Descending Octopaminergic Neurons in the Stick Insect:
Their Inputs and their Output Effects on the Locomotor System

Inaugural-Dissertation

zur

Erlangung des Doktorgrades der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln 2018

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Tag der mündlichen Prüfung: 12.06.2018

Abstract

The neural networks controlling locomotion (walking) must exhibit a high degree of flexibility for task-specific adaptation of behavior to environmental influences and changes in internal state. Neuromodulatory influences are very important for this flexibility, as they can regulate the activity of all neurons in the walking system and the strengths of their synaptic connections. To fully understand the neural control of walking, it is crucial to identify the neurons that release neuromodulators and to determine their activity patterns during behavior and analyze the properties of their output effects.

Octopamine, one such neuromodulator, is considered the invertebrate homolog to the vertebrate noradrenaline. It is a significant modulator in insect locomotor systems, both acting in the peripheral and central nervous systems. Octopamine modulates muscle metabolism, neuromuscular transmission, sensory sensitivity, excitability of motor neurons, and activity in the central pattern generating networks that control locomotion. The neural source of octopamine acting in the central nervous system of insect thoracic segments has not yet been identified. Thus, it is unknown to what extent effects of application of octopamine to thoracic ganglia in previous studies reflect the physiological role of octopamine.

In the current thesis, I hypothesized that dorsal unpaired median neurons with bilaterally descending axons (desDUM neurons) are a source of octopaminergic modulation of activity in thoracic neural networks for the control of walking in the stick insect *Carausius morosus*. I revealed the morphology of desDUM neurons in the gnathal ganglion by intracellular staining. Employing the newly developed method of direct MALDI-TOF mass spectrometry, I could show that stick insect desDUM neurons are octopaminergic.

Using semi-intact preparations and intracellular recordings of desDUM neurons, I found that they are phasically activated during six-legged walking and single-leg stepping. The phasic excitatory input to desDUM neurons during walking does not arise from coupling to activity of mesothoracic central pattern generating networks, but most likely from activation of mechanosensory organs of all six legs. Passive leg movement and stimulation of mesothoracic

campaniform sensilla excited desDUM neurons. Furthermore, stimulation of the mesothoracic femoral chordotonal organ (fCO) had a weak excitatory influence on their activity.

Further, I investigated the output effects of desDUM neurons on reflex-evoked, spontaneous, and centrally generated activity of mesothoracic motor neurons with activation of single desDUM neurons. I could show that distinct desDUM neurons mediate differential effects. Some neurons induce a decrease and others an increase, in the magnitude of reflex-induced motor neuron activity. The neurons which mediated an excitatory influence additionally increased the frequency of reversal of an fCO-induced postural reflex. Some desDUM neurons mediated an increase in the frequency of centrally generated rhythmic motor neuron activity. Collectively, the results of the current thesis provide a comprehensive characterization of desDUM neurons and their complex roles in the stick insect locomotor system.

The identity of direct neural target structures for the modulatory action of desDUM neurons, as well as the net output effects of the entire population of desDUM neurons during walking remain open questions. In future experiments, genetic access to desDUM neurons could aid in the activation, silencing, or visualization of their activity, which would collectively contribute to comprehensive answers to the open questions.

Zusammenfassung

Das neuronale Netzwerk zur Steuerung der Fortbewegung (Laufen) muss ein hohes Maß an Flexibilität für die aufgabenspezifische Anpassung des Verhaltens an Umwelteinflüsse und innere Zustandsänderungen aufweisen. Neuromodulatorische Einflüsse sind sehr bedeutend für die Flexibilität, da sie die Aktivität aller Neuronen des Laufsystems und die Stärke ihrer synaptischen Verbindungen modulieren können. Um die neurale Kontrolle des Laufens vollständig zu verstehen, ist es wichtig, die Neuronen zu identifizieren, die Neuromodulatoren freisetzen, ihre Aktivitätsmuster während des Verhaltens zu bestimmen und die Eigenschaften ihrer Ausgangseffekte zu analysieren.

Der Neuromodulator Octopamin, gilt als Invertebraten-Homolog zum Noradrenalin der Wirbeltiere. Octopamin ist ein signifikanter Modulator im Laufsystem der Insekten, wo es sowohl im peripheren als auch zentralen Nervensystem wirkt. Octopamin, moduliert den Muskelstoffwechsel, die neuromuskuläre Übertragung, die sensorische Sensitivität, die Erregbarkeit von Motorneuronen und die Aktivität in zentralen Muster erzeugenden Netzwerken, die die Fortbewegung steuern. Die neurale Quelle von Octopamin, das im Zentralnervensystem der thorakalen Segmente von Insekten wirkt, ist noch nicht identifiziert worden. Es ist daher nicht bekannt, inwieweit Effekte infolge der Applikation von Octopamin auf thorakale Ganglien, in früheren Studien, die physiologische Rolle von Octopamin widerspiegeln.

In der vorliegenden Arbeit stellte ich die Hypothese auf, dass dorsale ungepaarte mediane Neuronen mit bilateral absteigenden Axonen (desDUM-Neuronen) eine Quelle der oktopaminergen Modulation der Aktivität in thorakalen neuronalen Netzwerken zur Steuerung des Laufens bei der Stabheuschrecke *Carausius morosus* sind. Ich habe die Morphologie der desDUM-Neuronen im Unterschlundganglion durch intrazelluläre Färbung aufgedeckt. Mit der neu entwickelten Methode der direkten MALDI-TOF Massenspektronomie konnte ich zeigen, dass desDUM Neuronen in der Stabheuschrecke oktopaminerg sind.

Mittels semiintakter Präparate und der intrazellulären Aufnahme des Membranpotentials von desDUM-Neuronen fand ich heraus, dass die Neuronen während des sechsbeinigen Laufens

und des einbeinigen Schreitens phasisch aktiviert werden. Der phasisch exzitatorische Eingang in desDUM-Neuronen während des Laufens ergibt sich nicht aus einer Kopplung an Aktivität von mesothorakalen zentralen Muster erzeugenden Netzwerken, sondern höchstwahrscheinlich aus einer Aktivierung von meschanosensorischen Organen aller sechs Beine. Passive Beinbewegung und Stimulation der mesothorakalen campaniformen Sensillen waren erregend. Weiterhin hatte die Stimulation des mesothorakalen femoralen Chordotonalorgans (fCO) einen schwachen exzitatorischen Einfluss auf die Aktivität von desDUM Neuronen.

Ich untersuchte die Effekte von desDUM Neuronen auf reflexinduzierte, spontane und zentral generierte Aktivität von mesothorakalen Motoneuronen anhand der Aktivierung von einzelnen desDUM Neuronen. Ich konnte zeigen, dass verschiedene desDUM-Neuronen unterschiedliche Wirkungen vermitteln. Einige Neuronen induzieren eine Abnahme und andere eine Zunahme der Stärke der reflexinduzierten Aktivität von Motoneuronen. Die Neuronen, die einen exzitatorischen Einfluss vermittelten, erhöhten zusätzlich die Häufigkeit der Umkehrung eines fCO-induzierten Haltungsreflexes. Einige desDUM-Neuronen vermittelten eine Zunahme der Frequenz von zentral erzeugter rhythmischer Motoneuronenaktivität. Zusammenfassend liefern die Ergebnisse der vorliegenden Arbeit eine umfassende Charakterisierung der desDUM-Neuronen und ihrer komplexen Rolle im Laufsystem der Stabheuschrecke.

Die Identität direkter neuronaler Zielstrukturen für die modulatorische Wirkung von desDUM-Neuronen sowie der Nettoeffekt der gesamten Population von desDUM-Neuronen beim Laufen bleiben offen. In zukünftigen Experimenten könnte der genetische Zugang zu desDUM-Neuronen bei der Aktivierung, Hemmung und Aufzeichnung ihrer Aktivität helfen. Die könnte dazu beitragen die offenen Fragen zu beantworten.

Contents

Abbreviations

AEP	anterior extreme position
AP	action potential
CA	4-hydroxy-3-methoxycinnamaldehyde
cAMP	3',5'-cyclic adenosine monophosphate
CNS	central nervous system
CPG	central pattern generator
CS	campaniform sensilla
DAG	diacylglycerol
desDUM	descending unpaired median
DUM	dorsal unpaired median
EPSP	excitatory postsynaptic potential
ExtTi	extensor tibiae
fCO	femoral chordotonal organ
FT	femur-tibia
GABA	gamma-aminobutyric acid
GNG	gnathal ganglion
IP_3	inositol 1,4,5-triphosphate
MALDI-TOF	matrix assisted laser desorption/ionization-time of flight
MN	motor neuron
MS	mass spectrometry
OA	octopamine
OAR	octopamine receptor
PEP	posterior extreme position
PKA	protein kinase A

PNS	peripheral nervous system
ProCx	protractor coxae
RetCx	retractor coxae
RMP	resting membrane potential
TA	tyramine
TC	thorax-coxa
UM	unpaired median
VUM	ventral unpaired median

1 Introduction

Animal locomotion is often necessary for survival. With the ability to move, animals can access food sources and mating partners and escape predators and negative environmental influences. Effective locomotion requires both robustness to perturbations and flexibility of the locomotor system. This is particularly important for terrestrial legged locomotion, in which walking movements must be adapted to the irregular and unpredictable nature of the ground. Furthermore, temporal coordination of movement between different legs and different joints of single legs has to be adjustable to meet the demands of different gaits and changes in walking direction and speed as well as varying environmental influences in real time. Therefore, the underlying neural networks must exhibit a high degree of flexibility for motor control to be adaptive. In general, legged locomotion is based on contractions of a large number of skeletal muscles that are controlled by motor neurons (MNs). The activity of MNs is under the control of interneurons that are part of central pattern generating networks (central pattern generators; CPGs) located in the spinal cord in vertebrates or thoracic ganglia in arthropods (Bässler and Büschges, 1998; Brown, 1911; Büschges et al., 1995; Chrachri and Clarac, 1987; Kiehn, 2006; Marder and Bucher, 2001; Marder and Rehm, 2005). In legged animals, additional interneurons coordinate the activity of muscle groups between joints of one leg or coordinate muscle contractions among different legs (Bidaye et al., 2018). Feedback from leg sensory organs to the CPGs, coordinating interneurons, or directly to MNs plays an essential role in allowing for plasticity (Bässler and Büschges, 1998; Bidaye et al., 2018; Burrows, 1996; Büschges et al., 2011; Tuthill and Wilson, 2016; Windhorst, 2007). In addition, descending commands from the brain control coordination and the selection of task-specific behaviors (Bidaye et al., 2014; Bidaye et al., 2018; Gal and Libersat, 2006; Hsu et al., 2017; Martin et al., 2015; Mu and Ritzmann, 2008a; 2008b; Szczecinski et al., 2015). On top of that, the intrinsic properties of all these neuronal components of locomotor networks and the strengths of their synaptic connections can be shaped by neuromodulatory input (Katz, 1995; Katz, 1999; Katz and Frost, 1996; Marder, 2012; Miles and Sillar, 2011). Neuromodulators can substantially extend the working range of a

given neuronal network for locomotion. Thus, on one hand, deciphering the topology of locomotor networks (neurons, synapses) alone is not sufficient for the understanding of the mechanisms of locomotor control. On the other hand, knowledge of the constituents of motor circuits and their connectivity is the basis for studying neuromodulatory action. The current thesis was focused on neuromodulatory influences on the activity of thoracic neural networks for the control of walking in the stick insect. At first I want to introduce general aspects of network properties for the generation of locomotion in greater detail.

General mechanisms for flexibility of locomotor control networks

Nervous systems have been clearly shown to possess the ability for intrinsic generation of rhythmic locomotor-like activity in the absence of phasic or rhythmic input (e.g., locust flight: (Wilson, 1961); leech crawling: (Kristan and Calabrese, 1976); stick insect walking: (Bässler and Wegner, 1983); cat hindlimb movements: (Grillner and Zangger, 1979); turtle rostral scratch: (Stein, 2008)). Using the application of muscarinic acetylcholine receptor agonists such as pilocarpine to the isolated ventral nerve cord, it was revealed that the underlying CPG networks for the generation of rhythmic leg MN activity in insects are situated in thoracic ganglia (e.g., locust: (Ryckebusch and Laurent, 1993) stick insect: (Büschges et al., 1995); hawkmoth: (Johnston and Levine, 2002); cockroach: (Fuchs et al., 2011)). Homologous locomotor CPGs in vertebrates are located in the spinal cord (Grillner, 2003; Kiehn, 2006). In the lamprey, application of *N*-methyl-D-aspartate, a synthetic amino acid derivative that mimics glutamate, to an isolated spinal cord preparation induces a swimming-like motor rhythm (Grillner, 2003). For some physiological processes, in some systems, the neural components of the CPGs generating and controlling rhythmicity have been identified (e.g., pyloric rhythm in crustaceans: (Miller and Selverston, 1982); leech heartbeat: (Arbas and Calabrese, 1987); crayfish swimmeret movement: (Mulloney and Smarandache-Wellmann, 2012)). In contrast, the identities of the neural constituents of CPGs controlling terrestrial legged locomotion remain rather elusive (Büschges, 2005, Bidaye et al., 2018). Nevertheless, some aspects of the results from experiments on isolated nerve cord preparations (Büschges et al., 2004), as well as computational modelling approaches, suggest that the CPGs for walking resemble half-center oscillators that control alternating rhythmic activation via mutual inhibition (Daun-Gruhn and Büschges, 2011; Ekeberg et al., 2004). Furthermore the

pattern generators for leg movements appear to comprise multiple CPG networks combined with a modular organization. Some of the evidence for this assumption comes from experiments on pharmacologically induced rhythmic MN activity in the stick insect (Büschges et al., 1995) and the rostral scratch behavior in turtles (Stein, 2008; Stein et al., 2016). Specifically, investigations of rhythmic properties of pilocarpine-induced alternating MN activity in isolated ventral nerve cord preparations in the stick insect suggest that each of the three proximal leg joints is controlled by its own CPG (Büschges, 1995). In the underlying experiments, activity alternated between antagonistic MN pools that control movement at one leg joint, but coupling of the burst frequency to MN pools controlling movement at adjacent joints in the same leg was extremely rarely observed (Büschges, 1995; Büschges, 2005).

In some animals, this centrally generated rhythmic activity closely resembles activity during locomotion (e.g., crayfish fictive swimmeret movement: (Mulloney and Smarandache-Wellmann, 2012); lamprey fictive swimming: (Grillner, 2003); cockroach fictive walking: (Fuchs et al., 2011); hawkmoth fictive walking: (Johnston and Levine, 2002)). However, when flexibility of the locomotor system is demanded, it becomes obvious that the centrally generated output must be shaped by additional coordinating and adapting mechanisms. This holds especially true for the generation of six-legged walking in insects (Bässler and Büschges, 1998; Bidaye et al., 2018). From the perspective of a single leg, walking consists of two phases, stance and swing (Fig. 1.1A and B). During stance phase, the tarsus (foot, distal end of the leg) is placed on the ground, and force is exerted to propel the body in the walking direction. During swing phase, the leg is moved through the air to the initial position of the subsequent stance phase (Graham, 1972). Both phases, and the transitions between them, require appropriate temporal sequential activation of MNs that control contractions of muscles resulting in distinct movements around leg joints (Fig. 1.1C; (Rosenbaum et al., 2010). Additionally, the movements of adjacent legs need to be coordinated well for effective locomotion. When insects change gaits, walking speed, and direction, or when they make turns, the temporal sequential activation of MNs has to be adjusted in a task-specific manner (Borgmann and Büschges, 2015; Grabowska et al., 2012; Gruhn et al., 2016; Rosenbaum et al., 2010; Rosenbaum et al., 2015; Wosnitza et al., 2013). The question remains incompletely answered, though, of what neural mechanisms contribute to coordination and adaptation of CPG-derived output

for walking. In stick insects, for instance, temporal coordination of the movement of single legs is only weakly organized by central drive. In pilocarpine-activated isolated ventral nerve cord preparations, so-called spontaneous recurrent patterns that resemble the motor output during step phase transitions occur only rarely (Büschges et al., 1995). Furthermore, central influences coordinating CPGs supplying adjacent legs are only weak in isolated pharmacologically activated stick insect preparations and do not account for the generation of any of the motor patterns observed during walking (Mantziaris et al., 2017). In contrast, there appear to be rather strong direct connections between CPGs in animals where pharmacologically induced motor output closely resembles the motor output during walking (e.g., cockroach: (Fuchs et al., 2011); hawkmoth: (Johnston and Levine, 2002)).

Studies in insects highlight the importance of the integration of sensory signals with central motor commands for shaping the timing and magnitude of motor output (review: (Bässler and Büschges, 1998; Bidaye et al., 2018; Büschges, 2005; Tuthill and Wilson, 2016). Insect legs comprise a large number of extremely diverse and specialized sensory organs that update the motor system about intrinsic and external mechanical changes (Tuthill and Wilson, 2016). Much of the processing of information from leg sensory organs is already carried out in the PNS, or at least directly between afferents of peripheral sensory organs. There is, for example, presynaptic inhibition in sensory afferents of a stick insect proprioceptive sense organ (Sauer et al., 1997). In general, sensory afferents only rarely exhibit direct synaptic connections to MNs (Burrows, 1987; Parker and Newland, 1995; Pearson et al., 1976). Moreover, in insects, computation of distinct mechanoreceptive signals in the first layers of the central nervous system (CNS) is performed in parallel and locally in nonspiking interneurons of the thoracic ganglia (Bässler and Büschges, 1998; Burrows, 1987; Burrows, 1996; Burrows and Pflüger, 1986; Sauer et al., 1996; Tuthill and Wilson, 2016; Watkins et al., 1985). This allows for sensory information to be processed at the necessary speed and markedly reduces the number of high-level CNS neurons required. Furthermore, intercalated nonspiking interneurons that receive excitatory sensory input are themselves either excitatory or inhibitory. Thus, the actual motor output that controls leg movement is markedly shaped by weighting of these low-level antagonistic pathways of sensory information processing (Bässler and Büschges, 1998; Tuthill and Wilson, 2016). Modification of these local

control modules not only affects the magnitude, but also the sign of motor output in a state-dependent manner. In inactive stick insects, for example, tibial flexion signals, mediated by the femoral chordotonal organ (fCO) lead to an increase in extensor MN activity counteracting the imposed movement. This is and thought to help the animals maintain their posture while standing. This postural reflex is called the resistance reflex. In active animals, this reflex is reversed. Flexion signals now induce an inhibition in extensor MN activity, whereas extension signals induce further extension, which likely facilitates the continuation of movement (Bässler, 1976; 1993; Bässler and Büschges, 1998; Hellekes et al., 2012).

Sensory signals have been shown to be relevant for the patterning of MN activity across joints of a single leg (review: (Bidaye et al., 2018; Tuthill and Wilson, 2016)). In the stick insect, for instance, position and load information was shown to facilitate the generation of motor patterns for the contraction of muscles that underlie step phase transitions (Akay et al., 2004; Zill et al., 2017). Load signals at the coxa-trochanter joint have an influence on the magnitude of motor activity at the femur-tibia (FT) joint (Akay et al., 2001), and the processing of fCO mediated sensory signals of position of the femur in relation to tibia, and movement around the FT joint (Akay and Büschges, 2006). Furthermore, these inter-joint reflexes can couple the activity of distributed CPGs (Akay et al., 2001; Akay et al., 2004; Bässler, 1986; Bucher et al., 2003; Büschges et al., 2008; Hess and Büschges, 1999). In addition, there is a role for sensory signals in the intersegmental modulation of motor activity. Walking legs have been shown to increase and pattern the motor output to neighboring legs (Borgmann et al., 2007; Ludwar et al., 2005). These modulations have been shown to be, in part, based on sensory signals that result from front leg stepping and can entrain the activity of mesothoracic thorax-coxa joint CPGs (Borgmann et al., 2009). When a mesothoracic leg was left attached to the prepared animal, activity of metathoracic CPGs was also influenced (Borgmann et al., 2009). The authors concluded that intrasegmental sensory information appears to override intersegmental signaling (Borgmann et al., 2009; Borgmann et al., 2011).

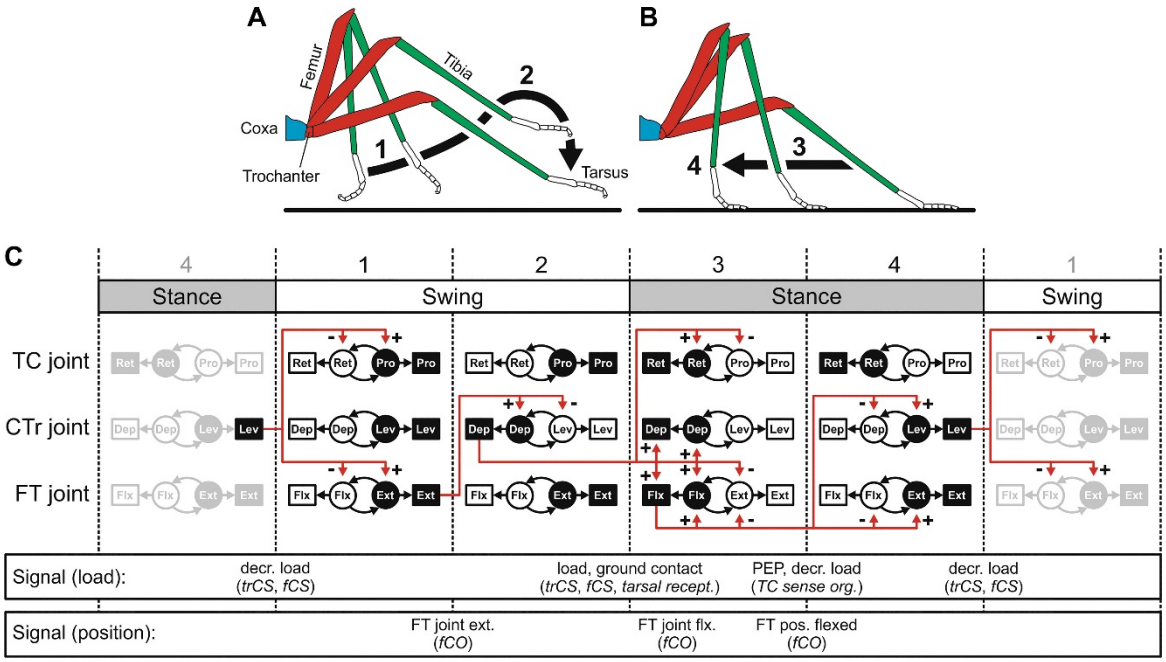

Figure 1.1 Control of single-leg stepping movements in stick insect. (**A**) Leg movement during swing phase. (**B**) Leg movement during stance phase. (**C**) Stepping consists of the cyclic repetition of swing and stance phases. Swing and stance phase can each be subdivided into two distinct phases. There is one CPG that drives MN activity which, in turn, activates muscles for each of the thorax-coxa (TC), coxa-trochanter (CTr), and femur-tibia (FT) joints. Sensory signals (load and position) have a pivotal role in coupling the activity of the distributed CPGs to enable transitions between the different phases of a step cycle. This figure was taken from Bidaye et al. (2018). For further detailed information see Büschges, 2005.

Based on these findings, we can assume that the basic neural circuitry for control of coordinated walking movements in insects is situated in the ventral nerve cord. At the thoracic level, motor control for coordinated legged locomotion is not achieved by a rigid hierarchical command structure but is due to a dynamic interplay between CPG-derived motor activity and sensory signals. However, descending signals from higher centers like the brain or gnathal ganglion (GNG) are crucial for the initiation, cessation, and modulation of effective thoracic motor activity (Bidaye et al., 2018). Furthermore, descending neurons are responsible for the task-specific selection of downstream motor programs (e.g., (Bidaye et al., 2014; Philipsborn et al., 2011; Reyn et al., 2014)). In insects, neurons in the GNG appear to facilitate the maintenance of ongoing behavior (Gal and Libersat, 2006; Kien and Altman, 1992; Ridgel and Ritzmann, 2005; Roeder, 1937). In stick insects, tonic depolarization of MNs contributes to their activity pattern during walking (Gabriel and Büschges, 2007; Ludwar et al., 2005; Westmark et al., 2009). It has been shown that this tonic depolarization does not derive from signals of leg sensory organs but appears to be the result of descending drive (Ludwar et al., 2005;

Westmark et al., 2009). With respect to the influence of the GNG on walking, this might be where the neural source for the tonic depolarization in MN membrane potential is situated.

Inputs from higher brain centers can influence sensorimotor loops in thoracic ganglia. Martin et al. (2015) showed that stimulation of central complex neurons induces the reversal of a postural reflex in the cockroach (Martin et al., 2015). In general, the task-dependent modulation of reflex responses, for example, during curve walking appears to depend on descending signals (Gruhn et al., 2016; Mu and Ritzmann, 2008a; 2008b). In addition, descending commands are crucial for the initiation and selection of motor activity in thoracic ganglia (e.g., fruit fly backward walking: (Bidaye et al., 2014; Sen et al., 2017); initiation of fruit fly voluntary walking: (Yellman et al., 1997); fruit fly courtship behavior: (Philipsborn et al., 2011); fruit fly escape behavior (Reyn et al., 2014; Reyn et al., 2017)). Overall, in animals, the selection of different motor programs does not appear to be accomplished by changes in high-level behavior, but rather by modification of low-level thoracic motor control networks by top-down commands (Bidaye et al., 2018). Basically, all of the neurons in thoracic motor control networks and the synaptic connections between them are subject to neuromodulation (Katz, 1999; Marder, 2012; Miles and Sillar, 2011). Thus, neuromodulatory neurons are an extremely important source of flexibility of the underlying neuronal networks for locomotion and the motor output they generate.

Neuromodulation largely extends the working range of motor control networks

A wide variety of chemical substances have been shown to exert effects on motor activity in both vertebrates and invertebrates. The major neuromodulators can be grouped into three categories. There are biogenic amines like serotonin, dopamine, and noradrenaline (octopamine in invertebrates); amino acids like gamma-aminobutyric acid (GABA) and glutamate; and peptidergic neuromodulators like substance P and proctolin (Katz, 1999; Miles and Sillar, 2011). With respect to the compartments these substances are released to, and the receptors they activate many of them can act as neuromodulators, neurohormones, and even neurotransmitters. Typically, neuromodulators exert their effects by acting as agonists of metabotropic receptors (Katz, 1999). With respect to the intracellular second-messenger cascades that modulators activate, the effects usually have a slow time course in contrast to the effects of synaptic neurotransmission via ionotropic receptors (Hultborn and Kiehn, 1992; Katz, 1995; Levitan, 1988; Lopez and Brown, 1992). Neuromodulators do not fixedly transmit excitation or inhibition to their target structures, but rather alter cellular or synaptic properties, which influences the impact of subsequent neurotransmission (Katz, 1995). Despite the aforementioned properties of neuromodulatory action, finding a rigid comprehensive definition for the term "neuromodulation" is difficult. This becomes apparent when a statement of Katz (1999) is considered, who claimed that "any communication between neurons, caused by release of a chemical, that is either not fast, or point-to-point, or not simply excitation or inhibition will be classified neuromodulatory."

Neuromodulators are released by neurons, and this can be distinguished between intrinsic and extrinsic at the level of integration of neuromodulatory neurons in neural control circuits (Katz, 1995). Intrinsic neuromodulation implies that the neuromodulatory neuron is an integral part of the neural circuit it modulates. One form of intrinsic neuromodulation is the simultaneous co-transmission of neuromodulatory substances and conventional fast transmitters at a synapse (Katz, 1999; Miles and Sillar, 2011). As an example for intrinsic neuromodulation, serotonergic neurons have been shown to be constituents of a CPG network that controls escape swimming in a mollusk, and these neurons modulate activity of other constituents of the same control network (McClellan et al., 1994). With regard to intrinsic neuromodulation, the release of a neuromodulator is

dependent on the activity of the neural circuit they are part of. This is in contrast to extrinsic neuromodulation, where the respective neuromodulatory neuron is located outside the neural circuit it affects. Thus, the release of neuromodulator is largely independent of the activity of the affected neural circuit. The best studied neural circuit influenced by extrinsic neuromodulation is the pyloric CPG of the spiny lobster stomatogastric ganglion (STG) (Marder, 2012). In this system, extrinsic modulatory input originates in central ganglia or sensory organs (Katz and Harris-Warrick, 1990a; 1990b).
In general, extrinsic modulatory neurons are not part of the circuits they modulate but are often activated in parallel to the neural circuits they affect (Katz, 1995). Octopaminergic efferent dorsal unpaired median (DUM) neurons in locust thoracic ganglia, for example, affect neuromuscular transmission to leg muscles and are activated in parallel to MNs responsible for walking (Baudoux et al., 1998; Duch et al., 1999). Extrinsic neuromodulation often comprises modulation of activity of more than one neural circuit (Katz, 1999). With respect to the sign and magnitude of neuromodulatory effects on a circuit level, not only the identity of the neuroactive substance but the timing and amount of its release, the type of receptor it activates, and the output properties of its target neurons are crucial (Katz, 1999; Marder, 2012; Miles and Sillar, 2011). The pyloric CPG of the spiny lobster, for instance, is differentially modulated by three neuromodulatory neurons that all contain proctolin (Nusbaum et al., 2001). Serotonin alike has been shown to exert different effects on pyloric CPG neurons as a result of activation of distinct serotonin receptors (Zhang and Harris-Warrick, 1994). Miles and Sillar (2011) note that all individual constituents of neural control networks for locomotion perpetually face a varying neuromodulatory environment, which is the pivotal aspect of flexibility.

Octopamine is a prominent neuromodulator in insect locomotor control

In the current thesis, I focused on the neuromodulatory substance octopamine (OA) and its effects on the generation of legged locomotion. This biogenic amine is considered to be the invertebrate homologue to the vertebrate noradrenaline, both with regard to its structure and physiological role (Roeder, 2005). Acting as a neurohormone, OA released to the haemolymph affects cell metabolism by mobilizing lipids and increasing glycolytic activity in muscles (Goosey and Candy, 1980; Mentel et al., 2003; Pflüger and Duch, 2011). Acting as a neuromodulator, OA has a prominent role in influencing multiple

physiological events both in the PNS and the CNS (Bräunig and Pflüger, 2001; Roeder, 2005; Verlinden et al., 2010). With respect to locomotion, the peripheral effects of OA are modulation of neuromuscular transmission and changes in the contraction kinetics of muscles (e.g., (Evans and O'Shea, 1977; Evans and Siegler, 1982)). Furthermore, OA affects the activity of sensory organs (Farooqui, 2007). In stick insects, for example, OA selectively increases the excitability of position-sensitive fCO neurons, but does not affect velocity-sensitive sensory neurons (Ramirez et al., 1993). Similarly, OA sensitizes sensory neurons of the locust forewing stretch receptor (Ramirez and Orchard, 1990). In addition to its effects in the PNS, OA has been shown to affect a wide array of complex behaviors. This has been demonstrated, for example, for fruit fly sleep/wake behavior (Crocker et al., 2010), aggression in crickets (Rillich et al., 2011; Rillich and Stevenson, 2011), division of labor in honeybee and ant colonies (Schulz and Robinson, 2001; Seid and Traniello, 2005), and learning and memory in honeybees (Hammer, 1993; Mercer et al., 1983; Schwaerzel et al., 2003).

With respect to locomotor activity generated in thoracic ganglia, OA has been shown to affect the activity of MNs, coordinating neurons, and CPG neurons (review: (Bräunig and Pflüger, 2001; Marder, 2012; Roeder, 2005; Verlinden et al., 2010)). OA was, for example, shown to induce bursting and plateau potentials in interneurons of the locust flight pattern generator (Ramirez and Pearson, 1991a; 1991b). Additionally, it mediates sensitization and dishabituation in a locust postural reflex (Sombati and Hoyle, 1984a), and it can evoke a fictive flight motor pattern in locusts (Claassen and Kammer, 1986; Rillich et al., 2013). Moreover, OA decreases the inhibitory postsynaptic potentials evoked by stretch receptor activity in locust flight MNs (Leitch et al., 2003). In inactive stick insects, OA selectively alters the response properties of a leg proprioceptive feedback system toward those that characterize the active state of animals (Büschges et al., 1993). Furthermore, OA modulates the tonic depolarization ubiquitous in mesothoracic leg MNs during walking in stick insects (Westmark et al., 2009). In summary, OA modulates muscle metabolism, neuromuscular transmission, sensory sensitivity, MN excitability, and activity in CPG circuits that control locomotion.

The neuromodulatory effects of OA are mediated by octopamine receptors (OARs) that are expressed in pharmacologically distinct forms and localized to the membrane of target

neurons. OARs are coupled to G proteins and activate various second-messenger cascades that can include 3',5'-cyclic adenosine monophosphate (cAMP), inositol 1,4,5-triphosphate (IP_3), and diacylglycerol (DAG). These second messengers not only modulate levels of intracellular Ca^{2+} but also regulate phosphorylation of various signaling proteins involved in differential fine-tuning of neuronal output. The second messenger cAMP activates protein kinase A, IP_3 mobilizes calcium from intracellular stores, and DAG activates protein kinase C. Through the various effects of their activation, OARs can markedly influence the electrical properties of target neurons. On a cellular level, OAR activation can modulate persistent inward currents, which can depolarize the neuron to facilitate AP generation or induce plateau potentials. OA can broaden action potentials (APs) and modulate calcium-dependent potassium currents that shape AP afterhyperpolarizations (Blenau and Baumann, 2001; Evans, 1993; Evans and Robb, 1993; Farooqui, 2007; Grohmann et al., 2003; Roeder et al., 1995; Roeder, 2005). In drosophila sleep/wake behavior, for example, OA signals via cAMP and protein kinase A (PKA) to selectively decrease the potassium current from calcium-dependent Slowpoke channels in target neurons, which leads to increased excitability (Crocker et al., 2010).

Classification and distribution of octopaminergic neurons

OA and octopaminergic neural structures (dendritic processes) are remarkably abundant in the insect CNS and PNS (Axelrod and Saavedra, 1977; Evans, 1978). In contrast, the number of octopaminergic neurons in the nervous system is surprisingly small. Roeder (2005) suggests that there are only about 100 such neurons in large insects like locusts (Roeder, 2005). Busch et al. (2009) note that the number of octopaminergic neurons in the relatively small fruit fly is also about 100. The major cellular source for OA acting in the PNS of thoracic and abdominal segments is the unpaired median (UM) neurons. These neurons appear as individuals (unpaired) and have symmetrical bilaterally projecting axons with extensive branches. Their large somata are located either at the dorsal (DUM) or ventral (VUM) midline of the respective ganglia (Bräunig and Pflüger, 2001). Most of the thoracic UM neurons were only shown to possess peripheral release sites for OA (Kononenko and Pflüger, 2007; Stocker et al., 2018; Watson, 1984). They directly affect contraction properties of muscles, neuromuscular transmission, muscle metabolism, or activity of sense organs in the periphery (Bräunig and Pflüger, 2001; Roeder, 2005).

Efferent DUM neurons are activated in parallel to locomotion and are responsive to mechanosensory signals in a multimodal fashion (Duch et al., 1999; Field et al., 2008; Gras et al., 1990; Mentel et al., 2008). In addition to UM neurons, there are relatively few paired octopaminergic neurons with small somata in the thoracic and abdominal ganglia (Stevenson and Spörhase-Eichmann, 1995); their physiological functions are unknown. Similarly, the neural source of OA acting as a neuromodulator in the CNS of thoracic segments has not yet been identified.

Further octopaminergic UM neurons are situated in the GNG, but not in the brain. The majority of GNG UM neurons project intersegmentally (Bräunig, 1991; Bräunig and Burrows, 2004; Bräunig and Pflüger, 2001; Busch et al., 2009). In the posterior locust GNG, for instance, there are six DUM neurons that send bilaterally descending axons to the thoracic ganglia (abbreviated desDUM neurons; Fig.1.2 (Bräunig and Burrows, 2004)). Bräunig and Burrows (2004) showed that these neurons arborize in regions of neuropil where information arising from leg sensory organs is processed. The population of six locust desDUM neurons comprises three morphologically heterogeneous subpopulations (Bräunig and Burrows, 2004). The presence of homologues neurons was demonstrated in several other insects like the hawk moth (Cholewa and Pfluger, 2009; Dacks et al., 2005); honeybee (Schröter et al., 2007; Kreissl et al., 1994; Sinakevitch et al., 2005); fruit fly (Busch et al., 2009; Selcho et al., 2012); and cockroach (Sinakevitch et al., 2005; Stevenson and Spörhase-Eichmann, 1995). With regard to the number of desUM neurons and their basic morphological properties, there are marked similarities between different insect species. Despite an investigation of input to locust desDUM neurons from electrical stimulation of leg nerves (Bräunig et al., 2004), as well as coupling of larval hawk moth desVUM neurons to a centrally generated motor rhythm (Cholewa and Pfluger, 2009), the physiology of desUM neurons has not been addressed to date. This holds especially for the output physiology of the neurons.

Retrograde labeling of the posterior connectives in stick insects revealed that there are putative desDUM neurons with large somata and bilaterally descending axons in the posterior part of the GNG (Fig. 1.2B; (Heß, 2008). On the assumption of morphological homology to locust desDUM neurons (Fig. 1.2A), these neurons are promising candidates for the octopaminergic modulation of thoracic local control networks for legged locomotion.

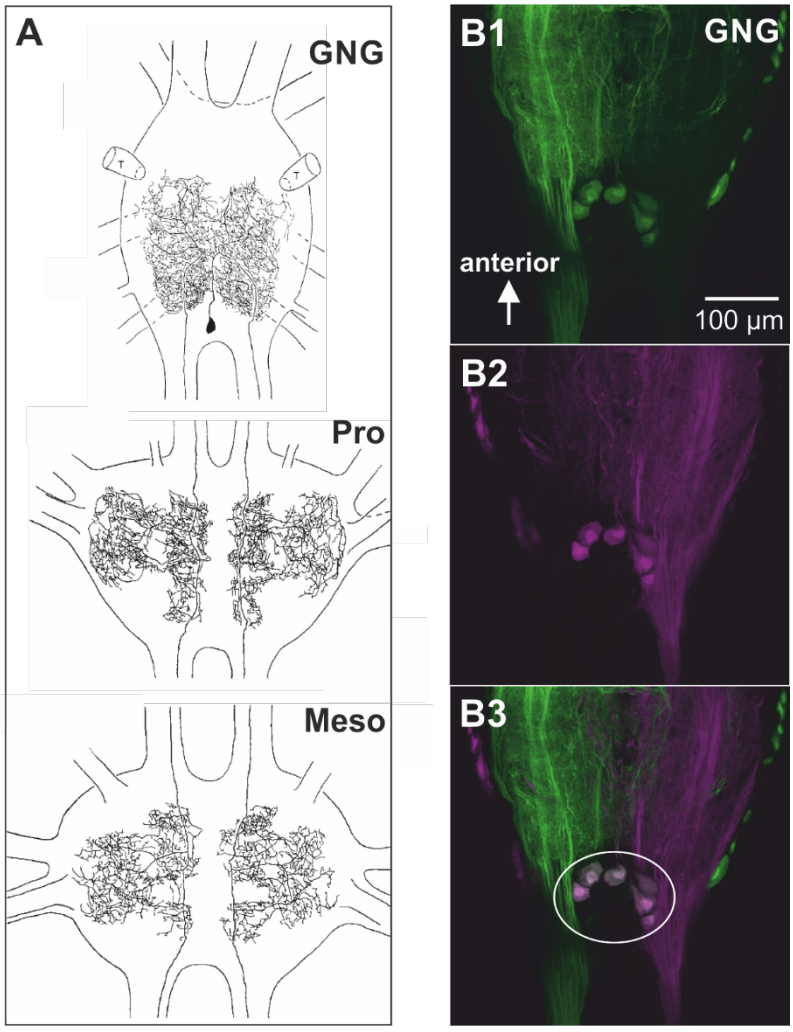

Figure 1.2 Morphology and somata location of desDUM neurons. (A) Morphology of one of six locust desDUM neurons. The large somata of desDUM neurons are located in the posterior GNG. A primary neurite is projecting anteriorly and bifurcates into two bilaterally symmetrical axons. The axons extensively branch in the posterior half of the GNG, but do not send projections to the periphery. Two bilaterally symmetrical axons project to thoracic ganglia, where they are extensively branching. (B) In the stick insect GNG, six large somata of neurons with bilateral symmetrically descending axons were labeled by retrograde fills of the posterior connectives. The figure was modified from (Heß, 2008). (B1) Labeling of the left posterior connective. (B2) Labeling of the right posterior connective. (B3) Co-localization of fluorescent dyes in six large somata.

This thesis

MNs are at the final level of neural processing for the generation of walking. Their activity reflects their intrinsic properties and, moreover, the net output of the upstream neural circuitry and effects of neuromodulatory neurons. In stick insects, the activity of MNs is patterned by tonic depolarizing drive as well as rhythmic excitatory and inhibitory inputs (Büschges, 1998; Ludwar et al., 2005; Rosenbaum et al., 2015; Schmidt et al., 2001; Westmark et al., 2009). To fully understand the mechanisms of neural circuit function and neuromodulation for walking, it is crucial to decipher the neural basis for the activity pattern of MNs. In particular, the underlying neural sources of the depolarizing drive to MNs during walking are unknown. Westmark et al. (2009) targeted the pharmacological background of the tonic depolarization and highlight a modulatory role for OA. As outlined in the previous sections, OA is a major neuromodulator in locomotor behavior, but the neural source of OA acting in the CNS of thoracic segments of insects has remained elusive. Most of the findings on OA action in the insect thoracic nerve cord are based on systemic application of OA or blocking OAR action. The question arises, then, of to what extent these rather unspecific alterations in behavior or physiological processes reflect the physiological action of OA in the intact animal. Systemic application of OA does not consider the timing of natural modulator release in a behavioral context, the physiological amount of the modulator, or the specific target sites the modulator is released to. The need for physiological modulator release, for example, by stimulation of identified neuron becomes apparent in a study by Flamm and Harris-Warrick (1986). The sign of octopaminergic effects on constituents of the pyloric CPG in the lobster was dependent on the applied concentration of OA (Flamm and Harris-Warrick, 1986). In a study by Westmark et al. (2009), the sign of OA action on tonic depolarization in MN membrane potential was dependent on the circuit level of OA application. Bath application of OA to the entire mesothoracic ganglion induced an increase in the tonic depolarization in MNs, whereas application of OA to isolated MN somata decreased an inward current that contributes to the tonic depolarization in MN membrane potential (Westmark et al., 2009). The current thesis is based on the hypothesis that the activity of local control networks for walking in thoracic ganglia of insects is modulated by octopaminergic desDUM neurons.

The first part of this thesis is dedicated to the identification of desDUM neurons in stick insects. I used intracellular recordings of desDUM neurons to characterize their electrical properties, and investigated these neurons' morphology using intracellular labeling. Furthermore, I employed a newly developed method of mass spectrometry (matrix assisted laser desorption/ionization-time of flight mass spectrometry; MALDI-TOF MS) to analyze the OA content of electrophysiologically identified stick insect desDUM neurons.

In the second part of this thesis, I asked if the activity of desDUM neurons is coupled to walking behavior. Activation of these neurons during walking is a precondition for their neuromodulatory role on locomotor circuit action. In addition, knowledge of the magnitude and timing of desDUM neuron activation is a prerequisite for physiologically relevant stimulation of the neurons to test their output properties. By modifying established semi-intact preparations in combination with intracellular recording, I studied the activity of desDUM neurons during six-legged walking and single-leg stepping. Furthermore, I studied the neural background of walking-related activity. Using pharmacological activation of leg joint CPGs, I tested the effects of central synaptic drive on desDUM neurons. Finally, with specific stimulation of leg sensory organs, I tested the role of sensory signals in activating desDUM neurons.

In the third part of this thesis, I analyzed the modulatory role of desDUM neurons on activity of mesothoracic MNs. I activated single desDUM neurons by current injection and studied their effects on fCO-evoked reflexes. I used this approach, because stimulation of the fCO can be easily done in a controlled fashion. Furthermore stimulation of the fCO induces resistance reflexes in ExtTi MNs (Bässler, 1976; 1983; Bässler and Büschges, 1998). that are very stereotypic after a short time for habituation (Stein and Sauer, 1998). Thus, ExtTi MN activity induced by stimulation of the fCO can function as a stable control in comparison to putative alteration upon desDUM neuron activation. The reflex response to fCO stimulation is dependent on the state of the animal (active vs. inactive;(Bässler, 1976)). Thus, it may be possible to detect effects of desDUM neuron stimulation on the state-dependent processing of reflex responses. Büschges et al. (1993) demonstrated that bath application of OA specifically suppressed neural pathways involved in the generation of resistance reflexes. The neuronal source of OA is not

known. Therefore, my experiments could possibly target the question of whether OA application might mimic the effects of desDUM neuron activity. To test whether other modes of sensorimotor interaction are modulated by des DUM neurons, I studied their effects on ProCx and RetCx MN activity evoked by stimulation of campaniform sensilla (CS). In addition, I asked whether desDUM neurons have an influence on centrally generated MN activity. Collectively, these methods allow for a comprehensive characterization of desDUM neurons and their complex roles in locomotor networks.

2 Materials and Methods

All experiments were carried out on adult female stick insects (*Carausius morosus*) from a colony maintained at the University of Cologne. Experiments were conducted under dimmed light conditions at room temperature (20-24°C).

2.1 Basic semi-intact stick insect preparation

All legs except one middle leg were cut at the level of the mid coxa. Animals were affixed to a platform dorsal side up using dental cement (Protemp II, 3M ESPE Dental AG, Seefeld, Germany). The head capsule and thorax were opened dorsally by cutting a rectangular window into the cuticle. The gut, fat tissue, and small tracheae were removed to gain access to the gnathal ganglion (GNG), the mesothoracic ganglion, and mesothoracic lateral nerves. The body cavity of the animal was rinsed and filled with saline according to Weidler and Diecke (in mmol: NaCl 180; KCl 18; $CaCl_2$ 8; $MgCl_2$ 25; HEPES buffer 10; pH 7.2; (Weidler and Diecke, 1969)).

2.2 Intracellular recordings

For intracellular recordings from desDUM neurons, the GNG was lifted onto a wax coated steel platform while still connected to the animal. Loose tissue surrounding the ganglion was removed with forceps. To ease electrode penetration, small crystals of a proteolytic enzyme (Pronase E; Merck, Darmstadt, Germany) were placed on the ganglionic sheath for about 10-20 s. The enzyme was thoroughly washed off with saline. Intracellular recordings from desDUM neurons were made from their somata located in the posterior part of the GNG at the origin of the posterior connectives. Borosilicate glass micropipettes (GB100-TF8P; Science Products, Hofheim, Germany) with resistances of 15-35 MΩ were manufactured on a filament puller (P-97; Sutter Instruments, Novato, CA, USA). The electrodes were filled with a mixture of 0.1 M KCl and 3 M CH_3CO_2K. Cells were identified by their large-amplitude (>70 mV), overshooting soma action

potentials (APs), pronounced underhoot (>7 mV) and the generation of APs in response to gentle touch of the abdomen with a paint brush. Membrane potentials were amplified with an intracellular amplifier (SEC-10L; NPI Electronics, Tamm, Germany) in bridge mode. Recordings were stored on a PC using Spike 2 software (Version 7.09, Cambridge Electronic Design, Ltd., Cambridge, UK).

2.3 Intracellular neurobiotin staining of desDUM neurons

In 30 experiments, desDUM neurons were successfully stained intracellularly. Electrodes were filled with 5% Neurobiotin™ (Vector Laboratories Inc., Burlingame, CA, USA) in 0.1 M KCl/3 M CH_3CO_2K. The tracer was injected into desDUM somata with depolarizing current pulses of 1-4 nA with 400-ms pulse duration at 1 Hz for 5-15 min. After tracer injection and 45-60 min incubation for tracer diffusion, the ganglia were removed from the animal and fixed in 4% paraformaldehyde in 0.1 M PBS for 2-16 h at 4 °C. The ganglia were then washed (3 x 15 min in 0.1 M PBS) and incubated in Cy3-conjugated streptavidin (1:500 in 0.1 M PBS; Sigma-Aldrich, St. Louis, MO, USA) with 0.5% Triton X-100 and 2-4% normal goat serum (NGS; Vector Laboratories Inc) for 5-16 h at 4 °C on a shaker. The ganglia were washed again (3 x 15 min with 0.1 M PBS) and dehydrated in an ascending ethanol series (50%, 70%, 90%, 2 x 100% EtOH; 10 min each). To clear the tissue, the ganglia were transferred to a microscope slide and embedded in methyl salycilate. Specimens were examined using a laser scanning confocal microscope (Zeiss 510 META; Carl Zeiss AG).

2.4 Extracellular recording from leg-nerves

Extracellular recordings from leg nerves were done using modified monopolar hook electrodes (Schmitz et al., 1988). Recordings were amplified (500x-2000x, depending on the recording quality) and filtered (250Hz- 5kHz). The recordings were digitized by a MICRO 1401 A/D converter (sampling rate: 12.5 kHz). Leg nerves were labeled according to literature (Bässler, 1983; Marquardt, 1940).

2.5 Analysis of octopamine/tyramine content with MALDI-TOF MS[1]

Individual somata were marked by tetramethylrhodamine dextran (5%, 3000 Dalton (MW), Invitrogen, Eugene, Oregon, USA) injection after electrophysiological recording. The somata were dissected and treated after Diesner and Neupert (2018 in prep.). For this, isolated GNGs were transferred to a new dissection dish filled with saline containing 33% glycerol (v/v). Marked cell bodies were visualized and dissected under a fluorescence stereomicroscope (Lumar V12; Carl Zeiss AG) using fine forceps (Dumont #5; Fine Science Tools, Heidelberg, Germany) and, immediately after isolation, transferred onto a MALDI sample plate using a glass capillary. Any excess dissection buffer was removed with the same glass capillary. Subsequently, remaining glycerol was washed off with 50% MeOH/H_2O using a fresh glass capillary. Furthermore, 18.4 nl of isotopically marked internal standard, containing 100 nM (±)-p-octopamine-α,β,β-d3 hydrochloride (OA[*d3*]) and 100 nM 2-(4-Hydroxyphenyl) ethyl-1,1,2,2-d4-amine hydrochlorid (TA[*d4*]) dissolved in 50% MeOH/H_2O (CDN Isotopes, Pointe-Claire, Canada) was applied to the samples. Each preparation was air-dried in darkness at room temperature. After that, 9.2 nl of 4.27 mM 4-hydroxy-3-methoxycinnamaldehyde (CA; in 50% MeOH) was applied and samples were air-dried again. In the last step, 18.4 nl of saturated α-cyano-4-hydroxycinnamic acid diluted 1:6 in 80% MeOH/H_2O (Sigma-Aldrich) was applied as matrix solution to the sample. For a more consistent distribution of matrix crystals, the sample spot was directly dried under an air stream at room temperature. All solutions were pipetted using a micro injector (Nanoliter 2000; World Precision Instruments, Sarasota, FL, USA) under a stereomicroscope (STEMI 2000; Carl Zeiss AG) equipped with a KL 1500 LED light source (Schott, Mainz, Germany).

Samples were analyzed with an ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik Gmbh, Bremen, Germany) under manual control in reflectron positive

[1] Experiments of this section were performed in collaboration with Max Diesner and Dr. Susanne Neupert (University of Cologne, Department of Biology, Institute for Zoology). Contributions: M.D. and S.N. designed research, M.D. and T.S. performed experiments, M.D. analyzed the data, M.D. and T.S. created the figures and wrote the text.

ion mode in a mass range of m/z 0-400. All mass spectra were acquired with a fixed laser intensity. The instrument was calibrated using prominent matrix signals ($[M+H-CO_2]^+$: m/z 146.0606, $[M+H-H_2O]^+$: m/z 172.0399, $[M+Na]^+$: m/z 212.0324, $[M+K]^+$: m/z 228.0063, $[2M+H-CO_2]^+$: m/z 335.1032, $[2M+H]^+$: m/z 379.093) with a mass accuracy below 20 ppm. Two thousand laser shots per sample spot were accumulated for one MS spectrum. Fragmentation data to verify OA and OA(*d3*) were acquired in "LIFT" mode. MS^2 experiments to identify TA and TA(*d4*) were recorded in collision-induced dissociation (CID)-on mode, with argon as the collision gas. All masses given herein and masses labelling ion signals are monoisotopic masses.

2.6 Single-legged stepping and six-legged walking preparations and passive leg movement

To monitor the activity of desDUM neurons during single-legged stepping, a low-friction treadmill (Gabriel et al., 2003) was positioned under the middle leg parallel to the longitudinal axis of the animal. Stance phase of the middle leg led to acceleration of the treadmill. Upward deflection of the tachometer trace represented the begin of stance phase during forward middle-leg stepping. Downward deflection indicated begin of a stance phase during backward middle-leg stepping. The maximum deflection in the tachometer trace refers to the end of a stance phase. When stepping sequences did not occur spontaneously, they were induced by brief tactile stimulation of the abdomen with a paint brush to activate the animal. Steps that occurred during, and just after tactile stimulation were not considered in the evaluation of their effect on desDUM neurons. Passive movements of single middle legs that were standing on the treadmill were induced by movement of the treadmill via the motor or by hand. The initial position of the femur was anteriorly directed at about 70° inner angle in respect to the longitudinal axis of the animal. From this position, the leg was moved backwards by about 40°. Passive backward movement was followed by a pause of 1-2 s and subsequent passive forward movement of the leg to the initial position. During movements, the leg remained on the treadmill. To study the effects of six-legged walking on activity of desDUM neurons , animals were glued dorsal side up on a piece of balsa wood. All legs were left intact, and leg movement was not restricted. One treadmill on each side of the animal was placed

parallel to the animals' longitudinal axis. Coordination of the legs during six-legged walking on two treadmills was mainly that of a tetrapod coordination pattern.

2.7 Pilocarpine-evoked rhythmic motor neuron activity

Influence of CPGs on activity of desDUM neurons

The following experiments were performed according to Borgmann et al. (2007). The thoracic cuticle was opened dorsally to gain access to the prothoracic and mesothoracic leg nerves, and the leg nerves were cut. A 3-mm segment of cuticle between the prothoracic and mesothoracic ganglia was removed. Tracheae and connectives were left intact. The resulting gap was filled with vaseline to create two separated wells, and both wells were filled with saline. The connectives were cut posterior to the mesothoracic ganglion. Activity in the mesothoracic nl2 and nl5 leg nerves was recorded with hook electrodes. The nl2 nerve contains axons of *protractor* (ProCx) MNs, and nl5 contains axons of the antagonistic *retractor* (RetCx) MNs. As soon as a stable intracellular recording from a desDUM neuron was obtained, the posterior well was filled with the muscarinic acetylcholine (ACh)-receptor agonist pilocarpine (5 mmol in saline). About 2-5 min after pilocarpine application, rhythmic oscillatory activity could be observed in the antagonistic MN pools. This activity was used as an indicator for ongoing activity in leg-joint CPGs (Büschges et al., 1995). Responses in desDUM neurons were recorded intracellularly.

Output effects of desDUM neurons on CPG activity

The same preparation was used to study both inputs to desDUM neurons as well as the neurons effect on CPG neurons. After at least 5 min of pilocarpine-induced MN activity as control action potential (AP) generation in a single desDUM neuron was induced by depolarizing current injection to somata for 60 s. After that again followed at least 5 min of control, before the same desDUM neuron was activated again. For evaluation of nl2 and nl5 burst properties I used custom written spike2 scripts. Thereby, APs in the respective MN pools were identified by signal falling through a threshold that was adjusted manually fitting the signal to noise ratio as well as signal amplification in any given experiment. APs were thereupon transformed to uniform events for further analysis. Bursts were defined as temporal aggregation of APs of excitatory MNs comprising at

least five APs with a maximum interspike-interval of 0.5 s. Period was the time from onset of one burst to onset of the next burst of a MN pool. Only experiments in with stable rhythmicity (alternating activity of antagonistic MN pools, maximum burst duration of 30 s) were considered for evaluation.

2.8 Stimulation of the femoral chordotonal organ (fCO)

Effect of fCO stimulation on desDUM neurons

Stimulation of the femoral chordotonal organ (fCO) of the mesothoracic leg was performed according to Hellekes et al. (2012). The intact middle leg was amputated at the middle of the tibia. All other legs were removed at the level of mid-coxa. The remaining leg was fixed perpendicular to the longitudinal axis of the animal and glued to a platform resulting in an angle of about 120° between femur and tibia. A small window was cut in the dorsal cuticle of the femur to gain access to the nerve F2 containing *extensor tibiae* (ExtTi) MN axons, and the receptor apodeme of the fCO. A vaseline well around the leg was filled with saline (Weidler and Diecke, 1969), the apodeme of the fCO was cut, and the distal ending was attached to a clamp. Mechanical displacements of the apodeme parallel to the leg and towards or away from the body were produced by a piezoelectric actuator driven by a ramp generator (Electronics Workshop, Zoological Institute, University of Cologne). The fCO was activated with ramp-and-hold stimuli (2s duration) which produced displacements of the apodeme of 300 µm from the starting position. These displacements corresponded to changes in FT joint angle (inner angle) from 120° to 60° (Weiland et al., 1986). ExtTi MN activity was monitored extracellularly from the leg nerve F2, which contains axons of the excitatory fast ExtTi MN (FETi), the slow ExtTi MN (SETi), and the inhibitory MN common inhibitor 1 (CI_1) (Goldammer et al., 2012). The single units of all three MNs could be readily distinguished in the extracellular recording trace by their characteristic AP amplitude. Successful stimulation of the fCO was monitored by the occurrence of resistance reflex responses characterized by activation of ExtTi MNs during elongation of the apodeme (Bässler, 1976; 1983). Responses of desDUM neurons were recorded intracellularly. For evaluation of the effects of fCO stimulation by means of EPSP amplitude and latency of occurrence, averages of the time course of desDUM neuron membrane potential were created using spike2 software.

Output effects of desDUM neurons on fCO induced ExtTi MN activity

Reflexes in extensor MNs were induced by continuous fCO stimulation with afore mentioned properties. After at least 2 min of time for habituation of the reflex responses, a single desDUM neuron was activated by intracellular depolarizing current injection for 30 s (Fig. 3.1 B and C). Within 30 s there occur 15 cycles of fCO stimulation. In detail, desDUM neurons were stimulated with rectangular depolarizing current pulses with a frequency of 20 Hz and duty cycle of 20 ms. The amplitude of injected current was adjusted to yield AP generation in the stimulated desDUM neuron at a mean frequency of 25- 40 Hz. In some experiments the parameters of activation were altered. This is stated in the corresponding sections of the results part. In the experiments, 30 s of control prior to desDUM neuron activation (control), 30 s of desDUM neuron activation (desDUM stim.) and two consecutive 30 s sections (post1, post2) after desDUM neuron stimulation resembled one trial (Fig. 2.1B). Each experiment consisted of at least three trials with intervals of at least 4 min between single trials (Fig2.1C). For evaluation of the effects of desDUM neuron activation a custom written spike2 script was used. APs of FETi, SETi and CI_1 MNs were identified by voltage signal falling through a threshold that was adjusted manually, fitting the signal to noise ratio, as well as signal amplification in a given experiment. APs of the respective MN units were then transformed to uniform events. As a basis for further evaluation, the APs of the three extensor MNs were each binned in 200-ms intervals, for every 2-s cycle of fCO stimulation (Fig. 2.1A). The effect of desDUM neurons on extensor MN activity 44 experiments was evaluated and illustrated in three different ways.

(I) Results section 3.3.1.1.3. In order to assess the effects on properties of fCO-induced reflex responses, histograms of activity of the single MN units over the 2-s time course of fCO stimulation were generated. APs of MNs were summed in 200 ms intervals for control section, desDUM neuron stimulation, post1, and post2 in the three trials of an experiment. To be able to compare in between animals and single experiments, the activity was normalized to the average activity of the 3rd control bin in each experiment

(II) Results section 3.3.1.1.1. For investigation of the time course of effects of desDUM neuron stimulation, SETi APs were summed up over the 2-s of single cycles of fCO stimulation (Fig. 2.1A). Averages of the activity over trials were created for each of the 60 fCO stimulation cycles of one trial. To be able to compare in between experiments and

animals the activity during every fCO stimulation cycle was normalized to the average activity of the 15 control stim. cycles in each trial. Here the normalization was only possible for SETi MNs, because APs of FETi MNs and CI_1 neurons were not generated during all control fCO stim. cycles.

(III) Results section 3.3.1.1.2. To better assess the net output of a given desDUM neuron subpopulation on all units of the ExtTi MN pool, the APs of the extensor MN units generated in the four 30-s sections of each trial were summed up and averaged over trials. To be able to compare in between single experiments, the activity was normalized to control in each experiment.

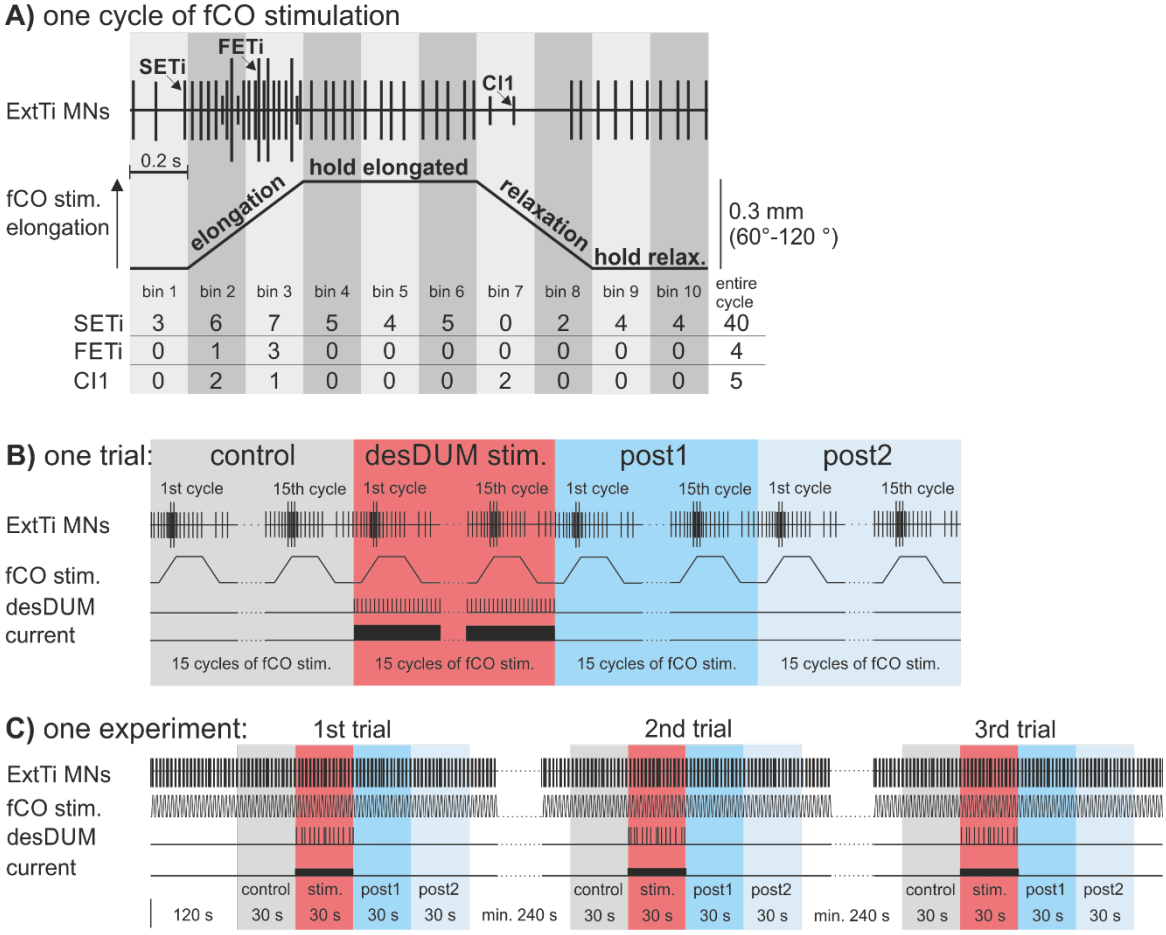

Figure 2.1 Stimulation of the femoral chordotonal organ. (**A**) The fCO was stimulated by repetitive elongation and relaxation of the fCO receptor apodeme. The applied stimuli followed a 2 s ramp-and-hold function. Reflex responses in extensor MNs were monitored by recording from leg nerve F2. The single units of the extensor MN pool can be distinguished according to their distinct AP amplitudes in the extracellular recording (for details: see text). (**B**) and (**C**) I studied the effect of desDUM neuron activation on fCO stimulation-induced extensor MN activity. One experiment consisted of three trials (C). For evaluation of the effects, each trial was subdivided in 4 sections that each consisted of 15 fCO stim. cycles (for details: see text).

2.9 Stimulation of campaniform sensilla (CS)

Effect of CS stimulation on activity of desDUM neurons

Campaniform sensilla (CS) were stimulated according to Akay et al. 2007. All legs were amputated except for one middle leg. The remaining middle leg was cut at two-thirds of the length of the femur. The leg was positioned perpendicular to the animals' longitudinal axis and parallel to the horizontal plane. Leg movement was prevented by gluing the coxa to the platform using dental cement. The femur of the middle leg was bent with a piezoelectric actuator driven by a ramp generator (Electronics Workshop, University of Cologne). Stimuli followed a ramp-and-hold function (4-s duration) and resulted in posterior bending of the leg by 200 µm. Successful stimulation of CS was monitored by the occurrence of phasic tonic responses in extracellularly recorded ProCx and RetCx MNs (Schmitz et al. 1993). DesDUM neurons were recorded intracellularly, and ProCx and RetCx MN responses to continuous CS stimulation were simultaneously recorded extracellularly.

Output Effects of desDUM neurons on CS-induced ProCx and RetCx MN activity

After an initial 200 s of CS stimulation, single desDUM neurons were activated by injection of positive current pulses (20 ms duration, 20 Hz) for 30 s. The amount of injected current was adjusted such that desDUM neurons generated APs at a mean frequency of 20-40 Hz. This stimulation protocol of a 200-s pause and 30-s stimulation was repeated at least 3 times per neuron. For evaluation, the APs of MNs generated in seven cycles of CS stimulation before (control), during, and after desDUM neuron activation were summed over the three trials of a single experiment and normalized to control. For an alternative display of data, APs were summed in 500-ms intervals and normalized to the first control bin.

2.10 General information on data analysis and statistics

Data were analyzed using Spike2 software using custom scripts, Microsoft Excel (2007; Microsoft Corp., Redmond, WA, USA), Origin 6.1 (OriginLab Corp., Northampton, MA, USA) and GraphPad Prism. Figures were prepared with CorelDrawX6 (Corel

Corporation, Ottawa, Ontario Canada). All data sets were tested for normality with Kolmagarow-Smirnow test, D´Agostino & Pearson omnibus test, and Shapiro-Wilk test. Correspondent to the result of the normality tests appropriate statistical tests were used for further evaluation of data. The applied statistical tests are mentioned in the respective figure legends. Additionally they are noted once for every data set in the text of the result section. In the text, data points are noted as mean ± SD. "N" resembles the number of experiments (intracellularly recorded desDUM neurons) and "n" refers to the number of trials, bursts, steps, or stimuli. The particular application of "N" and "n" is defined in the figure legends and in the text of the results section.

3 Results

3.1 Identification of stick insect desDUM neurons

The existence of desUM neurons in the GNG has been demonstrated in various hemi- and holometabolous insects (e.g., locust: (Bräunig, 1991; Bräunig and Burrows, 2004); sphinx moth: (Cholewa and Pfluger, 2009; Dacks et al., 2005); honeybee: (Schröter et al., 2007); fruit fly: (Busch et al., 2009; Selcho et al., 2012). With retrograde labeling of the two posterior connectives using two different fluorescent dyes, putatively homologous neurons have also been labeled in the stick insect (Heß, 2008). There are on average six large somata of neurons with bilaterally descending axons in the posterior stick insect GNG (Fig. 1.1). In the current thesis I first wanted to assess the morphology, octopamine content and electrical properties of these neurons to prove that they are indeed desDUM neurons.

3.1.1 Morphology of desDUM neurons

Using intracellular Neurobiotin™ staining of the neurons, I checked for morphological similarities of putative stick insect desDUM neurons to locust desDUM neurons, in the current thesis. In total, 30 stick insect desDUM neurons were intracellularly labeled after they had been identified by their electrical and physiological properties (70-120 mV APs; afterhyperpolarizations > 8 mV; response to tactile stimulation of the abdomen). The morphologies of 13 of these neurons are presented in Fig. 3.1. Somata of stick insect desDUM neurons were located at the dorsal surface of the posterior GNG (Fig. 3.1L). All of the stained neurons projected a primary neurite anteriorly along the midline of the GNG (Fig. 3.1A-K). The primary neurites bifurcate into two bilaterally symmetrical axons, and these axons give rise to numerous arborizations in the GNG but not to efferent axons in the lateral nerves of the GNG. Two axons (one axon per hemiganglion) descend to thoracic ganglia through the posterior connectives. The dendritic aborization patterns of intracellularly labeled desDUM neurons (N = 30) are similar to each other, or rather

not consistently different. There was no correlation between soma location and physiological differences of neurons. Therefore, I was not able to identify single desDUM neurons across animals using morphological characteristics. The descending axons of 3 desDUM neurons were successfully stained in the prothoracic ganglia (e.g., Fig. 3.1M1-M2). However, in these specimens, the signal to noise ratio was very low.

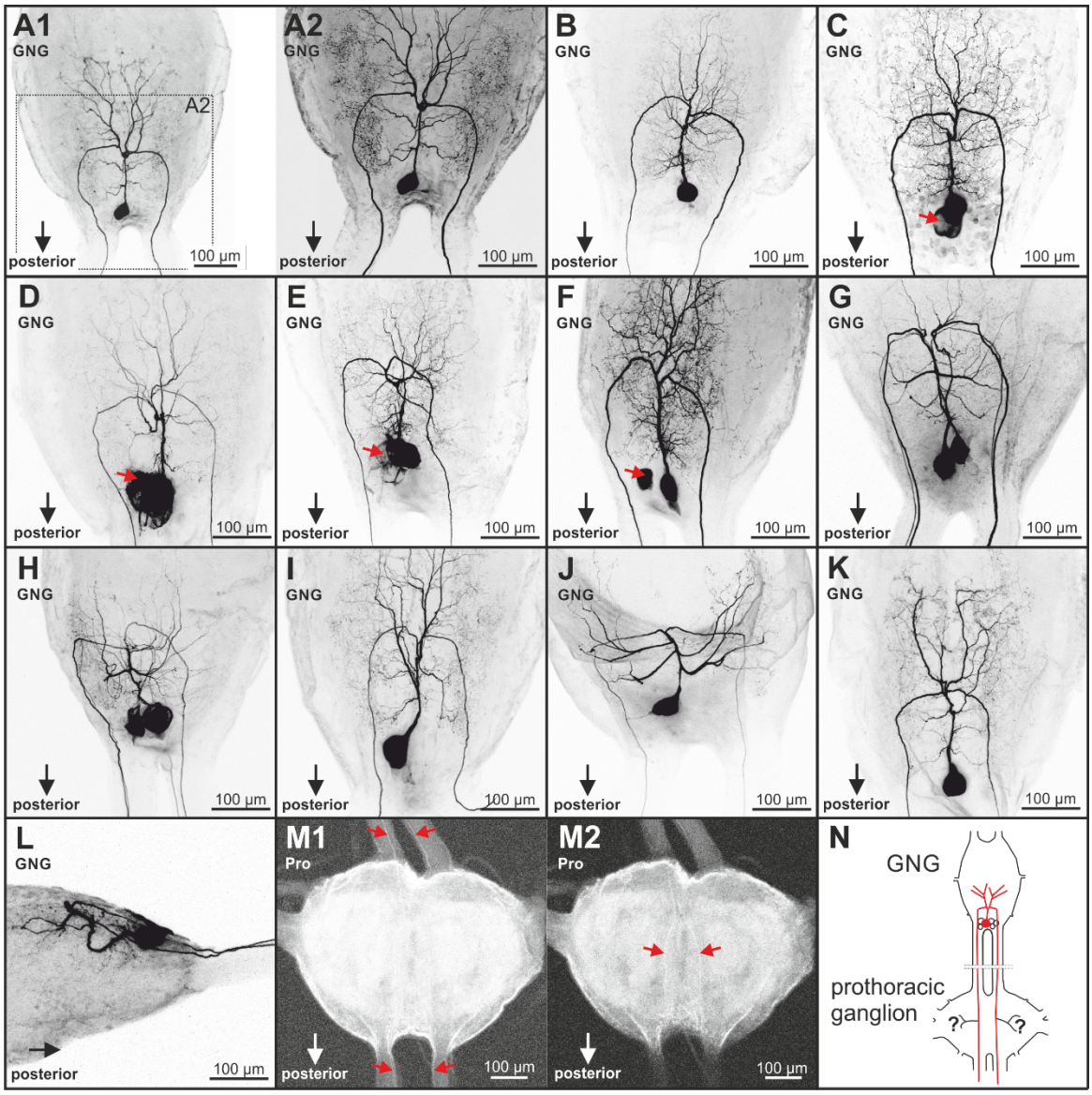

Figure 3.1 Morphology of stick insect desDUM neurons. The large somata of desDUM neurons were found in the posterior gnathal ganglion (GNG). The neurons project a primary neurite anteriorly, where it bifurcated into bilaterally symmetrical axons that descend the thoracic nerve cord at least to the mesothoracic ganglion (**N**). (**A-K**) Morphology of 13 examples of 30 intracellularly labeled desDUM neurons; collapsed z-stack images of one or two desDUM neurons in the GNG. Red arrows indicate cell bodies of desDUM neurons that had been stained as a side effect of intracellular recordings of their physiological activity in the same experiment. Neurites of the indicated neurons were not labeled. (**A2**) Magnified version of (**A1**). (**G** and **H**) Two desDUM neurons with similar morphology were labeled in the same ganglion. (**J**) The ganglion shrank during dehydration. (**L**) Side-view of a desDUM neuron. (**M1**) Collapsed z-stack images of the axons of a desDUM neuron (red arrows) in the anterior and posterior connectives of a prothoracic ganglion. (**M2**) Collapsed z-stack images of the axons of the desDUM neuron (red arrows) shown in

(**M1**) in the dorso-medial region of a prothoracic ganglion. (**N**) Schematic of cell body location and projections of a desDUM neuron. The projection pattern of secondary neurites within the prothoracic ganglion could not be assessed.

Further arborizations in the prothoracic ganglion, as well as in more posterior ganglia, were not detectable. This is likely due to the small diameter of desDUM axons and the relatively long distances between ganglia in stick insects over which the dye needed to diffuse. The demonstration of axons of desDUM neurons in the posterior connectives of the prothoracic ganglion (Fig. 3.1M1) shows that the neurons project at least as far as to the mesothoracic ganglion. Overall, the results indicate that stick insect desDUM neurons are very similar to locust desDUM neurons regarding their large soma size, number, locations, and morphology (Bräunig and Burrows, 2004).

3.1.2 Octopamine content of desDUM neurons[2]

To test whether desDUM neurons contain OA, I collaborated with Max Diesner and Dr. Susanne Neupert to establish a protocol for MALDI-TOF MS. Single desDUM neurons were identified electrophysiologically and labeled with tetramethylrhodamine dextran (Fig. 3.2A). The cell bodies of identified neurons were removed from the ganglia and transferred to a sample plate (Fig. 3.2B). For better detection of OA and tyramine (TA) by direct MALDI-TOF MS, isolated somata were on-plate derivatized with CA (***Manier et al., 2014, Diesner and Neupert, 2018***). The derivatization resulted in a mass shift of m/z 160.0, due to the carbonyl group of CA forming a stable Schiff base with the amine moiety. A deuterated internal standard containing 100 nM OA(*d3*) and 100 nM TA(*d4*) was additionally applied onto native samples to monitor successful derivatization reactions. A representative mass spectrum from an isolated desDUM cell body is shown in Fig. 3.2C. Our single-cell results show ion signals for putative derivatized OA and TA (OA-CA, m/z 314.1, N = 14; TA-CA, m/z 298.1, N = 5; Fig. 3.2C) as well as ion signals for the deuterated internal standard (OA[*d3*]-CA, m/z 317.2; TA[*d4*]-CA, m/z 302.2; Fig. 3.2C). Observed ion masses from putative derivatized OA-CA and TA-CA matched recorded ion masses from synthetic OA-CA and TA-CA (OA-CA, m/z 314.1; TA-CA,

[2] Experiments of this section were performed in collaboration with Max Diesner and Dr. Susanne Neupert (University of Cologne, Department of Biology, Institute for Zoology). Contributions: M.D. and S.N. designed research, M.D. and T.S. performed experiments, M.D. analyzed the data, M.D. and T.S. created the figures and wrote the text.

m/z 298.1; Fig. 3.2F). To confirm the ion identity of putative OA-CA and TA-CA from desDUM somata, OA-CA and TA-CA were fragmented from the same sample spot using MS^2. Recorded fragmentation spectra of putative OA-CA/TA-CA ion signals revealed distinct ion fragments for both molecules (OA-CA, m/z 296.1, TA-CA, m/z 120.8; Fig. 3.2E). These fragment spectra were in accordance with fragment spectra obtained from derivatized synthetic OA and TA (Fig. 3.2G). Hence, we could prove the presence of OA and TA in the dissected desDUM somata.

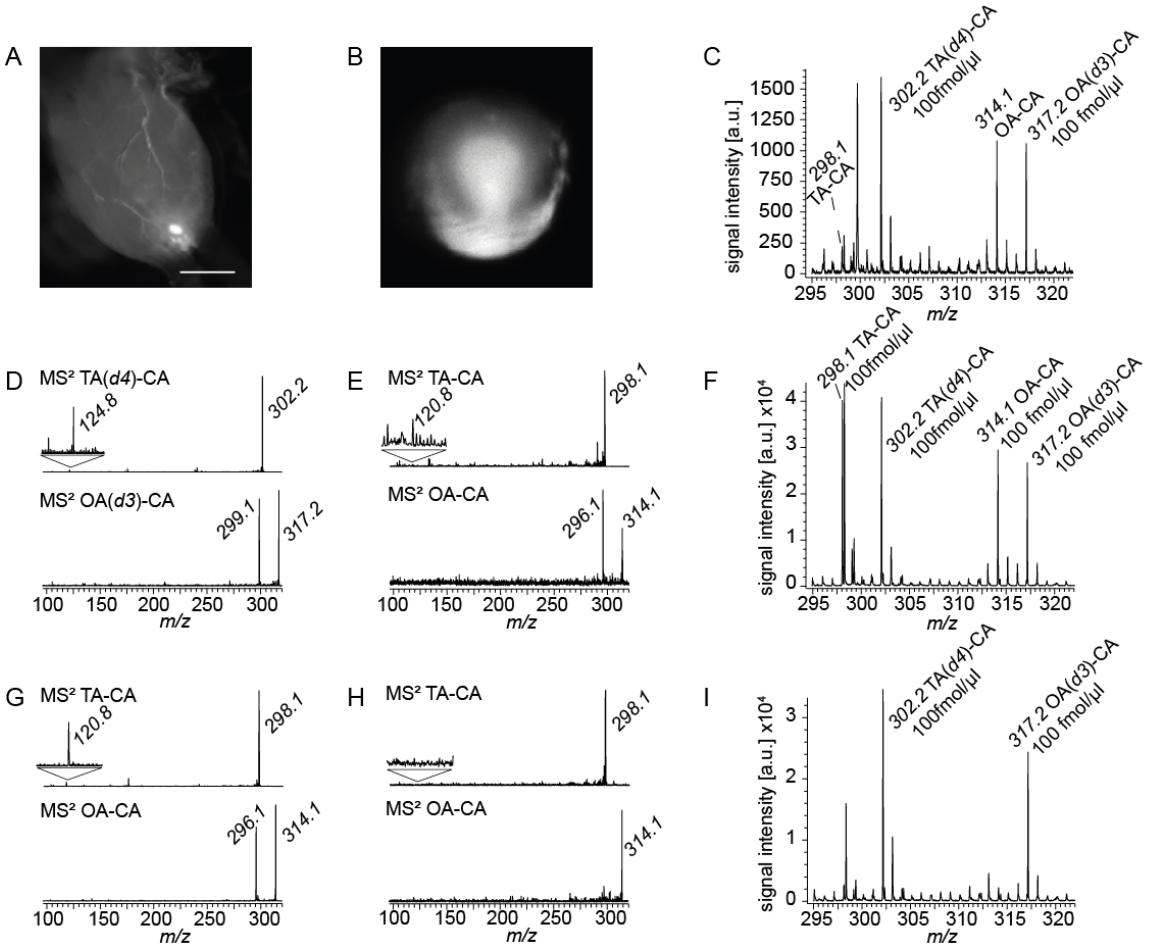

Figure 3.2 Detection of OA and TA from an individual desDUM soma from the GNG of *C. morosus* by direct MALDI-TOF MS after intracellular recording and tetramethylrhodamine dextran injection. **(A)** Isolated GNG with a tetramethylrhodamin dextran-injected desDUM soma after electrophysiological characterization; scale. Scale bar = 100 µm. **(B)** Marked desDUM soma after dissection und transfer to a MALDI sample plate. **(C)** Representative MALDI-TOF MS spectrum of a dissected single desDUM neuron soma after internal standard addition (m/z 302.2 TA[*d4*]-)-CA, m/z 317.2 OA[*d3*]-CA), derivatization with CA, and matrix application. A clear ion signal for OA-CA (m/z 314.1) and a weak ion signal for TA-CA (m/z 298.1) were observed. **(D)** To confirm a successful derivatization reaction, both internal standards were fragmented. MS/MS spectra of TA(*d4*)-CA revealed a distinct ion signal at m/z 124.8, while MS^2 experiments of OA(*d3*)-CA showed an ion fragment at m/z 299.1. **(E)** Validation of putative OA-CA/TA-CA by tandem MS. Fragment spectra showed unique fragments for each substance in accordance to standards (TA-CA, m/z 120.8; OA-CA, m/z 296.1). **(F)** Mass spectrum obtained of a mixture of synthetic 100 fmol/µl TA/OA and TA(*d4*)/OA(*d3*) after derivatization with CA. **(G)** Fragmentation spectra of derivatized synthetic OA-CA and TA-

CA with the major fragment ion signals for OA-CA (m/z 269.1) and TA-CA (m/z 120.8). **(H)** Recorded MS^2 mass spectra of putative OA-CA/TA-CA ion signals showed no ion fragments. **(I)** Control mass spectra of 100 fmol/µl deuterated TA(*d4*)-CA and OA(*d3*)-CA after chemical derivatization with CA.

3.2 Input physiology

3.2.1 Activity of desDUM neurons during walking

As a precondition for the modulation of motor activity in thoracic ganglia, the activity of desDUM neurons must be coupled to locomotor activity. I tested whether this requirement is met by recording intracellularly from single desDUM neurons of tethered animals that were walking with all six legs intact on two passive treadmills (schematic: Fig. 3.3A1). In resting animals, desDUM neurons displayed resting membrane potentials of -39 mV to -58 mV (N = 6 desDUM neurons). The neurons generated APs very rarely or not at all. In most cases, walking was initiated by brief tactile stimulation of the animal's abdomen. This mechanosensory stimulus was excitatory to desDUM neurons (indicated by arrows in Fig. 3.3A2, Fig. 3.4A1). Walking sequences consisted of stance and swing phases of single legs that occurred in defined coordination patterns. The inter-leg coordination pattern during walking most frequently resembled that of a tetrapod gait, where one leg of each body side is in swing phase and the other four legs are on the ground at any given moment (Cruse et al., 2009; Graham, 1972). During stance phases, the legs pulled on the belt of their respective treadmill. This was indicated by an upward deflection in the treadmill velocity traces (e.g., Fig. 3.3A2). When animals were walking, desDUM neurons received excitatory input and generated APs (e.g., Fig. 3.3A2). APs were overshooting and had amplitudes of 70 to 120 mV from baseline to peak. The APs displayed pronounced afterhyperpolarizations larger than 8 mV. When animals started walking spontaneously, desDUM neuron activity did not consistently precede these events. These results suggest that desDUM neurons are activated during walking, but their activity is not coupled to the initiation of spontaneous walking sequences.In order to study the timing of inputs to desDUM neurons during walking in greater detail, I used a preparation in which all legs except for one (mesothoracic leg) were amputated (Fig. 3.3 B1 and C1). During single-legged forward stepping, desDUM neurons received excitatory input and generated APs (e.g., Fig. 3.3B2). In most experiments, the animals showed stepping activity only on rare occasions. For better comparison of the excitatory effects of stepping across animals, I only evaluated desDUM neuron activity that occurred during the first 5 steps (without simultaneous tactile stimulation) in single experiments.

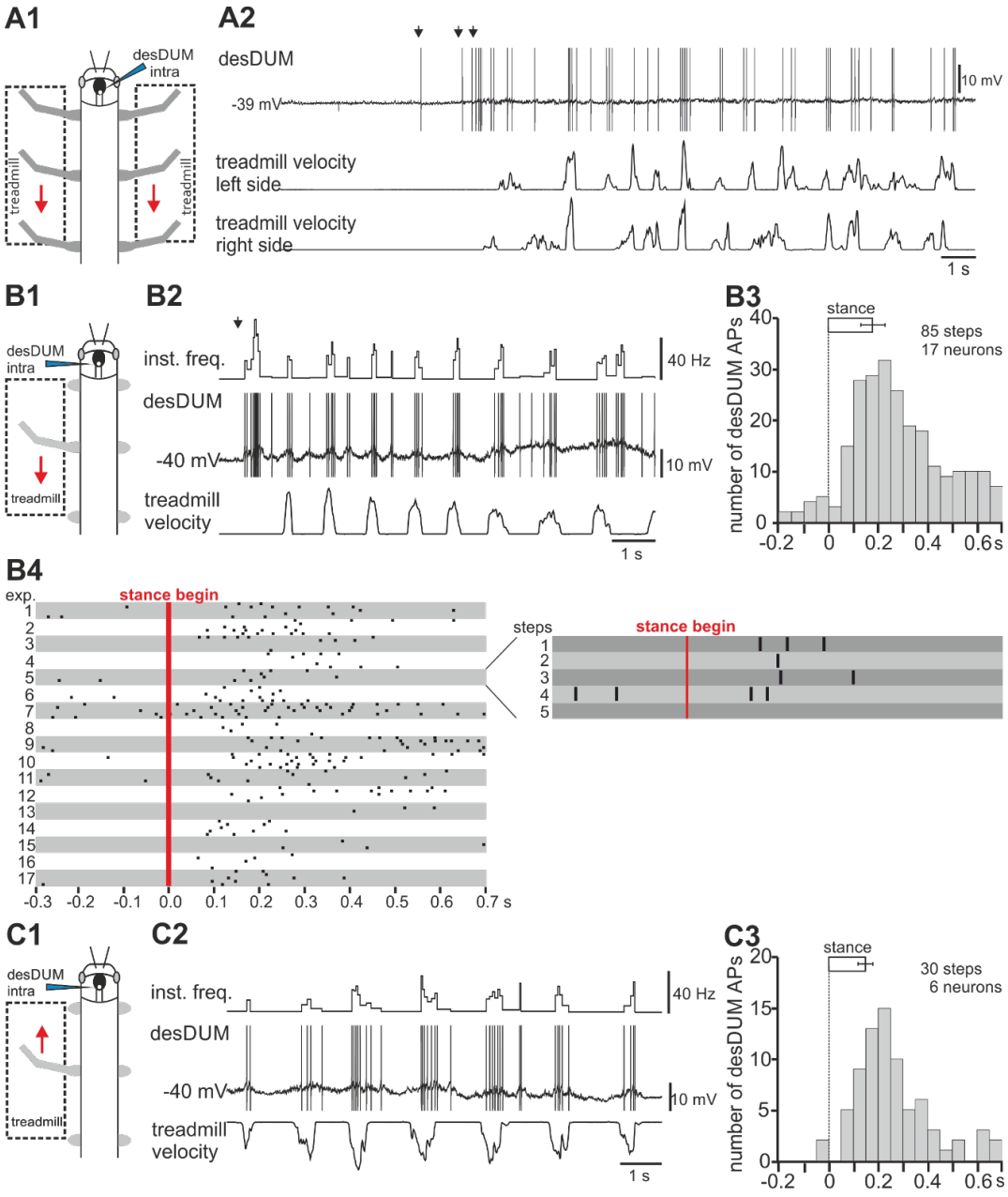

Figure 3.3. Activity of desDUM neurons during walking. (**A1**) Schematic of the experimental setup: A desDUM neuron was recorded intracellularly while the animal was walking with six legs on two treadmills. During forward stepping the treadmill-belt was pulled backwards. (**A2**) Membrane potential (middle trace) and instantaneous action potential (AP) frequency (top trace) of a desDUM neuron that was activated during six-legged forward walking on two treadmills. The APs were generated in response to stance phases, indicated by upward deflections in the treadmill velocity traces (bottom trace). The arrows indicate brief tactile stimulation of the abdomen with a paint brush in order to induce walking sequences. Single leg stepping: (**B1** and **C1**) Schematics of the experimental setup. All legs except one middle leg were removed. A desDUM neuron was recorded intracellularly while the animal was stepping forward (**B1**) or backward (**C1**) on a treadmill. (B2) Forward stepping with a single middle leg on a treadmill activated a desDUM neuron. (**B3**) desDUM neurons were generating APs triggered by stance phases of forward steps. The vertical line indicates the onset of stance phases. The horizontal bar indicates the mean stance phase duration ± SD (0.14 ± 0.04 s, n=85 steps). (**B4**) Rasta plot of APs of 17 desDUM neurons during single leg forward steps. Each grey and white line represents a single experiment consisting of 5 individual steps (see enlarged right panel). The red vertical line indicates onset of stance phases. 92 % steps induced APs in desDUM neurons. (**C2**) Single leg backward stepping caused APs in a desDUM neuron. (**C3**) APs were triggered by stance phases of backward steps (stance phase duration: 0.14 ± 0.04 s, mean ± SD, n=30). The amplitude of APs of desDUM neurons displayed in raw data traces was clipped for better visualization of inputs**.**

Quantitative analysis of desDUM neuron activity in relation to step cycles revealed that APs were generated in response to stance phase, where middle legs were pulling the treadmill belt backwards (Fig. 3.3B3). All of the 17 desDUM neurons investigated produced APs. The latencies for AP generation varied between single steps and between animals. The first distinct responses occurred 50-100 ms after stance phase onset. A maximum in average desDUM activity could be observed 200-250 ms after the onset of stance phase. Hyperpolarization or tonic depolarization of desDUM neuron membrane potential was not observed. Fig. 3.3B4 depicts the generation of desDUM APs during single steps (n = 85) in 17 animals. DesDUM neurons generated APs in response to 92% of all steps (78/85). On average, every step induced 2.7 ± 1.8 APs (mean ± SD, N = 17 neurons, n = 85 steps) in single desDUM neurons. Backward single-leg stepping sequences were most often elicited by tactile stimulation of the antennae. This kind of stimulation was excitatory to desDUM neurons (Fig. 3.4A1, arrows). During stance phases of backward steps, the treadmill-belt was pulled forwards (indicated by downward deflections in the treadmill velocity trace, Fig. 3.3C2). Backward stepping induced a phasic generation of APs in response to stance phases in all 6 investigated desDUM neurons (e.g., Fig. 3.3C2). Quantitative analysis demonstrated that the number of APs elicited by desDUM neurons during a single step was, on average, 2.5 ± 1.6 (mean ± SD, N = 6 neurons, n = 30 steps). This illustrates that both forward and backward stepping were equally efficient in activating desDUM neurons ($p = 0.778$, Mann-Whitney U test). During backward stepping, the first distinct responses of desDUM neurons occurred 50–100 ms after stance phase onset (Fig. 3.3C3). Maximum desDUM neuron activity could be observed 200–250 ms after the onset of stance phases. This finding indicates that the latencies of activation during forward and backward stepping were very similar and, thus, might have involved similar sensory and neuronal pathways.

I was able to show that desDUM neurons phasically generate APs during forward and backward stepping of the mesothoracic leg. Overall, the detailed results from single-leg stepping experiments indicate that, during six-legged stepping, the activity of desDUM neurons also relates to stance phases of the single legs. This suggests that desDUM neurons may receive excitatory input from all six legs during walking.

3.2.2 Influence of CPGs on activity of desDUM neurons

To locate the integration of desDUM neurons within locomotor networks, I investigated the origin of their phasic recruitment during walking. Step-coupled excitatory input to desDUM neurons might be centrally driven by activity in CPGs that control leg joint movements during walking. To test for this possibility, I activated leg joint CPGs with application of the muscarinic acetylcholine receptor agonist pilocarpine (5 mM) exclusively to the deafferented mesothoracic ganglion (schematic: Fig. 3.4B1).

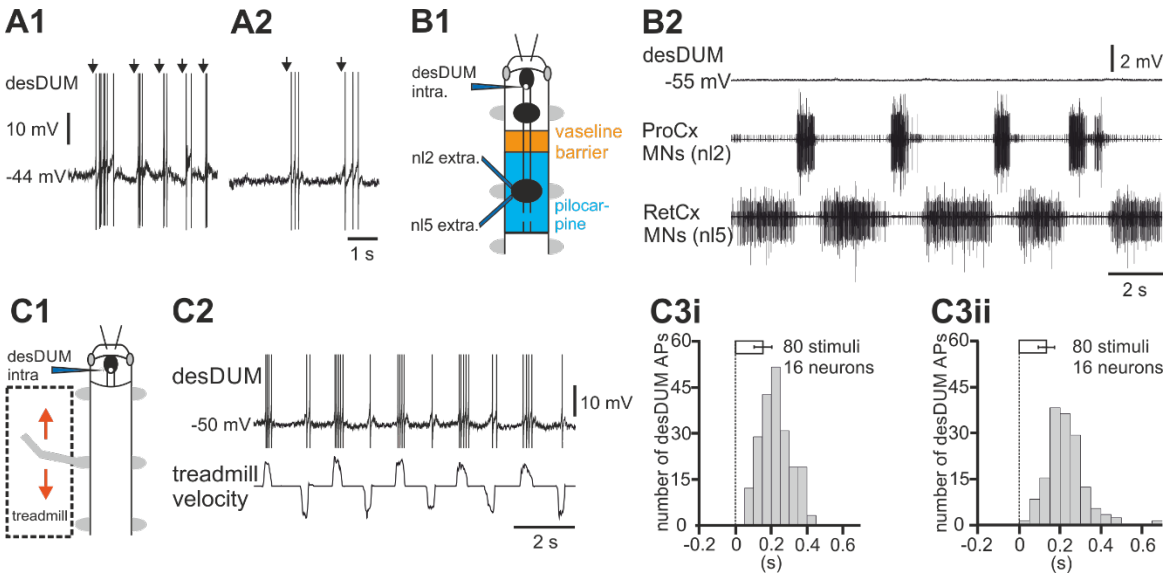

Figure 3.4 Influence of CPG activity and passive leg movments on desDUM neurons.
(A1) Brief tactile stimulation (arrows) of the animal´s abdomen led to the generation of APs in a desDUM neuron. **(A2)** Brief tactile stimulation (arrows) of the animal´s antennae with a paint brush induced APs in a desDUM neuron. **(B1)** The membrane potential of a single desDUM neuron was recorded intracellularly while alternating rhythmic activity in extracellularly recorded ProCx (nl2) and RetCx (nl5) MNs had been induced by application of the ACh receptor agonist pilocarpine. Pilocarpine was exclusively applied to the deafferented mesothoracic ganglion. **(B2)**. Activation of leg-joint central pattern generating networks (CPGs) did not affect the activity of a desDUM neuron. **(C1)** Passive movement of legs was induced by forward and backward movement of a treadmill, on which the tarsus of a leg was standing. Single desDUM neurons were recorded intracellularly. **(C2)** A desDUM neuron generated APs during passive forward and backward movement of a middle leg. **(C3)** The excitatory effect of passive leg movement was observed in 16/16 neurons. The vertical line indicates the onset of passive backward (left panel) and forward (right panel) leg movement. The horizontal bar indicates average duration of passive leg movement (mean ± SD). The amplitude of APs of desDUM neurons displayed in raw data traces was clipped for better visualization of inputs.

The reliable occurrence of alternating rhythmic activity in antagonistic ProCx and RetCx MN pools was taken as a proxy for the activation of all mesothoracic leg joint CPGs. However, activation of other leg joint CPGs was not explicitly monitored by extracellular recording, but, at a pilocarpine concentration of 5 mM, all mesothoracic leg joint CPGs should have been activated (Büschges et al., 1995; Mantziaris et al., 2017). During

pilocarpine-induced rhythmic MN activity, none of the intracellularly recorded desDUM neurons (N = 14) received phasic excitatory input or generated APs related to the rhythmic activity of MNs (e.g., Fig. 3.3 B2). Moreover, the membrane potential of desDUM neurons did not change after pilocarpine application. There was no tonic depolarization or hyperpolarization of desDUM neuron membrane potential. The results suggest that desDUM neurons are not activated by central drive from mesothoracic leg-joint CPGs.

3.2.3 Effect of passive leg movement on activity of desDUM neurons

Walking activates leg sensory organs (Bidaye et al., 2018; Tuthill and Wilson, 2016). In order to examine their role as a putative source of input to desDUM neurons, I moved middle legs of resting animals to activate sense organs in absence of CPG activity (Fig. 3.4C1). For the interpretation of the results, the fact that sensory information is processed differently in active and inactive animals must be taken into account. Figure 3.4C2 shows the membrane potential of a desDUM neuron that was generating APs in response to passive forward and backward movements of a mesothoracic leg. Quantitative analysis demonstrated that passive leg movement was excitatory in all 16 investigated desDUM neurons. Imposed backward movement of legs approximately followed the leg movement trajectories of stance phase during active forward steps (Grabowska et al., 2012; Graham, 1972; Gruhn et al., 2006) from anterior extreme position (AEP) to posterior extreme position (PEP). On average, passive backward movement induced 2.5 ± 1.4 APs (mean ± SD, N = 16 neurons, n = 80 backward movements) in single desDUM neurons (Fig. 3.4C3i). Thus, passive backward leg movements were equally efficient in activating desDUM neurons in comparison to active forward steps ($p = 0.913$, Mann-Whitney U test). The first distinct responses of desDUM neurons occurred 50–100 ms after initiation of passive movement. Maximum desDUM neuron activity could be observed 200–250 ms after the beginning of passive backward movement (Fig. 3.4C3i). These latencies are similar to those during active forward stepping. Passive forwards movements of the legs approximately followed the leg movement trajectories of stance phase during active backward steps from PEP to AEP. The raw traces in Fig. 3.4C2 suggest that passive forward movement of legs (downward deflections) was excitatory to desDUM neurons to a lower extent than passive backward movement (upward deflections). Quantitative

analysis demonstrated that single passive forward movements of legs induced, on average, 1.9 ± 1 APs in desDUM neurons (mean ± SD, N = 16 neurons, n = 80 forward movements; Fig 3.4C3ii). Thus, the overall effects of forward movement did not significantly differ from those of passive backward movement ($p = 0.161$; Paired t-test). The first distinct responses of desDUM neurons occurred 50–100 ms after initiation of passive forward movement. Maximum desDUM neuron activity could be observed 150–200 ms after the beginning of passive forward leg movement (Fig. 3.4C3ii).

The activity evoked in desDUM neurons by imposed leg movements was similar to the activity induced during stance phases of an actively stepping leg (Fig. 3.4C3i and C3ii compared to Fig. 3.3B3 and C3). With respect to the absence of an influence of CPGs, this suggests that excitatory input to desDUM neurons may originate in leg sensory organs during walking.

3.2.4 Effect of femoral chordotonal organ stimulation on activity of desDUM neurons

Since stance phase triggered activation of desDUM neurons during forward and backward stepping, I investigated the effects of sensory organs active during this phase of the step-cycle in greater detail. The fCO is activated by movement of the FTi joint (Bässler, 1993). Flexion of the leg occurs during stance phase (Rosenbaum et al., 2010). The stimulation of the fCO by leg flexion was mimicked in the present study by mechanical elongation of the fCO receptor apodeme with a clamp connected to a piezoelectric actuator (schematic Fig. 3.5A1). Ramp stimuli were applied to mimic flexion of the tibia from 120°-60° FTi joint angle, followed by a stationary phase of elongation (elongated-hold-part) and successive relaxation of the receptor apodeme. Relaxation mimicked extension of the tibia from 60° to 120° FTi joint angle. In resting animals, fCO elongation activates ExtTi MNs (Bässler, 1993). This response is called the resistance reflex. In the experiments described herein (N = 55 desDUM neurons), resistance reflexes were reliably elicited upon fCO elongation, indicating that the fCO had been successfully stimulated. The resting membrane potential (RMP) of desDUM neurons did not tonically depolarize or hyperpolarize from the beginning of fCO stimulation or ongoing fCO stimulation. (e.g., Fig. 3.5A2).

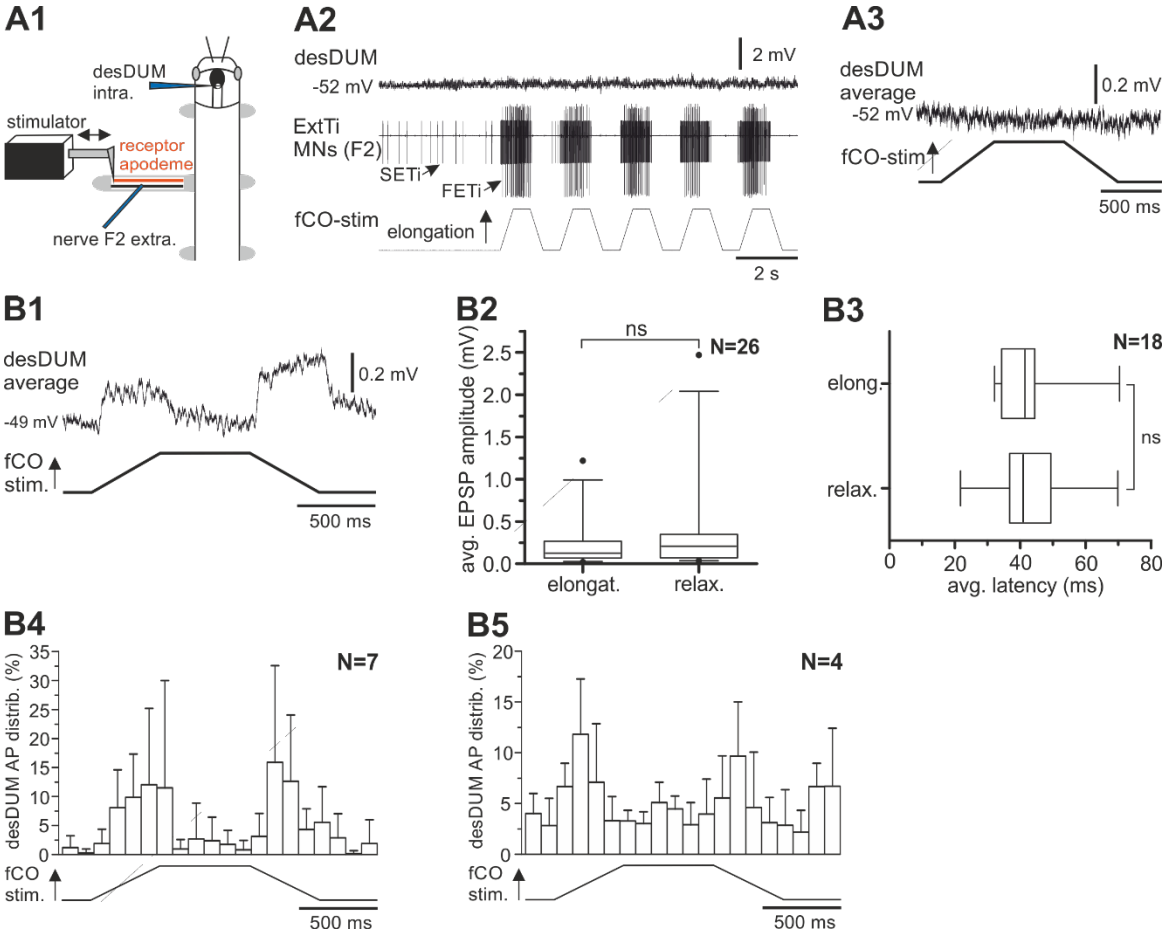

Figure 3.5 Effect of stimulation of the middle leg fCO on activity of desDUM neurons. (**A1**) The left middle leg femoral chordotonal organ (fCO) was stimulated by elongation and relaxation of the receptor apodeme, while resistance reflex responses in ExtTi MNs (nerve F2) were recorded extracellularly. (**A2**) The membrane potential of a desDUM neuron was not influenced by stimulation of the fCO. Ascending ramps indicate elongation of the fCO, descending ramps indicate relaxation. (**A3**) Average membrane potential of the desDUM neuron recorded in (**A2**) with no response over 150 cycles of fCO stimulation. Overall, 29 of 55 desDUM neurons showed no response to fCO stimulation (**B1**) Average membrane potential of a desDUM neuron over 250 cycles of fCO stimulation. The neuron displayed relatively pronounced (compared to average in B2) depolarizations in response to elongation and relaxation of the fCO. (**B2**) Comparison of the average amplitude of depolarizations of 26 desDUM neurons to elongation (elongate., n = 6500 ascending ramps) and relaxation (relax., n = 6500 descending ramps) of the fCO. There was no significant difference in average amplitudes (p = 0.0596, ns, Wilcoxon matched-pairs signed-rank test, N = 26 desDUM neurons). (**B3**) Average latencies from onset of fCO elongation and relaxation to occurrence of depolarization were not significantly different from each other (p = 0.570, Wilcoxon matched-pairs signed-rank test, N =18 desDUM neurons). (**B4**) Average distribution of APs (mean ± SD) of 7 desDUM neurons over fCO stimulation period. The neurons were recorded at depolarized membrane potentials in inactive animals. (**B5**) Average distribution of APs (mean ± SD) of 4 desDUM neurons over fCO stimulation period. The neurons were recorded during the occurrence of active reactions in animals that were activated by tactile stimulation of the abdomen. Boxes are from Q1 to Q3. The vertical line in the box depicts the median. Whiskers indicate 5th and 95th percentile.

Furthermore, desDUM neurons did not generate APs in response to the beginning of fCO stimulation. In order to investigate subthreshold inputs to desDUM neurons in detail, I averaged the time course of membrane potentials of single desDUM neurons over 150-

250 ramp-and-hold stimuli. In 53% (29/55) of desDUM neurons, the RMP was not affected by fCO stimulation (e.g., Fig. 3.5A3). In the other 26 desDUM neurons, depolarization of membrane potential occurred in response to the fCO elongation stimulus and in response to fCO relaxation (example for a relatively large effect: Fig. 3.5B1). The EPSP amplitude of the first depolarization was on average 0.21 ± 0.25 mV (mean ± SD, N = 26 desDUM neurons). The depolarization in response to fCO-relaxation had an average amplitude of 0.34 ± 0.5 mV (mean ± SD, N = 26 desDUM neurons). The average EPSP amplitudes in response to elongation and relaxation were not significantly different from each other ($p = 0.0596$, N=26, Wilcoxon matched-pairs signed rank test; Fig. 3.5B2).

For 18 of 26 desDUM neurons, it was possible to precisely determine the onset of the depolarizations and calculate their latencies from the start of elongation and relaxation (Fig. 3.5B3). The latency from the start of elongation to depolarization was on average 44.4 ± 12.7 ms (mean ± SD, N = 18 desDUM neurons). The latency from the start of relaxation to depolarization was on average 43.4 ± 11.7 ms (mean ± SD, N = 18 desDUM neurons). The latencies were not significantly different from each other ($p = 0.570$, Wilcoxon matched-pairs signed rank test; Fig. 3.5B3). In 7 neurons, the RMP depolarized at the end of experiments to values between -44 and -38 mV. In this condition, the neurons generated APs. The occurrence of APs was correlated to the previously described depolarization during fCO elongation and relaxation (Fig. 3.5B4). This observation, as well as the ~44 ms latencies, indicates that the depolarization in response to fCO elongation and relaxation was not a mechanical side effect of stimulation but a physiological response.

FCO signals are processed differentially in inactive and active animals (Bässler, 1976; Bässler and Büschges, 1998), and the previous experiments were performed on resting animals. To test whether fCO signals also contribute to excitatory input to desDUM neurons in active animals, I stimulated activity using random tactile stimulation of animals' abdomens with a paint brush. Activation was demonstrated by the occurrence of reflex reversals. This means that fCO elongation was inhibitory to ExtTi MNs and relaxation was excitatory. I analyzed the distribution of desDUM neuron APs over fCO stimulation in the presence of active reactions. Tactile stimulation alone was excitatory to

desDUM neurons; nevertheless, there was a distinct relationship of the occurrence of desDUM neuron APs with fCO elongation and relaxation (Fig. 3.5B5). The distribution of desDUM neuron APs in active animals was similar to that in inactive animals. This indicates that the drive to desDUM neurons from fCO stimulation is of relevance in active animals.

While establishing the parameters for fCO stimulation used in the 55 experiments reported herein (see: Section 2), I also stimulated the fCO with a variety of different ramp-and-hold stimuli in five preliminary experiments. In these experiments, I used slower and faster elongation and relaxation velocities for fCO stimulation, and fCO stimulation also only had no or only weak effects on desDUM neurons (data not shown). In summary, these results demonstrate that elongation and relaxation of the fCO has weakly excitatory effects on a fraction of desDUM neurons. Particularly with regard to the occurrence of excitatory input related to fCO stimulation in activated animals, fCO signals may contribute to the excitatory input to desDUM neurons during walking. Tibial extension occurs during swing phase and was mimicked by mechanical fCO relaxation in these experiments. As fCO relaxation was weakly excitatory, this input may be responsible for the generation of APs in desDUM neurons during walking that do not occur in response to stance phase (see Fig. 3.3B2).

3.2.5 Effect of campaniform sensilla stimulation on activity of desDUM neurons

CS are sense organs receptive to strain produced in the cuticle ***(Pringle 1938).*** They are predominantly activated during stance phase, when self-generated muscle forces are resisted and the leg is loaded (Schmitz, 1993; Zill et al., 2004; Zill et al., 2012). Different groups of CS on the leg demonstrate varied sensitivity to the direction of strain in the cuticle (Zill et al., 2013). This allows for the activation of distinct groups by bending a fixed leg in a specific direction (schematic: Fig. 3.6A1). Caudal bending of the mesothoracic femur stimulates CS of Group 1, which activate RetCx MNs (Schmitz, 1993). In the current experiments, caudal leg bending reliably induced reflexes in RetCx MNs. This indicates that stimulation of CS had been achieved (e.g., Fig. 3.6A2). In response to the first cycles of leg bending, 5 of 8 desDUM neurons generated APs (e.g.,

Fig. 3.6A2). AP generation in desDUM neurons ceased after about 10-15 cycles of CS stimulation. However, EPSPs were evoked by CS stimulation throughout experiments with durations of up to 1.5 h. Figure 3.6A3 shows that the latency from the onset of CS stimulation to depolarization of desDUM neuron RMP was, on average, 90 ± 40 ms (mean ± SD; N = 6 desDUM neurons, n = 90 CS stimulations). The amplitude of EPSPs was, on average, 1.7 ± 0.4 mV (mean ± SD; N = 6 desDUM neurons, n = 90 CS stimulations). The results indicate that CS represent a potential source of excitatory input to desDUM neurons during walking.

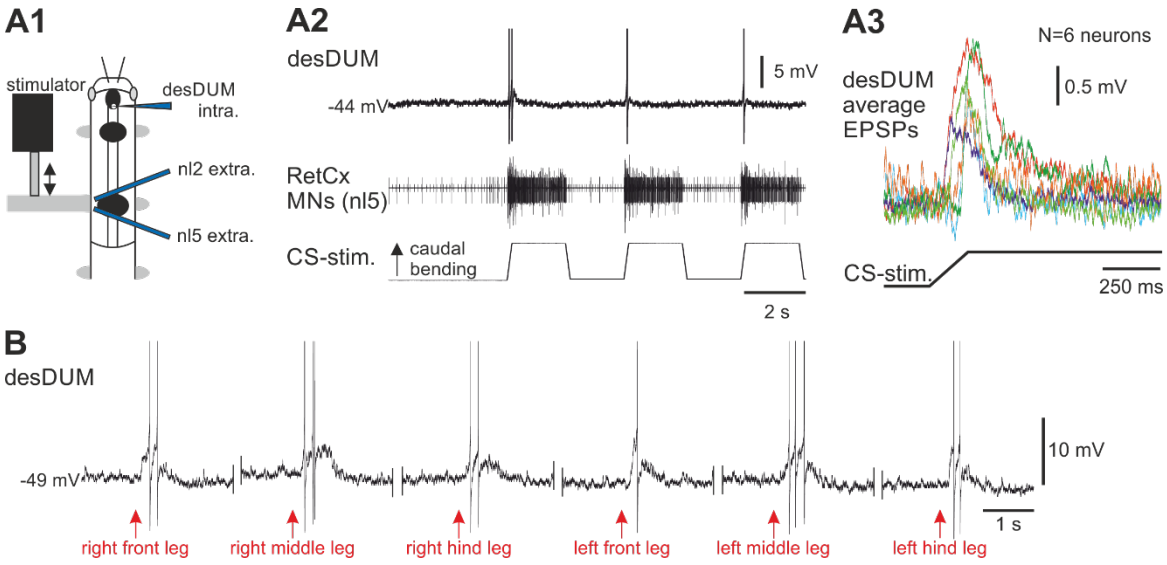

Figure 3.6 Effect of stimulation of Campaniform sensilla on the activity of desDUM neurons. (**A1**) Campaniform sensilla (CS) were stimulated by caudal leg bending, while responses in ProCx and RetCx MNs were recorded extracellularly. (**A2**) A desDUM neuron generated APs in response to CS stimulation by caudal leg bending. (**A3**) Superimposed average membrane potentials of 6 desDUM neurons during 15 (for each neuron) cycles of CS stimulation. The neurons were recorded at resting membrane potentials from -52 to -56 mV about 60 s after the start of CS stimulation. (**B**) Activation of a desDUM neuron by brief tactile stimulation of the tibia of right and left front-, middle-, and hind legs with a small wooden stick (approximate time points of stimulation are indicated by red arrows). The amplitude of APs of desDUM neurons displayed in raw data traces was clipped for better visualization of inputs.

To investigate whether desDUM neurons receive input from sense organs of all six legs, I briefly and gently touched the dorsal cuticle of tibiae of single legs with a wooden stick. This kind of stimulation should activate CS other than those in Group 1, as strain is induced in the cuticle in a different manner than caudal leg bending. Notable, of course, is that this unspecific stimulation might also activate other sense organs. Fig. 3.6B shows an exemplary recording of a desDUM neuron that generated 1-3 APs in response to brief and gentle touching of the dorsal cuticle of the tibiae of single legs. The absolute number of

elicited APs was not related to stimulation of distinct legs and varied between single trials within an animal. This qualitative approach revealed similar observations in all four desDUM neurons tested. The time points of touching the tibiae were only roughly documented, which is why the latencies from tibia stimulation to AP generation in desDUM neurons could not be precisely determined. Together, the results indicate that single desDUM neurons can integrate sensory signals from all 6 legs.

3.3 Output effects of desDUM neurons

So far the results suggest that desDUM neuron activity is phasically increased during walking, presumably as a result of the excitatory influence of strain detecting leg sensory organs. To some extent, also fCO signals could contribute to the walking-related excitatory influence. Next, I asked the question, whether the output of desDUM neurons in turn can modulate activity of neural control networks for the generation of walking in thoracic ganglia.

3.3.1 Effects of desDUM neurons on activity of ExtTi MNs

3.3.1.1 Modulation of fCO-induced reflex responses in ExtTi MNs

To test the possible modulatory role of desDUM neurons, I activated single desDUM neurons by current injection and studied their effects on fCO-evoked reflexes of mesothoracic ExtTi MNs. Previous results in the current thesis show that fCO stimulation had no pronounced effect on the generation of APs in desDUM neurons. Hence, the experimental design allows for full control over the generation of APs in single desDUM neurons by depolarizing current injection into somata. Furthermore, stimulation of the fCO induces resistance reflexes in ExtTi MNs of inactive animals (Bässler, 1976; 1983; Bässler and Büschges, 1998) which are very stereotypic (Sauer et al., 1996). Thus, ExtTi MN activity induced by stimulation of the fCO functions as a stable control. The ExtTi muscle is only innervated by three MNs (Goldammer et al., 2012) that can be readily distinguished in extracellular recordings (Bässler, 1983). Therefore, putative effects of desDUM neurons can be compared in between single excitatory and inhibitory units of the ExtTi MN pool. In the experiments, I continuously stimulated the fCO and extracellularly recorded ExtTi MN responses as control. During continuous fCO

stimulation single desDUM neurons were stimulated by 30-s depolarizing current injection to generate APs. I asked five questions regarding the influence of desDUM neurons on reflex-induced ExtTi MN activity: (I) Do desDUM neurons modulate ExtTi MN activity? (II) Do distinct neurons of the desDUM population mediate different effects? (III) What is the time course of effects? (IV) Are the single units of the ExtTi MN pool affected in similar fashion? (V) To what extend do the neurons influence processing of fCO signals? I used three different approaches of quantitative analysis of a data set from 44 experiments to address the distinct questions (see section 2.8).

3.3.1.1.1 Classification and time course of modulatory effects on reflex-evoked SETi MN activity mediated by desDUM neurons

The first experiments of the current section already suggested that single desDUM neurons have an effect on fCO stimulation-evoked ExtTi MN activity. Moreover, these results indicate that the effects may differ between single desDUM neurons. Some desDUM neurons seemed to increase the reflex-evoked ExtTi MN activity (e.g., Fig. 3.8A), some appeared to decrease reflex-evoked ExtTi MN activity (e.g., Fig. 3.9 A), and others seemed to be rather ineffective (e.g., Fig. 3.10 A). To test these assumptions on differential effects, I conducted experiments on 44 desDUM neurons in 42 animals in total. First, I tested whether there are any substantial effects of single neurons and to what extend the neurons' effects can be grouped according to their characteristics. Each experiment consisted of three trials. For each of the trials (30-s desDUM neuron activation) of an experiment, I assessed the average ExtTi MN activity (number of APs) during each of 60 consecutive 2-s ramp-and-hold stimulations of the fCO (fCO-stim. cycles). In detail, the single trials comprised recording of ExtTi MN activity during 4 subsequent sections of 15 fCO-stim. cycles each: (I) a control section prior to desDUM neuron stimulation; (II) a section in which a single desDUM neuron was stimulated for 30 s to produce APs at 25-40 Hz (desDUM-stim.), (III and IV) and two consecutive sections after desDUM-stim. (post1 and post2) that functioned as follow-up controls (for more details see section 2.8). For comparison of effects between animals, the number of ExtTi MN APs during single 2-s fCO stim. cycles was summed and normalized to the average sum of ExtTi MN APs of the 15 fCO stim. cycles of the control section. This normalization was only possible for SETi MN activity, because FETi MNs and CI_1

neurons were often not recruited during all fCO stim. cycles in the control section. Every data point in Fig. 3.7.1A represents average SETi MN activity over three fCO-stim. cycles (one for each trial) at a given time point (normalized to control) in a single experiment. The figure shows that the average SETi MN activity in single experiments in the control period did not largely diverge from 100% of control (data points of one experiment are represented by symbols with the same color and shape). This demonstrates that the SETi MN activity was stable during the control periods in all the experiments. Directly after onset of desDUM neuron stimulation, most of the data points deviated from 100% average SETi MN activity of the control section. Upon a closer look, the average SETi MN activity consistently increased, decreased, or remained unaffected in single experiments during the first 9 fCO-stim. cycles of desDUM neuron-stimulation. After these 9 fCO-stim. cycles, SETi MN activity, which appeared to be completely unaffected by desDUM neuron-stimulation at first, decreased slightly.

These findings were the basis for the classification of desDUM neurons according to their effects on SETi MN activity (Fig. 3.7.1B). Fourteen desDUM neurons were grouped with respect to their excitatory influence on SETi MN activity (increase), 19 neurons were grouped according to their inhibitory influence (decease), and 7 neurons were grouped based on their slight inhibitory influence on SETi MN activity (slight decrease).

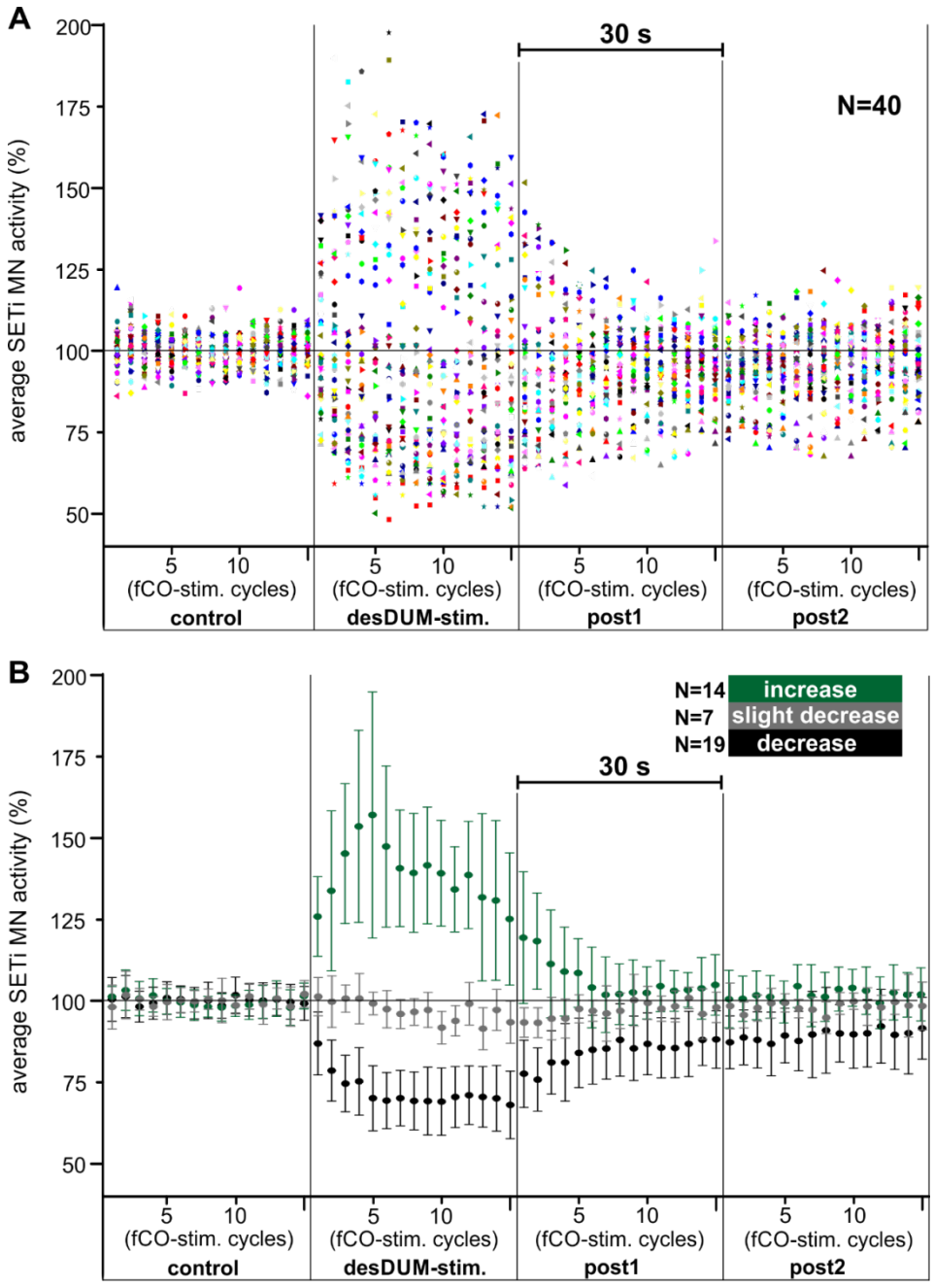

Figure 3.7.1 Time course of differential effects on reflex-induced SETi MN activity mediated by desDUM neurons. (**A**) Normalized SETi MN activity (in %) over 60 consecutive cycles of fCO-stimulation. The vertical lines separate SETi MN activity during 15 cycles of control, 15 cycles during stimulation of single desDUM neurons (desDUM-stim.), 15 cycles directly after desDUM neuron-stimulation (post1), and 15 subsequent cycles (post2). Each symbol represents the averaged and normalized SETi MN activity in an individual experiment during a distinct cycle of fCO stimulation. Symbols with the same color and shape resemble SETi MN activity in one of 40 experiments. In 33 experiments, the MN activity clearly deviated from 100% of control during the first 9 cycles from the start of desDUM neuron-stimulation (desDUM-stim.). In 14 experiments, the MN activity was increased, in 19 experiments it was decreased. In 7 experiments, the MN activity remained unaffected at around 100% of control in this period. (**B**) Data of 14 experiments that displayed an increase in MN activity (green), 19 experiments with decrease (black), and 7 experiments with no obvious change (grey) were each pooled and averaged. The graph displays normalized SETi MN activity (mean +/- SD) of the three distinct groups across 60 cycles (in 4 sections) of fCO stimulation.

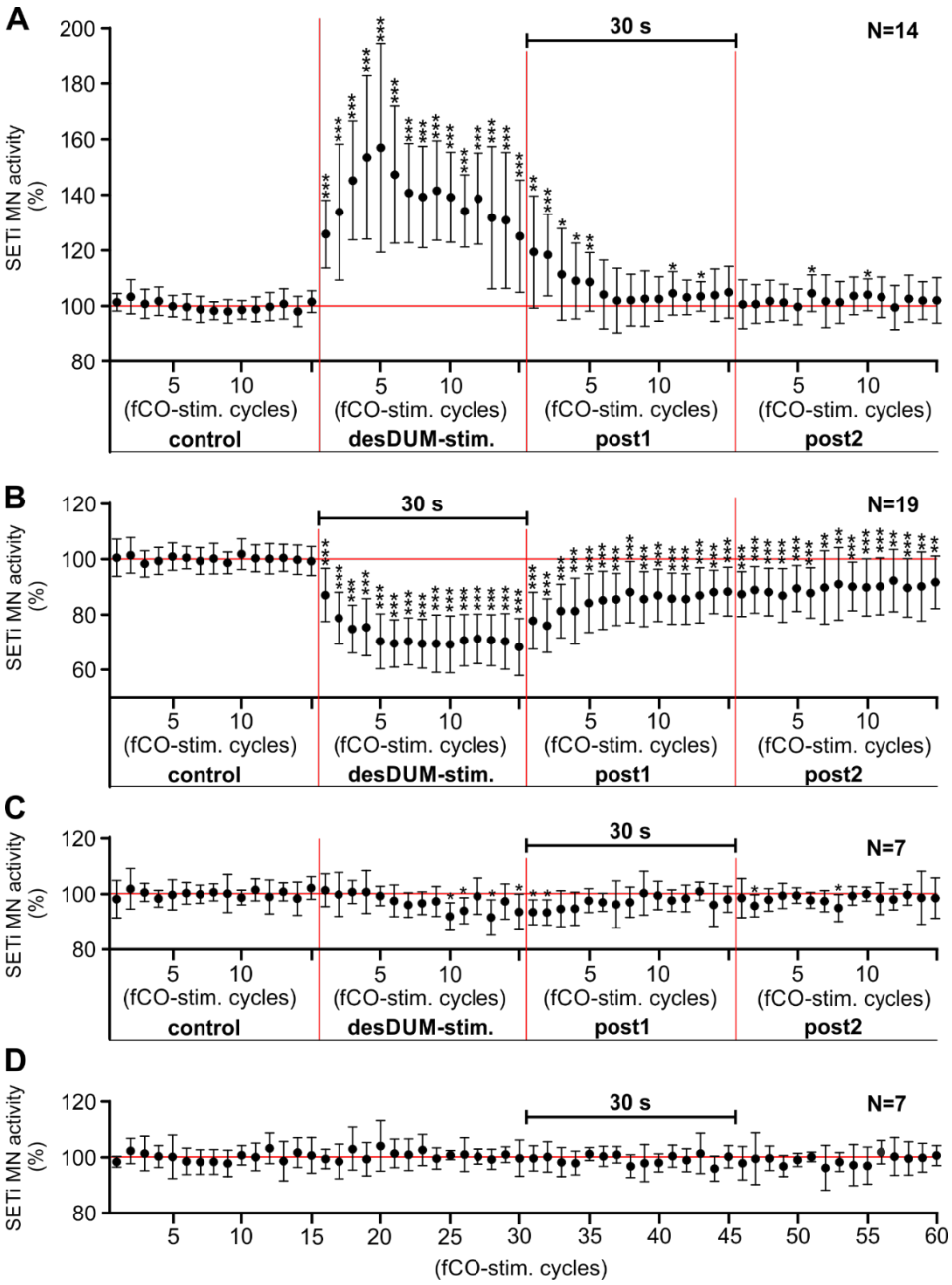

Figure 3.7.2 Time course of differential effects on reflex-induced SETi MN activity mediated by desDUM neurons.
(A-D) Normalized SETi MN activity (mean +/- SD) over 60 consecutive cycles of fCO-stimulation.
(A) In 14 experiments, the mean SETi MN activity significantly increased in direct response to stimulation of single desDUM neurons. The excitatory effect of desDUM neuron activity decreased during stimulation (desDUM-stim.) and ceased 6 cycles after the end of stimulation (post1). **(B)** The mean SETi MN activity significantly decreased in 19 experiments directly upon stimulation of single desDUM neurons. The inhibitory influence of desDUM neuron activity was constant throughout the stimulation of desDUM neurons and decreased slightly after stimulation (post1), but it was still present 30 cycles later (post2). **(C)** In 7 experiments, there was no obvious change in MN activity at the beginning of desDUM neuron-stimulation. Towards the end of stimulation, and shortly thereafter, a slight decrease in MN activity became apparent. **(D)** 7 control experiments were conducted without desDUM neuron-stimulation. The mean SETi MN activity in these experiments never significantly differed from the theoretical control median of 100% throughout the 60 cycles of fCO stimulation. **(A-D)** Statistics: The differences between the actual medians of MN activity to the theoretical control median of 100% were tested with the Wilcoxon signed-rank test. Statistical significance is denoted as follows: no mark above error bar: not significant, $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N indicates number of recorded desDUM neurons.

Time course of excitatory output effects

To assess the time course of the effects of the single desDUM neuron classes, I tested for significant differences in average SETi MN activity of the single classes during each fCO-stim. cycle against the theoretical control median of 100% (Fig. 3.7.2A-C; Wilcoxon signed-rank test). Fig. 3.7.2A shows that the excitatory effect was significant at the beginning of desDUM neuron stimulation (SETi MN activity: 125.9 ± 12.2%, mean ± SD, p = 0.0002, N = 14 experiments). The increase in average SETi MN activity was maximal during the fifth fCO-stim. cycle during desDUM neuron activation (SETi MN activity: 156.9 ± 37.63%, mean ± SD, p = 0.0001, N = 14 experiments). Over the subsequent fCO-stim. cycles, the increase in average SETi MN activity declined. The excitatory effects outlasted the activation of the desDUM neurons for five fCO-stim. cycles until it ceased during the sixth fCO-stim. cycle (post1; SETi MN activity: 104.2 ± 12.2%, mean ± SD, p = 0.286, N = 14 experiments). Thereafter, average SETi MN activity was only slightly increased during four fCO-stim. cycles in post1 and post2.

Time course of inhibitory output effects

Fig. 3.7.2B demonstrates that the inhibitory influence of 19 desDUM neurons on SETi MN activity was significant at the beginning of desDUM-stim. (SETi MN activity: 86.9 ± 9.6%, mean ± SD, p = 0.0004, N = 19 experiments). The decrease in average SETi MN activity peaked during the fifth fCO-stim. cycle of desDUM neuron-activation (SETi MN activity: 70.3 ± 9.9%, mean ± SD, p = 0.0001, N = 19 experiments). Over the subsequent fCO-stim. cycles, the decrease in average SETi MN activity was constant. SETI MN activity recovered about 10% directly after desDUM-stim. (SETI MN activity: 81.2 ± 9.7%, mean ± SD, p = 0.0001, N = 19 experiments). Still, the decrease in average SETi MN activity was highly significant during all fCO-stim. cycles of post1 and post2. Fig.

Time course of slightly inhibitory output effects

3.7.2C depicts the slight delayed inhibitory influence of 7 desDUM neurons on average SETi MN activity. SETi MN activity maximally decreased to 91.53 ± 6.4% of control (mean ± SD, p = 0.031, N = 7 experiments). A significant decrease was only observed during eight fCO-stim. cycles.

In 7 experiments I did not stimulate desDUM neurons, but assessed average fCO stimulation-evoked SETi MN activity in the same way as in the experiments described above. Fig. 3.7.2D demonstrates that average SETi MN activity did not spontaneously deviate from the theoretical control median of 100% during 60 stimulus cycles in absence of desDUM neuron activation. This demonstrates that there were no significant rundown or habituation effects in the course of single trials. In addition, the results of the control experiments indicate that the faint inhibitory effects depicted in Fig. 3.7.2C can be attributed to desDUM neuron activation.

Taken together, these results demonstrate that individual desDUM neurons exhibit distinct effects on SETi MN activity. There are neurons that mediate an excitatory influence and neurons that mediate an inhibitory influence. Pronounced differences in the time course and size of effects of desDUM neurons with inhibitory influence suggest the further subdivision into two separate classes.

3.3.1.1.2 Net-output effects on FETi, SETi and CI_1 MNs mediated by desDUM neurons

In the previous analysis of output effects, I focused on the time course of effects of desDUM neurons and could only consider the influence on SETi MN activity. Next, I wanted to address the question whether FETi MNs and CI_1 neurons of the ExtTi MN pool are affected in a similar desDUM-class-specific fashion as the SETi MNs are. Furthermore, I wanted to investigate if the output physiology of desDUM neurons can be delineated by means of further characteristics exclusive to the distinct classes. For this, I evaluated the data differently to get a measure for the net effect size mediated by the distinct neuron classes over longer, but physiologically relevant, periods of 30s. This duration is in the range of walking sequences that I previously described. Furthermore, the evaluation of desDUM neuron effects on longer periods of time enables the normalization of FETi MN and CI_1 neurons (see section 2.8). This was not possible in the previous section because, these motor units are not recruited during most fCO stim cycles of control.

Characterization of the excitatory net-output effect on FETi, SETi, and CI_1 MNs

Fig 3.8A shows an example recording of a desDUM neuron that significantly increased fCO-evoked SETi MN activity. Moreover, a FETi MN that did not generate APs in absence of desDUM activation was recruited upon desDUM neuron stimulation. At a closer look, it becomes apparent that the MN activity not only increased, but the activity of the desDUM neuron triggered the occurrence of active reactions (Bässler, 1976; 1986; 1988) (detail Fig. 3.8B1 and B2). In contrast to resistance reflexes before desDUM neuron stimulation (Fig. 3.8B1), ExtTi MNs were inhibited during fCO elongation and excited during fCO relaxation (raw and mean frequency trace, Fig. 3.8B2) in a fraction of reflex responses during desDUM neuron stimulation (Fig. 3.8A). Quantitative analysis tested whether this effect is commonly mediated by desDUM neurons of the class with excitatory influence on ExtTi MN activity. The average frequency for occurrence of the active reaction was compared between control sections and during desDUM neuron stimulation (Fig. 3.8C). The frequency of occurrence of active reactions during control fCO-stim. cycles was, on average, 0.37 ± 0.85% (mean ± SD, N = 18 experiments, n = 810 fCO-stim. cycles; Fig. 3.7C, “control”). There were only three experiments in which a single active reaction occurred in control sections in absence of desDUM neuron activation. In all other experiments, no active reactions were observed during control. The average frequency for occurrence of active reactions increased to 13.95 ± 17.48% upon desDUM neuron stimulation (mean ± SD, N = 18 experiments, n = 810 fCO-stim. cycles; Fig. 3.8C, “stimulation”). This increase was statistically significant ($p = 0.0005$, Wilcoxon matched-pairs signed-rank test, N = 18 experiments, n = 810 fCO-stim. cycles). In fact, in only 2 (of N = 18) experiments, stimulation of desDUM neurons mediating an excitatory effect did not induce the generation of active reactions. The results suggest that desDUM neurons with an excitatory influence may mediate a specific effect on pathways involved in the generation of reflex reversal.

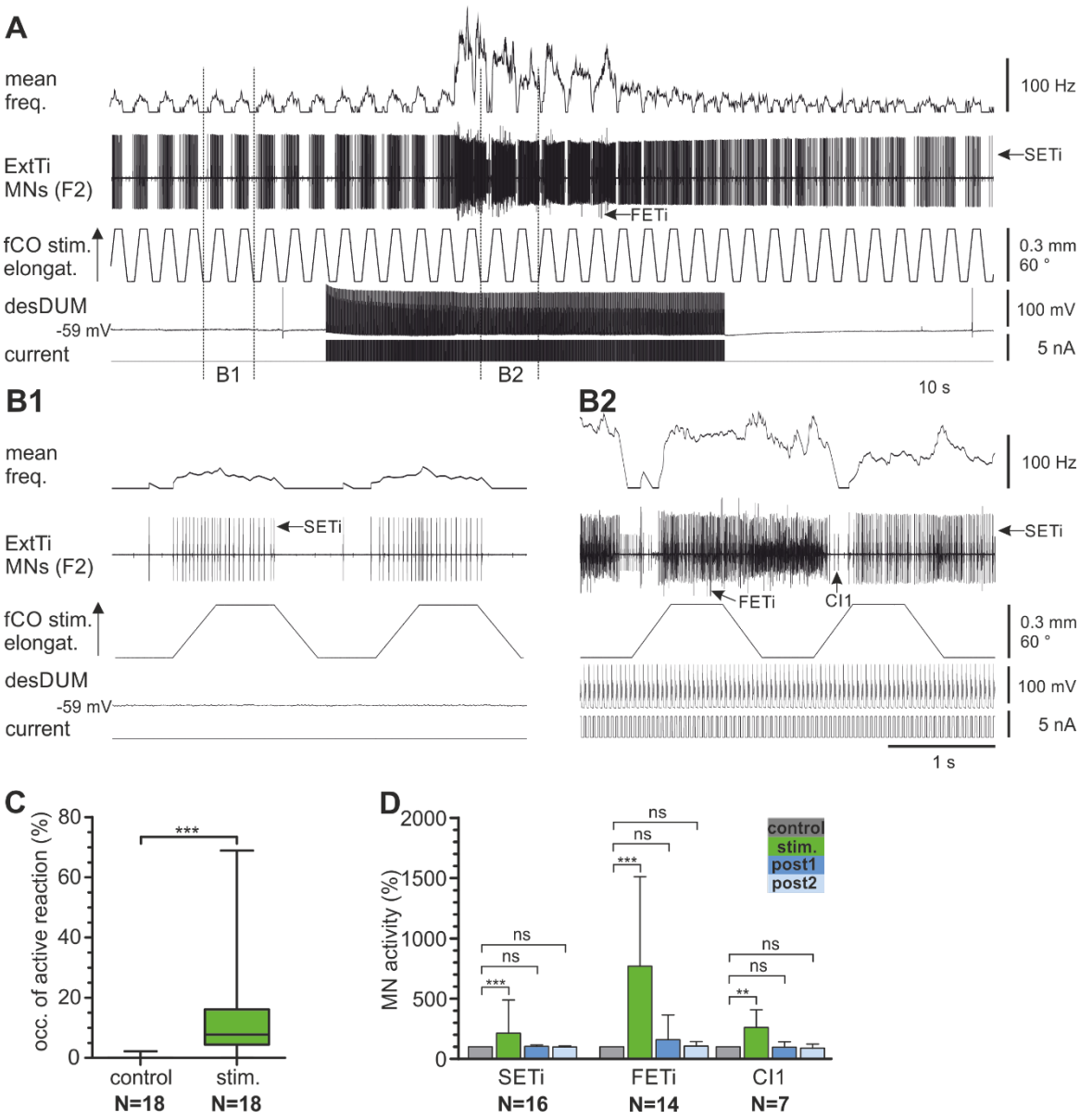

Figure 3.8 Increase in the activity of ExtTi MNs and frequency of occurrence of reflex reversal mediated by desDUM neurons. (**A**) Reflex responses in ExtTi MNs were induced by elongation (upward ramp) and relaxation (downward ramp) stimuli of the fCO. The mean frequency of SETi MN APs increased upon activation of a single desDUM neuron. (**B1** and **B2**) Details of the recording in (**A**). In addition to the increase in AP frequency, the desDUM neuron induced the occurrence of active reactions. During control, SETi MN activity was most pronounced in response to elongation-stimuli. This is characteristic for resistance reflexes (**B1**). During stimulation of a desDUM neuron, maxima in SETi MN activity shifted towards relaxation-stimuli of the fCO, which characterizes active reactions (**B2**). (**C**) The boxplots with whiskers illustrate the average frequency of occurrence of active reactions during 810 fCO stim. cycles of control (no desDUM stimulation) and 810 fCO-stim. cycles upon desDUM neuron activation in 18 experiments (statistics: Wilcoxon matched-pairs signed-rank test, p = 0.0005). Boxes are from Q1 to Q3, the vertical line in the box depicts the median, whiskers indicate 5th and 95th percentiles. (**D**) The bar graphs display the normalized average activity (mean ± SD) of excitatory and inhibitory ExtTi MNs in response to fCO stimulation during 30 s of control period (grey bars), 30 s of desDUM neuron stimulation (green bars), 30 s directly after desDUM neuron stimulation (post1, blue bars), and a consecutive 30 s section (light blue bars, post2). Each bar represents the average MN activity of n = (3*N) sections. DesDUM neurons significantly increased the activity of all MN units. The mean effects ceased after desDUM neuron stimulation (post1). The differences between control>stim., control>post1, and control>post2 were tested with the Friedman Test in combination with Dunn´s Multiple Comparison Test. Statistical significance is denoted as follows: (ns) not significant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N= number of experiments.

Figure 3.8D summarizes the effects of desDUM neurons that mediate an excitatory effect on the average activity of distinct units of the ExtTi MN pool. The figure depicts the average sum of ExtTi MN APs over 15 fCO stim. cycles before desDUM neuron stimulation ("control", grey bars), during desDUM neuron stimulation ("stim.", green bars), and after desDUM neuron stimulation ("post1", blue bars, and "post2", light blue bars). The average activity of SETi MNs was significantly increased upon desDUM neuron activation to 213.4 ± 274.9% of that in the control condition (mean ± SD, 1st green bar, $p < 0.001$, Friedman test with Dunn´s Multiple Comparison Test, N = 16 experiments). The average activity of SETi MNs was not significantly different from the control during post1 and post2. The average activity of FETi MNs was significantly increased upon desDUM neuron activation to 769.5 ± 742.7% of that in the control condition (mean ± SD, 2nd green bar, $p < 0.001$, N = 14 experiments). The average activity of FETi MNs was not significantly different from the control during post1 and post2. The average activity of the inhibitory CI_1 MNs was significantly increased upon desDUM neuron activation to 261.6 ± 146.0% of that in the control condition (mean ± SD, 3rd green bar, $p < 0.01$, N = 7 experiments). The average activity of FETi MNs was not significantly different from the control during post1 and post2.

These results demonstrate that the stimulation of desDUM neurons with an excitatory influence on SETi MN activity (Fig. 3.7.2A) triggered a similar increase in the average activity of FETi MNs and CI_1 neurons. In a large fraction of experiments, the absolute reflex-evoked activity of FETi MNs during the control was very low. This is why the increase during desDUM neuron stimulation appears very pronounced in the normalized averages. Fig. 3.7.2A showed that the excitatory influence of desDUM neurons on SETi MN activity ceased within 10 s after the end of desDUM neuron stimulation. These results indicate that this might also be the case for FETi MN and CI_1 neuron activity, as there were no significant differences between control and post1 (Fig. 3.8D). Each experiment consisted of three trials with sections of control, desDUM neuron stimulation, post1, and post2. In all the experiments, ExtTi MN activity was consistently increased in every trial from control to activation of desDUM neurons, triggering an excitatory effect.

Characterization of the inhibitory net-output effect on FETi, SETi, and CI1 MNs

Fig. 3.9.1A shows a representative recording of a desDUM neuron that decreased reflex-evoked SETi and FETi MN activity. The magnitude of resistance reflexes was reduced, but reflex reversals did not occur (detail: Fig. 3.9.1B1 and B2). Quantitative analysis demonstrated that desDUM neurons of the class with an inhibitory influence on SETi MN activity did not increase the frequency of occurrence of active reactions. There was no active reaction observed in any of 855 fCO-stim. cycles without desDUM stimulation (control) or during 855 fCO-stim. cycles during desDUM neuron stimulation (N = 19 experiments, data not shown). Fig. 3.9.1C summarizes the effects of inhibitory desDUM neurons on the average activity of distinct units of the ExtTi MN pool. The figure displays the average sum of ExtTi MN APs over all 15 fCO stim. cycles before desDUM neuron stimulation ("control", grey bars), during desDUM neuron stimulation ("stim.", green bars), and after desDUM neuron stimulation ("post1", blue bars, and "post2", light blue bars). The average activity of the SETi MNs significantly decreased upon desDUM neuron activation to 77.1 ± 18.4% of that in the control condition (mean ± SD, 1st red bar, $p < 0.001$, Friedman test with Dunn´s Multiple Comparison Test, N = 19 experiments). The average SETi MN activity was significantly different from the control during post1 (85.0 ± 8.5%, mean ± SD, 1st blue bar, $p < 0.001$, N = 19 experiments). There was no significant difference in SETi MN activity between control and post2 (90.1 ± 8.8%, mean ± SD, 1st light blue bar, $p > 0.05$, N = 19 experiments). The average activity of FETi MNs significantly decreased upon desDUM neuron stimulation to 40.1 ± 25.7% of that in the control condition (mean ± SD, 2nd red bar, $p < 0.001$, N = 9 experiments). The average activity of FETi MNs was not significantly different from the control during the sections post1 and post2 (2nd blue and light blue bars). The average activity of the inhibitory CI_1 MNs increased slightly but not significantly during desDUM neuron activation (141.2 ± 31.4%, mean ± SD, 3rd red bar, $p > 0.05$, N = 4). The average activity of CI_1 neurons was not significantly different between control and the sections post1 (3rd blue bar) and post2 (3rd light blue bar).

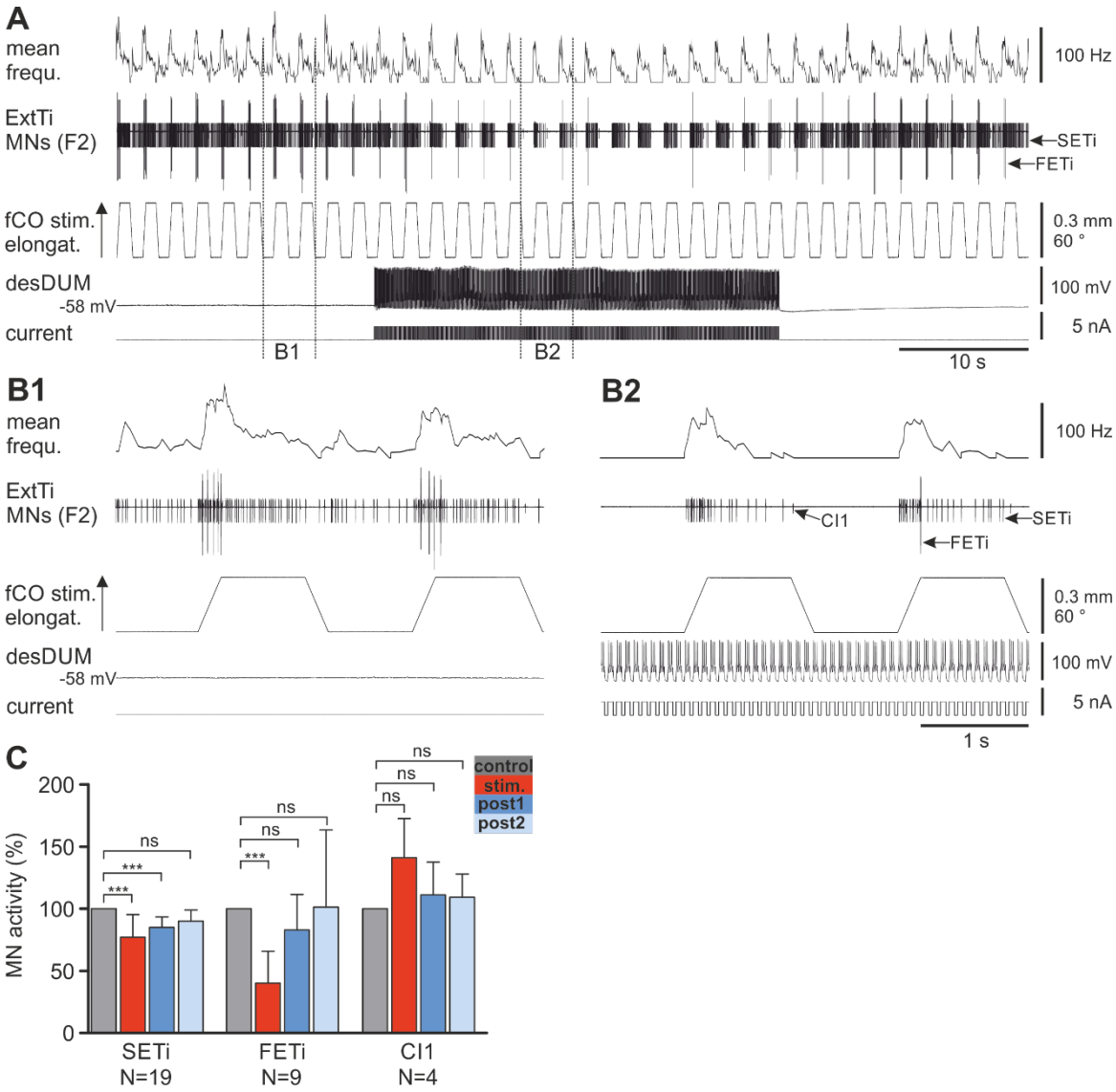

Figure 3.9.1 Decrease in the activity of reflex-induced excitatory ExtTi MNs mediated by desDUM neurons. (**A**) Resistance reflex responses in ExtTi MNs were induced by stimulation of a middle-leg fCO. The magnitude of SETi and FETi MN activity decreased after activation of a single desDUM neuron. (**B1** and **B2**) Details of recording (**A**). (**C**) The bar graphs display the normalized average activity (mean ± SD) of excitatory and inhibitory ExtTi MNs in response to fCO stimulation during 30 s of control (grey bars), 30 s of desDUM neuron stimulation (red bars), 30 s directly after desDUM neuron stimulation (post1, blue bars), and a consecutive 30 s section (light blue bars, post2). Each bar represents the average MN activity of n = (3*N) sections. DesDUM neuron activation significantly decreased the activity of SETi MNs (N = 19) and FETi MNs (N = 9). The decrease in SETi MN activity was still present after desDUM neuron stimulation (post1). CI1 neuron activity did not decrease, but rather showed a tendency to increase during stimulation of desDUM neurons (N = 4). The differences between control>stim., control>post1, and control>post2 were tested with the Friedman Test in combination with Dunn´s Multiple Comparison Test. Statistical significance is denoted as follows: (ns) not significant, $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N indicates number of experiments.

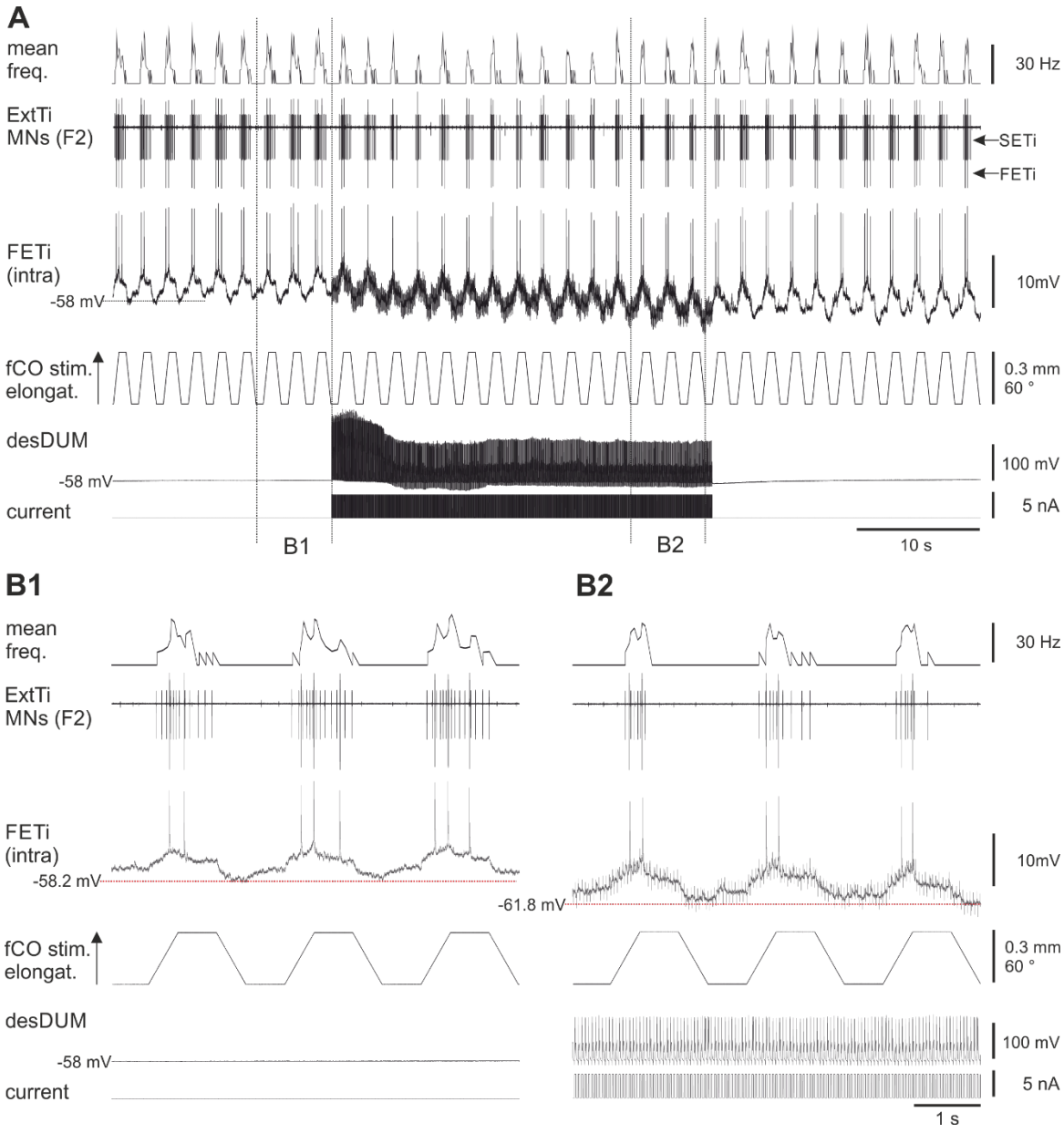

Fig. 3.9.2 Simultaneous intracellular recording of a desDUM neuron and a FETi MN (**A**) A desDUM neuron mediated a decrease in the AP frequency of SETi and FETi MNs (**B1** and **B2**) detail of A.). The FETi MN membrane potential hyperpolarized during desDUM neuron stimulation.

Fig. 3.9.2 shows a simultaneous intracellular recording of a desDUM neuron and a FETi MN. The membrane potential of the FETi MN was hyperpolarized upon desDUM neuron activation.

The results show that the activation of desDUM neurons that had previously been demonstrated to trigger an inhibitory effect on SETi MN activity (Fig. 3.7.2B) mediated a decrease in the average activity of FETi MNs. In contrast, the activity of CI_1 neurons was

not significantly affected by activation of these desDUM neurons. Each of the experiments consisted of three trials. SETi and FETi MN activity consistently decreased compared to the control upon activation of desDUM neurons that were classified as mediating an inhibitory effect in every trial.

Analysis of net-output of desDUM neurons that mediated a slightly inhibitory effect

One class of desDUM neurons was characterized to have a delayed and rather faint inhibitory influence on SETi MN activity during single cycles of fCO stimulation (Fig. 3.7.2C). Fig. 3.10A shows a recording of a desDUM neuron that did not have an explicit effect on reflex-evoked ExtTi MN activity (detail: Fig. 3.10B1 and B2). Quantitative analysis demonstrated that average reflex-evoked SETi MN activity did not significantly decrease upon activation of desDUM neurons of this class (SETi activity: 97.1 ± 2.7%, mean ± SD, Fig. 3.10C yellow bar, $p > 0.05$, Friedman test with Dunn´s Multiple Comparison Test, N = 7 experiments). There was also no significant delayed inhibitory effect as activity in post1 was not significantly different to control activity before desDUM neuron stimulation (SETi activity: 97.0 ± 3.3%, mean ± SD, blue bar, $p > 0.05$, N = 7 experiments). These results indicate that the delayed inhibitory influence on SETi MN activity that was apparent during just a few cycles of fCO stimulation is insignificant in the evaluation of the net effect on SETi MN activity over a physiologically relevant period of 30 s.

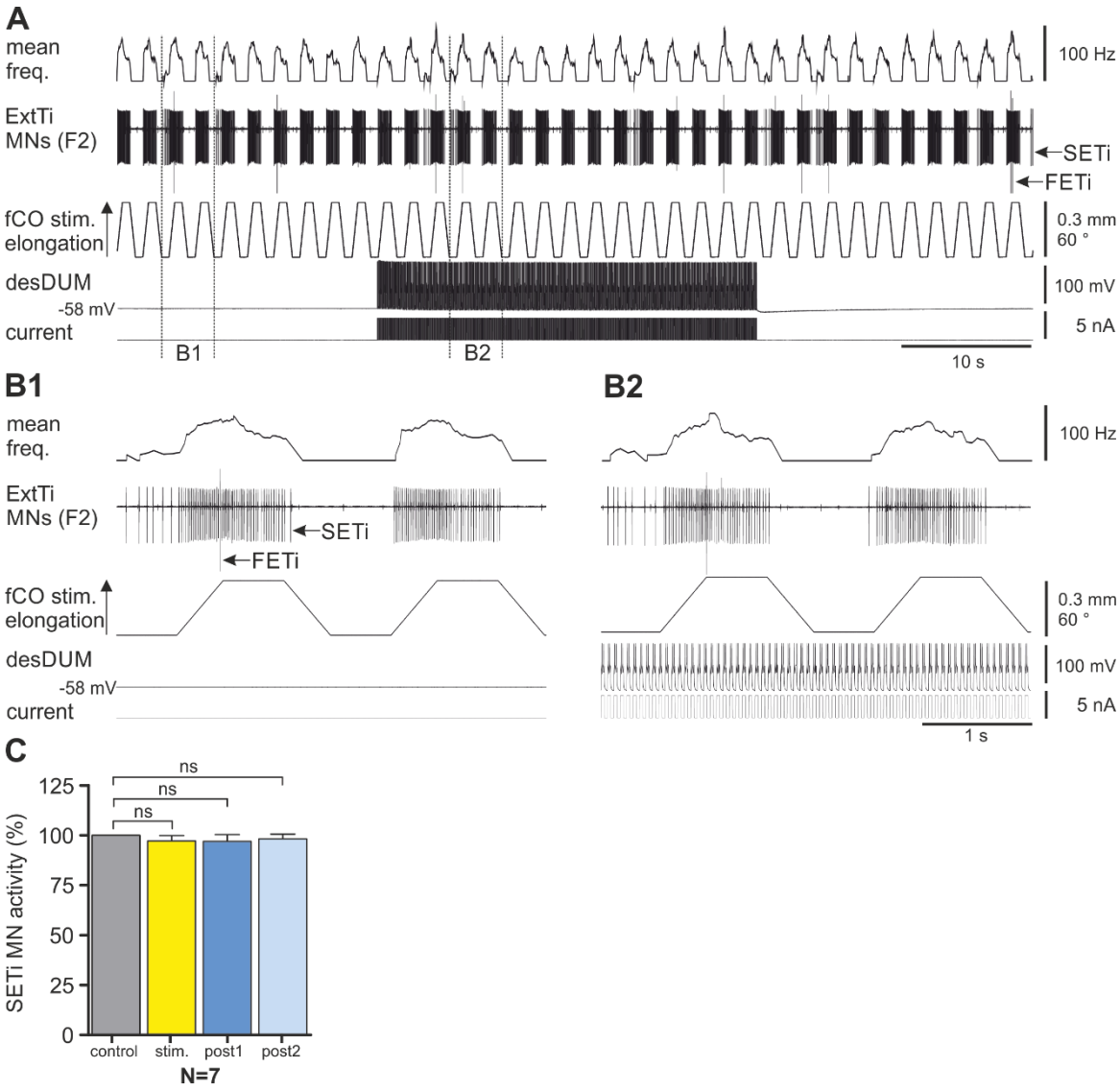

Figure 3.10 desDUM neurons with no significant effect on reflex-induced ExtTi MN activity. (**A**) Resistance reflex responses in ExtTi MNs were induced by stimulation of the left middle-leg fCO. The mean AP frequency of ExtTi MNs was not affected by the activation of a single desDUM neuron. (**B1** and **B2**): Details of recording (**A**). (**C**) The bar graph displays the normalized average SETi MN activity (mean ± SD) in response to fCO stimulation during 30 s of control (grey bar), 30 s of desDUM neuron stimulation (yellow bar), 30 s directly after desDUM neuron stimulation (post1, blue bar), and a consecutive 30-s section (light blue bar, post2). Each bar represents the average MN activity of 21 sections. On average, desDUM neurons (N = 7) had no effect on SETi MN activity. The differences between control>stim., control>post1, and control>post2 were tested with the Friedman test in combination with Dunn´s Multiple Comparison Test. Statistical significance is denoted as follows: (ns) not significant $p > 0.05$. N indicates number of experiments.

3.3.1.1.3 Characterization of output effects on properties of fCO-evoked reflex responses

The results of the previous sections on output physiology, suggest that desDUM neurons have an effect on mesothoracic ExtTi MN activity. Moreover, it appears that the population of desDUM neurons comprises distinct neuron classes that can be distinguished according to their influences on reflex-evoked ExtTi MN activity. In the process, it was sufficient to restrict the quantitative analysis of effects to the average sum of ExtTi MN activity over single cycles of fCO stimulation (Fig. 3.7.1 and 3.7.2) or average sum of ExtTi MN activity over sections of 15 cycles of fCO stimulation (Fig. 3.8D, 3.9C, and 3.10 C). This was done to investigate how properties of the reflex responses are affected by desDUM neuron stimulation. To address this question, I created histograms of averaged and normalized ExtTi MN activity before desDUM neuron stimulation ("control"), during desDUM neuron stimulation ("stim"), and after desDUM neuron stimulation ("post1", and "post2") over the 2-s time course of fCO ramp-and-hold stimulation (for details, Section 2.8.).

Excitatory influence on fCO-induced reflex responses

Figure 3.11A shows that the time course of average **SETi MN activity** during control (grey bars, N = 15) was typical for fCO stimulation-induced resistance reflex responses. There was a characteristic increase in activity in response to fCO elongation (upward ramp, 2nd and 3rd grey bars) and increased tonic activity during the elongated hold part of the stimulation (horizontal line, 4th, 5th, and 6th grey bars). FCO relaxation (downward ramp) induced a pronounced decrease in average SETi MN activity (7th and 8th grey bars), which slightly recovered during the relaxed hold part of the stimulation (9th and 10th grey bars). Upon stimulation of single desDUM neurons, there was a significant increase in average SETi MN activity throughout the entire time course of fCO stimulation, except for during elongation (2nd and 3rd green bar). The increase in average SETi MN activity was most pronounced during fCO relaxation and the relaxed-hold part (7th, 8th, 9th, and 10th green bars).

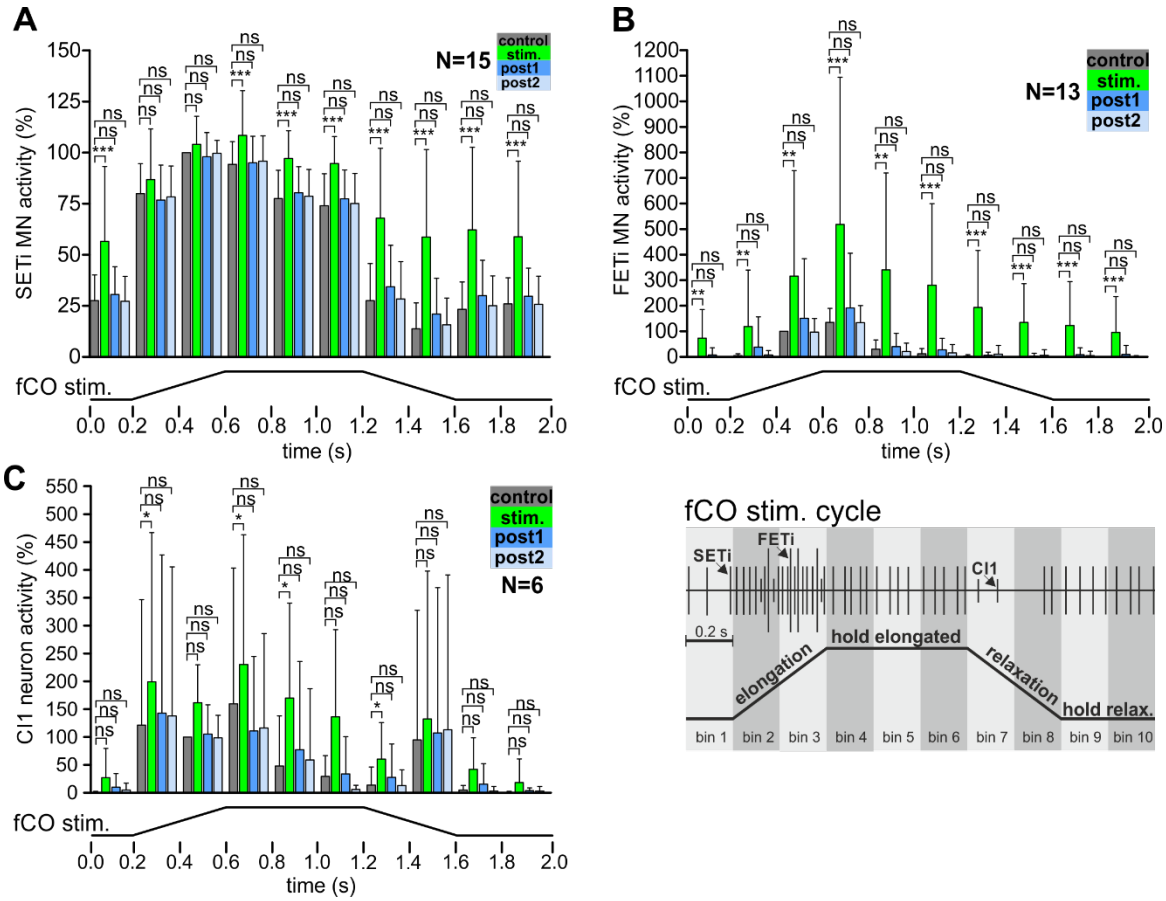

Figure 3.11 Excitatory influence on sensorimotor interaction mediated by desDUM neurons. (**A-C**) Histograms of normalized average fCO-induced ExtTi MN activity (mean +/- SD) of experiments in which an excitatory effect of desDUM neuron stimulation had been observed. ExtTi MN activity during control condition (control, grey bars), during stimulation of single desDUM neurons (stim., green bars), directly after desDUM neuron stimulation (post1, blue bars) and during 15 successive cycles of fCO stimulation (post2, light blue bars) is plotted over a cycle of fCO stimulation (200ms bin size). Each bar represents the average MN activity over n= 45*N fCO-stim. cycles. (**A-B**) The effect of desDUM neuron-stimulation on the activity of SETI and FETi MNs was highly significant during the elongated-hold part of ramp stimulation and during relaxation stimuli. When the fCO apodeme was elongated, there was no significant increase in SETi and FETi MN activity upon desDUM neuron stimulation. (**B**) The large standard deviations indicate big differences in the baseline activity and effect size in individual animals. In general, the excitatory effect ceased after desDUM neuron stimulation. (**C**) There was a tendency for an increase in CI1 neuron activity during desDUM neuron stimulation throughout the entire cycle of fCO stimulation, but the increase was only statistically significant for 4 bins. (**A-C**) The differences between control - stim., control - post1, and control - post2 were tested with Friedman Test in combination with Dunn´s Multiple Comparison Test. Statistical significance is denoted as follows: (ns) not significant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N indicates number of experiments.

The specific alterations in SETi MN activity upon desDUM neuron stimulation in part reflect the increased occurrence of active reactions, characterized by inhibition of SETi MNs during fCO elongation (Bässler, 1976; 1986; 1988; Bässler and Büschges, 1998). This would explain why there was no significant increase in SETI MN activity during fCO elongation upon stimulation of desDUM neurons (2nd and 3rd green bars). Furthermore, fCO relaxation is excitatory during the active reaction, and SETi MN

activity is tonically increased during the relaxed hold part. These were the time points in the experiments where the increase in SETi MN activity was most pronounced upon desDUM neuron stimulation (Fig. 3.11A 7th, 8th, 9th, and 10th green bars). The fact that the activity did not decrease in response to fCO elongation, as it would be expected for active reactions, suggests that there was a general excitatory influence of desDUM neurons on SETI MN activity during resistance reflexes on top of the increased frequency for reflex reversal. In the follow-up periods of post1 and post2, the average SETi MN activity was not significantly different from the control at any point during fCO stimulation.

Next, I wanted to investigate whether the previous observations on the effect of desDUM neurons on SETI MNs also hold for **FETi MNs** (Fig. 3.11B). The controls reflect typical FETi MN activity for resistance reflexes (Fig. 3.11B, grey bars, N = 13 experiments). FETi MN activity significantly increased upon desDUM neuron stimulation throughout the entire time course of fCO stimulation. The increase was most pronounced at the start of the elongated hold part (4th green bar) and during fCO relaxation (7th and 8th green bars). This is where FETi MNs receive excitatory input during active reactions (Bässler and Büschges, 1998). The recruitment of FETi MNs in response to desDUM neuron stimulation in many experiments indicates that desDUM neurons may influence the excitability of FETi MNs. Thus, the effects on the excitatory ExtTi MN units appear to be an increase in excitability and a specific influence on pathways that control the generation of active reactions during fCO stimulation.

The time course of average **CI_1 neuron activity** during the control (Fig. 3.11C, grey bars, N = 6) was typical for fCO-induced resistance reflex responses. There was a characteristic increase in activity in response to fCO elongation (fCO stim. upward ramp, 2nd and 3rd grey bars) and increased tonic activity during the elongated hold-part of stimulation (fCO stim. upper horizontal line, 4th, 5th, and 6th grey bars). Relaxation of the fCO (fCO stim. downward ramp), in contrast to SETi and FETi MN activity, induced an increase in average CI_1 activity (8th grey bar) that declined during the relaxed hold part of fCO stimulation (9th and 10th grey bars). Upon activation of desDUM neurons, average CI_1 activity was significantly increased during the first 200 ms of fCO elongation (2nd green bar), during the first 400 ms of the elongated-hold part (4th and 5th green bars), and during the first 200 ms of fCO relaxation (7th green bar). The specific alterations in average CI_1

activity upon desDUM stimulation in part reflect the increased occurrence of active reactions. In active animals, average CI_1 neuron activity is increased (Bässler, 1986).

Inhibitory influence on fCO-induced reflex responses

Figure 3.12A shows that the time course of average **SETi MN activity** during control (grey bars, N = 19 experiments) was typical for fCO stimulation-induced resistance reflex responses. There was a characteristic increase in activity in response to fCO elongation (upward ramp, 2nd and 3rd grey bars) and increased tonic activity during the elongated-hold part of stimulation (horizontal line, 4th, 5th, and 6th grey bars). Relaxation of the fCO (downward ramp) induced a pronounced decrease in average SETi MN activity (7th and 8th grey bars), which slightly recovered during the relaxed-hold part of stimulation (9th and 10th grey bars). Upon desDUM activation, the average SETi MN activity decreased throughout the entire time course of fCO stimulation except for the last 200 ms of relaxation (8th red bar) and first 200 ms of the relaxed-hold part (9th red bar). The decrease in average SETi MN activity was most pronounced during fCO elongation and the elongated-hold part (2nd to 6th green bars). This is where the SETi MNs receive inhibitory input during resistance reflexes (Bässler and Büschges, 1998). These observations suggest that desDUM neurons decrease the magnitude of resistance reflexes in SETi MNs. The absence of a significant decrease during fCO relaxation and shortly thereafter might be due to an overlap of desDUM neuron influence and general inhibitory input to SETi MNs at this point.

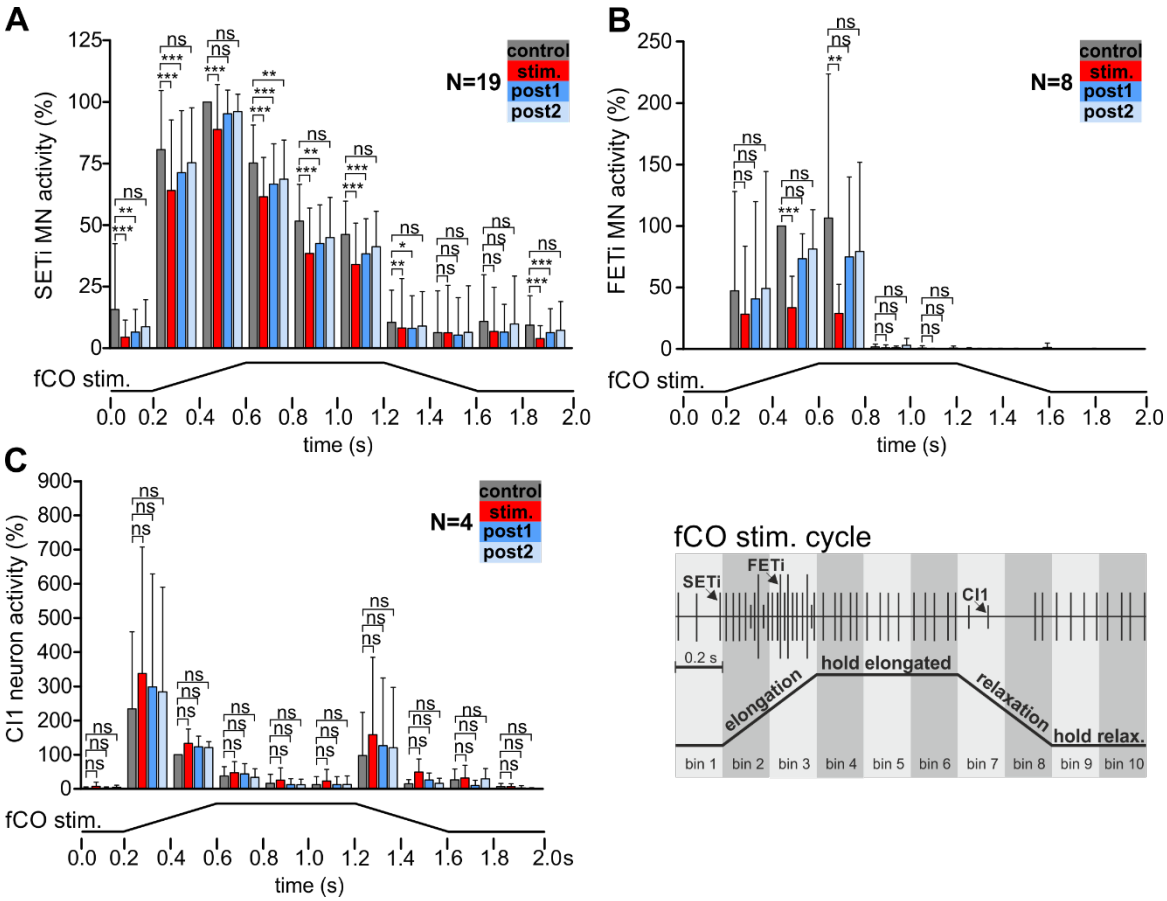

Figure 3.12 Influence of desDUM neurons on the magnitude of resistance reflexes. (**A-C**) Histograms of normalized average fCO-induced ExtTi MN activity (mean +/- SD) of of experiments where an inhibitory effect of desDUM neuron stimulation had been observed before. ExtTi MN activity during the control condition (control, grey bars), during stimulation of single desDUM neurons (stim., green bars), directly after desDUM neuron stimulation (post1post1, blue bars) and during 15 successive cycles of fCO stimulation (post2post2, light blue bars) is plotted over one cycle of fCO stimulation (200-ms bin size). (**A-B**) The inhibitory effect mediated by desDUM neuron stimulation was most prominent at time points where the control activity was high. That is, namely during fCO elongation and at begin the start of the elongated-hold part of fCO stimulation. (**B**) The large standard deviations indicate huge large differences in the baseline activity and effect size in individual animals. In general, the inhibitory effect ceased after desDUM neuron stimulation. (**C**) There was a tendency for an increase in CI1 neuron activity during desDUM neuron stimulation throughout the entire cycle of fCO stimulation, but the increase was not significant. (**A-C**) The differences between control> stim., control>post1, and control>post2 were tested with the Friedman Test in combination with Dunn´s Multiple Comparison Test. Statistical significance is denoted as follows: (ns) not significant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N indicates number of experiments.

FETi MN activity was observed during fCO elongation (Fig. 3.12B, 2nd and 3rd grey bars, N = 8) and within the first 200 ms of the elongated hold-part of stimulation during the control period (3rd grey bar). During the rest of the time course of fCO stimulation, there was no or only very faint FETi MN activity in the control condition. This is the characteristic appearance of FETi MN activity during resistance reflexes (Bässler and Büschges, 1998). Activation of desDUM neurons induced a significant decrease of FETi

MN activity during the last 200 ms of fCO elongation (3rd grey bar) and during the first 200 ms of the elongated-hold part of stimulation (4th grey bar).

The time course of average **CI_1 neuron activity** during the control condition (Fig. 3.12C, grey bars, N = 4) was typical for fCO stimulation-induced resistance reflex responses. DesDUM neuron stimulation did not have a statistically significant effect on CI_1 neuron activity. In contrast to the decrease of activity caused in the excitatory ExtTi MNs, the activity of CI_1 increased slightly.

3.3.1.1.4 Output effects of desDUM neurons upon physiological activation

For the previous examination of desDUM neuron effects, I induced AP generation in desDUM neurons with depolarizing current pulses to their somata. In the process, the elicited frequency of APs in desDUM neurons was about 25-45 Hz. I wanted to test whether significantly lower AP frequency of desDUM neurons is as effective as the induced high-frequency activity. Current injection was adjusted to produce mean AP frequencies of 2-10 Hz in desDUM neurons. The average activity induced in desDUM neurons during single-leg forward stepping ranged from 0.7-9.3 Hz (lowest and highest animal mean; average: 3.05 ± 2.20 Hz, mean ± SD, N = 17 desDUM neurons). This demonstrates that the induced low-frequency activity was in a physiological range. In 6 of 9 experiments, the phasic nature of the input to desDUM neurons during single-leg stepping and six-legged walking was mimicked by appropriately patterned current injection. Trials with low-frequency activation were performed on desDUM neurons that had been classified as having either an inhibitory or excitatory influence under the induced high-frequency activity in the same experiment.

Excitory output effect upon physiological activation

In three prior trials with induced high-frequency activity (30 Hz), the desDUM neuron displayed in Fig. 3.13A induced a significant increase in reflex-evoked SETi MN activity. When the mean AP frequency of the desDUM neuron was reduced to 5 Hz, there was still a significant increase in SETi MN activity.

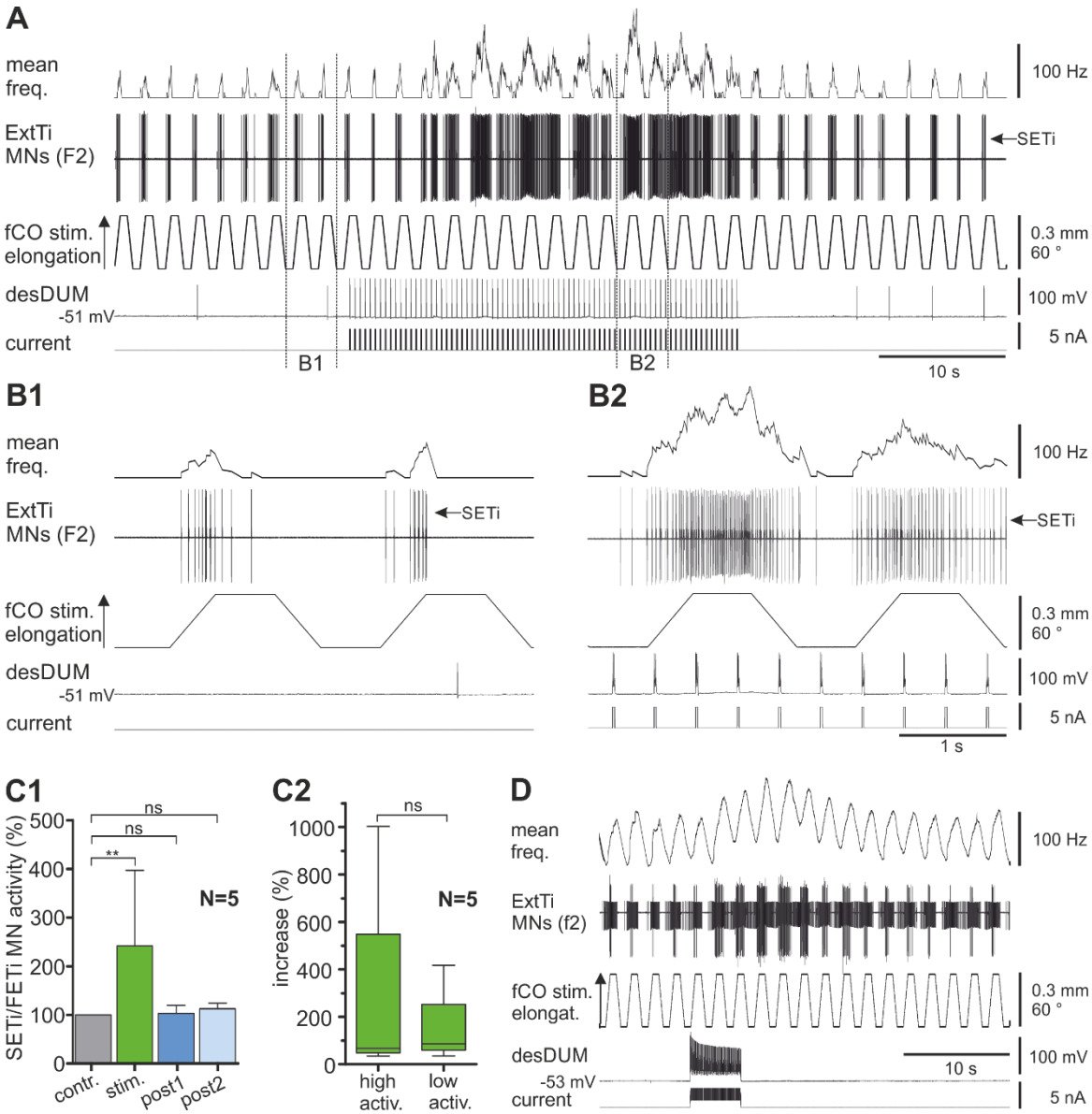

Figure 3.13 Excitatory effect on refle-induced ExtTi MN activity mediated by physiological stimulation of desDUM neurons. (**A**) Example recording of a desDUM neuron that was activated to produce a low AP frequency of 5 Hz but still had a pronounced excitatory effect on fCO-induced SETi MN activity. (**B1** and **B2**) details of (**A**). (**C1**) The bar graph displays the normalized average SETI MN activity (mean ± SD) in response to fCO-stimulation during 30 s of the control condition (grey bar), 30 s of induced low-frequency desDUM neuron activity (green bar), 30 s directly after desDUM neuron stimulation (post1, blue bar), and a consecutive 30-s section (post2, light blue bar). Each bar represents the average MN activity over 15 sections. Induced low-frequency activity in desDUM neurons caused a significant increase in the activity of SETi MNs. The mean effects ceased after desDUM neuron stimulation (post1, post2). The differences between control>stim., control>post1, and control>post2 were tested with the Friedman Test in combination with Dunn´s Multiple Comparison Test. (**C2**) The average percent increase induced by the same 5 desDUM neurons either with high AP frequencies (high active.) or low AP frequencies (low activ.) was not significantly different ($p = 0.813$, Wilcoxon matched-pairs signed-rank test). Boxes are from Q1 to Q3, the vertical line in the box depicts the median, whiskers indicate 5th and 95th percentiles. (**C1-C2**) Statistical significance is denoted as follows: (ns) not significant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N indicates number of experiments. (**D**) Brief (5 s) activation of a desDUM neuron induced a long-lasting increase in ExtTi MN activity.

In this example, complete reflex reversals did not occur, but SETi MN activity increased during the elongated-hold part and during fCO relaxation relative to the control condition before desDUM neuron stimulation (detail: Fig. 3.13B1 and B2). Quantitative analysis demonstrated that average SETi and FETi MN activity significantly increased to 241.6 ± 55.5% of that in the control condition upon low-frequency activity of desDUM neurons (mean ± SD, $p \leq 0.01$, Friedman test with Dunn´s Multiple Comparison Test, N = 5 experiments; Fig. 3.13C1). There was no significant difference between the percent increase upon induced high-frequency activity in desDUM neurons (252.2 ± 419.8%, mean ± SD, N = 5 experiments, Fig. 3.13C2) and low-frequency activation of the same neurons (141.6 ± 155.5%, mean ± SD, $p = 0.813$, Wilcoxon matched-pairs signed-rank test, N = 5 experiments).

Fig. 3.13D displays a recording of a desDUM neuron that increased SETi and FETi MN activity upon brief (5 s) activation. The increase in ExtTi MN activity outlasted the desDUM neuron activation for about 15 s (mean frequency trace, Fig. 3.13D). In fact, the peak mean frequency of FETi and SETi MNs occurred about 4 s after termination of desDUM neuron stimulation. The time course of the excitatory effect upon brief stimulation was reminiscent of the average time course of excitatory influences displayed in response to 30 s induced high-frequency activity in desDUM neurons (see Fig. 3.7.2A). Pronounced and long-lasting effects of brief activation were observed in 6 of 7 investigated desDUM neurons. The results demonstrate that activation of desDUM neurons at a frequency close to the normal physiological frequency triggered effects that were similar to the effects of induced high-frequency activity in previous experiments.

Inhibitory output effect upon physiological activation

Next, I tested whether inducing activity at a more physiologically-relevant frequency was just as effective as induced high-frequency activity in desDUM neurons that caused a decrease in ExtTi MN activity. Fig. 3.14A shows a recording of a desDUM neuron that had an inhibitory influence on SETi MN activity upon induction of activity at an AP frequency of 10 Hz (detail: Fig. 3.14B1-B2). Quantitative analyses of the effect of induced low-frequency activity (Fig. 3.14C) revealed that there was a significant decrease in SETi MN activity to 78.6 ± 14.9% of the control condition before desDUM neuron

stimulation (red bar, mean ± SD, $p = 0.012$, repeated measures ANOVA with Bonferroni correction, $N = 4$). During post1 (blue bar) the decrease to 89.5 ± 15.4% (mean ± SD) of the control condition was not statistically significant ($p > 0.05$, $N = 4$). The effect of induced low-frequency activity was smaller and more transient than the effect of induced high-frequency activity in desDUM neurons. This became apparent in the differences in the influence on MN activity during post1.

Together, these results demonstrate that the output properties of desDUM neurons upon induced high-frequency activity were also observed during physiologically relevant induced low-frequency activity and brief activation to a large extent.

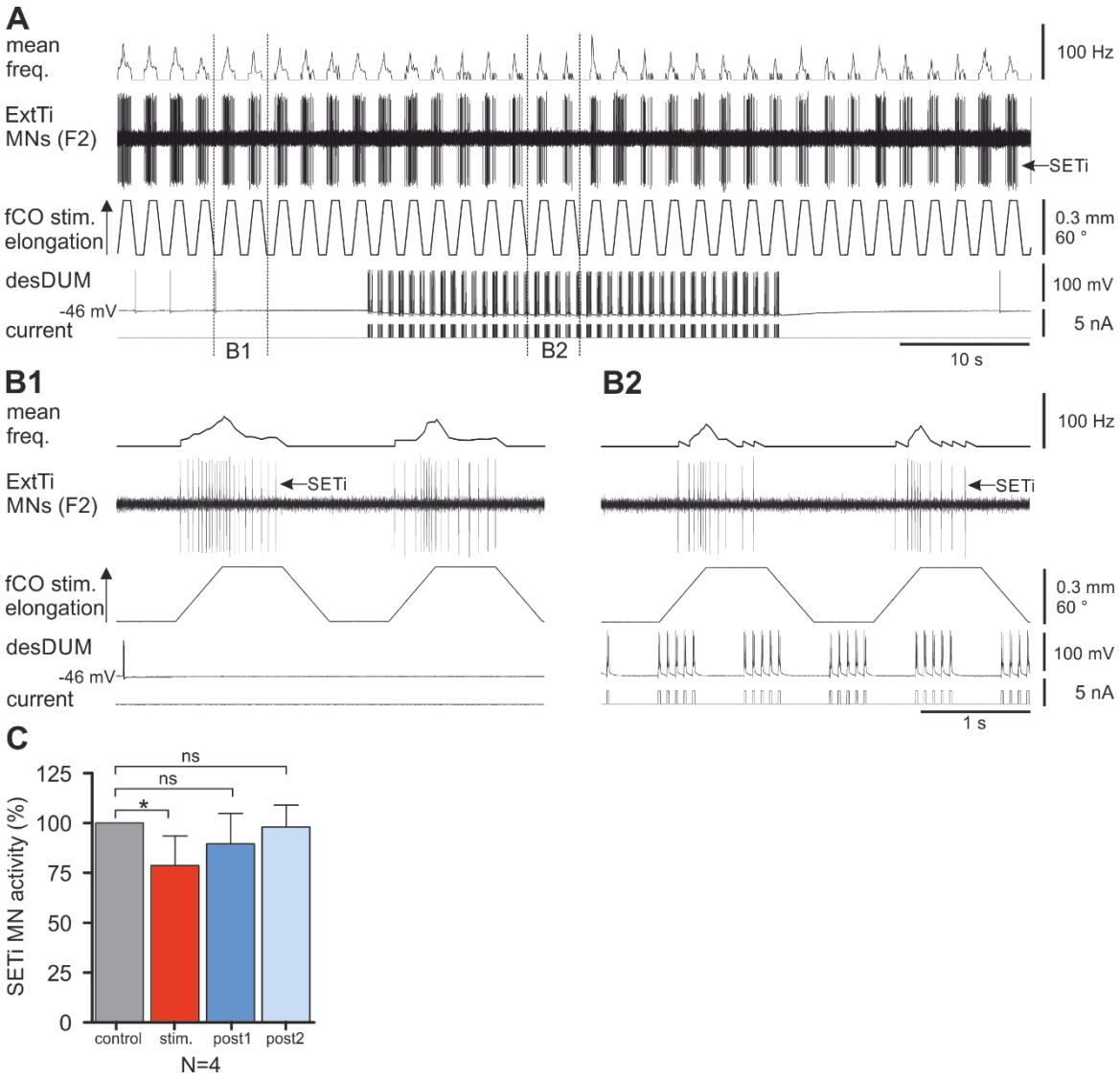

Figure 3.14 Inhbitory effect on reflex-induced ExtTi MN activity mediated by physiological stimulation of desDUM neurons. (**A**) Representative recording of a desDUM neuron that was induced to produce an AP frequency of 10 Hz and had an inhibitory influence on fCO stimulation-induced SETi MN activity. (**B1** and **B2**) details of (**A**). (**C**) The bar graph displays the normalized average SETI MN activity (mean ± SD) in response to fCO stimulation during 30 s of the control condition (grey bar), 30 s of induced low-frequency desDUM activity (red bar), 30 s directly after desDUM neuron stimulation (post1, blue bar), and a consecutive 30-s section (light blue bar, post2). Each bar represents the average MN activity of 12 sections. Induced low-frequency activity of desDUM neurons induced a significant decrease in the activity of SETi MNs. The differences between control>stim., control>post1, and control>post2 were tested with a repeated measures ANOVA with Bonferroni correction. Statistical significance is denoted as follows: (ns) not significant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. N indicates number of experiments.

3.3.1.2 Effects of desDUM neurons on spontaneous ExtTi MN activity

Some desDUM neurons mediated a decrease in the amplitude of resistance reflexes in ExtTi MNs, while other desDUM neurons induced an increase in ExtTi MN activity during resistance reflexes and additionally triggered the occurrence of a reflex reversal. I

asked whether these effects only occur when the MN activity was generated in response to fCO stimulation. To address this question, I investigated whether the general excitatory and inhibitory influences of desDUM neurons also affect spontaneous ExtTi MN activity in the absence of fCO stimulation. Trials to test effects on spontaneous MN activity were performed on desDUM neurons that had been classified as either having an inhibitory or excitatory influence due to induced high-frequency activity in the same experiment.

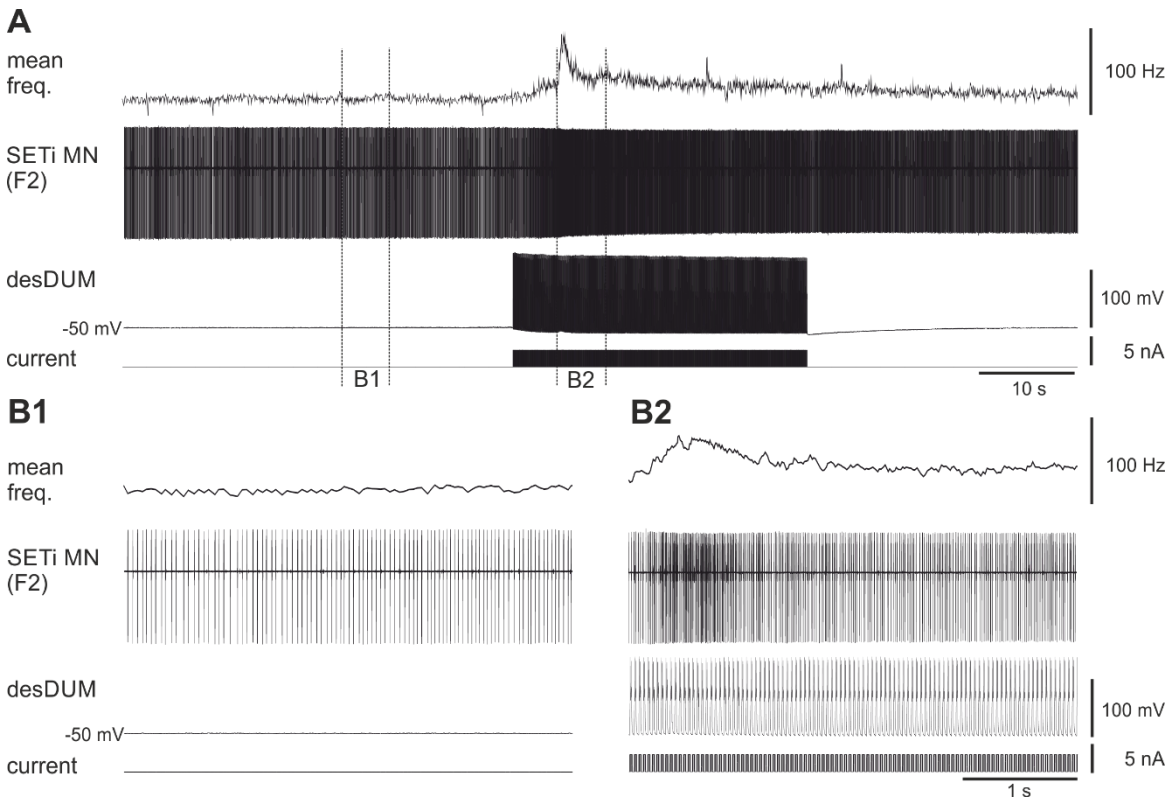

Figure 3.15 Excitatory effect on spontaneous ExtTi MN activity mediated by desDUM neurons. (**A**) The activity of a single desDUM neuron that has previously been shown to mediate an excitatory effect on reflex evoked MN activity, increased spontaneous tonic SETi MN activity. The increase peaked at about 10 s of desDUM neuron stimulation. (**B1** and **B2**) details of (**A**).

When animals were at rest, spontaneous tonic SETi MN activity was observed. Figure 3.15A shows a recording in which a SETi MN spontaneously generated APs at a mean frequency of ~18 Hz (control). Upon stimulation of a desDUM neuron, the AP frequency steadily increased until, at about 5 s after the beginning of stimulation, a drastic increase in the AP frequency to 90 Hz was observed (detail: Fig. 3.15B2). After this transient 1 s bout of high-frequency activity, the SETi MN frequency decreased throughout the

continuous desDUM neuron stimulation to 30 Hz at the end of the stimulation. From there, the activity did not return to the control AP frequency (18 Hz) before desDUM until 70 s after stimulation. Similar observations were made in 4 of 7 experiments that each contained at least 2 trials.

Figure 3.16A shows an intracellular recording of a desDUM neuron in addition to an extracellular recording of spontaneous SETi MN activity, which had a AP frequency of ~0.5 Hz before desDUM neuron activation (control). After about 5 s of desDUM neuron activation, bouts in SETi MN activity with a peak AP frequency of 50 Hz occurred. These burst-like increases in activity were observed throughout desDUM neuron activation.

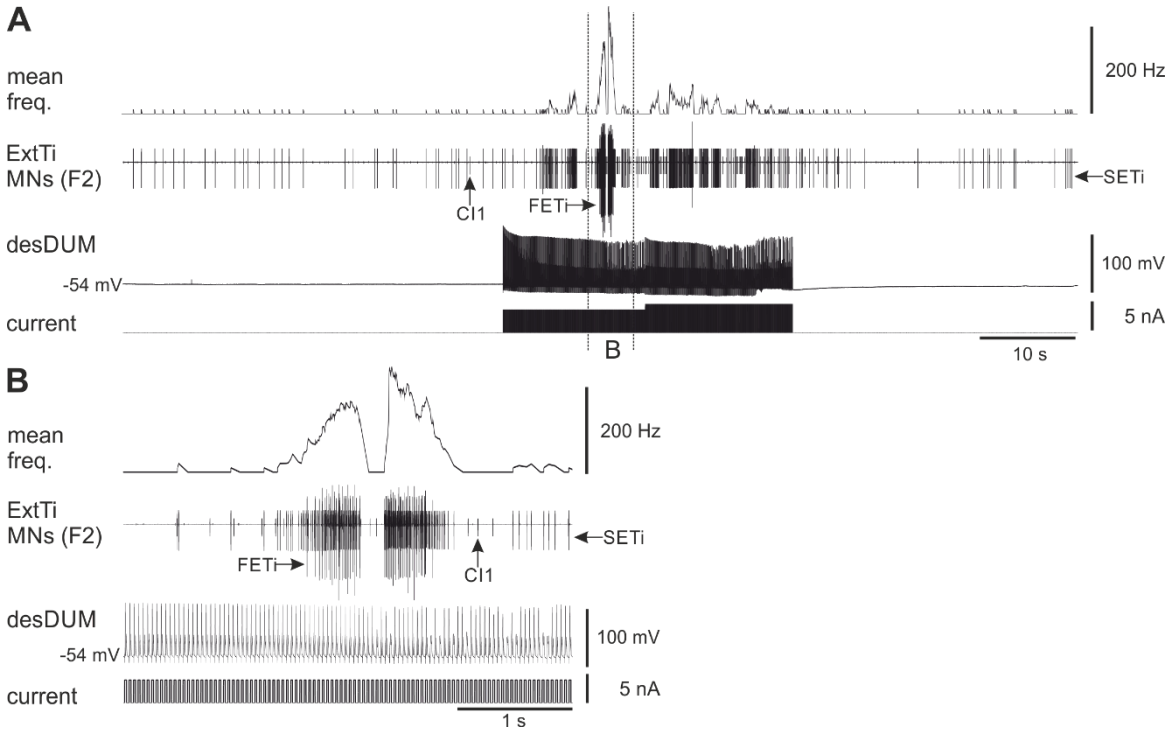

Figure 3.16 Excitatory influence on spontaneous ExtTi MN activity mediated by desDUM neurons. (**A**) The activation of a single desDUM neuron that was previously shown to mediate an excitatory influence on reflex-evoked MN activity, induced an increase in spontaneous tonic SETi MN activity. The increase was not only characterized by higher tonic AP frequency but also featured bouts of activity in a SETi MN and the recruitment of a FETi MN. (**B1** and **B2**) details of (**A**).

The mean AP frequency within these bouts decreased during the last 10 s of the 30-s desDUM neuron stimulation. In addition to an increase in SETi MN activity, a FETi MN was recruited upon stimulation of the desDUM neuron. There were no FETi MN APs

before desDUM neuron activation. Similar observations were made in 3 of 7 experiments that each contained at least 2 trials. In general, there was no correlation between high or low SETi AP frequency in the control and occurrence of tonic (N = 4 experiments) or burst-like increases (N = 3 experiments) in activity upon desDUM neuron stimulation.

Figure 3.17A shows a recording of a desDUM neuron that previously had an inhibitory influence on fCO stimulation-evoked ExtTi MN activity and also led to a decrease in spontaneous SETi MN activity in the current experiment. In this experiment, the mean AP frequency of the SETi MN was about 2 Hz before desDUM neuron activation (control). At 5 s after the start of desDUM neuron activation, the mean frequency was 0.2 Hz. At the end of the 30-s desDUM neuron stimulation, the activity had completely ceased. SETi MN AP generation recovered to the control level 12 s after the end of desDUM neuron activation. Similar observations were made in 11 of 11 experiments that each included at least 3 trials.

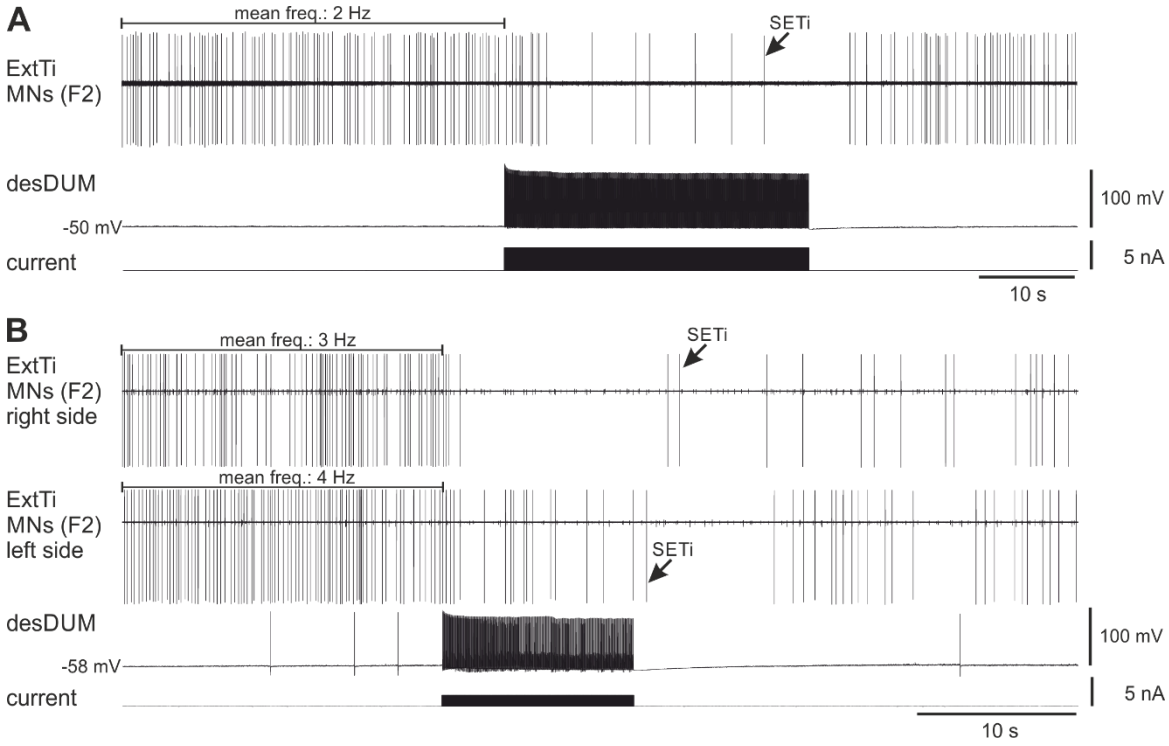

Figure 3.17 Inhibitory effect on spontaneous MN activity mediated by desDUM neurons. (**A**) The activity of a single desDUM neuron that had been previously shown to mediate an inhibitory influence on reflex-evoked MN activity, decreased spontaneous tonic SETi MN activity. (**B**) The activity of a single inhibitory desDUM neuron induced a bilateral decrease in spontaneous tonic SETi MN activity in left and right ExtTi nerve F2.

The primary neurites of desDUM neurons bifurcate into two bilateral symmetrically descending axons (see: Fig. 3.1). This morphology suggests that the neurons exert a modulatory influence on ExtTi MNs of both the left and right sides of the body. To test for this possibility, I performed extracellular recordings of SETi MN activity in the ExtTi nerves (F2 nerves) of the left and right middle legs and activated single desDUM neurons. Fig. 3.17B shows a recording of spontaneous SETi MN activity at about 3 Hz for the right middle leg and 4 Hz for the left middle leg before desDUM neuron stimulation (control). Upon desDUM neuron stimulation, the SETi MN activity in both ExtTi nerves simultaneously decreased after 1 s. In fact, SETi MN activity on the right side completely diminished. On the left side, SETi MN activity decreased to a mean AP frequency of ~1 Hz. Activity in both nerves almost simultaneously recovered to control levels over 50 s after the end of the 10-s desDUM neuron activation. Similar observations were made in 3 of 3 experiments.

In summary, the results indicate that desDUM neurons modulate the magnitude of spontaneous activity in resting animals. This modulation was simultaneously seen on ExtTi MNs of both body sides of the mesothoracic segment.

3.3.2 Effect of desDUM neurons on activity of ProCx and RetCx MNs

The previously described experiments reveal a modulatory influence of desDUM neurons on the magnitude (AP frequency) of MN activity exclusively at the example of reflex responses of ExtTi MNs. This triggered the question of whether the output of desDUM neurons is restricted to this MN pool or whether the influence on magnitude is rather unspecific and the activity of other leg MN pools is affected as well. Furthermore, the previous experiments do not give an indication as to the synchronous modulation of MNs innervating the antagonistic *flexor tibiae* (FlxTi) muscle. The questions thus arose whether activity in antagonistic MNs is similarly modulated, whether possible co-contractions of muscles would occur, or whether activity of antagonistic MN pools is alternating. Additionally, desDUM neurons with an excitatory influence increased the frequency of occurrence of reflex reversals during stimulation of the fCO. Therefore, I wanted to test whether sensorimotor interactions involving other sense organs are

modulated as well. In the previous experiments, ExtTi MN activity either occurred spontaneously or as a reflex response to stimulation of the fCO.

3.3.2.1 Effect on CS stimulation-induced MN activity

In the following experiments, I stimulated leg CS by bending the leg caudally to evoke reflex responses in ProCx and RetCx MNs (see section 2.9).

Figure 3.18A1 displays extracellular recordings of ProCx and RetCx MN activity evoked by stimulation of CS. During the control period, before desDUM neuron stimulation, only CI_1 activity was seen in recording of the nerve containing ProCx MN axons (nerve nl2). Activity in the nerve containing RetCx MN axons (nerve nl5) was characteristic of reflex responses to activation of CS group1 (Schmitz, 1993; Zill et al., 2012). Upon stimulation of a desDUM neuron, RetCx MN activity increased (mean frequency trace). Furthermore, additional units (large amplitude APs) of the RetCx and ProCx MN pools were recruited. The appearance of large ProCx MN units was observed in 4 of 6 experiments. The increase in RetCx MN activity was observed in 8 of 8 experiments. The excitatory effect of desDUM neurons on RetCx MN activity occurred after about 4-8 s of activation.

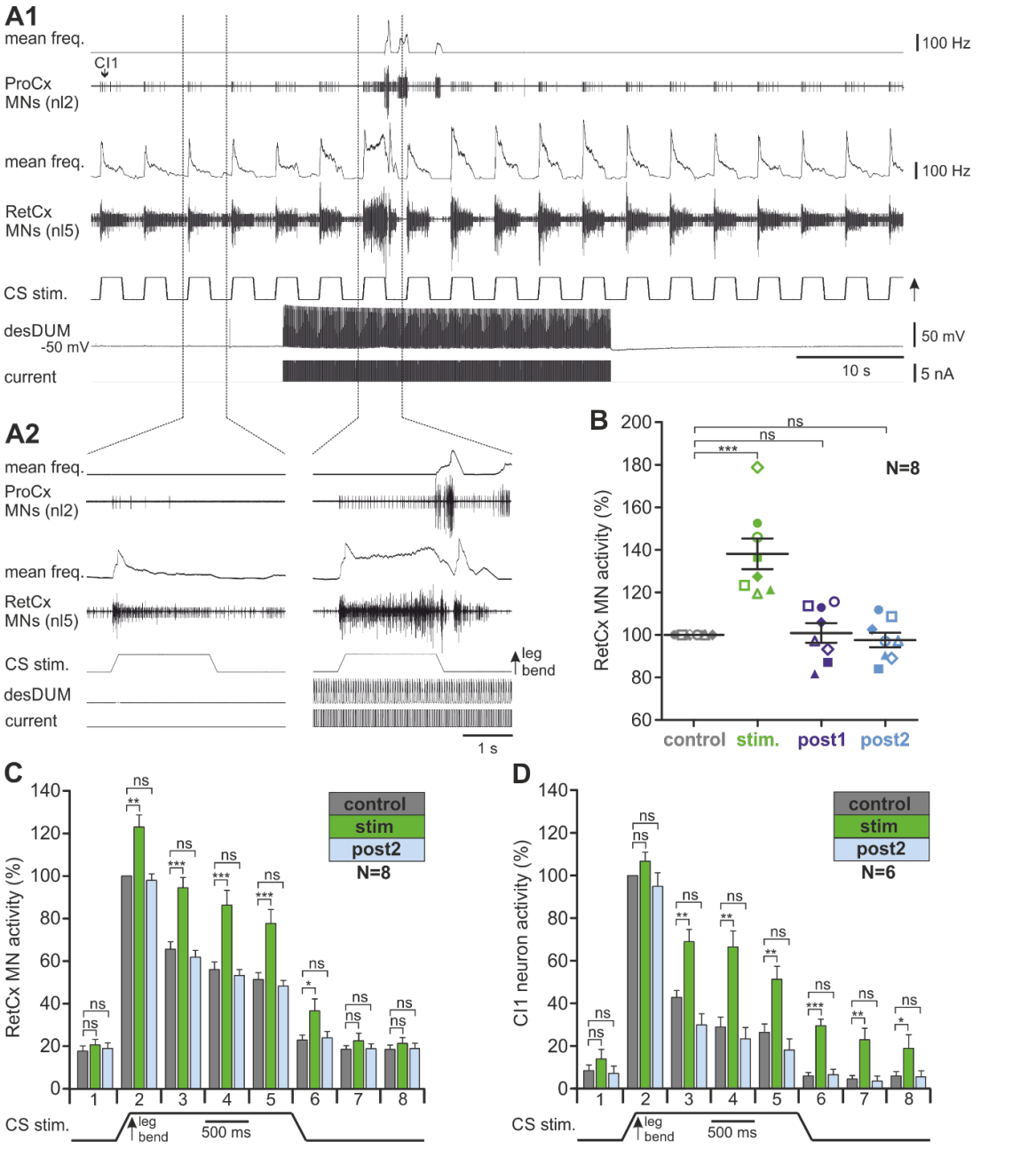

Figure 3.18 Effects of DesDUM neurons on ProCx and RetCx MN activity evoked by stimulation of campaniform sensilla (CS). (**A1**) Mean AP frequency of ProCx and RetCx MNs was increased by activation of a single desDUM neuron. (**A2**) Excerpts of the recording trace show recruitment of large excitatory units in ProCx and RetCx MN recordings during desDUM neuron stimulation. The antagonistic MN pools displayed alternating activity. (**B**) Normalized RetCx MN activity of single experiments (N = 8) during CS stimulation, as well as before (control, grey symbols), during (stim., green symbols), directly after (post1, blue symbols), and 30 s after (post2, light blue symbols) desDUM neuron stimulation. The black horizontal lines indicate mean RetCx MN activity (± SD). Symbol shape denotes affiliation to single experiments. There was a significant increase in RetCx MN activity upon desDUM neuron stimulation ($p < 0.0001$, repeated measures ANOVA with Bonferroni correction, N = 8 neurons, n = 24 sections of desDUM neuron stimulation). (**C**) Histogram of normalized RetCx MN activity before (control, grey bars), during (green bars), and 28 s after (post2, light blue bars) desDUM neuron stimulation plotted over CS stimulation (bottom trace). Each bar represents average activity over 168 CS stimulation cycles (bin size: 500 ms). (**C2**) Histogram of normalized CI1 MN activity before (black bars), during (red bars), and 30 s after (blue bars) desDUM neuron stimulation plotted over CS stimulation (bottom trace). (**C1**, **C2**) Differences between control>stim. and control>post2 were analyzed with a repeated measures ANOVA with Bonferroni correction. Statistical significance denoted as follows: (ns) not significant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Quantitative analysis (Fig. 3.18B) revealed a significant increase in RetCx MN activity to 138.1 ± 20.3% of the control level (mean ± SD, $p < 0.0001$, repeated measures ANOVA with Bonferroni correction, N = 8 experiments, n = 24 sections of desDUM neuron stimulation) upon desDUM neuron stimulation. After desDUM neuron stimulation (post1 and post2), average RetCx MN activity decreased to the control level within 28 s. Fig. 3.18C displays the excitatory influence on RetCx MN activity over the course of CS stimulation in detail. The average increase in RetCx MN activity upon desDUM neuron activation was statistically significant during leg bending (2nd green bar, upward ramp, $p < 0.01$, repeated measures ANOVA with Bonferroni correction, N = 8 experiments, n = 168 CS stimuli) and during release of the leg (6th green bar, downward ramp, $p < 0.05$, N = 8 experiments, n = 168 CS stimuli). The increase was most pronounced during the hold part of the stimulation (3rd, 4th, and 5th green bars, upper horizontal line, each data set: $p < 0.001$, N = 8 experiments, n = 168 CS stimuli). The excitatory effect ceased within 30 s after desDUM neuron stimulation (blue bars, each data set: $p > 0.05$, N = 8 experiments, n = 168 CS stimuli). In addition to the effects on excitatory MNs, modulation of CI_1 was also investigated. CI_1 activity increased in 6 of 6 experiments during desDUM stimulation (data not explicitly shown, but see Fig. 3.18D). Quantitative analysis revealed that CI_1 neuron activity was modulated by desDUM neurons similarly to RetCx MN activity over the course of CS stimulation (Fig. 3.18D). From these data, it can be concluded that activation of single desDUM neurons has an excitatory influence on the activity of excitatory and inhibitory MNs that innervate antagonistic ProCx and RetCx muscles. In contrast to differential effects on reflex responses in ExtTi tibiae MNs (N = 40 experiments), desDUM neurons only had an excitatory influence on reflex responses of ProCx and RetCx MNs (N = 8 experiments).

3.3.2.2 Effects on spontaneous RetCx and ProCx MN activity

Additionally, I investigated the effect of desDUM neurons on spontaneous ProCx and RetCx MN activity in five experiments. Fig. 3.19A shows extracellular recordings of spontaneous activity in ProCx (nl2) and RetCx MNs (nl5). During control, rhythmic activity was observed in addition to tonic CI_1 activity in the ProCx MN recording (2nd trace, detail: Fig. 3.19B1). The occurrence of spontaneous rhythmic activity has been observed in most experiments in the current thesis, however, the underlying physiology

has not yet been revealed. This is why I do not consider the spontaneous rhythmic activity with respect to the evaluation of effects. The RetCx MN recording only displayed CI_1 activity during the control period (4th trace). Upon stimulation of a desDUM neuron, CI_1 neuron activity increased (2nd and 4th trace). Furthermore, additional units (large-amplitude APs) of the ProCx MN pool and the RetCx MN pool were recruited. The appearance of large ProCx MN units was observed in 3 of 5 experiments. The increase in RetCx MN activity was observed in 3 of 5 experiments. An increase in CI_1 activity was observed in 5 of 5 experiments. The recruitment of additional units occurred about 6-9 s after the beginning of desDUM neuron activation.

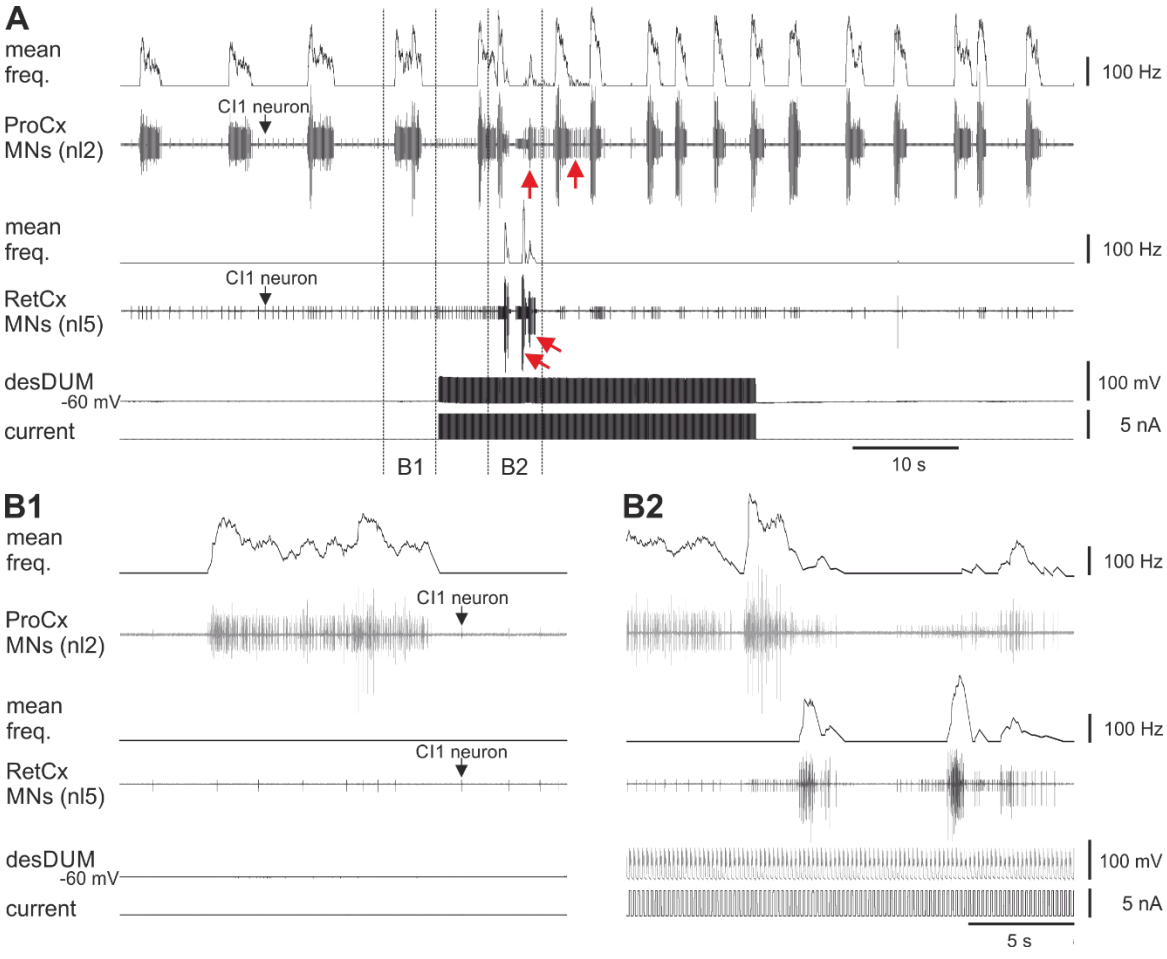

Figure 3.19 Effects of desDUM neurons on spontaneous ProCx and RetCx MN activity. (**A**) desDUM neuron increased spontaneous ProCx and RetCx MN activity. In addition to the effect on CI1 neuron AP frequency, activation of the desDUM neuron triggered the recruitment of additional large units in the ProCx MN pool (arrows) and the RetCx MN pool. (**B1** and **B2**) details of recording (**A**).

Together, these results demonstrate that desDUM neurons mediate similar excitatory effects on the antagonistic ProCx and RetCx MN pools. The activity of antagonistic MN pools alternated, which suggests that co-contraction of the muscles they innervate would not occur.

3.3.2.3 Effects on centrally generated RetCx and ProCx MN activity

The previous experiments show that the magnitude of reflex-evoked and spontaneous activity in ExtTi MNs, ProCx MNs and RetCx MNs is modulated by desDUM neurons. Next, I investigated whether desDUM neurons not only modulate the magnitude of MN activity but also alter the timing of centrally generated rhythmic activity. To target this question, I activated mesothoracic leg joint CPGs via application of pilocarpine (see mat met, (Büschges et al., 1995)). Fig. 3.20 A shows a representative recording in which pilocarpine application induced alternating rhythmic activity in the antagonistic ProCx- and RetCx MN pools. Stimulation of a desDUM neuron triggered the occurrence of a prolonged RetCx burst (about 8-s duration). Subsequently, the frequencies of ProCx- and RetCx bursts (1st and 3rd traces) were increased (burst period decreased). Both observations appeared in every trials upon activation of the desDUM neuron within the displayed experiment, and in every trial of two further experiments. I evaluated alterations in parameters related to rhythmicity (period and burst duration) and magnitude (AP frequency) of RetCx MN bursts in 5 experiments (Fig. 3.20B) and RetCx MN bursts in 3 experiments. Each experiment consisted of 2-5 trials with control sections, and sections of 60-s desDUM neuron stimulation. For each trial properties of 10 bursts before desDUM neuron stimulation (control), and 10 bursts starting 10 s from begin of desDUM neuron stimulation were evaluated. The analysis revealed that there was an influence of desDUM neurons on parameters of the rhythm in 3 of 5 experiments (Fig3.20 B1 and B2, exp.1-3). In exp.1 the average period of RetCx bursts was significantly decreased from 3.13 ± 0.76 s of the control section (mean ± SD, n=45 periods) to 2.38 ± 0.48 s (mean ± SD, n=45 periods) during desDUM neuron stimulation ($p<0.0001$, Mann Whitney U test, Fig. 3.20B1, exp.1). The RetCx burst duration decreased from 2.04 ± 0.59 s (mean ± SD, n= 50 bursts) of the control to 1.50 ± 0.48 s (mean ± SD, n=50 bursts) during desDUM neuron stimulation ($p<0.0001$, Fig. 3.20B2, exp.1). These parameters were also significantly decreased for ProCx bursts (nl2) in the same experiment (data not shown).

The AP frequency in RetCx MN bursts was significantly increased (p=0.0091) from 40.4 ± 11.9 Hz (mean ± SD, n=50 bursts) of the control to 46.7 ± 10.8 Hz (mean ± SD, n=50 bursts) during desDUM neuron-stimulation Fig3.20 B3, exp.1). The AP frequency within ProCx MN bursts was also significantly increased (p=0.0007) from 79.2 ± 19.6 Hz of the control (mean ± SD, n=50 bursts) to 93.3 ± 14.9 Hz (mean ± SD, n=50 bursts) during desDUM neuron stimulation (data not shown). In two other experiments, I only evaluated rhythmic properties of RetCx bursts (because I recorded extracellularly from nl3 (extensor nerve) and nl5 (retractor nerve) in these experiments). In the experiments (Fig. 3.20B1 and B2 exp.2 and exp.3) both the period and duration of RetCx bursts significantly decreased upon desDUM neuron stimulation. In contrast to exp.1 the AP frequency within RetCx and ProCx MN bursts was not affected. In two further experiments parameters of rhythmicity were not influenced by desDUM neuron activation (Fig. 3.20B1 and B2 exp.4 and exp.5). In exp.4 the AP frequency in RetCx bursts was significantly decreased ($p<0.0001$) from 54.1 ± 17.5 Hz (mean ± SD, n=30 bursts) of the control to 35.7 ± 8.8 Hz (mean ± SD, n=30 bursts) during desDUM neuron stimulation in another one of the 3 experiments (Fig3.20 B3, exp.4).

In summary these results suggest that some desDUM neuron have an influence on activity of CPG neurons as 3 of 5 neurons increased the frequency of pharmacologically-induced rhythmic MN activity. Furthermore there was the consistent occurrence of a prolonged retractor MN burst at begin of desDUM neuron stimulation in these 3 experiments. Two neurons had an influence on the magnitude (AP frequency) of MN activity.

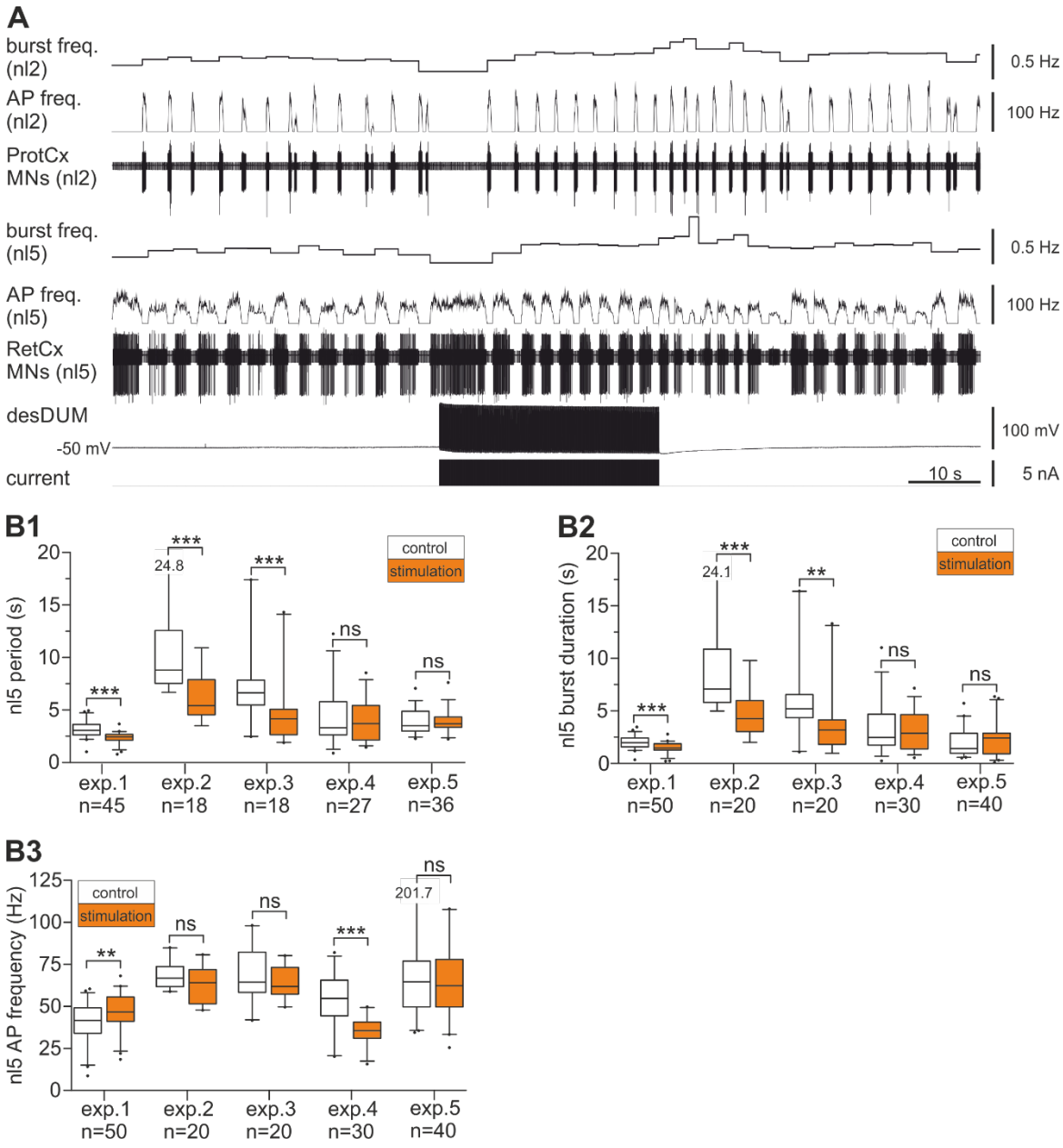

Figure 3.20 Effects of desDUM neurons on CPG-derived MN activity. (**A**) Alternating rhythmic activity in pro- and retractor coxae MNs was induced by bath application of pilocarpine to the mesothoracic ganglion. Stimulation of a single desDUM neuron induced the occurrence of a prolonged (8-s) RetCx MN burst in the beginning of stimulation. The burst frequency of ProCx- and RetCx MNs increased as a consequence of desDUM neuron stimulation. (**B1**) and (**B2**) The period and duration of RetCx MN bursts (nl5) decreased during des DUM neurons stimulation in three of five experiments. (**B3**) The AP frequency of RetCx MNs (nl5) increased in one (exp.1) experiment and decreased in a second experiment (exp.4). (**B1-B3**) Boxes are from Q1 to Q3, the vertical line in each box depicts the median, whiskers indicate 5th and 95th percentile. The differences between control and stimulation of desDUM neurons were analyzed with Mann Whitney U test. Statistical significance is denoted as follows: (ns) not significant $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

4 Discussion

In this thesis, I aimed at a comprehensive survey on a modulatory role for a population of octopaminergic descending neurons located in the stick insect GNG. By modification of well-established stick insect preparations and utilization of newly developed techniques, I took an extensive approach to investigate the neurons' morphology, octopamine content, input physiology and properties of output.

4.1 Identification of stick insect desDUM neurons

4.1.1 Morphology of desDUM neurons

Unpaired octopaminergic neurons with either dorsally (dorsal unpaired median; DUM) or ventrally (ventral unpaired median; VUM) located neurons were demonstrated at the midline of gnathal (GNG) and thoracic and abdominal ganglia in every insect species investigated (review: (Bräunig and Pflüger, 2001). These UM neurons share unique morphological features across different insect species, such as location of large somata and bilaterally symmetrical axons. The vast majority of UM neurons in the thoracic ganglia project their neurons to the periphery of the ganglia in which their cell bodies are located (review: (Bräunig and Pflüger, 2001)). In the current thesis, these local UM neurons are referred to as efferent UM neurons. In contrast to those in the thoracic and abdominal ganglia, the majority of UM neurons in the GNG are intersegmentally projecting interneurons (review: (Bräunig and Pflüger, 2001; Stevenson and Spörhase-Eichmann, 1995)). Some of the GNG UM neurons project their axons anteriorly (ascending UM neurons) to innervate principal neuropil and peripheral nerves of the brain. Another population of UM neurons exhibits axons that descend (desUM neurons) to segmental ganglia via the neck connectives. The existence of desUM neurons has been assessed in various studies on hemi- and holometabolous insects (e.g, locust: (Bräunig, 1991; Bräunig et al., 2004; Bräunig and Burrows, 2004; Kien et al., 1990); cricket:

(Spörhase-Eichmann et al., 1992); honeybee: (Kreissl et al., 1994; Schröter et al., 2007); moth: (Cholewa and Pfluger, 2009; Dacks et al., 2005); cockroach: (Sinakevitch et al., 2005); and fruit fly (Busch et al., 2009; Hsu and Bhandawat, 2016; Monastirioti et al., 1995)). Most of these studies addressed the distribution of octopaminergic neurons in the GNG and had their morphological and physiological focus on ascending UM neurons. In the stick insect, retrograde labeling of both neck connectives revealed six neurons with large somata in the posterior GNG and the existence of bilaterally descending axons in the neck connectives (Heß, Bachelor thesis).

In this thesis, these neurons were labeled intracellularly to verify their identity as desDUM neurons by means of their typical morphological characteristics. The dye fills show neurons with large somata in the posterior GNG. The cell bodies give rise to anteriorly directed primary neurites that all bifurcate at roughly the same location into two bilaterally symmetrical descending axons. The neurites display a dense branching pattern throughout the GNG, but the neurons do not project axons to side nerves of the GNG innervating muscles of the mouthparts. The neurons' descending axons project at least as far as to the mesothoracic ganglion. The most detailed morphological description of desDUM neurons to date was in the locust (Bräunig and Burrows, 2004). Six locust desDUM neurons have cell bodies in the posterior GNG. Their somata are not exclusively located medially, but also appear laterally displaced. The six neurons project a primary neurite anteriorly, where it bifurcates into two bilaterally symmetrical axons that descend to the thoracic ganglia via the neck connectives. Additionally, these neurons display an extensive branching pattern in the GNG but do not send axons to the periphery. With respect the morphological properties of soma size and location, branching patterns in the GNG, and the existence of bilateral symmetrically descending axons, there is high consistency between locust and stick insect desDUM neurons. Bräunig and Burrows (2004) stained two additional UM neurons in the posterior locust GNG cluster. One of these neurons sends axons to the circumoesophageal connectives, and a second cell has both descending and ascending axons, which is characteristic of the so-called H-cell (Bräunig, 1991). In contrast, none of the neurons that I labeled in the posterior stick insect GNG display ascending axons. In the locust, Bräunig and Burrows (2004) identified descending axons as well as branches of desDUM neurons in the prothoracic and mesothoracic ganglia Through identifying differences in the branching pattern in the

segmental ganglia, three types of desDUM neurons (two neurons of each type) were distinguished. In the current thesis, I was not able to conduct a classification of desDUM neurons based on morphological heterogeneity. I could assess the neurons' morphology in the GNG in great detail, but there were no consistent morphological differences in branching pattern between neurons. Labeling of neurites in the prothoracic or even mesothoracic ganglion was too faint for unambiguous identification of desDUM neuron branches in the current experiments. Nevertheless, I was able to show the presence of descending axons of desDUM neurons within the prothoracic ganglion and its posterior connectives. This demonstrates that stick insect desDUM neurons project at least as far as to the mesothoracic ganglion. The major problem of labeling neurites of stick insect desDUM neurons in segmental ganglia despite highly defined staining in the GNG was most likely the relatively long distances between ganglia. Even allowing 2-6 d for dye diffusion within the long descending axons did not yield acceptable results.

Immunocytochemistry for octopaminergic neurons revealed that there are seven desVUM neurons in the labial neuromere of adult hawk moth (Dacks et al., 2005). However, the branching patterns of these neurons in the segmental ganglia were not investigated in this study. The morphology of 7 desVUM neurons in the hawk moth was solely assessed using immunocytochemical techniques. Thus, the labeling was not as detailed as in intracellular dye fills of single neurons that have been done in the locust (Bräunig and Burrows, 2004) and stick insects (in this thesis). Nevertheless, it is apparent that hawk moth desVUM neurons share great morphological similarities with locust and stick insect desDUM neurons. In hawk moth larvae, there are three desVUM neurons at the posterior end of the GNG (Cholewa and Pfluger, 2009). Interestingly, these neurons can be distinguished in larvae by means of their soma location at the ventral midline, strung from anterior to posterior. As the axons of these neurons bifurcate at about the same location, the primary neurites vary in length. I did not observe such specific localization of cell bodies nor marked differences in the length of primary neurites in the current thesis. Apart from the differences outlined above, Cholewa and Pflüger (2009) do not report on further morphological characteristics of heterogeneity in the larval hawk moth desVUM neuron population.

In adult fruit flies, the population of desVUM neurons was shown to comprise three morphologically different types of neurons (Busch et al., 2009). Detailed morphology was

assessed for single neurons in the GNG and revealed similarities to previously described desUM neurons (see above). The genetic methods such as the Flp-out technique (Wong et al., 2002) applied in the study by Busch et al. (2009) did not reveal absolute numbers of fruit fly desVUM neurons.

Studies on UM neurons in the cockroach (Sinakevitch et al., 2005) and honeybee (Kreissl et al., 1994; Schröter et al., 2007; Sinakevitch et al., 2005) have demonstrated the existence of desUM neurons in the labial neuromere of the GNG but did not elaborate on their morphology. The exact numbers of desUM neurons in both insect species remain unclear. For cockroaches, the number is assumed to be between 4 and 6. For honeybees, there is no proposed number.

Together, these studies indicate that desUM neurons, which are the focus of the current thesis, are a common feature of insects. Moreover, stick insect desDUM neurons share great morphological similarities with desUM neurons of other insect species. These similarities are most obvious between stick insect and locust desDUM neurons. Given the generally large homology between stick insect and locust neurons (e.g., (Burrows, 1996); (Schmitz et al., 1991); (Büschges and Wolf, 1995), I acted on the assumption that the branching patterns of stick insect desDUM neurons in segmental ganglia, which I was unable to assess, may be very similar to those demonstrated for locust desDUM neurons by Bräunig and Burrows (Bräunig and Burrows, 2004).

4.1.2 Electrophysiological identification of desDUM neurons

In some experiments in the current thesis, intracellular labeling of desDUM neurons was not possible because the GNGs were used in further experiments. Therefore, I used distinct electrical features to substitute morphological evidence for the identification of desDUM neurons. In general, the somata of desDUM neurons in the stick insect are not visible during experiments; identification of neurons based on electrical properties facilitated the search for desDUM neurons during experiments. Nevertheless, this begged the question of whether the criteria applied for identification are valid. Efferent DUM neurons in stick insect have been shown to generate large-amplitude APs of up to 100 mV and pronounced, long-lasting afterhyperpolarizations in a previous study (Mentel et al., 2008). This appears to be a common feature of efferent UM neurons across insect species

(review: (Bräunig and Pflüger, 2001)). In addition, it has been shown that the somata of locust UM neurons can actively generate APs (Crossman et al., 1971; Grolleau and Lapied, 2000; Heidel and Pflüger, 2006). This is the reason why I aimed for neurons in the posterior part of the stick insect GNG that displayed APs with amplitudes larger than 70 mV and afterhyperpolarizations larger than 8 mV and that responded to weakly depolarizing current injection by generating APs. All neurons that were electrically identified according to these properties exclusively revealed the morphological properties of desDUM neurons presented above when they were filled with dye. While searching for desDUM neurons, I often recorded other neurons with APs of up to 80 mV but only small afterhyperpolarizations of ~3 mV and no significant responses to depolarizing current injection. Staining of these neurons revealed only unilateral descending axons and small anteriorly located cell bodies. These properties were reminiscent of descending neurons of the stick insect antennal mechanosensory pathway (Ache and Dürr, 2013) that project one descending axon past the location of some desDUM neuron cell bodies. Overall, these findings indicate that the method of electrical identification of desDUM neurons was valid. Interestingly, the only other studies that touch upon electrical properties of desUM neurons (locust: (Bräunig, 1991; Bräunig and Burrows, 2004) larval hawk moth: (Cholewa and Pfluger, 2009)) claim that desUM neurons exhibit membrane properties dissimilar to efferent UM neurons. The neurons spontaneously generate APs at a faster rate, with significantly smaller amplitudes (locust: 21 mV, hawkmoth: 3 mV), briefer durations, and much smaller afterhyperpolarizations in comparison to efferent UM neurons (Heidel and Pflüger, 2006) in the respective species. These conclusions, however, were drawn from only a few intracellular recordings. In the current thesis, I never came across neurons showing such properties in, by far, more than 1000 attempts to record from desDUM neurons in the posterior stick insect GNG. For locusts, it was shown that the large soma APs are carried by Na^+ and Ca^{2+} currents (Grolleau and Lapied, 2000). With respect to the electrical properties revealed in my experiments, stick insect desDUM neurons are likely to exhibit membrane properties and current compositions typical of efferent UM neurons, rather than desUM neurons, of adult locust and hawk moth larvae.

4.1.3 Octopamine content

In the current thesis, I investigated the potential neuromodulatory influence of desDUM neurons on thoracic locomotor network activity. Until now, it had not been demonstrated that stick insect desDUM neurons are indeed octopaminergic. In other insect species, the OA content of homologous neurons has been analyzed either directly by distinct labeling of desDUM neurons with fluorophore-coupled antisera against OA (Busch et al., 2009; Cholewa and Pflüger, 2009; Dacks et al., 2005) and genetic approaches (Busch et al., 2009) or indirectly with OA immunocytochemisty labeling tissue in areas where desUM neurons are putatively located (Bräunig, 1991; Hsu and Bhandawat, 2016; Kreissl et al., 1994; Stevenson and Spörhase-Eichmann, 1995). In the stick insect, previous attempts to establish immunocytochemistry against OA had failed or yielded inconclusive results. Thus, I employed a recently established direct approach (Diesner et al., 2018) to assess the OA content of single identified neurons in collaboration with Max Diesner and Dr. Susanne Neupert (University of Cologne, Department of Biology, Institute for Zoology) . In brief, desDUM neurons were identified by their electrical properties in intracellular recordings. After electrophysiological experiments, cell bodies were labelled with fluorescent dye, dissected out of the animal, and analyzed for their OA content using MALDI-TOF MS. In some cases, whether the neurons coexpressed the OA precursor and neuroactive biogenic amine TA was additionally investigated. Using this direct analytical tool, we could clearly demonstrate that all desDUM neurons investigated herein were octopaminergic.

4.2 Input physiology of desDUM neurons

4.2.1 Activity of desDUM neurons during walking

A precondition for a neuromodulatory role of desDUM neurons in locomotor activity is their activation in conjunction with walking behavior. The results of experiments correlating these two events demonstrate that stick insect desDUM neurons are activated during walking. The phasic coupling of AP generation to stance phases of legs was indicated during restrained six-legged walking and became obvious during single-leg stepping of a middle leg. Activation of desUM neurons during semi-intact walking has not been previously reported. In general, information on the activity patterns of

descending neuromodulatory neurons during legged-locomotion, or even in response to leg movement, is scarce. Bräunig et al. (2004) claim that electrical stimulation of hind-leg ExtTi muscles induces leg movements which trigger the generation of EPSPs in desDUM neurons (Bräunig et al., 2004). The corresponding data and number of experiments, however, are not shown in the paper. Tschida and Bhandawat (2015) show that the firing rate of a pair of descending dopaminergic neurons located in the fruit fly GNG increased during leg movements in a speed-dependent fashion (Tschida and Bhandawat, 2015). In the experiments underlying this statement, the flies were tethered such that legs had no ground contact and the flies were stimulated to produce leg movements while descending dopaminergic neurons were electrophysiologically recorded. The leg movements were grossly grouped into "kicking" movements, which induced a burst-like increase in activity, and "rubbing" movements, which induced a tonic increase in activity.

In vertebrates, multi-channel recordings have revealed that putative serotonergic descending neurons in the dorsal raphe nucleus of freely moving rats exhibit low tonic firing rates and a low sensitivity to specific locomotor activity, except during the transition from sleep to wakefulness (Waterhouse et al., 2004). In freely moving cats, extracellular single-unit recordings showed that activity of serotonergic neurons of the caudal raphe nuclei is increased by locomotor activity (Veasey et al., 1995). In conclusion, the results of the current thesis novelly demonstrate a phasic activation of single descending neuromodulatory neurons during legged locomotion. For efferent thoracic DUM neurons in cricket and stick insect, activation during walking or single-leg stepping has been demonstrated (Gras et al., 1990; Mentel et al., 2008). Overall, the results support the idea (Baudoux et al., 1998; Duch et al., 1999) that octopaminergic UM neurons are recruited in parallel to locomotor activity in a task-specific manner.

4.2.2 Activity of desDUM neurons during centrally generated rhythmic leg MN activity

In the current thesis, I investigated the neuronal origin of excitatory input to desDUM neurons during walking. It is generally accepted that rhythmic activity in MNs that drives muscle activity during walking is the result of an interplay between activity of CPGs and feedback from sensory organs in response to leg movement (Bidaye et al., 2018). Thus, these influences are potential drivers of the activation of desDUM neurons during

walking. First, I studied the potential influence of CPGs on desDUM neuron activity. Bath application of pilocarpine to the mesothoracic ganglion to activate leg joint CPGs did not affect the membrane potential of desDUM neurons. This result is contrasts with observations made in hawk moth larvae, where activity in all 3 morphologically different desVUM neurons is coupled to depressor/flexor MN bursts during a pilocarpine-evoked fictive crawling motor pattern (Cholewa and Pfluger, 2009). Varying experimental approaches might be responsible for this discrepancy between stick insects and hawk moth larvae. Cholewa and Pflüger (2009) applied pilocarpine to the entire isolated nerve cord, whereas I applied pilocarpine only to a single thoracic ganglion. This might have caused a lower level of overall activation insufficient for the activation of desDUM neurons. Furthermore, there is strong intra- and intersegmental cycle-to-cycle coupling of pilocarpine-induced MN activity in hawk moth larvae (Johnston and Levine, 1996). In each cycle, alternating bursts in levator/extensor nerves and depressor/flexor nerves of all three pairs of thoracic legs are bilaterally synchronized with respect to left or right body side. This indicates that there is rather strong coupling of central drive in the hawk moth. This strong rhythmic central drive to depressor/flexor MNs might also be the source of rhythmic depolarization in larval desUM neurons. In contrast, pilocarpine application to the isolated nerve cord of adult stick insects revealed no stereotyped cycle-to-cycle coupling of activity in MNs innervating muscles of different leg segments (Büschges et al., 1995). Apart from alternation of activity in MNs innervating antagonistic leg muscles, pilocarpine-induced MN activity cannot be assigned to specific events in the step cycle (stance phase, swing phase) during active walking. Moreover, intersegmental coupling of MN activity is also rather weak in stick insects (Büschges et al., 1995; Mantziaris et al., 2017). This weak central coupling between leg joint CPGs in stick insects might result in rather weak central drive to desDUM neurons. In addition to desVUM neurons, activity of larval efferent VUM neurons of thoracic ganglia is also coupled to a fictive crawling motor pattern (Johnston et al., 1999). Baudoux et al. (1998) have shown that the activity of some locust efferent DUM neurons that innervate leg muscles is coupled to bursts of MNs innervating stance phase muscles during established pilocarpine-induced rhythmic motor patterns (Baudoux et al., 1998). Here it must be noted that the pilocarpine-induced motor pattern (fictive locomotion) in locusts is considered structurally similar to that during legged-locomotion in intact animals (Ryckebusch and Laurent, 1993; 1994). In the stick insect, efferent DUM neurons are rhythmically active in isolated pilocarpine-

activated nerve cord preparations (Goldammer et al., in preparation). In this case, the activity is not driven by the same central source of activation as to RetCx MNs (innervating fictive stance phase muscles). The results in the current thesis show that stick insect desDUM neurons are not activated by the rhythmic central drive that shapes the rhythmic activity of MNs during walking, in contrast to UM neurons in other insects and body segments. This finding substantiates the idea that the source of rhythmic activation in stick insect desDUM neurons lies rather in sensory organs.

4.2.3 The role of sensory organs in recruiting desDUM neurons

The results herein demonstrate that passive movements of middle legs on a treadmill are excitatory to desDUM neurons in a similar fashion to stance phases during single-leg stepping on a treadmill. Together with the observation that central drive has no obvious influence on desDUM neuron activity, this finding highlights the putative role of leg sensory organs in activating desDUM neurons. Due to the multitude of potential leg sensory organs (Tuthill and Wilson, 2016) activated by rather unspecific passive leg movement, these results only weakly contributed to the identification of the distinct source of excitation to desDUM neurons during walking. In a more detailed survey of the effects of single sensory organs, I demonstrated that elongation and relaxation of the fCO induces EPSPs in some desDUM neurons in resting animals. The amplitude of depolarization was, in most cases, below the threshold required for generation of APs at stable RMPs of desDUM neurons. When desDUM neurons were recorded at a depolarized RMP, APs were generated on top of the fCO stimulation-induced EPSPs. The latency to activation was about 44 ms from the onset of fCO elongation or relaxation. This long latency, together with the occurrence of AP generation in depolarized desDUM neurons, demonstrates that the small alterations in desDUM neuron membrane potential were not artifacts of the mechanical stimulation, but physiological responses to fCO stimulation. When animals were activated by tactile stimulation of the abdomen to induce reflex reversal, desDUM neurons generated APs in relation to fCO elongation and relaxation. The distribution of APs during fCO stimulation in activated animals was similar to the distribution of APs in depolarized desDUM neurons in inactive animals. Information from leg sensory organs is processed in a state-dependent fashion, which has

most elaborately been investigated in the stick insect FT control loop (review: (Bässler and Büschges, 1998; Tuthill and Wilson, 2016)). The results indicate that fCO signals may play a role in activating desDUM neurons in active walking animals. From the results of the current experiments, it cannot be exactly concluded which of the fCO sensory neurons are involved in the weak activation of desDUM neurons. Presumably 80 of about 480 fCO neurons in locusts and stick insects are differentially sensitive to parameters of tibia position and movement (review: (Tuthill and Wilson, 2016; Field and Pflüger, 1989; Kittmann and Schmitz, 1992)). This population of 80 fCO neurons can be distinguished by their firing and encoding of leg movement parameters. Tonically firing neurons were shown to encode the position of the tibia relative to the femur. Phasically firing neurons, in contrast, encode the velocity and acceleration of tibial movement. In regards to the bimodal responses in desDUM neurons with equal amplitude, the velocity and/or acceleration-sensitive fCO neurons described by Büschges (1994) might be activating desDUM neurons during ramp stimuli mimicking tibia flexion and extension. This could be further examined in future experiments by recording desDUM neuron responses to fCO stimulation with more systematic variation of velocity and acceleration. In comparison to fCO stimulation, stimulation of middle-leg CS induced a more significant activation in desDUM neurons. CS are mechanosensory organs responsive to strain in the leg cuticle (Tuthill and Wilson, 2016; Zill, 1985; Zill et al., 2004). Therefore, CS sensory neurons can encode loading of legs as well as leg muscle forces that are resisted (Zill et al., 2013). Loading of legs and resistance of muscle forces occur during leg touchdown and stance phase (Dallmann et al., 2016; Dallmann et al., 2017). On the legs, CS cluster in groups that exhibit varying directional sensitivity to cuticular strain. Caudal leg bending has been shown to activate CS of group 1 on the trochanter (Zill et al., 2012), which leads to excitation of RetCx MNs and inhibition of ProCx MNs (Schmitz, 1993). Caudal leg bending was performed in resting animals and does not resemble activation of CS during walking. With respect to the activation of CS during stance phase, which was excitatory to desDUM neurons during walking, the data show that CS represent a potential source of activation. Rather unspecific tactile stimulation of single leg tibiae revealed that desDUM neurons receive input arising from sensory organs of all 6 legs. In the underlying experiments, the exact identity of leg sensory organs that were stimulated remains elusive. Touch to the tibiae might have induced strain in the cuticle that activated tibial CS, as described by Zill et al. (2011). However, APs in desDUM

neurons might also have, for example, been induced in response to stimulation of tactile hairs (Pringle, 1938) on the cuticle.

Bräunig et al. (2004) report that electrical stimulation of nerves that innervate the fCO and the subgenual organ, a leg stretch receptor, did not elicit responses in locust desDUM neurons (Bräunig et al., 2004). Similarly, stimulation of the fCO did not evoke EPSPs or APs in locust efferent DUM neurons (Field et al., 2008). In general, however, efferent DUM neurons are responsive to multimodal mechanosensory stimulation of various body parts (Bräunig and Pflüger, 2001; Field et al., 2008; Mentel et al., 2008).

The question of to which extent the magnitude and pattern of activity in single desDUM neurons during six-legged walking, in the current thesis, represented the actual activity in freely walking stick insects remains open. In the six-legged walking experiments, stick insects were tethered to enable intracellular recording of the desDUM neuron membrane potential. The tethering was such that the distance between the body and the belts of a pair of independent treadmills resembled the height of animals during free walking (Gruhn et al., 2006). Because three legs of each body side were stepping on an independent treadmill, there was no substrate coupling between legs of both body sides. Nevertheless, Graham (1981) found that stick insects walking on two independent treadmills exhibit fully coordinated walking movements, similar to those seen in freely walking animals (Graham, 1981). In the current thesis, the inertia of leg retraction during stance phase was adjusted to physiological conditions using a servomotor (for detailed information: (Gabriel and Büschges, 2007)). Dallmann et al., (2016 have shown that depression of the leg to lift the body and counteract gravitational forces induces the most significant ground reaction forces and joint torques during stick insect walking. Due to tethering, animals did not have to carry their own body weight in the experiments described herein. Thus, the absolute value of depression forces in the experiments of the current thesis could be different from the forces that would occur during free walking. This could have had an influence on the resulting strain in the leg cuticle responsible for activating CS. I found that CS play an important role in activating desDUM neurons. Thus, substrate coupling of both body sides, as well as divergent strain in the cuticle from supporting body weight during free walking, might activate desDUM neurons with different magnitudes in free-walking animals. Still, the results show that desDUM

neurons receive input from leg sense organs of all six legs during walking in a phasic manner.

4.2.4 Possible mechanisms of signal integration

I found that the origin of input to desDUM neurons during walking is in leg mechanosensory organs, but it remains unclear where exactly and through which neural structures this input is conveyed to desDUM neurons. The afferents of most leg meachnosensory organs terminate in the ventral neuropil of thoracic ganglia in the stick insect and locust (Burrows, 1996; Goldammer et al., 2012; Pflüger et al., 1988; Schmitz et al., 1991). Intracellular labeling in the current thesis shows that stick insect desDUM neurons have an extensive bilaterally symmetrical branching pattern in the GNG, which was also observed for locust desDUM neurons (Bräunig and Burrows, 2004). Additionally, it has been shown that locust desDUM neurons have a vast bilaterally symmetrical projection pattern in both hemispheres of thoracic ganglia. Four of the six locust desDUM neurons have extensive branches close to ventral mechanosensory neuropil. However, there is no ultrastructural or immunocytochemical evidence for synaptic inputs from sensory afferents or any other neurons to desDUM neurons in the thoracic ganglia of locusts and stick insects. The same accounts for output structures of desDUM neurons. It is, therefore, unknown whether neurites of desDUM neurons in the thoracic ganglia resemble axon terminals, dendritic branches, or a mixture of both. Likewise, the identity of the neurite projections in the GNG is elusive. In the experiments of this thesis, APs and EPSPs in desDUM neurons were recorded from their electrically excitable somata located in the GNG. In addition to somatic excitability, another physiological feature of locust efferent DUM neurons is the existence of multiple spike-initiation zones (Crossman et al., 1971; Heitler and Goodman, 1978). This may also be a physiological property of stick insect desDUM neurons. The distinct morphological and physiological features of desDUM neurons suggest two generally different possibilities of signal integration: (I) DesDUM neurons receive input originating in leg sensory organs locally in hemiganglia of each thoracic segment; (II) information from leg-sensory organs ascends to the GNG, where it is integrated by desDUM neurons.

The first possibility implies that desDUM neurons would generate APs locally in thoracic ganglia, where local spike initiation zones might be situated. APs could be conducted to

somata in the GNG via the neurons' own axons in an ascending fashion. Consequently, desDUM neurons could be descending neurons only by morphological definition. The existence of neurons that display such a discrepancy in the direction of axonal projections and AP conduction was demonstrated by Pflüger (1984). Intersegmental interneurons with somata in the locust 4th abdominal ganglion receive direct sensory input from various wind-sensitive hairs via synaptic connections in the prothoracic ganglion (Pflüger, 1984). Additionally, APs were elicited by current injection into somata of these neurons and were conducted anteriorly. Therefore, these neurons are ascending in a morphological sense, but both descending and theoretically ascending in a functional sense. On the hypothetical basis of such a mechanism, desDUM neurons could function as integral parts of local microcircuits in hemisegments of thoracic ganglia. Mechanosensory signals could be integrated locally within thoracic ganglia to induce AP generation and OA release by local output structures. In this scenario, electrical signals recorded in somata in the experiments of the current thesis could have been a mere byproduct of desDUM neuron activity in thoracic ganglia.

The second possibility of signal integration would imply that sensory signals are first conducted to the GNG in ascending pathways. It has been shown in the stick insect that most leg mechansensory afferents terminate in ventral neuropil of the ganglion of the thoracic segment of the leg (Goldammer et al., 2012; Schmitz et al., 1991). This suggests that in stick insects, leg sensory information would be most likely transferred to the GNG polysynaptically in interneuron pathways and not by acending sensory afferents. In the GNG, information originating in mechanosensory organs of all six legs could then be conveyed to desDUM neurons. APs could then, for example, be generated in the GNG at spike initiation zones at the primary neurite or both descending axons. The APs would then propagate to output zones in thoracic ganglia. The morphologically descending axons would, thus, be functionally descending. Support for this mechanism comes from evidence of efferent locust DUM neurons that project their axons bilaterally to the lateral nerves of the ganglion that houses their cell bodies (Field et al., 2008). Lesion experiments have shown that mechanosensory input from the legs to these local efferent DUM neurons is preprocessed in the GNG. According to this finding, the GNG appears to be an integration hub for leg-mechanosensory information to neuromodulatory octopaminergic neurons. Bräunig and Burrows (Bräunig et al., 2004) propose that

desDUM neurons could function as parts of long feedback loops of leg-mechanosensory signals influencing local motor activity via the GNG. In fruit flies it was shown that some afferents of the leg chordotonal organ and wing CS directly project to the brain (Tsubouchi et al., 2017). This finding provides further evidence that meachosensory signals ascend to the GNG or brain, and are not exclusively integrated or processed locally in thoracic ganglia.

A study on the functional morphology of desVUM neurons in the fruit fly specifies the mode of signal integration but does not rule out any of the afore-mentioned possibilities. Using genetic markers, Busch et al. (2009) show that the descending neurons display a clear separation of dendritic and presynaptic regions. This polarity is also expressed morphologically, as spiny arborizations are formed in the GNG around the esophagus, and varicose nerve terminals can be found in the target regions of neurons. However, these target regions are within the posterior GNG. Busch et al. (2009) did not assess the functional morphology of the areas of the fruit fly ventral nerve cord to where desVUM neurons project.

I attempted to address the question of the direction of AP propagation and signal integration in desDUM neurons with monopolar hook electrode recordings from connectives between the GNG and thoracic ganglia in combination with intracellular recordings of somata. These experiments were not successful because of the AP amplitudes of desDUM neurons were too small for identification in extracellular recordings. Elucidation of the mechanisms of signal integration in desDUM neurons on the basis of identification of underlying neural structures remains an interesting target for further experiments. The lack of information on presynaptic partners not only accounts for stick insect desDUM neuron but for DUM neurons in insects in general.

4.3 Output effects of desDUM neurons

At the heart of the current thesis was the question of whether desDUM neurons have a neuromodulatory influence on thoracic locomotor network activity. I found that all of the desDUM neurons investigated affected reflex-induced and spontaneously occurring MN activity. The effects of desDUM neuron activation on MN activity varied in their sign (increase or decrease), magnitude (slight decrease or significant decrease), and specificity

(general effects on amplitude of MN activity vs. increased occurrence of active reactions and effects on rhythmicity). Here, I would like to discuss the output characteristics of some of the neurons in detail.

4.3.1 Involvement of OA in mediating the effects of desDUM neuron

In the current thesis, depolarizing current injection was used to induce the generation of APs in desDUM neuron somata. As a result of AP generation in single desDUM neurons, I observed a modulation of the activity of mesothoracic MNs. MALDI-TOF MS revealed that the somata of all tested desDUM neurons, independent of the magnitude or sign of their effects, contained OA. For some of the neurons, it was possible to demonstrate the additional presence of the OA precursor TA. Although I could show that the neurons contain OA, I would like to discuss whether OA was in fact involved in mediating the effects of desDUM neuron stimulation.

Hoyle and Barker (1975) demonstrates that insect DUM neurons synthesize and contain OA (Hoyle and Barker, 1975). The release of OA from efferent DUM neurons was first demonstrated using high K^+ depolarization of neurons and a radioenzyme assay to measure the subsequent accumulation of OA in target tissues (Evans et al., 1975; Howell and Evans, 1998). Mentel et al. (2003) triggered release of OA to a flight muscle using antidromic electrical stimulation of an efferent DUM neuron. It is generally accepted that OA is released from UM neurons via Type II varicosities at nerve terminals (Bräunig and Pflüger, 2001; Farooqui, 2007; Roeder, 2005; Stocker et al., 2018). This mode of exocytosis has been studied in great detail using an optogenetic approach to stimulate Type II boutons in fruit fly larvae (Majdi et al., 2015). Evidence for OA release from intersegmentally projecting UM neurons comes from a study by Hammer (1993). Intracellular current injection to induce AP generation in an ascending VUM neuron mimicked OA effects on associative olfactory learning in honeybees (Hammer, 1993). Thus, there is evidence that suggests that depolarizing current injection to trigger AP generation in desDUM neurons in the current experiments induced the release of OA. It remains to be clarified whether OA was the only neurotransmitter released from these neurons. TA is a precursor of OA, but also appears to be a neuroactive substance on its own (Kononenko et al., 2009; Lange, 2009; Rillich et al., 2013; Roeder, 2005). The MALDI-TOF MS data in Section 3.2 show that somata of desDUM neurons contain both

substances. Similarly, immunocytochemistry for OA and TA revealed co-expression of both neurotransmitters in locust efferent DUM neurons (Kononenko et al., 2009). Donini and Lange (Donini and Lange, 2004) suggest the co-release of tyramine from efferent DUM neurons of the locust abdominal ganglion. Using fast-scan cyclic voltammetry and an optogenetic approach to stimulate all neurons that co-express TA and OA, it was shown that OA is supplied to the ventral nerve cord of fruit fly larvae to a much greater extent compared to TA (Pyakurel et al., 2016). These findings suggest that TA could also be released from desDUM neurons; this would most likely occur to a much more minor degree compared to OA. Co-release of both OA and TA could have further physiological implications than the release of OA alone, and other substances could also be released from desDUM neurons.

A conceivable experiment to test the involvement of OA in the effects on MN activity would be the application of an OA receptor antagonist to the mesothoracic ganglion. Significant declines in desDUM neuron-mediated effects in such experiments could demonstrate the necessity of OA. I attempted to perform this kind of experiment in the current thesis, but did not succeed. When conducting the experiments, it was first necessary to demonstrate the effects of the recorded desDUM neuron under control conditions before the application of the OA receptor antagonist. Then, it was necessary to test how bath application of the antagonist affects MN activity in the absence of desDUM neuron stimulation to set a new control condition due to possible side effects. After that, desDUM neurons needed to be activated with sufficiently long intervals between single stimulations. Finally, the blocker needed to be washed out to return to the initial control condition for desDUM neuron stimulation. In my approach, I used epinastine (Roeder et al., 1998) to selectively block OA receptors. However, the incubation time for the antagonist to be effective in stick insects was too long for maintaining stable intracellular recordings of desDUM neurons and extracellular leg nerve recordings throughout the experiments.

4.3.2 Individual neurons of the desDUM neuron population have differential effects on MN activity

The effects of desDUM neuron activation on fCO stimulation-induced ExtTi MN activity were different between individual neurons. Some desDUM neurons mediated a decrease

in the magnitude of resistance reflexes; others triggered an increase in ExtTi MN activity and the occurrence of active reactions. These major differences in the polarity of the effects were accompanied by marked differences in the duration of said effects. Nevertheless, the output properties of an individual neuron were consistent throughout an experiment. The question arises, then, of how these differences come about; they can likely be explained by two possibilities.

(I) Effects could differ because the respective desDUM neurons exhibit nonhomogeneous transmitter expression or release. With MALDI-TOF MS, we could specifically analyze the OA and, in some cases, TA content of desDUM neurons after characterization of their output physiology. All tested neurons, independent of their mediation of a decrease, slight decrease, or increase in ExtTi MN activity, contained OA and TA. This finding indicates that there is no difference in the content of these substances in single neurons of the stick insect desDUM neuron population. A study in locust suggests that some efferent DUM neurons most likely release OA and TA (Donini and Lange, 2004). Furthermore, TA was shown to mediate effects on motor network activity contrasting the effects of OA (Hardie et al., 2007; Rillich et al., 2013). Therefore, the differences in effect might be due to a differential proportion of OA and TA released from neurosecretory structures of different desDUM neurons. Some neurons could release more TA than OA and vice versa. This is an issue that could not be addressed in the current thesis. Additionally, the inhibitory influence of a desDUM neuron subpopulation on MN activity could be due to the corelease of gamma aminobutyric acid (GABA). However, colocalization of OA with other chemical mediators any other than TA occurs extremely rarely (Bräunig and Pflüger, 2001; Hsu and Bhandawat, 2016). Stevenson et al. (Stevenson et al., 1992) showed that there are putative intersegmentally projecting DUM neurons in the locust thoracic ganglia that demonstrate GABA-like immunoreactivity but do not contain OA(Stevenson et al., 1992). These results were substantiated by Thompson and Siegler (Thompson and Siegler, 1993), who demonstrated that there are intersegmentally projecting DUM neurons in locust thoracic ganglia that have relatively small cell bodies and exclusively express GABA immunoreactivity (Thompson and Siegler, 1993). Moreover, Watkins and Burrows (1989) showed that there are GABA-like immunoreactive cell bodies in the labial neuromere of the locust GNG. Although this is where desDUM neuron somata are located, GABA-like immunoreactivity could not be

ascribed to these neurons (Watkins and Burrows, 1989). In the fruit fly, there are descending GABAergic neurons with cell bodies in the posterior GNG (Hsu and Bhandawat, 2016). However, these neurons appear to have only unilaterally descending axons and are, thus, not desUM neurons. Together, these studies suggest that putative GABAergic DUM neurons most likely do not express OA and have significantly smaller cell bodies in comparison to desUM neurons. Still, direct evidence for this assumption is missing in stick insects.

(II) Effects could also differ because single desDUM neurons specifically supply OA to different neural targets. In the current experiments, I could not asses the projection patterns of stick insect desDUM neurons in the thoracic ganglia. Therefore, it remains elusive whether individual stick insect desDUM neurons have different OA release sites. In contrast, Bräunig and Burrows (Bräunig and Burrows, 2004) showed that locust desDUM neurons innervate different neuropils in the thoracic ganglia. Hence, they might mediate different effects, which could also be the case for stick insect desDUM neurons. The differences in effects on MN activity could be a result of different output properties of target neurons within the locomotor network. For example, an excitatory octopaminergic effect on an inhibitory interneuron could result in the inhibition of MN activity. In this context, not only the site of OA release, but also the distinct expression of OA receptors in the membrane of target neurons, is particularly predictive of the modality of the effect. OA effects are mediated by various types of G protein-coupled receptors that activate distinct second-messenger pathways (Evans and Maqueira, 2005; Farooqui, 2007; Roeder, 2005). Fundamental differences in receptor type-dependent second-messenger pathways are an intracellular increase in either adenosine 3',5'-cyclic monophosphate (cAMP) or Ca^{2+} levels. These messengers in turn activate differential downstream second-messenger cascades in a tissue- or cell-type-specific manner. As a result, OA can, for example affect membrane resistance, AP frequency, and the strength of synaptic output of target neurons (Farooqui, 2007; Marder, 2012). In addition, there are receptors that are sensitive to TA to a greater extent than they are to OA. These induce a decrease in intracellular cAMP upon binding of TA and a Ca^{2+} increase when OA is present at high concentrations.

In the experiments of the current thesis, desDUM neurons that mediated a decrease in the activity of FETi and SETi MNs, did not decrease, but rather increase the activity of CI_1

MNs. This indicates that the general differences in effect of single desDUM neurons on MNs are rather the result of a different function of upstream neurons specifically targeted by OA, or differential expression of OARs on target neurons.

There is a plethora of studies on the distribution of different OA receptor classes in the peripheral nervous system, as well as the brain and GNG of the honeybee, locust, cockroach, and fruit fly (review: (Evans and Maqueira, 2005; Farooqui, 2007; Roeder, 2005). With few exceptions (Degen et al., 2000; Roeder et al., 1995), there is scarce information on the distribution of OA receptors in insect thoracic ganglia. The underlying studies are often based on whole-tissue analysis and do not allow for identification of putative target neurons of OA. Thus, there is a lack of direct evidence for the identity of putative OA receptors, which OA that is released from desDUM neurons likely activates. Nevertheless, differences in the effects of desDUM neurons may be mediated by the activation of different OA receptors, potentially coupled to different types of G proteins. In particular, differences in duration of inhibitory and excitatory influences of desDUM neurons could, thus, be a result of target-tissue-dependent differential induction of second-messenger cascades.

With respect to both chemical structure and physiological effects, the vertebrate noradrenaline and noradrenaline receptors are very similar to the invertebrate OA and OAR (Roeder, 2005). The assumption that OA effects differ because of activation of different OAR is supported by evidence for receptor-dependent differential activity of noradrenaline in the vertebrate spinal cord. For example, the frequency of pharmacologically activated motor activity in the isolated rat spinal cord is reduced by the activation of α2-adrenoceptors, with the magnitude of MN bursts remaining unaffected. Activation of α1-adrenoceptors, in contrast, mediates an acceleration of the motor rhythm and further reduction in the magnitude of MN bursts (Sqalli-Houssaini and Cazalets, 2000). In general, spinal neurons involved in the generation of locomotion appear to be activated by noradrenaline acting on α1-adrenoceptors to induce increased excitability. In contrast, binding of noradrenaline to α2-adrenoceptors triggers an inhibition of motor activity (Chau et al., 1998; Fedirchuk and Dai, 2004; Giroux et al., 1998; Miles and Sillar, 2011; Ono and Fukuda, 1995; Sqalli-Houssaini and Cazalets, 2000; Tartas et al., 2010; White et al., 1991).

To fully unravel the mechanism that underlies differential effects of desDUM neurons, recording of membrane potential of putative target neurons and analysis of OAR-expression in the thoracic ganglia would be necessary.

4.3.3 Functional implications for desDUM neuron output

The experiments of the current thesis were based on the stimulation of desDUM neurons in inactive animals. Some aspects of the results, however, nevertheless provide an account of how these neurons might function in the intact walking stick insect.

The most detailed approach addressing the output of desDUM neurons was in the survey of the neurons' effects on fCO stimulation-induced extensor MN activity. I found that they modulate the magnitude of the reflex responses in two distinct ways; some neurons mediated a general increase in extensor MN activity, whereas others decreased extensor MN activity. The latency of about one to six seconds from the beginning of desDUM neuron stimulation to the onset of modulatory effects is remarkably short. Regarding previous studies, the impact of octopaminergic modulatory effects was expected to be on the scale of minutes. For instance, the effects of a GNG ascending DUM neuron on a motor rhythm of the locust stomatogastric nervous system occurred about 3-4 min after stimulation of the neuron (Rand et al., 2012). Similarly, activation of a locust efferent DUM neuron was effective after 20 min (Mentel et al., 2003). In contrast, direct iontophoretic release of OA to regions of neuropil comprising FETi dendrites triggered a potentiating effect after 12 s in the locust (Sombati and Hoyle, 1984a). Studies, however, in which OA was bath-applied to whole-tissue specimens, are ignored in the current discussion, because the time required for OA to diffuse to OA receptors artificially prolongs effect times.

Furthermore, the excitatory effects of some desDUM neurons in particular, but also the inhibitory effects of others, were relatively transient in the current thesis. The desDUM neuron stimulation-induced increase in MN activity peaked at about 10 s (of 30 s stimulation) and ceased shortly after the termination of stimulation. The inhibitory influence lasted at maximum for about 2 min. The time courses and direction of the effects of stick insect desDUM neurons, in the current experiments, persisted when neurons were activated to produce a lower, more physiologically relevant spike rate and

when neurons were activated for short periods. This suggests that the properties of the output are persistent even when the absolute amount of neuromodulator release varies. In summary, these varying output properties suggest that desDUM neurons are relevant for the short term flexibility of the locomotor system. In contrast, effects of DUM neuron activation or OA iontophoresis in previous studies lasted much longer (Bräunig and Pflüger, 2001; Farooqui, 2007; Mentel et al., 2003; Parker, 1996; Ramirez and Pearson, 1991b; Rand et al., 2012; Sombati and Hoyle, 1984a; 1984b).

It was further investigated, in the current thesis, how specific the increase or decrease in the magnitude of MN activity is to fCO or CS stimulation. In detail, I wanted to decipher whether desDUM neurons modulate the processing of sensory signals at the level of sensory afferents or premotor interneurons as part of joint control loops, whether they rather directly affect the excitability of MNs, or both. Evidence suggesting that the modulation might be rather independent of sensory stimulation comes from the effects of desDUM neurons on spontaneous ExtTi, ProCx, and RetCx MN activity. In the absence of stimulation of sensory organs, desDUM neurons mediated either an increase or decrease of ExtTi, as well as ProCx and RetCx, MN activity with characteristics of direction and time course reminiscent of the effects on reflex-induced MN activity. In the underlying experiments on excitatory desDUM neurons, additional units, in particular fast MNs, of the respective MN pool were recruited. This suggests that activity in desDUM neurons may have increased the excitabilitythe threshold for AP generation in the respective MN pools. This assumption is substantiated by previous studies in which OA application potentiated the activity of insect MNs (Parker, 1996; Sombati and Hoyle, 1984a; 1984b; Westmark et al., 2009). Furthermore, activation of two of five desDUM neurons modulated the magnitude of pharmacologically-induced MN activity in deafferented ventral nerve cord preparations in the current thesis. In the absence of sensory information, such an alteration in the amplitude of MN activity is generally ascribed to modulation at the level of the final stage of processing of motor activity, which comprises MNs or last-order interneurons (Miles and Sillar, 2011; 2011). In my experiments on the magnitude of reflex-induced MN activity, in total about 86% of desDUM neurons triggered significant modulation. This percentage is markedly higher than the 40% (2 of 5) of desDUM neurons that significantly modulated the magnitude of centrally generated MN activity. The overall variability in burst strength in the isolated

pilocarpine preparations, in contrast to the stereotypic reflex responses in fCO- and CS-stimulation experiments, could be responsible for this difference in the percentage of significant effects.

Excitatory desDUM neurons specifically increased the occurrence of active reactions during fCO stimulation (without tactile stimulation) on average from almost 0% to 14%. This suggests that some desDUM neurons specifically, but not exclusively, act on premotor pathways involved in the state dependent processing of fCO signals. It is difficult, however, to elaborate on the identity of putative direct targets for these kind of effects, due to the plethora of putative target neurons (Bässler, 1993; Bässler and Büschges, 1998) and the generally scarce information (exception: (Driesang and Büschges, 1996)) on the exact neural control of reflex reversal. The role of desDUM neurons in modulating reflex activity is substantiated by previous studies on effects of OA application. In the locust, iontophoretic OA application mediates sensitization and dishabituation of a resistance reflex (Sombati and Hoyle, 1984a). In inactive stick insects, properties of reflex-induced extensor MN activity are changed to those of the active state by bath application of OA (Büschges et al., 1993). Futhermore, it has been shown that descending drive is crucial for the state-dependent changes in fCO-mediated sensorimotor interaction (Martin et al., 2015; Mu and Ritzmann, 2008a; 2008b). Overall, desDUM neurons might contribute to the descending drive that affects state-dependent alterations of sensory information processing. In walking stick insects, the probability for occurrence of active reactions is dependent on the behavioral state, but is on average about 60% (Bässler, 1988; Hellekes et al., 2012). The 14% probability of occurrence of reflex reversal in my experiments appears comparably low, but this was induced by only activating single desDUM neurons. This value might be markedly increased when the activity of the whole desDUM neuron population is considered. Furthermore, it has been shown that load signaling contributes to an increase in the occurrence of reflex reversal in walking stick insects (Akay and Büschges, 2006). With respect to absence of these sensory influences in my experiments, the contribution of a single desDUM neuron to reflex reversal is, in fact, remarkably high. If the neurons that mediated a decrease in resistance reflex responses in the current thesis would mediate a similar effect in the walking animal they could contribute to the state dependent suppression of active reactions that occurs during curve walking (Hellekes et al., 2012).

I found that three of five desDUM neurons had an influence on parameters of the rhythmicity of pharmacologically induced rhythmic MN activity. The major influence was an increase of the burst frequency. In general, alterations in rhythmicity of centrally generated MN activity are ascribed to modulation of neurons that are constituents of CPGs (Miles and Sillar, 2011). Similar to the present results, the frequency of pharmacologically induced motor activity in the isolated rat spinal cord was increased by noradrenergic activation of α1-adrenoceptors (Sqalli-Houssaini and Cazalets, 2000). To date, there has been no comprehensive identification of the neural constituents of insect CPGs for the control of walking (Bidaye et al., 2018), which prevents assumptions about any affected targets. One further aspect of desDUM neuron effects on centrally generated MN activity, in the current thesis was the occurrence of one prolonged retractor MN burst before acceleration of the rhythm. As the only significant alteration to rather stable alternating activity in antagonistic MN pools, Büschges et al. (Büschges et al., 1995) describe the occurrence of so-called spontaneous recurrent patterns (SRPs) in the isolated, pilocarpine-activated mesothoracic ganglion in stick insects. SRPs resemble the patterns of MN activity during stance phase transitions in walking animals. However, these SRPs were not correlated with such markedly prolonged retractor bursts that occurred upon activation of desDUM neurons in the experiments in this thesis. Moreover, in contrast to the experiments of Büschges et al. (1995), the brain and GNG were attached to the thoracic ventral nerve cord. With respect to the physiological consequences of long retractor bursts for the intact walking stick insect, it must be taken into account that the centrally generated rhythm is shaped by sensory signals (review: (Bidaye et al., 2018)). Activation of leg sensory organs in the stick insect, for example, has been shown to be able to entrain the centrally generated rhythm (Akay et al., 2007; Borgmann et al., 2009). This suggests that, during walking in intact animals, long bursts like the ones observed in the experiments herein would most likely be terminated by the integration of sensory signals.

4.3.4 Assumptions on the net output of the desDUM neuron population

The experiments of the current thesis were focused on the effects of single desDUM neurons themselves on MN activity; the final stage of neural control networks for walking. Therefore, considerations on the net effects of the entire desDUM neuron

population remain hypothetical. If, for example, the effects on magnitude of MN activity would be rather direct to MNs, the inhibitory desDUM neurons would counteract excitatory desDUM neurons. On the other hand, different desDUM neurons could be targeting different premotor interneurons of the FT control loop. Thus, a decrease in postural reflexes like the fCO-mediated resistance reflex would add up to produce an increase in the probability for active reactions to result in a net output of facilitation or reinforcement of walking activity. Regardless of their output effect, all desDUM neurons were activated by tactile stimulation of the abdomen and antennae. Both stimuli are known to induce walking, directed either forwards or backwards (Graham, 1972; Gruhn et al., 2006). With regard to the aforementioned net output, desDUM neurons might be involved in stimulus-triggered initiation of walking sequences. During walking, desDUM neurons are most likely activated by input from leg sensory organs mediating load signals as well as velocity and acceleration signals from all six legs. Due to the short latency and transient nature of occurrence of their effects, desDUM neurons might in turn be involved in facilitating ongoing walking sequences.

4.4 Summary and Conclusions

There are six desDUM neurons in the posterior part of the stick insect GNG. These neurons contain OA and send bilaterally symmetrical axons to the thoracic ganglia. The activity of all stick insect desDUM neurons is coupled to walking in a phasic manner. APs are generated during stance phases of all stepping legs. Load signals (mediated by CS), and to a lower extent, velocity and acceleration of joint movement (mediated by the fCO) are responsible for the excitatory input to desDUM neurons during walking. This suggests that desDUM neurons integrate multimodal sensory signals. In addition, tactile stimulation of antennae and the abdomen of animals also had an excitatory influence on all desDUM neurons.

The output effects of single desDUM neurons on reflex-evoked, spontaneous, and centrally generated activity of MNs were investigated at the level of the mesothoracic segment, and there were major differences in effects of single desDUM neurons. Some desDUM neurons mediated an excitatory influence on the activity of MNs and others mediated an inhibitory influence on the activity of MNs. DesDUM neurons with a general excitatory influence on MN activity, for example, facilitated the reversal of a postural

reflex. Some desDUM neurons accelerated the rhythm of centrally generated MN activity. This suggests that they affected the activity of CPG neurons. With respect to the projection pattern of desDUM neurons, these effects are likely to occur also in the prothoracic and metathoracic segments. The neurons could thus function as parts of long feedback loops from sensory structures of all legs to the exertion of modulatory effects on neural circuits that control movements of all legs. Overall, the population of stick insect desDUM neurons has a marked influence on the activity of thoracic neural circuits for the generation of walking on multiple levels of neural control. Neurons that are homologues to stick insect desDUM neurons have been identified in every insect species in which they have been investigated. With respect to homology in the walking control network topology (review: (Smarandache-Wellmann, 2016) and function (review: (Bidaye et al., 2018; Smarandache-Wellmann, 2016; Tuthill and Wilson, 2016) between different insect species, the basic mode of action of stick insect desDUM neurons revealed in the current thesis could apply to insects in general. Moreover, some of the desDUM output properties are similar to effects of noradrenaline on motor activity generated in the vertebrate spinal cord (review: (Miles and Sillar, 2011)). The majority of neurons that release noradrenaline to the vertebrate spinal cord are descending from the locus coeruleus in the brainstem (Commissiong et al., 1978; Nygren and Olson, 1977; Westlund et al., 1981; 1983; 1984). With regard to the physiological homology of OA and noradrenaline (Roeder, 2005), desDUM neurons that descend from the GNG to the thoracic ganglia could function as the insect homologue of vertebrate noradrenergic neurons that descend from the brainstem to the spinal cord.

4.5 Outlook

In the current thesis, I investigated the effects of single desDUM neurons on extracellularly recorded MN activity. This enabled only hypothetical assumptions on the direct neural targets for desDUM neuron output. For more detailed insight, recordings of membrane potential from putative neural targets would be necessary. I attempted to address this topic with intracellular current injection for the activation of single desDUM neurons and simultaneous intracellular recordings of ExtTui MNs or non-spiking interneurons of the FT control loop (Bässler and Büschges, 1998) to monitor their responses. These experiments turned out to be unfortunately too time-consuming for the

progress of this thesis. As a solution to this problem, the stimulation of desDUM neurons could be made more reproducible by genetic access to the neurons. The toolkit for genetic targeting of neurons on the basis of the transgenic split-GAL4 system (e.g., Sen et al., 2017) cannot yet be applied to the stick insect. In contrast, *Drosophila melanogaster* split-GAL4 driver lines can, for example, already be used for the targeted expression of optically activated cation channels, such as CsChrimson (e.g. Klapoetke et al., 2014). Upon optical activation of CsChrimson, the membrane potential of the neurons that express the channel is depolarized and APs are produced. After designing appropriate driver lines, such an optogenetic approach (Cande et al., 2017) coupled with the split-Gal4 system could be used to stimulate single fruit fly desVUM neurons or the entire population. This, in particular, could help to assess the net output of the entire desVUM neuron population. In the genetically accessible fruit fly, electrical recording of membrane potential of putative target neurons is possible (e.g. (Reyn et al., 2017; Tschida and Bhandawat, 2015)), but stimulation of specific sense organs (as in the current thesis) as well as detailed behavioral assays (e.g., (Berg et al., 2015)) are more easily carried out in large insects like the stick insect. This is why it would be beneficial to target the genetic accessibility of neurons in the stick insect. One promising approach is the use of CRISPR-based genetics (Fineran and Charpentier, 2012; Wiedenheft et al., 2012) to create transgenic stick insects.

With the genetic toolkit ready to use in stick insect, it could be also possible to silence desDUM neurons, for example, by targeted expression of anionic channelrhodopsins (Mohammad et al., 2017) or temperature-sensitive expression of the tetanus toxin light chain (Bidaye et al., 2014). By silencing desDUM neurons, the influence of the neurons on walking could be assessed in an intact, behaving animal. In addition, the expression of calcium indicators (Russell, 2011) or voltage-sensitive dyes could aid in deciphering the distinct activity patterns of individuals of the desDUM neuron population.

List of Figures

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Danksagung

Ich möchte folgenden Personen danken:

PD Dr. Jochen Schmidt für die Vergabe des Themas, die große Motivation, die hervorragende Betreuung, große Hilfsbereitschaft und seine entspannte nordische Art

Prof. Dr. Peter Kloppenburg für die freundliche Übernahme des Zweitgutachtens

Prof. Dr. Ansgar Büschges dafür, dass ich Teil seiner Arbeitsgruppe sein konnte und für seine ansteckende Begeisterung für die Wissenschaft

Dr. Elzbieta Hammel, Joscha Schmitz, Sander Liessem und Volker Berendes für Diskussionen, Hilfeleistungen und Spaß in unserem Büro

Alexander Chockley und Dr. Till Bockemühl für das schnelle Korrekturlesen der Arbeit

Dipl. Ing. Michael Dübbert, Jan Sydow, Hans-Peter Bollhagen, Sima Seyed-Nejadi, Sherylane Seeliger, Corinna Rosch, Laura Nitzold und Andrea Hagenbruch für ihre immer freundliche und äußerst hilfreiche Unterstützung

Den gesamten Arbeitsgruppen Büschges, Wellmann und Gruhn für die anregende und lustige Atmosphäre

Meinen Eltern, Geschwistern und Freunden für ihren Rückhalt, ihre Kreativität und ansteckende Freude am Leben, ihre mitreißende Motivation und ihr Interesse an meiner Arbeit

Ganz besonders Julia für ihre direkte motivierende Art, ihre Hilfsbereitschaft und die schöne Zeit während der Doktorarbeit

Dankeschön

Erklärung

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