The role of the femoral chordotonal organ in motor control, interleg coordination, and leg kinematics in *Drosophila melanogaster*

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The years of work that went into
this dissertation are dedicated to my mother,

Lynn Michelle Chockley,

who always encouraged me
to go anywhere possible
and do what I love

Abstract

Legged locomotion in terrestrial animals is often essential for mating and survival, and locomotor behavior must be robust and adaptable in order to be successful. The behavioral plasticity demonstrated by animals' ability to locomote across diverse types of terrains and to change their locomotion in a task-dependent manner highlights the flexible and modular nature of locomotor networks. The six legs of insects are under the multi-level control of local networks for each limb and limb joint in addition to over-arching central control of the local networks. These networks, consisting of pattern-generating groups of interneurons, motor neurons, and muscles, receive modifying and reinforcing feedback from sensory structures that encode motor output. Proprioceptors in the limbs monitoring their position and movement provide information to these networks that is essential for the adaptability and robustness of locomotor behavior.

In insects, proprioceptors are highly diverse, and the exact role of each type in motor control has yet to be determined. Chordotonal organs, analogous to vertebrate muscle spindles, are proprioceptive stretch receptors that span joints and encode specific parameters of relative movement between body segments. In insects, when leg chordotonal organs are disabled or manipulated, interleg coordination and walking are affected, but the simple behavior of straight walking on a flat surface can still be performed. The femoral chordotonal organ (fCO) is the largest leg proprioceptor and monitors the position and movements of the tibia relative to the femur. It has long been studied for its importance in locomotor and postural control. In *Drosophila melanogaster*, an ideal model organism due its genetic tractability, investigations into the composition, connectivity, and function of the fCO are still in their infancy. The

fCO in *Drosophila* contains anatomical subgroups, and the neurons within a subgroup demonstrate similar responses to movements about the femur-tibia joint. Collectively, the experiments laid out in this dissertation provide a multi-faceted analysis of the anatomy, connectivity, and functional importance of subgroups of fCO neurons in *D. melanogaster*.

The dissertation is divided into four chapters, representing different aspects of this complex and intriguing system. First, I present a detailed analysis of the composition of the fCO and its connectivity within the peripheral and central nervous systems. I demonstrate that the fCO is made up of anatomically distinct groups of neurons, each with their own unique features in the legs and ventral nerve cord. Second, I investigated the neuropeptide profile of the fCO and demonstrate that some fCO neurons express a susbtance that is known to act as a neuromodulator. Third, I demonstrate the sufficiency of subsets of fCO neurons to elicit reflex responses, highlighting the role of the *Drosophila* fCO in postural control. Lastly, I take this a step further and look into the functional necessity of these neuronal subsets for intra- and interleg coordination during walking. The importance of the fCO in motor control in *D. melanogaster* has been considered rather minor, though research into the topic is very limited. In the work laid out herein, I highlight the complexity of the *Drosophila* fCO and its role in the determination of locomotor behavior.

Zusammenfassung

In den meisten terrestrischen Tieren ist zwei- oder vierbeinige Fortbewegung essentielle Grundlage für Verhaltensweisen wie Paarung und Überleben. Um dessen Erfolg zu garantieren, muss das Fortbewegungsverhalten robust und anpassungsfähig sein. Die Fähigkeit eines Tieres, seine Fortbewegung spezifisch gemäß den Ansprüchen diverser Untergründe zu verändern, demonstriert die Plastizität dieses Verhaltens und unterstreicht den flexiblen und modularen Aufbau der Fortbewegungsnetzwerke.

Bei Insekten, sowie bei vielen anderen Wirbellosen und Wirbeltieren, wird jedes Gelenk und jedes Bein, zusätzlich zur übergreifenden zentralen Kontrolle, von mehrstufigen lokalen Netzwerken kontrolliert. Letztere bestehen aus Rhythmus-generierenden Gruppen von Interneuronen, Motoneuronen und Muskeln, deren Aktivität von sensorischen Strukturen modifiziert und verstärkt wird. Propriozeptoren in den Gliedmaßen versorgen diese Netzwerke mit Informationen zu Bewegung und Position, welche essentiell für die Anpassungsfähigkeit und Robustheit des Verhaltens sind.

Propriozeptoren der Insekten formen eine diverse Klasse und die exakte Funktion jedes einzelnen Typs für die Kontrolle der Motorik ist weiterhin offen. Chordotonalorgane, funktional analog zu den Muskelspindeln der Wirbeltiere, sind propriozeptive Streckrezeptoren an den Gelenken, die spezifische Parameter der relativen Bewegungen zwischen Segmenten signalisieren. Wenn die Chordotonalorgane im Insektenbein funktional ausgeschaltet oder manipuliert werden, beeinflusst dies das Laufen und die Koordination zwischen den verschiedenen Beinen; das simple geradeaus Laufen auf einer flachen Ebene ist jedoch weiterhin möglich. Das femorale Chordotonalorgan (fCO) ist der größte Propriozeptor im Bein

und misst Position und Bewegungen der Tibia relativ zum Femur. Zahlreiche Studien belegen seine Relevanz für Fortbewegung und zur Kontrolle der Körperhaltung. Untersuchungen zur Zusammensetzung, Konnektivität und Funktion des fCO sind bei *Drosophila melanogaster*, einem aufgrund seiner genetischen Formbarkeit idealen Modellorganismus, noch in den Kinderschuhen. Das fCO bei *Drosophila* ist in anatomische Untergruppen unterteilt und die Neuronen innerhalb einer produzieren ähnliche Signale in Reaktion auf Bewegungen des Femur-Tibia Gelenks. Zusammengenommen liefern die Experimente dieser Dissertation eine vielseitige Analyse der Anatomie, Konnektivität und funktionalen Relevanz der Neuronen in Untergruppen des fCO in *D. melanogaster*.

Die vorliegende Dissertation ist in vier Kapitel unterteilt, welche sich mit unterschiedlichen Aspekten dieses komplexen und faszinierenden Systems beschäftigen. Im ersten Kapitel stelle ich eine detaillierte Analyse der Zusammensetzung des fCO und seiner Konnektivität im peripheren und zentralen Nervensystem dar. Ich zeige, dass das fCO aus anatomisch abgetrennten Gruppen von Neuronen besteht, jede mit individuellen Merkmalen im Bein und im ventralen Nervensystem. Der zweite Abschnitt beschäftigt sich mit dem Neuropeptid-Profil des fCO und belegt, dass einzelne Neurone des fCOs ein Neuropeptid exprimieren, welches ein bekannter Neuromodulator ist. Im dritten Teil demonstriere ich, dass Teilmengen der fCO Neurone ausreichend sind, um Reflexantworten auszulösen. Letzteres unterstreicht die Rolle des *Drosophila* fCOs für die Kontrolle der Körperhaltung. Im letzten Abschnitt erweitere ich dies auf die funktionale Notwendigkeit der neuronalen Untergruppen für die Kontrolle innerhalb eines und zwischen allen Beinen während des Laufens.

In der motorischen Kontrolle von *D. melanogaster* wurde das fCO bisher als von geringer Relevanz eingeschätzt, trotz oder wegen der begrenzten Anzahl der Studien

zu diesem Thema. In dieser Arbeit verdeutliche ich die Komplexität des *Drosophila* fCOs und seine Bedeutung für die Kontrolle des Laufens.

List of Abbreviations

AEP	Anterior extreme position
BDSC	Bloomington Drosophila Resource Center
BL	Body length
ChAT	Choline acetyltransferase
CNS	Central nervous system
CO	Chordotonal organ
CPG	Central pattern generator
CS	Campaniform sensilla
DAPI	4',6-diamidino-2-phenylindole
DLC	DeepLabCut
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DN	Descending neuron
EMG	Electromyogram
fCO	Femoral chordotonal organ
Flp	Flippase
FRET	Fluorescence resonance energy transfer
FRT	Flippase recognition target
GECI	Genetically encoded calcium indicator
GFP	Green fluorescent protein
GtACR1	*Guillardia theta* anion channelrhodopsin
IN	Interneuron
IR	Infrared

MN	Motor neuron
NOMPC	No mechanoreceptor potential C
PBS	Phosphate-buffered saline
PBT	PBS with Triton X-100
PEP	Posterior extreme position
PFA	Paraformaldehyde
RFP	Red fluorescent protein
RMSE	Root mean squared error
RNA	Ribonucleic acid
ROI	Region of interest
RT	Room temperature
TA	Tyramine
tCO	Tibial chordotonal organ
TNT	Tetanus toxin
TRP	Transient receptor potential (channel)
UAS	Upstream activating sequence
VNC	Ventral nerve cord

1   General Introduction

1.1   Locomotion and Sensorimotor Networks in Insects

Locomotion, be it flight, swimming, or walking, is necessary for the survival of animals. Legged locomotion in terrestrial animals is a ubiquitous, flexible, and highly optimized behavior (Alexander, 1989). With the ability to move, animals can access mates and resources, escape predators or find prey, and seek out habitats and environments that fit their current needs. Highlighting the importance of locomotion, most animals exhibit some degree of motility in their life cycle, and even sponges have been shown to move across the substrate (Bond and Harris, 1988).

In order to be effective, locomotion must be both resistant and resilient to environmental factors, such as unexpected perturbations, as well as internal factors, such as mismatch between the "intended" and actual output of the locomotor system (af Klint et al., 2010; Blaesing and Cruse, 2004; Hellekes et al., 2012). This behavioral plasticity is reflected in the ability of animals to change the employed type of locomotion in a task-dependent manner (Ashley-Ross and Lauder, 1997; Islam et al., 2006; Orger et al., 2008; Pick and Strauss, 2005). Particularly in terrestrial legged locomotion, walking movements must almost continuously be adapted to the irregular and often unpredictable nature of the substrate. Legged animals encounter many types of terrain and walking situations, so the networks and appendages supporting locomotion must be quite flexible to be successfully used under different conditions. In addition to the dynamic appendages and physical characteristics that contribute to this flexibility, the underlying neuromuscular networks contain multiple levels of control loops and exhibit experience-dependent plasticity (Bidaye et al., 2018; Hooper and Bueschges, 2017).

The neural networks underlying limb movements contain many interneurons (INs), motor neurons (MNs), and sensory neurons. These networks are largely under the influence of central descending neurons (DNs), and additionally provide ascending innervation to these brain networks. In insects, movements of the six legs are under the multi-level control of local networks for each limb and limb joint in addition to over-arching central control of the local networks. These networks controlling locomotion consist of pattern-generating networks that activate MNs, which, in turn, activate the muscles of the appendages. These central pattern generators (CPGs) consist mainly of INs located in the thoracic ganglia, analogous to the spinal cord of vertebrates (Bassler and Buschges, 1998; Bidaye et al., 2018; Brown, 1911; Kiehn, 2006). Additional INs coordinate the activity of groups of muscles between segments of individual legs or between different legs. CPG activity is responsible for the rhythmic activation of MNs, and these MNs regulate the contractions of skeletal muscles that are attached to the skeleton either directly or via connective tissue, resulting in propulsion of the animal. This loop circuit is completed by various sensory structures in the limbs that monitor their position and movement, providing modifying or reinforcing feedback signals directly onto CPGs, coordinating INs, or MNs (Figure 1.1; Bidaye et al., 2018; Burrows, 1996; Buschges et al., 2011; Tuthill and Azim, 2018; Tuthill and Wilson, 2016).
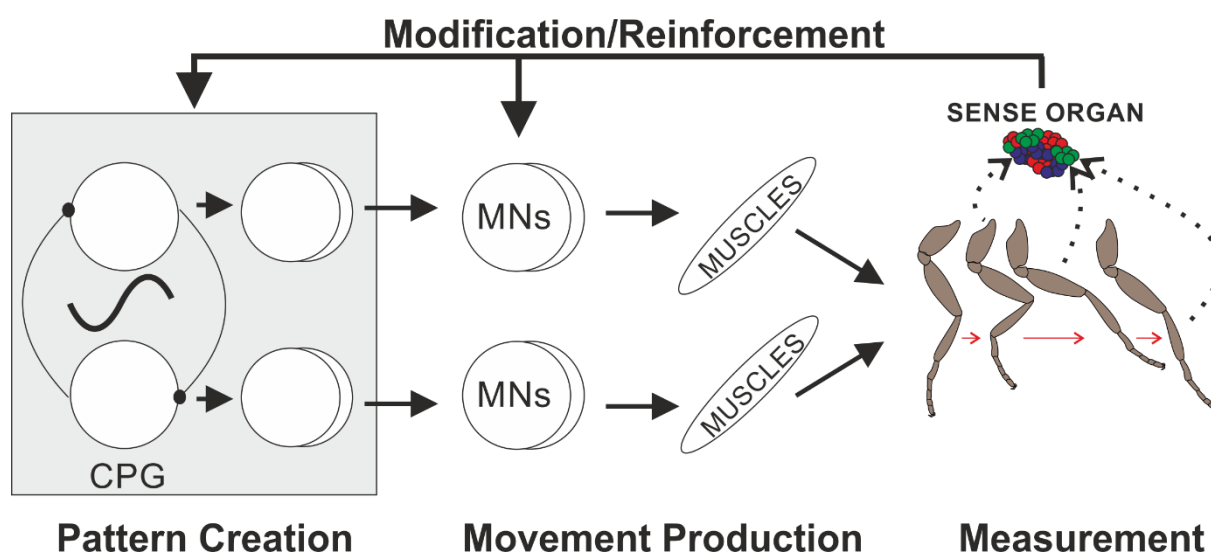
Figure 1.1 Schematic of neuromuscular networks underlying movement creation and modification. Neurons (circles) making up central pattern generators (CPGs) create patterns of neural activity, which in turn activate or inhibit motor neurons (MNs). MNs activate muscles, producing movement. Sensory organs (multi-colored circles) read out the position and movement of segments, and project onto MNs and CPG neurons, modifying or reinforcing their activity. In this manner, movements are fine-tuned, and posture is adjusted.

The neuromuscular networks underlying stepping in insect limbs contain all of these parts; this has been shown in larger insects such as stick insects and locusts but has yet to be empirically determined in *Drosophila*. A step, consisting of a swing phase (Figure 1.2A) and a stance phase (Figure 1.2B), requires the coordination of many CPGs, MNs, and muscles (Figure 1.2C). Muscles in antagonistic pairs, such as depressors and levators or extensors and flexors, alternate their activity to lift legs up, push them away from the body, move them down, and pull the body in the direction of movement. These phases of stepping and the transitions between them require precise temporal sequential activation of the MNs controlling the muscles that produce movement around each leg joint (Figure 1.2C). Signals from the locomotor network components

are used to coordinate activity between limb segments and whole limbs, and additional

control is introduced by load and position sensors that signal the state of the limb or

transitions between the phases of stepping (Bidaye et al., 2018; Mantziaris et al., 2017)
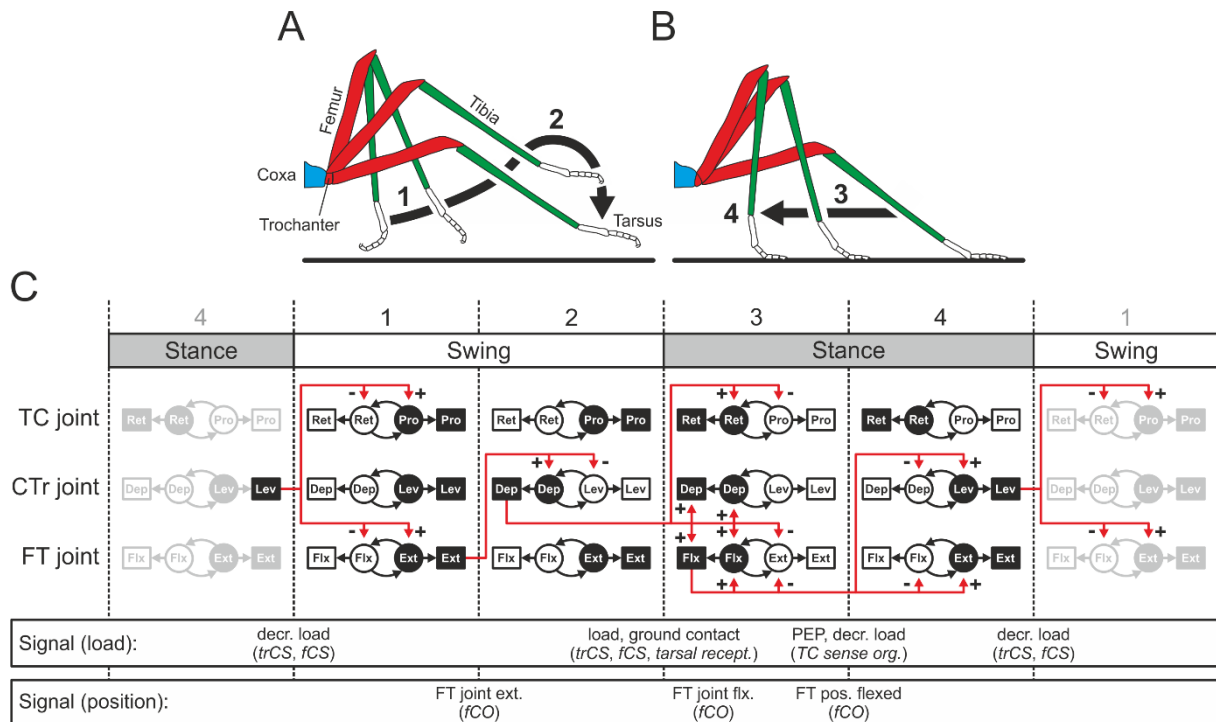
Figure 1.2 Schematic of stepping in an insect leg. A stepping cycle consists of a swing phase (A), in which the tarsus is lifted (1) and moved over the substrate (2); and a stance phase (B), in which the tarsus is placed on the substrate (3) and the body is moved relative to the tarsus (4). The muscles (squares) and CPGs (circles) required for these movements are shown in C, with antagonistic pairs in white and black. Movements around each joint are controlled by individual CPGs, which drive motor neuron and muscle activity. Sensory signals transduced by CS and the fCO are indicated in the lower rows. The fCO, phasically active during extension and flexion of the femur-tibia joint and tonically active at positions throughout the movement range, signals transitions between the beginning and end of each stepping phase (1-2 and 3-4) as well as during the transitions between the phases, signaled by a reduction in tibial extension (2-3). Figure used with permission from Bidaye et al. (2018)

This feedback from sensory organs monitoring the position and state of limbs, as well

as from exteroceptive organs monitoring the surrounding environment, is essential for

the adaptability and robustness of locomotor behavior. Exteroception can, in principle,

provide feedback about and modify motor output, especially during goal-directed movement. This is exemplified by optic flow, the pattern of apparent motion of the visual scene. As animals move, the movement of their surroundings relative to their body is represented as optic flow across photoreceptors; visual neurons have even been shown to encode information about locomotion behavior independently of vision, suggesting ascending signals from locomotor networks to the visual system (Fujiwara et al., 2017). When an animal is moving forward and the optic flow does not match forward motion, descending neurons from central brain networks provide modulatory signals to local locomotor networks; these locomotor networks can modify their output accordingly to match the optic flow (Poggio and Reichardt, 1976; Reichardt and Poggio, 1976). A prime example of this is the optokinetic reflex response in *Drosophila*, where animals will orient toward the direction of optic flow stimuli (Götz, 1968). Further, when animals are following olfactory plumes when foraging, the olfactory stimulus can provide feedback about self-motion and modulate motor programs (Jung et al., 2015). Regarding precise positions and movements of the effectors and limbs producing locomotion, however, these systems cannot be effectively employed. Constant visual monitoring of limb position and movement, for example, is highly inefficient, and makes successful locomotion slow and difficult (McNeill et al., 2010). Humans with sensory neuropathy affecting proprioception can learn to perform coordinated movements, but still show large deviations in executed movement trajectories (Ghez et al., 1990; Ghez and Sainburg, 1995; Miall et al., 2018). This highlights the importance of limb proprioceptors in the production of robust, adaptive movements.

1.2 Proprioception

Proprioception provides information on the positions and movements of body parts that is crucial for the neural control loops regulating appendages, enabling precise and appropriate adjustments of posture and movements (Bassler, 1993; Bässler et al., 1982). Originally thought to be based on precise monitoring of motor output commands, postural control, fine motor control, and limb coordination are all highly dependent on specific mechanosensitive proprioceptors (Graham and Bassler, 1981; Pearson, 1995; Steeves and Pearson, 1982). Proprioceptors exist throughout the animal kingdom, serving as sensors of movement, load, and body position (Tuthill and Azim, 2018; Tuthill and Wilson, 2016).

Vertebrates have three main classes of limb proprioceptors, each with distinct function. Muscle spindles are stretch receptors located within skeletal muscle fibers and encode changes in length of the muscle (Ellaway et al., 2015). Golgi tendon organs act as load sensors by encoding relative tension at tendons (Mileusnic and Loeb, 2006). Lastly, joint receptors, or joint afferents, encode extreme positions of joint rotation (Burgess and Clark, 1969). In recent decades, evidence has additionally been put forth that cutaneous stretch receptors also play a significant role in proprioception (Collins and Prochazka, 1996; Collins et al., 2005; Edin and Abbs, 1991). It seems that there are evolutionary advantages of having proprioceptors that encode information about these aspects of body position and movement, as invertebrates have many analogous proprioceptors in their limbs and appendages.

In insects, proprioceptors are highly diverse, and the exact role of each type in motor control has yet to be determined. Insect legs contain five main classes of proprioceptors: campaniform sensilla (CS), hair plates, bristles, multipolar receptors, and chordotonal organs (COs). CS, analogous to Golgi tendon organs, are the major

load sensors in insects. Compression of the cuticle is transduced by CS neurons when self-generated movements are resisted or body load is changed (Delcomyn et al., 1996). Bristles, or trichoid sensilla, are mainly exteroceptive mechanosensors that function as tactile surface receptors on the cuticle. Some bristles contain blunt, hollow shafts that are connected to gustatory neurons; these bristles belong to a different class called basiconic sensilla (Hannah-Alava, 1958; Tuthill and Wilson, 2016). Hair plates, structurally similar to mechanosensory bristles, are small regions of many densely packed mechanosensitive "hairs" that activate their underlying neurons when they are deflected. Using a transduction mechanism similar to bristles, hair plates get their main function from their location on the body—on the cuticular surface near the creases of joints—and are deflected when joint angles reach their extremes (Tuthill and Wilson, 2016). Multipolar receptors are a rather under-researched class of mechanosensors. They are non-ciliated neurons with multiple dendritic branches and are found throughout the body embedded in different tissues and structures. Their main function as proprioceptors is through their encoding of parameters related to stretching of tissue, though their exact role and details of their transduction and signaling mechanisms are not well understood. COs, functionally analogous to vertebrate muscle spindles (and mildly to cutaneous stretch receptors), are proprioceptive stretch receptors that span joints and encode positions of and relative movements between body segments. CO neurons encode specific parameters of these relative movements and positions, and as such are rather diversely employed proprioceptors. Among the most well-researched COs are the Johnston's organ of the antenna, the largest CO in *Drosophila* and main detector for sound, gravity, and wind; and the femoral chordotonal organ, a major player in the control of locomotion (Field and Matheson, 1998). Upon first glance, these many proprioceptor types appear to

have their own function; however, the leg proprioceptive system of insect contains a surprising amount of redundancy of function.

Theoretically, some of these proprioceptors could effectively replace others while maintaining a functional proprioceptive system. Bristles, for example, could effectively be replaced by CS; bristle neurons are activated by their deflection, via exogenous particles coming into contact with the legs or the legs touching against something. CS are highly sensitive to cuticular deformations and could functionally perform the same task, assuming the stimulus deforms the cuticle. Hair plates are responsible for measuring extreme joint positions, but CO neurons encoding joint angles do the same. CS can read out muscle activity, and CO neurons provide a proxy of this in encoding actual movement about joints. Lastly, in elegant experiments artificially increasing body load in *Drosophila melanogaster*, Mendes et al. (2014) demonstrated that flies can adapt their posture and locomotion to two-fold body load increases. They further demonstrate the necessity of the fCO in these postural adaptations. Despite these redundancies in the proprioceptors of the legs, these many different organs exist. This suggests that they, as individual organs, provide specific information to the system that may be lost without them. If these signals were somehow pooled together by a more generalized proprioceptive organ, specific information might be lost; while a hair plate can signal an extreme position in a joint, it cannot signal anything between the extreme positions—that is the role of the fCO.

In insects, when leg proprioceptors are disabled, interleg coordination and walking are affected, but the simple behavior of straight walking on a flat surface can still be performed (Cruse et al., 1984; Mendes et al., 2013; Mendes et al., 2014; Usherwood et al., 1968). Disabling leg COs alone, however, produces effects similar to disabling multiple leg sensory structures (Field and Matheson, 1998; Mendes et al., 2013; Mendes et al., 2014). Only two COs can be found on the legs, the small tibial CO (tCO)

and the much larger fCO; many of the detrimental effects from disabling leg proprioceptors likely come from disabling the fCO.

1.3 The Femoral Chordotonal Organ

The femoral chordotonal organ (fCO; Bässler, 1965) has long been studied for its involvement in locomotor control. It consists of a group of cells with mechanosensitive dendrites in the femur and encodes various movement parameters (position, velocity, and acceleration) about the femur-tibia joint (Figure 1.3A; Field and Matheson, 1998). fCO dendrites attach to either a receptor apodeme attached to the tibia or to surrounding muscles and the distal femoral epicuticle. When the tibia rotates about the femur-tibia joint and strain on the receptor apodeme changes, mechanosensitive ion channels (Akitake et al., 2015; Cheng et al., 2010) in the dendritic tips open, leading to changes in membrane potential in the neurons (Field and Matheson, 1998). Due to its functional connectivity with various components of locomotor networks, signals from the fCO play a major role in inter- and intraleg coordination during walking.

1.3.1 Signal Transduction and Transmission

The fCO, as all COs, consists of one or more closely packed groups (scoloparia) of sensory neurons embedded in a matrix of supporting cells. In *Drosophila*, around 152 primary sensory neurons are grouped into three scoloparia attached to femoral muscles and epicuticle via connective apodemes (Figure 1.3B; Mamiya et al., 2018; Shanbhag et al., 1992). Each scoloparium consists of paired sensory neurons organized into scolopales. At the proximal end of the scolopales lie the neurons' cell bodies, and they project their dendrites distally into a scolopale cell, which attaches to the apodeme via an attachment cell (Field and Matheson, 1998). fCO dendrites contain a cilium at

their distal end within the scolopale cell, and it is at the level of this cilium where signal transduction occurs.

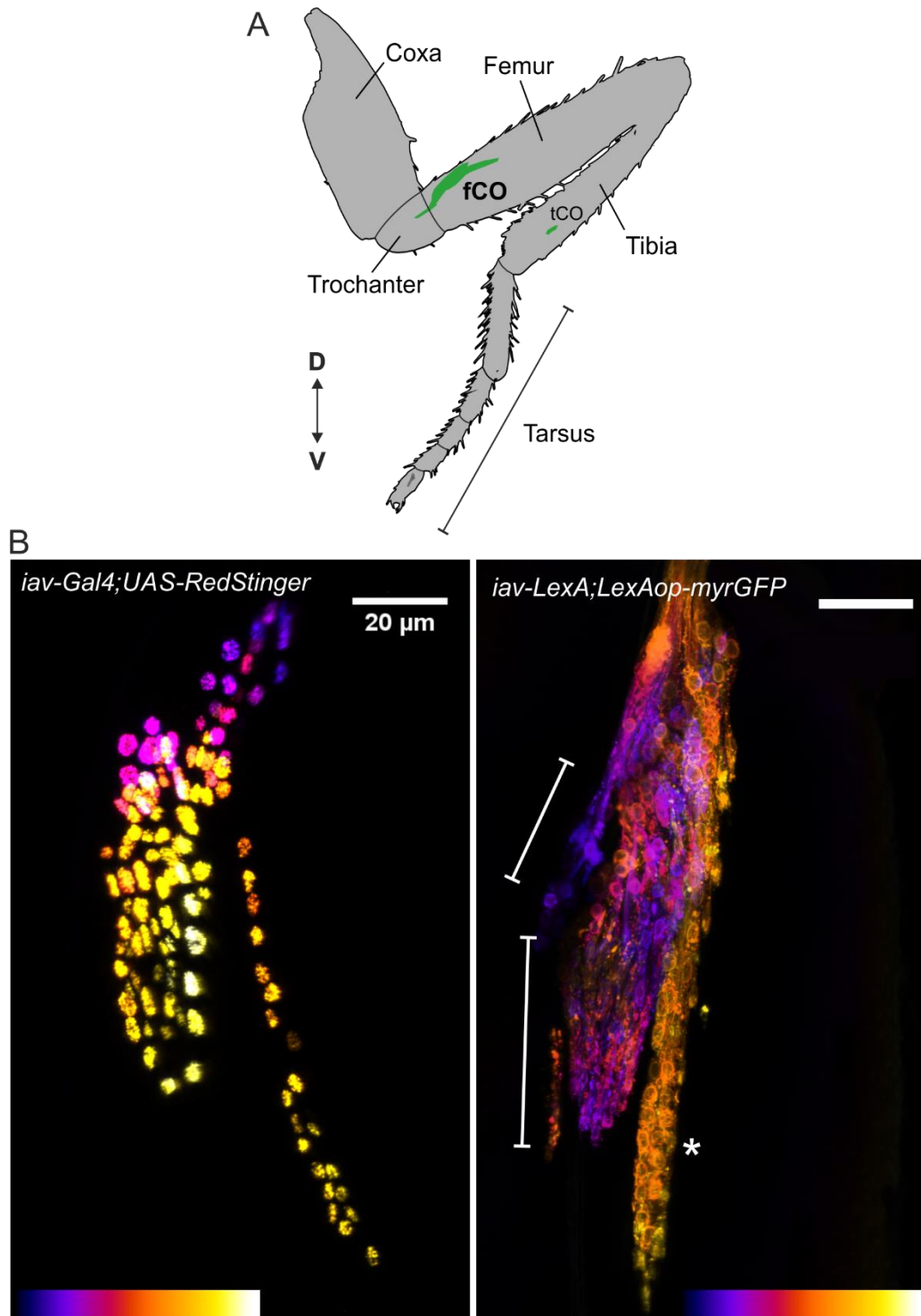**Figure 1.3. Schematic and detailed view of the femoral chordotonal organ in *D. melanogaster*.** (A) Schematic showing segments of the leg and the placement of the femoral chordotonal organ (fCO) and tibial chordotonal organ (tCO), as labeled by *iav*-GAL4. (B) Confocal laser scanning microscope view of *iav*-GAL4; UAS-RedStinger (left) showing the locations of fCO cell nuclei, and *iav*-LexA; LexAop-myrGFP (right) showing

the membranes of fCO neurons; three lobes of the fCO can be seen—one elongated, thin lobe (asterisk), and two more round, clustered lobes (brackets); color in (B) represents relative z-stack depth in maximum intensity z-projections.

Exactly how mechanical signals are transduced by COs remains debated, but both theories include mechanosensitive ion channels located in the cilium of the dendrite. Several such channels have been implicated in the proper functioning of COs, mostly in the Johnston's organ, and they belong to the transient receptor potential (TRP) superfamily of ion channels. The two channels in question are NOMPC (no mechanoreceptor potential C; Gong et al., 2013; Göpfert et al., 2006) and a heterodimeric TRPV channel that is formed by the proteins Inactive and Nanchung (iav/nan; Warren and Matheson, 2018). Both of these are mechanically activated, cation permeable ion channels localized to CO dendritic cilia. It has been proposed, but not empirically determined, that when the cilia are stretched or relaxed, mechanical gates on the ion channels open or close and thus regulate the influx of cations into the dendrite. In this way, the mechanical stimulus is coupled to the sensory neuron, transduced into a receptor potential, and the receptor potential is coded into an action potential (Field and Matheson, 1998; French, 1992).

The fCO contains primarily excitatory neurotransmitters, and evidence hints toward the presence of biogenic amines and neuropeptides. In the locust *Schistocerca gregaria* (Lutz and Tyrer, 1988), some afferent neurons from the fCO are immunoreactive to choline acetyltransferase (ChAT) antiserum. In *Drosophila,* Johnston's organ neurons are cholinergic (Sivan-Loukianova and Eberl, 2005), and a driver line under control of *ChAT* labels the fCO (Mamiya et al., 2018). Further, a subset of fCO neurons in *D. melanogaster* can be labeled using genetic driver lines based on *tdc2* (tyrosine decarboxylase) expression (Pauls et al., 2018). Tyrosine decarboxylase is necessary for the synthesis of Tyramine (TA) from tyrosine.

Interestingly, TA can function as a neurotransmitter and a neuromodulator depending on the receptor to which it binds, and it is known to regulate a variety of physiological functions in invertebrates (Zhang and Blumenthal, 2017). Another biogenic amine, serotonin, has also been found in the fCOs of *S. gregaria* and *D. melanogaster* and has been shown to affect locomotor behavior (Howard et al., 2019; Lutz and Tyrer, 1988). Lastly, the presence of neuropeptides, a family of molecules that act as neurohormones and neuromodulators, has been suggested due to the finding of dense-core vesicles in an electron microscopy study of the fCO of *D. melanogaster* (Shanbhag et al., 1992).

The finding of dense-core vesicles in fCO axon by Shanbhag et al. (1992) was coupled with the discovery of a peripheral processing center of sorts between fCO neurons. A transmission electron microscopy analysis of the fCO in *D. melanogaster* led to the discovery that all axons from the fCO form a peripheral glomerulus before entering the leg nerve. This glomerulus contains reciprocal and serial synapses, and rarely contained monosynapses between fCO axons. It was in this glomerulus that dense-core vesicles were identified in addition to the clear vesicles, in which classical neurotransmitters are found. The complex neurochemical and neuroanatomical composition of the fCO, specifically in *Drosophila*, deserves the attention of researchers, especially when looking at the transmission of signals from the fCO into the central nervous system. It remains to be investigated exactly what occurs in this glomerulus, but peripheral transformations of signals before they reach axon terminals could throw a wrench into our young understanding of the Drosopholid fCO.

1.3.2 Encoding of Proprioceptive Signals

Extracellular single-unit recordings in *Cuniculina impigra* were the first to show that fCO neurons encode various parameters of joint movement and position. Cells were found that responded to positions, velocities, and accelerations, with some cells sensitive even to positive or negative velocities or accelerations (Bueschges, 1994; Hofmann and Koch, 1985; Hofmann et al., 1985). Position sensitive neurons respond tonically with increased firing rates as the joint reaches their preferred angle, and velocity sensors respond phasically to their preferred velocity (Bueschges, 1994; Hofmann et al., 1985). Acceleration receptors tend to respond with single or a few spikes (Hofmann and Koch, 1985). Intriguingly, many cells respond to combinations of these parameters while still maintaining directional selectivity (Bueschges, 1994; Hofmann et al., 1985). In a study in *S. gregaria*, white-noise stimuli were applied to the fCO and responses were analyzed with a Wiener kernel analysis to investigate non-linear dynamics in neural response characteristics. In their analysis, (Kondoh et al., 1995) concluded that fCO neurons can change stimulus encoding properties depending on the patterns of stimuli applied. This is also reflected in the presence of response hysteresis in fCO neurons, a phenomenon in which neurons fire at a certain rate for a given joint position when approached from one direction and fire at a different rate when the position is approached from the other direction (Field and Matheson, 1998; Mamiya et al., 2018).

Little work has been done on the encoding properties of fCO neurons in *Drosophila*, but a recent seminal study used calcium imaging to demonstrate similarities to other insects studied. In larger insects, such as the stick insect and locust, the fCO is normally stimulated by opening the leg, clamping the receptor apodeme of the fCO around a hook, and moving the hook back and forth with a piezoelectric actuator to simulate the

natural stretching and relaxation of the fCO during tibial movement. Because of its small size, this is nearly impossible in *D. melanogaster*; however, one advantage of this animal is the ability to express genetically encoded calcium sensors that change their fluorescence intensity in response to the internal calcium concentration of a neuron. In this manner, Mamiya et al. (2018) were able to record activity of fCO neurons at their axon terminals in the VNC while moving the tibia with a magnet. They showed that the fCO also contains tonically and phasically active neurons responding to position and velocity, as well as some cells that are directionally sensitive velocity sensors. Unfortunately, they were unable to address the presence of acceleration-sensitive neurons due to the kinetics of the calcium sensor.

Intriguingly, they imaged activity of a majority of fCO neurons (using a driver line based on *inactive*) and deduced all patterns of activity using a correlation-based K-means clustering analysis. This analysis provided an unbiased grouping of response patterns based on pixel intensity changes throughout fCO axon terminals in a single hemiganglion. From this, the authors found that pixels that were grouped in their responses corresponded to different anatomical projection patterns of fCO afferents.

These projection patterns, termed *club*, *claw*, and *hook* (Phillis et al., 1996), were shown to make up the entire pattern of *inactive* expression in the VNC (Mamiya et al., 2018). Similar to the stick insect and locust, the *D. melanogaster* fCO contains tonic position sensors and phasic velocity sensors, in addition to phasically-responding vibration-sensitive neurons, as can be found in the dorsal scoloparium of the stick insect fCO (Kittmann and Schmitz, 1992). From the analysis described above, no phasic-tonic responses were noted, though this could be an artefact of the clustering analysis. Interestingly, fCO neuron projection patterns clearly corresponded to response types. C*law* neurons encode the angle of the femur-tibia joint with tonic responses. *Club* neurons act as phasically responding, directionally insensitive

movement sensors. *Hook* neurons also respond to movement, but exhibit directional sensitivity; the *hook* neurons tested by (Mamiya et al., 2018) were mostly flexion sensitive, though a more detailed response analysis using other subpopulations of *hook* neurons is still needed to parse out their preferred stimuli.

Matheson (1992) demonstrated in a locust that neurons in the fCO that have similar response characteristics demonstrate morphological similarities. The morphological distinction between response types in the locust, however, is not as clear as that seen in *D. melanogaster* (Mamiya et al., 2018; Shanbhag et al., 1992). In addition to this morphological grouping, *Drosophila* is thus far unique regarding the presence of a peripheral hub of fCO synapses in the leg; its role in fCO signal processing, however, has not been investigated (Shanbhag et al., 1992). Whether the different neuron types are represented differently in the glomerulus remains an open question.

1.3.3 fCO Connectivity and Signal Processing

In the stick insects *Carausius morosus* and *C. impigra* and the locust species *Locusta migratoria* and *S. gregaria*, the animals in which pioneering work on the fCO was done, fCO afferents project ipsilaterally within their segmental ganglion (Braunig et al., 1981; Hustert, 1978; Pfluger et al., 1988). In *D. melanogaster*, the majority of fCO neurons project locally to their respective hemiganglion, some have interhemiganglionic projections, and some (~3%) ascend directly to the brain (Tsubouchi et al., 2017). In the local projection areas in the VNC, fCO neurons have been shown to affect MNs, spiking and non-spiking local INs, and intersegmental INs (Bassler, 1993; Burrows, 1985b, 1994). In *S. gregaria*, it has been shown that fCO neurons connect with tibial extensor and flexor MNs, sometimes monosynaptically, fitting with their role of the major mediators of femur-tibial joint movements (Burrows, 1987; Field and Matheson, 1998). Connections, both direct and indirect,

with local INs are where transformations of fCO signals and effects on other local locomotor and proprioceptor networks occur, with these largely being responsible for the fCO's role in intraleg coordination. Individual fCO afferents have also been shown to synapse with both MNs and INs; additionally, afferents from multiple fCO neurons sometimes converge on single INs (Burrows, 1985a; Field and Matheson, 1998). The role of the fCO in interleg coordination likely results from connections with intersegmental INs, and the fCO has been shown to have excitatory effects on such cells (Laurent and Burrows, 1988). On top of these local connections between the fCO and MNs or INs, another layer of processing occurs between pools of INs.

There are many parallel pathways of information processing that exist between INs that receive direct input from fCO neurons, and it is thought that behavioral flexibility is largely dependent on this level of signal processing (Field and Matheson, 1998). By way of polysynaptic connections via inhibitory INs, the fCO can exert inhibitory effects on MNs (Burrows and Siegler, 1982). It has further been shown that a single non-spiking IN can simultaneously excite one MN while inhibiting another, adding more degrees of freedom to the potential effects of fCO signals on locomotor networks (Burrows, 1989). Taken together, these examples illuminate only a portion of the myriad parallel processing pathways from fCO neurons via local and intersegmental INs onto other ascending and descending INs and MNs controlling leg muscles. In this regard, understanding the entirety of the locomotor networks receiving and transforming signals from the fCO is quite a daunting task.

1.4   fCO neurons in locomotor control

In *D. melanogaster*, owing to recent advances in neurogenetic techniques, research addressing the role of the fCO in locomotor behavior is just getting underway. In the larger insects studied, however, experiments using reduced, tethered preparations

have shed light on the functional connectivity between the fCO and other locomotor network components. Moreover, investigations of the fCO's involvement in interleg coordination and reflexes has set the stage for studies on the roles of individual fCO neuron types in motor control.

Drosophila leg proprioceptors have been shown to affect coordination parameters during walking. Using broad manipulations with tetanus toxin light chain (TNT), an inhibitor of synaptic transmission, and mechanoreceptor mutants, it has been shown that removal of leg sensory signals and, specifically, fCO signals leads to modified kinematic parameters during walking (Mendes et al., 2013). Synaptic inhibition using a pan-sensory driver line (5-40-GAL4) with expression restricted to the femur and proximal tibia (*dac*-Flp, UAS-FRT-stop-FRT-TNT; see section 3.2) caused flies to walk with longer and less linear steps, as well as increased swing duration. In flies expressing the Nanchung mutation nan[36a] and, therefore, with non-functional COs, step length and swing duration were also increased, and stances were less linear compared to wild-type flies, though not to the extent caused by the pan-sensory manipulations. Represented by gait patterns, which were defined by the number of tarsi concurrently on the ground at any given time during walking, flies exhibited largely normal interleg coordination. The authors deduced that, because "gait parameters and interleg swing phase coupling were largely unimpaired", interleg coordination in the fly is "not dominated by sensory feedback" (Mendes et al., 2013). While these conclusions are arguable based on the clear walking impairments shown, the main takeaway from this study is that removal of leg sensory activity and leg CO mechanotransduction does result in altered leg movement and coordination parameters, if not to a surprisingly large extent.

Intriguingly, the changes seen in CO-deficient flies largely matched those seen in flies lacking functional CS, hair plates, bristles, and COs. The functional role of CS, hair

plates, and COs in motor networks has been previously demonstrated in multiple insects (see Tuthill and Wilson, 2016); these results thus beg the question of the usefulness of these sensors in the walking behavior of the fly. This could partially be explained by the methodology used—the authors restricted TNT expression to the limbs using a transcription factor that is active in leg imaginal discs, dachshund (*dac*; Giorgianni and Mann, 2011; Rauskolb, 2001). Expression of *dac* starts in the larval stage of development, leaving open the possibility of plasticity in the functionally redundant sensory networks of the leg (Mardon et al., 1994). Thus, such experiments would ideally be repeated using transient inhibition of these sensory neurons. However, the results laid out in Mendes et al. (2013) suggest that these leg sensory organs—COs excluded—play only a minor role in locomotor coordination in *D. melanogaster*.

As sensory signals representing leg joint angles and movements are thought to mediate coupling between local motor control circuits and interleg coordination (Brunn and Dean, 1994) and the fCO is clearly necessary for normal walking behavior (Mendes et al., 2013), the question remains of the importance of the distinct types of fCO proprioceptive neurons in this behavior. As only broad manipulations of fCO activity are possible in larger insects, *Drosophila* provides a novel way to investigate sub-population level effects of fCO neurons in freely behaving animals (Mamiya et al., 2018).

The fCO of *Drosophila* contains neurons that respond to position and velocity, as well as some velocity-sensitive cells that are directionally selective. Mathematically speaking, velocity and acceleration are the first and second derivatives of position, and neural networks theoretically have the ability to perform this calculation (Areas et al., 2001). However, this would likely require a few processing steps within central networks and, consequently, extra time. *Drosophila* walk at a range of speeds between

roughly 4 and 15 body lengths (BL) per second, or roughly 8-30 mm/s, leading to a step cycle duration of 60-300 ms (Wosnitza et al., 2013). In the shortest of these, signal transfer through a few chemical synapses would simply take too long (up to about 5 ms per synapse). Coupled with the presence of neurons that encode individual first- and second-order derivatives of positional information, this suggests that specific information on speed, velocity, and acceleration of leg movements are useful for the regulation of locomotion.

1.5   Linking Encoding and Function

The encoding of tibial position, velocity, and acceleration by fCO neurons suggests distinct roles of these cells in the processing and regulation of limb movements. First, tonically active position sensors are likely less important for walking as they are for postural control. Simply put, signaling the current angle of a joint during stepping movements seems to be not very informative unless changes in angle can be inferred from these signals. In *D. melanogaster*, *claw* neurons fire with increasing frequency as the femur-tibia joint reaches its extreme positions and are less active (or not active) in the middle of the joint's range of motion (Mamiya et al., 2018). Response hysteresis seen in these neurons can provide information to locomotor networks about the direction of movements based solely on positional information, though this would pose a computational issue for downstream circuits without combined input from directionally selective *hook* neurons. As such, *claw* neurons alone are likely not important for signaling the current phase of the step cycle to other limbs and to central networks. Interestingly, communication of extreme joint angles is already done by hair plates, so the necessity of such signals from fCO neurons during walking is still unclear.

Phasically responding speed sensors (no directional sensitivity), *club* neurons in *Drosophila*, likely simply signal that the femur tibia joint is changing its angle. This could be used to distinguish between the phases of the stepping cycle; however, without directional information, these signals can only communicate that a change in position is occurring. It is likely that signals from these neurons are used to signal to neighboring legs, for example, when it is appropriate to initiate or end a swing or stance phase. Directionally sensitive speed sensors, on the other hand, could signal when a leg or leg joint is approaching the end of a step phase or when the leg is in stance or swing phase. Encoding of this by *hook* neurons in *Drosophila* is likely to be important for interleg coordination and for determining the switch from stance to swing (and swing to stance). Notably, these two phases involve different movements in *Drosophila* legs because the three leg types (front, middle, and hind) are not parallel to each other with respect to their relative angles to the thorax. The swing phase in front legs, for example, involves an extension movement as the femur-tibia joint changes from fully flexed to fully extended; in hind legs, the swing phase involves movement from an extended to a flexed position. Therefore, signals from neurons encoding the same velocities in these different limbs would need to be differentially processed in central networks.

Acceleration sensors have yet to be demonstrated in the fCO of *D. melanogaster*, but they are likely to play a role in signal lift-off and touch-down events of the tarsus in addition to vibration-related stimuli from the substrate. These neurons could be useful for stability during walking, but it is likely that they are not essential for walking behavior. It has been shown, however, that vibration-sensitive neurons of the fCO (*club* neurons) exhibit a topographical frequency tuning along their VNC axon terminals, so information about vibrational stimuli in the femur tibia joint must be useful to the underlying neural networks (Mamiya et al., 2018).

It is currently unclear if these signals from fCO neurons are preprocessed before entering VNC. Potential transformation of signals in the glomerulus, for example, indicate that the calcium activity induced by tibial movements as seen by (Mamiya et al., 2018) could represent signals that have already been shaped by synaptic transmission or neuromodulation. Further, it remains to be determined if all fCO neurons are important for walking or if some are specifically important for other behaviors. The present dissertation addresses these questions.

1.6   Aims of the Present Work

In the present dissertation, I aimed to answer some of the open questions regarding the fCO of *D. melanogaster* and its importance for locomotor behavior. The dissertation is divided into four chapters, representing different aspects of this complex and intriguing system. In the first chapter, I aimed to investigate the composition of the fCO and its connectivity within the peripheral and central nervous systems. For this, I identified various transgenic driver lines with expression within subsets of fCO neurons. Such driver lines provide the ability to break up the fCO into parts and allow for investigations into the functional and anatomical characteristics of the organ as a whole. Using anatomical techniques befitting transgenic model organisms, I demonstrate that the fCO is made up of anatomically distinct groups of neurons, each with their own unique features in the legs and VNC. Moreover, I investigated the peripheral and central connectivity of these neurons. In the second chapter of this dissertation, I investigated the neuropeptide profile of the fCO and demonstrate that, in addition to its central and peripheral connectivity, the fCO contains a neuropeptide that acts as a neuromodulator. In the third chapter of this dissertation I demonstrate the sufficiency of subsets of fCO neurons for the production of reflex responses. For this, I activated specific neurons in the fCO and measured leg

movements and muscle activity to demonstrate that stimulation of parts of the fCO is sufficient for producing reflex movements and muscle activity. In the fourth chapter, I take this a step further and look into the functional necessity of these neuronal subsets. For this, I inhibited these neurons in fully intact, behaving flies during natural walking behavior and investigated their effects on leg movements and coordination. I demonstrate that some, but not all, fCO neurons are important for normal walking behavior. In the final chapter of this dissertation, I describe my attempts to perform live imaging of neural activity in the periphery. While these experiments were cut short due to methodological difficulties, I have set the stage for future investigations into the activity of fCO neurons at the level of their cell bodies. Based on the peripheral connectivity of the fCO, it is essential that we understand encoding of proprioceptive stimuli in the periphery. Collectively, the experiments laid out in this dissertation provide a multi-faceted analysis of a proprioceptor that has previously received much attention in other insects. Moreover, they highlight the importance of the fCO in locomotor behavior of *D. melanogaster*.

2   General Methods

The following section contains a detailed description of the methods that were employed in the experiments described in this dissertation. In cases where methods were used only for experiments described in an individual chapter, the applicable methods are described in the chapter itself.

2.1   Choice of Model Organism

Despite their small size, *Drosophila* make excellent model organisms for locomotion research thanks to the extensive genetic tools available, low cost, ease of access, and very large foundation of knowledge of their development and physiology. Moreover, they are highly active animals, and they locomote often even when confined or restrained (e.g., Ali et al., 2011; Jones and Grotewiel, 2011; Woods et al., 2014; Wosnitza et al., 2013). *D. melanogaster*, thanks to decades of work in genetics, provides us with an almost fully tractable model nervous system. There are countless, ever-developing genetic tools available in *D. melanogaster* that make manipulations of the animal's anatomy and physiology rather straight-forward. The primary genetic tool used in the present work is the GAL4/UAS system (review: Duffy 2002). GAL4 is a transcription factor found in yeast that binds DNA and activates transcription via recruitment of the RNA polymerase transcriptional complex. The GAL4 sequence can be inserted into the *Drosophila* genome behind known promoters, for example, to restrict expression to a certain cell type or population. The GAL4 protein then binds DNA in those cells in which it is expressed and activates the transcription of downstream sequences. The DNA binding domain of GAL4 binds specifically to a so-called upstream activating sequence (UAS), which can be inserted into the genome artificially. Downstream of the UAS sequence, we can place a reporter gene, effector,

or other genes of interest. Once the DNA binding domain is bound to the UAS sequence, the activation domain of GAL4 then recruits RNA polymerase. Further, due to the two domains being necessary to activate UAS transcription, it is possible to express each part of GAL4 behind different promoters to find overlap and to create very sparse labeling (the split-GAL4 system; Luan et al. 2006). Additionally, another yeast transcription factor, GAL80, can be used to suppress GAL4, adding another level of control to the system (Lee and Luo, 1999). This can be further modulated via selective expression or using a temperature-sensitive variant of GAL80, GAL80^{ts}, which is inert below temperatures of around 30 °C (McGuire et al., 2004). The binary GAL4/UAS system can also be used concurrently with the LexA/LexAop system, a similar binary transcription system, without any cross-effects (Yagi, Mayer, and Basler 2010). A multitude of reporter genes and genes of interest can be driven by these systems, including interfering RNAs, channelrhodopsins, fluorescent calcium or voltage indicators, fluorophores, and various proteins of interest. These can all be used for both morphological and functional studies of multiple systems in *Drosophila*. Importantly, these systems allow researchers to alter the activity of neurons in a completely non-invasive manner—this is the main basis for using this organism in the present work.

2.2 Experimental Animals

The genotypes and sources of all transgenic fly lines used in the present work are listed in Table 1. For all experiments described herein, I used female flies aged 3-8 d post-eclosion. Animals were reared on a standard yeast-based medium (Backhaus et al., 1984) at 25 °C on a 12-h/12-h light/dark cycle.

Table 1. Transgenic animals

Line	Stock Number	Source	Genotype
GAL4 Lines			
R86D09-GAL4	40459	BDSC	w[1118]; P{GMR86D09-GAL4}attP2
R15A08-GAL4	48673	BDSC	w[1118]; P{y[+t7.7] w[+mC]=GMR15A08-GAL4}attP2
piezo-GAL4	59266	BDSC	w[*]; P{w[+mC]=Piezo-GAL4.1.0}III
R10H03-GAL4	47845	BDSC	w[1118]; P{y[+t7.7] w[+mC]=GMR10H03-GAL4}attP2
R27E02-GAL4	49222	BDSC	w[1118]; P{GMR27E02-GAL4}attP2
R46H11-GAL4	50284	BDSC	w[1118]; P{GMR46H11-GAL4}attP2
R47B12-GAL4	50296	BDSC	w[1118]; P{y[+t7.7] w[+mC]=GMR47B12-GAL4}attP2
iav-GAL4	52273	BDSC	W[*]; P{iav-GAL4.K}3
R55B03-GAL4	39101	BDSC	w[1118]; P{y[+t7.7] w[+mC] GMR55B03-GAL4}attP2
R93A02-GAL4	40635	BDSC	W[1118]; P{GMR93A02-GAL4}attP2
AstC-GAL4	52017	BDSC	w[1118]; P{w[+mC]=AstC-GAL4.TH}1M/TM6B, Tb[1]
Burs-GAL4	40972	BDSC	w[*]; P{w[+mC]=Burs-GAL4.P}P12
Capa-GAL4	51969	BDSC	w[1118]; P{w[+mC]=Capa-GAL4.TH}4F
FMRFa-GAL4	51990	BDSC	w[1118]; P{w[+mC]=FMRFa-GAL4.TH}1M
Dh31-GAL4	51988	BDSC	w[1118]; P{w[+mC]=Dh31-GAL4.TH}2M
Dh44-GAL4	51987	BDSC	w[1118]; P{w[+mC]=Dh44-GAL4.TH}2M
Dsk-GAL4	51981	BDSC	w[1118]; P{w[+mC]=Dsk-GAL4.TH}3M
ETH-GAL4	51982	BDSC	w[1118]; P{w[+mC]=ETH-GAL4.TH}1M

Lk-GAL4	51993	BDSC	w[1118]; P{w[+mC]=Lk-GAL4.TH}2M
MIP-GAL4	51984	BDSC	w[1118]; P{w[+mC]=Mip-GAL4.TH}2M
Ms-GAL4	51986	BDSC	w[1118]; P{w[+mC]=Ms-GAL4.TH}6Ma
Proc-GAL4	51972	BDSC	w[1118]; P{w[+mC]=Proc-GAL4.TH}6M
sNPF-GAL4	51991	BDSC	P{w[+mC]=sNPF-GAL4.TH}2, w[1118]
UAS Lines			
UAS-mCD8::GFP	32189	BDSC	y1 w* P{10XUAS-IVS-mCD8::GFP}su(Hw)attP8
UAS-GtACR1	-	A. Claridge-Chang	w1118;;P{20x-UAS-GtACR1-EYFP}attp2, Sb[1]
UAS-FRT-stop-FRT-mCD8::GFP	30032	BDSC	y[1] w[1118]; Pin[1]/CyO; P{w[+mC]=UAS(FRT.stop)mCD8-GFP.H}14, P{w[+mC]=UAS(FRT.stop)mCD8-GFP.H}21B
UAS-syt.eGFP	6926	BDSC	w*; P{ UAS-syt.eGFP}3
UAS-RedStinger	8545	BDSC	P{w[+mc]=UAS-RedStinger}3, w[1118]
UAS-trans-Tango	77124	BDSC	y[1] w[*] P{y[+t7.7] w[+mC]=UAS-myrGFP.QUAS-mtdTomato-3xHA}su(Hw)attP8; P{y[+t7.7] w[+mC]=trans-Tango}attP40
LexA/LexAop Lines			
iav-LexA	52246	BDSC	y[1] w[*]; wg[Sp-1]/CyO, P{Wee-P.ph0}Bacc[Wee-P20]; PBac{y[+mDint2] w[+mC]=iav-lexA::p65}VK00013
LexAop-myrGFP (III)	32209	BDSC	y[1] w[*]; wg[Sp-1]/CyO, P{Wee-P.ph0}Bacc[Wee-P20]; PBac{y[+mDint2] w[+mC]=iav-lexA::p65}VK00013
Other			
Dac[RE]-Flp	-	R. Mann	Genotype not provided
Berlin-K	8522	BDSC	Wild type

BDSC, Bloomington Drosophila Resource Center, Bloomington, IN, USA

2.3   VNC Preparation

For the dissection, staining and visualization of VNCs, flies were anesthetized on ice and briefly (< 1 min) soaked in 70% EtOH to de-wax the cuticle. VNCs were dissected out in 0.1 M PBS and fixed in 4% paraformaldehyde (PFA) for 30 min on ice. Following three 15-min washing steps in 0.5% Triton X-100 in 0.1 M PBS (0.5 % PBT), VNCs were blocked in 10% normal goat serum (blocking solution; ThermoFisher Scientific, Waltham, MA) in PBT for 20 min at RT and incubated for 48 h at 4° C in primary antibodies diluted in blocking solution (mouse anti-nc82, 1:500; rabbit anti-GFP, 1:500). After three washes in PBT, they were incubated for 48 h at 4° C in secondary antibodies (goat anti-mouse AlexaFluor 633, 1:500; goat anti-rabbit AlexaFluor 488, 1:500) followed by three washes in PBT, before being mounted in Vectashield (Vector Laboratories, Burlingame, CA) and coverslipped.

2.4   Leg Preparation

Whole flies were skewered on insect pins, briefly soaked in 70% EtOH to de-wax the cuticle, fixed in 4% PFA for 45 min on ice, and washed in 0.1 M PBS (3 x 15-min). After washing, the tibia, tarsus, and distal femur were removed with microscissors. Legs were then mounted in Vectashield (Vector Laboratories) and coverslipped. For immunohistochemistry experiments in legs, the following was done after fixation in PFA: flies were then incubated in a blocking solution of 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 5% PBT for 2 h at RT with nutation then incubated with primary antibody (mouse anti-Choline acetyltransferase [ChAT], 1:50) in 0.5% PBT and 1% donkey serum for 72 h at 4° C. After 4 washes in 0.5% PBT of 1.5-2 h each at RT, flies were incubated in the secondary antibody (anti-mouse AlexaFluor 488, 1:50) 0.5% PBT and 1% normal donkey serum for 96 h at 4° C. Nuclei were then stained with DAPI (5 mg/ml solution in DMSO)

diluted in 0.1 M PBS (1:10000) overnight at 4° C, after which samples were washed 3 x 1 h in PBS, dissected off of the thorax, mounted in Vectashield, and coverslipped.

2.5 Fluorescence Microscopy and Image Processing

Confocal stacks (SP8; Leica, Wetzlar, Germany) were taken of samples with a 63x oil immersion objective (Legs) or a 20x glycerol immersion objective (VNCs). Maximum intensity projections were created using Fiji (http://fiji.sc; Schindelin et al., 2012). All figures containing microscopy images were compiled using CorelDraw (X6; Corel Corporation, Ontario, Canada).

Some confocal stacks of VNCs were registered to a standard VNC to ensure comparability between them. This followed a process similar to that described by Boerner and Duch (2010) and Boerner and Godenschwege (2010).

2.6 Optogenetics Experiments

Light-activatable ion channels (channelrhodopsins) provide researchers with a tool for transiently activating and inactivating cells using pulses of light (Riemensperger et al., 2016). Here, I take advantage of two such channels: Chrimson and GtACR1. Chrimson, a red-shifted, cation-permeable channelrhodopsin, is useful for fast activation of neurons (Klapoetke, 2014). With an activation wavelength in the red spectrum, it has an activation spectral peak at 590 nm; in this dissertation, I activated it with 658-nm light. In contrast, GtACR1 (*Guillardia theta* anion channelrhodopsin) provides a sensitive and fast way to inhibit neurons in behaving flies (Mohammad et al., 2017). GtACR1 is sensitive to wavelengths in the green spectrum and was activated using 525-nm light in the experiments described herein.

For experiments employing light-activatable channelrhodopsins, flies (0-2 d post-eclosion) were transferred to vials containing food and 0.14 mM all-trans retinal for

three days before experiments. These vials were wrapped in aluminum foil to block out

light and kept at 25 ℃ for at least two days before experiments were performed.

3 Chapter 1: Anatomy of the *Drosophila* fCO

3.1 Identifying Driver Lines

The fCO is a multi-faceted proprioceptive organ and, as such, contains many diverse cell types, as described above. Attempting to decipher and manipulate the function of each of the cells in the fCO is a daunting task, and being able to only manipulate the entire fCO (or most of it) can only inform us about the general role of the fCO in locomotor networks. To be able to test the different types of neurons within the fCO and their specific morphologies and functions, it is necessary to put together a driver-line library of lines with distinct expression patterns. This has been done for other similar systems, in which creating a modular system of driver lines has proven essential in discoveries of circuit construction and functions. Catalogues of GAL4 driver lines have been created for larval motor neurons (Pérez-Moreno and O'Kane, 2019), the mushroom body (Aso et al., 2009), and the Johnston's organ (Kamikouchi et al., 2006), among others.

The FlyLight project at the Howard Hughes Medical Institute's Janelia Research Campus (HHMI Janelia, Ashburn, VA, USA) has generated more than 10,000 transgenic *D. melanogaster* lines. These are enhancer trap lines in which GAL4 is expressed under the control of different transcriptional enhancers that are often expressed only in small groups of neurons (Jenett et al., 2012; Pfeiffer et al., 2008). These GAL4 lines, as described in section 2.1, are essential tools for neuroscience researchers studying *D. melanogaster*.

To find GAL4 lines with expression in the fCO, I manually screened thousands of images from the FlyLight database of expression patterns in the VNC of various GAL4 lines. Based on the expression pattern of *iav*-GAL4, I manually searched through

images and selected GAL4 lines with *iav*-GAL4's characteristic patterns (Mamiya et al., 2018). In addition to this manual search, I also found several lines in the literature that were not previously identified as labeling the fCO, such as several lines with labeling in the Johnston's organ. After identifying candidate lines, I crossed them with a UAS-mCD8::GFP line and checked for expression in the legs (see sections 2.4 and 2.5).

3.2   Results

3.2.1 Leg Labeling

In total, I found about 20 GAL4 lines with variable expression patterns in the legs. Of these, nine had labeling restricted to the fCO (and the tCO in one case; Figure 3.1). These lines range in the amount of fCO labeling from very few cells (Figure 3.1D,E,G,J) to many cells (Figure 3.1C,I). Because there are many GAL4 lines that label fCO neurons, it can also be said that the fCO exhibits a high degree of genetic diversity.
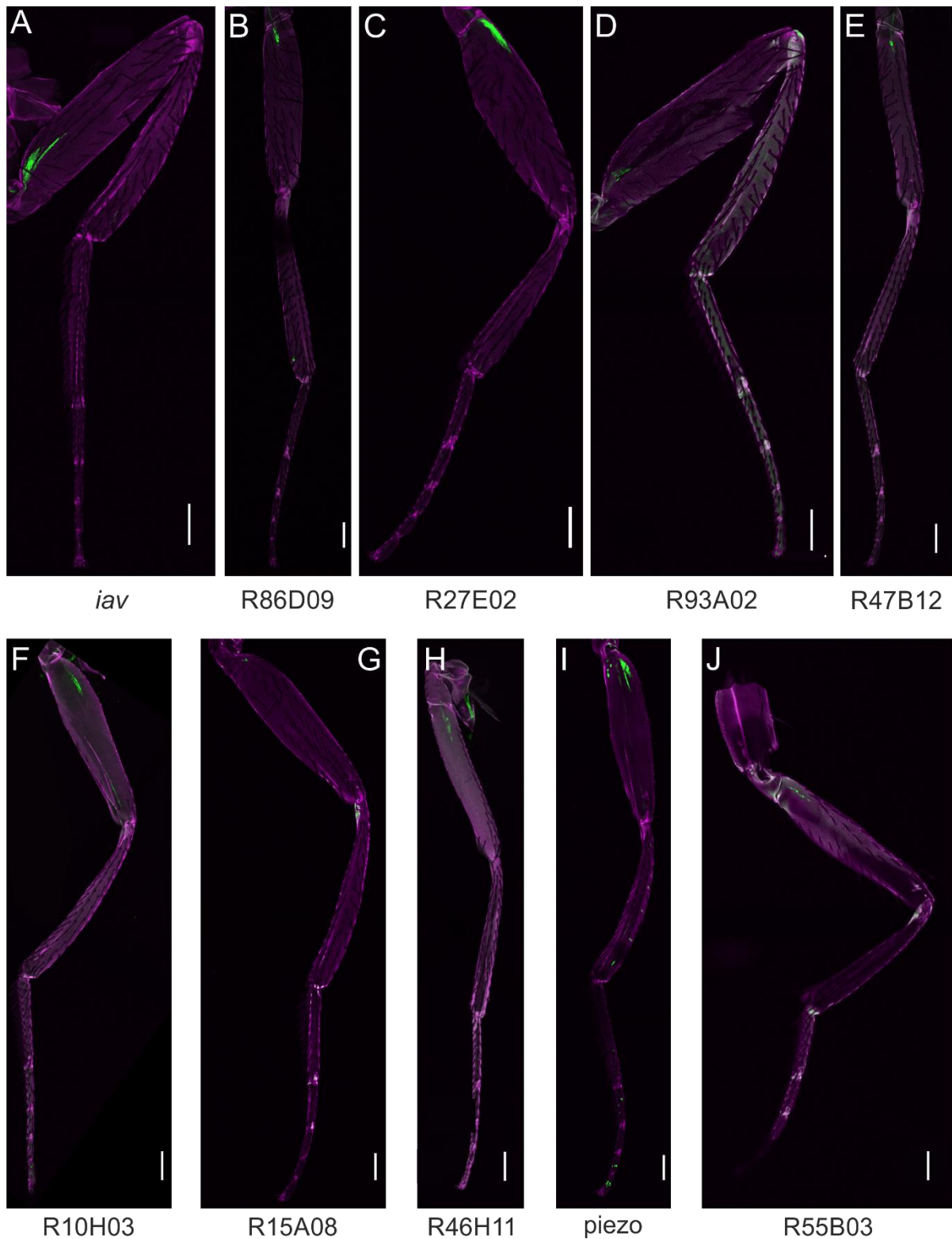
Figure 3.1 GAL4 driver lines expressing GFP in the fCO. (A-L) maximum intensity z-projections of confocal laser scanning microscope stacks of single legs from each driver line used to label the fCO; flies expressing mCD8::GFP (green) under GAL4 control were dissected as described in section 2.4 cuticular autofluorescence (633 nm excitation; magenta) shows outline of leg; *iav*-GAL4 (A) used as reference for labeling of fCO and tCO;

aside from *iav*-GAL4, only R86D09-GAL4 also labeled the tCO. Labeling of fCO neurons varied from very few (G, I, L) to many (E).

3.2.2 VNC Labeling

The VNC expression patterns of the fCO-GAL4 lines described in 3.2.1 can be seen in Figure 3.2. The expression pattern of *iav*-GAL4 (Figure 3.2A) contains all types of neurons in the fCO, and VNCs from the different subset lines demonstrate that this broad expression pattern can be broken down into parts. Of the nine fCO-GAL4 lines used in this dissertation, one contained neurons of the *claw* type (R55B03-GAL4; Figure 3.2C), four contained *hook* neurons (R10H03-GAL4, R47B12-GAL4, R86D09-GAL4 and R15A08-GAL4; Figure 3.2D-G), and two contained *club* neurons (R46H11-GAL4, R93A02-GAL4; Figure 3.2H-I). In addition to driver lines clearly labeling distinct types of fCO neurons, I found one line with fCO labeling in addition to off-target labeling from wings and halteres (*piezo*-GAL4, Figure 3.2J) as well as one line labeling a combination of *hook* and *club* neurons (R27E02-GAL4, Figure 3.2B). R47B12-GAL4 (hook, Figure 3.2E) also labeled many cell bodies within the VNC and was thus not used for optogenetic experiments.
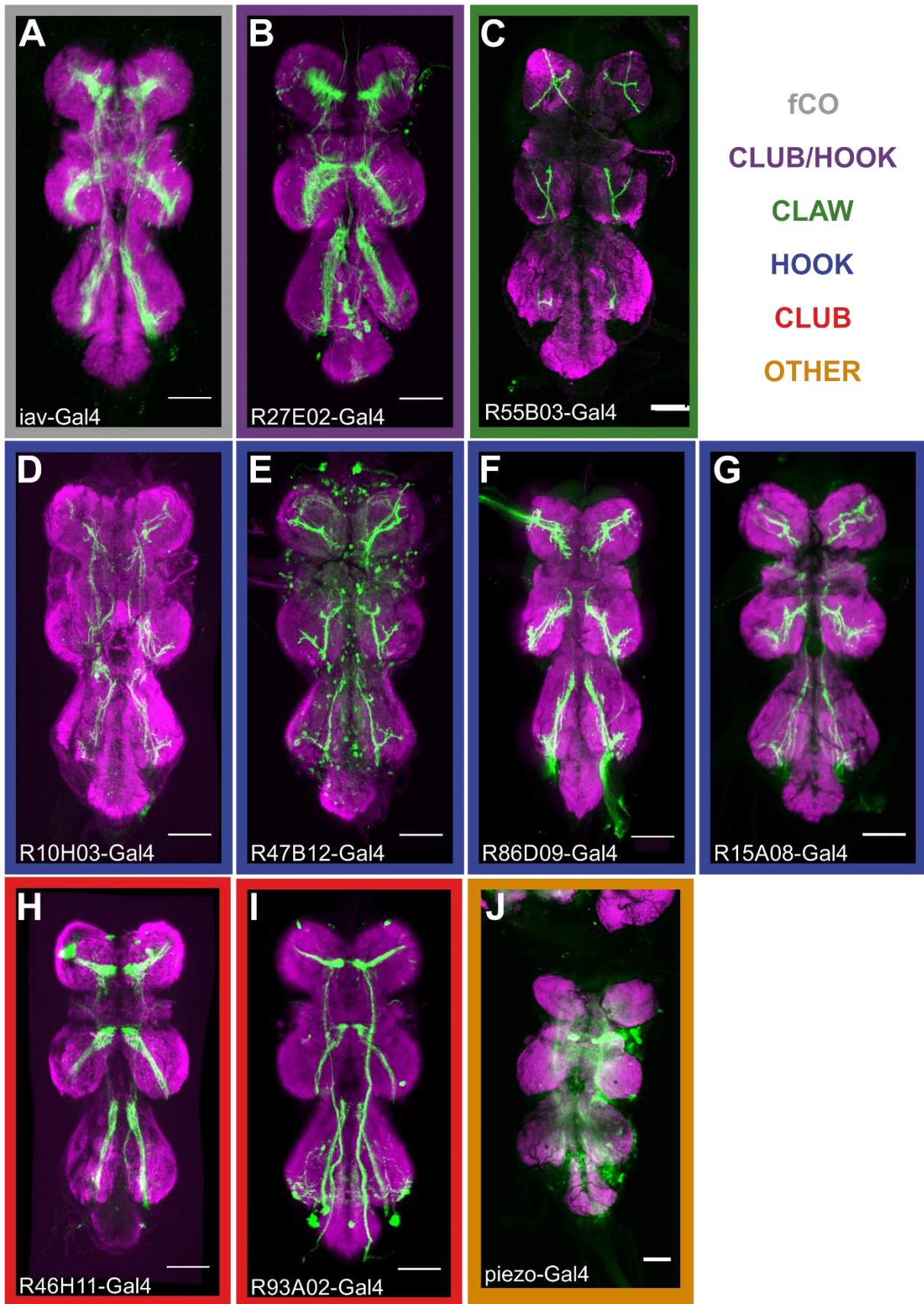
Figure 3.2. Central expression patterns of fCO GAL4 driver lines. Confocal laser scanning microscope images (maximum intensity z-projections) of ventral nerve cords (VNCs) dissected and prepared as described in section 2.3. *iav*-GAL4 (A) shows central

expression pattern of a majority of fCO and tCO neurons; fCO subset driver lines showed a few patterns of central expression, such as a combination of the so-called *club* and *hook* patterns (B), *claw* pattern (C), the *hook* pattern (D-G), the *club* pattern (H-I), and one undefined pattern (J). green – mCD8::GFP, magenta – anti-nc82 immunofluorescent staining; scale bars = 100 µm

3.2.3 Leg restriction

In the next step, I wanted to restrict expression in the VNC to projections from fCO primary sensory neurons. To avoid the labeling of any descending projections from the Johnston's organ or from the wings and halteres, I restricted expression of our UAS reporter to the legs using a cis-regulatory element from the *dachshund* (*dac*) gene to drive the expression of an Flp recombinase (Giorgianni and Mann, 2011; Mendes et al., 2013). *dac,* a gene expressed only in the retina and leg imaginal discs, is an excellent candidate to use to inhibit transcription of UAS-driven reporters outside of the legs (Mardon et al., 1994; Rauskolb, 2001). *dac* is expressed only in the femur and proximal tibia, so restricting expression to the intersection between this area and that of *iav* leaves only the fCO. Using a UAS-FRT-stop-FRT-mCD8::GFP line, the stop cassette is only excised in cells where *dac* is expressed. This results in GFP expression in cells expressing *iav* and *dac* concurrently, excising the expression from neurons outside of the legs and the distal tibia and tarsus.

The removal of expression from the Johnston's organ and the tCO revealed ascending connections directly from the fCO to the brain (Figure 3.3A). It has been said that roughly 3% of fCO neurons project to the gnathal ganglion, with one report of direct projections to the an area near the antennal mechanosensory motor center (Liu et al., 2007; Tsubouchi et al., 2017). A detailed view of the prothoracic ganglia demonstrates the different types of projections making up the *iav*-GAL4 VNC pattern (Figure 3.3B).

Further, expression in the tCO was also removed, as *dac* is only expressed in the femur and proximal tibia (Figure 3.3C). As a further control, imaging of the antenna in these flies revealed no labeling in the JO (Figure 3.3D). Beyond experiments in morphology, this restriction technique could be used to localize the expression of many reporter genes to the fCO only. This would be quite useful, for example, in optogenetics or RNA interference experiments.
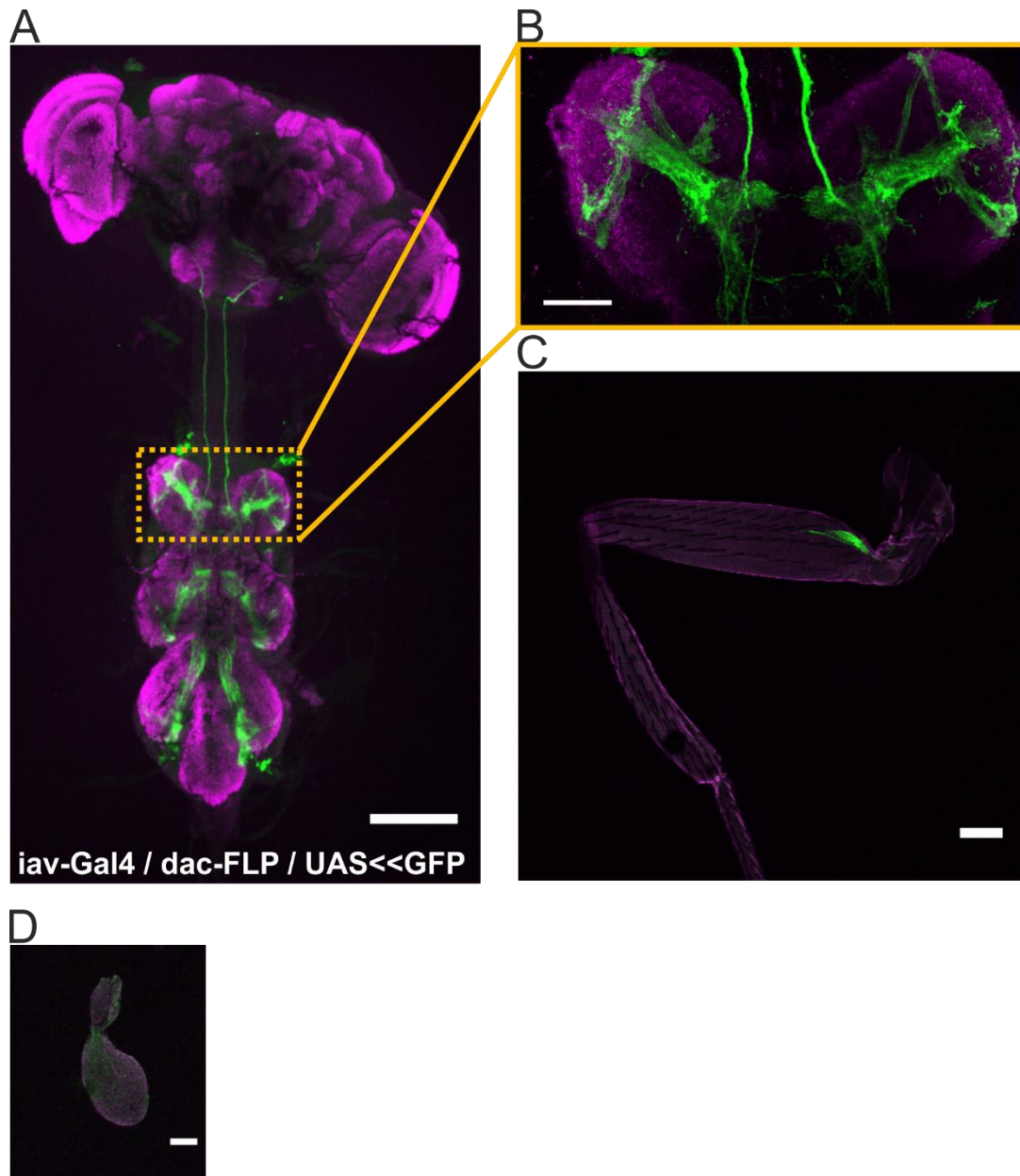
Figure 3.3. Leg-restricted expression pattern of *iav*-GAL4 in leg and VNC. UAS expression was restricted to the legs using the Flp-FRT system under control of the *dachshund* promotor. *Iav*-GAL4 was combined with *dac*-Flp and UAS-FRT-stop-FRT-mCD8::GFP. (A) image of brain and VNC showing mCD8::GFP expression in the VNC and direct projections of fCO primary sensory neurons to the gnathal ganglion; (B) 63x magnification of prothoracic ganglia showing detailed VNC expression pattern of fCO neurons; (C) leg showing expression of mCD8::GFP in the fCO only; (D) control of non-leg GFP expression shows no labeling in Johnston's organ of the antenna, usually labeled by *iav*-GAL4. Scale bars: A, 100 µm; B, 50 µm; C, 100 µm; D, 20 µm

3.2.4 Glomerulus

Confirming the existence of the glomerulus identified by Shanbhag et al. (1992) was as simple as crossing two fly lines and imaging their progeny. As can be seen in Figure 3.4, *iav*-GAL4>UAS-syt.eGFP, which expresses synaptotagmin-bound GFP, shows a small ballooning of fCO axons just proximal to the fCO (Figure 3.4). Interestingly, these flies also showed many presynapses in the thicker scoloparia of the fCO, but not in the longer, thin scoloparium. Further, the shape of the glomerulus indicates that it could be receiving input from other leg neurons, which send their axons through the main leg nerve in the midline of the leg (Nottebohm et al., 1994).
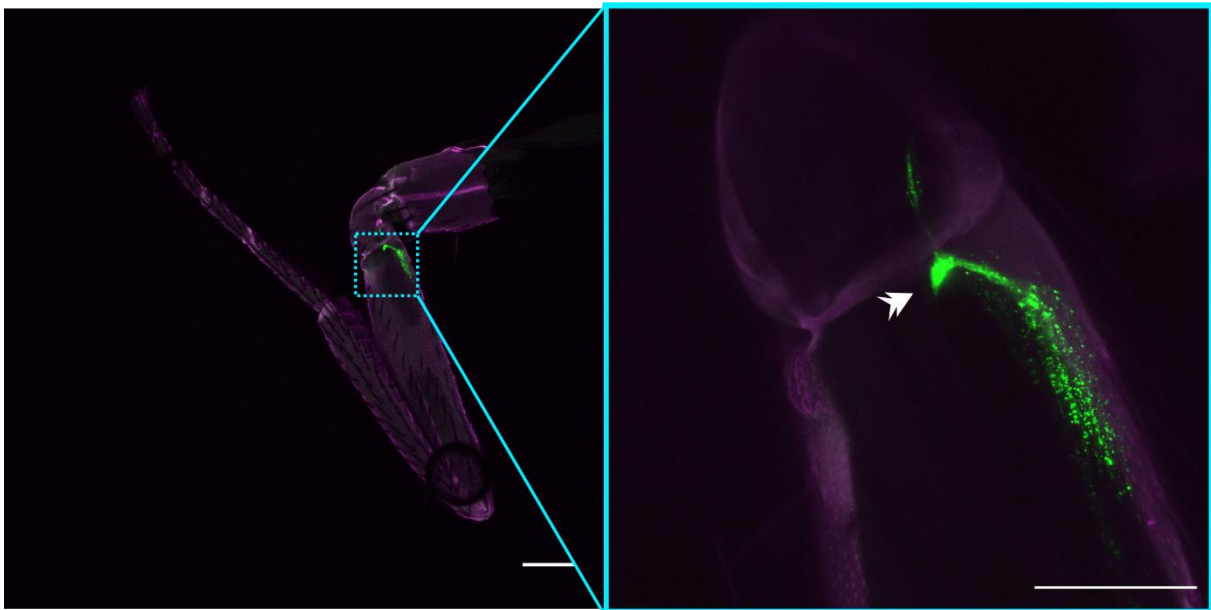

Figure 3.4. Confocal images of presynapses in and around the fCO. syt.eGFP expression under the control of *iav*-GAL4 shown in the whole leg (left); high-magnification view (right) shows synapses within the fCO (GFP puncta) as well as the glomerulus, a strongly-labeled cluster of synapses (arrowhead).

3.2.5 fCO connectivity

The labeling by *iav*-GAL4; UAS-syt.eGFP provokes a few further questions regarding the connectivity of fCO neurons. First, what are the post-synaptic partners of these presynapses located in the fCO? To test this in a rather broad manner, I used the trans-Tango method introduced by Talay et al. (2017). Taking advantage of another binary expression system, trans-Tango induces the expression of a reporter gene under the control of GAL4, in addition to a second reporter under control of the QF/QUAS system, the activity of which is induced by the activation of a pan-neuronally driven exogenous receptor by its ligand, driven by GAL4. This allows for labeling or manipulation of the secondary synaptic partners of neurons labeled by a GAL4 line. In this case, I used this method to check if the presynapses seen in fCO neurons potentially connect to other fCO neurons. This clearly demonstrated that, indeed, some fCO neurons are the secondary synaptic partners of other fCO neurons (Figure 3.5A,C). At least some of this intra-fCO connectivity involves the *club* neurons labeled by R46H11-GAL4 (Figure 3.5C-D).
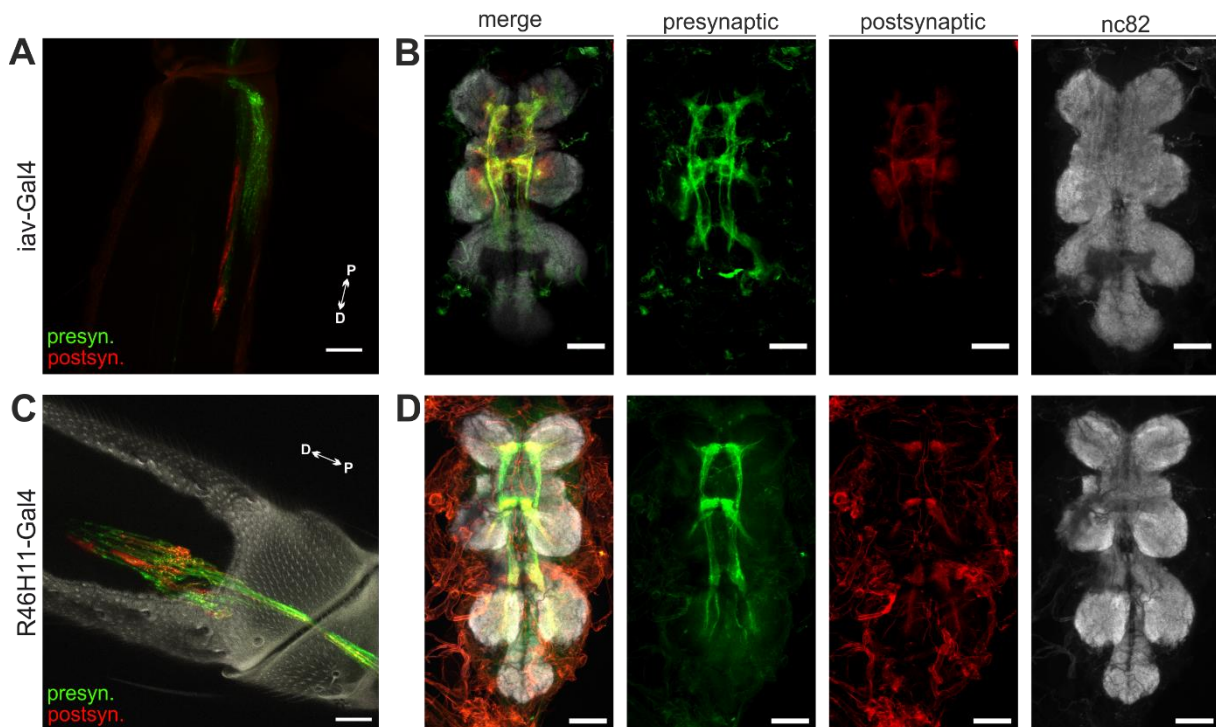
Figure 3.5. Primary and post-synaptic neurons of the fCO labeled using trans-Tango. Trans-Tango, a tool to label neurons downstream of neurons of interest, expressed under control of *iav*-GAL4 (A,B) and the *club* subset driver R46H11-GAL4 (C,D); fCO primary neurons (green) synapse directly onto other fCO neurons (red) as seen in A and C. VNC projection patterns show secondary neurons not labeled by *iav*-GAL4 (B, postsynaptic); neurons labeled by R46H11-GAL4 seem to synapse only onto other fCO primary neurons (D). anti-nc82 labeling shown for orientation. Scale bars: A and C, 50 μm; B and D, 100 μm.

VNC images of these flies show that there are second-order neurons within the expression pattern of the primary neurons, but there are also other second-order neurons outside of this projection pattern (Figure 3.5B,D). Interestingly, it does not seem that there are direct connections between fCO neurons and motor neurons, whose large cell bodies are characteristically located to the anterolateral side of each hemiganglion (Baek and Mann, 2009).

Direct connections between fCO neurons have not been demonstrated in other insects, but the circuitry suggests indirect functional connectivity. fCO neurons in *C. morosus*, for example, have direct connections to spiking and non-spiking INs (Sauer et al.,

1995). These INs, in turn, have connections to the fCO (Sauer et al., 1997; Stein and Schmitz, 1999). In this manner, fCO neurons could effectively transform signals of other fCO neurons, though this has not been exclusively demonstrated.

Little is currently known about the transformations applied to leg proprioceptive signals in *D. melanogaster*. Studies in other species of insects have shown direct connections between proprioceptors and central MNs and INs (Burrows, 1996). At these central stages, signals from proprioceptors are transformed and integrated, resulting in modification of the timing and magnitude of generated motor output (Bässler, 1983; Buschges, 2005). As no evidence exists in other insects for peripheral preprocessing of fCO signals, the idea that proprioceptive signals enter the CNS already transformed or modulated is rather provocative and should be investigated thoroughly.

3.3   Summary

In summary, the preceding experiments recapitulate identified properties of *D. melanogaster* fCO neurons, while shedding new light on their complexity. Since ist first mention, the fCO's glomerulus has been often cited as one potential aspect to consider when working with this organ, but little attention has been paid to its presence and significance. This evidence of direct, monosynaptic connections between fCO neurons is the first to my knowledge to confirm with state-of-the-art methods the existence of the glomerulus. Moreover, the presynapses of fCO neurons within the fCO itself suggests yet another layer of added processing to proprioceptive signals. It is of utmost importance to consider these connections when investigating the encoding properties of fCO neurons, as well as their effects on downstream components of locomotor networks. Now that much more is known about the anatomy and

connectivity of this organ, one could begin to investigate presynaptic processing of fCO proptioceptive information.

4 Chapter 2: Neuropeptides in Leg Sensory Organs

fCO neurons show complex patterns of connectivity, with some synapsing in the periphery onto neighboring fCO neurons. The function of this intra-fCO connectivity is unknown, but in its initial description, Shanbhag et al. (1992) demonstrated that, in addition to clear vesicles of multiple types, the glomerulus also contains dense-core vesicles, suggesting the presence of neuropeptides. Neuropeptides, before being released at synapses, are transported into neurosecretory vesicles, and these vesicles are densely stained in electron micrographs due to the accumulated peptides (Russo, 2017). Neuropeptides are not always released synaptically, and are sometimes released direclty into the circulating fluids of the body (Predel et al., 2004). In neurons, peptides are often co-released with neurotransmitters, sometimes even from the same vesicles (Jonas et al., 1998; Vilim et al., 2000), and can even modulate their own release dynamics (Burnstock, 2004; Merighi, 2002). Mostly dependent on the receptors present in a tissue or network, neuropeptides can have a variety of effects, ranging from direct activation of ion channels or metabotropic receptors to more subtle changes, regulatory actions, and complex feedback loops (Burnstock, 2004). In this manner, the presence and release of neuropeptides can indicate the presence of modulated signal processing and neuronal activity, adding another layer of complexity to circuit dynamics. Moreover, peptides have been shown to be involved in the generation and modulation of motor activity. Coupling this with the proposed role of proprioceptors in motor control, it makes intuitive sense that proprioceptive organs could employ neuropeptidergic signaling.

Research on neuropeptides and their roles in insects has taken off in the last few decades, with many new peptides and peptide precursor genes being identified. In

invertebrates, many neuropeptide precursor genes have been identified, with variable expression patterns in different species (Nässel and Zandawala, 2019). *D. melanogaster* has about 40 neuropeptide-encoding genes. Of these, many have been shown to have multiple functions ranging from regulation of muscle activity, ecdysis, and feeding behavior, to locomotor activity, growth, and circadian rhythms (Nässel and Zandawala, 2019). Multiple peptides have been shown to affect muscle contractions in some way, namely Allostatin-C (Ast-C), Capability (Capa), Diuretic Hormone 31 (Dh31), Leucokinin (Lk), Myoinhibitory Peptide (MIP, a.k.a. Allostatin-B), Myosupressin (Ms), Proctolin (Proc), and Tachykinin (Tk; Altstein and Nässel, 2010; Isaac et al., 2004; Nässel and Winther, 2010; Nichols, 2003; Schooley et al., 2012; Verlinden et al., 2015; Walker et al., 2009). Short Neuropeptide F (sNPF) has even been shown to directly regulate locomotor activity, along with growth, feeding, circadian rhythms, and immune responses (Nässel and Wegener, 2011).

4.1 Sensory structures and neuropeptides

Adding to the myriad functions known for neuropeptides, they have also been found throughout the animal kingdom in sensory organs, where they seem to play diverse roles in modulation sensory signal processing. In humans, for example, neuropeptides have been shown to modulate the processing of nociceptive signals in the trigenimal nerve. These actions take place in the periphery, and it has been suggested that the responsible neuropeptides also modulate the function of the sensory organs themselves (Carr and Frings, 2019). Moreover, Proctolin has been indentified in neurosecretory organs of a lobster, where it is co-released with Acetylcholine in sensory neurons innervating a mechanoreceptor (Siwicki et al., 1987; Siwicki et al., 1985). sNPF has also been shown to be a modulator of sensory signals in the periphery of *D. melanogaster*. In the gustatory system, it can control sensitivity for specific tastes, and

this mechanism is recruited when animals are hungry, indicating that the drive to consume specific types of food can be modulated at the level of the periphery (Inagaki et al., 2014). Further, sNPF can cause presynaptic facilitation leading to starvation-induced foraging behavior, and can also modulate olfactory memory in central Kenyon cells (Knapek et al., 2013; Root et al., 2011).

Considering the findings of dense-core vesicles in the fCO and the myriad roles of neuropeptides in *D. melanogaster*, especially regarding peripheral signal processing, I decided to investigate the presence of neuropeptide-producing neurons in leg sensory organs. In a collaboration with Dr. Sander Liessem inspired by his recent work on insect neuropeptides (Liessem et al., 2018), I screened multiple neuropeptide GAL4 driver lines for expression in the legs. The results revealed novel evidence for the presence and absence of certain peptides in the periphery of *D. melanogaster*.

4.2   Methods

GAL4 driver lines for peptides of interest were obtained from BDSC (see Table 1). These driver lines were crossed with UAS-mCD8::GFP in order to label the membranes of neurons where peptide-related enhancers are active. For the sake of simplicity, I used mated female flies aged 3-8 days post-eclosion, though this could have limited the scope of the results. Legs were dissected, prepared, and imaged according to sections 2.4 and 2.5 of this dissertation.

4.3 Results

Of the 14 driver lines screened, two showed expression in leg sensory structures: Proc-GAL4 and MIP-GAL4 (Table 2). Surprisingly, no expression in legs was seen for the other neuropeptide driver lines tested.

Table 2. Genes screened for expression in leg sensory organs

Gene	Annotation ID	Abbreviation	Leg Expression
Allatostatin C	CG14919	AstC	-
Bursicon	CG13419	Burs	-
Capability	CG15520	Capa	-
FMRFamide	CG2346	FMRFa	-
Diuretic hormone 31	CG13094	Dh31	-
Diuretic hormone 44	CG8348	Dh44	-
Drosulfakinin	CG18090	Dsk	-
Ecdysis triggering hormone	CG18105	ETH	-
Leucokinin	CG13480	Lk	-
Myoinhibiting Peptide Precursor	CG6456	Mip	CS
Myosupressin	CG6440	Ms	-
Proctolin	CG7105	Proc	fCO
Short Neuropeptide F Precursor	CG13968	sNPF	-
Tachykinin	CG14734	Tk	-

CS, campaniform sensilla; fCO, femoral chordotonal organ

Proc-GAL4 showed GFP expression in some fCO neurons (Figure 4.1A-B). Proc has been shown to affect muscle contractions, and stimulation of motor axons mobilizes dense-core vesicles and leads to release of neuropeptides (Ormerod et al., 2018; Ormerod et al., 2016). With this in mind, I expressed synaptotagmin-bound GFP in

Proctolinergic neurons to visualize potential localizations of Proc-containing presynaptic vesicles in the legs (Figure 4.1C-D). Proc-GAL4 neurons seem to have synaptic vesicles throughout all segments of the leg (Figure 4.1D) as well as within the fCO itself (Figure 4.1C). These vesicles look to be placed along the muscle fibers, suggesting proctolinergic modulation of muscle activity in *D. melanogaster* legs. Currently, it is unclear whether Proctolin release from the fCO directly affects muscles.
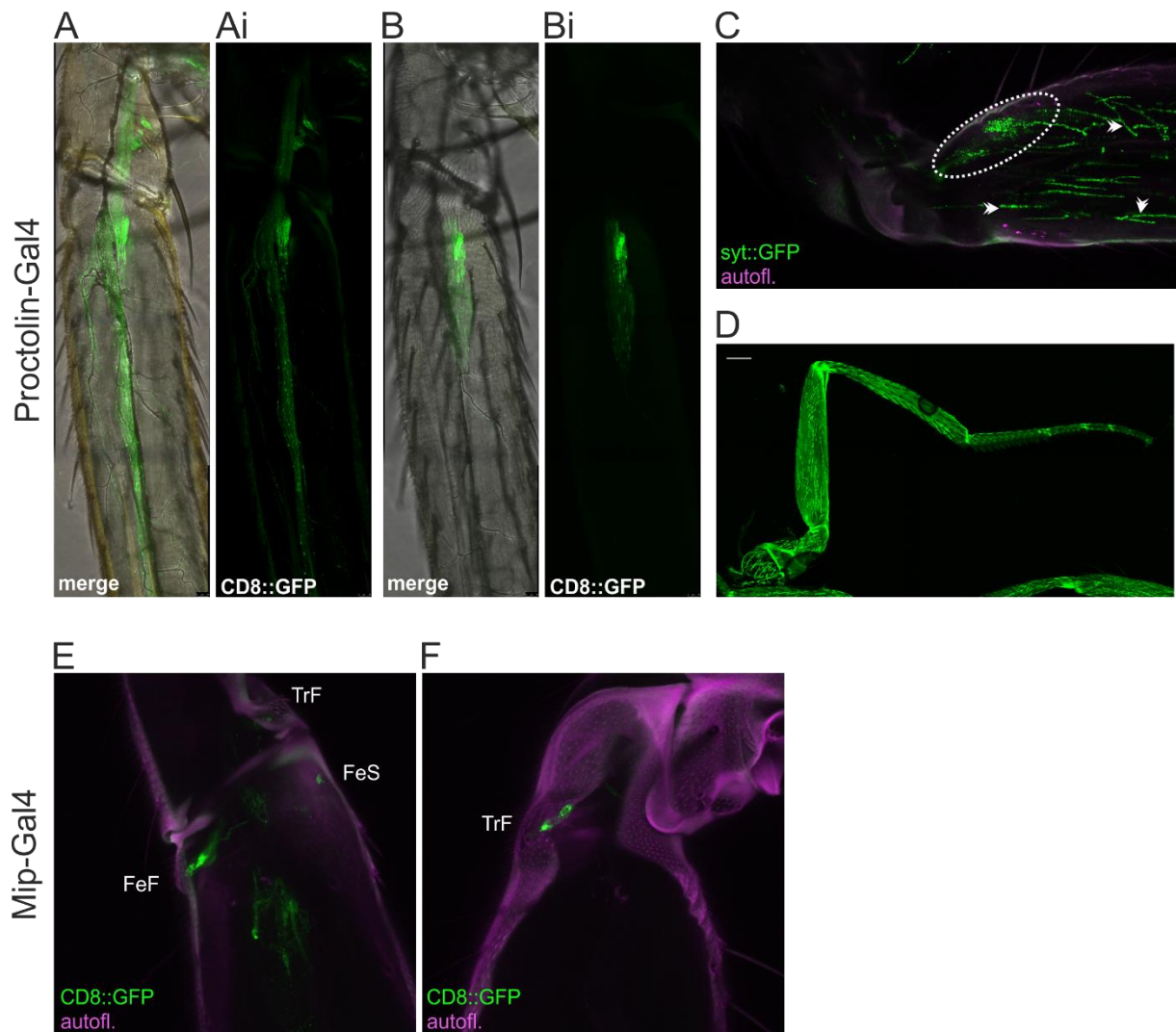
Figure 4.1. Expression of GFP under control of Proctolin-GAL4 and MIP-GAL4. Expression of mCD8::GFP under the Proctolin-GaL4 driver is located in the fCO (A-B). syt.eGFP expression in this line (C,D) shows labeling of muscles (arrowheads in C) as well as the fCO (circle in C). Muscles throughout leg demonstrate this punctate labeling (D). MIP-GAL4 is expressed in campaniform sensilla (CS) of the femur and trochanter. TrF, trochanteral field; FeF, femoral field; FeS, femoral single

MIP-GAL4 exhibited GFP expression in some CS of the trochanter and femur (Figure 4.1E-F). CS of the trochanteral field (TrF), femoral field (FeF), and the single femoral CS (FeS) showed labeling, indicating that these CS produce MIP (naming based on Dinges et al., in press). MIP expression patterns vary slightly between mated and

unmated female flies, along with willingness to mate again. However, no difference in leg expression of MIP-GAL4 was seen between virgin and mated female flies (not shown).

4.4   Discussion

MIP-GAL4 and Proc-GAL4 showed expression within some leg CS and the fCO, respectively, indicating that these peptides are produced within these cells. Proctolin is neurohormone and neuromodulator that is co-released often with glutamate (Orchard et al., 1989). It has been shown to increase levels of cAMP, IP3, and calcium, indicating that it likely acts as an excitatory molecule. Further, Proc is present in motor neuron axons and terminals innervating muscles in larvae (Anderson et al., 1988). Regarding its function, it enhances contraction in ovipositor muscles of the locust, and is suggested to be required for normal functioning of the ovipositor system (Belanger and Orchard, 1993). Moreover, it has been shown to induce sustained contractions in *D. melanogaster* larval body wall muscles (Ormerod et al., 2016). Overexpression of Proc in *D. melanogaster* pupae also leads to increases in heart rate, and it is generally thought to be neuromodulator in MNs and INs (Taylor et al., 2004).

The expression pattern of Proc-GAL4 seen here could mean that proctolinergic fCO neurons induce sustained muscle contractions or prime muscle fibers for contraction. To test this, one could express a fluorescent-protein-tagged variant of Proc in fCO neurons and image it's release (Watkins et al., 2002). The effects, however, of neurohormones and neuromodulators can be rather slow; the time course of proctolin's potential effects on leg muscles needs to be investigated along with its role. Considering the presence of dense-core vesicles in the glomerulus, it is likely that Proc

is released locally; however, the expression pattern of the Proc-receptor within the glomerulus, fCO, and leg in general is still unknown.

In addition to expression of Proc-GAL4 in the fCO, I found that MIP-GAL4 expresses in leg CS. MIP, also Allostatin-B, is a neuropeptide that seems to play very diverse roles in the nervous system and metabolism of insects. It has been shown to be involved in the regulation of satiety and ecdysis (Kim et al., 2006; Min et al., 2016), in addition to its myoinhibitory actions. As a myoinhibitor, MIP has been shown in non-drosopholids to mostly act on smooth muscle (Blackburn et al., 1995; Lange et al., 2012). Recent unpublished evidence from the stick insect *C. morosus*, however, indicates that it can also reduce contractions in slow muscle fibers of the leg, allowing for potentially faster cycling between antagonistic muscle groups (Sander Liessem, personal communication).

In *D. melanogaster*, the *mip* gene encodes five MIPs expressed in the CNS and intestine (Vanden Broeck, 2001), and one MIP receptor has been identified, but it's distribution is not known (Johnson et al., 2003). As for its function as a myoinhibitor in *Drosophila*, one can only speculate that it has similar effects as in other animals. Its presence in CS, and only in a few CS, is certainly interesting, but one would need to investigate the sites of its release and action before hypothesizing about its function. If it were to act on local muscles, as in some other insects, it could potentially act as a gating mechanism of fast contractions. As CS signal the presence and magnitude of body load and act as proprioceptors in walking, they could be modulating the excitability of muscles or even other CS to optimize signal processing. However, at present, the exact roles of individual CS in walking have yet to be determined. Further experiments could be done to investigate the localization and function of the MIP receptor in *D. melanogaster*, as well testing the effects of MIP on CS and muscle activity.

In summary, the presence of two different neuropeptides in two classes of sensory organs in the leg of *D. melanogaster* further emphasizes the complexity of signal processing in these systems. Proc, potentially being co-expressed with excitatory neurotransmitters in the fCO, likely plays a direct role in modulating muscle excitability. MIP, seen in a handful of CS of the proximal leg, could be playing a similar role in priming the system for locomotion, but more work is needed to understand these circuits.

5 Chapter 3: fCO activation

5.1 Introduction

After completing the anatomical characterization experiments described above, I tested basic functional effects of activating groups of fCO neurons. Stimulation of the entire fCO is known to elicit reflex responses, but the sufficiency of fCO neuronal subsets for this has never been demonstrated (Akitake et al., 2015; Bassler, 1993; Bässler, 1988; Clarac et al., 2000; Jin et al., 1998; Reddy et al., 1997). The ideal method for these investigations was using light-activated channelrhodopsins, as they are a completely non-invasive tool for transient (on the order of milliseconds) activation of neurons (Riemensperger et al., 2016; Simpson and Looger, 2018). Neuronal activation studies answer the question of sufficiency—they can tell us if particular cells or circuits are sufficient to elicit or modify behavior.

Optogenetic activation has been proven useful in a variety of screening studies, where researchers attempt to find driver lines expressing in neurons that are sufficient for a behavior. In a famous large-scale screen that resulted in the discovery of neurons whose activity induces backwards walking, flies at rest expressing Chrimson, a potent cation-permeable channelrhodopsin, were illuminated with red light (Klapoetke, 2014). This allowed the authors to easily identify any lines with interesting effects on locomotor behavior, leading to the discovery of the moonwalker descending neurons (Bidaye et al., 2014). In a more exploratory neuronal screen, Chrimson was activated in freely behaving flies, and the authors were able to use the resulting effects to generate a database of descending neuron driver lines that are sufficient to produce a variety of behaviors (Cande et al., 2018). This artificial activation, however, is not

always ideal. Providing a non-physiological activation to a neuronal circuit runs the risk of eliciting activity that is not normally present, or only occurs under certain circumstances. Therefore, results of activation screens should be thoroughly controlled and taken with a grain of salt.

Based on data from other insect species, activation of the fCO could lead to activation in multiple INs in the ventral nerve cord (see Burrows, 1996). Artificial stimulation of the fCO in quiescent *C. morosus*, via mechanically stretching the fCO receptor apodeme, leads to the generation of a resistance reflex (Bassler, 1993; Bässler, 1988; Clarac et al., 2000). However, in actively locomoting animals, the same stimulation produces a the opposite reflex, known as an assistance reflex (Bässler, 1988). These reflexes are thought to be the product of direct fCO-MN connections (Driesang and Buschges, 1996). This reflex upon fCO stretching or relaxation has been demonstrated in *D. melanogaster*, but the neuronal circuitry underlying this has not been investigated (Akitake et al., 2015; Jin et al., 1998; Reddy et al., 1997).

As no direct connections between fCO neurons and MNs have been found in *D. melanogaster*, it is unclear if optogenetic activation of the fCO is sufficient to produce reflex movements or activate leg muscles. There is a crucial difference between optogenetic activation and mechanical stretching of the receptor apodeme. In the former, cationic channels are rapidly opened in the neurons of interest, leading to quick depolarization and the release of action potentials. In the latter, however, mechanosensitive ion channels are naturally opened, leading to activation or potential changes only in the neurons that are tuned to that movement. In this sense, optogenetic activation can only inform us of the potential underlying circuitry, and not the mechanisms behind fCO-mediated reflexes at the level of the proprioceptor.

In the experiments detailed below, I used optogenetic stimulation with a cationic channelrhodopsin (Chrimson) to activate subsets of fCO neurons. Through video recordings of behavior as well as electrophysiological recordings of muscles, I demonstrate that activation of subsets of fCO neurons results in movements of the legs and depolarizations of femoral muscles.

5.2   Methods

Preliminary tests showed that, using a 25-μm optical fiber, the femur of a single leg of a fly could be stimulated without stimulating other areas. For these tests, I tethered the thorax of an intact fly to a wire using UV-activated glue (Sinfony; 3M ESPE, MN, USA), removed the head to prevent grooming behavior, and illuminated the femur of the right mesothoracic leg. R86D09-GAL4 expressing Chrimson showed a very rapid flexion of the tibia upon illumination. *Piezo-GAL4; UAS-CsChrimson*, on the other hand, showed a rapid extension of the tibia. These reflex reactions were seen at a stimulation intensity of about 250 μW/mm^2, and reactions could be seen with pulse durations down to 100 μs. These preliminary tests were very exciting and demonstrated that subsets of fCO neurons alone can produce rapid reflex reactions.

To standardize these experiments and quantify the resulting reflexes, flies were mounted on a small aluminum block with a sort of wall on one side of the thorax (Figure5.1A-A'). With the dorsal side of the thorax glued to the aluminum block, the femur could rest on the wall so that it was horizontal to the block, and the entire fly could be filmed from the anterior side. With the femur-tibia joint fixed in place, the tibia could move freely, and the femur-tibia joint angle could be tracked. In addition to these experiments with a fixed, unloaded fly, I repeated similar experiments with headless flies standing on a platform. To test any influence of other sensory signals,

such as load, on the elicited reflexes, these flies were freely standing and thus under natural load conditions. Video of these tests was recorded with a high-speed camera (100 fps; Marlin;) with infrared (IR) illumination (5-ms exposure time).
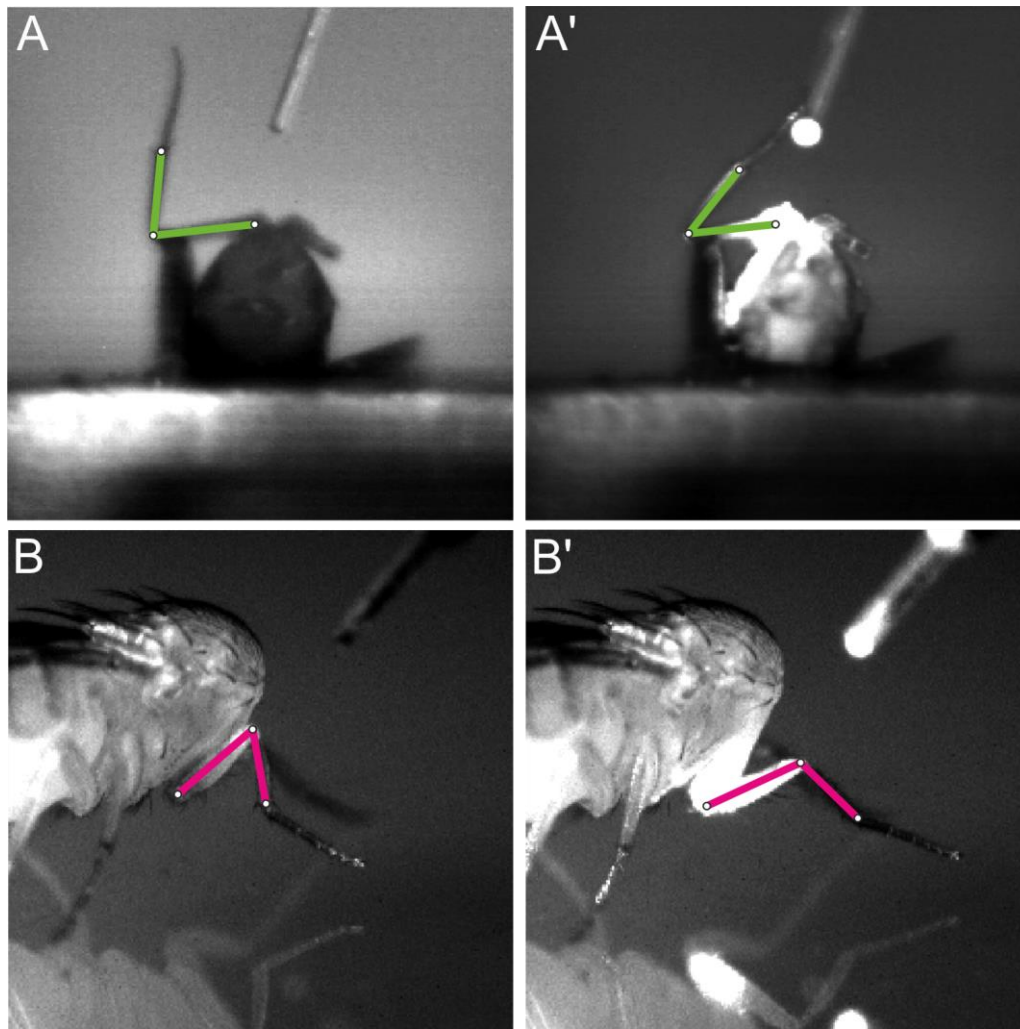
R86D09-Gal4; UAS-CsChrimson

Figure 5.1. Stills from videos capturing motor response of optogenetic activation of fCO neuron subset. UAS-CsChrimson, a red-shifted cationic channelrhodopsin, was expressed in R86D09-GAL4, and the cells were stimulated with red laser light. (A and A') Optical stimulation of the fCO in a fly without body load acting on the legs caused flexion of the tibia (A, before stimulation; A' immediately after). In a fly with normal body load (B), Chrimson activation of the same neurons caused extension of the tibia (B').

5.2.1 *Electromygrams*

To measure muscle activation, I recorded electromyograms of the tibial flexor and tibial extensor muscles. For this, borosilicate capillary electrodes with filaments (#BF100-50-10; Science Products GmbH, Hofheim, Germany) were filled with a solution of 3 M potassium acetate and 0.1 M KCl and attached to an electrode. Signals from the recording and reference electrodes were sent through a pre-amplifier (gain 100; MA101; Electronics Workshop, University of Cologne), and were then amplified and filtered through a differential amplifier (gain 100, 80-KHz high-pass filter, 7-KHz low-pass filter; MA102; Electronics Workshop, University of Cologne). After this, signals were digitized in an A/D converter (micro1401; Cambridge Electronic Design, Cambridge, UK) and recorded in Spike2 software (CED). EMGs were processed using custom-written code in Matlab (The Mathworks, Natick, MA, USA). Signals were smoothed with a gliding Gaussian kernel (25-ms standard deviation), and trials from each animal and conditon were averaged.

5.3   Results

Preliminary tests demonstrated that activating subsets of fCO primary sensory neurons can produce rapid leg movements in the stimulated leg (Figure 5.1). Some subsets produced extension movements (*piezo*-GAL4 and R27E02-GAL4, Figure 5.2), while some produced flexion movements (R86D09-GAL4, Figure 5.1).

5.3.1 EMG results

Optogenetic stimulation of primary proprioceptive neurons in *D. melanogaster* (Figure 5.2A) caused reflex responses in tibial extensor muscles (Figure 5.2C-F). EMG recording in the tibial flexor muscle during optogenetic activation of fCO neurons showed no muscle activation (Figure 5.2G,Gi). Both R27E02-GAL4 and *piezo*-GAL4 demonstrated very short latency tibial extensor activity upon fCO activation, and stimulus duration did not seem to largely affect response dynamics. However, 500-ms stimulation in *piezo*-GAL4 led to extensor activity that continued after stimulus offset (Figure 5.2E,Ei). This does not seem to be characteristic of activating fCO neurons, however, as 1000-ms stimulation caused a similar pattern in R27E02-GAL4.

Extensor activity generally followed a phasic pattern (Figure 5.2D-F), but also showed tonic activation in R27E02-GAL4 (Figure 5.2C-Ci).
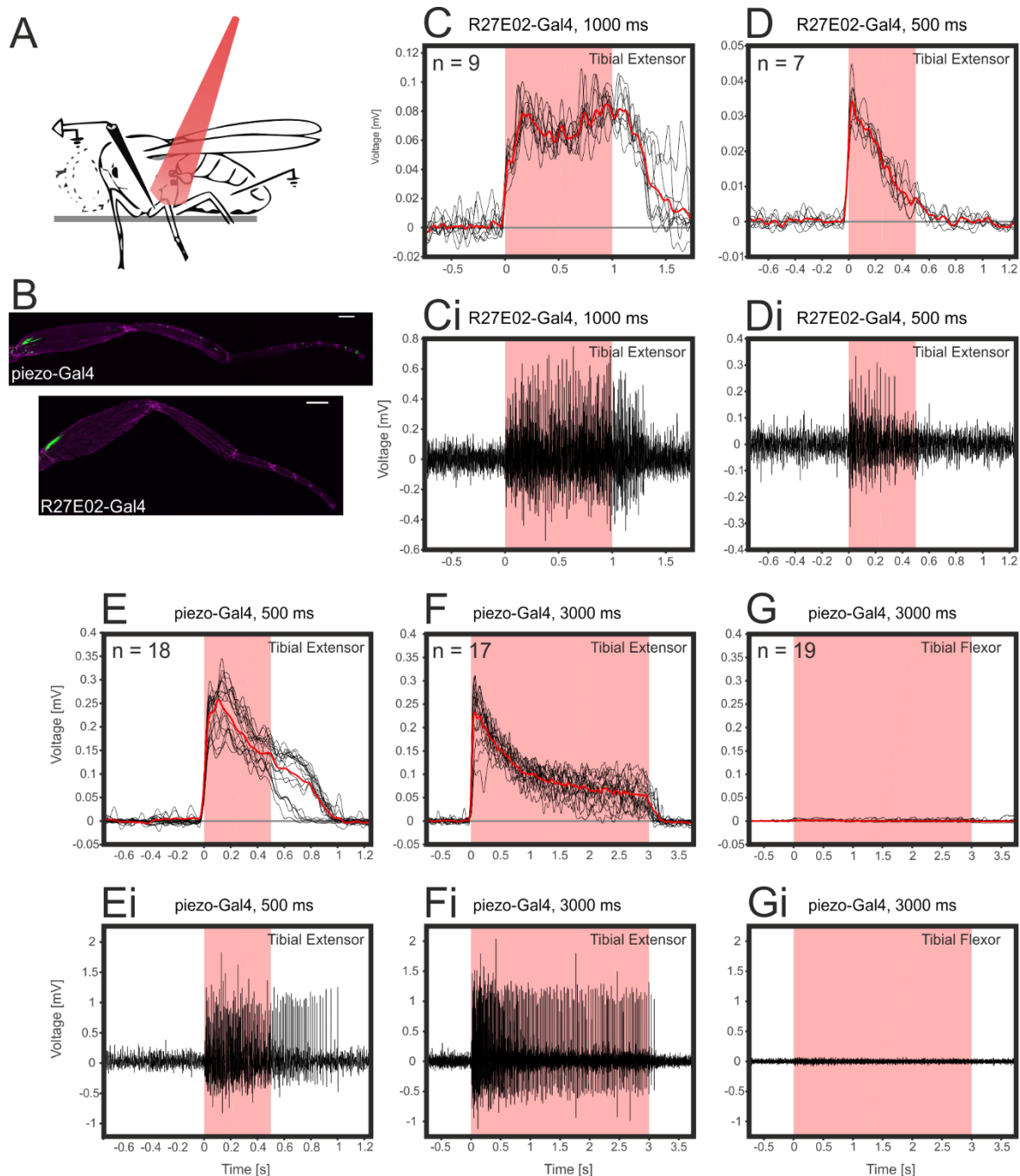
Figure 5.2. Electromyograms of muscles controlling the tibia during optogenetic activation of fCO subsets. Schematic diagram of electromyogram (EMG) setup shown in (A), modified from (Jin et al., 1998). EMGs were recorded in flies expressing Chrimson under control of *piezo*-GAL4 or R27E02-GAL4 (B). The fCO was stimulated with a red laser (658 nm) for 500, 1000, or 3000 ms (C-G) red shaded areas). Optogenetic activation of fCO subsets produced sustained activity in tibial extensor muscles (C-F), while no activity was observed in the tibial flexor (G-Gi). Red traces shown are smoothed and averaged over multiple trials (C-G), with representative single-trial recordings for each condition (Ci-Gi)

5.4 Discussion

Optogenetic activation of fCO neurons leads to the activation of tibial extensor muscles and resulting movement of the leg. Mechanical activation of the fCO via movement of the tibia has been shown to elicit reflex activity in the tibial extensor muscle (Akitake et al., 2015; Jin et al., 1998; Reddy et al., 1997). Importantly, mechanical stimulation of fCO neurons by moving the tibia reflects the natural activation conditions of the fCO. Here, I show that artificial activation of some fCO neurons is also sufficient to produce these responses. The short latencies of the tibial extensor responses suggest direct connections to MNs, but this is not supported by the fCO connectivity experiments laid out in section 3.2.

It is clear from tibial flexor recordings that *club/hook* neuron activation (R27E02-GAL4) leads to active movements rather than adjustments of tonic activity. Moreover, the lack of activation of tibial flexor muscles indicates that this artificial activation leads to a natural response, as reflected in the behavioral data. The anatomical connectivity from these fCO neurons to tibial extensor MNs has not been investigated, and this evidence of functional connectivity provides a solid basis for such experiments. Interestingly, the reflex response to activation of *hook* neurons labeled by R86D09-GAL4 was different in a standing fly than in upside-down flies resting on their thorax. This implies that other sensory signals, either from CS measuring load or from tactile sensors in the tarsi, can lead to the modification of motor responses elicited by the fCO.

Fitting with literature from *C. morosus*, fCO neurons in *D. melanogaster* are sufficient to elicit active reflex responses and are thus important in postural control (Bassler, 1993; Bässler, 1988). The work herein, however, does not consider potential effects of fCO activation on other legs. In addition to this local network activity, other leg joints, neighboring legs, and descending neurons from the CNS can affect motor output of the

femur-tibia joint network. In *C. morosus*, front leg stepping, and thus activation of the fCO, affects activity in MNs controlling middle leg movements (Borgmann and Buschges, 2015; Borgmann et al., 2009; Borgmann et al., 2007). These networks underly limb coordination and add yet another level of complexity to the interactions between fCO neurons and the neuromuscular locomotor networks.

Little is known in *Drosophila* about how leg proprioceptors are connected to locomotor INs and MNs in the VNC, both functionally and anatomically. Ideally, one could use electron microscopy volumes of the *D. melanogaster* VNC to trace the anatomical connectivity patterns of fCO neurons, INs, and MNs. Such data has yet to be released for the adult VNC, but similar approaches have been used to map the adult brain and larval nervous system (Schneider-Mizell et al., 2016; Zheng et al., 2018). Once neurons of interest have been identified in this manner, one could begin to record their activity via live imaging (Chen et al., 2018) or in-vivo whole-cell recordings (Azevedo et al., 2020). In this manner, we can begin to map out and investigate locomotor circuitry in *D. melanogaster*. Further, no work has been done in *D. melanogaster* investigating the functional importance or necessity of subsets of fCO neurons, and it is crucial that we perform ablation-style experiments to fully understand the underlying dynamics of these locomotor networks.

6 Chapter 4: The role of the fCO in walking

The contents of this chapter have been compiled into a manuscript that will be submitted for peer review and publication. The manuscript has the working title "Leg proprioceptors differentially affect leg kinematics in walking *Drosophila melanogaster*" and is authored by Alexander S. Chockley, Gesa F. Dinges, Giulia di Cristina, Sara Ratican, Till Bockemühl, and Ansgar Büschges.

6.1 Introduction

The previous chapters lay the groundwork for demonstrating that fCO neurons can be grouped genetically (section 2) and functionally (section 4) and exhibit complex connectivity via the glomerulus (section 2), intra-fCO presynapses, and potential neuromodulation via Proctolin (section 3). Further, I have demonstrated the sufficiency of subsets of fCO neurons for producing leg movements. However, the necessity of these neurons for the functionality of locomotion—specifically leg coordination and kinematics–have yet to be investigated on the subpopulation level in the fCO.

Previous studies have used ablation in reduced preparations and broad genetic manipulations to show that the fCO is important for normal walking behavior (Cheng et al., 2010; Cruse et al., 1984; Mendes et al., 2013). However, transient, sub-population-level manipulations of fCO function have not been possible so far due to methodological limitations. A major advantage of doing these experiments in *Drosophila*, aside from the ability to manipulate networks at sub-population levels, is the ability to perform closed-loop experiments in intact, freely behaving animals. In

larger insects without genetically tractable nervous systems, manipulations of the fCO involve removing the whole organ or artificial activation via physical stretching and relaxing of the receptor apodeme. Inherent in these methods is the requirement to stabilize the animal by fixation to a platform and open parts of the cuticle to gain access to the nervous system. In these animals, inactivation of the fCO can only be accomplished by complete ablation of the organ or cutting the receptor apodeme (Cruse et al., 1984; Takanashi et al., 2016).

Here, I took advantage of the genetic toolkit available for *D. melanogaster* to perform transient, sub-population-level manipulations of the fCO in fully intact, freely behaving flies. I identify and describe in detail four of the fCO subsets driver lines introduced in Chapter 1 of this dissertation. Further, I test their functional role in the control of locomotion using optogenetic inhibition in unrestrained, naturally behaving and walking flies. The results demonstrate that some, but not all, subsets of fCO neurons are necessary for natural forward walking, underlining the importance of the fCO for successful leg movements and coordination. These results are discussed in the context of leg kinematics and processing of proprioceptive signals.

6.2   Methods

6.2.1 Free-walking Assay

To investigate the involvement of neuronal subsets in the fCO in walking behavior, I drove expression of a green light-activated anion channelrhodopsin (GtACR1; Govorunova et al., 2015; Mohammad et al., 2017) under control of some of the previously described GAL4 driver lines in the fCO of freely walking flies (Figure 6.1A). This enabled us to inhibit specific fCO subpopulations during natural locomotion. This

use of transient, light-based neuronal inhibition allowed us to use each fly as its own control to make clearer the effects of fCO inhibition over the natural inter-individual variability in walking.

Flies (0-2 d post-eclosion) expressing GtACR1 (or wild-type>UAS-GtACR1 flies in control experiments; Mohammad et al., 2017) were transferred to vials containing food and 0.14 mM all-trans retinal for three days before experiments. Some flies were starved for 18-24 h prior to testing; for this, they were placed in a 2-ml Eppendorf tube with a small piece of wet tissue. A schematic of the free-walking setup is shown in Figure 6.1A. It consisted of an inverted glass petri dish used as a transparent arena (diameter 100 mm) held by a circular frame with a cutout below the dish. The cutout provided an unobstructed view from under the arena. To prevent flies from escaping, the arena was covered with a watch glass that established a dome-shaped chamber, similar to an inverted FlyBowl (Simon and Dickinson, 2010). To prevent flies from walking upside down on the watch glass, its inside surface was coated with siliconizing reagent (Sigmacote; Sigma-Aldrich, RRID:SCR_008988). A surface mirror was placed below the arena at a 45° angle. Using this mirror and an infrared (IR)-sensitive high-speed camera (model VC-2MC-M340; Vieworks, Anyang, Republic of Korea), we captured a bottom view of a central quadratic area on the surface of the arena of approximately 30 by 30 mm (Figure 6.1A), with a resolution of 1000 by 1000 pixels, a frame rate of 250 Hz, and an exposure time of 200 μs. The arena was illuminated by ring of 60 IR LEDs (wavelength: 870 nm) arranged concentrically around the arena; light from these LEDs was mainly emitted parallel to the surface of the arena. This resulted in a strong contrast between the background and the fly and facilitated video acquisition (see Figure 6.1B). A second ring of 60 green LEDs (wavelength: 525 nm) positioned above the IR LED ring provided the light necessary to transiently activate GtACR1, thereby hyperpolarizing targeted neurons. Contrast and homogeneity were

further increased by equipping the camera's lens with an IR-pass filter (cut-off frequency: 760 nm) that blocked all ambient visible light and the green light from the stimulation LEDs. IR LEDs were pulsed and synchronized to the frame acquisition of the camera (250 Hz, 200 µs acquisition time). A computer fan was used to cool the LEDs and arena from below.
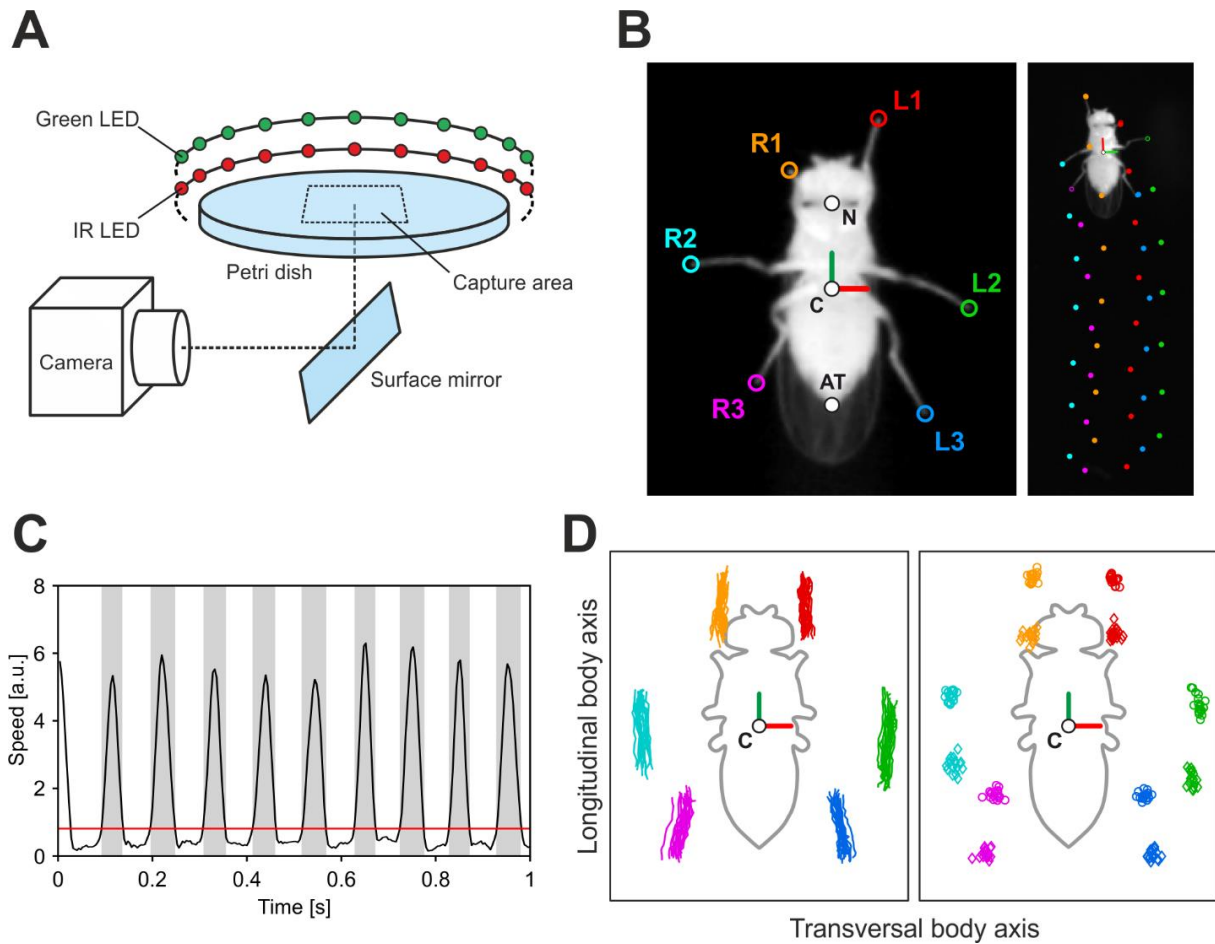
Figure 6.1. Free-walking assay and analysis (A) Setup schematic; Flies walked on top of an inverted petri dish while being video recorded from below via a surface mirror. Only straight walking sequences within a sub-region of the arena (capture area) were saved for analysis. Illumination was provided by an IR-LED ring encircling the arena. A ring of green LEDs above the IR-LED ring provided the illumination necessary for GtACR1 activation; entire rings not shown for clarity. A watch glass (not shown) placed onto the petri dish prevented flies from escaping. (B) Example ventral views of a fly in the setup showing a whole trial (top) and cropped video frame used for annotation (bottom); Illuminated by IR light, the fly appears bright on a black background. The neck (N) and the posterior tip of the abdomen (AT), as well as the tarsal tips of all six legs (R1-R3, L1-L3) were automatically annotated in each video frame using DLC. (C) Detection of swing (gray areas) and stance movements. Based on the relative speed between tarsus and substrate lift-off and touchdown times and positions were determined automatically. (D) Average stance trajectories of all legs in a fly-centered coordinate system (black dot indicates body center) of six individual control flies. Data in D and E formed the basis for all subsequent kinematic analyses.

Prior to an experiment, a single fly was extracted with a suction tube from its starvation or rearing vial and placed onto the arena, which was then covered with the watch glass. Flies walked spontaneously for a few hours in the arena and frequently crossed the capture area. Video data of this area was continuously recorded into a frame buffer of five seconds. Custom-written software functions evaluated the recorded frames online and determined if a fly was present in the recorded area at a particular time and if it had produced a continuous walking track that had a minimum length of 7 body lengths (BL) with a minimum walking speed of 2 BL·s^{-1}. Once the fly had produced such a track and then either stopped or left the capture area, the contents of the frame buffer were committed to storage as a trial. After this, acquisition started anew. Video acquisition and online evaluation during experiments were implemented in MATLAB (2014b, The Mathworks).

During an experimental session, flies either walked in darkness (Control condition) or under green-light illumination (Inhibited condition). These two conditions were alternated in the following way: after a fly produced a valid Control trial, the green light was switched on for 60 s, during which the recording system was primed to record a trial. Once this happened, the light was switched off again and the system would be primed for the next valid Control trial, and so on. If the fly failed to produce a valid trial during the on-time of 60 s, the recording system would be paused, and the light would be switched off again for 60 s. We used this cooldown to prevent extended periods of neuronal inhibition and temperature increases from the green LEDs. After the cooldown period, the green light was switched on again and the system was primed for the acquisition of a valid trial (again for 60 s). Switches from green light to cooldown were repeated until the fly produced a valid trial during green light illumination, after which the system was primed for the next valid Control trial. Trial acquisition was completely automated and implemented in MATLAB (2014b; The Mathworks). The

green LEDs were controlled using a digital I/O device (USB-6501; National Instruments, Austin, TX).

Video trials of straight walking were captured under IR illumination only (Control condition) or green LED illumination (525 nm, Inhibited condition) to activate GtACR1. Videos (Figure 6.1B) were cropped, and the positions of tarsal tips were determined using DeepLabCut (DLC; Mathis et al., 2018). Touch-down and lift-off events were automatically detected (Figure 6.1C), allowing us to extract parameters related to leg kinematics and coordination (Figure 6.1D).

6.2.2 Quantification and Statistical Analysis

Free-Walking Data annotation

Prior to data analysis, we selected walking sequences that had low intra-trial variability in walking speed, in which flies walked in a straight line, and that contained at least five consecutive steps. To exclude random walking speed-dependent differences between conditions, these trials were further selected so that the two conditions (Control and Inhibited) had similar speeds (within 6 mm/s of each other). These pre-selected sequences served as the basis for further analysis of low-level walking-related parameters. First, the position of the fly throughout a sequence was determined automatically. Each video frame was converted into a binary image (black background, bright fly), in which the fly was detected as the largest bright area following a simple threshold operation. Walking speed was calculated as changes of the center of mass of this area over time. We then used this positional information to crop the fly from the original 1000 x 1000-pixel video. These smaller and fly-centered video sequences were used for annotation of eight different body parts in every video frame: the tarsal tips of all six legs, the neck, and the posterior tip of the abdomen (Figure 6.1B). This step of

the annotation was done automatically in DLC; we trained and evaluated DLC with a data set of 1000 manually annotated video frames (10 flies, 100 frames each), that were similar to the ones we recorded during the experiments described herein, but were not part of the present study. One half (10 flies, 50 frames each) of this set was used for training, and the other half was used to evaluate its performance. Performance of DLC was generally good; however, to ensure high-quality annotations we inspected the results visually and, if necessary, manually corrected mis-annotations.

Free-Walking Kinematic analysis

To determine the times of lift-off and touch-down for each leg in a walking sequence, the DLC-determined positions of the tarsal tips were transformed into a world-centered coordinate system. In this coordinate system, a leg tip is stationary (i.e., has a speed of 0 mm/s) when the leg is touched down (here, defined as the stance movement) and moves markedly respective to the ground when it is lifted off (here, defined as the swing movement). These measures and an empirically determined threshold were used to distinguish swing and stance movements. Transitions between these two were defined as touch-down and lift-off events, and the positions of the tarsal tip at these times were defined as the anterior and posterior extreme positions (AEPs and PEPs) in fly-centered coordinates. A single step of a particular leg was then defined as its movement between two subsequent PEPs; its period was defined as the time difference between two subsequent PEPs. Swing movement and duration were defined as the movement and the time difference, respectively, between a PEP and the subsequent AEP; stance movement and duration were defined as the movement and the time difference, respectively, between an AEP and the subsequent PEP. A stance trajectory was defined as the complete path of a tarsal tip in fly-centered coordinates

between an AEP and the subsequent PEP. Step amplitude was defined as the distance between a PEP and the subsequent AEP. Stance linearity was calculated as the root mean squared error (RMSE) between an actual stance trajectory and a straight line between this stance trajectory's AEP and PEP; the higher this measure is, the stronger the deviation from a straight line. Average AEPs and PEPs were defined as the arithmetic mean of all AEP and PEP position vectors, respectively; the standard deviation of these positions were estimated as a bivariate distribution. Stance trajectories were averaged by first resampling all n trajectories to 100 equidistant 2-dimensional positions (100-by-2 data points) and then calculating the arithmetic mean for each set of 100-by-2-by-n data points. To facilitate comparison between control and inhibited conditions we normalized all individual step periods, swing durations, stance durations, stance amplitudes, and stance linearity values to the arithmetic mean of the control condition.

Free-Walking Phase analysis

Phase relationships (i.e., phase differences) were calculated for all ipsilaterally or contralaterally adjacent leg pairs. This resulted in seven phase relationships: three contralateral leg pairs (front, middle, and hind legs) and four ipsilateral leg pairs (hind and middle legs, middle and front legs). A reference leg was selected for each leg pair. For contralateral phase relationships, this was always the left leg; for ipsilateral phase relationships, this was the posterior leg. For each complete step of the reference leg (PEP to PEP) we calculated its instantaneous phase as a value that linearly increased from 0 to 1 during the step. We then calculated the phase relationship between the two legs as the phase value at the times of PEPs in the non-reference leg; this was equivalent to the phase difference between these legs. A value of 0 indicated synchronous lift off,

while a value of 0.5 indicated an anti-phase relationship. The phase between two legs is written as, for instance, R3>R2, where leg R3 is the reference leg and R2 is the non-reference leg.

Aside from DLC-based functions, all annotations and calculations for the kinematic as well as the phase analysis were carried out with custom-written functions in MATLAB 2018b (The Mathworks, see also section *6.4.1 - Statistical Analyses*).

3D reconstruction of Confocal Data

Confocal z-stacks of fCO subset lines expressing nuclear-localized RFP were reconstructed using Amira (6.0.1; Thermo Fisher Scientific). These samples were prepared according to section 2.4. First, nuclei of the RedStinger-labeled *iav*-GAL4 neurons were traced by hand. These outlines were then checked and corrected using the signal from DAPI-stained nuclei. The outer shell of each fCO was generated by tracing the outermost edge of the anti-ChAT signal in each slice of the z-stack. All labels were smoothed along the three dimensions. Once smoothed, a surface mesh was generated from the data. These were then imported into Blender (2.79; www.blender.org), where they were re-meshed, smoothed, and rendered. PNGs were exported from Blender.

Statistical Analyses

Phase data was calculated, processed, and plotted using custom-written functions in MATLAB 2018b (The Mathworks) and the MATLAB CircStat toolbox (Berens, 2009). Boxplots were calculated and plotted with built-in functions in MATLAB 2018b (The Mathworks).

6.3 Results

6.3.1 The fCO consists of multiple anatomical subpopulations

To tease apart the role of the fCO (Figure 6.2) and subsets of its neurons in leg movements and coordination during walking, we first identified several GAL4 driver lines labeling subpopulations of fCO neurons via a manual search of the FlyLight database (HHMI Janelia Research Campus, http://flweb.janelia.org/cgi-bin/flew.cgi) and the relevant literature, as described in Chapter 1 of this dissertation. For these behavioral experiments, we used four driver lines of interest that were selected using a broad chordotonal organ driver line as a reference (*iav*-GAL4; Figure 6.2B). These driver lines labeled different subsets of fCO neurons, allowing for a differential analysis of the contributions of fCO neuron subtypes to leg movements and coordination. We used driver lines labeling *club* and *hook* neurons (R27E02-GAL4, Figure 6.2C), *claw* neurons (R55B03-GAL4, Figure 6.2D), *club* neurons (R46H11-GAL4, Figure 6.2E), and *hook* neurons (R86D09-GAL4, Figure 6.2F). Three-dimensional surface reconstructions based on driver-line-expressed nuclear-localized RFP (UAS-RedStinger), anti-cholinergic acetyl-transferase antibodies (ChAT), and DAPI staining to label mechanosensitive neurons and all nuclei, respectively, show that these driver lines label varying numbers of cells within the fCO itself (Figure 6.2B-F).
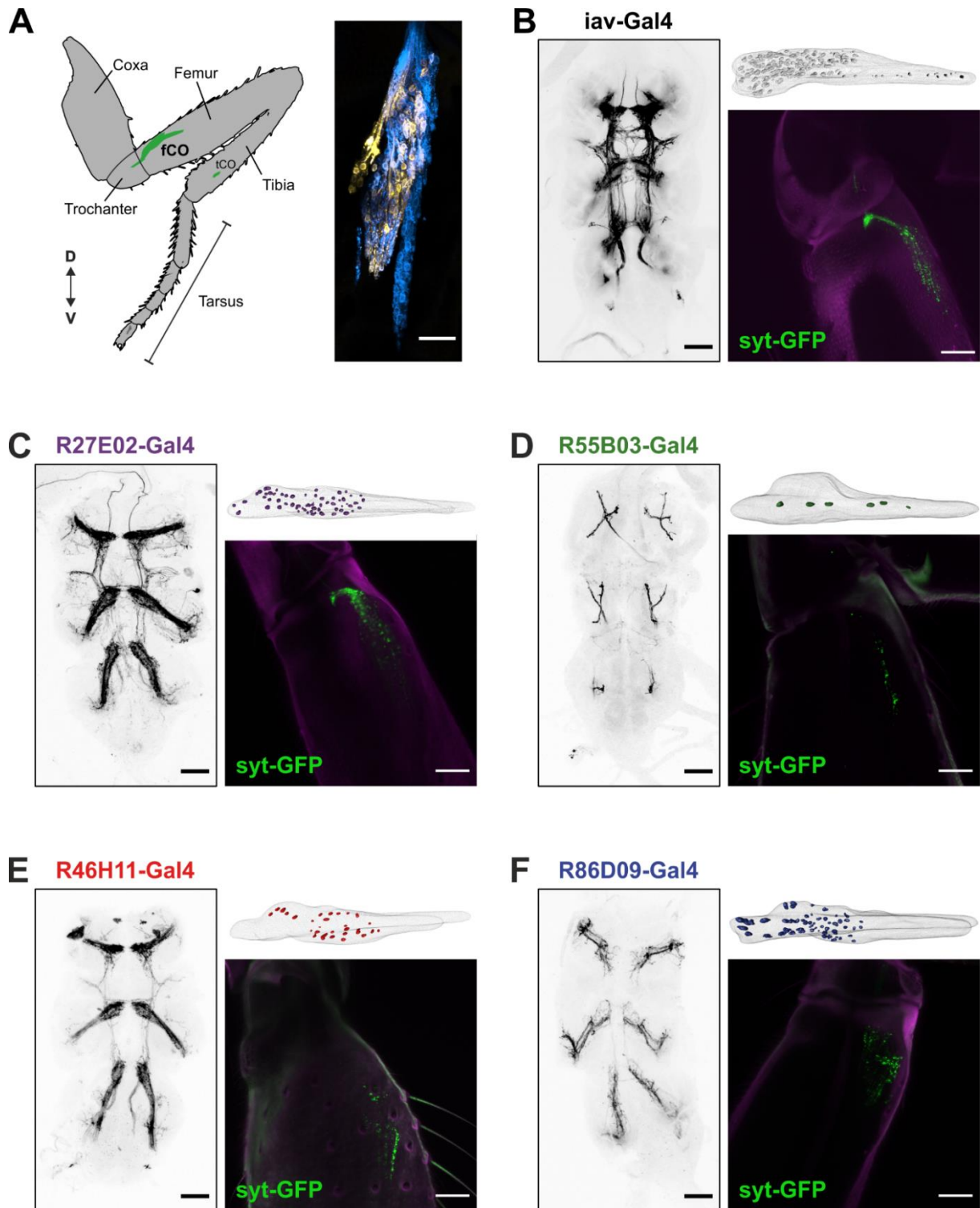
Figure 6.2. Anatomy of fCO labeling and VNC projection patterns of fCO GAL4 driver lines. (A) (left) schematic of D. melanogaster leg showing the femoral (fCO) and tibial chordotonal organs (tCO); (right) depth-color-coded confocal stack of fCO in *iav*-LexA expressing myrGFP; (B-F) Z-projections of confocal stacks of VNCs of flies expressing fCO-GAL4 > UAS-mcd8::GFP show the projection patterns of the entire fCO (B) as well as fCO subsets (C-F). 3D reconstructions show nuclei of labeled cells (UAS-RedStinger, DAPI staining) within the fCO (anti-ChAT immunolabeling). Expression of syt.eGFP shows presynapses of the glomerulus in *iav*-GAL4 and R27E02 as well as intra-fCO presynapses in all lines. Magenta is cuticular autofluorescence for orientation. VNC scale bars: 50 μm. fCO scale bars: 25 μm

The chordotonal organ primary sensory neurons labeled by *iav*-GAL4 and the club/hook line project both intersegmentally and locally (Figure 6.2B,C). These projection patterns can be teased apart when inspecting the expression patterns of the fCO subset lines used herein (Figure 6.2D-F). *Club* neurons exhibit direct inter-ganglionic connectivity (between thoracic ganglia T1-T2 and T2-T3; Figure 6.2E). *Hook* neurons show inter-hemiganglionic connectivity (left-right; Figure 6.2F). *Claw* neurons are unique among these three subset driver lines in that they only contain local VNC projections from each leg nerve (Figure 6.2D).

These subset lines label different numbers of cell bodies in the fCO, but no clear spatial clustering can be seen in our analysis (Figure 6.2B-F). Further, projections going directly to the brain (anterior ventrolateral protocerebrum) can be seen in *iav*-GAL4 (Tsubouchi et al., 2017) and in the *club/hook* neurons (Figure 6.2B,C), but ascending neurons were not labeled by the other subset lines shown here (Figure 6.2D-F). This indicates that some but not all *club* or *hook* neurons project to the brain. In addition, it is important to note that these driver lines do not exclusively label neurons in the fCO; some labeling is also seen in the Johnston's organ of the antenna as well as chordotonal organs associated with the coxa (data not shown). Furthermore, the *hook* driver line also shows some labeling in the tibial chordotonal organ.

Aside from potential connections between fCO neurons in the VNC, many presynapses from fCO neurons are present within the fCO itself as well as in the glomerulus, a synaptically dense structure through which fCO axons pass just before they join the leg nerve (section 3.2; Shanbhag et al., 1992). Synaptotagmin-bound GFP expression in *iav*-GAL4 and our subset driver lines shows presynapses within the fCO in all lines tested as well as labeling in the glomerulus in *iav*-GAL4 and R27E02-GAL4 (Figure 6.2B-F). Intra-fCO connections seem to therefore be a general feature among these

neurons, while glomerulus-associated connections seem to be restricted to certain neurons. Here, the neurons labeled by R27E02-GAL4 are *club* and *hook* neurons, which might suggest that synapses in the glomerulus are associated with either or both of these neuron types but not *claw* neurons. Interestingly, however, neither driver lines labeling *club* nor *hook* neurons alone show any synaptic labeling in the glomerulus, indicating that the *club/hook* driver line contains a set of neurons that have additional glomerulus-associated presynaptic connectivity. In addition, this finding also suggests that the morphological categorization is not entirely congruent with putative functional categories.

6.3.2 Inhibition of fCO and subsets disrupts leg kinematics and spatial coordination in walking

Control flies (Berlin-K > UAS-GtACR1) fed all-trans retinal were remarkably consistent under IR and green light illumination but exhibited interindividual variability (Figure 6.3).
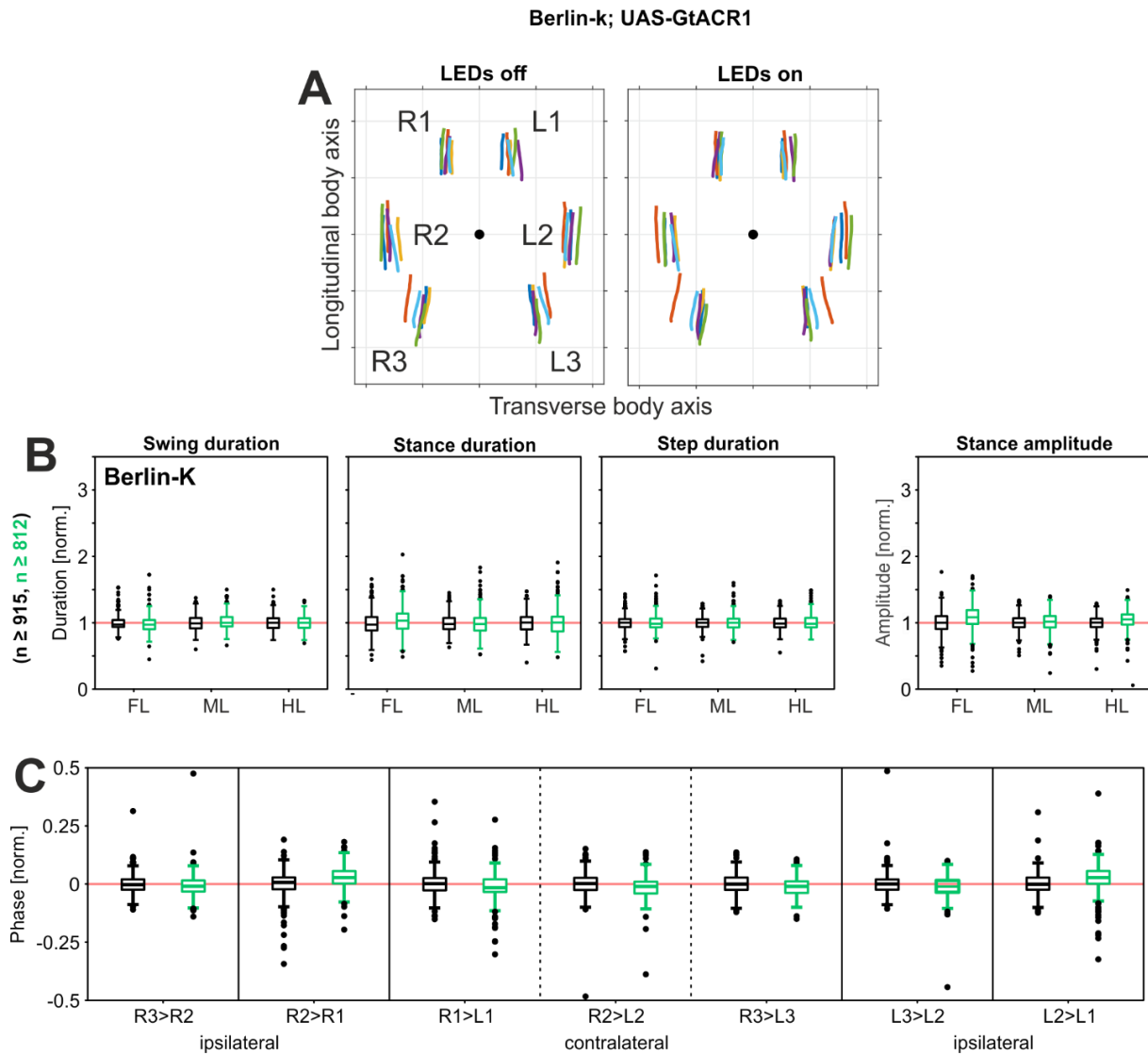
Figure 6.3. Kinematic and temporal coordination data from control experiments. Berlin-K > UAS-GtACR1 flies fed retinal were tested in the free-walking assay; the conditions of "Green LEDs off" and "Green LEDs on" correspond to the Control and Inhibited conditions, respectively. Stance trajectories (A) and kinematic parameters (B) show no differences between lights on and off (green and black, resp., in B). (C) Interleg phase coupling is slightly shifted between middle and front legs under illumination condition (R2>R1 and L2>L1).

Parameters reflecting spatial control and coordination, such as the anterior (AEP) and posterior extreme position (PEP) of each step, stance amplitude (distance between AEP and PEP; Figure 6.1D) as well as stance, swing, and step duration, were extracted from the tarsus tracking data. As a first step towards understanding the functional role of the fCO in locomotion, we inhibited a majority of fCO neurons using the broad *iav-*

GAL4 driver line (Kwon et al., 2010; Tsubouchi et al., 2017). Flies were still able to walk in a coordinated manner, suggesting that the fCO is not indispensable during walking. A similar finding has been reported by Mendes et al. (2013) for even stronger manipulations during walking. Unlike in this previous study, however, our temporally precise and rather strong manipulation led to pronounced deficiencies mainly in kinematics and to a small extent in coordination. This is reflected in increased stance amplitude (Figure 6.4A,B), which was most pronounced in the front legs. Moreover, swing, stance, and step duration were also increased (Figure 6.4B). Swing duration was increased less in front legs than in hind legs, while the other changes were equal for all legs. These retardations in stepping frequency were accompanied by increases in step amplitudes, so the overall walking speed was not affected (trials matched for walking speed); in essence, walking speed is only dependent on the movement rate of the legs during stance, not the duration. Interestingly, this seemed to be unaffected.
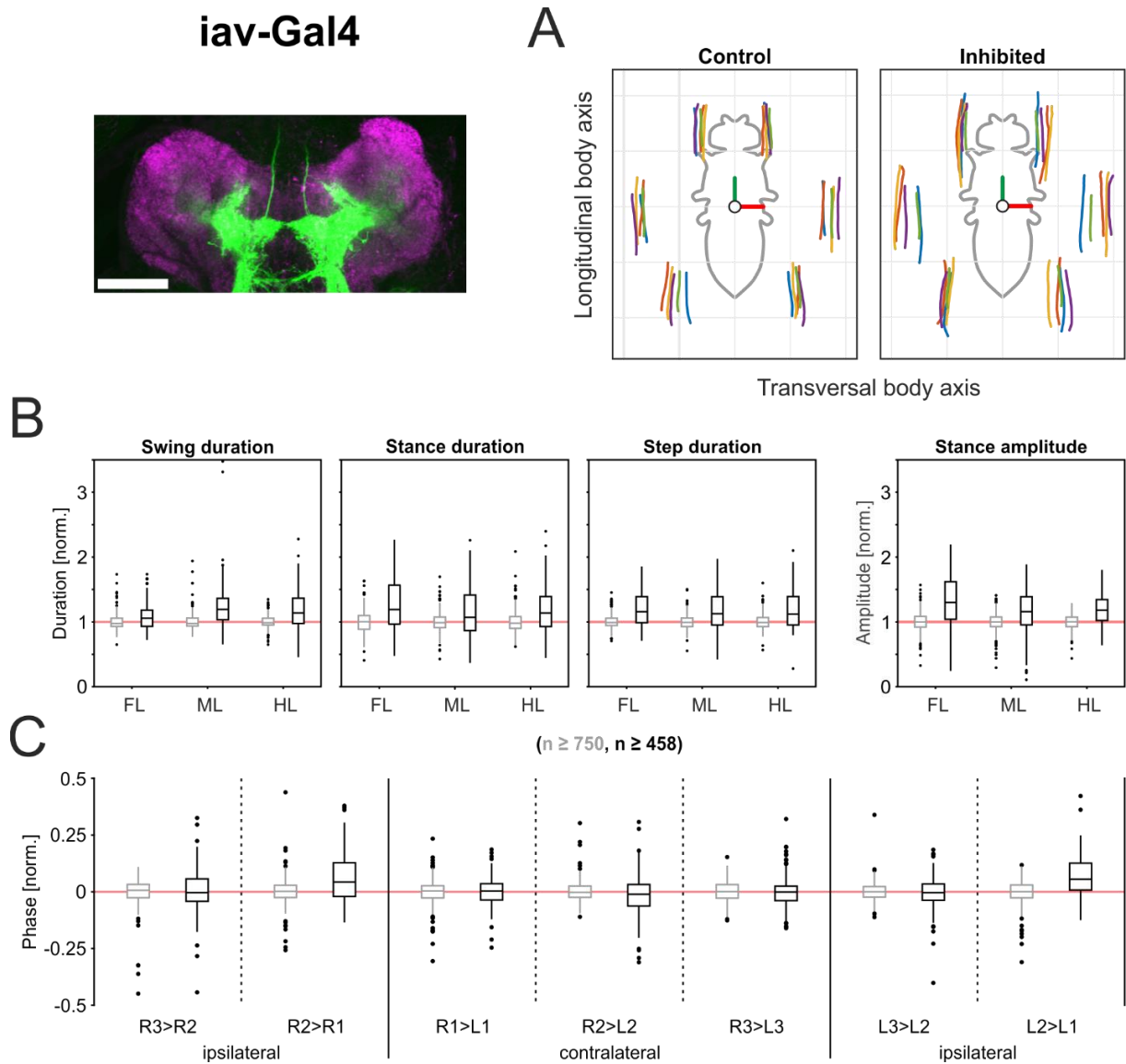
Figure 6.4. Stance trajectories and kinematic parameters. Free Walking Inhibition of fCO neurons in *iav*-GAL4; flies expressing GtACR1 were tested in the walking assay. (A) average stance trajectories per trial shown in fly-centered coordinate space for Control and Inhibited conditions; (B) Kinematic parameters calculated from tracked tarsal positions for Control (grey) and Inhibited (black) conditions; (C) Phase relationships between leg pairs for both conditions

Inhibition of *club/hook* neurons (R27E02-GAL4) resulted in elongations of stance trajectories similar to those seen in *iav*-GAL4 (Figure 6.5A). Interestingly, although this line labels fewer neurons, these effects were either equally strong, if not stronger, compared to the broader *iav*-GAL4 line. Further, swing and stance duration increased

in all legs, with some differences between leg types (Figure 6.5B). Swing duration increased most in hind legs and least in front legs, whereas stance duration increased most in front legs and least in hind legs. Stance amplitude was increased in a pattern similar to stance duration. Step duration in this line was increased somewhat equally in all legs. Flies still walked in a coordinated manner, but it is clear that the effects of inhibiting these fCO neurons affected mostly flexion movements—in the anteriorly oriented front legs, flexion is associated with stance movement as flexion in the femur-tibia joint pulls the fly forward. In the posteriorly oriented hind legs, flexion is associated with swing movements; here, extension of the femur-tibia joint pushes the fly forward. In the middle legs, flexion is used to a smaller extent in both stance and swing movements, and both of these movements seem to be somewhat affected.
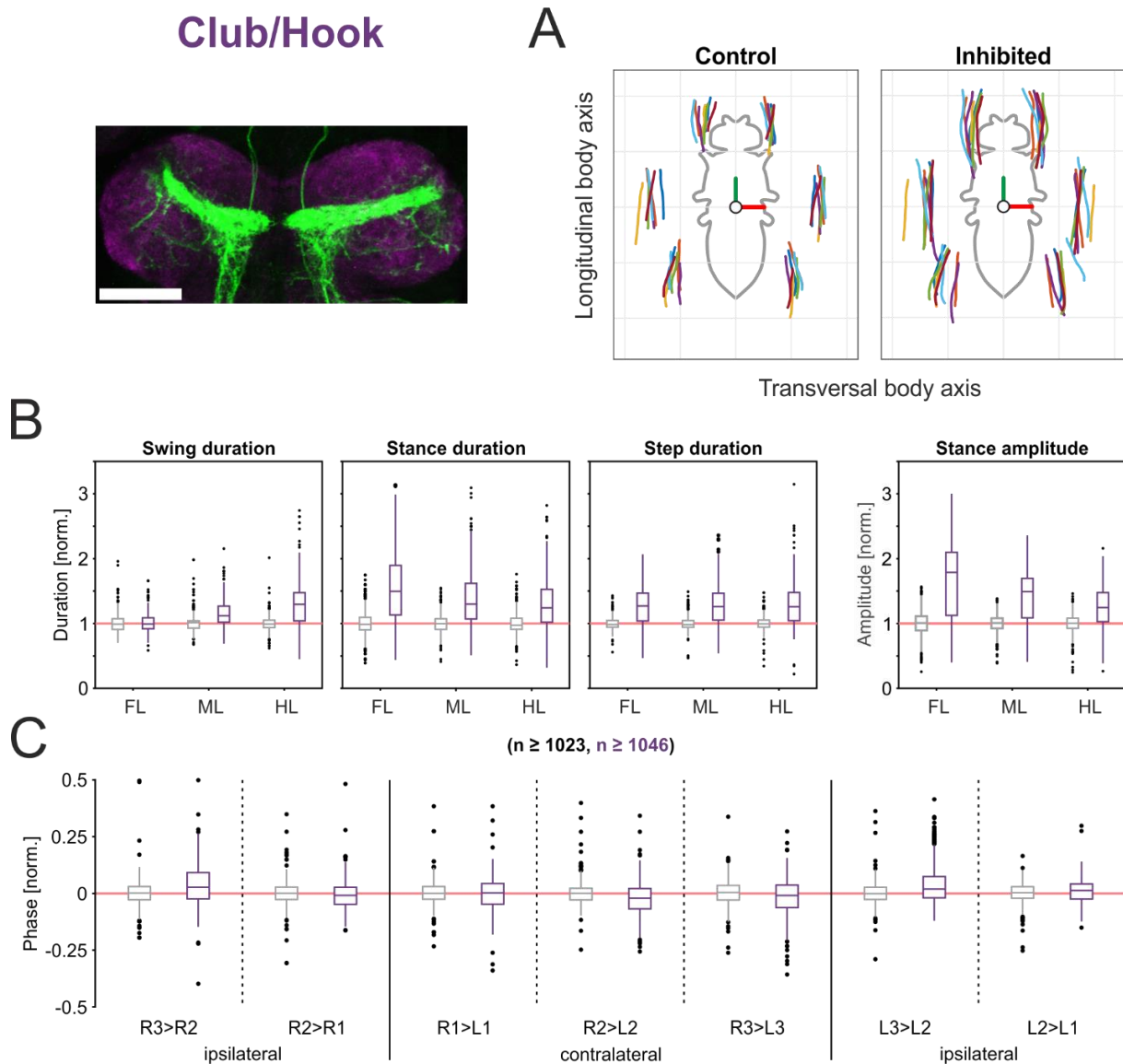
Figure 6.5. Leg Trajectories and kinematic parameters. Free Walking Inhibition of *club/hook* neurons in R27E02-GAL4; flies expressing GtACR1 were tested in the walking assay. (A) average tarsal trajectories per trial recreated in fly-centered coordinate space for Control and Inhibited conditions; (B) Kinematic parameters calculated from tracked tarsal positions for Control (grey) and Inhibited (purple) conditions; (C) Phase relationships between leg pairs

Surprisingly, inhibition of *club*, *hook*, and *claw* neuron types individually did not ostensibly affect stance trajectories or kinematic parameters (Figure 6.6). Interestingly, this coincides with the apparent absence of synapses from these neurons in the glomerulus (see Figure 6.2).
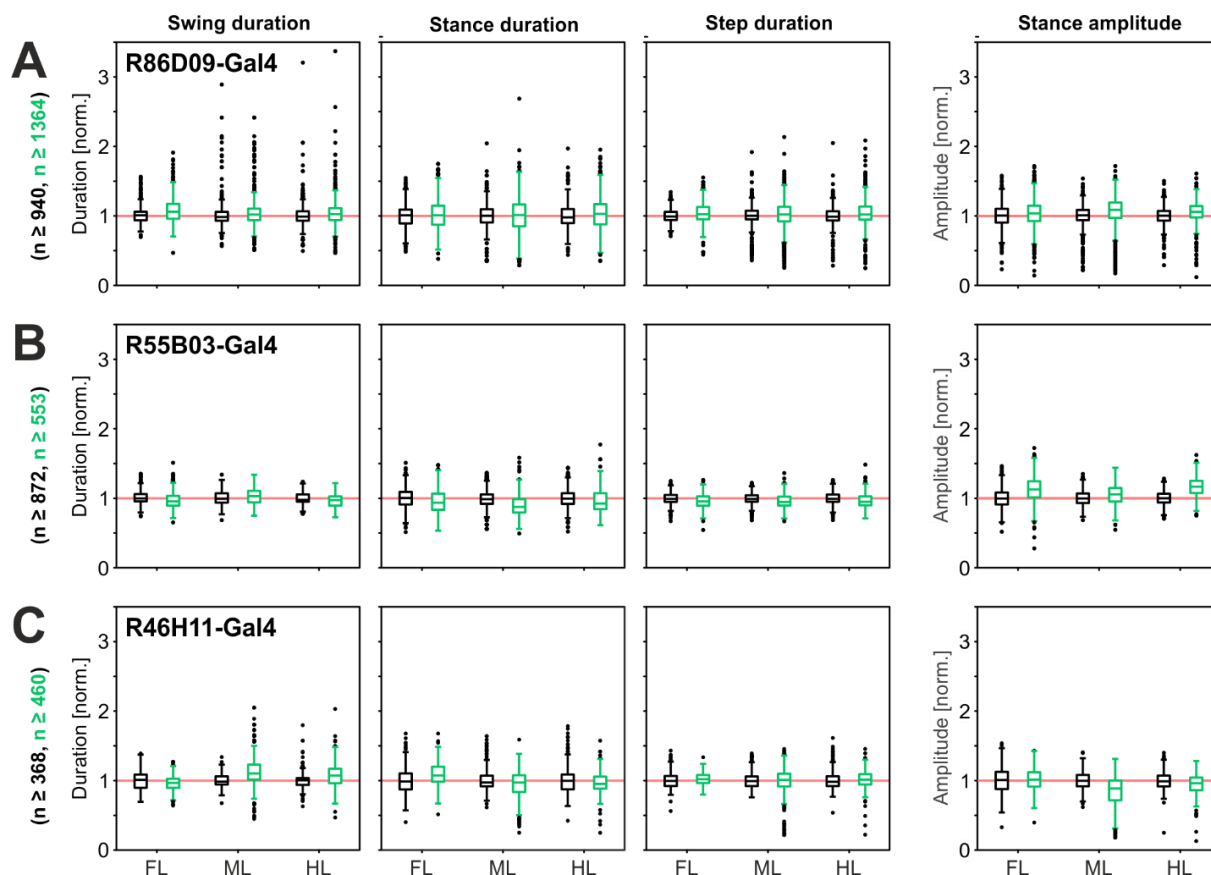
Figure 6.6. Kinematic parameters of flies expressing GtACR1 in fCO neuron subsets. Annotated video data from Control trials (black) and Inhibited trials (green) are shown for (A) *hook* neurons, (B) *claw* neurons, and (C) *club* neurons.

6.3.3 fCO inhibition disrupts interleg coordination in walking

Temporal interleg coordination is determined by the phase relationships between adjacent leg pairs (ipsi- and contralateral). We defined a complete step cycle in a leg as the movement between two consecutive liftoff events (PEPs) and normalized that time span to 1, the reference phase. To then determine the phase relationship between two legs, we expressed the PEP of a dependent leg in terms of normalized phase of the reference leg; for instance, R3>R2 describes the phase relationship of the right middle leg in reference to the right hind leg. Thus, a phase relationship of 0 indicates stepping in unison (i.e., liftoff events occur at the same time). A value of 0.5 means anti-phase alternation. We then defined the mean phase relationship during the control condition

as a reference phase of 0 and expressed the changes during inhibition as the difference from the control. Positive values of this measure thus indicate a later PEP in the dependent leg during inhibition.

Demonstrating the functional relationship between the fCO and coordination, *iav*-GAL4-mediated fCO inhibition caused mild phase delays (~5%) in swing onset between ipsilateral front and middle legs (R2>R1 and L2>L1; Figure 6.4C). Interestingly, effects on coordination were not seen between ipsilateral hind and middle legs, suggesting that changes in the movement of the front legs, more specifically a delayed liftoff, are responsible for this effect. In the data from *iav*-GAL4, this delay might also be reflected in the front leg stance amplitudes being the most strongly elongated (Figure 6.4A,B). The phase relationships between hind and middle legs, however, were unchanged (R3>R2 and L3>L2, Figure 6.4C), while the stance amplitudes in these legs were also larger. This suggests uniform shifts in the absolute phases in these legs, thus negating any shifts in their relative temporal coordination.

This was also seen for *hook*, *claw*, and *club* subset neurons (Figure 6.7). It should be noted that this pattern was also seen in control flies (Figure 6.3) and it was not seen in the *club/hook* driver line (Figure 6.5C). Compared to the effects found for the spatial parameters, these effects are small, indicating a lesser role of the fCO for temporal coordination.
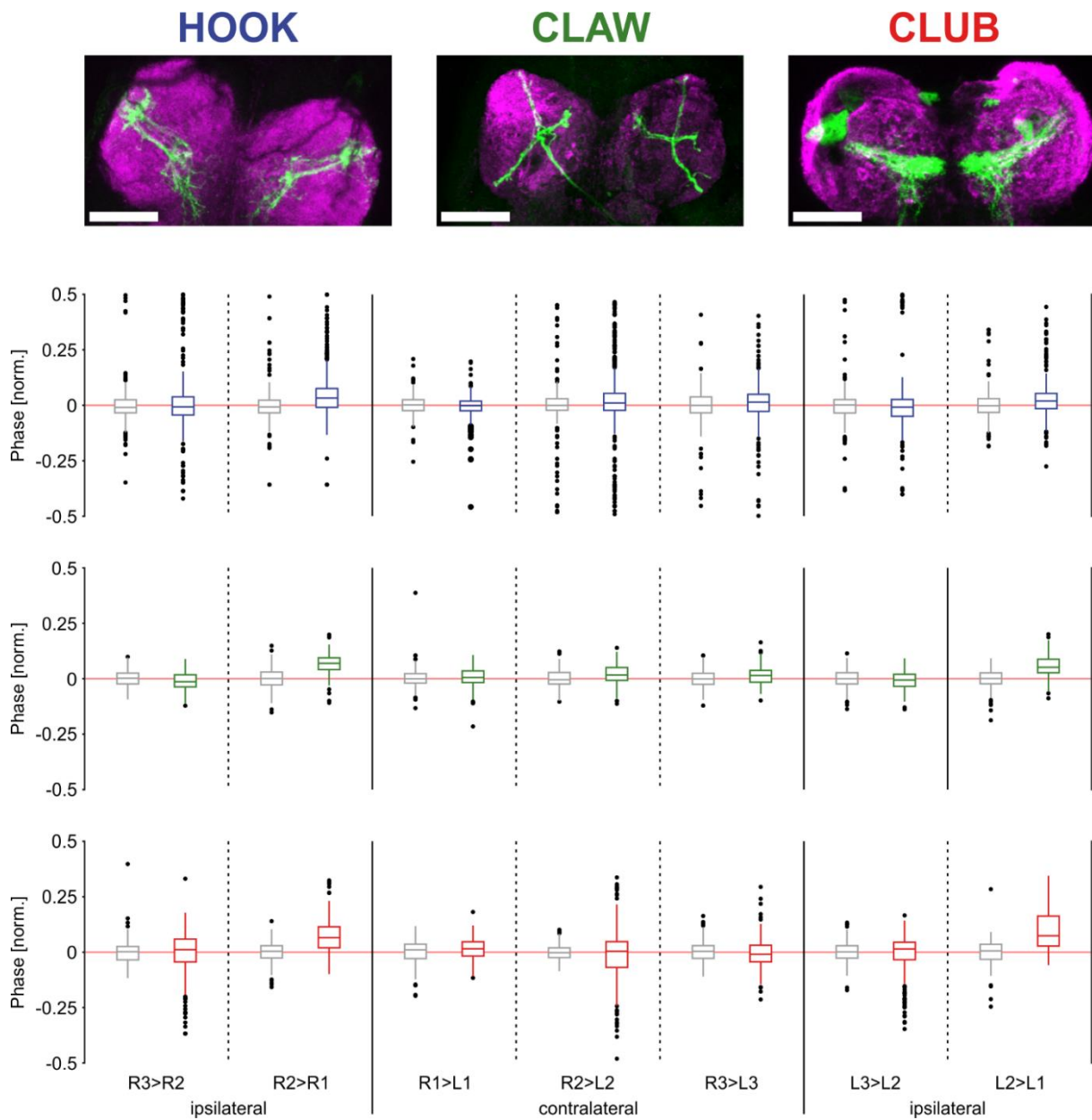
Figure 6.7. Temporal Coordination in free-walking flies. Relative normalized phase of posterior extreme positions plotted for all adjacent leg pairs for the Control (grey) and Inhibited (colors) conditions, shown for the *hook* (blue), *claw* (green), and *club* (red) driver lines.

6.4 Discussion

In these experiments, we used a free-walking assay and optogenetic inhibition to probe the functional role of the fCO and its neuronal subgroups in leg kinematics and interleg coordination during walking. Our results confirm previous findings that the fCO is necessary for normal single-leg kinematics and spatial coordination; our data further suggest that the fCO plays role, albeit minor, in the temporal coordination between legs. fCO subsets displayed no effects on kinematics or leg coordination when inhibited alone, but when inhibited in combination, a group of *club* and *hook* neurons showed effects similar and more exaggerated than those of inhibiting a majority of fCO neurons. This suggests a non-linear interaction of fCO signals with locomotor networks, in addition to a functional segregation of the temporal and spatial aspects of walking with regard to the influence of sensory information.

6.4.1 fCO neurons are mainly involved in spatial aspects of leg kinematics

It has been demonstrated that signals from the fCO are crucial for the control of the femur-tibia joint during resistance reflexes and active movements (Bässler, 1988; Bässler and Büschges, 1990; Burrows, 1987, 1988; Burrows et al., 1988). Furthermore, it has been demonstrated that interrupting synaptic transmission from leg COs broadly affects walking kinematics (Mendes et al., 2013). Coupled with our data, it becomes clear that, during walking, fCO neurons are mainly involved in leg kinematics and postural control. When inhibiting a majority of fCO neurons using *iav*-GAL4, we found that tarsal touch-down and lift-off positions were shifted and that kinematic parameters were affected similarly in all leg types. Coordination between adjacent leg pairs, however, was only mildly affected (Figures 3C, 4C, 5). This is also true for the neurons labeled by the *club/hook* driver line, but not for *club*, *claw*, or *hook* neurons when inhibited alone (Figure 6.6).

As inhibition of the entire fCO and a large group of *club/hook* neurons (R27E02-GAL4) both increased stance trajectory length, it is clear that these neurons are important for the determination of AEPs and PEPs as well as the stepping frequency during walking.

Transient CO inhibition produced effects similar to broader inhibition of leg sensory structures and mutations of CO-associated mechanosensitive ion channels. Using *nanchung* mutants as well as tetanus-toxin-mediated silencing in leg sensory neurons (5-40-GAL4), Mendes et al. (2013) demonstrated that walking speed decreased, step length increased, stance trajectories became less linear, and AEP and PEP placement became more variable compared to wild-type flies. Our data show the same findings for step length, stance linearity, and AEP/PEP placement, however only for the broader fCO inhibition seen in *iav*-GAL4 and the *club/hook* line. The data in this previous study indicated rather small effects, even though their manipulation applied chronic neuronal silencing (tetanus toxin light chain, TNT) to virtually all sensory neurons in the leg. The difference to the present study might be found in the contrast between transient and chronic inhibition—expressing TNT in leg sensory neurons allows much more time to compensate for a reduction in sensory information, and these animals might have been able to recover behavioral functionality to some degree. It has been shown in the locust, for example, that gain changes in the fCO can be recovered (Page and Matheson, 2009); this process is relatively slow, however, occurring over the span of a few days.

Null mutants of *transient receptor potential-γ* (TRPγ), which encodes an ion channel found in mechanosensors and COs, are also impaired in fine motor control and gap crossings (Akitake et al., 2015). Moreover, flies with *nompc* (TRPN) mutations, another common CO ion channel, have demonstrated increased step duration, while step amplitude was unchanged compared to wild-type flies (Cheng et al., 2010). Our findings confirm that the effects seen in other studies are the result of CO inhibition

and further demonstrate that they can be caused by the inhibition of subsets of fCO neurons.

6.4.2 Front, middle, and hind legs show differential effects

Some of the kinematic effects seen in *iav*-GAL4 and R27E02-GAL4 differ between the three leg types (front, middle, hind). The types of legs in insects differ in their involvement in certain behaviors, and walking is no exception (Cruse, 1976; Dallmann et al., 2016; Seeds et al., 2014; Zumstein et al., 2004). In *D. melanogaster*, the phases of the step cycle are performed differently in different legs. Swing phase in hind legs, for example, requires flexion of the tibia as the tarsus is lifted and returned toward the thorax, stretching the fCO; in front legs, it is represented by tibial extension and fCO relaxation as the tarsus is lifted and moved away from the thorax. The same behavior (i.e., forward stepping) is thus encoded by different proprioceptive signals and would require appropriately different post-processing. Middle legs occupy the middle ground here, as the fCO is not strongly engaged during the mainly retraction and protraction movements of stance and swing.

When we inhibited a group of *club/hook* neurons, we found that the different leg types were affected differently (Figure 4). Swing and stance duration were affected in all legs, but swing duration was highest in front legs, while stance duration was highest in hind legs. Front leg swing and hind leg stance have one movement in common: flexion. One group of hook neurons has been shown to be directionally sensitive, with "direction" here being between extension and flexion (Mamiya et al., 2018). As the neural coding of fCO neurons has only been shown for front legs, however, it is difficult to say whether the directional sensitivity, or even presynaptic signal processing, of the same neurons could vary between the leg types.

6.4.3 fCO inhibition has mild effects on coordination

Mendes et al. (2013) did not see effects on left-right or intersegmental temporal coordination, which is in accordance with our findings. The small effects on phase relationships seen here are likely due to animals walking in a well-lit arena compared to a dark arena, as control flies also showed the same effects. The phase shifts seen here seem to be caused by changes in front leg stepping. It has been shown that blow flies (*Calliphora vicina*) seem to use their front legs as tactile probes and change the pitch of their body while walking in darkness (non-visible IR illumination; Kress and Egelhaaf, 2012); this might have been the case in the present study, too, but this has not been tested in *D. melanogaster*. The evidence herein argues for the strongest role of the fCO being in leg kinematics during walking, and while the data show a mild effect on coordination, it is difficult to tease these two apart. That said, the lack of a strong role of the fCO subsets tested here in temporal coordination could suggest a functional segregation between temporal and spatial aspects of walking.

6.4.4 Some club and hook neurons, but not all, involved in leg coordination

Inhibiting most of the fCO or a subset of *hook* and *club* neurons changed stepping parameters and altered the movements of legs. Inhibiting *hook*, *claw*, or *club* neurons alone, however, did not have effects on the parameters tested here. This could be due a number of factors, but it could be that these neurons are simply not involved in the behavior of straight walking at moderate speed on a flat surface.

Moreover, the roles of these fCO subsets in walking correlates with their differential expression of presynapses in the glomerulus and fCO. Intra-fCO connectivity, whether functional or physiological, is likely to be different between the neuron types, as seen

by the syt.eGFP labeling. Only two driver lines tested here, *iav*-GAL4 and the *club/hook* line, showed presynapses in the glomerulus; these are also the only two where inhibition affected leg kinematics. Intra-fCO connectivity seems to be strongest in the neurons labeled by the *club/hook* driver line (Figure 6.2C), with labeling in the glomerulus and the fCO, while the individual *club*, *hook*, and *claw* driver lines show presynapses only in the fCO itself. It does not seem as if glomerular labeling changes with neuron number; rather, some fCO neurons likely do not have presynapses in the glomerulus. This immediately implies a particular role of the glomerulus for walking. It is conceivable that sensory information important for the central control of walking behavior is pre-processed in the glomerulus, where signals might be transformed before they enter the CNS. However, a detailed and well-controlled input-output characterization would be necessary to test this and the putative transfer function of the glomerulus.

Peripheral chemical synapses in sensory nerves like those shown in the fCO and glomerulus have been demonstrated for mechanosensors in spiders (Fabian-Fine et al., 1999; Foelix, 1975), as well as in the retina of horseshoe crabs (Fahrenbach, 1975), where they have been shown to play a role in lateral inhibition. In *Drosophila melanogaster*, peripheral synapses have been shown in the escape circuitry between interneurons and motor neurons (King and Wyman, 1980) as well as in the glomerulus of the fCO (Shanbhag et al., 1992). Considering their functions in other organisms, peripheral synapses in the glomerulus could play a role in presynaptic inhibition (Stein and Schmitz, 1999); the current data correlate presynapses in the glomerulus with effects of fCO subset inhibition on leg motor control. Moreover, the presynapses seen on fCO primary sensory neurons within the fCO itself add yet another layer of complexity to the encoding and processing of leg proprioceptive signals.

6.5   Conclusions

In summary, transient optogenetic inhibition of proprioceptor subsets in intact flies causes changes in walking kinematics, and these changes vary depending on the neurons that are silenced. Considering that fCO neurons seem to be functionally grouped based on their response profiles, we expected a similar functional grouping regarding effects on behavioral output. Interestingly, inhibition of individual neuronal types did not affect leg kinematics but caused mild phase delays in front legs. Further, peripheral presynapses are seen in the fCO itself and in a glomerulus located where fCO axons enter the leg nerve, with fCO subset driver lines displaying different patterns of presynaptic labeling in both areas. Further research moving forward should test intra-fCO connectivity within and outside of the glomerulus in addition to the functional relevance of proprioceptive signals in a wider range of behaviors.

7 Chapter 5: fCO encoding of proprioceptive stimuli

7.1 Introduction

The previous chapter demonstrates that some, but not all, fCO neurons are necessary for normal walking kinematics. In order to correlate neuronal encoding properties with this necessity for walking, it is crucial to be able to measure their activity during movements about the femur-tibia joint and, thus, their stimulus-encoding properties. Owing to the small size of the legs of *D. melanogaster*, this is best done using non-invasive imaging techniques, such as calcium or voltage imaging (Hod Dana et al., 2018; Stadele et al., 2012; Yang and Yuste, 2017).

Calcium imaging, in which calcium levels can be visualized as a proxy for the activity of neurons, is an ideal technique for investigating response properties of fCO neurons. Owing to the leg's small size, electrophysiological recordings of fCO neurons before their axons exit the leg prove rather difficult. Additionally, the high spatial resolution offered by calcium imaging could allow for recording synaptic activity in the glomerulus. Using genetically encoded calcium indicators (GECIs), one can visualize changes in intracellular calcium concentrations via changes in fluorescence intensity of emission wavelength. GECIs can be expressed under the control of the GAL4/UAS system, allowing for targeted visualization of changes in Ca2+ concentration. This can be done in the subsets of fCO neurons described herein in order to determine the response properties of these neurons to movements at the femorotibial joint.

Previous work has demonstrated that GECIs can be used to image neural activity in the peripheral nervous system directly through the cuticle (Kamikouchi et al. 2010). Further, recording of calcium signals has been elegantly performed using GCaMP6f in fCO axon terminals in the VNC (see section 1.3.2; Mamiya et al., 2018). Using a setup

inspired by Mamiya et al. (2018) with the help of the Electronics Workshop in the Institute of Zoology (University of Cologne), I developed a device based off of that used in Mamiya et al. (2018) to stimulate the fCO with controlled velocity and acceleration while performing live imaging.

7.2 Methods

7.2.1 Animals

Preliminary tests of the calcium imaging setup were done on female R27E02-GAL4 flies expressing GCaMP6s.

7.2.2 Device and Stimulation

I designed and created a device based on that used in Mamiya et al. (2018) consisting of a magnet attached to a stepping motor so that it could be rotated around the motor's center while maintaining a fixed distance from the center (Figure 7.1). An aluminum platform was attached near the motor so that a fly could be placed on the platform with the femur extending over the edge. The fly was positioned so that the femur-tibia joint was aligned with the rotational center of the stepping motor (with the help of a pin extending from the motor), and screws on the platform allowed for minor positional adjustments. UV glue was used to attach a small piece (~.5 mm) of steel wire to the tibia-tarsus joint, so as to fix them in place relative to each other. The magnet attached to the motor was then brought closer to the leg until it was clear that it was pulling on the steel pin. In this manner, we could manipulate the angle of the femur-tibia joint

simply by rotating the magnet, and the fly was positioned so that we could image from the dorsal side of the proximal femur.

Rotation of the stepping motor was controlled via a custom-built electronic controller. Command scripts were sent to the controller via Mach3 computer numerical control software (version R3.043.066; Newfangled Solutions, Livermore Falls, ME, USA). Two stimulus protocols were created for this. In the ramp-and-hold stimulus protocol, the tibia was extended to 180 degrees (parallel with the femur) and held there for 5 s. It was then flexed stepwise in 18-degree steps with a 3-s pause between steps until it reached an angle of 18 degrees and was then returned to 90 degrees in the same stepwise fashion. In the swing motion stimulus protocol, the tibia, starting at 90 degrees, was flexed to 18 degrees, held for 3 s, extended fully to 180 degrees and held for 3 s, and then flexed again to 18 degrees. This was repeated 6 times per trial. Stimuli were measured by an angular position sensor on the stepping motor, which read out via an A/D converter (Micro1401; Cambridge Electronic Design) into Spike2 software (Cambridge Electronic Design) at a sampling rate of 10 KHz.

7.2.3 Imaging

Imaging was performed using a Zeiss Axio-Imager M2 upright widefield fluorescence microscope (Carl Zeiss AG, Jena, Germany). Images were recorded through a Zeiss AxioCam MRm (1388 x 1040 px; Carl Zeiss AG) in Zen Pro software (2012; Carl Zeiss AG) at 9.773 Hz with 100-ms exposure time. A broad-spectrum light source (Polychrome V; Till Photonics, Kaufeberen, Germany) run through a filter cube was used for imaging (488 nm for GCaMP excitation). Still images of DsRed expression were used for region of interest (ROI) creation.
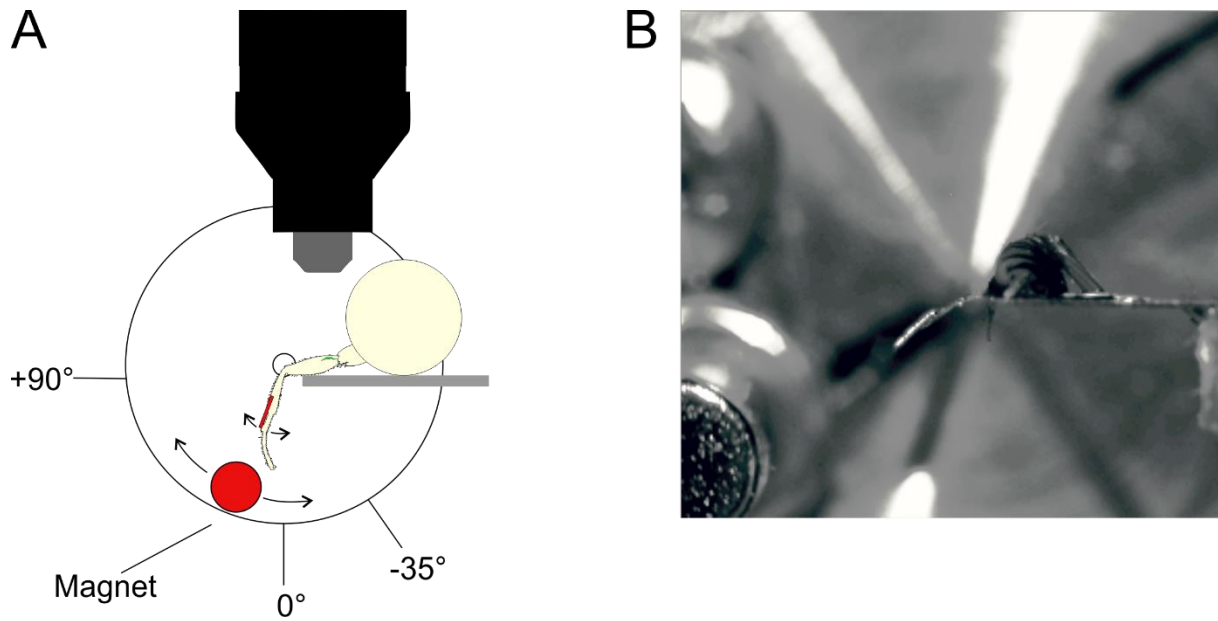
Figure 7.1. Stimulus apparatus used for stimulation of fCO neurons. Schematic (A) shows the stimulus apparatus for recording fCO neurons (green). The fly was placed on a platform (grey) so that the femur extended over the edge to the center of the stepping motor (large circle). A pin (red rectangle) attached to the tibia was brought in proximity of a magnet (red circle) so that the femur-tibia joint angle could be precisely manipulated. (B) shows a photograph of this setup from the same angle, with the magnet in the lower left corner**.**

7.2.4 Data Analysis

Pixel intensites were extracted for each frame of the recordings in Fiji (Schindelin et al., 2012) and exported to Matlab (The Mathworks). Average pixel values from a background ROI were subtracted from the fCO ROI, and these resulting ΔF values were divided by the average background ROI values to get a percentage change in fluorescence signal ($\Delta F/F$). The derivative of the output of the motor position sensor was taken to give the instantaneous angular velocity of the stimulus.

7.3   Results

Pixel intensity values changed with changes in femur-tibia angle, indicating that relative changes in fluorescence intensity could be measured using this setup (Figure 7.2A-B). However, no clear preferred stimuli were seen in neurons labeled by R27E02-GAL4 in these initial tests. Step-wise flexion and extension of the tibia (Figure 7.2A) showed the highest changes in fluorescence intensity around the most extended joint angles, and the swing stimulus protocol in the same fly (Figure 7.2B) demonstrated fluorescence signal increases during flexion movements. Notably, however, the difference in relative fluorescence intensity between these two trials was alarming (~1.2% change in ramp-and-hold trial, ~15% change in swing trial). To test if this was simply artifacts being measured, I selected six ROIs inside and outside of the fCO, as well as one entirely outside the leg. Data from these ROIs (Figure 7.2D) show rather large (~20%) changes in fluorescence intensity in all ROIs measured, indicating that these changes seen were indeed movement artifacts.
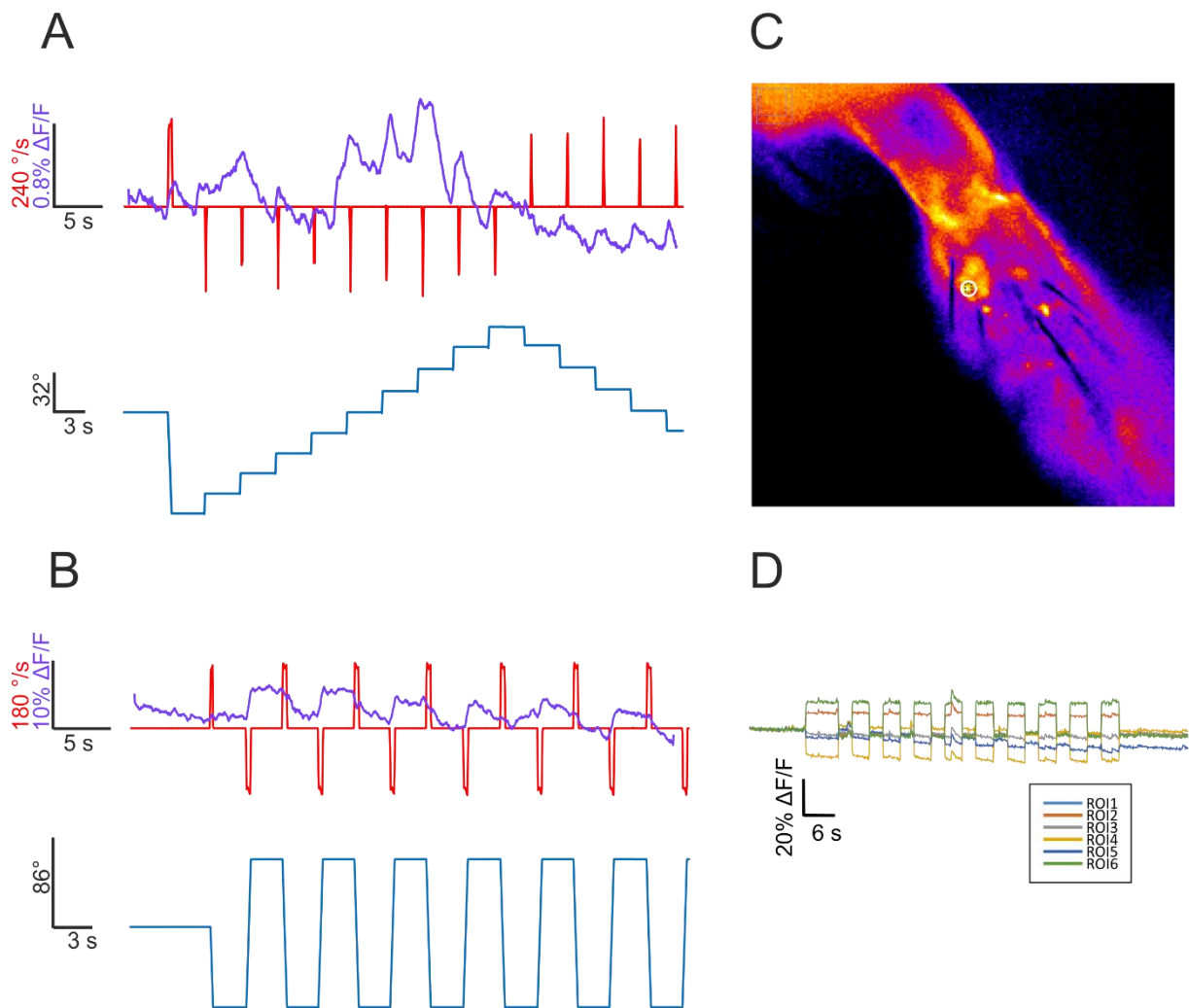
Figure 7.2. Calcium signals and stimulus traces in R27E02-GAL4. Flies expressing GCaMP6s were stimulated with the apparatus described above during calcium imaging acquisition. (A and B) Relative changes in fluorescence intensity (ΔF/F; purple) plotted as function of time along with the angular velocity of magnet movement (red) and a trace of the femur-tibia joint angle (blue). An image of DsRed expression (C) shows the fCO and the ROI used for the measurements in A and B (white circle in C). Data from another swing motion trial are shown in D, with the different colored lines representing 6 ROIs within and outside of the fCO.

7.4   Discussion

These experiments demonstrated that recording calcium signals through the cuticle is rather difficult. Looking into the relevant literature on the subject, a few potential reasons why this did not work become clear. First, the only study demonstrating

calcium imaging in the periphery used a rather bright calcium sensor called Cameleon (Kamikouchi et al., 2010). Cameleon is a fluorescence resonant energy transfer-based (FRET) calcium sensor that changes it's emission wavelength when bound with calcium (Miyawaki et al., 1999). The kinetics of these fluorescences changes are much slower than GCaMP sensors (on the order of seconds rather than milliseconds), making these sensors less than ideal for measuring fast calcium transients, such as those seen in proprioceptors (Helassa et al., 2016; Kerr et al., 2000). The success of Kamikouchi et al. (2010) in measuring calcium signals transcuticularly is likely due to the relatively high brightness of Cameleon, which comes at the high cost of the very slow kinetics. Further, successful calcium imaging in fCO axons as performed by Mamiya et al. (2018) could be due to their use of a 2-photon imaging setup and imaging within the VNC. These technological advantages provide much higher spatial resolution and signal-to-background ratio, as this imaging was not performed transcuticularly. In the current experiments, a widefield microscope with standard fluorescence illumination was used to image a rather low-baseline-intensity fluorophore through the cuticle, providing an explanation for the low fluorescence intensity changes and less-than-clear images (Figure 7.2A,C).

In addition to these issues, it is rather obvious from the data that movement artifacts of the femur during stimulation led to an inability to properly subtract background pixel values from the ROI data (Figure 7.2D). In an attempt to remedy this, I further fixed the femur in place using a thin wire wrapped around the femur and mounting plate. While this did reduce the motion artefacts in the data, it did not completely remove them. This could simply be a difficulty inherent in imaging the fCO during stimulation. Using a 2-photon imaging setup could provide higher spatial resolution and allow for better 3-dimensional alignment of the calcium sensor data with control

data (in this case from DsRed expressed in the same neurons), though these movement artefacts are likely to still pose a problem.

In conclusion, performing transcuticular calcium imaging of the fCO using the setup described herein is less than ideal. Considering the intra-fCO connectivity provided in the glomerulus and at presynapses in the fCO, as well as the presence of modulatory neuropeptides in fCO neurons, it is crucial that we get an idea of the encoding of movement stimuli by fCO neurons before these signals are potentially transformed in the glomerulus. More precise stimulation and imaging techniques, ideally with a 2-photon imaging setup, should be performed to this end.

8   Summary and Conclusions

8.1   Anatomy and Connectivity of the fCO

The fCO of *D. melanogaster* consists of multiple subpopulations of neurons, whose connectivity and function are investigated in this dissertation. These subpopulations can be grouped based on their genetic signatures (section 3.1) as well as their morphology (section 3). The genetic and morphological grouping conveniently corresponds to functional groupings, as outlined in (Mamiya et al., 2018). Some fCO neurons project to the brain (section 3.2), and at least one subset driver line labels ascending *club* or *hook* neurons (R27E02-GAL4, section 6.5). The axons of fCO neurons form many synapses with each other in the glomerulus, a highly intriguing peripheral processing center unique to the fCO and potentially *Drosophila* among the insects (sections 3.2, 6.5; Shanbhag et al., 1992). fCO neurons exhibit heterogeneity in their neurotransmitter and neuropeptide profiles; all are cholinergic, but there are subsets of neurons containing serotonin (Howard et al., 2019), tyramine (Pauls et al., 2018), and proctolin (section 4.3). As biogenic amines and neuropeptides are known to have a wide variety of functions in neurotransmission and the modulation of neural activity, further investigations should focus on this aspect of fCO neuronal grouping. Additionally, this dissertation demonstrates that a few leg CS are also neuropeptidergic; this could be a feature of other leg sensory structures, and its role in signal processing and motor control should be investigated.

8.2 Role of the fCO in leg movements and motor control

It has long been known that fCO neurons play a major role in the coordination of legs and leg joints during motor behavior (Field and Matheson, 1998). The importance of the fCO in motor control in *D. melanogaster*, however, has been considered only minor. In the work laid out herein, I demonstrate that some subsets of fCO neurons are sufficient to generate reflex movements and tibial extensor activity (section 5.3). Moreover, subsets of fCO neurons vary in their importance for locomotor coordination and walking behavior. When *club* and *hook* neurons are inhibited, at least in one driver line with expression in both types, flies exhibit changes to coordination parameters similar to when the whole fCO is inhibited (section 6.5). Some subsets of neurons seem to not play a role in intraleg coordination and leg kinematics during walking but do seem to affect temporal coordination between limbs. The differences in necessity for locomotion between morphological subset types begs the question of whether the morphological grouping of fCO neurons always coincides with functional grouping. However, if morphology and function go hand-in-hand, the results of the inhibition experiments described herein suggest that fCO neurons could have behavioral specificity, even if their proprioceptive encoding properties are the same (Section 6.5).

8.3 Outlook and Future Investgations

Research on the role of the fCO in motor control and locomotion has a long history; investigations into the subpopulation dynamics, however, have only begun in the last decade. This can be mostly attributed to the methods available in the insects studied— in animals without genetic tractability, only crude manipulations of the fCO have been

possible. *D. melanogaster*, however, finally provides researchers with an extensive toolkit to investigate the anatomical and functional characteristics of this organ on a subpopulation, and even single-cell, level. The results presented in this dissertation should only serve as a jumping-off point for future studies into the details of this fascinating proprioceptive organ.

It should be stated that our understanding of the encoding of stimuli by the *Drosophila* the fCO is still in its infancy. Beautiful work has recently been done to highlight the usefulness of transgenic organisms and binary expression systems in such investigations (Mamiya et al., 2018), but this has only scratched the surface. Now that we know that groups of neurons that should have similar encoding characteristics play different roles in the locomotor system, future work should focus on characterizing, functionally and otherwise, all neurons in the fCO of *Drosophila*. While calcium imaging provides an excellent tool for this, electrophysiological recordings are ideal for uncovering the stimulus encoding and potential peripheral preprocessing of fCO signals. Moreover, such methods are likely necessary for understanding details of vibration sensitivity in the fCO and uncovering which, if any, fCO neurons in *D. melanogaster* are sensitive to acceleration.

First identified in the early 1990s, the glomerulus has been largely ignored in the research of the last 30 years. Since its discovery, its existence has been briefly mentioned in multiple publications, but no direct investigations into its presence or characteristics have been published. Peripheral preprocessing of proprioceptive stimuli is an inherently interesting phenomenon and has never been demonstrated in an insect. Understanding the transformation of signals that is potentially taking place inside this structure will prove crucial for our understanding of the role of the fCO in motor control.

Lastly, the neuromuscular networks that signals from leg proprioceptors feed into have been thoroughly investigated in both vertebrates and invertebrates. However, even as *D. melanogaster* has proven itself as an ideal model organism for this research, we still know very little about the central pattern generators, interneurons, motor neurons, and ascending and descending influences that are involved in locomotor behavior in flies. These neurons all comprise a highly complex network, and cracking these large circuits is no easy task. However, the tools developed in *Drosophila* are very promising for the future of such investigations. I look forward to the future of this research, where large-scale functional and anatomical characterizations of *Drosophila* locomotor networks and the wildly complex and inherently intriguing fCO are no longer things of dreams.

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References

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Erklärung

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Köln, den

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Curriculum vitae

PERSONAL

Date of Birth: May 18, 1988
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EDUCATION

University of Cologne, Germany
Ph.D. in Zoology/Neurobiology
Expected completion: September 2020
Dissertation: The role of the femoral chordotonal organ in motor control, interleg coordination, and leg kinematics in *Drosophila melanogaster*
Supervisors: Prof. Dr. Ansgar Büschges, Prof. Dr. Kei Ito

University of Bonn, Germany   2014
M.Sc. in Neurosciences
Cognitive Neuroscience

Michigan State University, E. Lansing, MI, USA   2010
B.S. in Zoology
Specialization: Ecology, Evolution, and Organismal Biology

PUBLICATIONS

Chockley, AS, Dinges GF, Ratican S, di Cristina G, Büschges A, Bockemühl T. Femoral Chordotonal Organ Neurons Differentially Affect Leg Movements and Coordination During Walking in *Drosophila melanogaster*. (*in preparation*)

Dinges GF, **Chockley AS**, Bockemühl T, Ito K, Blanke A, Büschges A. 2020. Location and arrangement of campaniform sensilla in *Drosophila melanogaster*. *J Comp Neurol (in press)*

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RESEARCH EXPERIENCE

Research Associate   2020 - present
Junior Research Group Ache, Department of Neurobiology and Genetics, University of Würzburg, Germany
Context-dependent flexibility in sensorimotor networks in Drosophila; Patch-clamp electrophysiology, behavioral genetics, optogenetics, calcium imaging

Research Associate   2014 - 2015
Experimental Psychology, University of Regensburg, Germany
Experimental design; EEG testing, participant recruitment, course supervision (EEG Data Collection and Analysis)
Supervisor: PD Dr. Gregor Volberg

Intern   2014
Lab of PD Dr. med. Nikolai Axmacher, Institute for Epileptology, University of Bonn, Germany
fMRI data pre-processing; behavioral piloting of studies with humans; experimental design; learning and memory

Intern   2013
Lab of Prof. Dr. Susanne Schoch-McGovern, Institute for Epileptology, University of Bonn, Germany
Molecular mechanisms of malformations of cortical development; analysis of co-localization patterns of proteins involved in cell migration and synaptic development; molecular cloning, cell culture, confocal fluorescence microscopy

Intern   2013
NEMO (Neuromodulation of Emotion) Research Group, Dept. of Psychiatry, University of Bonn, Germany
Signal processing and data analysis of human skin conductance response data in a fear-conditioning paradigm, development of a method to remove fMRI artifacts
Supervisor: Dr. Benjamin Becker

Student Assistant   2009 - 2010
Sakai Lab, Michigan State University, MI, USA
Created 3D virtual endocasts of avian brains to investigate relationship between sociality and brain evolution
Supervisor: Dr. Sharleen Sakai

Student Assistant   2009 - 2010
Holekamp Lab, Michigan State University, MI, USA
Created ethograms from videos of captive carnivores learning to open puzzle boxes; analyzed data to investigate phylogenetic relationships and cognition

TEACHING EXPERIENCE

Graduate Assistant   2015 - 2020
Department of Animal Physiology, University of Cologne, Germany
Teaching Assistant: General Physiology (B.Sc.), Intro to Neuroscience (B.Sc.), Neural Function II (M.Sc.)
Supervision of students during practical internships and thesis projects in the lab.

Instructor 2015
Center for Language and Communication, University of Regensburg, Germany
Developed and taught two English as a Second Language courses: Scientific Writing
and English for Psychologists and Biologists

INVITED TALKS/POSTERS

Germany Neuroscience Society Meeting 2019
Göttingen, Germany
Talk: Subgroups of femoral chordotonal organ neurons differentially affect leg
movements and coordination in *D. melanogaster*

International Brain Research Organization World Congress 2019
Daegu, Korea
Poster: Subgroups of femoral chordotonal organ neurons differentially affect leg
movements and coordination in *Drosophila melanogaster*

Neural Circuits of the Insect Ventral Nerve Cord 2018
HHMI Janelia
Ashburn, VA, USA
Poster: Neuronal subgroups in the femoral chordotonal organ of D. melanogaster and
their influence on locomotion

Society for Neuroscience Meeting 2017
Washington D.C., USA
Poster: Anatomy and function of distinct groups of sensory neurons in the femoral
chordotonal organ of *Drosophila*

German Neuroscience Society Meeting 2017
Göttingen, Germany
Poster: Effects of optical activation of groups of sensory neurons in the femoral
chordotonal organ of *D. melanogaster*

NeuroFly 2016
Chania, Greece
Poster: Anatomical and functional characterization of sensory neurons in the femoral
chordotonal organ of *D. melanogaster*

OTHER

Languages – English (native), German (fluent/C1), Mandarin Chinese (beginner/A1)

Computer Skills – Confocal Microscope Image Acquisition (LASX), Microsoft Office,
MATLAB, Image Processing (Fiji, CorelDraw)

Scientific Copyediting 2015 - 2020
Freelance;
Scientific and language editing of scientific manuscripts for clients from Asia, Europe,
and South America; Management and advising of manuscript construction from initial
drafting through to revisions and publishing

Doctoral Candidate Representative   **2016 - 2019**
Research Training Group Neural Circuit Analysis, Graduate School for Biological
Sciences, University of Cologne

Student Council President   **2012 - 2014**
M.Sc. Neurosciences Program
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