed line) is currently unresolved due to limited data.



A
Tyrosine
Tyrosine Hydroxylase (TH)
Tyrosine Decarboxylase (TDC)
L-3,4-dihydroxyphenylalanine (L-DOPA)
DOPA Decarboxylase (DDC)
Dopamine (DA)
Dopamine β-Hydroxylase (DβH)
Norepinephrine (NE)
Phenylethanolamine N-methyltransferase (PNMT)
Epinephrine (E)
CATECHOLAMINES
Tyramine (TA)
Tyramine β-Hydroxylase (TβH)
Octopamine (OA)
PHENOLAMINES
B
Tryptophan
Tryptophan Hydroxylase (TPH)
5-Hydroxytryptophan (5-HTP)
5-Hydroxytryptophan Decarboxylase (5-HTP DC)
Serotonin (5-Hydroxytryptamine, 5-HT)
INDOLEAMINE
C
Histidine
Histidine Decarboxylase (HDC)
Histamine (HA)
IMIDAZOLEAMINE

Figure 1.2. Biosynthetic pathways of the seven major biogenic amines. **A.** Synthesis of amines derived from tyrosine. Tyrosine is converted to L-L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH). Dopamine (DA) is synthesized from L-DOPA via DOPA decarboxylase (DDC), and can be converted to norepinephrine (NE) via dopamine β-hydroxylase (DβH). Phenylethanolamine N-methyltransferase (PNMT) synthesizes epinephrine (E) from NE. Collectively, DA, NE, and E are known as the catecholamines on the basis of a shared catechol moiety (highlighted). Notably, TH is the rate limiting enzyme in catecholamine synthesis. Tyrosine also serves as the precursor to the phenolamines via a separate synthetic pathway. Tyrosine decarboxylase (TDC) converts tyrosine into tyramine (TA), which can be further converted to octopamine (OA) via tyramine β-hydroxylase (DβH). TA and OA are known as the phenolamines because they share a phenol moiety (highlighted in blue). **B.** Synthesis of serotonin (5-hydroxytryptamine or 5-HT) from tryptophan. Tryptophan is converted to 5-hydroxytryptophan via the enzyme tryptophan hydroxylase (TPH), and can be converted to 5-HT via 5-hydroxytryptophan decarboxylase (5-HTP DC). 5-HT belongs to a class of compounds known as the indoleamines, on the basis of its indole moiety (highlighted). **C.** Synthesis of histamine (HA) from histidine. This reaction is catalyzed by the enzyme histidine decarboxylase (HDC). The presence of an imidazole moiety (highlighted) signifies that HA is an imidazoleamine.

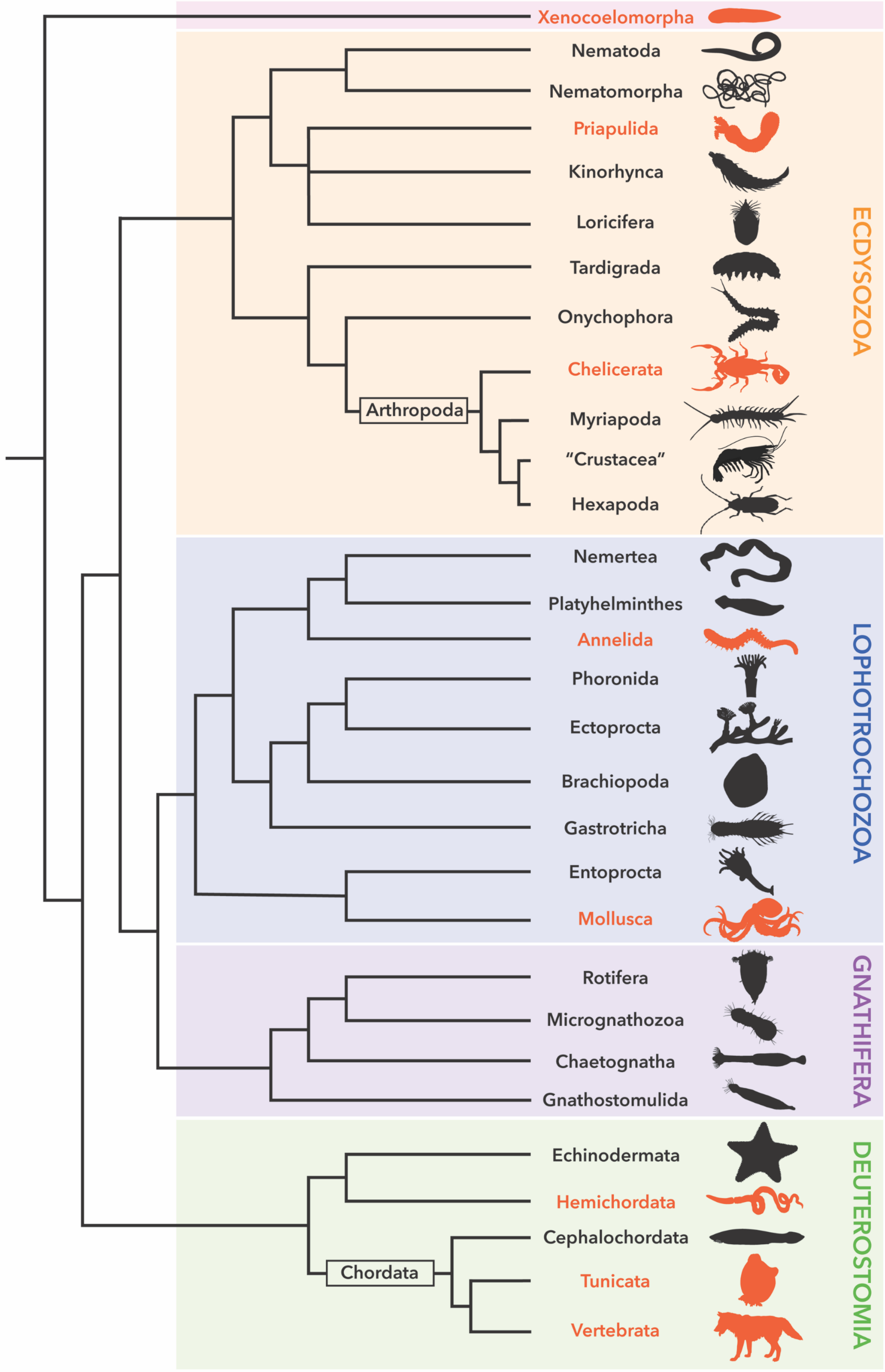

Xenocoelomorpha
Nematoda
Nematomorpha
Priapulida
Kinorhynca
Loricifera
Tardigrada
Onychophora
Chelicerata
Arthropoda
Myriapoda
"Crustacea"
Hexapoda
ECDYSOZOA
Nemertea
Platyhelminthes
Annelida
Phoronida
Ectoprocta
Brachiopoda
Gastrotricha
Entoprocta
Mollusca
LOPHOTROCHOZOA
Rotifera
Micrognathozoa
Chaetognatha
Gnathostomulida
GNATHIFERA
PROTOSTOMIA
Echinodermata
Hemichordata
Cephalochordata
Chordata
Tunicata
Vertebrata
DEUTEROSTOMIA

Figure 1.3. Phylogenetic tree showing the relationships among the phyla of Bilateria. Tree topology is a synthesis of the topologies reported by Cannon et al. (2016), Giribet and Edgecombe (2017), Marlétaz et al. (2019), and Simakov et al. (2015). Excluded from the tree are several minor phyla for which phylogenetic data are unavailable. Subphyla are shown for the Arthropoda and Chordata. Highlighted taxa contain at least one species for which distinct adrenergic receptors have been identified (based on the findings of Bauknecht & Jékely, 2017; Sukumar et al., 2018). Notably, these taxa are widely dispersed throughout the Bilateria.

CHAPTER 2

Tyrosine hydroxylase immunolabeling reveals the distribution of catecholaminergic neurons in the central nervous systems of the spiders *Hogna lenta* (Araneae: Lycosidae) and *Phidippus regius* (Araneae: Salticidae).

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2.1 I Synopsis

With over 48,000 species currently described, spiders (Arthropoda: Chelicerata: Araneae) comprise one of the most diverse groups of animals on our planet, and exhibit an equally wide array of fascinating behaviors. Studies of central nervous systems (CNSs) in spiders, however, are relatively sparse, and no reports have yet characterized catecholaminergic (dopamine- or norepinephrine-synthesizing) neurons in any spider species. Because these neuromodulators are especially important for sensory and motor processing across animal taxa, we embarked on a study to identify catecholaminergic

neurons in the CNS of the wolf spider *Hogna lenta* (Lycosidae) and the jumping spider *Phidippus regius* (Salticidae). These neurons were most effectively labeled with an antiserum raised against tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis. We found extensive catecholamine-rich neuronal fibers in the first- and second-order optic neuropils of the supraesophageal mass (brain), as well as in the arcuate body, a region of the brain thought to receive visual input and which may be involved in higher-order sensorimotor integration. This structure likely shares evolutionary origins with the dopamine-enriched central complex of the Mandibulata. In the subesophageal mass, we detected an extensive filigree of TH-immunoreactive (TH-ir) arborizations in the appendage neuromeres, as well as three prominent plurisegmental fiber tracts. A vast abundance of TH-ir somata were located in the opisthosomal neuromeres, the largest of which appeared to project to the brain and decorate the appendage neuromeres. Our study underscores the important roles that the catecholamines likely play in modulating spider vision, higher-order sensorimotor processing and motor patterning.

2.2 l Introduction

Spiders (Arthropoda: Chelicerata: Araneae) are one of the most diverse groups of organisms on Earth, with over 48,000 species currently described, including many species of medical, economic, and environmental importance (World Spider Catalog, 2019). However, our understanding of how spider

nervous systems are organized and function still lags behind our knowledge of nervous systems in other arthropod groups, such as the Insecta. Recent research suggests that many spider behaviors are highly complex and flexible (Herberstein, 2011); for example, spiders have been shown to exhibit multimodal communication (Elias et al., 2006; Gordon & Uetz, 2011; Uetz & Roberts, 2002; Uetz et el., 2009), maternal care and sociality (Avilés, 1997; Lubin & Bilde, 2007; Whitehouse & Lubin, 2005), spatial navigation (Hoefler & Jakob, 2006; Jackson & Wilcox, 1993a; Nørgaard, 2005), behavioral mimicry (Ceccarelli, 2008; Cushing, 1997; Jackson & Whitehouse, 1986; Jackson & Wilcox, 1993a), and a multitude of versatile hunting, mating, and anti-predator strategies (Blackledge & Wenzel, 2001; Lohrey et al., 2009; Persons & Rypstra, 2001). Certain behaviors known from jumping spiders are exceptionally elaborate and rare among arthropods, and can arguably be considered examples of invertebrate cognition, such as the trial-and-error learning, object categorization, and object permanence exhibited by jumping spiders in the genus *Portia* (Araneae: Salticidae) (Jackson & Cross, 2011; Jackson & Nelson, 2011; Jackson & Wilcox, 1990, 1993a,b; Tarsitano & Jackson, 1994, 1997).

The behavioral complexity and versatility of spiders, like that of other animals, is facilitated by intricate neuromodulatory systems that can finely adjust those behaviors to meet specific environmental demands (Marder, 2012). Although such systems are well described in several arthropod groups, they remain poorly characterized in others—including the subphylum Chelicerata,

which contains the spiders and other arachnids. The Chelicerata occupy a key position at the base of the arthropod phylogeny (Giribet & Edgecombe, 2017; Meusemann et al., 2010; Misof et al., 2014; Reiger et al, 2010; Rota-Stabelli et al., 2011; Schultz, 2007), and thus a better understanding of the neuromodulatory systems in spiders and other chelicerates is critical for understanding the evolution of neuromodulatory systems and the neural bases of behavior in the Arthropoda as a whole.

Among the most important modulators of behavior are the catecholamines, a class of biogenic amines that includes both dopamine (DA) and norepinephrine (NE) (Gallo et al., 2016). DA is universally present in all animal taxa whose nervous systems have been studied and is a vital driver of behavior in other arthropods (Barron et al., 2010; Gallo et al., 2016; Mustard et al., 2010); thus it is a prime candidate to shape some of the complex behavioral patterns that spiders exhibit. NE is synthesized from DA via dopamine β-hydroxylase in vertebrates, and is a major modulator of several vertebrate behaviors; however, NE has long been thought to be absent in invertebrate nervous systems, where the phenol analogue octopamine (OA) is used instead to perform similar functions (Farooqui, 2012; Gallo et al., 2016; Verlinden et al., 2010). Interestingly, recent evidence is challenging this notion that invertebrates lack NE, as receptors for NE have been shown to exist in several invertebrate taxa, including the spider *Cupiennus salei* (Trechaleidae) (Bauknecht & Jékely, 2017; Sukumar et al., 2018). Thus, both DA and NE have the potential to

modulate key behaviors in the spider. The presence of DA in the spider central nervous system (CNS) has been confirmed by radiometric assays and high-pressure liquid chromatography (Meyer et al., 1984; Schmid et al., 1992). Yet, the characterization of dopaminergic or adrenergic neurons (i.e., those that synthesize NE) in the CNS of spiders—or any other chelicerate taxon— remains unreported. The identities and distributions of other key neuroactive substances in the spider CNS have been known for decades, including other biogenic amines such as OA, histamine (HA), and serotonin (5-hydroxytryptamine, 5-HT), as well as other small-molecule neurotransmitters (e.g., acetylcholine, GABA, and glutamate) and a variety of neuroactive peptides (Becherer & Schmid, 1999; Fabian-Fine et al., 2015; Fabian-Fine et al., 2017; Meyer & Poehling, 1987; Schmid & Becherer, 1999; Schmid & Duncker, 1993; Seyfarth et al., 1990; Seyfarth et al., 1993). Thus, it is somewhat surprising that the catecholaminergic neurons of spiders have not yet been described.

In this study, we used immunocytochemical methods in wholemounted and sectioned preparations of the spider CNS to label neurons expressing immunoreactivity to tyrosine hydroxylase (TH, also known as tyrosine-3-monooxygenase), which is the rate-limiting enzyme in catecholamine synthesis (Molinoff & Axelrod, 1971). TH converts the amino acid L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA), which is the direct precursor to DA, and thus numerous studies in insects have used anti-TH labeling as a proxy for the selective labeling of dopaminergic neurons, as DA is not known to be converted

to NE in insects (Gallo et al., 2016). However, since the possibility exists that DA may not be the sole cellular end product of catecholaminergic neurons in the Araneae, we chose to use anti-TH labeling to reveal the comprehensive set of all catecholaminergic neurons, including both dopaminergic and, potentially, adrenergic cells. In doing so, we are able to provide the first anatomical road map to establish where these catecholaminergic neurons reside and project in the spider CNS. Our study serves as a foundation upon which future hypotheses concerning the functional roles of DA (and possibly NE) may be based, and thus is a crucial first step towards illuminating the understudied neurochemical bases of behavioral complexity in the Chelicerata.

2.3 I Materials and Methods

2.3.1 I Study Animals

The spiders used in the majority of experiments were small adult or large juvenile/subadult Floridian wolf spiders, *Hogna lenta* (Lycosidae). Spiders were collected in central Florida between March and November and supplied by Todd Gearhart (tarantulaspiders.com). For comparative purposes, we also utilized adult regal jumping spiders, *Phidippus regius* (Salticidae), which were collected by the same supplier. These taxa were selected because the Lycosidae and Salticidae exhibit complex and fascinating visually-guided behaviors (Herberstein, 2011), which could be modulated in part by catecholamines (as has been shown in other organisms).

Preliminary examinations revealed no discernible differences in TH-immunolabeling between the CNSs of male and female spiders; thus we utilized both male and female specimens for all studies described here. The spiders were individually housed in aerated plastic containers (18 cm diameter) containing approximately 1-2 cm of EcoEarth substrate, and were fed once per week on cockroaches (*Blatta lateralis*), crickets (*Gryllodes sigillatus*), or mealworms (*Tenebrio molitor*). The enclosures were kept at 23-26°C and the substrate was periodically misted with water; this procedure was done to mimic the temperatures and humidity levels of the spiders' native habitat. All spiders selected for dissection were first thoroughly examined to ensure that they showed no signs of poor health or behavioral anomalies.

2.3.2 I Primary Antiserum Characterization

The primary anti-TH antiserum used in our experiments was a mouse monoclonal anti-TH (ImmunoStar, Cat# 22941, RRID: AB_572268). This antibody was generated against TH purified from rat PC12 cells and found not to cross-react with purified phenylalanine hydroxylase or tryptophan hydroxylase (Immunostar, 2019). The manufacturer's specifications indicate that, based on protease chymotrypsin digests of TH, the antiserum recognizes the 34 kDa catalytic core of TH, which shares extensive homology across both invertebrate and vertebrate species. SDS-PAGE and Western blotting confirmed that only HEK293 cells transfected to express TH were identified by the 60 kDa

immunolabeled band of the purified TH protein. The use and specificity of this antibody for TH detection has been well-documented in a wide array of organisms. As of early 2019, 1,561 references have reported using this particular antiserum (CiteAb 22941, 2019). Below is a small sampling of other organisms in which this antiserum has been successfully used: the fruit fly *Drosophila melanogaster* (Greer et al., 2005; Hartenstein et al., 2017; Nässel & Elekes, 1992), the locust *Locusta migratoria* (Lange & Chan, 2008), the honey bee *Apis mellifera* (Tedjakumala et al., 2017), the crab *Pugettia producta* (Dickinson et al., 2008), the snail *Lymnaea stagnalis* (Voronezhskaya et al., 1999; Wyeth & Croll, 2011), the zebrafish *Danio rerio* (Puttonen et al., 2013), the mouse *Mus musculus* (Heyer et al., 2012; Tóth & Mezey, 2007; Tritsch et al., 2012), the rat *Rattus norvegicus* (Pienaar & van de Berg, 2013; Zhang et al., 2013), and the water buffalo *Bubalus bubalis* (Mirabella et al., 2003), among many others.

Although this TH antibody has been shown to act with high specificity for TH in so many other organisms, we are the first to use it to label TH-synthesizing cells in a spider. Thus, to confirm that the antibody binds specifically to TH in the spider nervous system as well, we performed our own Western blot analysis using *H. lenta* tissue. Briefly, the complete spider CNS (n = 3) was sonicated in Laemmli loading buffer (Bio-Rad, Cat# 161-0737) with a protease cocktail inhibitor (Sigma Aldrich, Cat# 11697498001) for whole-protein extraction. Protein extracts were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (ThermoFisher Scientific, Cat# 88025). The transferred membrane

was blocked for 30 minutes at room temperature in Tris-buffered physiological saline with 5% nonfat dried milk. It was then incubated in a 1:1000 dilution of the primary TH antiserum for 1 hour at room temperature (22-23 ºC), followed by incubation in a 1:10,000 secondary goat anti-mouse antiserum (Promega, Cat# S3721, RRID: AB_430871) for 30 minutes at room temperature. The blots were then developed using 2% NBT/BCIP (Roche, Cat# 11861451001) in AP buffer until bands were visible. A ladder containing fragments of known size (Bio-Rad, Cat #161-0377) was used to estimate the size of bands in the spider. The resulting blot revealed a single labeled band in our spider tissue extracts, approximately 60 kDa in size (Figure 2.1, arrowheads); this band corresponds to the mass of the TH isoform that the TH antiserum is known to bind to in other organisms (Immunostar, 2019). Thus, this result indicates that the antibody does indeed bind specifically to one protein (TH) in *H. lenta* tissue.

To confirm further that our spider tissue did not contain a novel epitope to which the antibody might cross-react, we also conducted protein-to-protein BLASTs to compare the amino acid sequences of THs from several well-established model organisms against the entire proteome of a representative spider. The four model organisms used were *D. melanogaster*, *A. mellifera*, *M. musculus*, and *Da. rerio*. These species were chosen because, as noted earlier, the TH antiserum we used has been used extensively in these animals and has been confirmed to bind very specifically to TH. Furthermore, the full genomes and proteomes of these animal models are readily available via NCBI GenBank

for easy comparison. Because the genomes of *H. lenta* and *P. regius* are presently unavailable, the house spider *Parasteatoda tepidariorum* (Araneae: Theridiidae) was chosen as a representative for these analyses. The genome of *Pa. tepidariorum* has been fully sequenced by Schwager et al. (2017) and deposited into GenBank as part of BioProject PRJNA167405 (Accession # AOMJ00000000). The specific query sequences used in our BLASTs can be accessed in GenBank under the following accession numbers: NP_476898.1 (*D. melanogaster* TH, annotated as *pale* isoform B), XP_006565138.1 (*A. mellifera* TH isoform X1), NP_033403.1 (*M. musculus* TH), and NP_571224.1 (*Da. rerio* TH). These particular sequences were confirmed by the antibody manufacturer to be the exact isoforms to which the antibody is known to bind. The search set for the BLASTs were all non-redundant protein sequences from *Pa. tepidariorium* (taxid: 114398), and the BLAST algorithm used was blastp (protein-protein BLAST). Protein sequences from the BLAST output were considered homologous if they showed an E value below $1x10^{-150}$ with a query cover of 70% or greater. We chose these strict cutoff criteria based on the known levels of sequence similarity among the TH sequences of the four well-established model organisms used in our analyses.

Among all sequences in the *Pa. tepidariorum* proteome, a protein annotated as tyrosine 3-monooxygenase-like protein (i.e. TH) (Accession # XP_015911830.1) consistently showed the highest levels of sequence similarity to the TH proteins from the four species we compared (E= 0.0 against *D.*

melanogaster and *A. mellifera* TH; E= 6x10^{-170} against *M. musculus* TH; E= 4x10^{-165} against *Da. rerio* TH). A second protein, annotated as *Pa. tepidariorum* tyrosine 3-monoxygenase (Accession # XP_015925695.1), also showed high levels of sequence similarity in our analyses (E= 9x10^{-163} against *D. melanogaster* TH; E= 8x10^{-166} against *A. mellifera* TH; E= 8x10^{-157} against *M. musculus* TH; E= 9x10^{-153} against *Da. rerio* TH). Using the cutoff values that we stated previously, these two spider THs are presumed to be homologous to the THs of the four other animals we analyzed, and are the only proteins in the spider proteome that meet the criteria for homology. These data support the idea that spiders have a TH that is highly similar and likely homologous to the THs of other organisms in which our anti-TH antibody has been used with great success and specificity. Taken together, these findings indicate that the TH antiserum we used does, indeed, bind specifically to TH (and only TH) in our spider tissue.

2.3.3 I Dissection and Immunolabeling of Wholemounted Tissue

To localize catecholamine-synthesizing neurons in the spider CNS, we employed a modified version of the anti-TH immunolabeling protocols used previously in our lab (Crisp et al., 2002; Mesce et al., 2018; Mesce et al., 2001; Mustard et al., 2012; Tedjakumala et al., 2017), which have been shown to label DA-containing neurons reliably and specifically in wholemounted preparations of the hawkmoth, honeybee, and medicinal leech. First, the spiders were anesthetized in a -4 ºC freezer for 5-8 minutes. As unfixed spider tissue is

especially fragile, it was necessary to pre-fix the CNS prior to dissection. To accomplish this objective, a solution of freshly made 4% paraformaldehyde in iso-osmotic Millonig's buffer (13 mM NaH_2HPO_4, 86 mM Na_2HPO_4, 75 mM NaCl, pH 7.8) was injected into the anesthetized spiders in both the dorsal prosoma and the dorsal opisthosoma (directly above the dorsal vessel). Following pre-fixation, the entire CNS was dissected out of the spider under a bath of spider saline (recipe adapted from Maier et al., 1987), which was buffered to a pH of 8.0 to match the measured pH of *H. lenta* hemolymph. The dissected tissue was then submerged in 4% paraformaldehyde and fixed for 2.5-3 hours at room temperature (22-23 ºC). The tissue was then subjected to three 10-minute rinses in iso-osmotic Millonig's buffer and placed in a blocking solution (1.5mL Normal Goat serum + 13.5mL of 1% Triton-X Buffer in iso-osmotic Millonig's buffer) overnight at room temperature. Following the blocking procedure, the CNS was incubated in a 1:100 dilution of mouse anti-TH antiserum for 4-7 days at 4º C. Tissues were subjected to a 2-hour rinse in a dilutant solution (a 1:2 dilution of blocking solution in iso-osmotic Millonig's buffer), followed by incubation in a 1:200 Cy3-conjugated goat anti-mouse antiserum (ThermoFisher Scientific, Cat# A10521, RRID: AB_2534030) for 2 days at 4 ºC. Incubation of spider CNS in this secondary antiserum only (without the primary antiserum) yielded no TH-specific labeling (n = 3). Following incubation, the tissue was treated with three 20-minute rinses in iso-Millonig's buffer. It was then dehydrated and cleared via 30-minute treatments in each of the following: 70% EtOH, 80% EtOH, 85% EtOH, 90%

EtOH, 95% EtOH, 100% EtOH, a 1:1 mixture of methyl salicylate in 100% EtOH, and finally pure methyl salicylate. The tissue was then wholemounted between two glass coverslips in DEPEX mounting medium (Electron Microscopy Sciences).

To establish whether DA was the end product of TH synthesis, we labeled a subset of spider CNSs with a variety of commercially available anti-DA antisera. We also performed these anti-DA protocols in the medicinal leech (*Hirudo verbana*) as labeling controls. The leech has well-described DA-synthesizing neurons (Crisp et al., 2002; Mesce et al., 2018), and possesses a highly penetrable CNS that has facilitated strong immunolabeling in the past, especially with previously manufactured anti-DA antisera that are no longer available (e.g., from Diasorin, Stillwater, MN). The leech tissue was treated exactly as the spider tissue, with the exception of the pre-fixation step, which was omitted. We tested three commercial rabbit polyclonal anti-DA antisera (Abcam, Cat# ab6427, RRID: AB_305458; Abcam, Cat# ab8888, RRID: AB_306841; Genetex, Cat #GTX12511), one commercial mouse monoclonal anti-DA antiserum (Genetex, Cat# GTX21001, RRID: AB_384594), and several rabbit polyclonal anti-DA antisera generously provided by the lab of H.W.M. Steinbusch (Steinbusch et al., 1991). Tissue was pre-fixed, dissected, and fixed as described above, using a glutaraldehyde-picric acid (GPA) fixative instead of paraformaldehyde, following a protocol adapted from Crisp et al. (2002) and Spivak et al. (2003). Tissue was incubated for 3-7 days in a 1:100 dilution of

primary antiserum, followed by 1-2 days in secondary antiserum. The secondary antiserum used was either a 1:200 dilution of Cy3-conjugated goat anti-rabbit antiserum (ThermoFisher Scientific, Cat# A10520, RRID: AB_10563288) or a 1:200 dilution of Cy3-conjugated goat anti-mouse antiserum (ThermoFisher Scientific, Cat# A10521, RRID: AB_2534030). Although multiple attempts were made to obtain positive DA immunolabeling in both the leech and spider, we were unsuccessful in the spider even when we achieved positive—albeit very weak— immunolabeling in the leech tissue, which was only recovered using the Steinbusch antiserum (n = 12 spiders; n = 20 leeches). Thus, until we can procure a DA antiserum that can first adequately label the easily accessed and well-characterized dopaminergic neurons in the leech CNS, we opt not to continue sacrificing any additional spiders for this aspect of our study.

2.3.4 I Staining and Immunolabeling of Sectioned Tissue

To visualize additional cellular details that might not be accessed in wholemounted specimens, a subset of *H. lenta* specimens (n = 3) was selected for sectioning and subsequent staining with hematoxylin and eosin (H & E) or immunolabeling with our anti-TH antiserum. Dissection and fixation protocols for these samples were identical to those used in our wholemounted preparations. Following fixation, tissue was rinsed for 3 hours in iso-osmotic Millonig's buffer before being fully dehydrated via a graded ethanol series. Tissue was then immersed three times in a bath of xylene before being embedded in paraffin at

58° C. Paraffin blocks were sectioned to a thickness of 5 μm on a microtome, and mounted on gelatin-coated histological slides. For H & E staining, the tissue was deparaffinized and rehydrated, and the slides then submerged in alum haematoxylin for 4 minutes. Slides were then rinsed and dipped 3 times in 0.3% acid alcohol. Following another rinse, the slides were then stained with eosin for 2 minutes, and then dehydrated, cleared, and mounted. For TH immunolabeling, following deparaffinization, rehydration, and microwave-assisted antigen retrieval, the tissue sections were then incubated in a 1:1,000 dilution of the mouse anti-TH antiserum for 24 hours. The tissue was then rinsed several times and incubated in 1:500 dilution of Cy3-conjugated goat anti-mouse antiserum (ThermoFisher Scientific, Cat# A10521, RRID: AB_2534030) for 24 hours at 4 ºC before being mounted.

2.3.5 I Glyoxylic Acid Histology

To establish that neurons within the spider CNS contain catecholamines, per se, we employed a modification of the glyoxylic acid protocol described by Crisp et al. (2002), de la Torre and Surgeon (1976), and Lent (1982). This amine reaction method reliably results in the somata of catecholamine-containing neurons emitting a dominant blue emission (478-480 nm). In contrast, indolamine-containing (i.e., serotonergic) cells fluoresce yellow (518-526 nm) and decay more rapidly. Due to its lack of a hydroxyl group in the 3 position of the 6-carbon ring, OA does not produce a fluorophore during this reaction process

(Corrodi & Jonsson, 1967; Lindvall & Björklund, 1974). To perform this assay, the opisthosomal neuromeres were removed and carefully flattened out on a glass slide and dried with a hair dryer for a minimum of 10 minutes. We chose to perform this assay only in the opisthosomal neuromeres, because they were the only region of the CNS that was thin enough to be sufficiently flattened and dried for the reaction to proceed; the assay is not feasible in thicker tissues (c.f., preparations of thin embryonic insect tissue in Budnik et al., 1986). A glyoxylic acid solution was freshly prepared by mixing 1 g glyoxylic acid monohydrate, 6.8 g sucrose, and 3.2 g monobasic potassium phosphate in 62.5 ml of distilled water. The pH was adjusted to 7.4 via titration of a 1 M NaOH solution, and the solution was then diluted to a final volume of 100 ml with distilled water. The completely dried slide was briefly dipped 3 times into this glyoxylic acid solution, and then dried in a stream of cool air for 15 minutes before being heated to 92 °C for 3 minutes. The tissue was then cooled to room temperature before being placed in glycerol or cleared in methyl salicylate and mounted on a glass slide under a coverslip in DEPEX mounting medium for immediate viewing.

2.3.6 I Microscopy and Image Analysis

A total of n = 73 mounted *H. lenta* tissue samples yielded TH-immunoreactivity when inspected using a compound fluorescence microscope equipped with a Zeiss Atto Arc HBO 110W mercury vapor lamp. Of those, a total of n = 16 were selected for imaging at the University of Minnesota Imaging

Center on a Nikon Ti2 inverted microscope equipped with an A1 confocal scan head and a Plan Apo lambda 10x, 20x, or 40x dry objective. The excitation wavelength for all Cy3 labeled specimens was 562 nm, and emission was collected at 570-620 nm. The step size for optical sections was 0.95 μm. Laser power and PMT voltage were adjusted for best contrast and images were acquired at 1.2 μs/pixel and 4x averaging to improve SNR. Images were later viewed and analyzed using FIJI (ImageJ) version 2.0.0 (RRID: SCR_002285). When appropriate, the brightness and contrast of the images were increased in Adobe Photoshop CS6 (RRID: SCR_014199); no other manipulations were conducted. These imaging protocols were also used to visualize n = 4 *P. regius* anti-TH preparations and n = 3 sectioned *H. lenta* anti-TH preparations. A total of n = 10 *H. lenta* glyoxylic acid samples were viewed using a DAPI filter set (359 nm excitation and 461 nm emission) on the compound fluorescence microscope, or imaged on the confocal microscope with an excitation wavelength of 405 nm and emission in two channels (425-475 nm and 500-550 nm). H & E preparations were imaged in bright field on a Nikon 90i digital microscope.

2.4 I Results

2.4.1 I Anatomical Orientation and Nomenclature

Before we describe the presence of conspicuous TH-immunoreactive (TH-ir) neurons in *H. lenta*, it is necessary to present briefly the gross organization of the spider body plan and CNS, shown in Figure 2.2 A-B and summarized

diagrammatically in Figure 2.2 C. The CNS of araneomorph spiders consists of a single condensed mass of tissue that results from the complete fusion of ganglia during early neurodevelopment. This is a relatively derived condition compared to the nervous systems of most other arthropods, which more closely resemble the ancestral arthropodan ground plan consisting of a paired ventral nerve cord with an anterior brain and numerous discrete segmental ganglia (Babu, 1965; Babu & Barth, 1984; Schmidt-Rhaesa et al., 2015; Strausfeld et al., 2006; Weygoldt, 1985; Whitington & Mayer, 2011). In spiders, the esophagus passes through the central mass of the CNS, thereby dividing it into two broad regions that have been termed the supra- and sub-esophageal masses (Babu & Barth, 1984). Despite shared terminology, these regions do not strictly correspond to the supraesophageal and subesophageal ganglia of insects. Within each of these regions are several distinct substructures, the functions of which are only just beginning to be understood. The nomenclature we used to describe such substructures was based on that of Babu and Barth (1984). Examples of such structures include the corpora pedunculata and the arcuate body of the supraesophageal mass, which share several structural similarities to the mushroom bodies and central complex of insects, respectively (Babu & Barth, 1984; Barth, 2002).

2.4.2 I The Supraesophageal Mass

Robust and extensive TH-immunoreactivity was observed within the supraesophageal mass (Figure 2.2 B). Several of the more noteworthy structures are outlined below.

2.4.2.1 I First- and Second-Order Optic Neuropils

The anterior-most region of the supraesophageal mass, which lies directly posterior to the optic nerves, displayed relatively bright TH-ir processes. Within this region, the immunolabeling revealed four distinct, bilaterally symmetrical pairs of neuropils (Figure 2.3 A-E), the location and gross morphology of which corresponded to the first optic neuropils (ON1s) described for *Cu. salei* by Strausfeld et al. (1993) and reviewed by Barth (2002). The eight identifiable neuropils that we detected appear to correspond to each of the eight eyes, as consistent with the hypothesis of Strausfeld et al. (1993) that photoreceptors from each eye transmit information directly to a distinct ON1. The eight ON1s were arranged in a close cluster and did not appear to be connected to each other through their labeled processes. We were unable to identify which ON1s were associated with each of the eight eyes, but future studies could trace the path of each eye from the photoreceptors to their respective first-, second-, and third-order optic neuropils.

The gross morphology of each ON1 was similar (Figure 2.3 A-E). In transverse and sagittal sections, they were roughly boomerang-shaped, and appeared as domed mushroom cap-shaped structures in 3D. Within each ON1,

we found numerous cuboid-like structures approximately 3 μm in diameter (Figure 2.3 B, arrows) associated with dense bundles of TH-ir fibers that accounted for the majority of the TH-labeling in the ON1s. The fibers were arranged in a parallel fashion, running anterior to posterior, which gave the ON1s a distinctly striated appearance. Numerous bright, smaller punctate TH-ir structures, less than 1 μm in diameter, were also found along the length of each of these fibers (Figure 2.3 B, arrowheads). Both of these smaller and larger structures were presumed to be synaptic boutons. Similar substructures were detected in all eight of the ON1s.

In addition to the ON1s, TH-immunoreactivity was observed in the second optic neuropils (ON2s) (Figure 2.3 D), though it was not as conspicuous as the TH-labeling in the ON1s. There were four pairs of ON2s, corresponding to the four pairs of ON1s (again, corresponding to the four pairs of eyes). The gross morphology of the ON2s was similar to that of the ON1s, although they were generally smaller in volume. ON2s, like the ON1s, were also striated in their appearance and contained parallel TH-ir fibers with conspicuous synaptic boutons. A summary diagram, which highlights the relative positions of the ON1s and ON2s in the spider CNS, is shown in Figure 2.3 F.

We did not detect any TH-ir somata within the optic neuropils, and were unable to identify the cells that give rise to the TH-ir striations in the ON1s and ON2s. We hypothesize that they may arise from local TH-ir interneurons whose

somata were obscured by the dense collection of bright synaptic boutons in these regions (and thus were not visible in our samples).

2.4.2.2 I The Arcuate Body

The arcuate body, alternatively known as the third optic neuropil of the anterior median eyes (AM-ON3), is a large, crescent-shaped unpaired midline neuropil situated posterior to the ON2s in the supraesophageal mass. Our anti-TH protocols revealed extensive labeling of the arcuate body neuropil, highlighting its distinctive multilayered organization; at least five discrete arcuate body layers were visible (Figure 2.4 A). One of the most conspicuous of these layers (designated Layer IV in Figure 2.4 A) contained numerous small, densely packed punctate structures, each measuring between 0.7 and 1.0 µm in diameter, which we presumed to be synaptic boutons (Figure 2.4 A-B, arrows). Associated with these structures were numerous fine processes (Figure 2.4 A-B, arrowheads), presumably the axon terminals extending from the more anterior layers of the arcuate body. Also noteworthy was the presence of six large TH-ir somata (8-9 µm in diameter each), arranged in two triads anterior to the arcuate body (Figure 2.4 A, asterisks). A subset of projections from these cells appeared to feed into the anterior strata of the arcuate body, while other projections appeared to contribute to a large arc-shaped commissure ventral to the arcuate body, which spans nearly the entire length of the supraesophageal mass (Figure 2.4 C). Still other projections from these neurons extended downwards towards

the posterior-most regions of the supraesophageal mass (Figure 2.4 C, arrowheads). The relative positions of these structures within the CNS of *H. lenta* is represented by Figure 2.4 D.

In the jumping spider *P. regius* (Figure 2.5 A), we found similarly high levels of TH-immunoreactivity in the arcuate body and were able to identify many substructures similar to those we observed in *H. lenta*. The jumping spider arcuate body is also comprised of multiple distinct TH-ir layers, although only three layers were visible (as opposed to five in the wolf spider arcuate body) (Figure 2.5 B). Layer IV, which contains the small, densely packed punctate structures assumed to be synaptic boutons, was labeled in the jumping spider arcuate body as well (Figure 2.5 B, arrow). Anterior to the arcuate body were four faintly labeled TH-ir somata (Figure 2.5 C, asterisks), each approximately 6 µm in diameter, which are assumed to be homologous to the paired triads of TH-ir somata associated with the wolf spider arcuate body, on the basis of their positions and projection patterns. The axons of these cells appeared to contribute to commissural fibers (Figure 2.5 C, arrowheads) that lie ventral to the arcuate body and which may be related to the large arc-shaped commissure seen in the wolf spider (Figure 2.4 C). Curiously, a large subset of the commissural fibers in *P. regius* appeared to branch and project downwards to more posterior regions of the supraesophageal mass (Figure 2.5 C, arrows), to a much greater extent than observed in *H. lenta*. Figure 2.5 D shows the relative positions of these structures in the CNS of *P. regius*.

2.4.2.3 I Additional Cells and Projections in the Supraesophageal Mass

In addition to the optic neuropils and the arcuate body, other features of interest in the supraesophageal mass included three distinct populations of TH-ir neurons that lie in more ventral planes (Figure 2.6 A-E). The most dorsal of these populations (designated Group 1 in Figure 2.6 A) is comprised of bilaterally paired clusters of relatively large and bright TH-ir somata that lie along the lateral edges of the supraesophageal mass (Figure 2.6 E, arrowheads). Each cluster contained at least 6 distinct cells, the somata of which measured 7-10 µm in diameter. The projections of some of these cells appeared to extend dorsally and contribute to the large arc-shaped commissure associated with the arcuate body (Figure 2.6 E, arrows).

A separate population of neurons (designated Group 2 in Figure 2.6 A) consisted of paired clusters of smaller TH-ir neurons at an intermediate depth within the supraesophageal mass (Figure 2.6 B, arrowheads). Each of these clusters contained at least 12 distinct cells, the somata of which measured 4-6 µm in diameter. The projections of these neurons were clearly visible in our samples, and appeared to fasciculate and descend to a posterior and dorsal region of the supraesophageal mass (Figure 2.6 B, arrows) before looping back to more anterior regions of the mass (Figure 2.6 C, arrows).

The ventral-most population of neurons (designated Group 3 in Figure 2.6 A) were positioned adjacent to the midline just above the esophagus at the base of the supraesophageal mass. Their somata (Figure 2.6 D, arrowheads) were

arranged in paired clusters as well, with each cluster consisting of 3-4 neurons measuring 7-8 µm in diameter. The axons of these neurons (Figure 2.6 D, arrows) appeared to contribute to a dense plexus of fine TH-ir structures clustered around the esophagus. This region appeared to be a potential site of intense synaptic activity, as numerous and conspicuous synaptic boutons were visible among these processes (Figure 2.6 D). The relative positions of all three of these neuronal populations are summarized diagrammatically in Figure 2.6 F.

2.4.3 l The Subesophageal Mass

Extensive collections of catecholaminergic processes were observed in the subesophageal mass as well. Outlined below are several of the more noteworthy TH-labeled structures we observed.

2.4.3.1 l Plurisegmental Catecholaminergic Fiber Tracts

One of the most striking features of the subesophageal mass was the presence of three intensely-labeled TH-ir fiber tracts that spanned across multiple segmental neuromeres (Figure 2.7 A-C). The most dorsal of these tracts consisted of paired bundles of TH-ir fibers that run longitudinally adjacent to the midline (Figure 2.7 A, arrows). Based on the relative position of these fibers, they may correspond to the mid-central (MC) tract in *Cu. salei* as described by Babu and Barth (1984). The TH-ir fibers of this tract also produced an iterated pattern of robustly labelled commissural tracts, which projected dorsally and laterally in a

rib-like manner and fasciculated into distinct lateral longitudinal branches (Figure 2.7 A, arrowheads). In total, there were six commissures associated with this tract, which correspond to the six pairs of appendages (four pairs of walking legs, 1 pair of pedipalps, and 1 pair of chelicerae). The lateral longitudinal branches of these tracts were found to extend through multiple neuromeres associated with the appendages as well as the opisthosomal neuromeres. Based on the relative position of these lateral longitudinal fibers, they may be part of the mid-dorsal (MD) tract of Babu and Barth (1984). The distal-most branches of these fibers eventually gave rise to a fine meshwork of arborizations that decorate each of the appendage neuropils (Figure 2.7 D, arrows). It is not immediately clear from our images which cell populations contribute to this tract.

At an intermediate depth in the subesophageal mass is a second tract of robustly-labeled TH-ir fibers. This tract appeared to originate from the large (13-15 µm diameter) bilaterally paired somata located in the opisthosomal neuromeres (Figure 2.7 B & E, arrowheads). The axons of these neurons fasciculate to form large ascending bundles of fibers (Figure 2.7 B & E, arrows), which crossed the midline just anterior to the opisthosomal neuromeres (Figure 2.7 B & E, asterisk). These fiber bundles then passed through the medial edges of the appendage neuromeres and continued through the lateral edges of the supraesophageal mass. As they ascend, several of the TH-ir fibers that comprise this tract appear to branch and possibly contribute to the fine arborizations that innervate the appendage neuromeres. Based on the relative position of this tract,

it may correspond to either the central (CT) or centro-lateral (CL) tract described by Babu and Barth (1984).

The ventral-most tract that we observed consist of paired bundles of TH-ir fibers that run longitudinally adjacent to the midline (Figure 2.7 C, arrows), and may correspond to the mid-ventral (MV) tract of Babu and Barth (1984). These fibers that comprise this tract pass through all of the neuromeres associated with the walking legs and pedipalps, and the two sides of the tract appear to fuse in a loop (Figure 2.7 C, dagger) just above the esophagus. Prominent branches (Figure 2.7 C, arrowheads) extend laterally from this tract and project to each of the appendage neuromeres, where they give rise to notably dense collections of arborizations and synaptic boutons (Figure 2.7 C, asterisks). As with the dorsal-most tract, it was not clear from our images which cell populations give rise to fibers of this tract. The relative positions of these three tracts in the spider CNS is summarized by the diagram in Figure 2.7 F.

2.4.3.2 I Cells of the Opisthosomal Neuromeres

The opisthosomal neuromeres were found to contain a vast abundance of TH-synthesizing neurons. Each sample examined contained over 80 TH-ir somata in this region, which we have grouped into ten distinct populations on the basis of their relative positions and morphology. These populations (summarized in Figure 2.8) are numbered in ascending order from the most dorsal (Population 1) to the most ventral (Population 10).

The most dorsal of these populations (designated Population 1 in Figure 2.8 A) consists of four neurons arranged in a bilaterally symmetrical pair of two neurons each. Their somata (Figure 2.8 A, arrowhead 1) measure 9-10 µm in diameter, and their axons (Figure 2.8 A, arrow 1) ascend to more anterior regions of the opisthosomal neuromeres, where they appear to form a conspicuous coiled structure (Figure 2.8 A, asterisk) before descending back to the posterior. A second population of neurons (Figure 2.8 A, arrowhead 2) occurs slightly ventral to Population 1 in a more lateral region of the opisthosomal neuromeres. This population consists of bilaterally paired clusters of at least 6 neurons each, whose somata measure 7-8 µm in diameter. The primary neurites of these somata appear to extend upwards (Figure 2.8 A, arrow 2), indicating that these cells may also produce ascending projections; however, these projections were difficult to resolve in our images. A third population of neurons (Figure 2.8 B, arrowhead 3) consists of bilaterally paired clusters of 3 neurons each, whose somata measure 8-9 µm in diameter and whose primary neurites also appear to ascend (Figure 2.8 B, arrow 3). The fourth population of neurons (Figure 2.8 B, arrowhead 4) lies along the lateral edges of the opisthosomal neuromeres. These cells are relatively large (8-10 µm in diameter) and bright, and are arranged in paired clusters of at least three neurons each. Their axons (Figure 2.8 B, arrow 4) appear to fasciculate and ascend along the lateral margins of the opisthosomal neuromeres. The fifth cell population (Figure 2.8 C, arrowhead 5) consists of paired clusters of at least 4 neurons each which lie just adjacent to

the midline at an intermediate depth within the opisthosomal neuromeres. The somata of these cells measure 6-8 µm in diameter, and their axons (Figure 2.8 C, arrow 5) clearly ascend to more anterior and lateral regions of the opisthosomal neuromeres. The sixth population of neurons (Figure 2.8 C, arrowhead 6) consists of paired clusters of 4 neurons each, which lie lateral relative to Population 5 but at a similar depth within the tissue. The somata of these cells measure 6 µm in diameter, and they also give rise to ascending projections (Figure 2.8 C, arrow 6). The seventh population of neurons (Figure 2.8 C, arrowhead 7) is among the most intensely labeled neurons in the opisthosomal neuromeres. The somata of these cells measure 9-10 µm in diameter, and are situated in bilaterally symmetrical pairs of at least 6 neurons each along the lateral margins of the neuromeres. Like most cells of the opisthosomal neuromeres, their axons (Figure 2.8 C, arrow 7) appear to ascend. More ventrally in the tissue, the eighth population of neurons (Figure 2.8 D, arrowhead 8) consists of four somata arranged in a bilaterally symmetrical pair of two somata each. These cells measure 6 µm in diameter, and their projections were not visible in our images. The ninth population of neurons (Figure 2.8 D, arrowhead 9) consists of a single pair of large somata that sit adjacent to the midline in the posterior region of the opisthosomal neuromere. These neurons are the largest of the opisthosomal neurons, with somata measuring 15 µm in diameter. Their projections (Figure 2.8 D, arrow 9) ascend and contribute to the intermediate plurisegmental tract described earlier (Figure 2.7 B). The dorsal-

most population of neurons (Figure 2.8 D, arrowhead 10) also consists of a single pair of large somata positioned adjacent to the midline; in contrast to Population 9, however, these cells are more anterior and slightly more ventral in the neuromere. The somata of these cells measure 13 µm in diameter, and their axons (Figure 2.8 D, arrow 10) also ascend and join the intermediate plurisegmental tract.

Figure 2.8 E highlights the relative positions of some of these opisthosomal neurons in the spider CNS. In addition to these ten populations of opisthosomal neurons, we also observed numerous randomly distributed TH-ir somata throughout the opisthosomal neuromeres. These neurons did not appear to form ascending projections, and thus are likely intrinsic neurons of these neuromeres.

2.4.3.3 l Additional Cells and Projections in the Subesophageal Mass

Our labeling also uncovered several relatively large (8-10 µm diameter) TH-ir somata in relatively ventral regions of the appendage neuromeres (Figure 2.9 A, arrowheads). Subsets of these neurons were located near the base of the neuromeres, adjacent to the center of the subesophageal mass, whereas others were located along more lateral margins of the neuromeres. Within individuals, these cells did not appear to be arranged in a discernible pattern (e.g. bilaterally symmetrical), although many of them were found in unilateral pairs. The axons of some of these cells (Figure 2.9 A, arrows) appeared to innervate the individual

appendage neuromeres and contribute to the meshwork of fine TH-ir arborizations there (Figure 2.9 A, asterisks). Somata of similar size were also observed in the ventral aspect of the appendage neuromeres in our paraffin-embedded tissue sections (Figure 2.9 B, arrowheads). Although these cells were clearly visible in the sectioned tissue preparations, they and their projection patterns were more easily and completely observed in wholemounted preparations. Our H & E stained sections revealed numerous somata surrounding the appendage neuromeres (Figure 2.9 C, arrowheads), as well as nuclei of partial somata near the base of those neuromeres (Figure 2.9 C, arrows). The TH-ir somata highlighted in Figures 2.9 A-B are in the same general region as the many H & E stained somata in Figure 2.9 C, which highlights that only a relatively small percentage of the somata in this region of the CNS appear to contain TH. A summary diagram highlighting this region of the CNS is presented in Figure 2.9 D.

Lastly, our TH labeling revealed conspicuous serially repeating structural motifs, formed by the commissural fibers of the dorsal TH-ir tract described earlier, which represent the repeated neuromeres of the six paired appendages (chelicerae, pedipalps, and legs) (Figure 2.10 A, arrowheads). Structurally similar motifs were also found within opisthosomal neuromeres, again demarcated by fibers crossing the midline (Figure 2.10 B, arrowheads).

2.4.4 I Glyoxylic Acid Histology

During the glyoxylic acid protocol, catecholamines react with glyoxylic acid, which induces a cyclization of their ethylamine side chains and results in the generation of blue-green fluorescent compounds (Björklund et al., 1972; de la Torre & Surgeon, 1976; Lent, 1982; Lindvall & Björklund, 1974). We observed bright glyoxylic acid-induced, blue-green fluorescence (dominant blue emission, 478-480 nm) in catecholamine-containing neurons residing in the opisthosomal neuromeres using both a fluorescence microscope and by detecting its emission on the spectral confocal microscope. Numerous large somata within the opisthosomal neuromeres were observed to emit the blue-green fluorescence as shown under the confocal microscope in Figure 2.11 A (arrowheads). These somata corresponded in both location and size to some of the ascending populations of TH-ir neurons we observed in the opisthosomal neuromeres (Figure 2.11 B). A summary of these structures, showing their relative positions in the spider CNS, is represented by Figure 2.11 C.

2.5 I Discussion

2.5.1 I Overview

We found that TH-ir neurons are in abundance and richly innervate key regions of the spider nervous system, including those involved in visual and sensorimotor integration, strongly supporting the idea that catecholamines (most likely DA, but possibly NE as well) play vital roles in spider behavior and its modulation. Our TH immunocytochemical protocols resulted in reliable and

intense labeling of neurons and their projection patterns even within deeper tissues of the spider CNS (e.g., the tracts of the subesophageal mass). Wholemounted preparations were favored for imaging over sectioned material because we could more easily observe the three-dimensional structures of labeled neurons while avoiding the time-consuming process of serial-section reconstruction. Direct confirmation that a representative subset of TH-labeled neurons contain catecholamines was demonstrated by a classical histofluorescence method utilizing the glyoxylic acid reaction (Lent, 1982). While not studied as in depth, the CNS of the jumping spider (*P. regius)* was also labeled with the anti-TH antiserum to determine if the general pattern of TH immunolabeling in *H. lenta* was more or less generalizable to other species, which it was (e.g., in the arcuate body, shown in Figures 2.4 & 2.5). Although the precise nature of catecholaminergic modulation of spider behavior remains largely unknown, the anatomical data we have provided here will provide a foundation upon which to formulate specific hypotheses regarding the functional roles of DA (and possibly NE) in the Chelicerata.

2.5.2 l Optic Neuropils 1 and 2

One of the major findings of our study is that the visual system of *H. lenta* is enriched with catecholaminergic processes throughout multiple stages of visual integration. The precise ways in which catecholamines affect visual perception, however, must await future investigations; nevertheless, our results

remain informative especially in the context of previous findings. For example, Schmid and Duncker (1993) have shown strong HA-immunoreactivity in photoreceptors of the trechaleid spider *Cu. salei*, which belongs to the sister family (Trechaleidae) of the Lycosidae and is thus very closely related to *H. lenta* (Piacentini & Ramírez, 2019). The histaminergic projections from these photoreceptors terminate in the first optic neuropils (ON1s) of each eye, which are believed to be the first way-station for visual processing in the spider (Barth, 2002; Strausfeld et al., 1993). Our TH-immunolabeling has shown that the ON1s of each eye also contain a dense meshwork of catecholaminergic processes. The striated arrangement of these fine TH-ir fibers in the ON1 is highly reminiscent of the columnar organization of the afferent histaminergic fibers described by Schmid and Duncker (1993). In conjunction with the observation that TH-ir labeled fibers show numerous and conspicuous synaptic boutons, it is possible that they are synapsing directly onto the histaminergic afferents from the eyes, offering the potential for visual gain control.

In the hoverfly *Eristalis*, octopaminergic inputs to the visual system have been shown to modulate contrast sensitivity (de Haan et al., 2012). The immunolabeling studies of Seyfarth et al. (1993), however, indicate that OA is absent in the visual system of the spider *Cu. salei*; thus, one exciting hypothesis is that the catecholamines in the spider ON1s may perform similar roles to those of OA in the insect visual system. Another possibility is that the catecholaminergic fibers in the ON1s may modulate dark sensitivity, as has been

shown in the visual system of the fiddler crab *Uca pugilator* (Kulkarni & Fingerman, 1986). Indeed, for a nocturnal, visually-guided predator such as *H. lenta*, this type of modulation would be beneficial. Further studies of the spider visual system are needed to test such hypotheses.

2.5.3 I The Arcuate Body

The arcuate body of spiders receives input from the ON1s and ON2s of the principal (i.e., anterior median) eyes; for this reason, the arcuate body is sometimes also referred to as AM-ON3, or the third optic neuropil of the anterior median eyes (Barth, 2002; Strausfeld et al., 1993). The finding that the arcuate body contains extensive innervation by catecholaminergic fibers thus suggests that these amines may not only be important modulators of early visual processing, but also of the later processing of visual and other sensory information.

The arcuate body of spiders bears many structural similarities to the central complex of insects (Homberg, 2008; Loesel et al., 2002; Loesel et al., 2011; Strausfeld et al., 1993). Both structures are relatively large, unpaired midline neuropils in a region of the CNS that corresponds to the protocerebrum. Furthermore, there are striking parallels between the multilayered organizational patterns of both structures. Our study highlights for the first time a third important similarity between the two neuropils; i.e., that both are very rich in catecholaminergic innervation (Pfeiffer & Homberg, 2014). This finding could lend

further support to the idea that the arcuate body of the Chelicerata and the central complex of the Mandibulata share a common evolutionary origin (Homberg, 2008; Loesel et al., 2002; Pfeiffer & Homberg, 2014; Strausfeld et al., 2006). The structural and neurochemical similarities between the two structures make it tempting to posit that they perform similar functions as well. Recent studies have shown that the insect central complex is a remarkably sophisticated structure involved in many visual and motor processes, including visual pattern recognition and memory (Pan et al., 2009), visual spatial memory and place learning (Kuntz et al., 2012; Ofstad et al., 2011), locomotor behaviors (Poeck, et al., 2008; Strauss & Heisenberg, 1993), sleep, arousal, and general activity levels (Liu et al., 2012; Ueno et al., 2012), spatial orientation and goal-directed locomotor behaviors (Harley & Ritzmann, 2010; Ridgel et al., 2007; Ritzmann et al., 2012; Strauss, 2002), and even courtship (Popov et al., 2005). Sites in the spider CNS in which these processes originate and are controlled are unknown, but based on similarities to the central complex, the arcuate body is a prime candidate. In insects, several of these processes—e.g., locomotory behaviors and sleep-arousal cycles—are modulated, at least in part by DA, and could be similarly modulated in spiders as well. Recent advances by Menda et al. (2014) may enable electrophysiological recordings from the arcuate body to be more easily conducted in the near future, thereby allowing researchers to discover its various functions.

2.5.4 | Plurisegmental Catecholaminergic Tracts and Associated Neurons

The three plurisegmental fiber tracts of the subesophageal mass were among the most prominent features detected using our TH-labeling protocols, and shared structural similarities with tracts observed in studies of other spiders. The pioneering work of Babu and Barth (1984), which remains the most comprehensive neuroanatomical treatment of the spider CNS to date, described six major longitudinal tracts in the subesophageal mass of *Cu. salei*. These tracts, in order of the most dorsal to most ventral, are: the mid-dorsal (MD) tract, the mid-central (MC) tract, the central (CT) tract, the centro-lateral (CL) tract, the ventro-lateral (VL) tract, and the mid-ventral (MV) tract. Although we cannot be certain that these same tracts exist in wolf spiders as well, we nevertheless found striking similarities between them and the plurisegmental TH-ir tracts we describe for *H. lenta*, likely due to the close phylogenetic relationship between the two species. The dorsal-most TH-ir tract we observed (Figure 2.7 A) shares many features with the MC tract of *Cu. salei*, with its lateral projections feeding into a structure reminiscent of the MD tract. The intermediate TH-ir tract (Figure 2.7 B) is somewhat more difficult to place, but its position suggests that it could either be related to the CT tract or the CL tract of *Cu. salei*, both of which are at intermediate depths in the tissue. The ventral-most TH-ir tract (Figure 2.7 C) may well be homologous to the MV tract of *Cu. salei*, as both are ventral and relatively close to the midline.

We observed that the lateral branches of the dorsal-most TH-ir tract (potential MD tract of Babu & Barth, 1984) ramified further to innervate the appendage neuromeres (Figure 2.7 D). In *Cu. salei*, the dorsal neuropil of the appendage neuromeres is hypothesized to be primarily motor in function (Babu & Barth, 1984; Barth, 2002); thus, given the dorsal position of these TH-ir arborizations, it is likely that they represent sites of catecholaminergic modulation of motor networks. Indeed, DA is a well-known modulator of locomotion in many other invertebrate taxa (Mesce & Pierce-Shimomura, 2010; Mustard et al., 2010; Puhl & Mesce, 2008), and may performs similar functions in the spider. Schmid and Duncker (1993) found extensive HA-immunoreactivity in the MD tract of *Cu. salei*, the pattern of which closely mirrors the TH-immunoreactivity we observed in the lateral branches of our dorsal-most tract. The dorsal HA-ir fibers of Schmid and Duncker (1993) also formed ipsilateral branches that innervated the dorsal neuropil of the appendage neuromeres, and were also likely involved in the modulation of motor networks. Similar OA-ir structures were found in the MD tract and dorsal appendage neuropils as well (Seyfarth et al., 1993). It is presently unclear if these histaminergic and/or octopaminergic fibers form synapses with the TH-ir fibers we observed in the same regions, but their proximity to each other suggests that they may have a close association. The many parallels and potential interactions between the histaminergic and catecholaminergic systems of spiders, both here and in the visual centers of the supraesophageal mass, warrant future examination, as does the potential

interplay between octopaminergic and catecholaminergic innervation of the subesophageal mass.

The intermediate TH-ir tract is of special interest, as it is the only tract of the three we observed in which we could discern the particular neurons that give rise to it. These neurons (designated as Populations 9 and 10 in Figure 2.8) were also the largest TH-ir somata detected. The function of these large ascending neurons remains unknown, but their long-distance projection pattern suggests that they may modulate populations of cells in the cephalic regions of the CNS and play important motor-related behavioral roles, reminiscent of similar ascending cells in insects (Bidaye et al., 2014; Cardona et al., 2009; Novicki & Weeks, 1995).

The TH-ir fibers of our ventral-most tract do not share notable similarities with any other fibers highlighted by HA, OA, or 5-HT immunolabeling. We did observe, however, that the fibers of our ventral TH-ir tract did directly innervate the ventral regions of the appendage neuropils, which are hypothesized to be primarily sensory centers (Babu & Barth, 1984; Barth, 2002). Thus, the dense collection of TH-ir arborizations and synaptic boutons we observed in these regions (Figure 2.7 C, asterisks) may represent areas of intense modulation of sensory input by catecholaminergic interneurons. Indeed, the appendage neuromeres of the spider CNS, like the thoracic ganglia of insects, are sites where sensory information, especially mechanosensory stimuli from legs, is processed so as to enable the spider to move flexibly through its environment

(Barth, 2002; Tuthill & Wilson, 2016). Our observations raise the possibility that local sensorimotor neural circuits in these neuromeres, comprising afferent inputs, interneurons, and motoneurons, receive paracrine catecholaminergic modulation at multiple stages of sensory and motor processing, as is known in insects and other arthropods (Burrows, 1996). Such central catecholaminergic modulation may complement the extensive efferent neuromodulation that spider mechanoreceptors have previously been shown to receive (Fabian-Fine et al., 2000; Pfeiffer, Torkkeli, & French, 2012; Sukumar et al., 2018; Torkkeli et al., 2012).

2.5.5 I Segmental Organization of the Spider CNS

By characterizing TH-ir neurons in wholemounted tissue, other organizational features of the spider CNS were revealed. In particular, we observed conspicuous serially-repeating TH-ir structural motifs within the subesophageal mass associated with the pedipalps, legs, and opisthosoma (Figure 2.10). The CNS of araneomorph spiders such as *H. lenta* is highly cephalized to form a single mass of tissue in the prosoma, but the TH-ir labeling we observed highlights the developmental origin and segmental nature of the spider CNS that has been well documented in other spider species (Doeffinger et al., 2010; Paese et al., 2018; Stollewerk et al., 2003; Stollewerk et al., 2001). This developmental pattern mirrors the segmental ground plan organization thought to exist in the common ancestor of all arthropods (Schmidt-Rhaesa et al.,

2015; Strausfeld et al., 2006; Weygoldt, 1985; Whitington & Mayer, 2011). We believe that our data and immunocytochemical protocols may provide assistance to others studying the genetic, developmental, and evolutionary origins of body segmentation in spiders.

2.5.6 I Utility and Specificity of the TH Antiserum

The characterization of many key neuroactive compounds in the spider CNS has been known for decades, which stands in sharp contrast to the dearth of DA-related studies in spiders. This absence of previous studies may stem not from a lack of interest, but perhaps from the inherent difficulty of obtaining positive immunolabeling via DA antisera that have been commercially available. Although we tried a number of different DA antisera, we were unsuccessful in our attempts to obtain positive DA immunolabeling in the spider CNS; other researchers may have experienced similar difficulties. The DA antiserum (Diasorin) we used previously to label dopaminergic neurons in insects and annelids is no longer available (Crisp et al., 2002; Mesce et al., 2001). The leech CNS, with its well described DA-containing neurons and readily penetrable nerve cord, served as a positive control for the DA antisera we tested in the spider and confirmed that all of the DA antisera we tested were unreliable. In contrast, the TH antiserum we used here worked exceptionally well in both leech and spider CNS. Our Western blot controls showed that it bound specifically to TH in *H. lenta* tissue, and the protein-to-protein BLASTs indicate that there are no other

potential epitopes in spider tissue to which the antibody could bind. This same antiserum has been shown to strongly label DA-containing neurons in almost 100 different invertebrate and vertebrate taxa. Thus, we are confident that the TH immunolabeling we obtained in the spider accurately and completely reflects the profile of catecholaminergic neurons in the spider CNS. It remains to be determined, however, if any of these neurons convert DA into NE as their end product, which would be highly atypical for an arthropod based on current scientific consensus, but which cannot be ruled out given the presence of α1- and α2-adrenergic receptors in *Cu. salei* (Sukumar et al., 2018). Although we suspect that the vast majority of TH-ir neurons in the spider CNS are dopaminergic, the TH-labeled neurons we have described here will include any potential adrenergic neurons as well, thus providing a more comprehensive road map for future studies of catecholaminergic modulation in the Chelicerata.

2.5.7 I Conclusions

Our study is first to report on the anatomical distribution patterns of catecholamine-synthesizing neurons not only in spiders, but rather in any member of the subphylum Chelicerata. Catecholamines are clearly important neuromodulators of sensory and motor processing, and influence an array of diverse behaviors in invertebrates (e.g., Alekseyenko et al., 2013; Barron et al., 2010; Kulkarni & Fingerman, 1986; Kume et al., 2005; Mercer & Menzel, 1982; Neckameyer, 1998; Pfeiffer & Homberg, 2014; Puhl & Mesce, 2008; Unoki et al.,

2005, 2006; van Swinderen & Andretic, 2011). NE, in contrast, has long been considered to be absent from invertebrate nervous systems (Farooqui, 2012; Gallo et al., 2016; Verlinden et al., 2010), but as previously mentioned, adrenergic receptors in diverse invertebrate taxa is challenging this notion (Bauknecht & Jékely, 2017; Sukumar et al., 2018). As such, NE may also prove to be a behaviorally important catecholamine in spiders and other arachnids. However, experiments demonstrating that DA can influence neural activity are rare in arachnids; for example, we found a report of DA modulating electroretinogram recordings in wolf spiders (Muñoz-Cuevas & Carricaburu, 2000), and a more recent study indicating that DA can influence the firing properties of peripheral mechanoreceptors in a jumping spider (Sukumar et al., 2018). Based on the anatomical results presented here, it is clear that DA and/or NE have the potential to modulate behavior at all stages of neural processing—including early visual processing (evidenced by labeling in optic neuropils), sensorimotor integration (labeling in arcuate body), and motor patterning and activity (labeling in the appendage neuromeres). Functional studies, which incorporate neuroethological methods in conjunction with electrophysiology and pharmacology, are needed to examine further the putative roles of catecholamines in these (and other) behaviors. It is hoped that the anatomical data we have acquired will serve as a strong foundation to help inform such future studies of the spider CNS, and bring us one step closer to understanding the neurobiology of these fascinating yet relatively understudied organisms.

2.6 l Figures

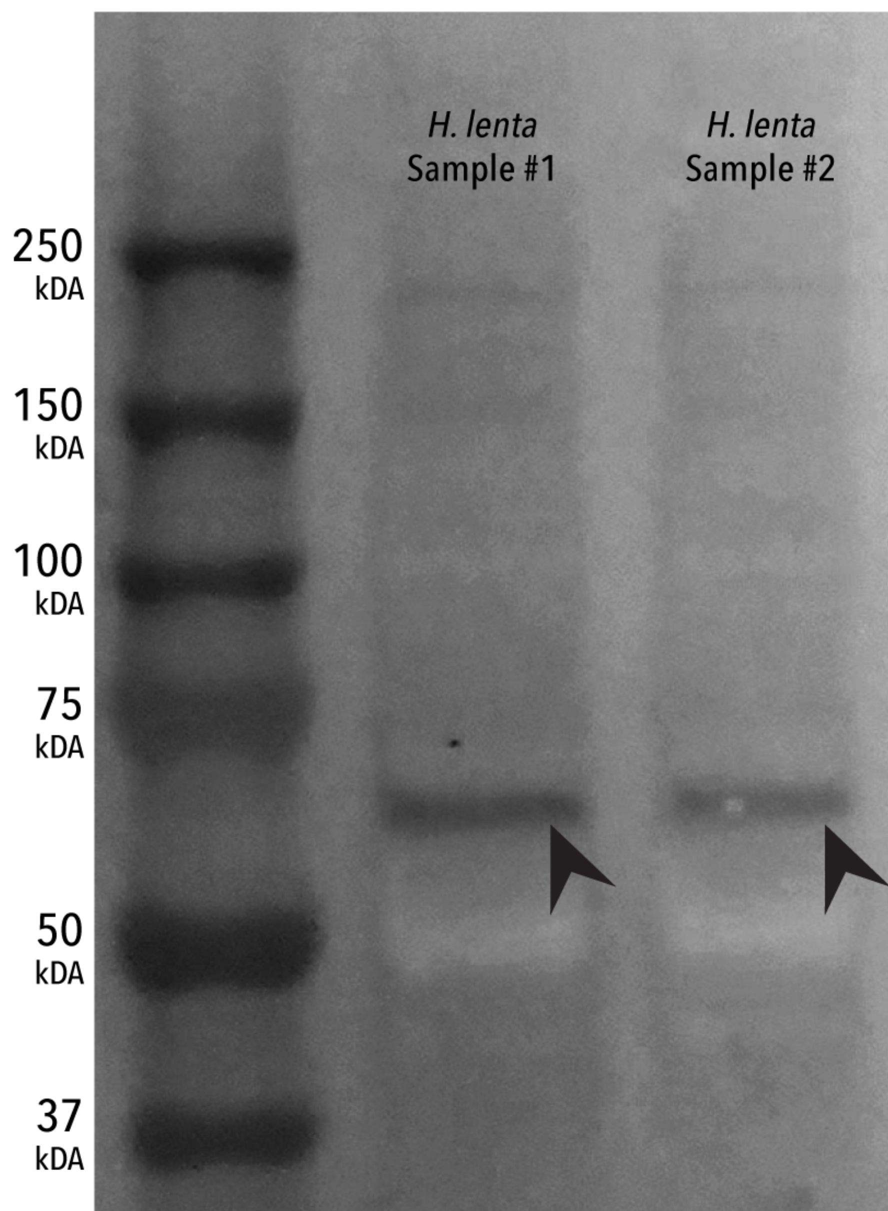
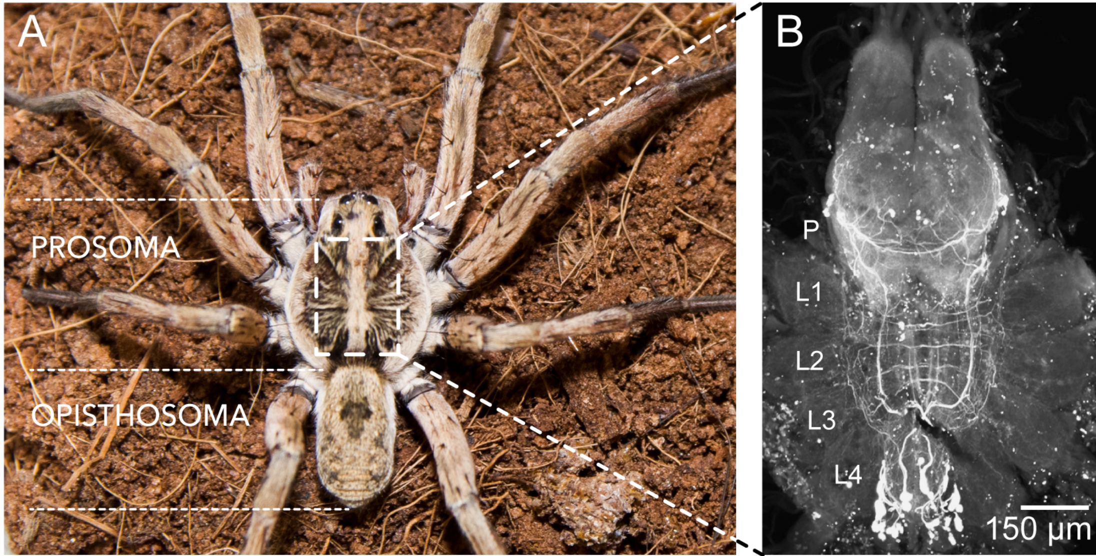


Figure 2.1. Western blot of central nervous system (CNS) protein extracts from *Hogna lenta* treated with the primary mouse anti-tyrosine hydroxylase (TH) antiserum. In each of the two spider samples, a single band of approximately 60 kDa was recovered (arrowheads), corresponding to the known molecular weight of the TH protein. No other bands were visible in any of the spider samples, indicating that the antibody binds specifically to TH in *H. lenta*.


A
PROSOMA
OPISTHOSOMA
B
P
L1
L2
L3
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150 µm

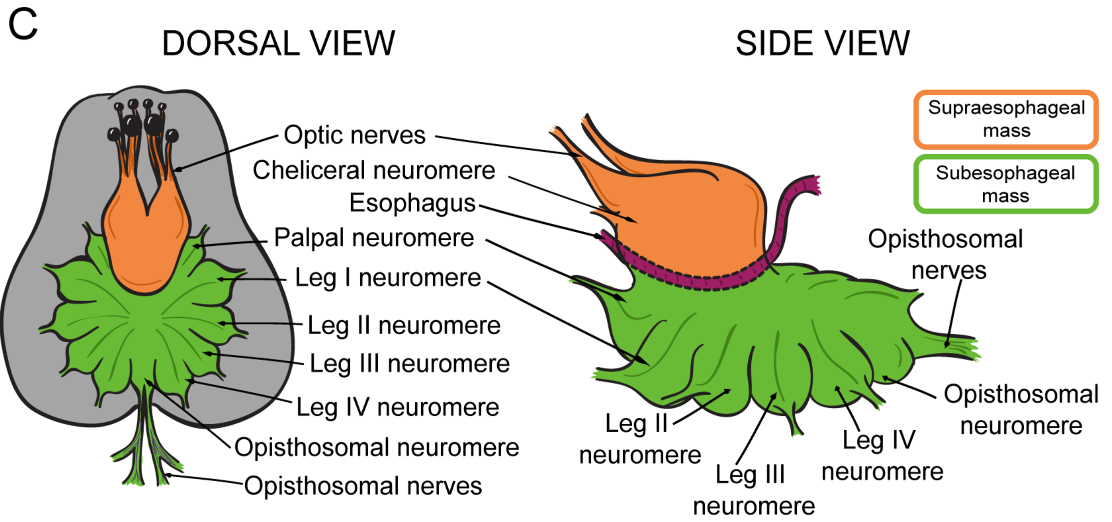

C
DORSAL VIEW
SIDE VIEW
Optic nerves
Cheliceral neuromere
Esophagus
Palpal neuromere
Leg I neuromere
Leg II neuromere
Leg III neuromere
Leg IV neuromere
Opisthosomal neuromere
Opisthosomal nerves
Supraesophageal mass
Subesophageal mass
Opisthosomal nerves
Opisthosomal neuromere
Leg II neuromere
Leg III neuromere
Leg IV neuromere

Figure 2.2. An overview of the CNS of *H. lenta*. **A.** Photograph of an adult male *H. lenta*, highlighting the two tagmata of the spider, i.e. the prosoma (cephalothorax) and opisthosoma (abdomen). **B.** Whole-mounted sample of the entire *H. lenta* CNS, which contains numerous and conspicuous tyrosine hydroxylase-immunoreactive (TH-ir) somata and projections in both the supraesophageal (brain) and subesophageal masses. Neuromeres corresponding to the pedipalps (P) and four pairs of walking legs (L1-4) are marked for orientation purposes. Optic lobes are anterior (top). **C.** Schematic diagrams showing the organization and major divisions of the spider CNS in dorsal (transverse) and side (sagittal) views.

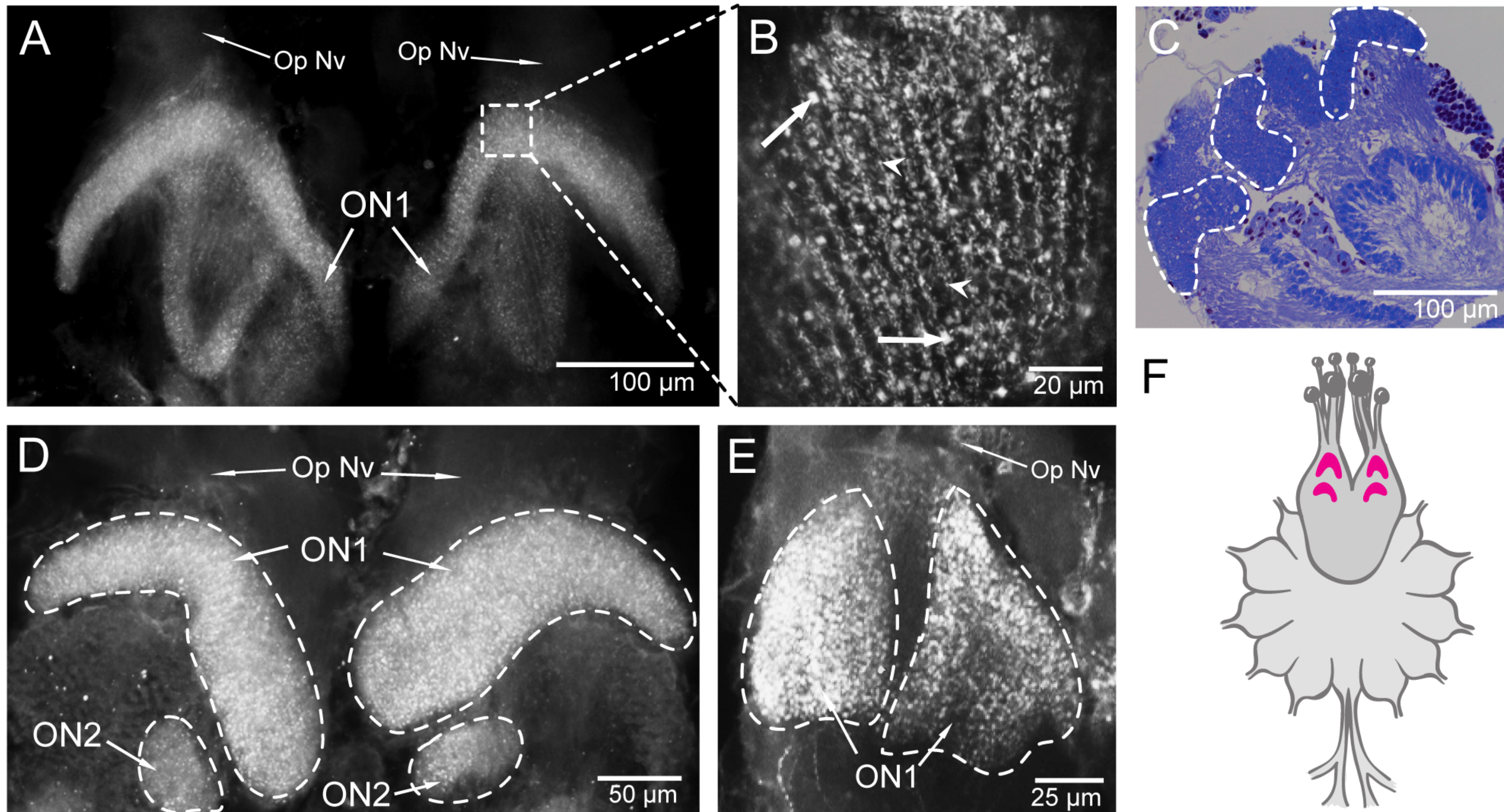

A
Op Nv
Op Nv
ON1
100 µm
B
20 µm
C
100 µm
D
Op Nv
ON1
ON2
ON2
50 µm
E
Op Nv
ON1
25 µm
F

Figure 2.3. TH-immunoreactivity in the first- and second-order visual neuropils of *Hogna lenta*. Each of the eight spider eyes was associated with its own first- and second-order visual neuropil. **A.** The first optic neuropils (ON1s) exhibit strong TH-immunoreactivity compared to surrounding structures (e.g., optic nerves, Op Nv). **B.** A magnified view of the outlined structures (dashed box) in Panel A, which reveals distinct striations composed of TH-ir fibers. Arrows denote conspicuous structures approximately 3 µm in diameter, which are likely congregations of synaptic boutons. Arrowheads indicate smaller structures (1 µm or less in diameter), which may also represent synaptic boutons. No TH-ir somata were visible in this region of the CNS. **C.** A sagittal section of the anterior supraesophageal mass, stained with hematoxylin and eosin. Dashed outlines indicate several representative ON1s. **D.** A second pair of ON1s (outlined), showing similarly high levels of TH-immunoreactivity. Also visible are portions of the second optic neuropils (ON2s) that correspond to the ON1s shown in this panel. The ON2s were found to be strongly labeled as well, indicating rich catecholaminergic innervation. **E.** The leftmost members of the third and fourth pairs of ON1s (outlined). The contralateral ON1s for these pairs are not shown. **F.** A schematic diagram showing the relative positions of a representative pair of ON1s (top) and ON2s within the spider CNS.

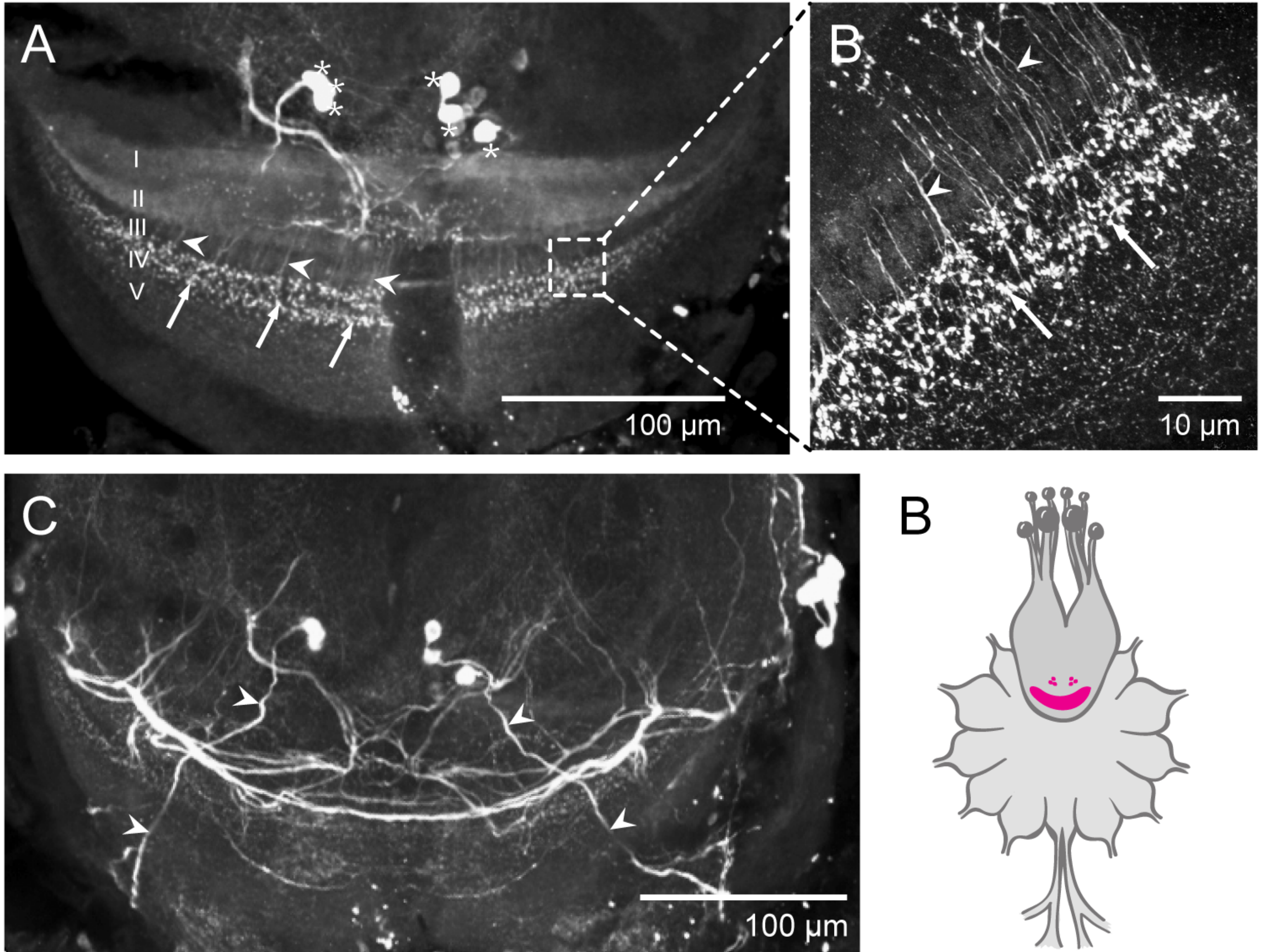
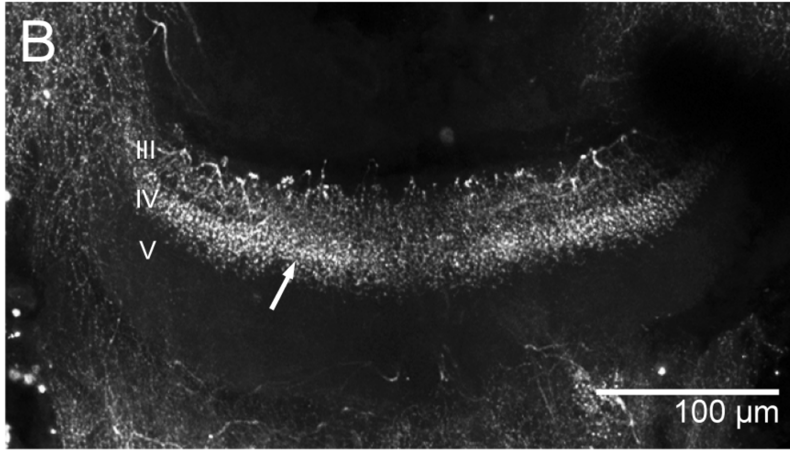

A
I
II
III
IV
V
100 µm
B
10 µm
C
100 µm
B

Figure 2.4. TH-immunoreactivity in the arcuate body of *H. lenta*. **A.** Overview of the arcuate body, showing extensive TH labeling and a characteristic layered organization. At least 5 distinct layers of the arcuate body were observed, indicated by Roman numerals. Of special note is Layer IV, which contains densely-packed punctate structures, each of which is 0.7-1 µm in diameter (arrows). Associated with these structures are numerous fine fibers, indicated by the arrowheads, which connect the punctate structures of Layer IV to more anterior layers of the arcuate body. Also of interest are paired triads of large (8-9 µm diameter) TH-ir somata, indicated by asterisks, some of which appear to project to more posterior layers of the arcuate body. **B.** A magnified view of the punctate structures (arrows) and their fibers (arrowheads) noted in Panel A. These structures appear to represent large synaptic boutons and their associated axon terminals. **C.** More ventral view of the arcuate body, showing thick commissural fibers. One bilaterally pair of somata (indicated by asterisks in Panel A) appears to send projections (indicated by arrowheads) beyond the arcuate body to more posterior regions of the supraesophageal mass. The remaining somata appear to contribute to the conspicuous commissure that lays just ventral of the arcuate body, and which spans the entire width of the neuropil. **D.** Schematic diagram showing the relative positions of the arcuate body and the associated paired triads of somata within the spider CNS.

A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III
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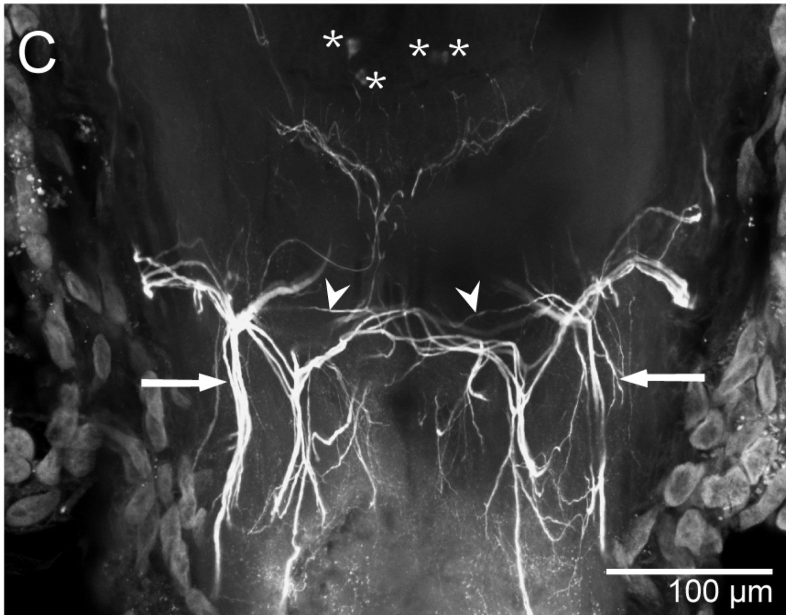
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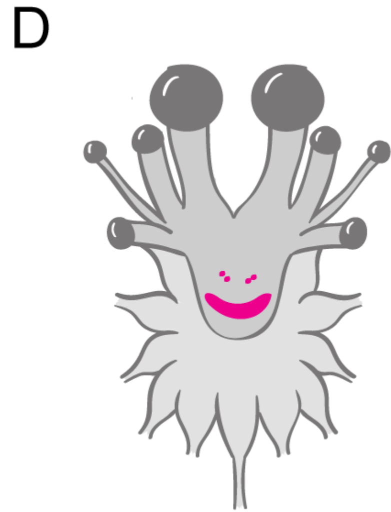
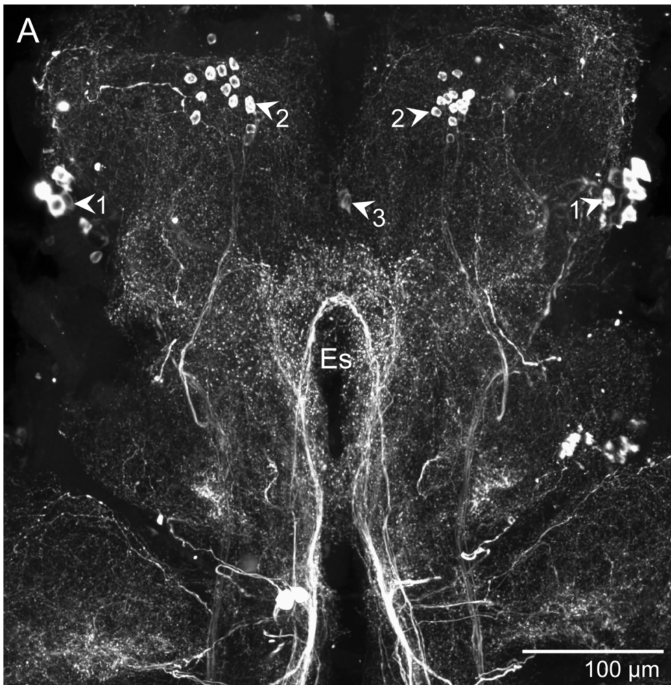

D

Figure 2.5. TH-immunoreactivity in the arcuate body of *Phidippus regius*. **A.** Photograph of an adult female *P. regius* [Original photo by Thomas Shahan, and reproduced here in accordance with the Creative Commons Attribution 2.0 Generic license, https://creativecommons.org/licenses/by/2.0/deed.en]. **B.** Overview of the *P. regius* arcuate body, showing TH immunolabeling and a similar layered organization to that of *H. lenta* (see Figure 2.4). Three distinct layers of the arcuate body were visible and indicated by Roman numerals that correspond to matching layers in the arcuate body of *H. lenta*. **C.** Ventral to the arcuate body is an extensive collection of TH-ir fibers, some of which are commissural (arrowheads) and may be homologous to the arc-shaped commissure seen in *H. lenta*. Asterisks indicate the somata of TH-ir neurons, each approximately 6 µm in diameter, whose projections appear to contribute to these commissural fibers. These cells may be homologous to the paired triads of TH-labeled cells associated with the arcuate body of *H. lenta*. Arrows indicate descending TH-ir projections, which are more numerous and pronounced in *P. regius* than in *H. lenta*. **D.** Schematic diagram showing the relative positions of the arcuate body and the associated somata within the jumping spider CNS.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2
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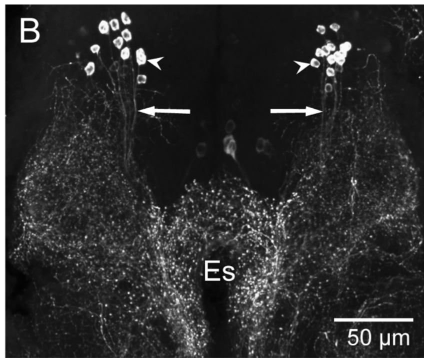
B
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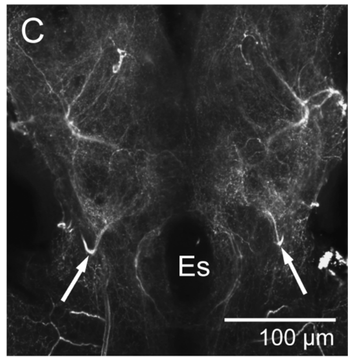
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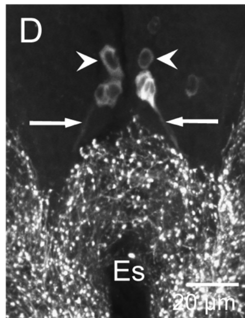
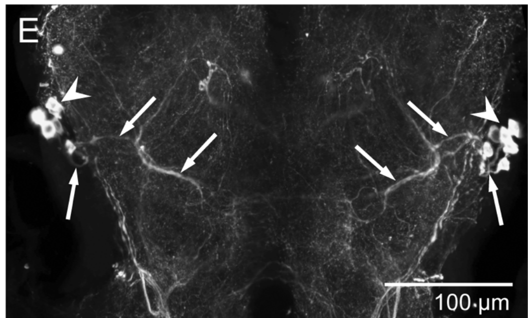
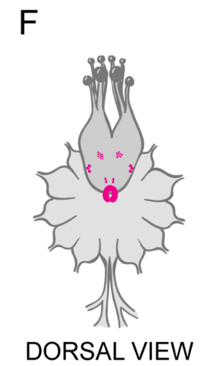

D
Es


E
100 µm


F
DORSAL VIEW

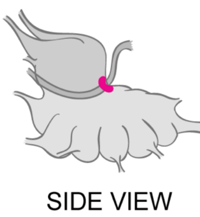

SIDE VIEW

Figure 2.6. Additional notable TH-ir neurons in the supraesophageal mass of *H. lenta*. **A.** Lower magnification view of the supraesophageal mass, showing the relative distribution of three distinct populations of bilaterally paired TH-labeled neurons (arrowheads), which are marked as cell groups 1-3 (cluster 3 is partially out of view). The position of the esophagus, which separates the supra- and subesophageal masses, is indicated as Es. **B.** Cluster-2 cells in the medial supraesophageal mass. The somata of these neurons (arrowheads) range from 4-6 µm in diameter, and their projections (arrows) appear to descend to more posterior regions of the CNS. **C.** Many of the descending fibers highlighted in Panel B appear to fasciculate and curve upwards, thus ascending back to more anterior regions of the supraesophageal mass. Arrows indicate the turning points of these fiber bundles. **D.** Medial cluster-3 cells sit above the esophagus (Es) in a more ventral plane of the CNS. Their somata (arrowheads) measure 7-8 µm in diameter, and their projections (arrows) appear to descend and contribute to the dense plexus of fine TH-labeled fibers surrounding the esophagus. Numerous small punctate structures are visible around the esophagus, likely representing synaptic boutons. **E.** Along the lateral margins of the supraesophageal mass are the paired cluster-1 cells, which are relatively large (7-10µm diameter); the TH-ir somata are denoted by arrowheads. At least six somata were detected in each cluster; importantly, their axons (arrows) appear to contribute to the large commissure that sits ventral to the arcuate body (see Figure 2.4 C). **F.** A pair of schematic diagrams showing the relative positions of these three neuronal populations as well as the dense plexus surrounding the esophagus within the spider CNS.

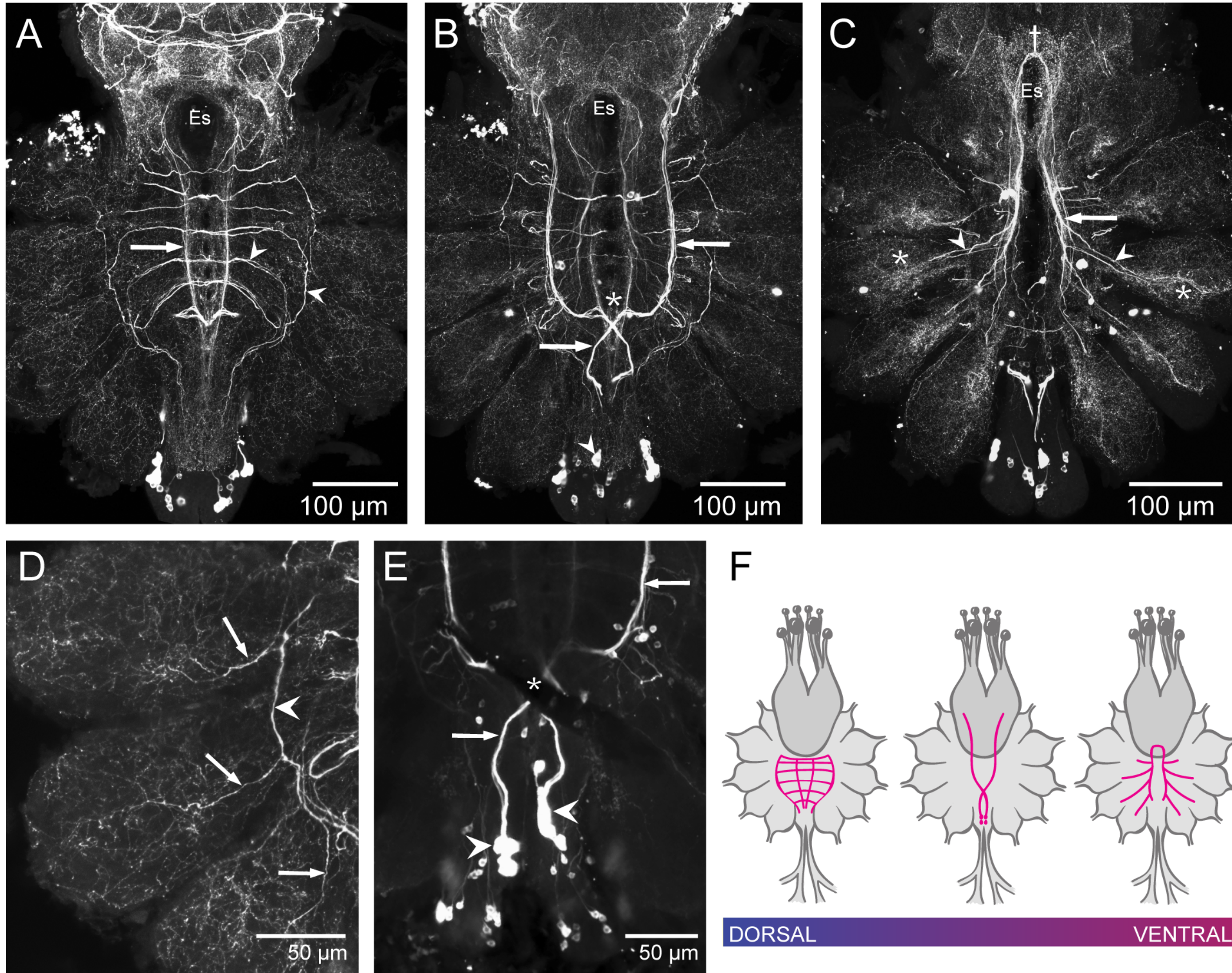
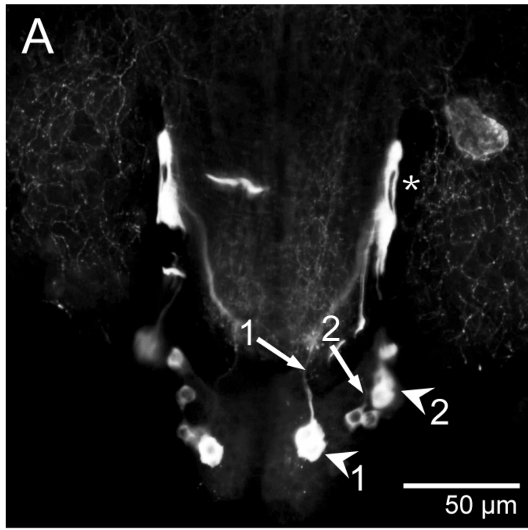

A
Es
100 µm
B
Es
100 µm
C
Es
100 µm
D
50 µm
E
50 µm
F
DORSAL
VENTRAL

Figure 2.7. Major catecholaminergic tracts in the CNS of *H. lenta*. **A.** The dorsal-most tract of TH-ir fibers consists of paired bundles of axons that run longitudinally adjacent to the midline (arrow), and may correspond to the mid-central (MC) tract described by Babu and Barth (1984). Six distinct commissural tracts, corresponding with the six pairs of appendages (chelicerae, pedipalps, and walking legs), project dorsally and laterally to fasciculate into distinct lateral longitudinal branches (arrowheads). These lateral longitudinal branches project through multiple appendage neuromeres as well as the opisthosomal neuromere, and may be part of the mid-dorsal (MD) tract of Babu and Barth (1984). **B.** The intermediate TH-ir tract arises from conspicuous, large (13-15µm diameter) TH-labeled somata in the opisthosomal neuromeres (arrowhead). The axons of these neurons fasciculate and ascend in a large tract (arrows), which crosses the midline just anterior to the opisthosomal neuromere; the point at which the projections cross the midline is denoted by an asterisk. This tract extends through all appendage neuromeres and appears to terminate in the lateral margins of the supraesophageal mass. Based on position, this tract may correspond to the central (CT) or centro-lateral (CL) tract of Babu and Barth (1984). **C.** The ventral-most TH-ir tract consists of paired bundles of fibers (arrow) that run longitudinally through the margins of the appendage neuromeres adjacent to the midline. These bundles connect in a loop (dagger) just above the esophagus (Es). Lateral projections from this tract (arrowheads) extend into all appendage neuromeres and give rise to dense arborizations (asterisks) in the ventral neuropil of the appendage neuromeres. This tract may be the mid-ventral (MV) tract of Babu and Barth (1984). **D.** A magnified view of a lateral branch (arrowhead) of the dorsal-most TH-ir tract shown in Panel A. Fibers from the lateral branches (arrows) project into the dorsal neuropil of the appendage neuromeres, giving rise to an intricate meshwork of fine arborizations. **E.** A magnified view of the large ascending TH-ir neurons of the opisthosomal neuromeres that give rise to the intermediate TH-labeled tract. This view better shows the connection between their somata (arrowheads) and the fibers that comprise the tract (arrows). Asterisk denotes the point at which the fibers cross the midline. **F.** Summary schematic diagrams showing the relative positions of the three TH-ir fiber tracts within the spider CNS.



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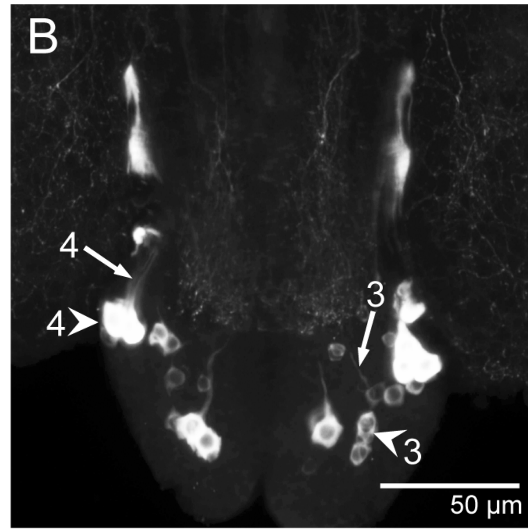

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4
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3
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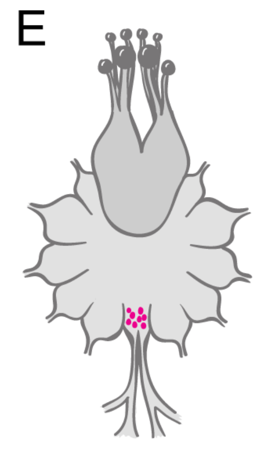

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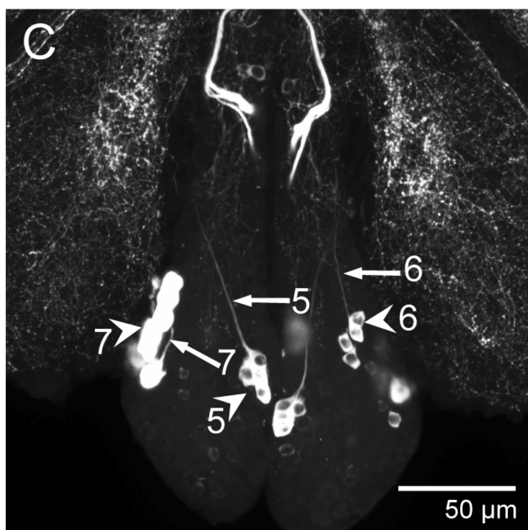

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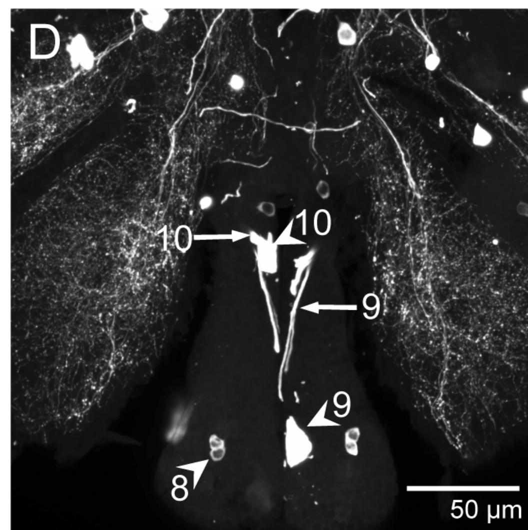

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10
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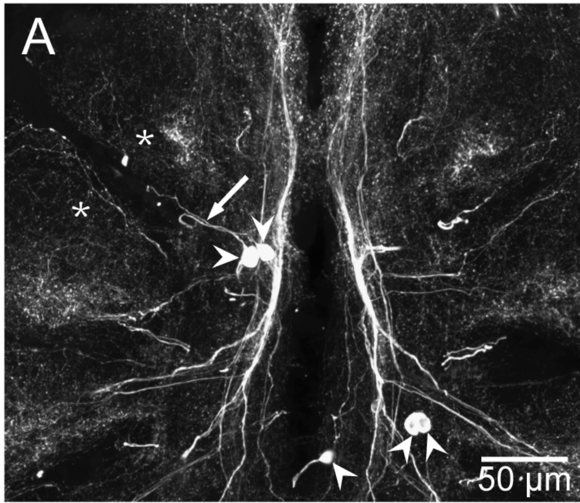
9
9
8
50 µm

Figure 2.8. Populations of TH-ir neurons in the opisthosomal neuromeres of *H. lenta*. Ten populations were identified, numbered in ascending order of dorsal-most (Population 1) to ventral-most (Population 10). In all panels, arrowheads denote somata and arrows denote their projections. **A.** Cell populations of the dorsal opisthosomal neuromere. Population 1 consists of four neurons (bilaterally paired), with ascending projections that form a tight coil (asterisk) in the anterior opisthosomal neuromere. Somata measure 9-10 µm in diameter. Population 2 consists of 6 neurons (bilaterally paired) with ascending fibers. Somata measure 7-8 µm in diameter. **B.** Cell populations of the mid-dorsal opisthosomal neuromere. Population 3 consists of paired clusters of 3 neurons each with ascending fibers. Somata measure 8-9 µm in diameter. Population 4 consists of paired lateral clusters of at least 3 neurons each, with fasciculating ascending fibers. Somata measure 8-10 µm in diameter. **C.** Cell populations of the mid-ventral opisthosomal neuromere. Population 5 consists of paired clusters of at least 4 midline-adjacent neurons each with ascending fibers. Somata measure 6-8 µm in diameter. Population 6 consists of paired clusters of 4 neurons each with ascending fibers. Somata measure 6 µm in diameter. Population 7 consists of paired clusters of at least 6 lateral neurons each with ascending fibers. Somata measure 9-10 µm in diameter. **D.** Cell populations of the ventral opisthosomal neuromere. Population 8 consists of 2 neurons per paired cluster, with somata measuring 6 µm in diameter. Projections from these cells were not visible in our samples. Population 9 consists of a single pair of large somata that measure 15 µm in diameter. Projections from these neurons ascend and contribute to the intermediate TH-labeled tract (see Figure 2.7 B & E). Population 10 consists of a single pair of somata along the ventral-most plane of the neuromere. These somata measure 13 µm in diameter, and their projections also ascend to contribute to the intermediate TH-ir tract. **E.** Schematic diagram showing the general positions of the opisthosomal populations of somata within the spider CNS.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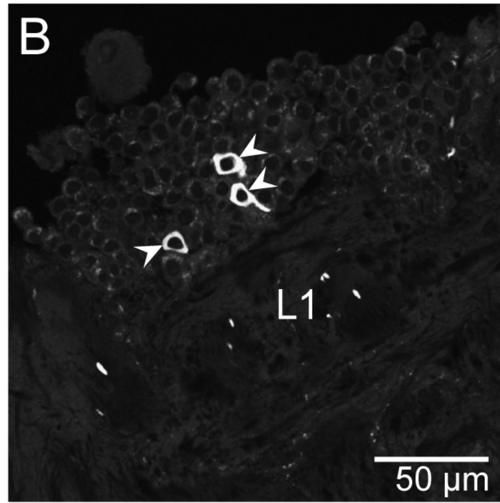
B
L1
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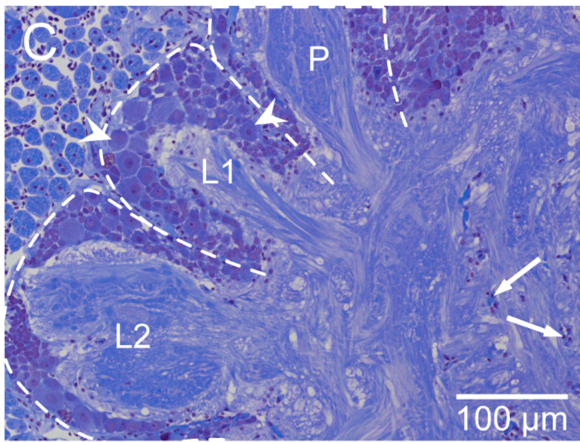
C
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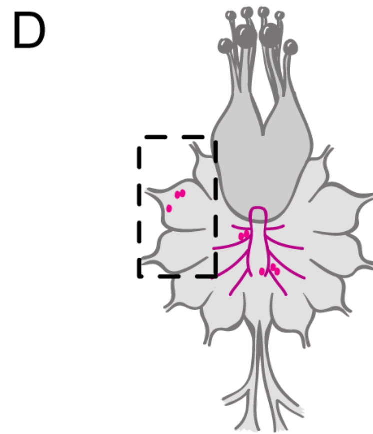
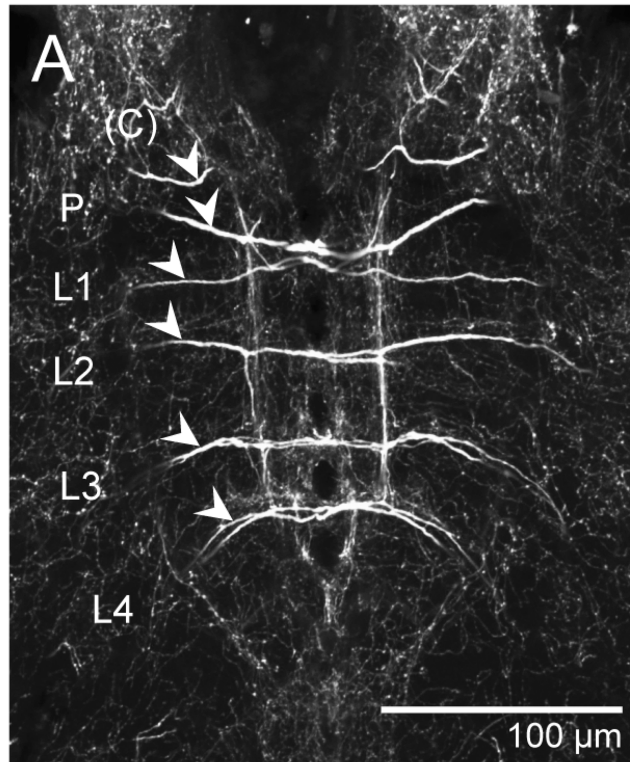

D

Figure 2.9. Intrinsic neurons of the appendage neuromeres of *H. lenta*. **A.** Within each animal, intrinsic TH-ir neurons appeared randomly distributed along the ventral regions of the appendage neuromeres. The somata of these neurons (arrowheads) may be paired or unpaired, and at least some of them send projections (arrow) to the ventral neuropil of the appendage neuromeres, where they contribute to a meshwork of fine TH-ir arborizations (asterisks). **B.** TH-ir neurons of similar position and size were also observed in the ventral regions of the appendage neuromeres in our paraffin-embedded sectioned material. Arrowheads indicate neurons along the ventrolateral margin of the leg 1 neuromere. These neurons presumably belong to the same class of intrinsic neurons that we observed in wholemounted preparations. **C.** Hematoxylin and eosin staining of paraffin-embedded sectioned tissue provides anatomical context for the locations of cells associated with the leg appendages. Dashed lines indicate the approximate borders between the left pedipalpal neuromere (P), leg 1 neuromere (L1), and leg 2 neuromere (L2). Arrowheads denote relatively large somata within these appendage neuromeres, in the same general region as the TH-ir neurons in Panel B. Arrows denote nuclei of partially-visible somata closer to the midline, in the same general region as the TH-ir neurons of Panel A. **D.** A schematic diagram indicating the relative positions of some of these intrinsic TH-ir neurons in the spider CNS. The ventral TH-ir tract is shown for orientation. Dashed outline indicates the region of the CNS represented in Panel C.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(C)
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L3
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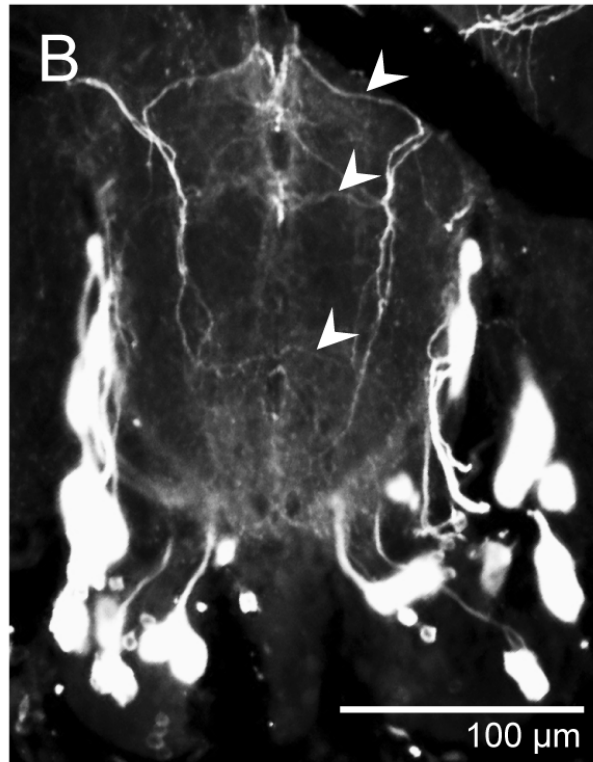

B
100 µm

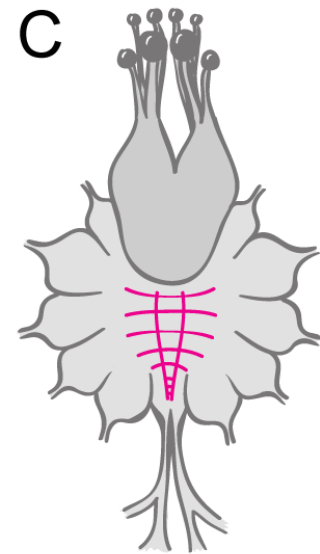
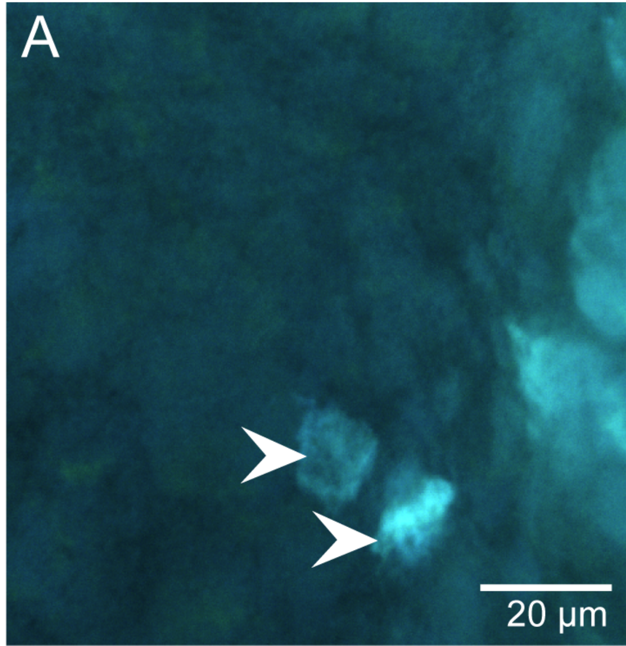

C

Figure 2.10. TH labeling highlights the segmental organization of the spider CNS. **A.** Conspicuous, serially-repeating structural motifs (arrowheads), formed by the commissural fibers of the dorsal TH-ir tract, are visible in the subesophageal mass and correspond to the neuromeres of the six paired appendages (chelicerae, pedipalps, and legs). These motifs are remnants of the segmental origin of the fused spider CNS. (C)= fibers projecting to the cheliceral neuromere, which is ventral to the plane showed in this figure; P= pedipalpal neuromere; L1-4= neuromeres of legs 1-4. **B.** Structurally similar motifs (arrowheads) are also seen in the opisthosomal neuromeres, again indicating the basal segmental organization of the CNS. Also visible are many of the conspicuous TH-ir somata described in Figure 2.8. **C.** Schematic diagram showing the relative positions of dorsal tract, which contains these repeating motifs, within the spider CNS.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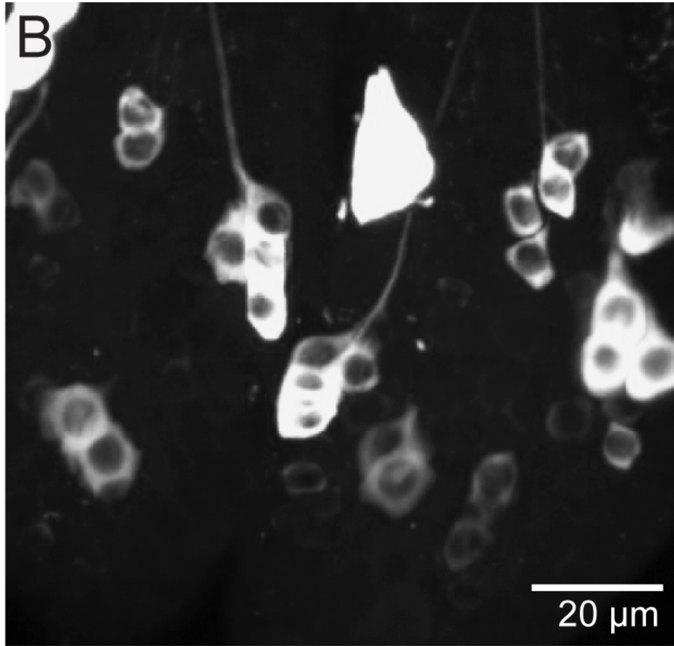
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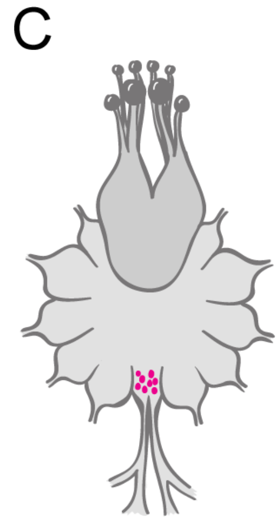

C

Figure 2.11. Glyoxylic acid histology confirms the presence of catecholamines in opisthosomal neurons. **A.** Several representative somata (arrowheads) in the opisthosomal neuromere test positive for the presence of catecholamines via the glyoxylic acid reaction, as indicated by their prominent blue fluorescence. **B.** TH immunolabeling reveals numerous catecholamine-synthesizing somata in the same region of the CNS as the somata in Panel A. **C.** A schematic diagram showing the relative positions of these opisthosomal cells within the spider CNS.

CHAPTER 3

A study of biogenic amines in the scorpion *Centruroides sculpturatus* (Scorpiones: Buthidae) with evidence for norepinephrine as an endogenous neuromodulator in the Chelicerata.

3.1 I Synopsis

Despite the astounding diversity and importance of the Chelicerata, the structure and functions of their nervous systems remain relatively understudied. Because the Chelicerata are phylogenetically basal to all other extant arthropods, knowledge of their neurobiology is crucial for understanding the evolution of nervous systems in the Arthropoda. To address this knowledge gap, we conducted a study of biogenic amines in the central nervous system (CNS) of the scorpion *Centruroides sculpturatus* (Scorpiones: Buthidae). Using immunocytochemistry, we localized and described neurons in the ventral nerve cord (VNC) that contain catecholamines (dopamine, DA, and norepinephrine, NE), octopamine (OA), and serotonin (5-hydroxytryptamine, 5-HT). Among our most salient findings were: (1) large efferent dopaminergic neurons that directly innervate peripheral respiratory structures, (2) serially iterated clusters of efferent serotonergic neurons, and (3) medial clusters of numerous octopaminergic cells. Although NE was not detected in the ganglia of the nerve cord, it was detected (along with OA) in the supraneural lymphoid glands, non-neural organs that are

closely associated with the CNS and whose functions remain enigmatic. The majority of aminergic neurons in the VNC were found in large clusters of 12+ somata, which mirrors the arrangement in other chelicerates, but is highly divergent from that in mandibulate arthropods. Additionally, we utilized a combination of quantitative chemistry, electrophysiology, and bioinformatics to test the hypothesis that NE is an endogenous signaling molecule in chelicerates, despite the widespread notion that NE is absent from invertebrates. We detected appreciable amounts of NE in both the prosomal CNS and VNC of *C. sculpturatus* using ultra-performance liquid chromatography/mass spectrometry. Furthermore, NE induced measurable changes in the physiological activity of the scorpion CNS that were distinct from the effects of related compounds (DA and OA), and genes coding for distinct NE, OA, and DA receptors were found in *C. sculpturatus*. Taken together, our results suggest that NE is a present and physiologically relevant signaling molecule in the Chelicerata. The source of this endogenous NE is presumed to be the prosomal CNS and lymphoid glands. These findings support the idea that NE signaling is not an autapomorphy of vertebrates, but rather an ancient phenomenon present in the common ancestor of all bilaterian phyla.

3.2 I Introduction

With over 130,000 species currently known and countless more yet to be described, the arthropod subphylum Chelicerata is one of the most diverse

lineages of animals on the planet (Garb et al., 2018; Lozano-Fernandez et al., 2019; Shultz, 2007). Indeed, the astounding diversity of the Chelicerata, which include the arachnids and their allies, is rivaled only by the Insecta (Misof et al., 2014). Equally diverse is the broad range of fascinating behaviors that chelicerates exhibit, such as intricate multimodal courtship displays (Elias et al., 2012; Girard et al., 2015; Gordon & Uetz, 2011), sophisticated social behaviors (Del-Claro & Tizo-Pedroso, 2009; Machado, 2002; Rayor & Taylor, 2006; Viera & Agnarsson, 2017; Walter & Bilde, 2015), spatial navigation (Gaffin & Brayfield, 2017; Ortega-Escobar & Ruiz, 2017; Wiegmann et al., 2016), behavioral mimicry (Ceccarelli, 2008; Cushing, 1997; Jackson & Whitehouse, 1986), various predatory and anti-predator strategies (Carlson et al., 2014; Hara & Gnaspini, 2003; Lohrey et al., 2009; Pékar & Toft, 2015), and even cognitive behaviors (Jackson & Cross, 2011; Jackson & Nelson, 2011; Jackson & Wilcox, 1990, 1993a,b; Tarsitano & Jackson, 1994, 1997). Although these behaviors (and many others) have been extensively studied in the Chelicerata, the neurobiological and neurochemical processes that underlie them have received relatively little attention and remain poorly understood.

Across animal phyla, many of these complex behaviors are modulated by biogenic amines, a class of neuroactive compounds that are derived from amino acid precursors (Evans, 1980; Libersat & Pflueger, 2004; Walker et al., 1996). Five major amines are known to influence behavior in the Arthropoda, by acting as neurotransmitters, neuromodulators, and neurohormones: dopamine (DA),

serotonin (5-hydroxytryptamine, 5-HT), histamine (HA), octopamine (OA), and tyramine (TA) (Barron et al., 2010; Evans, 1980; Farooqui, 2012; Gallo et al., 2016; Libersat & Pflueger, 2004; Pflüger & Stevenson, 2005; Roeder 2005; Verlinden et al., 2010; Vleugels et al., 2014; Walker et al., 1996; Weiger, 1997). Two other amines, norepinephrine (NE) and epinephrine (E), are synthesized from DA, and collectively these three compounds are known as the catecholamines; however, NE and E are thought to be physiologically active only in vertebrates, where they perform a multitude of important functions that mirror the functions of OA and TA in invertebrates (Adamo, 2008; Blenau & Baumann, 2001; Farooqui, 2012; Gallo et al., 2016; Pflüger & Stevenson, 2005; Roeder, 1999, 2005; Verlinden, 2010; Walker et al., 1996). As such, it is widely reported that DA is the only catecholamine present in arthropods (Adamo, 2008; Blenau & Baumann, 2001; Farooqui, 2012; Gallo et al., 2016; Pflüger & Stevenson, 2005; Roeder, 1999, 2005; Verlinden, 2010; Walker et al., 1996).

In the insects and crustaceans (collectively known as the Pancrustacea), the functional roles of the biogenic amines have been extensively studied using a broad range of behavioral and physiological experimental approaches (e.g., Alekseyenko et al., 2013; Djokaj et al., 2001; Hong et al., 2006; Jin et al., 2014; Kravitz, 2000; Ma et al., 2015; Neckameyer, 1998; Novak & Rowley, 1994; Perrot-Minnot et al., 2013; Selcho et al., 2012; Spivak et al., 2003; Vieira et al., 2018; Yamagishi et al., 2004). The anatomical distribution patterns of these compounds in the pancrustacean central nervous systems (CNSs) have also

been well characterized across an expansive array of species, via immunocytochemistry and other histological techniques (e.g., Antonsen & Paul, 2001; Hartline & Christie, 2010; Haselton et al., 2006; Hörner, 1999; Mesce at al., 2001; Nässel, 1996; Ponzoni, 2014; Spörhase-Eichmann et al., 1992; Stemme et al., 2013; Stemme et al., 2017; Tedjakumala et al., 2017; Thamm et al., 2017; Thompson et al., 1994). In contrast, our knowledge of how biogenic amines are anatomically distributed in the CNSs of chelicerates is extremely limited. The majority of this work has focused on just one species: the horseshoe crab *Limulus polyphemus* (Xiphosura: Limulidae), whose octopaminergic (Batelle et al., 1999; Lee & Wyse, 1991), serotonergic (Batelle et al., 1999; Chamberlain et al., 1986; Harzsch, 2004; Harzsch et al., 2005; Washington et al., 1994), and histaminergic (Batelle et al., 1991; Batelle et al., 1999; Harzsch et al., 2005) systems have been well documented. The distributions of OA, 5-HT, and HA have also been described for the wandering spider *Cupiennius salei* (Araneae: Trechaleidae) (Schmid & Becherer, 1999; Schmid & Duncker, 1993; Seyfarth et al., 1990; Seyfarth et al., 1993). Beyond these two organisms, immunocytochemical studies of biogenic amines in the Chelicerata are limited to isolated reports of 5-HT in a small handful of species (Breidbach & Wegerhoff, 1993; Brenneis & Scholtz, 2015; Harzsch, 2004; Hummel et al., 2007; Lees & Bowman, 2007; Wolf & Harzsch, 2012) and our previous study of catecholamines in the wolf spider *Hogna lenta* (Araneae: Lycosidae) (Auletta et al., 2019). This latter study is, to date, the only study of DA and catecholamines in the CNS of

any chelicerate, despite the widespread importance of DA in modulating behaviors in other animal groups (reviewed by Gallo et al., 2016). Indeed, the only other anatomical studies of catecholamines conducted in the Chelicerata have focused exclusively on its presence in the salivary glands of ticks (Binnington & Stone, 1977; Kaufman et al., 1999). Studies of how the biogenic amines actually function in the CNSs of Chelicerata are rare as well, and are again primarily limited to the horseshoe crab and a narrow assortment of other species (Batelle et al., 1999; Dalal & Battelle, 2010; James & Walker, 1979; Muñoz-Cuevas & Carricaburu, 2000; Wyse, 2010).

Thus, there is a clear need for further studies of biogenic amines in chelicerates, especially in taxa beyond the Xiphosura and Araneae. Since the Chelicerata occupy a key position at the base of the arthropod phylogeny (Giribet, 2018; Legg et al., 2013; Lozano-Fernandez et al., 2019; Meusemann et al., 2010; Misof et al., 2014; Regier et al, 2010; Rota-Stabelli et al., 2011; Sharma et al., 2014; Wheeler & Hayashi, 1998), such studies would be especially informative for comparative analyses of amine distribution and functions within the Arthopoda as a whole. Indeed, a better understanding of aminergic modulatory systems in the Chelicerata would provide unique and valuable insight into the evolution of those systems in arthropods, because chelicerates may retain ancient features of the arthropodan CNS that have been subsequently lost or modified in other subphyla. To address this need, we have conducted a broad structural and functional examination of several aminergic

systems in the Arizona bark scorpion, *Centruroides sculpturatus* (Scorpiones: Buthidae). This species was selected not only as a representative of an important yet understudied chelicerate lineage, but also because its nervous system is highly amenable to physiological manipulations and it is one of the few chelicerates for which genomic data is available (Garb et al., 2018; Schwager et al., 2017). Using immunocytochemical techniques, we localized neurons in the scorpion ventral nerve cord (VNC) that contained 5-HT, OA, NE, and tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis (Molinoff & Axelrod, 1971; Roberts & Fitzpatrick, 2013). In doing so, we present the first description of catecholaminergic and octopaminergic systems in the order Scorpiones, and greatly expand upon the preliminary descriptions of the scorpion serotonergic system initially reported by Harzsch (2004). These investigations also complement existing data for *L. polyphemus*, *Cu. salei*, and *H. lenta*, and will help to paint a better picture of the ground-plan organization of aminergic systems in the Chelicerata.

In addition, recent work by Bauknecht & Jékely (2017) has reported the presence of adrenergic (i.e., NE and E) receptors in multiple invertebrate taxa. These results challenge the commonly accepted notion that NE and E are autapomorphies (i.e., exclusive derived features) of the Vertebrata and are therefore absent in invertebrates (Adamo, 2008; Blenau & Baumann, 2001; Farooqui, 2012; Gallo et al., 2016; Pflüger & Stevenson, 2005; Roeder, 1999, 2005; Verlinden, 2010; Walker et al., 1996). A subsequent analysis by Sukumar

et al. (2018) has shown that the CNS of the spider *Cu. salei* contains adrenergic receptors as well, which implies that chelicerates may make use of NE as a signaling molecule. To examine this possibility further, we performed a comprehensive study of NE in *C. sculpturatus*, which complements our immunolabeling studies in this species. We integrated a varied suite of techniques, including analytical chemistry, histology, bioinformatics, and electrophysiological records, to quantify, localize, and identify the physiological effects of NE in the CNS of the scorpion*.* This approach takes full advantage of genomic resources and physiological manipulations that are available for *C. sculpturatus*, but not yet for *Cu. salei* (c.f., Garb, 2018; Menda et al., 2014). In doing so, we are the first to report that NE is not only present, but also physiologically relevant in the Chelicerata. Taken together, our analyses provide new insight into the organization and functional roles of aminergic systems in the scorpion CNS. These findings stand to inform future studies of the neurochemical bases of behavior in the Chelicerata, as well as the evolution of aminergic signaling across bilaterian animals as a whole.

3.3 I Materials and Methods

3.3.1 I Study Animals

The focal organism used throughout our study was the Arizona bark scorpion, *Centruroides sculpturatus* (Buthidae) (Figure 3.1 A). Adult or subadult scorpions were primarily used for all experiments, but some embryonic tissue

was used to examine the projections of TH-ir neurons. Specimens were collected in southern Arizona in 2017-2019 between March and October and supplied by Tim Burkhardt (TriprionCo). In the lab, scorpions were housed communally in a large (20 cm x 10 cm x 10 cm) glass terrarium containing approximately 2 cm of substrate (50 % EcoEarth, 50% aquarium sand) with numerous overlapping pieces of bark to serve as hiding places. The scorpions were fed once per week on either feeder cockroaches (*Blatta lateralis*) or house crickets (*Gryllodes sigillatus*). The enclosure was maintained at 23-26 °C and the side of the enclosure was lightly sprayed with water once per week; this procedure was performed to emulate the temperatures and humidity levels of habitat in which the scorpions were originally collected. All scorpions selected for experiments were thoroughly inspected to ensure that they were in good health and displayed no abnormal motor patterns. Because preliminary investigations did not reveal any distinct differences between male and female scorpions with regard to our histological or physiological experiments, we used both sexes throughout this study.

3.3.2 l Dissection and Immunolabeling

To visualize catecholaminergic neurons in the scorpion nerve cord, we employed a modification of the TH immunolabeling protocol used successfully in another arachnid, the wolf spider *H. lenta* (Auletta et al., 2019). This protocol was a modification of protocols used previously in our lab, which had been shown to

label catecholaminergic neurons consistently and specifically in wholemounted preparations of numerous invertebrates, including the hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae), the honeybee *Apis mellifera* (Hymenoptera: Apidae), and the medicinal leech *Hirudo verbana* (Hirudinea: Hirudinidae) (Crisp et al., 2002; Mesce et al., 2018; Mesce et al., 2001; Mustard et al., 2012; Tedjakumala et al., 2017). Immunolabeling protocols for individual amines (OA, NE, and 5-HT) were adapted from other studies conducted in our lab on a similarly broad array of invertebrates (Crisp et al., 2002; Gilchrist et al., 1995; Mesce et al., 1993; Spivak, et al., 2003).

Scorpions were first anesthetized in a -4 °C freezer until immobile (approximately 3-5 minutes) and then pinned dorsal side up in a wax dish under a bath of physiological saline. The saline recipe was an adaptation of that developed for the buthid scorpion *Mesobuthus martensii* by Kimura et al. (1988) and contained the following compounds per liter of distilled water: 17.06 g NaCl, 0.25 g KCl, 0.53 g $CaCl_2 \cdot 2H_2O$, 0.12 g $MgCl_2 \cdot 6H_2O$, 0.50 g glucose, and 1.50g Trizma Pre-set 7.4 crystals (Sigma-Aldrich). The final pH of the scorpion saline was 7.4, which matched our measurements of the pH of *C. sculpturatus* hemolymph. Two dorsal transverse cuts (directly posterior to the prosoma and directly anterior to the metasoma) and one midline cut down the length of the mesosoma and metasoma were made to expose the inner abdominal cavity of the scorpion, and non-neural tissue was carefully removed to expose the VNC.

Using fine dissecting scissors, the entire VNC (including its associated nerves) was carefully excised from the scorpion.

For TH and 5-HT immunolabeling, the dissected nerve cords were transferred immediately to a vial of freshly prepared PFA fixative, which consisted of 4% paraformaldehyde dissolved in iso-osmotic Millonig's buffer (13 mM NaH_2HPO_4, 86 mM Na_2HPO_4, 75 mM NaCl, pH 7.8). Samples for TH immunolabeling were fixed for 3 hours at room temperature (22-23 ºC), whereas those used for 5-HT labeling were fixed for 24 hours at 4 °C. After fixation, the tissue was thoroughly rinsed in iso-osmotic Millonig's buffer (three 10-minute washes) and then incubated for at least 2 hours and up to overnight in room-temperature blocking solution (1.5mL Normal Goat serum + 13.5mL of 1% Triton-X Buffer in iso-osmotic Millonig's buffer). The tissue was then incubated in the primary antiserum: either 1:100 mouse monoclonal anti-TH (ImmunoStar, Cat# 22941, RRID: AB_572268) or 1:200 rabbit polyclonal anti-5-HT (ImmunoStar, Cat# 20080, RRID: AB_572263) for 3-7 days at 4º C. The anti-TH antibody was generated against TH purified from rat PC12 cells and was found to bind with high specificity to TH only; a thorough report of the specificity of this antiserum is included in our previous study of TH immunolabeling in the wolf spider (Auletta et al., 2019). The anti-5-HT antibody was generated against purified 5-HT conjugated to bovine serum albumin (BSA) with paraformaldehyde, and was found not to cross-react with 5-hydroxytryptophan (5-HTP), 5-hydroxyindole-3-acetic acid (5-HIAA), or DA. The manufacturer's specifications indicate that

quality control testing of this antibody was conducted in rat hypothalamus, raphe nuclei, and spinal cord, where substantial positive immunolabeling was detected; however, pretreatment of the diluted antibody with 25 µg/ml of 5-HT/BSA completely abolished any labeling, which further confirmed the specificity of this antiserum. The use and specificity of these primary antisera have been well-documented in numerous organisms across a wide variety of studies; as of mid-2019, the anti-TH antiserum had been cited in 1,565 publications (CiteAb 22941, 2019) and the anti-5-HT antiserum had been cited in 1,385 publications (CiteAb 20080, 2019).

For OA and NE immunolabeling, nerve cords were placed in freshly prepared, chilled (4 °C) GPA fixative, which consisted of 5 ml 25% glutaraldehyde, 15 ml saturated picric acid, and 0.1 ml glacial acetic acid. Samples were fixed for 3 hours at 4 °C and then thoroughly rinsed in 70% ethanol until the tissue was no longer yellow. The tissue was then washed in a solution of 1% sodium metabisulfite in iso-osmotic Millonig's buffer (three 10-minute washes) and then in 1% sodium metabisulfite + 1% sodium borohydride in iso-osmotic Millonig's buffer (one 10-minute wash). The tissue was next incubated in blocking solution at room temperature, for at least 2 hours and up to overnight, before being transferred to a primary antiserum solution for 3-7 days at 4° C. The antisera used were a 1:100 rabbit polyclonal anti-OA (Genetex, Cat# GTX12562, RRID: AB_2800443) and a 1:100 rabbit polyclonal anti-NE (Millipore, Cat# AB120, RRID: AB_90481), diluted in iso-osmotic Millonig's buffer. The anti-

OA antibody was raised against purified OA conjugated to BSA and glutaraldehyde, and specificity tests performed by the manufacturer revealed minimal cross-reactivity with TA, tyrosine, NE, and DA. The anti-NE antibody was generated against purified NE conjugated to BSA and glutaraldehyde; it has been shown to recognize NE in human and rat tissues, and was found to have minimal cross-reactivity with OA, DA, E, L-DOPA, and TA.

After incubation in primary antiserum, the nerve cords were rinsed for 2 hours in dilutant (a 1:2 dilution of blocking solution in iso-osmotic Millonig's buffer) and then transferred to secondary antiserum for 2 days at 4 ºC. For the anti-TH samples, the secondary antiserum was 1:200 Cy3-conjugated goat anti-mouse (ThermoFisher Scientific, Cat# A10521, RRID: AB_2534030), and for all other samples, the secondary antiserum was 1:200 Cy3-conjugated goat anti-rabbit (ThermoFisher Scientific, Cat# A10520, RRID: AB_10563288). Secondary-only controls were conducted for both of these antisera (n = 2 per antiserum) and yielded no TH- or amine-specific labeling. Following secondary incubation, the tissue was rinsed in iso-osmotic Millonig's buffer (three 30-minute washes), and then dehydrated via a graded ethanol series (30 minutes in each of the following: 70% EtOH, 80% EtOH, 85% EtOH, 90% EtOH, 95% EtOH, 100% EtOH). Tissue was cleared for 30 minutes in a 1:1 mixture of methyl salicylate and EtOH followed by 30 minutes in pure methyl salicylate, and then wholemounted between two glass coverslips in DEPEX mounting medium (Electron Microscopy Sciences).

To visualize TH-immunoreactivity in peripheral tissues, we also created mesosomal fillets from adult scorpions and labeled them with the TH antiserum. To do so, scorpions were anesthetized, pinned, and their mesosomas were exposed as described earlier. The dorsal vessel, digestive tissues, hepatopancreas, and reproductive organs were then discarded, such that the only tissues remaining in the mesosoma were the nervous system, supraneural lymphoid glands, muscle, and epidermis. The prosoma and metasoma were then carefully cut away, leaving only the exposed mesosoma. These preparations were subsequently processed through the full TH-immunolabeling protocol described above. Prior to the dehydration steps, the mesosomal fillets were securely pinned such that they were as flat as possible for mounting.

In addition to the immunolabeling of subadult/adult scorpion tissue, we also adapted our protocols to label TH-synthesizing neurons and their targets in embryonic scorpion tissue. Embryos selected for these experiments were at or near the final stage of embryonic development, corresponding in morphology to the “Stage M” embryos of Laurie (1890), as evidenced by the presence of distinct segmented appendages, pigmented median and lateral eye spots, and a well-developed terminal segment (telson). At this stage, discrete ganglia become visible along the VNC (Anderson, 1973; Laurie, 1890; Polis & Sissom, 1990). Gravid adult female scorpions were anesthetized and pinned in a wax dish, and their mesosomal cavities exposed as descried earlier. The developing embryos were carefully extracted from the female’s ovariuterus and transferred to a bath

of physiological saline in a small Sylgard-coated dish, where they were secured dorsal-side up with minuten pins. Using ultrafine forceps, we carefully peeled away the prosomal carapace and mesosomal tergites of the embryos and removed extraneous tissue, creating a preparation consisting of the developing CNS, ventral muscles, and ventral integument. The saline was then pipetted from the dishes and replaced with freshly prepared fixative (4% paraformaldehyde in iso-osmotic Millonig's buffer). The preparations were fixed at room temperature for 45-60 minutes, then rinsed in iso-osmotic Millonig's buffer (three 10-minute washes) and incubated in blocking solution overnight. Following blocking, the preparations were carefully unpinned from the dish and incubated in 1:100 mouse anti-TH for 3 days. The remainder of the labeling protocol was identical to that used for adult tissue. Because the embryonic tissue was extremely fragile, great care was taken throughout the protocol to minimize damage to the structures of interest.

3.3.3 I Backfills of Segmental Nerves

To characterize the efferent neurons of interest, we backfilled segmental nerve roots in the mesosoma with Neurobiotin, in conjunction with either TH immunolabeling or 5-HT immunolabeling. This technique was adapted from protocols used previously in our lab to fill the nerve roots of both insects and the medicinal leech (Mesce et al., 1993; Puhl et al., 2018). The scorpion was anesthetized and pinned dorsal side up in a dish of physiological saline, as

described earlier. The mesosomal cavity was then exposed via two dorsal transverse incisions (directly anterior and posterior to the mesosoma) as well as two lateral incisions along the pleural membrane. The dorsal vessel, hepatopancreas, digestive system, and reproductive tissues of the mesosoma were all discarded, leaving only the exposed nervous system, supraneural lymphoid glands, musculature, and ventral cuticle. The volume of saline was then reduced, such that it just barely covered the preparation.

The posterior branch of the dorsal segmental nerve associated with the ganglion of interest was then carefully cut, and a small piece of Parafilm was secured with minuten pins near the site of the cut. On this film, a small well was constructed from a mixture of petroleum jelly and mineral oil, which was then filled with a solution of 5% Neurobiotin tracer dissolved in scorpion saline. Using ultrafine forceps, the cut nerve ending was gently placed in the well, such that it was in direct contact with the tracer. The preparation was placed in a humid chamber and covered, then allowed to sit at room temperature for 30-60 minutes before being transferred to a 4 °C refrigerator for overnight incubation. The mesosomal portion of the nerve cord, including the first three ganglia and their associated nerves, was then dissected out from the animal and fixed in PFA fixative at room temperature, either for 3 hours (if selected for TH labeling) or overnight (if selected for 5-HT labeling). The samples were then carried through the steps of our TH or 5-HT immunolabeling protocol, up to and including incubation in secondary antiserum (1:100 goat anti-mouse). The sample was

then rinsed in a solution of 1% Triton X-100 in iso-osmotic Millonig's buffer for 2 hours and incubated in 1:50 Cy5-conjugated streptavidin for 24 hours. Samples were then rinsed again, dehydrated, cleared, and mounted as described for our immunolabeling studies.

3.3.4 I Microscopy and Image Analysis

Preliminary inspection of histological samples was conducted using an upright fluorescence microscope connected to a Zeiss Atto Arc HBO 110W mercury vapor lamp. Sample sizes for each treatment are listed below: n = 36 TH-labeled nerve cords, n = 9 TH-labeled mesosomal fillets, n = 14 TH-labeled embryonic preparations, n = 14 backfills with TH labeling, n = 2 backfills with 5-HT labeling, n = 12 5-HT-labeled nerve cords, n = 9 OA-labeled nerve cords, and n = 6 NE-labeled nerve cords. Selected samples, which most clearly showed the structures of interest, were subsequently imaged at the University Imaging Centers (UIC) at the University of Minnesota, using a Nikon Ti2 inverted microscope equipped with an A1 confocal scan head and a Plan Apo lambda 4x, 10x, or 20x dry objective. The excitation wavelength of Cy3-labeled samples was 562 nm, with emission collected at 570-620 nm. For Cy5-labeled specimens, excitation was at 640 nm and emission was collected at 650-720 nm. The step size for all optical sections in the *z* plane was 0.95 µm. Laser power and PMT voltage were adjusted for best contrast and images were acquired at 1.2 µs/pixel and 4x averaging to improve the signal-to-noise ratio. Images collected from the

confocal microscope were later viewed and analyzed in FIJI (ImageJ) v2.0.0 (RRID: SCR_002285). The brightness and contrast of images was adjusted in Adobe Photoshop CS6 (RRID: SCR_014199) as necessary; no other manipulations were conducted.

3.3.5 I Ultra-Performance Liquid Chromatography and Mass Spectrometry

To confirm the presence of DA, NE, and OA in the scorpion CNS and quantify the absolute levels of these compounds, n = 20 scorpions were processed for ultra-performance liquid chromatography and mass spectrometry (UPLC-MS). The VNC of the scorpion was exposed following the same dissection protocol used in our immunolabeling studies. The entire nerve cord, containing all seven ganglia and their associated nerves, as well as the associated supraneural lymphoid glands, was then removed, placed into a cryotube, and immediately flash-frozen in liquid nitrogen. All 20 cords were then pooled in a 1.5 mL vial and stored at -80 °C until extraction. The prosomal portions of the CNS, including the supra- and subesophageal masses and their associated nerves, were also removed from these scorpions, to be processed separately from the nerve cords. To access this region of the CNS, two cuts along the lateral edges of the prosomal carapace were made and the carapace was then gently lifted up and forward to expose the internal tissue of the prosoma. Non-neural tissue and supporting apodemes were removed and discarded to reveal the tissues of the CNS, which were then carefully excised.

These tissues were flash-frozen, pooled in a separate 1.5 mL vial, and stored at -80 ºC until extraction.

In addition to the scorpion tissue, we also processed neural tissue from the wolf spider *H. lenta*. The spider was chosen for comparative purposes, as to date it is the only other arachnid species whose catecholaminergic neurons have been well-characterized (Auletta et al., 2019). Using the dissection protocols previously described by Auletta et al. (2019), we removed the entire fused CNS from n = 10 adult *H. lenta* and flash-froze, pooled, and stored them following the same procedure used for our scorpion samples.

For extraction, 250 µL of ice-cold 1% formic acid in deionized water was added to each of the three sample vials (i.e., scorpion nerve cords, scorpion prosomal CNS, and spider CNS) as the extraction solvent. The tissue was homogenized in an ice water bath with a Sonic Dismembrator (Model 120, Fisher Scientific) and centrifuged at 4 ºC and 20,000 RCF for 10 minutes. The supernatant was then collected and the remaining pellets were re-extracted twice with 200 µL of extraction solvent each. The combined supernatant was then dried down and resuspended in 0.1% formic acid in water, then centrifuged again before being collected. A total of 140 µL of tissue extract was obtained from each of the three samples, and 6 µL of internal standard (IS) was spiked into 60 µL of each tissue extract. The IS used was Dopa-(phenyl-d_3). A series of DA, NE, and OA standard mix solutions were also prepared for constructing calibration curves with a fixed IS concentration of 3.6 µM.

UPLC-MS analysis of the tissue extracts was conducted using a Dionex UltiMate 3000 LC system coupled with a Q-Exactive Orbitrap mass spectrometer. A reversed-phase column (Thermo Scientific Hypersil GOLD C18, 2.1 mm × 100 mm, 1.7 µm) was used for chromatographic separation at 35 ºC with a flow rate of 0.45 mL/min. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The LC gradient was set as follows: 0-2 min, 0% B; 2-5 min, 0-0.5% B; 5-8 min, 0.5-3.2% B; 8-11 min, 3.2-98% B; 11-14 min, 98% B; 14-16 min, 0% B. Injection volume was 10 µL for standards and 15 µL for scorpion/spider samples. Parallel reaction monitoring (PRM) acquisition was performed from 0-6 min at a resolution of 35 K, an automatic gain control of 1×10^6, a maximum injection time (IT) of 100 ms, and an isolation window of 0.4 m/z. The multiplexed target precursor ions were 154.0863 for DA, 170.0812 for NE, 154.0863 for OA, and 201.0949 for the IS. The collision energy was 30% with higher-energy collisional dissociation (HCD) fragmentation. From 6-16 min, full MS scan was acquired from 70-1000 m/z.

The identification of OA, NE, and DA in scorpion and spider samples was achieved using the standard retention times and monitoring ions for the standard compounds listed in Table 3.1. The absolute quantification of OA, NE, and DA was achieved via LC-MS/MS-based targeted PRM assay, using calibration curves that were made by plotting the monitoring ion area ratio (amine standard:IS) against the concentration ratio (amine standard:IS).

3.3.6 I Extracellular Electrophysiology

To determine if DA, NE, and OA could induce physiological responses in the scorpion nervous system, we conducted extracellular recordings of isolated terminal (i.e., telsonic) nerves of the seventh abdominal ganglion. The protocol for these electrophysiological experiments was adapted from similar protocols previously used to record extracellular activity in the medicinal leech (Puhl & Mesce, 2008; Puhl et al., 2018). The scorpion was first anesthetized and pinned dorsal-side up in a wax dish under a bath of physiological saline, and a dorsal midline incision was made down the length of the third, fourth, and fifth metasomal segments. The integument of these segments was then peeled away and, using fine-tipped forceps, extraneous muscle and digestive tissue were discarded to expose the VNC. Using fine dissection scissors, we then carefully extracted the seventh abdominal ganglion and all of its associated nerves. This tissue was transferred to a small Sylgard-lined recording dish filled with scorpion saline, and secured in place with minuten pins, thus forming an isolated preparation of the ganglion and nerve of interest.

The terminal nerve was then taken up via a custom-made plastic suction electrode (the tip of which measured approximately 50 µm in diameter), which was connected to an A-M Systems model 1700 AC-coupled differential amplifier. Electrophysiological signals were digitized at 10 kHz via a Digidata 1440A digital-to-analog converter (Molecular Devices) and recorded in pCLAMP Clampex v.10 (RRID: SCR_011323). An initial baseline recording of spontaneous extracellular

unit activity was performed for several minutes, after which the saline was gradually siphoned from the dish and replaced with a 100 µM solution of either DA, NE, or OA in scorpion saline. Changes in extracellular activity were continuously recorded for at least 60 minutes and up to 3 hours post-perfusion. In total, n = 5 preparations were recorded in DA, n = 6 in NE, and n = 4 in OA. An additional set of recordings (n = 3) was performed in a 25 µM solution of NE, to determine if the physiological effects observed at 100 µM could be elicited at more dilute concentrations as well. Extended controls, in which activity of the telsonic nerve was recorded in pure saline over a span 3 hours, were also conducted (n = 3). All recordings were saved on a computer and later analyzed in pCLAMP Clampfit v.10.

3.3.7 I Bioinformatics

We further explored the possibility that DA, NE, and OA coexist in the scorpion by searching NCBI GenBank for protein sequences from *C. sculpturatus* that were annotated as putative dopaminergic, adrenergic, and octopaminergic receptors. This was possible due to recently available genome sequence data for *C. sculpturatus*, first published by Schwager et al. (2017) and publicly accessible in GenBank under accession number PRJNA168116. In total, 41 such protein sequences were identified, as described below (GenBank accession numbers are given in parentheses). Thirteen of these sequences were putative adrenergic receptors: two α_1 adrenergic receptor-like proteins (XP_023217181.1,

XP_023217180.1), eight α_2 adrenergic receptor-like proteins (XP_023223923.1, XP_023223922.1, XP_023231566.1, XP_023231558.1, XP_023231552.1, XP_023216971.1, XP_023216970.1, XP_023216969.1), one β_2 adrenergic receptor-like protein (XP_023234755.1), and two β_2 adrenergic receptor-like proteins (XP_023218469.1, XP_023218468.1). Seventeen sequences were identified as putative octopaminergic receptors: eleven OAMB (= OA-α) receptor-like proteins (XP_023213977.1, XP_023213976.1, XP_023213975.1, XP_023240153.1, XP_023240149.1, XP_023240148.1, XP_023234747.1, XP_023234746.1, XP_023234745.1, XP_023234743.1, XP_023234742.1), three OA-β receptor-like proteins (XP_023212722.1, XP_023236844.1, XP_023226948.1), and three unspecified OA receptor-like proteins (XP_023213841.1, XP_023218555.1, XP_023231590.1). Finally, eleven sequences were determined to be putative dopaminergic receptors: six D_1 receptor-like proteins (XP_023213411.1, XP_023227694.1, XP_023227693.1, XP_023227692.1, XP_023218180.1, XP_023218179.1) and five D_2 receptor-like (XP_023244848.1, XP_023244847.1, XP_023244846.1, XP_023244845.1, XP_023231635.1). FASTA sequences for all 41 of these putative receptors were downloaded from NCBI GenBank. For comparison, FASTA sequences of known dopaminergic, adrenergic, and octopaminergic receptors from a wide variety of genetic model organisms were also downloaded. Representative sequences of receptors in the α1, α2, β1, and β2 adrenergic receptor families were selected from the following vertebrate taxa: the dog *Canis lupus familiaris*, the zebrafish

Danio rerio, the chicken *Gallus gallus*, the Rhesus monkey *Macaca mullata*, the mouse *Mus musculus*, and the clawed frog *Xenopus tropicalis*. Representative octopaminergic receptors, including those in the OA-α and OA-β receptor families, were selected from the following invertebrate taxa: the horseshoe crab *L. polyphemus*, the spider mite *Tetranychus urticae*, the mosquito *Aedes aegypti*, the honeybee *A. mellifera*, the fruit fly *Drosophila melanogaster*, the flour beetle *Tribolium castaneum*, the nematode *Caenorhabditis elegans*, and the flatworm *Schistosoma haematobium*. Dopamine receptor sequences, including representatives from the D1-like and D2-like receptor families, were taken from all of these vertebrate and invertebrate taxa. In addition, sequences of dopaminergic, adrenergic, and octopaminergic receptors from the organisms examined by Bauknecht and Jékely (2017)— i.e., *Platynereis dumerilii* (Annelida: Polychaeta), *Priapulus caudatus* (Priapulida), and *Saccoglossus kowalevskii* (Hemichordata)— were also included in our analyses. In total, 86 dopaminergic, adrenergic, and octopaminergic receptor sequences were utilized for comparison against the 41 putative scorpion receptors; the complete list of sequences, along with their corresponding GenBank accession numbers, is given in Table 3.2. Redundant sequences were removed prior to analysis via Cd-hit (Li & Godzik, 2006), using an identity cutoff value of 0.7. A sequence cluster map was then created using Pclust, an open access tool for visualizing all-against-all pairwise similarities between protein sequences (Li et al., 2013), with a cutoff E-value of $1x10^{-60}$. These parameters are similar to those used by Bauknecht and Jékely

(2017) to create cluster maps comparing the protein sequences of DA, NE, and OA receptors in other taxa.

3.4 I Results

3.4.1 I Anatomical Orientation and Nomenclature

To contextualize our findings, the scorpion body plan and gross structure of the scorpion CNS is shown diagrammatically in Figure 3.1. The body of the scorpion, like that of all arachnids, is divided into two distinct tagmata— the prosoma (or cephalothorax) and the opisthosoma (or abdomen). Unique to scorpions, however, is the subdivision of the opisthosoma into a broad, 7-segmented mesosoma and a narrow, 5-segmented metasoma. The fifth metasomal segment bears a terminal structure known as the telson, which includes the venom-delivering stinger. These body divisions are highlighted in Figure 3.1 A.

The CNS of the scorpion consists of a fused mass of neural tissue in the prosoma, as well as a VNC that extends down the length of the opisthosoma and contains discrete segmental ganglia. This arrangement is shown in Figure 3.1 B, and reviewed in more detail by Babu (1965, 1985) and Root (1990). The prosomal portion of the CNS is subdivided by the esophagus into two broad regions: a supraesophageal mass, which contains the protocerebrum (brain) and the cheliceral neuromeres, and a subesophageal mass, which consists of the fused ganglia of the pedipalps, the four pairs of walking appendages, and the first

two opisthosomal segments. In the opisthosoma, the VNC contains seven ganglia separated by paired connectives. The first three of these ganglia are located in the mesosoma, and ganglia 4-7 are located in the metasoma. Extending from each of these ganglia are paired dorsal and ventral segmental nerves that connect to the periphery of their respective segments. The seventh (terminal) ganglion results from the fusion of two developmentally distinct ganglia and contains several additional nerves that project to the fifth metasomal segment and the telson. Also of note are the supraneural lymphoid glands, which are irregularly-shaped masses of non-neural tissue that are connected to the dorsal surfaces of the first three ganglia and connectives in the mesosoma. The cellular contents and functions of these organs have not been extensively studied, but their structure is reviewed by Farley (1984).

The general arrangement of the CNS in arthropods consists of an anterior compound brain and numerous discrete segmental ganglia comprising a VNC. The CNS of non-scorpion arachnids, in contrast, is highly condensed, with most or all of these ganglia fusing during development to form a conspicuous, single mass of neural tissue in the prosoma (Babu, 1965, 1985; Babu & Barth, 1984; Schmidt-Rhaesa et al., 2015; Weygoldt 1985; Whitington & Mayer, 2011). Thus, the CNS of the scorpion, with its partial fusion of ganglia and retention of a nerve cord, is an intermediate state between the ancestral arthropod condition and the more derived condition of other arachnids.

3.4.2 I TH-ir Neurons in the VNC

We reliably detected robust TH immunoreactivity in all ganglia of the VNC (representative ganglia shown in Figure 3.2 A-B). A total of four distinct populations of TH-ir neurons were identified (highlighted in Figure 3.2 C-F). However, the pattern of labeling differed substantially between the mesosomal ganglia and the metasomal ganglia.

Each ganglion of the mesosoma contained three distinct populations of TH-ir neurons, labeled 1-3 in Figure 3.2 A. The most striking of these neurons were arranged in paired clusters of large somata located along the anterior lateral edges of the ganglion (Figure 3.2 A & C, arrowhead 1). Each cluster contained at least 14 distinct somata, with individual somata measuring approximately 17-20 μm in diameter. The axons of these neurons (Figure 3.2 A & C, solid arrow 1) fasciculated into a tight, conspicuous bundle of fibers, which descended along the lateral margin of the ganglion and eventually exited the ganglion through the segmental nerve. We were able to trace these projections reliably from the somata out through the nerves in all preparations of TH-immunolabeled mesosomal ganglia, thus confirming that these neurons are indeed efferent. These neurons are shown at an enhanced magnification in Figure 3.2 C, which more clearly highlights the individual somata that comprise the cluster and their associated axons.

Slightly posterior to these large efferent cells was a second population of TH-ir neurons, whose somata (Figure 3.2 A & D, arrowhead 2) were also

positioned along the lateral margins of the mesosomal ganglia. These somata were arranged in paired clusters of at least 12 somata, and were substantially smaller than the efferent cells, with diameters measuring between 7-9 μm. The axons of these neurons did not appear to exit the ganglion, but rather projected towards the midline (Figure 3.2 A & D, solid arrow 2), where they contributed to the anterior arms of a pronounced chiasma of fibers (Figure 3.2 A, asterisk). An enhanced view of one of these clusters that more clearly shows individual somata and their projections is shown in Figure 3.2 D.

Posterior to the central chiasma was the third population of TH-ir neurons that we detected in the mesosomal ganglion. The somata of these neurons were contained in paired clusters adjacent to the midline of the ganglion (Figure 3.2 A & E, arrowhead 3). Each of these clusters contained approximately 12 somata, measuring 6-8 μm in diameter each, making them comparable in size and morphology to the cells of Population 2. The axons of these neurons formed clear fasciculations (Figure 3.2 A & E, solid arrow 3), which initially descended diagonally towards more lateral and posterior regions of the ganglion before turning to ascend towards the midline and contributed to the posterior arms of the central chiasma. The somata of neurons in this population and their associated axons are highlighted more clearly in Figure 3.2 E.

The ganglia of the metasoma also contained three distinct populations of TH-ir neurons. Two of these populations, labeled 2′ and 3′ in Figure 3.2 B, were presumed to be homologous to Populations 2 and 3 (respectively) in the

mesosomal ganglia on the basis of similar morphologies and patterns of connectivity. Population 2′ of the metasoma consisted of paired clusters of somata along the anterior lateral margins of the ganglion (Figure 3.2 B, arrowhead 2′); each cluster contained at least 8 somata measuring 8-10 µm in diameter. The axons from these somata fasciculated and extended posteriorly towards the midline of the ganglion (Figure 3.2 B, solid arrow 2′), where they formed the anterior branches of a central chiasma (Figure 3.2 B, asterisk) that mirrored what was observed in the mesosomal ganglia. Population 3′ of the metasoma was marked by paired lateral clusters of somata along the posterior margins of the ganglion (Figure 3.2 B, arrowhead 3′). Within each of these clusters were at least 12 distinct somata, each of which measured approximately 6-8 µm in diameter. The axons of these neurons fasciculated and ascended towards the midline (Figure 3.2 B, solid arrow 3′), where they contributed to the posterior aspect of the central chiasma structure. Although the cells of this population are more laterally positioned than those of Population 3 in the mesosomal ganglia, the striking similarities in soma size, soma number, and projection patterns between the two populations nevertheless implies a strong homology.

The third distinct set of TH-ir neurons in the metasomal ganglia did not correspond to any neurons observed in the mesosomal ganglia. This population consisted of paired clusters of at least three somata along the lateral edge of the ganglion (Figure 3.2 B & F, arrowhead 4), positioned at an intermediate length

between the other two populations of TH-ir cells. Each of these clusters contained at least 3 somata that were in very close association with each other, with individual somata measuring approximately 10-11 μm in diameter. The axons of these cells projected towards the midline of the ganglion at a slight descending angle (Figure 3.2 B & F, solid arrow 4). The exact site at which these axons terminated was not discernible in our preparations; thus, it is unclear whether they also contribute to the central chiasma alongside the other populations of TH-ir cells in the metasoma.

We also detected lateral plurisegmental TH-ir projections that appeared to span the entire length of the VNC (Figure 3.2 A-B, open arrows). Although these projections were found in all ganglia observed, they were more prominent in the metasomal ganglia (Figure 3.2 B, open arrows). We were unable to identify any neurons in the VNC that gave rise to these projections, and thus it is likely that those somata lie in portions of the nervous system (e.g. the prosomal CNS and/or the periphery) that were not examined in this study. As such, it is presently unclear whether these are ascending fibers, descending fibers, or a mixture of both.

3.4.3 | Peripheral Projections of Efferent TH-ir Neurons

We achieved robust TH immunoreactivity in the whole-body preparations of late-stage scorpion embryos (Figure 3.3 A), and through these preparations, the peripheral projection patterns of the large TH-ir efferent neurons (cell

population 1 in Figure 3.2 A & C) were revealed. The bundled axons of these cells were shown to unambiguously exit each developing mesosomal ganglion (as well as the posterior portion of the developing prosomal CNS) via the segmental nerves and extend towards the lateral edges of the ventral mesosomal body cavity (Figure 3.3 A, arrows). There, the fibers of each nerve ramified extensively to form a dense plexus of TH-ir fibers (Figure 3.3 A, arrowheads). We also observed conspicuous TH-ir plexuses along the periphery in our TH-immunolabeled mesosomal fillets of adult scorpions (Figure 3.3 B, arrow). In all preparations, these plexuses were found in close association with the four pairs of book lungs (Figure 3.3 B, dashed outline), which are the respiratory organs of scorpions. The majority of projections were concentrated along the inner margins of the book lungs. Extending from the main arms of each plexus were numerous fine TH-ir processes that appeared to innervate the book lung tissue directly (Figure 3.3 C, arrowheads).

Although both the TH immunolabeled nerve cords (Figure 3.2) and embryonic scorpion preparations (Figure 3.3 A) allowed us to confirm that these large TH-ir neurons exited the CNS and were efferent, we were unable to detect double labeling in them when our TH immunolabeling protocol was paired with Neurobiotin backfilling of the segmental nerves. In these preparations, the large somata showed robust TH immunoreactivity, but did not show positive labeling with streptavidin-Cy5 (which would have bound to Neurobiotin) (Figure 3.4 A-B, solid arrowhead). In contrast, the somata of many other neurons that had efferent

projections (but were not TH-ir) showed strong, positive labeling with streptavidin-Cy5, demonstrating the presence of Neurobiotin in those structures. Among these positively labeled efferent neurons were large cells (approximately 25-30 µm in diameter) that sat adjacent to the large TH-ir somata (Figure 3.4 A & C, open arrowhead). Similarly, strong streptavidin-Cy5 labeling was detected in a large number of afferent axons in the filled nerve, as well as their sites of termination within the CNS.

In preparations that were treated with 5-HT immunolabeling in combination with Neurobiotin backfills of the segmental nerves, we did observe positive streptavidin-Cy5 labeling in somata that corresponded in position and size to the large efferent TH-ir neurons in other preparations (Figure 3.4 D, arrowheads). This result confirmed that the lack of co-localization observed in the anti-TH/backfill preparations was due to some kind of masking effect, whereby the anti-TH antibody possibly prohibited the streptavidin-Cy5 from binding or obscured its fluorescence.

3.4.4 I OA- and NE-ir Neurons in the VNC and Lymphoid Glands

Paired clusters of OA-ir neurons were found in all three ganglia of the mesosoma and were positioned adjacent to the midline at an intermediate length along each ganglion (Figure 3.5 A, arrowheads). Each cluster contained at least 35 somata, approximately one-third of which were relatively brightly labeled (Figure 3.5 B, solid arrowhead); the remaining cells were more faintly labeled

(Figure 3.5 B, open arrowhead). The somata of both types of cells measured approximately 8-10 µm in diameter. The axons of these OA-ir neurons were not visible in any of our preparations, and thus we were unable to discern their complete morphologies and projection patterns. However, our Neurobiotin backfills of nerves in the mesosomal ganglia did not reveal any positive labeling of cells that corresponded in size and position to those in these OA-ir clusters (Figure 3.4); thus, it is presumed that these neurons are not efferent, and may instead be local interneurons. These OA-ir clusters were not detected in any of the metasomal ganglia.

Using the anti-NE antiserum, we were unable to detect any positive labeling of ganglionic neurons within the VNC. However, we did detect numerous, small, and irregularly distributed NE-ir somata (Figure 3.6 A-B, arrowheads) in the supraneural lymphoid glands. The majority of these NE-ir cells measured between 5-6 µm in diameter, although some larger cells (up to 8-9 µm) were visible as well. Outlines of nuclei were clearly visible in these structures, confirming that they were indeed cells. They appeared distinctly non-neuronal in their morphology, devoid of any neurites or other projections, and thus they may be secretory (e.g. endocrine or paracrine) cells instead. Other cells in the supraneural lymphoid glands were positively labeled with the anti-OA antiserum (Figure 3.6 C, arrowheads), suggesting that these glands contain both adrenergic and octopaminergic cells. The OA-ir cells measured approximately 4-6 µm in diameter and showed a similar morphology to the NE-ir cells. However,

the OA-ir cells in the lymphoid glands appeared to be much more numerous and more densely packed compared to the NE-ir cells.

3.4.5 I 5-HT-ir Neurons in the VNC

We detected robust, serially repeating patterns of 5-HT immunoreactivity in all seven ganglia of the VNC (Figure 3.7 A-C). Notably, there were no discernible differences in the distribution of 5-HT-ir structures between the mesosomal (Figure 3.7 A) and metasomal (Figure 3.7 B) portions of the CNS, which contrasted with the patterns we observed for TH-ir and OA-ir cells. Conspicuous bilaterally paired clusters of bright 5-HT-ir neurons were found along the lateral margins of each ganglion, posterior to the segmental nerve roots (Figure 3.7 A-D, solid arrowheads). Within each cluster were at least 12 somata, each of which measured 7-8 µm in diameter. The axons of these cells fasciculated and ascended along the lateral portion of the ganglion before clearly exiting the ganglion through the segmental nerves (Figure 3.7 A-D, solid arrows). Neurobiotin backfills of segmental nerves, in combination with 5-HT immunolabeling, revealed that these neurons were reliably double labeled (showing Cy3 and Cy5 fluorescence) (Figure 3.4 D-F, arrow), further confirming that they are efferent. Notably, the terminal ganglion was shown to contain a second paired cluster of 5-HT-ir neurons, which was positioned directly anterior to the terminal nerve roots (Figure 3.7 C, open arrowheads). The projection patterns of these neurons were not clearly discernible in our preparations. Since

the cells in these clusters corresponded in number, size, and general appearance to those in the serially repeating clusters present in all ganglia, it was presumed that the two populations of neurons are homologous. The presence of two paired clusters in the terminal ganglion was likely due to the fact that the terminal ganglion results from the fusion of two neuromeres during development (Babu, 1965; Root, 1990).

Our 5-HT immunolabeling also revealed prominent bundles of serotonergic afferent fibers in the segmental nerves associated with all seven ganglia (Figure 3.7 A-D, open arrows). A subset of these afferent fibers (Figure 3.7 D, open arrow 1) appeared to terminate within the same ganglion that they entered, where they contributed to a dense and intricate meshwork of fine 5-HT-ir processes (Figure 3.7 D, asterisk). Other serotonergic afferents (Figure 3.7 D, open arrow 2) did not terminate in the ganglion that they entered, but instead ascended to the ganglion directly anterior to it, where they ramified and formed similar dense and intricate collections of fine 5-HT-ir fibers (Figure 3.7 A-B, asterisks). Along these fine serotonergic projections were many conspicuous and large punctate structures (Figure 3.7 E, arrowheads), the largest of which measured approximately 3 µm in diameter. Based on their size and general morphology, these structures appear to be large synaptic boutons.

3.4.6 I Quantification of Catecholamines and OA in the CNS

Our UPLC-MS assays revealed detectable quantities of DA, NE, and OA in the CNS of both *C. sculpturatus* and *H. lenta*. The mass spectra of each of these amines in the tissue extracts showed a high degree of correspondence to the mass spectra of the DA, NE, and OA standards used for comparison (Figure 3.8 A-C), thereby confirming the presence of these three amines in the tissues sampled. Within the scorpion, the average amount of DA detected was 15.29 pmol in the VNC and 79.44 pmol in the prosomal CNS, whereas the average amount of NE recovered was 1.09 pmol in the nerve cord and 9.55 pmol in the prosomal CNS. The average amount of OA was found to be 12.19 pmol per nerve cord and 87.48 pmol per prosomal CNS. Within the spider, the average amount of each amine per CNS was as follows: 90.78 pmol DA, 17.80 pmol NE, and 237.74 pmol OA. The calibration curves used to obtain these absolute quantifications of the three amines are shown in Figure 3.9 A-C. Six-point standard calibration curves were constructed for both DA and OA with excellent linearity across four orders of magnitude in the dynamic range (R^2 = 0.9990 for DA; R^2 = 0.9988 for OA). A five-point calibration curve for NE was made with excellent linearity as well (R^2 = 0.9975) across three orders of magnitude in the dynamic range.

3.4.7 I Physiological Responses to Catecholamines and OA

In all experiments, baseline extracellular neuronal recordings made of the telsonic nerve showed variable amounts of spontaneous small unit activity

(example trace in Figure 3.10 A). We never observed repeated patterns of neuronal activity (e.g., rhythmic bursting) in any of our baseline recordings, nor did we detect any large units in these traces. Notably, at a concentration of 100 µM, all three of the amines we tested (DA, NE, and OA) were able to evoke rhythmic patterns of bursting activity, as shown in Figure 3.10 B-D. Furthermore, the patterns of neuronal bursting elicited by each amine at this concentration were distinct from one another, suggesting that each amine has its own unique mode of action in the scorpion.

A representative trace of the patterned activity evoked by NE is shown in Figure 3.10 B. This pattern was marked by the the rhythmic bursting of two distinct units whose amplitudes were above baseline. The smaller unit bursts (Figure 3.10 B, arrowheads) lasted for a duration of approximately 4-5 seconds each and were almost always immediately followed by the larger unit bursts (Figure 3.10 B, arrows), which lasted for approximately 3-4 s each. The period between bursts of each type was approximately 12-15 s during the most rhythmic portion of the recordings. This pattern was observed in all of the NE recordings (at both 100 µM and 25 µM) and appeared to be unique to NE, as neuronal bursts of similar duration and frequency were not observed in the presence of DA or OA.

DA evoked two different types of patterned neuronal activity, exemplified by the representative traces in Figure 3.10 C. In n = 3 of our DA recordings, we observed regular bursting of large units (Figure 3.10 C top trace, arrowheads),

each of which was very brief in duration (approximately 0.2-0.3 s). The period between these bursts ranged from 5-15 s. In n = 2 DA recordings, this pattern was not evoked, and instead we observed a different bursting of large units (Figure 3.10 C bottom trace, arrows) that occurred for a much longer duration (approximately 8-10 s) and with a much larger period between them (4.25-5 minutes). These two types of DA bursts never co-occurred within a single recording, nor were they observed in any of our NE recordings. The units that comprised the DA bursts were also noticeably larger in amplitude than those in bursts evoked by NE.

Neuronal activity elicited by OA was also variable. In n = 2 of four OA recordings, we observed numerous overlapping bursts of multiple units in response to OA, as shown in Figure 3.10 D. These bursts included the largest units recorded from the telsonic nerve, as well as numerous smaller units. Due to the overlapping nature of these OA-induced bursts, it was difficult to determine their exact durations and periods, nor was it feasible to determine what relation (if any) they had to the patterns observed in the NE and DA recordings. In contrast to this intense activity, we did not observe any rhythmic bursting in the remaining n = 2 OA recordings. In those recordings, the effect of OA appeared to be minimal, and only slightly increased the rate of unpatterned firing relative to the baseline.

3.4.8 I Sequence Analysis of Aminergic Receptors

After redundancies were removed from the analysis, a total of 80 protein sequences for aminergic receptors remained in the sequence cluster map (Figure 3.11). Among these sequences were 12 from *C. sculpturatus*—four putative dopaminergic receptors (Accession #s XP_023213411.1, XP_023218179.1, XP_023231635.1, XP_023244848.1), four putative adrenergic receptors (Accession #s XP_023216971.1, XP_023217181.1, XP_023231566.1, XP_023234755.1), and four putative octopaminergic receptors (Accession #s XP_023212722.1, XP_023213977.1, XP_023226948.1, XP_023240153.1). Under the parameters used, the 68 non-scorpion receptor sequences showed a high degree of affinity for receptors of the same class, as revealed by their relative positions in the sequence cluster map. In total, seven discrete clusters were visible in the map, consisting of known D_1-like, D_2-like, α_1 adrenergic, α_2 adrenergic, β_1+ β_2 adrenergic, OA-α, and OA-β receptor sequences, respectively.

All four of the putative dopaminergic receptors of *C. sculpturatus* clustered tightly with known dopaminergic receptors from other organisms, indicating relatively high levels of sequence similarity between those sequences. Two of these scorpion sequences (XP_023213411.1, XP_023218179.1) fell within the cluster of known D_1-like receptors, whereas the other two (XP_023231635.1, XP_023244848.1) clustered with D_2-like receptors (albeit also very close to the α_1 adrenergic cluster). Of the four putative adrenergic receptors from *C. sculpturatus*, one (XP_023217181.1) was recovered well within the cluster of

known α_1 adrenergic receptors. Two other putative adrenergic receptors of the scorpion (XP_023216971.1, XP_023231566.1) clustered with known α_2 adrenergic receptors, although the margins of this cluster contained several known octopaminergic receptors as well. The final putative adrenergic receptor of the scorpion (XP_023234755.1) was located in relative isolation, equidistant from the D_1-like, β adrenergic, and OA-β clusters. Of the four putative *C. sculpturatus* octopaminergic receptors, two (XP_023212722.1, XP_023226948.1) clustered tightly with known OA-β receptor sequences. The remaining two putative OA receptors of the scorpion (XP_023213977.1, XP_023240153.1) were recovered between the α_1 adrenergic cluster and the OA-α cluster, but curiously showed higher similarity to the α_1 adrenergic sequences than to the OA-α sequences.

3.5 I Discussion

3.5.1 I Overview

We reliably detected multiple populations of TH-ir neurons in the VNC of *C. sculpturatus*. The abundance of TH-ir neurons that were detected and their intricate patterns of connectivity support the idea that catecholamines likely have important functional roles in scorpions, including possibly in the modulation of behavior. These neurons were not labeled by our anti-NE antiserum, which implies that they synthesize DA as their end product. Among these TH-ir neurons were prominent clusters of efferent cells, whose projections were visualized in both embryonic and adult tissue fillets; their close association with the book lungs

supports the possibity that DA may regulate aspects of respiratory activity in the scorpion. We also detected conspicuous, clustered populations of serotonergic and octopaminergic neurons in the VNC, suggesting that these compounds also contribute to neuromodulation in the CNS. Curiously, OA-ir and NE-ir somata were localized in the supraneural lymphoid glands directly above the VNC in the mesosoma, raising the possibility that these organs may have as-of-yet uncharacterized secretory functions, providing release sites for amines over the CNS and/or into the hemolymph. Finally, we not only detected appreciable quantities of NE in the scorpion CNS, but also showed that genes encoding distinct adrenergic receptors exist in the *C. sculpturatus* genome. We also demonstrated that NE can induce specific physiological changes in the activity of the scorpion's telsonic nerve. These results present a compelling case for NE as an endogenous signaling molecule in scorpions, and directly challenge the long-held belief that adrenergic signaling is limited to vertebrates. Although the precise functions of NE, DA, OA, and 5-HT in scorpions remain largely unknown, the anatomical, physiological, and quantitative results provided here will serve as a foundation for future investigations into the aminergic modulation of behavior, both in scorpions and across the Chelicerata more broadly. Additionally, our comprehensive and integrative study design provides a useful model strategy for future examinations of adrenergic systems in other invertebrates, which have only recently begun to be investigated in earnest.

3.5.2 | TH-ir Neurons in the VNC are Likely Dopaminergic

We detected robust and extensive TH-immunoreactivity in the VNC of *C. sculpturatus*, which implies potential widespread modulation of behavior and other phenomena by catecholamines in the mesosoma and metasoma. Among the structures we identified were four distinct populations of TH-ir neurons. Notably, we did not observe any positive labeling of these cells or their projections with the anti-NE antiserum, indicating that they likely do not produce NE (or its derivative E) as their end product; thus, they are all presumed to be dopaminergic neurons. Direct confirmation that these cells produce DA could theoretically be achieved via immunolabeling with an anti-DA antiserum; unfortunately, our previous attempts at DA immunolabeling in the wolf spider *H. lenta* were unsuccessful and highlighted a pressing need for new antisera that can reliably and effectively label DA in arachnid tissues (Auletta et al., 2019). Until such an antiserum becomes available, we have elected not to attempt direct DA labeling in the scorpion. Nevertheless, we believe that the lack of NE-immunoreactivity in these TH-ir cells is sufficient evidence that they synthesize DA as their sole end product.

This conclusion was further supported by our quantitative chemistry analyses, which indicated that NE is present in the VNC only in small quantities (1.09 pmol). Given the lack of NE-ir neurons in the ganglia, it is likely that this small amount of NE is entirely constrained to the supraneural lymphoid glands, where NE-ir cell bodies were observed; this possibility is discussed in more detail

later. In contrast, levels of DA in the nerve cord were over 14 times higher (15.29 pmol), and the presence of numerous dopaminergic neurons in the ganglia would account for this finding. The levels of NE detected in the prosomal mass (9.55 pmol) were higher than those in the nerve cord, which is likely due to the presence of adrenergic neurons in that region as there are no lymphoid glands in the prosoma. Thus, it appears that, in *C. sculpturatus*, potential NE-synthesizing neurons are exclusive to the prosomal CNS. Since the prosomal mass was not included in any of our immunolabeling experiments, future investigations are necessary to confirm that these hypothesized adrenergic neurons exist.

3.5.3 I Peripheral Modulation by Efferent Dopaminergic Neurons

Among the most salient structures observed across all of our immunolabeling studies were the clusters of efferent TH-ir neurons in the mesosomal ganglia. These cells are the largest dopaminergic neurons in the VNC of the scorpion and were consistently the most intensely labeled with the TH antiserum. We were able to reliably trace the bundled axons of these cells out to the periphery, thereby confirming that they are truly efferent neurons. This finding is of particular interest, because peripherally-projecting dopaminergic neurons are exceptionally rare across animal taxa. Although dopaminergic interneurons can be components of efferent pathways, the dopaminergic cells themselves do not typically leave the CNS, meaning that most of the modulatory actions of DA on peripheral processes are indirect; this trend is observed across

both invertebrates (Budnik et al., 1988; Crisp et al., 2002; Croll, 2001; Mesce et al., 2001; Nässel & Elekes, 1992; Orchard, 1990; Tedjakumala et al., 2018; Vömel & Wegener, 2008) and vertebrates (Dopeso-Reyes et al., 2014; Hatzipetros & Yamamoto, 2006; Moret et al., 2004; Pérez-Fernández et al., 2014). Our anatomical data oppose this trend, and instead support the hypothesis that DA can have direct modulatory effects on peripheral tissues in *C. sculpturatus*, in addition to possible indirect effects.

Although comparatively rare, there are nevertheless several examples of peripherally-projecting DA cells in other invertebrates. The dopaminergic salivary neuron (SN1) of certain insects has been shown to exit the CNS and directly innervate the salivary glands (Ali, 1997; Ali & Orchard, 1996; Elia et al., 1994; Hörner et al., 1995; Orchard et al., 1992). Notably, these efferent cells are only known from a small subset of insect orders in the Polyneoptera (e.g., Blattodea, Orthoptera, and Phasmatodea); they have not been observed in the other major insect groups, such as the Paraneoptera and Holometabola (Budnik & White, 1988; Mesce et al., 2001; Nässel & Elekes, 1992; Orchard, 1990; Tedjakumala et al., 2018). The dopaminergic anterior unpaired midline (AUM) cells of the crayfish *Procambarus clarkii* have also been observed exiting the CNS, and appear to innervate the surface of the hindgut (Mercier et al., 1991). Dopaminergic neurons have been observed leaving the CNS in several species of snail as well, and appear to innervate the albumen gland (Hernádi et al., 1993; Kiehn et al., 2001). In vertebrates, direct dopaminergic innervation of the periphery appears to be

limited to a small number cells that modulate the auditory system. Dopaminergic neurons in the diencephalon of teleost fishes were shown to exit the CNS and directly innervate the auditory saccule (Forlano et al, 2014; Ma, 2003; Perelmuter & Forlano, 2017), and similar innervation by dopaminergic efferents was detected in the mammalian cochlea, although the contribution of these DA cells was relatively minor compared to the intense adrenergic modulation of the inner ear (Darrow et al., 2006; Eybalin et al., 1993; Maison et al., 2012). These scarce examples emphasize that peripherally-projecting dopaminergic neurons are known in only a relatively few animal species, compared to the broad range of taxa whose dopaminergic systems have been investigated; furthermore, within each of these species, peripherally-projecting neurons constitute only a very small fraction of the total number of DA cells. From this perspective, the efferent dopaminergic cells of *C. sculpturatus*—which are relatively numerous in the VNC— are indeed exceptional.

The efferent DA cells of the scorpion were found to terminate in intricate plexuses directly over the book lungs, suggesting that DA directly modulates respiratory processes in *C. sculpturatus*. The precise nature of this proposed modulation is not currently known, however. The book lungs of scorpions are remarkably intricate respiratory organs. Their fine structure, as well as the mechanisms underlying the flow of air and hemolymph through them, have been examined in great detail across a broad range of scorpion taxa (Farley, 1990, 1999, 2008, 2011; Kamenz and Prendini, 2008; Scholtz & Kamenz, 2006). The

dorsal vessel (heart) and hemolymph vascular system of scorpions, which are intimately associated with the respiratory system, have also been thoroughly examined (Farley, 1985, 1999; Klußmann-Fricke et al., 2012; Klußmann-Fricke et al. 2014; Randall, 1966; Wirkner & Prendini, 2007). The findings of these structural and functional analyses, as they relate to the results of the present study, are briefly summarized below.

All scorpions possess four pairs of book lungs, located along the ventral surface of the mesosoma. At the posterior end of each book lung is a slit-shaped spiracle, which is opened and closed by the actions of the poststigmaticus muscle that extends from the sternum to the spiracle's posterior edge. Oxygen enters through the open spiracle and fills a large chamber (the atrium), which can be expanded or constricted via the actions of the dorsal and ventral atrial muscles that lie along the distal lateral margins of the book lung. From the atrium, oxygen passes through the air sacs that sit between the book lung lamellae, which are thin membranes of epidermal tissue that comprise the majority of the book lung. The inner lumen of each lamella is filled with hemolymph, and gas exchange occurs as oxygen crosses from the air sacs into this hemolymph space (via diffusion across the epidermal cell layer of the lamella); carbon dioxide is excreted via diffusion in the opposite direction. The lamellae are arranged in an overlapping fashion, similar to the pages of a book (hence, the name "book lung"). The oxygenated hemolymph is carried through the lamellae to the pneumocardial vein, which connects to the book lung at its

distal lateral margin. This vein transports the hemolymph along the lateral body wall up to the pericardial sinus that surrounds the dorsal heart. From there, it enters the heart itself to be circulated throughout the body. The heart is also connected to the book lungs via the hypocardial ligaments, which extend downwards and attach to a mass of connective tissue known as the fenestrated membrane, which covers the dorsal surface of each book lung directly above the lamellae.

The processes of the TH-ir plexuses that we observed did not appear to extend to the poststigmaticus muscle at the base of the spiracle, nor did they extend far enough laterally to reach the atrial muscles or pneumocardial vein. Thus, it is unlikely that the large efferent DA cells of the mesosomal ganglia directly modulate the opening of the spiracles, the contraction of the atrium (which assists in the flow of hemolymph to the lamellae), or the transport of oxygenated hemolymph to the heart. Rather, the thick arms of each plexus appear to ramify directly over the fenestrated membrane, with finer fibers appearing to branch off and directly innervate the lamellae below. It is presently unclear why these structures of the book lungs, in particular, would require such intense dopaminergic innervation and modulation. Possibily, the terminals of the plexuses release DA directly into the lamellae in a paracrine fashion; DA would then be transported directly to the heart via the pneumocardial vein, where it could either modulate the activity of the heart or be circulated more broadly as a hormone. Indeed, DA has been shown to play important roles in regularting the

heartbeat of crustaceans, both as a neuromodulator and a neurohormone (Cooke & Sullivan, 1982; Fort et al., 2004; Lane et al., 2018; Yamagishi et al., 2004). Dopaminergic modulation of the heartbeat has also been shown in insects, though it appears to play a minor role compared to 5-HT and OA (Collins & Miller, 1977; Johnson et al., 1997; Zornik et al., 1999). Thus, it is conceivable that DA could play similar roles in the scorpion, although the indirect route to the heart via the book lungs is somewhat unusual.

An intriguing alternative hypothesis is that some of the TH-ir fibers wrap around the base of the hypocardial ligament at its sites of attachment to the fenestrated membrane. The microscopy studies of Farley (1990) have revealed that the hypocardial ligaments contain numerous muscle fibers in addition to their connective tissues. Thus, it is possible that the efferent DA cells could modulate the activity of these muscle fibers, the contraction and relaxation of which would facilitate the movement of hemolymph through both the lamellae and the heart. Unfortunately, the hypocardial ligaments needed to be removed with the heart during our dissections. Future immunolabeling studies that include intact ligaments are therefore needed to determine if they are associated with the TH-ir plexuses. Other experiments, in which the effects of DA on the book lungs is observed directly, would be highly informative as well.

Comparative studies that examine potential dopaminergic control or modulation of respiration in other chelicerate taxa would also be relevant, as the respiratory systems of the Chelicerata are exceptionally diverse in morphology

and function. Book lungs are present in only five of the extant arachnid orders (Scorpiones, Araneae, Amblypygi, Thelyphonida, and Schizomida), which together form the proposed clade Arachnopulmonata (Howard et al., 2019, Klußmann-Fricke & Wirkner, 2018; Scholtz & Kamenz, 2006; Schultz, 2007; Sharma et al. 2014). In many araneomorph spiders, the posterior pair of book lungs are modified to form a network of tracheae that coexists as a separate respiratory system alongside the anterior book lungs; in rare instances, both pairs of book lungs may be modified in this way (Bromhall, 1987; Opell, 1998; Schmitz, 2013). The remaining arachnid orders are apulmonate (i.e., lacking book lungs); the majority of these taxa (Opiliones, Pseudoscorpiones, Solifugae, Acari, Ricinulei) possess intricate tracheael systems instead, which have evolved independently of spider tracheae (Franz-Guess et al., 2014; Klußmann-Fricke & Wirkner, 2018; Schultz, 2007; Sharma et al. 2014). The Palpigradi lack well-developed respiratory systems, and gas exchange likely occurs directly across the cuticle, although the enigmatic "ventral sacs" on the opisthosoma may represent reduced book lungs (Rowland & Sissom, 1980; Sharma et al. 2014; van der Hammen, 1982). Among the non-arachnid chelicerates, the Xiphosura utilize book gills that some researchers believe may represent the precursors to the book lungs of the Arachnopulmonata (Farley, 2012; Henry et al., 1996; Howard et al., 2019; Scholtz & Kamenz, 2006), whereas the Pycnogonida lack specialized respiratory structures and instead absorb oxygen across the cuticle and transport it internally using diverticula of the midgut (Woods et al., 2017).

Given the extensive dopaminergic innervation of the scorpion book lungs observed in the present study, it is expected that DA may also modulate book lung functions in other orders of the Arachnopulmonata, and potentially the book gills of the Xiphosura as well. It would be especially interesting to determine if the tracheal systems of spiders and apulmonate arachnids are also innervated by dopaminergic fibers. Future TH immunolabeling studies with a broader range of taxon sampling could shed light on these hypotheses.

Curiously, we failed to recover co-localization of Cy5-streptavidin and anti-TH immunolabeling (Cy3) in the efferent dopaminergic cells when the appropriate segmental nerve roots were filled with Neurobiotin. The exact reason for this result is unclear, because the axons of the efferent neurons unambiguously exit through the same nerves that were filled with the Neurobiotin tracer. We assume that they did, in fact, take up the Neurobiotin just as the neighboring non-TH-ir axons did. Thus, the lack of positive labeling is likely due not to an absence of Neurobiotin, but rather to the inability of the Cy5-streptavidin to successfully bind to Neurobiotin in these cells. Clear co-localization of Cy5-streptavidin and TH-immunoreactivity has been reported in neurons of other organisms (Crisp & Mesce, 2004; Nässel, 1996; Rosner et al. 2018; Vallejo et al., 2014), which shows that the two compounds are not fundamentally incompatible. We also recovered good co-localization of streptavidin and a different antiserum (anti-5HT) in our scorpion tissue, which indicates that double labeling with streptavidin is possible in *C. sculpturatus*. Thus, we propose that the lack of expected co-

localization in the efferent TH-ir neurons may be due to a masking effect that is unique to those cells. Intense TH-immunolabeling in the efferent neurons indicates that they contained very high concentrations of TH (and thus high concentrations of our primary and secondary antibodies). It is possible that these TH-antibody complexes, present in such high concentrations, physically impeded the Cy5-streptavidin molecules from accessing the Neurobiotin within the cells. Backfills using a different tracer molecule— ideally one that does not require a secondary compound like streptavidin for visualization— would be helpful in determining the validity of this hypothesis. Nevertheless, the lack of positive streptavidin labeling in these neurons should not be interpreted as evidence that they are not efferent, as our other lines of evidence strongly demonstrate that they do, in fact, project to the periphery.

3.5.4 I Comparisons to Catecholaminergic Neurons in Other Taxa

Given the paucity of research on catecholaminergic neurons in the Chelicerata, it is currently difficult to ascertain whether the patterns of TH-immunoreactivity observed in *C. sculpturatus* are typical of chelicerates, or if they are aberrant patterns that are unique to scorpions. To date, the only basis for comparison within the Chelicerata is the wolf spider *H. lenta*, for which the catecholaminergic profile of the CNS was described in our earlier study (Auletta et al., 2019). Curiously, there do not appear to be strong parallels between the projection patterns of TH-synthesizing neurons in the two species. In *H. lenta*,

numerous TH-ir somata were detected in the opisthosomal neuromeres, i.e., the portion of the spider CNS that is homologous to the entire VNC of the scorpion. These opisthosomal TH-ir neurons were almost always present in clusters, as was true of the TH-ir neurons in *C. sculpturatus;* however, the number of discrete clusters was higher and the number of somata per cluster was lower in the spider. Furthermore, the vast majority of the opisthosomal TH-ir neurons in *H. lenta* produced ascending fibers that projected to more anterior regions of the CNS, i.e., areas that are homologous to the prosomal mass of the scorpion. None of the TH-ir neurons in the ganglia of *C. sculpturatus* were observed to ascend in this manner; instead, their axons either exited the CNS as efferents or innervated more central regions of the ganglia in which they originated. Comparable projection patterns were not observed in any of the TH-ir neurons in the opisthosomal neuromeres of *H. lenta*. Such discrepancies suggest that the distribution, morphology, and connectivity of catecholaminergic neurons may be highly variable among the different orders of the Chelicerata. Comparative studies that incorporate other chelicerate taxa are sorely needed to characterize the full extent of this variability, and to determine if there are any phylogenetically meaningful patterns of TH-immunoreactivity among the different chelicerate orders.

The arrangement and connectivity of TH-ir neurons that we observed in the VNC of *C. sculpturatus* also differs dramatically from those reported for TH-ir (and DA-ir) neurons in the Pancrustacea (i.e., insects and crustaceans). The

conspicuous clusters of dopaminergic neurons that were so apparent in the scorpion are completely absent in the abdominal ganglia of insects; instead, these insect ganglia contain just 1-3 pairs of single lateral neurons arranged in a serially iterated pattern. This arrangement has been noted in a broad array of insect taxa, including the cricket *Gryllus bimaculatus* (Hörner 1999; Hörner et al., 1996), the locusts *Locusta migratoria* and *Schistocerca gregaria* (Orchard et al., 1992; Watson 1992), the cockroach *Periplaneta americana* (Elia et al., 1994), the stick insect *Carausius morosus* (Ali & Orchard, 1996), the kissing bug *Rhodnius prolixus* (Orchard, 1990), the hawk moth *M. sexta* (Mesce et al., 2001), and the fruit fly *D. melanogaster* (Budnik & White, 1988; Monastirioti, 1999). Similar repeated pairs of single DA neurons were also observed in the abdominal ganglia of several decapod crustaceans (Beltz, 1999; Cournil et al., 1994; Mercier et al., 1991; Ponzoni, 2014; Tierney et al., 2003). When projections of these lateral neurons were visible, many appeared to project to and innervate the center of their home ganglion, acting presumably as local dopaminergic interneurons. In some (but not all) of these organisms, axons from the paired DA neurons were seen projecting anteriorly and/or posteriorly to other ganglia (Budnik & White, 1988; Cournil et al., 1994; Mercier et al., 1991; Mesce et al., 2001; Monastirioti, 1999; Orchard, 1990; Orchard et al., 1992; Tierney et al., 2003; Watson 1992), which is a projection pattern that was not observed for any TH-ir neurons in our scorpion preparations. Presently, it is unclear if any of these single paired DA neurons of insects and crustaceans share a homology with the

paired clusters of non-efferent DA neurons observed in *C. sculpturatus*. Interestingly, unpaired medial dopaminergic neurons are found in the abdominal ganglia of several paraneopteran and holometabolous insects (Budnik & White, 1988; Mesce et al., 2001; Monastirioti, 1999; Orchard, 1990), as well as in lobsters and crayfish (Beltz, 1999; Cournil et al., 1994; Mercier et al., 1991; Tierney et al., 2003). No putative homologs of these neurons were observed in *C. sculpturatus*, as none of the TH-ir cells that we observed were unpaired.

Regardless of homology, an interesting pattern arises from these comparisons between *C. sculpturatus* and the representative Pancrustacea—in abdominal ganglia, the dopaminergic neurons of pancrustaceans are always found either singly or in single pairs, whereas they are typically found in large clusters of 12+ somata in the scorpion. A similar trend towards clustering of catecholaminergic neurons was observed in the opisthosomal neuromeres of *H. lenta*, even though the number of somata per cluster was smaller (Auletta et al., 2019). The OA-ir and 5-HT-ir cells of the scorpion were also found in clusters of many somata each, and the possibility that this may be a defining feature of aminergic systems in the Chelicerata is discussed further below.

3.5.5 | Octopaminergic Neurons in the VNC

In addition to the catecholamines, we were also able to visualize OA-immunolabeled neurons in the VNC of *C. sculpturatus*, although these cells were only visible in the three ganglia of the mesosoma in our preparations. Each

mesosomal ganglion displayed a distinct pair of medial clusters containing at least 30 OA-ir somata each. Approximately one-third of these somata— most of which were concentrated in the center of the cluster— were very brightly labeled, whereas the remaining two-thirds were much fainter; this pattern was consistently observed across different samples. This pattern suggests that some cells in these medial clusters may contain higher levels of OA than others, although we did not directly test this idea. Notably, we only detected the somata of neurons with the anti-OA antiserum; the axons of those cells were not visible in any of our preparations. This starkly contrasts with our TH and 5-HT antisera, both of which reliably labeled numerous fine arborizations in the scorpion tissue. This outcome is not unusual for OA immunolabeling studies, as the projections of OA-ir cells are often weakly detected or fully undetected in other animals (Eckert et al., 1992; Crisp et al., 2002; Gilchrist et al., 1995; Hörner, 1999; Lee & Wyse, 1991). Nevertheless, the lack of visible processes prevented us from making detailed conclusions about the projection patterns of these OA-ir neurons. Notably, these somata did not positively label with streptavidin-Cy5 in any of our Neurobiotin backfills, and thus they appear to be neither efferent nor electrically coupled to efferent cells. It is therefore likely that they are octopaminergic interneurons.

Aside from *C. sculpturatus*, octopaminergic neurons have been identified in only two other chelicerates— the horseshoe crab *L. polyphemus* and the wandering spider *Cu. salei*. In *L. polyphemus*, paired medial clusters of 12-24

octopaminergic neurons each were detected in each neuromere of the prosomal CNS (Batelle et al., 1999; Lee & Wyse, 1991). It is unclear if similar clusters were observed in the VNC, because it was not examined in those studies; however, given our findings in the scorpion, it might be expected that similar clusters would be found there as well. Midline-adjacent clusters of OA-ir neurons were also found throughout the neuromeres of the fused CNS of *Cu. salei* (Seyfarth et al., 1993). Taken together with the findings of the present study, it appears that serially repeating ventral medial clusters of octopaminergic neurons are likey a shared feature of chelicerate nervous systems. However, since OA-ir cells have only been localized in three chelicerate species, such a conclusion may be premature. Nevertheless, clusters of numerous OA cells do appear to be characteristic of at least three members of the Chelicerata.

In pancrustaceans, OA-ir somata in the VNC are also concentrated near the midline of each ganglion, which corresponds to the general position of octopaminergic neurons observed in the Chelicerata. However, the vast majority of OA-ir cells in the insect VNC are unpaired, as opposed to the bilaterally paired clusters of OA-ir cells that were detected in *C. sculpturatus* and other chelicerates (Eckert et al., 1992; Farooqui, 2012; Hörner, 1999; Monastirioti, 1999; Monastirioti et al., 1995; Pflüger & Stevenson, 2005; Spörhase-Eichmann et al., 1992). These OA cells are the dorsal unpaired median (DUM) and ventral unpaired median (VUM) neurons, whose structure and function have been well characterized in numerous insect groups (Bräunig & Pflüger, 2001; Duch et al.,

1999; Farooqui, 2012; Hoyle, 1975; Johnston et al., 1999; Pflüger & Stevenson, 2005; Sinakevitch et al., 1995). The vast majority of the octopaminergic DUMs and VUMs project to peripheral tissues, in contrast to the OA-ir cells in the nerve cord of *C. sculpturatus*, which do not appear to be efferent; thus, it is unlikely that the medial clusters of OA cells of the scorpion are homologous to the unpaired OA cells of insects. A small minority of octopaminergic cells in the insect VNC do, however, exist as bilateral pairs and appear to be local interneurons, which mirrors the arrangement in crustaceans, where octopaminergic somata exist exclusively as iterated single pairs of midline-adjacent neurons (Antonsen et al., 2001; Beltz, 1999; Pflüger & Stevenson, 2005; Schneider et al., 1993). These paired neurons may represent homologs of the scorpion OA cells, but further analyses are necessary to test this hypothesis. Notably, although the number of OA-ir cells present in the abdominal ganglia of insects and crustaceans is variable between different species, they are consistently fewer than the abundant clustered somata present in the ganglia of *C. sculpturatus* (Antonsen et al., 2001; Beltz, 1999; Eckert et al., 1992; Farooqui, 2012; Hörner, 1999; Monastirioti, 1999; Monastirioti et al., 1995; Pflüger & Stevenson, 2005; Schneider et al., 1993; Spörhase-Eichmann et al., 1992). The clustered arrangement of OA-ir somata in the scorpion, as well as in the horseshoe crab and spider, is also reminiscent of the clustered arrangement of chelicerate dopaminergic neurons described earlier, further suggesting that clusters of aminergic neurons may be a shared feature of the Chelicerata.

Although the medial clusters of OA cells were reliably labeled in the mesosomal ganglia of the scorpion, they were not detected in the metasomal ganglia, suggesting that octopaminergic cells are absent there. It is presently unclear if this pattern extends to other Chelicerata. OA immunolabeling studies of the horseshoe crab *L. polyphemus* have focused exclusively on the prosomal CNS, and thus the distribution of OA cells in the VNC of this species is unknown (Batelle et al., 1999; Lee & Wyse, 1991). The only other chelicerate whose octopaminergic system has been examined is the spider *Cu. salei*, whose CNS is extremely condensed, making comparisions with the VNC of scorpions difficult (Seyfarth et al., 1993). In other arthropods, it has been shown that OA cells are not uniformly distributed along the VNC. In the squat lobster *Munida quadrispina*, for example, octopaminergic cells were found in the cephalic and thoracic ganglia, but not in the abdominal ganglia along the VNC (Antonsen & Paul, 2001). Similarly, in the American lobster *Homarus americanus*, octopaminergic neurons were absent from all but two abdominal ganglia (Schneider et al., 1993). Yet, in many other arthropods, including numerous insects, OA cells appear to be present in every ganglion of the VNC (Beltz, 1999; Eckert et al., 1992; Farooqui, 2012; Hörner, 1999; Monastirioti, 1999; Monastirioti et al., 1995; Pflüger & Stevenson, 2005; Seyfarth et al., 1993; Spörhase-Eichmann et al., 1992). Perhaps a different, possibly more sensitive anti-OA antiserum might be helpful in determining if the metasomal ganglia of the scorpion do, indeed, lack octopaminergic cells.

3.5.6 I Serotonergic Neurons in the VNC

Our 5-HT immunolabeling allowed us to visualize clearly the serotonergic system of *C. sculpturatus* with excellent spatial resolution, including details (e.g., fine fibers and synaptic boutons) that might have been undetectable via other protocols. We detected a distinct metameric arrangement of 5-HT-immunoreactivity throughout the VNC, with each ganglion containing bilaterally paired clusters of 5-HT-ir efferent cells as well as a dense central meshwork of fine processes supplied by numerous 5-HT-ir afferents. This pattern suggests that 5-HT has widespread and important modulatory functions in the scorpion, including the modulation of both sensory (afferent) and motor (efferent) pathways. The peripheral targets of the efferent 5-HT neurons of *C. sculpturatus* are presently unknown, as are the somata that give rise to the numerous afferent serotonergic fibers. Future investigations, which include detailed 5-HT immunolabeling of peripheral tissues, will be necessary to identify these structures.

5-HT is the only biogenic amine whose anatomical distribution has been previously described in a scorpion. Harzsch (2004) reported 5-HT-immunoreactivity in the nerve cord of the emperor scorpion *Pandinus imperator* (Scorpionidae), which revealed the presence of very similar paired clusters of 5-HT-ir neurons in the opisthosomal ganglia, as well comparable levels of intense serotonergic innervation in the central neuropil of each ganglion. However, Harzsch (2004) concluded that the serotonergic neurons of *Pn. imperator* were

not efferents, but rather local interneurons that projected centrally and gave rise to the fibers of the central neuropil. Their observation stands in contrast to our findings in the *C. sculpturatus* CNS, where the clustered 5-HT cells unambiguously exit the ganglia without contributing to the central neuropil. Our Neurobiotin backfills further confirmed that the 5-HT cells of *C. sculpturatus* were efferent. We were also able to show that the afferent fibers, specifically, gave rise to the central neuropil (Figure 3.7 D). Unfortunately, the conclusions of Harzsch (2004) are supported only by a single low-resolution photomicrograph, in which the projections of the 5-HT cells were ambiguous. In contrast, the images that we present of the serotonergic system in *C. sculpturatus* are substantially higher in resolution, which permited unequivocal tracing of both efferent and afferent fibers.

The serotonergic systems of several other chelicerate taxa have been examined as well, including the horseshoe crab (Xiphosura), sea spiders (Pycnogonida), true spiders (Araneae), harvestmen (Opiliones), and ticks (Parasitiformes). Segmentally iterated clusters of lateral 5-HT-ir neurons were observed in the CNSs of all of these taxa, as were dense 5-HT-ir arborizations in the central regions of the ganglia (Breidbach & Wegerhoff, 1993; Brenneis & Scholtz, 2015; Harzsch, 2004; Harzsch et al., 2005; Hummel et al., 2007; Seyfarth et al., 1990; Washington et al., 1994). This high level of concordance across multiple taxa suggests that elements of the serotonergic system that we reported in *C. sculpturatus* are highly conserved throughout the Chelicerata.

Efferent projections from these 5-HT neurons were not observed in any of the non-scorpion chelicerates, although in many of these taxa (e.g., Parasitiformes and Xiphosura) it was not possible to fully trace the axons of all 5-HT neurons in the CNS. Thus, the possibility that these 5-HT cells are efferent in non-scorpion chelicerates cannot be ruled out, and further investigations are needed to characterize their projection patterns in more detail. Nevertheless, it is also possible that, within the Chelicerata, efferent 5-HT neurons in the VNC are a unique feature (autapomorphy) of the scorpions. Comparative studies that include taxa more closely related to the Scorpiones (e.g., the other orders of the Arachnopulmonata) would prove to be especially informative in this respect.

Regardless of whether or not the 5-HT project to the periphery, clustered pairs of 5-HT neurons are clearly characteristic of the Chelicerata, and this pattern is divergent from that observed in the Mandibulata (i.e., Pancrustacea and Myriapoda). Although there is some level of variability between individual species, the ganglia in the VNC of mandibulate arthropods typically contain relatively few serotonergic neurons, and these neurons are usually arranged in bilaterally symmetrical pairs of 1-2 somata each (rarely more) per ganglion (Harzsch et al., 2004). This arrangement has been noted in numerous insects (Bishop & O'shea, 1983; Haselton et al., 2006; Longley & Longley, 1986; Mesce et al., 1993; Nässel, 1988; Stemme et al. 2017; Vallés & White, 1988; van Haeften & Schooneveld, 1992), crustaceans (Antonsen & Paul, 2001; Beltz & Kravitz, 1983; Harrison et al., 1995; Harzsch, 2004; Harzsch & Waloszek, 2000;

Stemme et al., 2013; Thompson et al., 1994), and myriapods (Harzsch, 2004; Harzsch et al., 2005; Sombke & Stemme, 2017). A similar trend was noted earlier for dopaminergic and octopaminergic neurons, which appear in clusters within the Chelicerata but as single cells or pairs in Mandibulata. Furthermore, this trend is also apparent when comparing the histaminergic and GABAergic systems of chelicerates vs. other arthropods (Harzsch, 2004; Harzsch & Glötzner, 2002; Hörner et al., 1996; Wiens & Wolf, 1993; Wolf & Harzsch, 2002). Given that the Chelicerata is evolutionarily basal to all other extant arthropods, it is possible that the clustered arrangement of aminergic neurons characteristic of chelicerates is the ancestral condition for the Arthropoda, with a reduction in aminergic neuron count occurring later in the common ancestor of the Mandibulata. Alternatively, the patterns observed in the mandibulate arthropods may be truly ancestral for the whole phylum, which would imply that a proliferation of aminergic neurons is an autapomorphy of the Chelicerata. Large-scale comparative analyses that include numerous arthropod taxa as well as appropriate non-arthropod outgroups would be useful in testing these competing hypotheses.

3.5.7 I Potential Secretory Functions of the Supraneural Lymphoid Glands

One exciting, yet unanticipated, outcome of this research was the discovery of amine-synthesizing cell bodies in the supraneural lymphoid glands that lie directly above the CNS in the mesosoma. Early works on scorpion

anatomy noted the presence of these conspicuous glands and determined that they are formed by extensions of the supraneural artery that runs longitudinally along the dorsal surface of the VNC (Cuénot, 1897; Kollman, 1908, 1910; Millot & Vachon, 1949; Pavlovsky 1924). These early studies hypothesized that the supraneural lymphoid glands are sites of hemocyte production, based primarily on observations that the glands contain numerous cell types that appeared to be directly released into the hemolymph. This hypothesis was later supported by ultrastructural studies of the lymphoid glands in the vaejovid scorpion *Smeringurus mesaensis* (Farley, 1984). Several studies have indicated that the supraneural lymphoid glands may have detoxifying functions as well, as phagocytic cells inside the glands have been shown to absorb and degrade toxic foreign substances that the experimenters injected into the scorpion (Millot & Vachon, 1949; Pavlovsky 1924). Organs similar in structure to the supraneural lymphoid glands are found in the anterior lateral margins of the mesosoma in some scorpion families (Farley, 1984; Millot & Vachon, 1949; Pavlovsky 1924), but are absent in the Buthidae (including *C. sculpturatus*) (Volschenk et al., 2008). Based on their structural similarities, many authors have concluded that these lateral lymphoid glands may perform similar hemopoietic and detoxifying functions (Farley, 1984; Millot & Vachon, 1949; Pavlovsky 1924).

Unfortunately, the lymphoid glands have received relatively little attention since these pioneering anatomical studies, and the full extent of their functions remains enigmatic. Our finding that the supraneural lymphoid glands of *C.*

sculpturatus contain many bright OA-ir and NE-ir cells suggests that the glands may also have secretory functions, in addition to their previously documented hemopoietic and detoxifying roles. The idea for that these glands could be secretory was first proposed by George et al. (1960) on the basis that cells in the periphery of the glands were labeled via the chromaffin reaction. The chromaffin reaction is a histological assay that has long been used in mammalian tissues, where it notably labels adrenergic secretory cells of the adrenal medulla (Coupland, 1954; Hale, 1958). Based on these findings, George et al. (1960) hypothesized that the supraneural lymphoid gland may release hormones into the hemolymph, thus acting similarly to vertebrate adrenal glands; unfortunately, they did not subsequently identify their proposed hormones or characterize any secretory activities of the glands. Our findings suggest that the secretory products proposed by George et al. (1960) may be NE and/or OA; indeed, the NE we detected in the cells of these glands is likely what caused the positive chromaffin reaction that they observed in their preparations. Since *C. sculpturatus* lacks lateral lymphoid glands, we were unable to examine whether those structures also contain amines such as NE and OA, which would suggest that they may be secretory as well. However, given our discoveries in the supraneural lymphoid gland, we believe that immunolabeling against amines in the lateral lymphoid glands in other scorpion families may prove worthwhile.

Further experiments are needed to directly test the hypothesis that the supraneural lymphoid glands are capable of secreting amines (and possibly other

compounds); however, we believe that the clear evidence of amine-synthesizing cells in these structures make this hypothesis very compelling. If the supraneural lymphoid organs are indeed secretory, their very close association with the ganglia of the mesosoma render them a prime candidate for local (i.e., paracrine) release of amines that could directly modulate the activity of neurons in those ganglia. It is also possible that the amines synthesized in the supraneural lymphoid glands could be released into the hemolymph for more widespread (hormonal) circulation, in line with the endocrine hypothesis of George et al. (1960). Paracrine and endocrine release of NE and E are well documented in a variety of vertebrate taxa, where these compounds have a multitude of behavioral effects (De Diego et al., 2008; Mazzocchi et al., 1998; Palme et al., 2005; Tobari et al., 2014). Similarly, paracrine and endocrine release of OA has been observed in insects and other invertebrates (Adamo & Baker, 2011; Breen & Atwood, 1983; Koon et al., 2011; Orchard, 1982). It is thus very possible that similar processes occur in the scorpion and involve the actions of these supraneural lymphoid glands.

An alternate possibility is that the NE-ir and OA-ir cells observed in the supraneural lymphoid glands are developing hemocytes, and that some scorpion hemocytes are themselves aminergic. To date, there are no reports of invertebrate hemocytes that produce endogenous catecholamines or OA; however, the immune cells of vertebrates—including phagocytes such as macrophages and neutrophils—have been shown to synthesize and release NE

(Bergquist et al. 1994; Flierl et al., 2007). The phagocytic immune cells of vertebrates and invertebrates share many structural and functional similarities. Furthermore, they both express many of the same highly conserved immune proteins, which suggests a deep homology between the two classes of cells (Browne et al., 2013; Buchmann, 2014; Franchini et al., 1996). Thus, the possibility remains that the hemocytes of *C. sculpturatus* may produce NE (and OA), consistent with the hemopoietic function of the lymphoid glands proposed by earlier authors (Cuénot, 1897; Farley, 1984; Kollman, 1908, 1910; Millot & Vachon, 1949; Pavlovsky 1924). Additional experiments, including immunolabeling of free-floating hemocytes in the hemolymph, are needed to test this hypothesis. Indeed, the high levels of uncertainty regarding the functions of the lymphoid glands underscores a need for further investigations of these intriguing structures, with a special focus on the aminergic cells that we have identified in the present study.

3.5.8 l NE is an Endogenous Signaling Molecule in *C. sculpturatus*

The UPLC-MS assays we conducted revealed that NE is present in detectable quantities in the CNS of *C. sculpturatus*, which unambiguously confirms NE as an endogenous compound in this species. NE was also recovered from the CNS of the spider *H. lenta*, which supports the idea that endogenous NE may be a shared trait of the Arachnida or Chelicerata as a whole. Direct evidence that neural tissue of these two arachnids contains NE is a

particularly exciting result, because it opposes the widely-reported assumption that NE is absent from arthropods and other invertebrates (Adamo, 2008; Blenau & Baumann, 2001; Farooqui, 2012; Gallo et al., 2016; Pflüger & Stevenson, 2005; Roeder, 1999, 2005; Verlinden, 2010; Walker et al., 1996). This discovery highlights the need to reconsider the possibility that adrenergic signaling may be more prevalent in invertebrates than previously thought, which we discuss later in this chapter.

Our bioinformatics analyses revealed that *C. sculpturatus* also possesses the necessary molecular machinery to differentiate NE from other related compounds, via genes coding for distinct dopaminergic, adrenergic, and octopaminergic receptors. This result is a prerequisite for adrenergic signaling, because NE cannot produce physiological effects in the absence of appropriate receptors. The sequence cluster map generated during these analyses indicates that there is at least one receptor in *C. sculpturatus* that shares a high level of sequence similarity with confirmed α_1 adrenergic receptors in other organisms, and two other receptors are relatively similar to known α_2 adrenergic receptors. The evidence for a β adrenergic receptor in the scorpion was more ambiguous. Furthermore, based on sequence similarity, these adrenergic receptors appear to be distinct from dopaminergic and octopaminergic receptors. Together, these results further support the idea that not only do NE, DA, and OA coexist in *C. sculpturatus*, but that the scorpion's nervous system can distinguish between

these three compounds, implying that they may each have discrete functional roles.

On this front, we demonstrated, via our electrophysiological recordings of the telsonic nerve, that NE was able to elicit clear patterned activity in the scorpion CNS. This NE-evoked activity was qualitatively and quantitatively distinct from the activity patterns seen in the presence of DA and OA, which further indicates that NE has specific functional roles in *C. sculpturatus* that are separate from those of the other biogenic amines. The precise nature and extent of those functional roles is currently unknown, but given that the telsonic nerve projects solely to the telson (stinger) of the scorpion, we hypothesize that NE is likely involved in venom production and/or stinging behavior. In vertebrates, NE is involved in the acute “fight or flight” stress response (Adamo, 2008; Berridge et al., 2012; Cecchi et al., 2002; Keller et al., 2006; Singh et al. 2015; Wirz et al., 2017; Zhang et al., 2003), and so it is possible that NE may bias the scorpion to sting more readily when exposed to stressors (e.g., potential predators). In this case, the rhythmic bursting observed in response to NE may represent fictive pumping of the muscles that control the venom gland. Future studies that pair electrophysiology with behavioral assays would be especially informative in testing this hypothesis, as well as others relating to the specific modulatory roles of NE, DA, and OA in scorpions.

The goal of these experiments was to provide preliminary data regarding the functional relevance of NE in *C. sculpturatus*, which could form the basis for

more rigorous future investigations on the topic. As such, there are several limitations to our approach that must be addressed. Crucially, the majority of our experiments examined physiological responses to DA, NE, and OA at just one effective concentration (100 µM). As such, we cannot definitively rule out the possibility of dose-dependent effects, including potential cross-reactivity of NE with dopaminergic and/or octopaminergic receptors at higher or lower concentrations. However, the observation that NE evoked its characteristic patterned activity at a much lower concentration (25 µM) suggests that this response is, in fact, specific to NE; future experiments could aim to test this hypothesis more rigorously via complete dose-response curves for all three amines. Furthermore, although we demonstrated that several receptors in *C. sculpturatus* are highly similar to adrenergic receptors in other organisms, we must acknowledge that sequence similarity does not conclusively indicate functional similarity. Specificity tests that incorporate known antagonists of the different DA, NE, and OA receptor families would be highly informative in demonstrating that NE is, in fact, binding to adrenergic receptors at physiologically relevant concentrations. Such analyses were beyond the scope of our current study but represent an important next step for this research. Nevertheless, the preliminary data presented here are highly suggestive (if not definitive) evidence that *C. sculpturatus* meets all of the necessary requirements for endogenous adrenergic signaling, i.e., appreciable quantities of NE in the

CNS, genes coding for specific adrenergic receptors that can preferentially bind NE, and the ability of NE to induce clear, specific changes in neuronal activity.

Although we were able to successfully detect endogenous NE and provide preliminary evidence of its functionality in the CNS of *C. sculpturatus,* it is presently unclear where the NE is synthesized. Our NE immunolabeling protocol did not reveal any adrenergic cells in the seven ganglia of the VNC. Instead, NE-immunoreactivity was limited to cells scattered throughout the supraneural lymphoid glands, as discussed previously. Given the relatively modest amounts of NE detected in the VNC (1.09 pmol), it is conceivable that these lymphoid cells do, indeed, represent the full complement of adrenergic cells associated with this part of the CNS. We therefore hypothesize that the somata of adrenergic neurons in the scorpion CNS are located entirely in the prosomal CNS, which contains the majority of NE that was detected in our assays (9.55 pmol). It is possible that these proposed adrenergic neurons of the prosoma may send descending projections to the VNC that were not resolved with our antisera (c.f., the inability of our anti-OA antiserum to label neuronal projections); however, future investigations are needed to examine this possibility more fully.

As noted earlier, appreciable quantities of NE (17.80 pmol) were detected in the CNS of *H. lenta*, where DA was detected as well (90.78 pmol). Our earlier investigation of this species revealed an extensive array of TH-ir neurons in its CNS (Auletta et al., 2019). The quantitative data presented here strongly indicate that those labeled neurons include both dopaminergic and adrenergic cells, as

we had previously hypothesized. NE immunolabeling studies of *H. lenta* are now necessary to determine which subset of catecholaminergic neurons in the spider produce NE as their end product. Unfortunately, we were unable to test the hypothesis that NE elicits distinct functional responses in the spider CNS due to the inherent difficulties of sustaining and recording from excised spider tissue (Menda et al., 2014). It is hoped that novel techniques will make such electrophysiological manipulations in the spider more feasible in the future, thereby allowing researchers to determine if the endogenous NE we detected in *H. lenta* is, in fact, physiologically active.

3.5.9 I A Reassessment of Adrenergic Systems in Invertebrates

It has long been assumed that NE is exclusive to the vertebrates wherein it modulates many key behaviors and other phenomena, most notably those involved in the acute "fight or flight" stress response (e.g., Adamo, 2008; Berridge et al., 2012; Cecchi et al., 2002; Keller et al., 2006; Singh et al. 2015; Wirz et al., 2017; Zhang et al., 2003). OA, which is present only in trace amounts in vertebrates (Berry, 2004; Borowsky et al., 2001; Zucchi et al., 2006), has been proposed as the invertebrate analog of NE, because it is not only structurally similar to NE but also performs many of the same functions that NE does in vertebrates (e.g., Adamo & Baker, 2011; Dierick, 2008; Duch & Pflüger, 1999; Li et al., 2016; Mentel et al., 2003; Orchard, 1992; Orchard et al., 1993; Verlinden et al., 2010). Upon closer inspection, however, these claims appear to be based on

studies of a relatively small fraction of invertebrate taxa— most notably an assortment of insect species (e.g., Blenau & Baumann, 2001; Barreteau et al., 1991; Bubak et al., 2013; Geng et al., 1993; Nässel & Laxmyr, 1983; Sparks & Geng, 1992; Wright, 1987), but also several other well-known invertebrate model systems including the sea hare *Aplysia* (Mollusca) (Saavedra et al., 1974), the nematode *Ca. elegans* (Nematoda) (Donnelly et al., 2013; Horvitz et al., 1982; Sanyal et al., 2004), and the medicinal leech (Annelida: Clitellata) (Crisp et al., 2002; Lent et al., 1983), all of which have been shown to lack NE. In contrast, recent research has demonstrated the presence of distinct adrenergic receptors in a variety of other invertebrate taxa, including the Xenocoelomorpha, Priapulida, Annelida: Polychaeta, Mollusca, Hemichordata, and Tunicata (Bauknecht & Jékely, 2017), as well as in a representative chelicerate, the spider *Cu. salei* (Sukumar et al., 2018). Thus, the conclusion that invertebrates lack NE and utilize OA exclusively in its place does not appear to be generalizable to invertebrates as a whole. Our detection of NE in the nervous systems of *C. sculpturatus* and *H. lenta* corroborates this idea, and further highlights the pitfalls of overextrapolation from studies of a restricted few "model" systems.

Our study is not the first to report the presence of NE in the nervous system of an arthropod. Indeed, despite the widespread claim that NE is absent from invertebrates, it has been detected and quantified in a broad array of insect groups, including the Blattodea (Natsukawa et al., 1996; Shafi et al., 1989), Orthoptera (MacFarlane et al., 1991; Pyza et al., 1991; Robertson, 1976),

Hemiptera (Chvalova et al., 2014), Hymenoptera (Mercer, 1983; Punzo & Williams, 1994; Sasaki & Nagao, 2001), Trichoptera (Klemm & Björklund, 1971), and Lepidoptera (Matsumoto & Takeda, 2002; Naokuni et al., 1991). Importantly, the detection of NE in the nervous system is not sufficient evidence that it functions as a signaling molecule in these insects. Bauknecht & Jékely (2017) report that genes coding for adrenergic receptors were not found in the genomes of most insect species, which suggests that the NE detected in insects is not physiologically active, and may instead be a nonfunctional metabolic byproduct. This idea is further supported by the observation that NE, if present in insect tissues, is typically recovered in very small quantities relative to OA and DA. Endogenous NE has also been detected in several decapod crustaceans, but at similarly low concentrations (Elofsson et al., 1982; Fingerman & Kulkarni, 1993; Hsieh et al., 2006; Laxmyr, 1984; Ocorr & Berlind, 1983).

To our knowledge, functional studies of NE in arthropods are entirely limited to experiments that have correlated behavioral changes to hemolymph titres of NE in crustaceans (Chang et al., 2015; Hsieh et al., 2006; Wood et al., 1995; Yeh et al., 2006), or studies that failed to detect meaningful differences between the physiological effects of NE and those of DA or OA (Berlind, 2001; James & Walker, 1979; Muñoz-Cuevas & Carricaburu, 2000). These studies, although worthwhile, do not provide sufficient information to confirm that endogenous NE is a physiologically relevant signaling molecule in the nervous systems of arthropods. In contrast, our findings in *C. sculpturatus* demonstrate

that the scorpion CNS contains not only endogenous NE, but also the appropriate receptors for adrenergic signaling; furthermore, we showed unambiguously that NE can evoke changes in neuronal activity that are distinct from those elicited by other compounds (e.g., DA or OA). Thus, our work is the first to provide strong, comprehensive evidence that NE functions as a bona fide, physiologically-relevant signaling molecule in any arthropod. Indeed, the highly integrative, multi-pronged experimental approach that we used is a major strength of this study, and it is hoped that it will serve as a robust model for future functional studies of adrenergic signaling in other organisms.

3.5.10 I Conclusions

This study is the first to report the distribution of catecholaminergic and octopaminergic neurons in the CNS of any scorpion species, as well as the first high-resolution study of serotonergic neurons in the scorpion ventral nerve cord. As such, it complements the currently limited literature concerning biogenic amines in the Chelicerata, which has historically focused on just two taxa: the horseshoe crab *L. polyphemus* and the spider *Cu. salei*. By doing so, we have contributed a wealth of new knowledge about the structure and organization of aminergic systems, not just in scorpions, but in the Chelicerata as a whole. When viewed holistically, the results of our many anatomical experiments emphasize that the aminergic systems of *C. sculpturatus* display several unique and unusual features relative to those of other arthropods. Some of these unusual features,

such as the organization of aminergic neurons into large clusters instead of lone pairs or unpaired cells, appear to be general trends among the Chelicerata that deviate from the ground-plan patterns of the Mandibulata. Still other features, such as the apparent dopaminergic modulation of the respiratory system and the presence of a putative secretory organ (the lymphoid glands) in close association with the CNS, are completely unknown outside the scorpions. Detailed examinations of other chelicerate taxa are necessary to determine if such qualities are true autapomorphies of the Scorpiones.

Furthermore, our study is also the first to present strong evidence that NE is an endogenous and physiologically relevant signaling molecule in an arthropod, by incorporating a suite of experimental techniques that span quantitative chemistry, histology, physiology, and bioinformatics. This finding is particularly important because it has broad implications for our understanding of how aminergic signaling systems evolved in the Bilateria. Historically, it has been presumed that invertebrates, including arthropods, do not make use of NE as a signaling molecule. This assumption has largely been due to the observation that NE is absent in a number of well-studied invertebrate "model" organisms, including the fruit fly *D. melanogaster* and the nematode *Ca. elegans*. Interestingly, the lancelets (Cephalochordata) lack NE as well but utilize OA extensively (Moret et al., 2004; Pflüger & Stevenson, 2005). Since the lancelets comprise the basal-most lineage of the phylum Chordata, which also includes the vertebrates, many researchers have concluded that adrenergic signaling must

have evolved relatively recently in the common ancestor of the Vertebrata. Furthermore, it was assumed that the ancestral octopaminergic signaling system was subsequently lost in vertebrates, but retained in all other bilaterians. This hypothesis, however, is incompatible with our findings in the Chelicerata, as well as the results of several other recent studies (Bauknecht & Jékely, 2017; Sukumar, 2018), which together provide strong support for adrenergic systems across multiple invertebrate phyla. It is now clear that the erroneous notion that NE is absent from invertebrates resulted from a severe under-sampling of invertebrate taxa in early neurobiological research, and thus our study warns against an overreliance on a small number of “model” systems when making such broad conclusions.

In light of our findings, we join Bauknecht and Jékely (2017) in offering a new hypothesis, i.e. that the ancestral condition present in the common ancestor of all bilaterian animals was the co-existence of separate adrenergic and octopaminergic systems. We propose that, in several invertebrate groups (e.g., many insects, nematodes, and lancelets), adrenergic signaling was secondarily lost and replaced entirely by octopaminergic signaling. The reasons for this loss are unclear, but it was likely facilitated by the fact that OA and NE are so similar in their chemical structures and general functions. Likewise, the octopaminergic system is hypothesized to have been completely supplanted by the adrenergic system in vertebrates. Most salient is that many bilaterian lineages—including the Chelicerata—have retained the ancestral co-existence of both molecules,

thereby offering an informative window into the evolutionary origins of bilaterian signaling systems. Stemming from this hypothesis is an important functional implication, i.e. that the acute "fight or flight" stress response may modulated by different compounds (NE vs. OA vs. a combination of the two) in different lineages of animals. It is hoped that our study will inspire future investigations of NE, OA, and other biogenic amines across animal phyla, and thus help test these hypotheses to paint a more complete picture of the evolutionary origins of aminergic systems in the Bilateria.

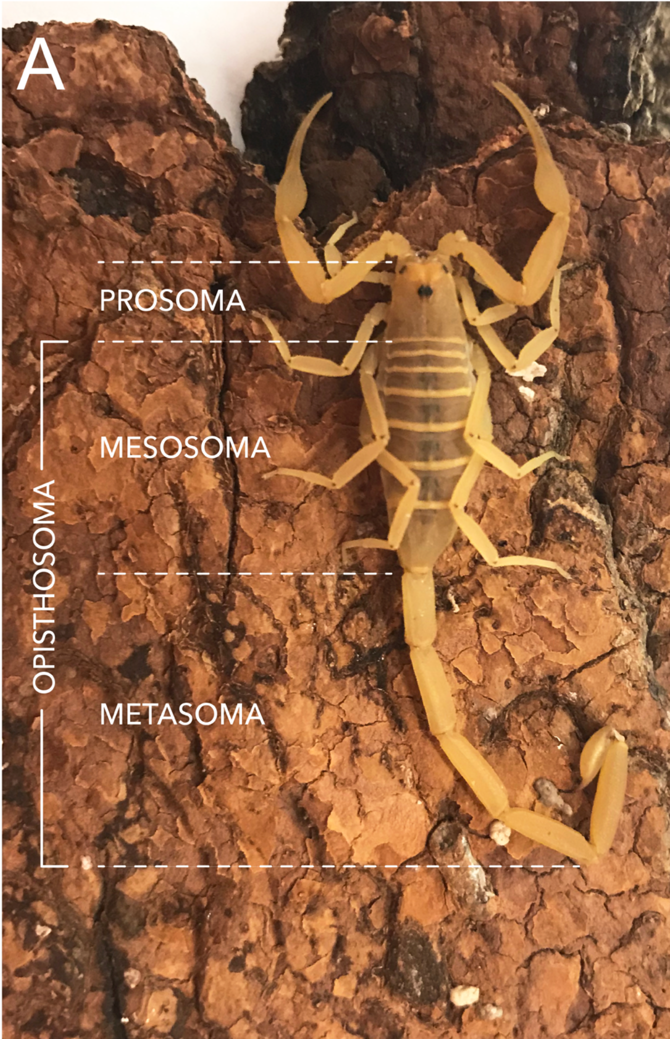
3.6 l Tables and Figures

Table 3.1. Retention times and m/z values for dopamine (DA), norepinephrine (NE), and octopamine (OA) standards, as well as the internal standard, used to identify compounds in UPLC-MS assays.

Compound	m/z	Monitoring Ion m/z	Retention Time (min)
DA	154.0863	137.0593	1.02
NE	170.0812	152.0702	0.68
OA	154.0863	136.0753	0.75
Internal Standard (Dopa-phenyl-d_3)	201.0949	154.0827	0.97

Table 3.2. NCBI Genbank accession numbers of the dopamine (DA), norepinephrine (NE), and octopamine (OA) receptors used for comparison against *Centruroides sculpturatus* receptors in protein sequence analyses.

Higher Taxon	Species	DA Receptors	NE Receptors	OA Receptors
Annelida	*Platynereis dumerilii*	----------	KX372342 KX372343	KU530199 KU886229
Arthropoda: Chelicerata	*Limulus polyphemus*	XP_013790897.2 XP_022241090.1	----------	XP_013782268.1 XP_022243119.1
	Tetranychus urticae	XP_015795378.1 XP_015791409.1	----------	XP_015785358.1
Arthropoda: Insecta	*Aedes aegypti*	XP_021693211.1 NP_001345854.1	----------	XP_021695040.1 XP_021695041.1 XP_021693342.1
	Apis mellifera	XP_026301048.1 NP_001014983.1	----------	XP_397139.3 XP_006558135.1
	Drosophila melanogaster	AAB08000.1 NP_001163607.1 AAX52463.2 NP_001027080.2	----------	NP_524669.2 AGB96094.1 NP_651057.1 NP_001247077.1
	Tribolium castaneum	NP_001280543.1 NP_001280503.1	----------	NP_001280501.1 NP_001164311.1
Chordata	*Canis lupus familiaris*	XP_005618635.1 NP_001003110.1	NP_001182772.1 XP_022267332.1 NP_001008713.2 NP_001003234.1	----------
	Danio rerio	XP_021336831.1 XP_005157558.1	XP_021334987.1 NP_997522.1 NP_001122161.1 NP_001082940.2	----------
	Gallus gallus	XP_015149309.2 XP_015153515.1	XP_015152983.1 XP_004942333.2 XP_015144447.1 XP_015149170.1	----------
	Macaca mullata	AAC27328.1 XP_001085571.1	NP_001108205.1 XP_014993395.2 NP_001276795.1 NP_001036239.1	----------
	Mus musculus	NP_038531.1 EDL41132.1 EDL25723.1 AAI05666.1	NP_001271310.1 NP_031443.4 EDL01765.1 NP_031446.2	----------
	Xenopus tropicalis	XP_017947694.1 XP_017951537.1	XP_017945765.1 NP_001120375.1 NP_001116897.2	----------
Hemichordata	*Saccoglossus kowalevskii*		ALR88680.1 XP_002734932.1	XP_006823182.1 XP_002733926.1
Nematoda	*Caenorhabditis elegans*	BAD01496.1 BAD01495.1	----------	NP_001024568.1 NP_001024569.1
Platyhelminthes	*Schistosoma haematobium*	KGB40594.1 XP_012792730.1	----------	XP_012792690.1
Priapulida	*Priapulus caudatus*	XP_014663918.1 XP_014679324.1	XP_014662992.1 XP_014681069.1	XP_014672936.1


A
PROSOMA
MESOSOMA
OPISTHOSOMA
METASOMA

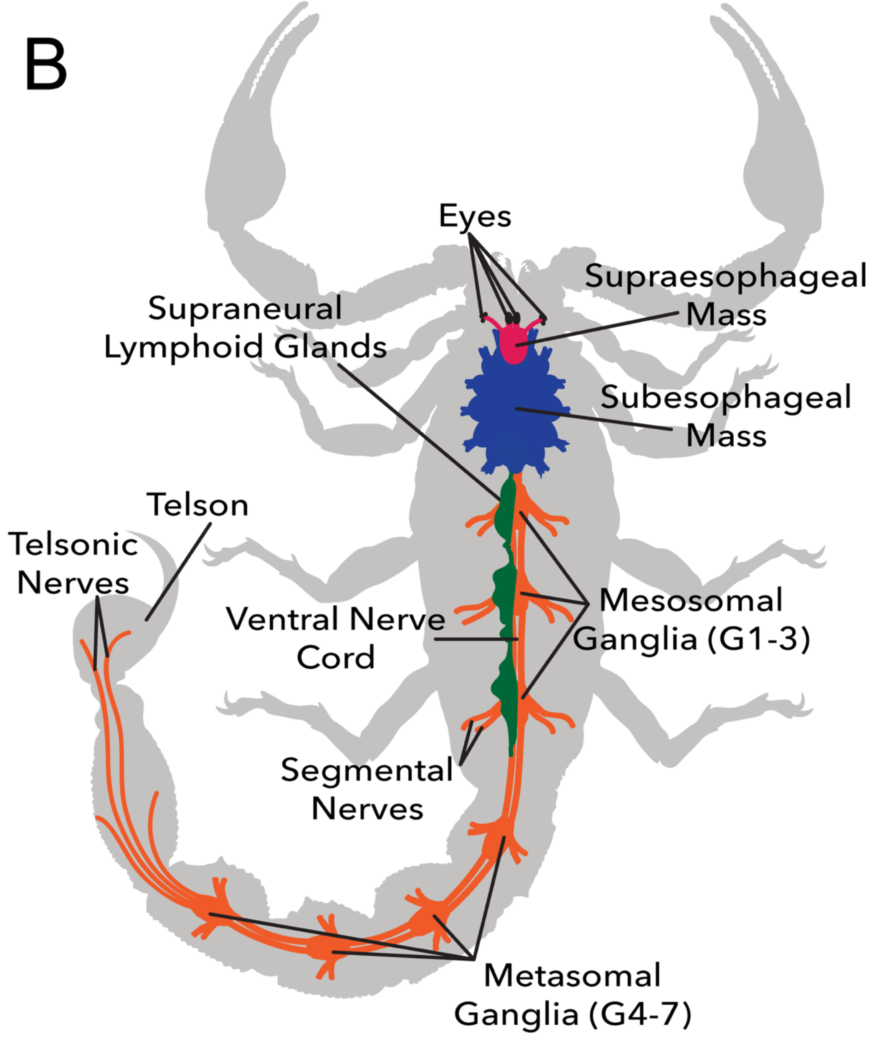
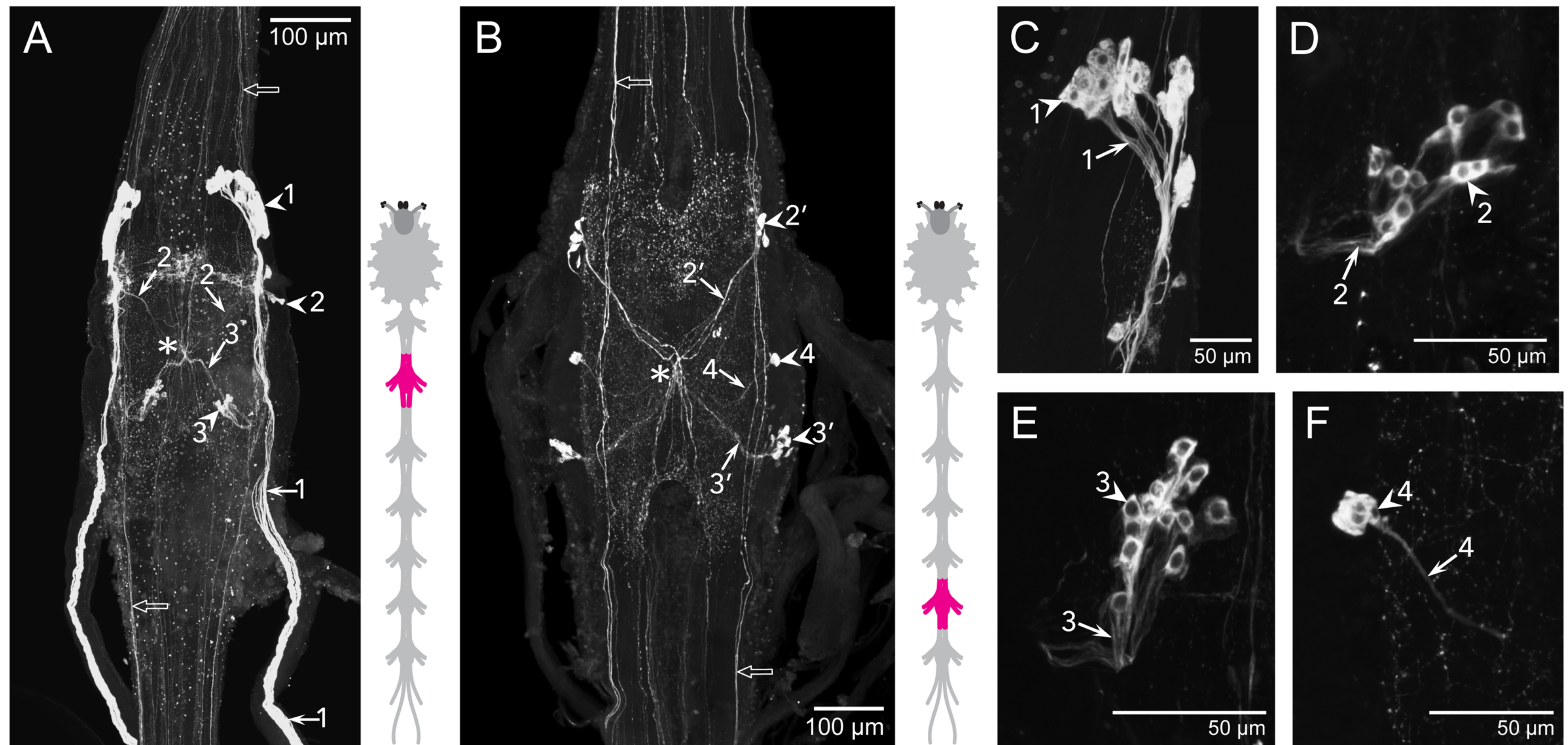

B
Eyes
Supraesophageal Mass
Supraneural Lymphoid Glands
Subesophageal Mass
Telson
Telsonic Nerves
Ventral Nerve Cord
Mesosomal Ganglia (G1-3)
Segmental Nerves
Metasomal Ganglia (G4-7)

Figure 3.1. An overview of the CNS of *Centruroides sculpturatus*. **A.** A photograph of an adult male *C. sculpturatus* specimen, highlighting the two tagmata of the scorpion—the prosoma (cephalothorax) and opisthosoma (abdomen). Unique to scorpions is the subdivision of the opisthosoma into a 7-segmented mesosoma and a 5-segmented metasoma, the last segment of which bears the telson (stinger). **B.** A diagrammatic representation of the scorpion central nervous system (CNS), highlighting its major subdivisions and structures. The prosomal portion of the CNS consists of a fused mass of tissue, which is subdivided into the supraesophageal mass (= brain) and subesophageal mass, the latter of which contains neuromeres associated with the appendages and opisthosomal segments 1-2. The opisthosomal portion of the CNS consists of a ventral nerve cord (VNC) that contains seven free ganglia, of which 3 are in the mesosoma and 4 are in the metasoma. Extending from each ganglion are paired segmental nerves that innervate the periphery. The terminal ganglion bears an additional pair of nerves that innervate the telson. Supraneural lymphoid glands, which are irregularly-shaped masses of non-neural tissue, lie on the dorsal surfaces of ganglia 1-3 as well as the portions of the nerve cord (= connectives) between them.



A
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B
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C
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1
50 µm
D
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2
50 µm
E
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3
50 µm
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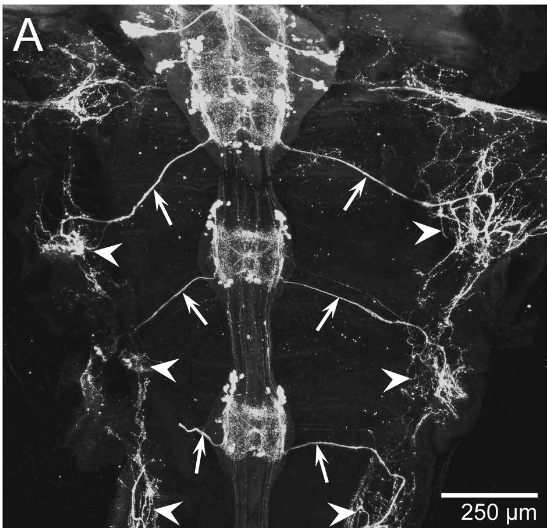
4
4
50 µm

Figure 3.2. Tyrosine hydroxylase (TH)-immunoreactivity in the VNC of *C. sculpturatus*. **A.** TH-immunoreactive (TH-ir) cells in a representative mesosomal ganglion (ganglion 2). Paired clusters of large TH-ir neurons (arrowhead 1) were detected along the anterior lateral margins of the mesosomal ganglia. Each cluster contained at least 14 somata which measured 17-20 µm in diameter. The axons of these cells (solid arrow 1) were shown to clearly exit the ganglion as efferents via the ipsilateral segmental nerves. A second population of TH-ir neurons (arrowhead 2) is positioned posterior to those efferent cells, also in paired clusters. Each cluster contained at least 12 neurons with somata measuring 7-9 µm in diameter, and their projections (solid arrow 2) descend to contribute to a pronounced chiasma structure (asterisk). A third population of TH-ir cells (arrowhead 3) consisted of paired clusters in a medial position within the ganglion, adjacent to the midline. Within each cluster were at least 12 cells whose somata measured 6-8 µm in diameter. Their projections (solid arrow 3) ascended to join the central chiasma. Also visible in the mesosomal ganglion were lateral plurisegmental TH-ir fibers (open arrows); the source of these fibers was not discernible. **B.** TH-ir cells in a representative metasomal ganglion (ganglion 6). Two populations of TH-ir cells (arrowheads 2′ and 3′) contain somata that correspond in size and number to cell populations 2 and 3 of the mesosomal ganglia; like those mesosomal cells, the projections of these metasomal neurons (solid arrows 2′ and 3′) also extend medially to form a conspicuous chiasma of TH-ir fibers (asterisk). Unique to the metasomal ganglia was another population of TH-ir neurons (arrowhead 4), which existed as paired clusters of at least 3 cells each. These clusters were positioned at an intermediate length along the lateral margin of the ganglion. The somata of the cells in these clusters measured approximately 10-11 µm in diameter, and sent projections to more central regions of the ganglion (solid arrow 4). The metasomal ganglia also showed the lateral plurisegmental TH-ir fibers (open arrows) that were seen passing through the mesosomal ganglia. **C.** An enhanced view of TH-ir cell population 1 from the mesosomal ganglion, showing more clearly individual somata (arrowhead 1) and their fasiculating axons (arrow 1). **D.** An enhanced view of TH-ir cell population 2 from the mesosomal ganglion, highlighting somata (arrowhead 2) and their projections (arrow 2). **E.** An enhanced view of TH-ir cell population 3 from the mesosomal ganglion, again highlighting somata (arrowhead 3) and their axons (arrow 3). **F.** An enhanced view of TH-ir cell population 4 from a metasomal ganglion, showing ttightly-packed somata (arrowhead 4) and their projections (arrow 4).


A
250 µm

B
500 µm

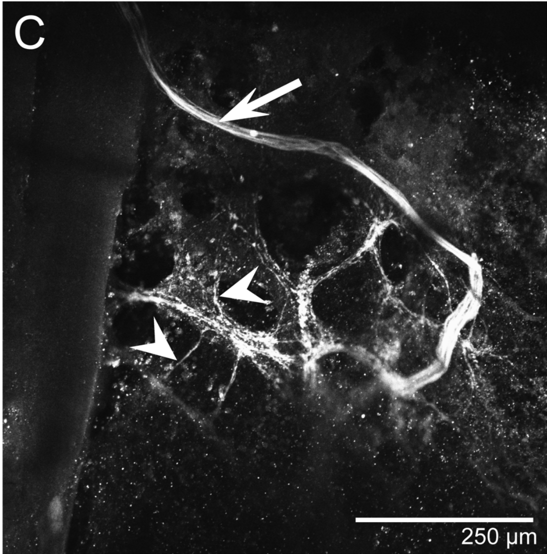

C
250 µm

Figure 3.3. TH-immunoreactivity in whole-body embryonic preparations and adult mesosomal fillets of *C. sculpturatus*, revealing the projection patterns of efferent TH-ir neurons of the mesosoma. **A.** A representative whole-body embryonic *C. sculpturatus* prep labeled with the anti-TH antiserum. The efferent projections (arrows) of the large TH-ir neurons (cell population 1 in Figure 3.2 A & C) were seen clearly exiting the developing mesosomal ganglia, as well as terminal portion of the developing prosomal mass. These projections terminated along the lateral edges of the ventral body cavity, where they formed extensively ramifying plexuses of TH-ir fibers (arrowheads). **B.** One such TH-ir plexus (arrow) as seen in a mesosomal fillet of an adult *C. sculpturatus* specimen. These plexuses were consistently found in close association with the book lungs (heavy dashed outline), directly above the fenestrated membrane. Arrowhead and light dashed line denote the spiracle (opening) of the book lung. **C.** An enhanced view of a TH-ir plexus from an adult mesosomal filet. The main branches of the plexus (arrow) ramified to form many smaller projections (arrowheads) that directly innervated the surface of the book lung tissues.

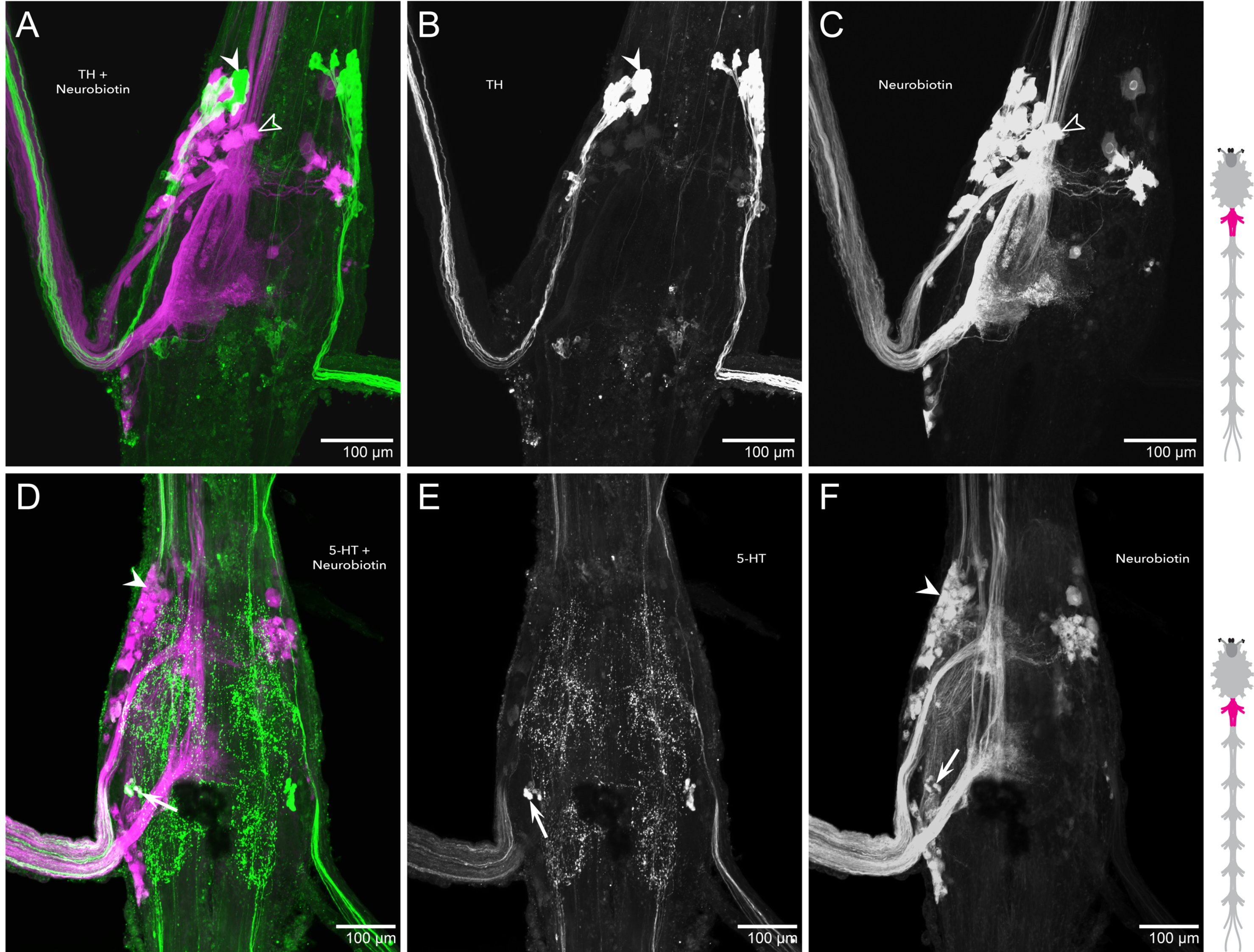
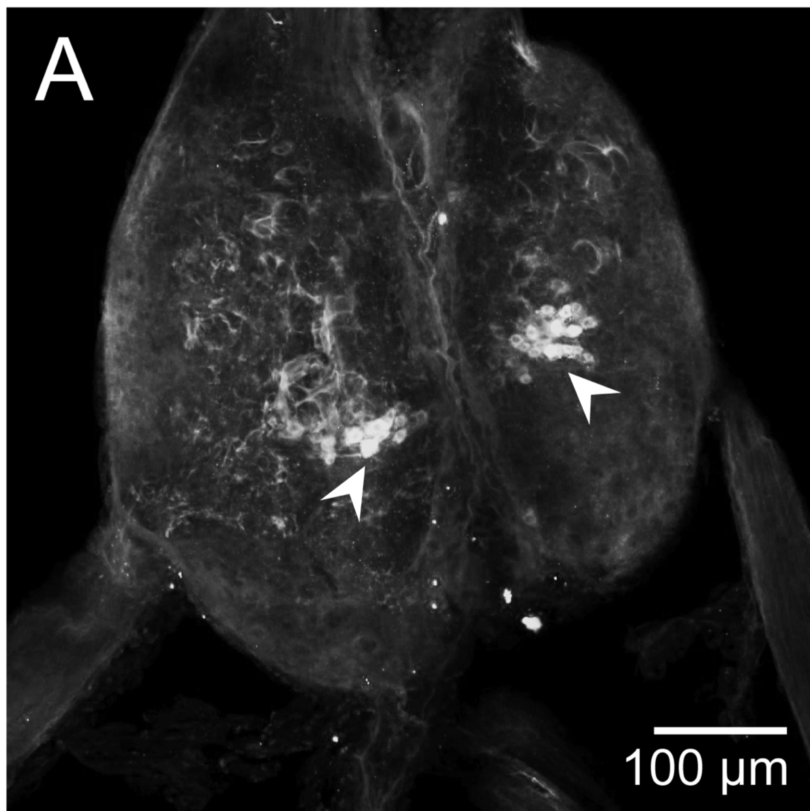

A
TH +
Neurobiotin
100 µm
B
TH
100 µm
C
Neurobiotin
100 µm
D
5-HT +
Neurobiotin
100 µm
E
5-HT
100 µm
F
Neurobiotin
100 µm

Figure 3.4. Neurobiotin backfills of segmental nerves in the mesosomal ganglia of *C. sculpturatus*. **A.** Backfilled mesosomal ganglion 1 labeled with anti-TH (green) and streptavidin-Cy5 (magenta). The large efferent TH cells were clearly labeled with the anti-TH antiserum (closed arrowhead), but not with streptavidin-Cy5, indicating that they did not contain Neurobiotin. This may be due to an incompatibility between the primary antiserum and the streptavidin-Cy5. The somata of other efferent neurons, which do not contain TH, were positively labeled with streptavidin-Cy5 (open arrowhead). Areas of bright white overlay do not indicate co-localization, but rather regions in which TH-labeled somata are positioned above streptavidin-Cy5-labeled somata. **B**. The same ganglion as in Panel A, showing only TH immunoreactivity. **C.** The same ganglion as in Panel A, showing only streptavidin-Cy5 labeling. **D.** Backfilled mesosomal ganglion 1 labeled with anti-serotonin (5-HT) (green) and streptavidin-Cy5 (magenta). Unlike in Panel A, somata corresponding in size and position to the large efferent TH cells were labeled with streptavidin-Cy5 in these preparations (arrowhead). Efferent 5-HT-ir somata (arrow), corresponding to those shown in Figure 3.7, were successfully double-labeled with both the anti-5-HT antiserum and streptavidin-Cy5, as indicated by areas of bright white overlay in the image. **E.** The same ganglion as in Panel D, showing only 5-HT immunoreactivity. **F.** The same ganglion as in Panel D, showing only streptavidin-Cy5 labeling.


A
100 µm

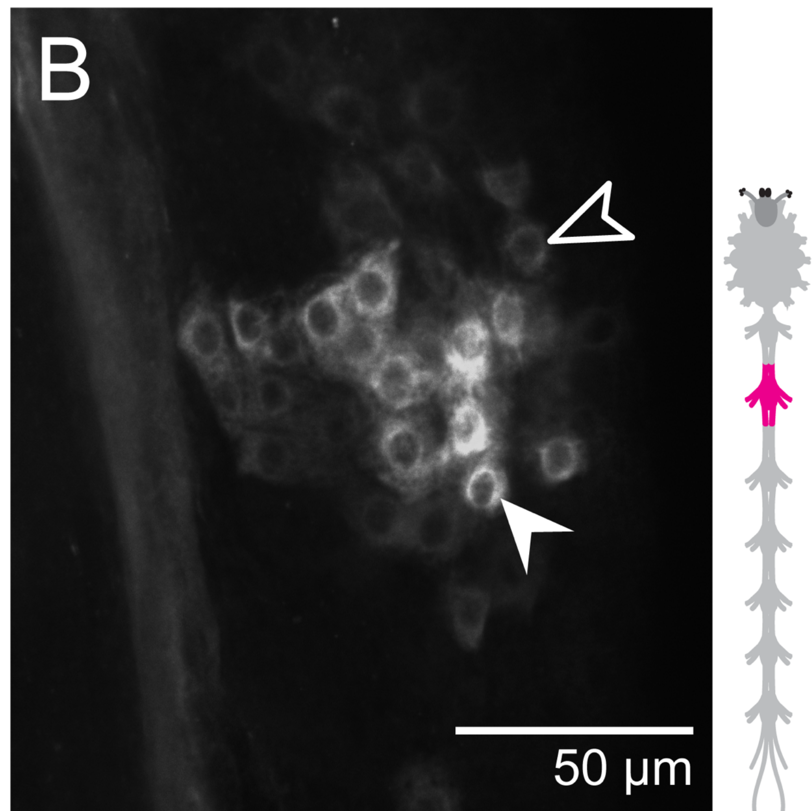
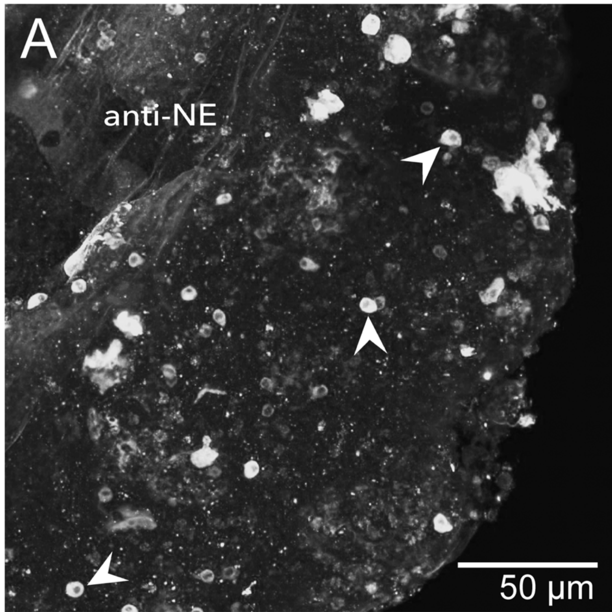
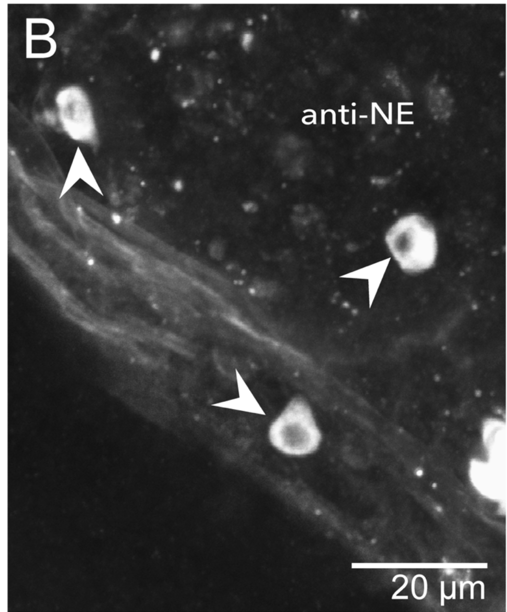

B
50 µm

Figure 3.5. Octopamine (OA)-ir neurons in the VNC of *C. sculpturatus*. **A.** OA-ir cells in a representative mesosomal ganglion (ganglion 2). Each mesosomal ganglion contained paired clusters of OA-ir neurons adjacent to the midline of the ganglion. Within each cluster were at least 35 somata measuring approximately 8-10 µm in diameter each. The projections of these cells were not discernible. **B.** An enhanced view of a representative cluster of mesosomal OA cells, highlighting both brightly-labeled (closed arrowhead) and more faintly-labeled (open arrowhead) somata.


A
anti-NE
50 µm


B
anti-NE
20 µm

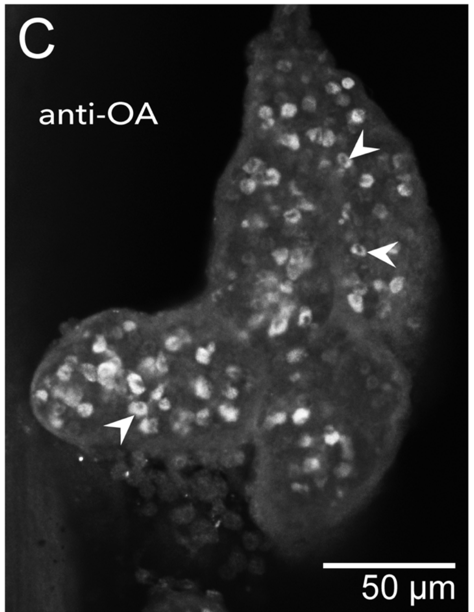
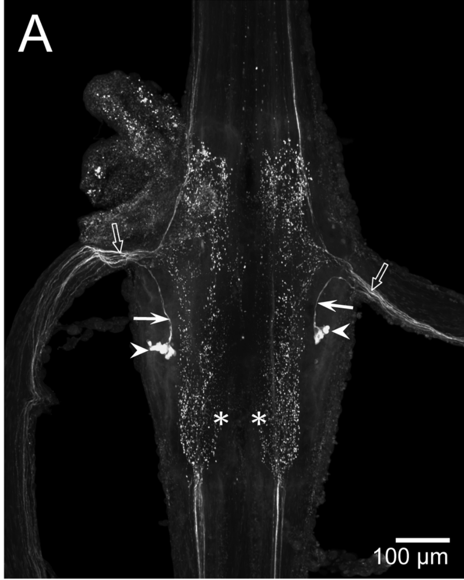
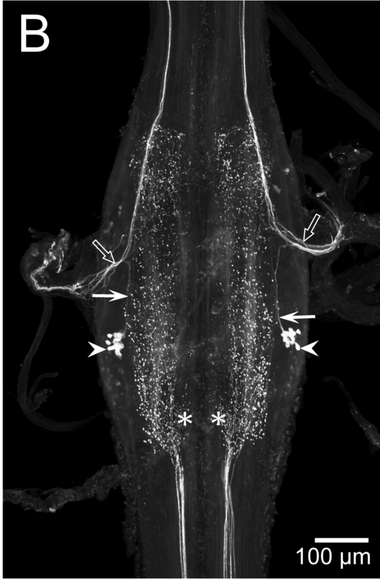
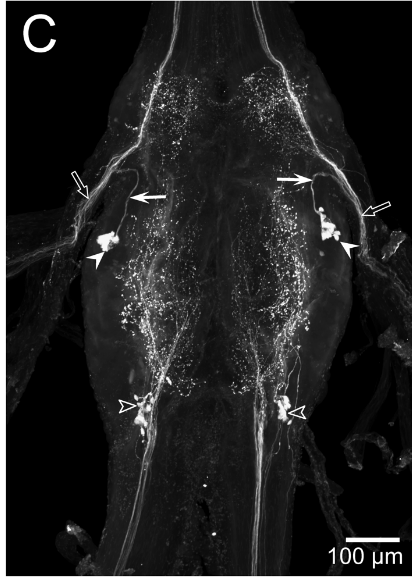
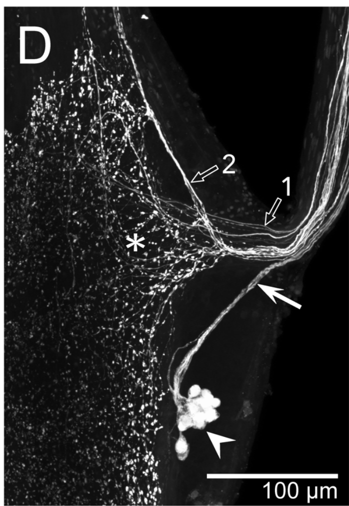

C
anti-OA
50 µm

Figure 3.6. Aminergic cells in the supraneural lymphoid glands of *C. sculpturatus*. **A.** Numerous small norepinephrine (NE)-ir cells (arrowheads) were detected in the supraneural lymphoid glands associated with the VNC in the mesosoma. The majority of these NE-ir cells measured approximately 5-6 µm in diameter, although some cells were as large as 8-9 µm. Their somata were found to be randomly distributed within the gland. **B.** A magnified view of several of the NE-ir cells (arrowheads) shown in Panel A, showing clear outlines of their nuclei. These cells did not appear to form any projections, and are rather non-neuronal in general appearance. **C.** OA-ir cells in a supraneural lymphoid gland. These cells measured approximately 4-6 µm in diameter, and were similar in general appearance to the NE-ir cells shown in Panels A-B. Like those NE-ir cells, these OA-ir cells were randomly distributed within the gland, although they appeared to be present at a higher density than the NE-ir cells.


A
100 µm


B
100 µm


C
100 µm


D
2
1
100 µm

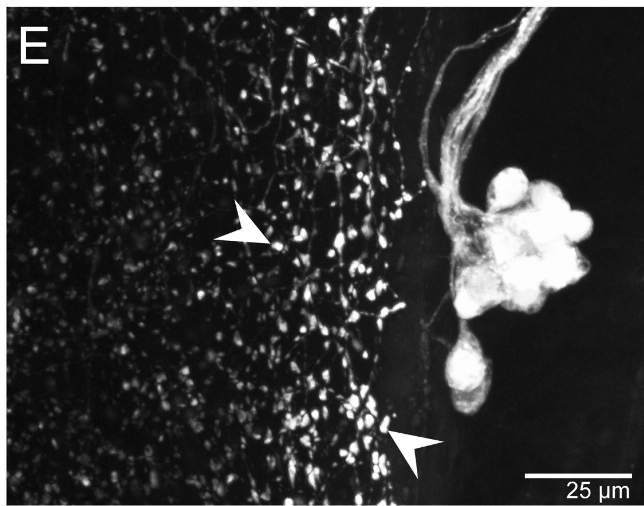
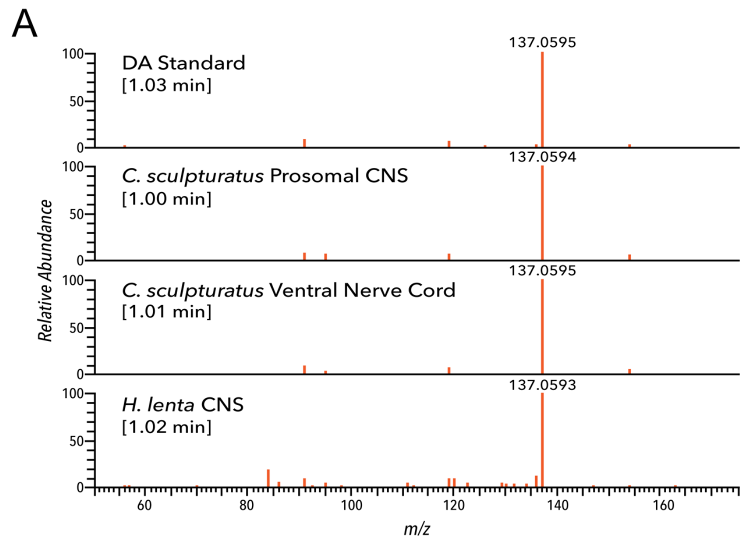
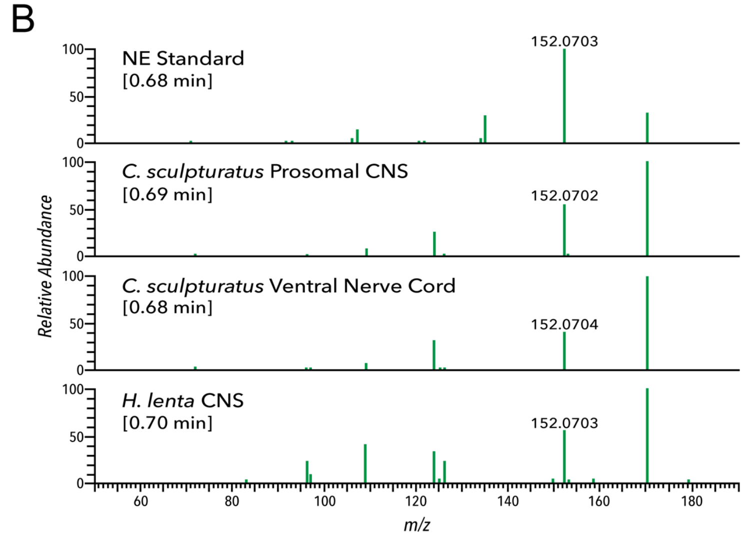

E
25 µm

Figure 3.7. 5-HT-immunoreactivity in the VNC of *C. sculpturatus*. **A.** 5-HT-ir cells in a representative mesosomal ganglion (ganglion 1). Along the lateral margin of each mesosomal ganglion, posterior to the segmental nerve roots, were bilaterally paired clusters of small 5-HT-ir neurons (arrowheads). Each of these clusters contained at least 12 somata, each measuring 7-8 µm in diameter. The axons of these neurons ascended in a bundle (solid arrows) and exited the ganglion via the ipsilateral segmental nerve. Also visible were large bundles of afferent 5-HT-ir fibers (open arrows) that entered the ganglion via the segmental nerves. A portion of these fibers appeared to contribute to a dense meshwork of 5-HT-ir processes within the ganglion, while others immediately ascended to and terminated in anterior ganglia. Asterisks denote dense collections of 5-HT-ir fibers originating from ascending afferent fibers. **B.** 5-HT-ir cells in a representative metasomal ganglion (ganglion 5). The pattern of 5-HT-immunoreactivity in the metasoma was reminiscent of that seen in the mesosoma, showing similar paired clusters of 5-HT-ir neurons (arrowheads) and their efferent projections (solid arrows), as well as pronounced afferent 5-HT-ir fibers (open arrows) that contribute to a dense meshwork of finer 5-HT-ir processes (asterisks). **C.** 5-HT-immunoreactivity in the terminal ganglion (ganglion 7) reveals a similar complement of clustered serotonergic neurons (solid arrowheads), their efferent projections (solid arrows), and numerous 5-HT-ir afferent fibers (open arrows). Unique to the terminal ganglion is the presence of a second population of 5-HT-ir neurons (open arrowheads), arranged in bilaterally paired clusters immediately anterior to the terminal nerve roots. The cells that comprise these clusters are similar in size and number to the efferent 5-HT-ir neurons observed in all ganglia, and are thus likely homologous to them. The projection patterns of these neurons were not discernible. **D.** An enhanced view of several 5-HT-ir structures in a mesosomal ganglion, showing more clearly the individual somata of the serotonergic neurons (arrowhead) and their fasciculated axons (solid arrow), which can be seen definitively exiting the ganglion. This enhanced view also clearly shows the separation of serotonergic afferent fibers; a portion of these afferents (open arrow 1) contribute to a complex assemblage of fine 5-HT-ir fibers (asterisk) in the same ganglion that they enter, whereas a separate portion of the afferents (open arrow 2) immediately ascend to more anterior ganglia. **E.** A highly magnified view of the meshwork of fine 5-HT-ir fibers in a mesosomal ganglion. Densely-packed punctate structures measuring 1-3 µm in diameter (arrowheads) can be seen along these fibers, and are presumed to be large synaptic boutons.


A
DA Standard
[1.03 min]
137.0595
C. sculpturatus Prosomal CNS
[1.00 min]
137.0594
C. sculpturatus Ventral Nerve Cord
[1.01 min]
137.0595
H. lenta CNS
[1.02 min]
137.0593
Relative Abundance
m/z


B
NE Standard
[0.68 min]
152.0703
C. sculpturatus Prosomal CNS
[0.69 min]
152.0702
C. sculpturatus Ventral Nerve Cord
[0.68 min]
152.0704
H. lenta CNS
[0.70 min]
152.0703
Relative Abundance
m/z

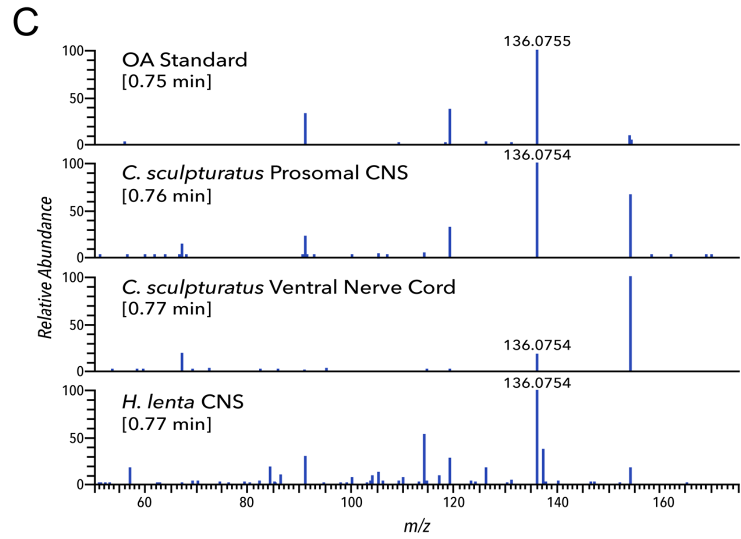
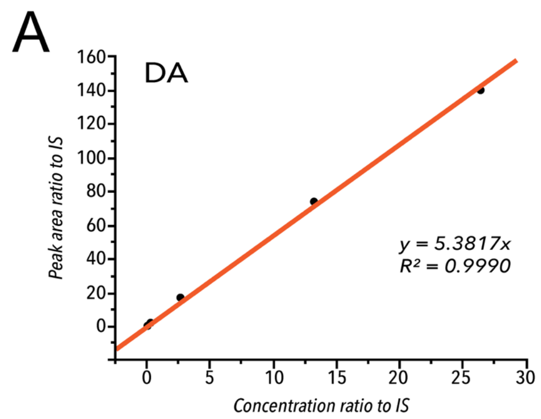
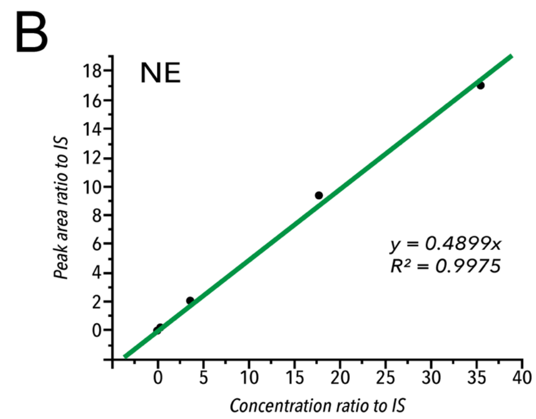

C
OA Standard
[0.75 min]
136.0755
C. sculpturatus Prosomal CNS
[0.76 min]
136.0754
C. sculpturatus Ventral Nerve Cord
[0.77 min]
136.0754
H. lenta CNS
[0.77 min]
136.0754
Relative Abundance
m/z

Figure 3.8. Ultra-performance liquid chromatography and mass spectrometry confirms the presence of DA, NE, and OA in the CNS of *C. sculpturatus* and *Hogna lenta*. **A.** The mass spectrum of a known DA standard (top) compared to the spectra obtained from extracts of the *C. sculpturatus* prosomal mass, the *C. sculpturatus* VNC, and the CNS of *H. lenta*. In all three tissue extracts, the patterns of fragmentation and retention times (as indicated in brackets above each spectrum) show a high level of correspondence to those observed for the DA standard. This result indicates that DA is present in all three tissue samples. **B.** The mass spectrum of a known NE standard (top) compared against mass spectra obtained from the same set of arachnid tissue extracts. The high level of correspondence in fragmentation pattern and retention time indicates that NE is present in all three tissue samples as well. **C.** The mass spectrum of a known OA standard (top) compared against mass spectra obtained from the three arachnid tissue extracts tested. As was true of DA and NE, there is a high level of correspondence in fragmentation pattern and retention time, which indicates that OA is also present in all three tissue samples.

A



B



C

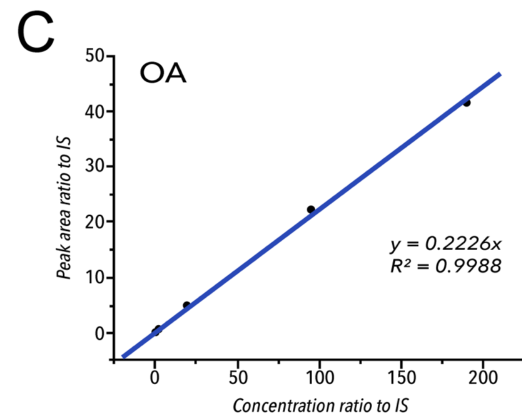
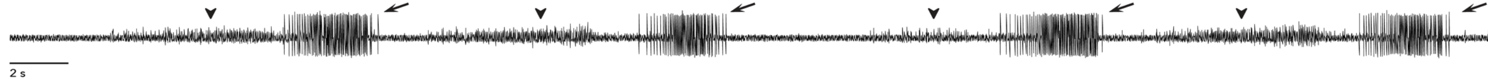
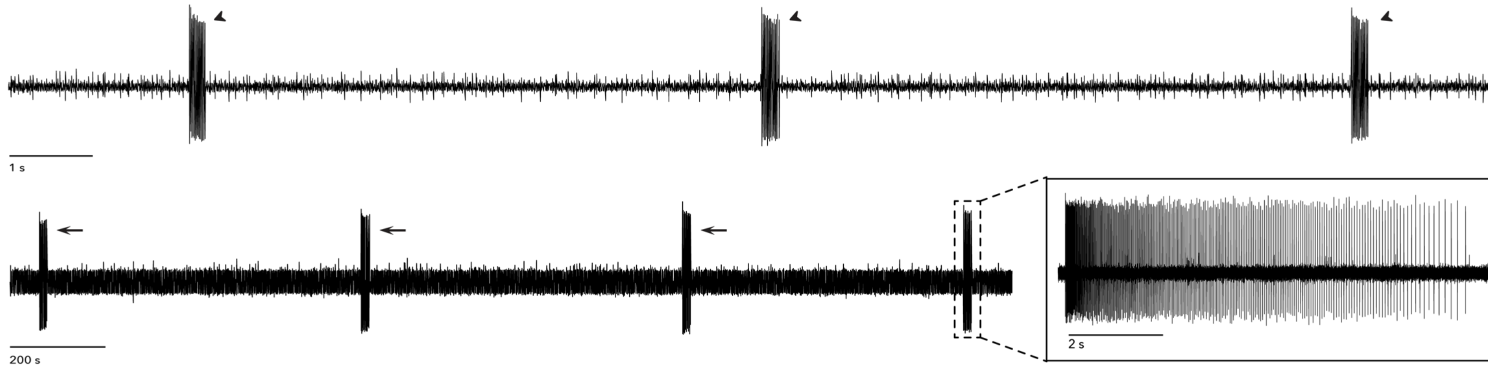


Figure 3.9. Calibration curves for DA, NE, and OA standards that were used to quantify the levels of these amines in *C. sculpturatus* and *H. lenta* tissue extracts. On the x-axis of each plot is the concentration of the amine standard relative to that of the internal standard (IS). On the y-axis is the peak monitoring ion area of the internal standard relative to that of the IS. **A.** Calibration curve for DA (R^2 = 0.9990) **B.** Calibration curve for NE (R^2 = 0.9975). **C.** Calibration curve for OA (R^2 = 0.9988).

A
Baseline activity:
1 s


B
100 µM NE:
2 s


C
100 µM DA:
1 s
200 s
2 s

D
100 µM OA:
2.5 s

Figure 3.10. DA, NE, and OA elicit distinct physiological responses in the telsonic nerve of *C. sculpturatus*. Each trace presented here is from a unique specimen, and the scales of each trace have been normalized along the y-axis. **A.** Baseline recording of the telsonic nerve in the absence of biogenic amine solutions. At baseline, the nerve exhibited varying amounts of spontaneous small unit activity, but distinct patterned activity (e.g., bursting) was always absent. **B.** Representative trace showing patterned activity evoked by a 100 µM solution of NE. This pattern was characterized by repeating 4-5 s bursts of small units (arrowheads) followed immediately by 3-4 s bursts of larger units (arrows). The period between bursts of each type was approximately 12-15 s. The same pattern was also elicited by 25 µM solution of NE. **C.** Representative traces showing two types of patterned activity evoked by a 100 µM solution of DA. Top trace: In a subset (n = 3) of recordings, DA elicited brief (0.2-0.3 s) bursts of large units (arrowheads) with a period of 5-15 s. Bottom trace: In n = 2 recordings, the brief DA-evoked bursts were not observed; instead, DA produced 8-10s bursts with a period of 4.25-5 minutes. Insets show the individual units that comprise these bursts. **D.** Representative trace showing patterned activity evoked by a 100 µM solution of OA. OA-evoked activity was marked by multiple overlapping bursts of many different units, including the largest units recorded from the telsonic nerve. Notably, this pattern was only observed in a subset (n = 2) of OA recordings.

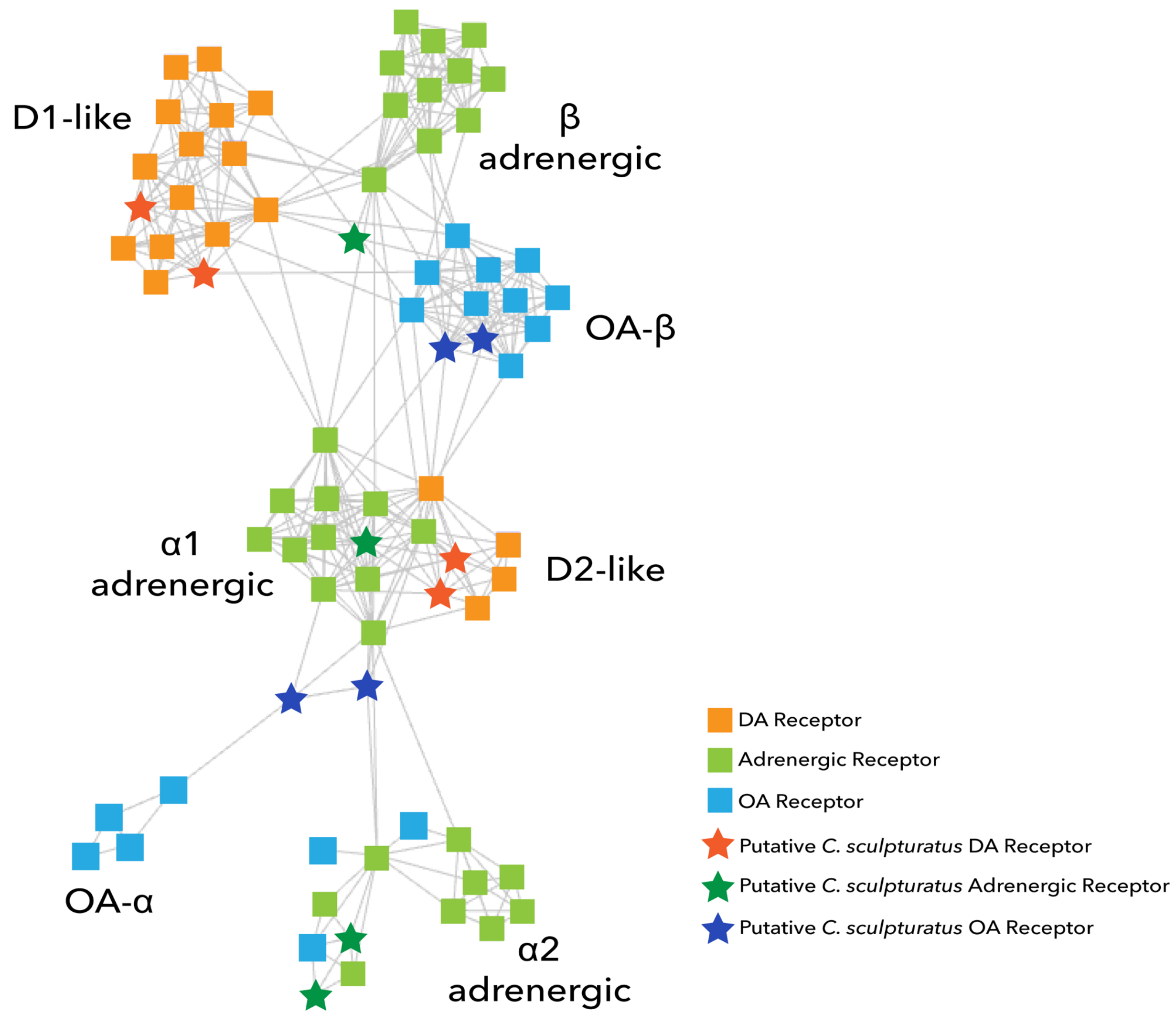

D1-like
β
adrenergic
OA-β
α1
adrenergic
D2-like
OA-α
α2
adrenergic
DA Receptor
Adrenergic Receptor
OA Receptor
Putative *C. sculpturatus* DA Receptor
Putative *C. sculpturatus* Adrenergic Receptor
Putative *C. sculpturatus* OA Receptor

Figure 3.11. Sequence cluster map of putative DA, NE, and OA receptor sequences from *C. sculpturatus* and known receptor sequences from a variety of other organisms. The distance between receptors on this map indicates their level of sequence similarity, with highly similar sequences clustering closer to each other. Of the four putative DA receptors in the scorpion, two clustered tightly with known D1-like receptors, while the other two were clustered between D2-like receptors and α1 adrenergic receptors. Of the four putative OA receptors in the scorpion, two clustered tightly with known OA-β receptors, while the other two were clustered between OA-α receptors and α1 adrenergic receptors. Of the four putative NE receptors in the scorpion, one clustered tightly with known α1 adrenergic receptors, two clustered with a mix of α2 adrenergic and OA-α receptors, and one was positioned equidistant from clusters of β adrenergic, OA-β, and D1-like receptors.

CHAPTER 4

Conclusions and prospects for future research

My dissertation has contributed valuable new knowledge to the fields of entomology and neurobiology, by providing detailed anatomical maps of biogenic amine-synthesizing neurons in the central nervous systems (CNSs) of two chelicerate orders: the Araneae (spiders) and Scorpiones (scorpions). My tyrosine hydroxylase (TH) immunolabeling studies in the wolf spider *Hogna lenta*, the jumping spider *Phidippus regius*, and the bark scorpion *Centruroides sculpturatus* constitute the first descriptions of catecholaminergic neurons in any member of the Chelicerata, and thus provide an important first step towards understanding how catecholamines are distributed and function in this diverse and important group of animals. Furthermore, the octopamine (OA) and serotonin (5-hydroxytryptamine, 5-HT) immunolabeling I conducted in *C. sculpturatus* complement previous studies of these amines in chelicerates, which are few in number and have been primarily limited to studies of just two species (the horseshoe crab *Limulus polyphemus* and the wandering spider *Cupiennius salei*). Taken together, my anatomical data have established a strong foundation upon which future studies of biogenic amines in the Chelicerata can be based.

Although the distributions and structures of catecholaminergic, octopaminergic, and serotonergic neurons in representatives of the Chelicerata have been further elucidated by my dissertation work, the functional roles of

these amines in chelicerates remain poorly understood. Of the relatively few studies that have examined the effects of biogenic amines in the chelicerate CNS, most are correlative in nature, and focus solely on OA in spiders and the horseshoe crab. For example, experimentally altered levels of OA in the hemolymph have been correlated with aggressive behaviors (DiRienzo et al., 2015; Hebets et al., 2015), defensive behaviors (Jones et al., 2011), and general activity levels (DiRienzo et al., 2015) in spiders, as well as an increase in sensitivity of their trichobothria to windborne stimuli (Torkkeli et al., 2011; Widmer et al., 2005). In the horseshoe crab *L. polyphemus*, OA has been shown to modulate aspects of the visual system (Dalal & Battelle, 2010; Kass & Barlow, 1984; Lim-Kessler et al., 2008; Renninger et al., 1989), heartbeat (Augustine et al., 1982), and motor patterning (Rane et al., 1984; Wyse, 2010). Dopamine (DA) levels have been linked to aggression in widow spiders (DiRienzo & Aonuma, 2017) and changes in electroretinogram recordings of wolf spiders (Munoz-Cuevas & Carricaburu, 2000), as well as heartbeat in *L. polyphemus* (Augustine et al., 1982). 5-HT titers have also been associated with defensive behaviors in orb-weaving spiders (Jones et al., 2011). These studies have provided valuable initial insights into the potential functional roles of catecholamines, OA, and 5-HT in the Chelicerata, but it is clear that further studies are needed.

Thus, an important future direction stemming from my anatomical data will be to investigate the precise ways in which these amines modulate chelicerate behavior. Of particular interest are the potential modulatory roles of

catecholamines in the visual system, as suggested by the intense levels of TH immunoreactivity in the first- and second-order optic neuropils and arcuate body of *H. lenta.* A second fascinating avenue for future research would be to characterize the ways in which catecholamines (likely DA) modulate respiratory processes in scorpions, as suggested by the direct innervation of the book lungs by catecholaminergic efferents in *C. sculpturatus*. An integrative approach, which combines behavioral assays with electrophysiological recordings, could shed light on these hypotheses, and many others that have arisen from my dissertation research. Such an approach is already feasible in the scorpion, whose CNS is highly amenable to physiological manipulations. In contrast, the inherent difficulties of conducting electrophysiological recordings in spiders has long been a stumbling block to understanding the neural bases of spider behavior, but recent advances in methodology are beginning to make these sorts of experiments more achievable in spiders as well (e.g. Menda et al, 2014; Shamble et al., 2016).

The finding that endogenous norepinephrine (NE) was present in the CNSs of both *H. lenta* and *C. sculpturatus* raises an interesting question: where are the adrenergic (i.e. NE-synthesizing) neurons located in the chelicerate CNS? It is presumed that a subset of the TH-immunoreactive neurons identified in *H. lenta* are adrenergic, but this was not directly tested in my present study. Thus, an important next step would be to conduct further immunolabeling studies in the CNS of *H. lenta*, using an antiserum against NE specifically. Curiously, no

NE-ir neurons were detected in the ventral nerve cord (VNC) of *C. sculpturatus*, which implies that the full complement of adrenergic neurons in the scorpion is limited to the prosomal regions of the CNS, which were not examined in the present study. Thus, NE immunolabeling experiments in the prosomal scorpion CNS are also needed to better determine where in the CNS NE is synthesized. Antibodies raised against dopamine β-hydroxylase, the enzyme which converts DA to NE, might also be informative in this regard. If NE-ir neurons are detected, it would also be important to determine if any of those cells convert that NE to epinephrine (E); this can be achieved via specific antisera against E and its synthetic enzyme phenylethanolamine N-methyltransferase. The potential roles of E in the chelicerate nervous system were not a major focus of this dissertation, but they should not be ignored in future studies.

With the distribution of catecholamines in the CNS of spiders and scorpions now revealed by my doctoral research, tyramine (TA) remains the only major biogenic amine whose distribution has not been examined in any chelicerate species. This is perhaps not surprising, because TA has only recently been recognized as a bona fide modulator of behavior in other invertebrates (Roeder, 2005; Roeder et al., 2003). Nevertheless, to fully understand the complex interplay between different aminergic systems in the Chelicerata, it is important to know the distribution and morphology of tyraminergic neurons as well. This could be achieved via immunolabeling with a specific antiserum

against TA, and comparing the resulting distribution patterns with those known for OA, which is synthesized directly from TA.

An interesting outcome of this dissertation was the finding that aminergic neurons in the Chelicerata are consistently arranged in large clusters of somata. This pattern was observed for catecholaminergic neurons in both *H. lenta* and *C. sculpturatus*, as well as for octopaminergic and serotonergic neurons in *C. sculpturatus*, and is consistent with patterns observed by other researchers in *L. polyphemus* and *Cu. salei*. Within the Arthropoda, this clustered arrangement of aminergic neurons appears to be unique to the Chelicerata, as aminergic neurons typically exist in single pairs (or as unpaired cells) in the Mandibulata. However, to determine fully if this pattern (as well as others I have described) is characteristic of the Chelicerata as a whole, additional immunolabeling studies of biogenic amines in other chelicerate taxa are necessary. To date, the vast majority of our knowledge of aminergic systems in the Chelicerata are limited to just three orders—the Xiphosura, Araneae, and Scorpiones. Investigations into the other chelicerate orders would prove to be highly informative. Taxa such as the Opiliones (harvestmen), Solifugae (camel spiders), and Acari (mites and ticks) are prime candidates for future investigations, as they are easily collected in the habitats where they are found and are relatively distantly related to the other chelicerates that have already been examined. Studies in the Thelyphonida (whip scorpions) and Amblypygi (whip spiders) would also be useful in determining if the patterns observed in spiders and scorpions are more

generalizable to the clade Arachnopulmonata as a whole; this would be especially important in relation to the possible roles of catecholamines in modulating book lung activity, as book lungs are unique to the Arachnopulmonata.

The availability of genomic data for *C. sculpturatus* allowed for the identification of putative adrenergic, octopaminergic, and dopaminergic receptors in the scorpion, which would not have been feasible otherwise. Aside from *C. sculpturatus*, reference genomes are available for a small handful of other chelicerates: five spider species (*Latrodectus hesperus*, *Loxosceles reclusa, Nephila clavipes, Parasteatoda tepidariorum,* and *Stegodyphus mimosarum*), one other scorpion (*Mesobuthus martensii*), and 15 species of mites and ticks (Acari) (Garb et al., 2018). It would be especially worthwhile to examine the genomes of these other chelicerates to determine if they also contain genes for distinct adrenergic receptors, as such a finding would further support the hypothesis that NE signaling is present throughout the Chelicerata as a whole, and not just a unique feature of *C. sculpturatus*. It is, of course, also imperative that genomes continue to be sequenced from other chelicerate species, especially those in orders that are completely unrepresented in this regard; the availability of more genomic information would drastically increase the toolkit available to researchers seeking to conduct broad-scale comparative studies of biogenic amines in the Chelicerata.

My electrophysiological recordings in *C. sculpturatus* highlighted that NE could reliably elicit patterned activity in the telsonic nerve that was distinct from the patterns elicited by other amines, which strongly implies that NE is a physiologically relevant signaling molecule in the scorpion. This effect was observed at the standard dose of 100 µM, which is comparable to doses used in other invertebrate preparations (c.f., Leitch et al., 2003; Price & Berry, 2006; Puhl & Mesce, 2008; Wood, 1995), as well as at a lower concentration of 25 µM. However, my present study did not attempt to strictly determine the optimal or effective doses of NE, DA, or OA in these preparations. As such, future studies in this system must include robust dose-response curves for these three amines, which would be very helpful in determining if there are any dose-dependent effects, including potential cross-reactivity of NE with dopaminergic and/or octopaminergic receptors at other concentrations. The specificity of the NE response could also be further tested by conducting these recordings in the presence of highly specific pharmaceutical blockers of adrenergic, octopaminergic, and dopaminergic receptors; indeed, the receptor genes highlighted in this dissertation could assist in the selection of specific antagonists that are likely to be effective in *C. sculpturatus*. The available genomic information also opens up the possibility for other experimental approaches that would not otherwise be possible, e.g., selective knockouts of particular receptors via RNAi.

One of the major strengths of my dissertation was the comprehensive approach I used to examine the possibility that NE is an endogenous signaling molecule in *C. sculpturatus*, via the integration of immunocytochemistry, analytic chemistry, electrophysiology, and genomics. I propose that this approach should be adopted as a model to investigate the potential presence and functions of NE in other invertebrate taxa as well. An ambitious, yet exciting, goal would be to apply this methodology in representatives of every major phylum of bilaterian animals and map the presence and absence of NE signaling onto a phylogenetic tree of the Bilateria. Such an undertaking would undoubtedly require the concerted efforts of many research groups over an extended period of time, but it would provide invaluable insight into the evolutionary origins of adrenergic signaling, as well as help identify lineages in which it has been lost and completely supplanted by octopaminergic systems.

Finally, my hope is that my dissertation will exemplify the importance of comparative studies in "non-model" organisms. It is now clear, from my results and those of others (e.g., Bauknecht & Jékely, 2017; Sukumar et al, 2018), that the previously established notion that NE is absent from invertebrates is not well supported. Instead, this idea likely arose from a severe under-sampling of invertebrate taxa in early neurobiological studies. As such, we as scientists must always be mindful of the limitations associated with an overreliance on a small handful of "model" organisms. It is hoped that this dissertation will be joined in the near future by similar investigations into other underrepresented organisms,

both within and beyond the Chelicerata. It is only through such studies that we will be able to fully understand the evolution of aminergic signaling systems in the animal kingdom, and better elucidate the full range of their roles in modulating animal behavior.

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