THE MODULATION OF LOCOMOTOR CENTRAL PATTERN GENERATORS BY OCTOPAMINE AND TYRAMINE IN *DROSOPHILA* LARVAE

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2011

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Final word count: 55401

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ABSTRACT

Movement is controlled by neuronal central pattern generator (CPG) networks that are segmentally organised in organisms across the animal kingdom. The precise role of neuromodulators in the function, development and, particularly, the maintenance of these circuits is currently unresolved. This study investigates the effects of chronically altered signalling of tyramine and/or octopamine, two well established neuromodulators, in *Drosophila* larval locomotion. It shows that tyramine reduces crawling speed in larvae, whereas octopamine increases speed up to a physiological maximum. Changes in crawling speed are mediated by modulating stride duration, whilst stride length remains constant. These two neuromodulators also affect segmental muscle contraction and relaxation rates, indicative that the effects on crawling speed are likely to be at least partially due to modulatory effects on muscle physiology.

Muscle recordings from muscle M6 in two adjacent segments, during fictive forward locomotion show that stride duration is influenced by a variable time delay between segmental CPG outputs. Frequency and duration of individual segmental outputs, by contrast, remains constant. The behavioural and electrophysiological data suggest, therefore, that the segmental locomotor CPG outputs remain constant in response to chronically altered neuromodulatory signalling. This study also identified a close spatial proximity of motor neuronal dendritic branches and putatively octopaminergic and/or tyraminergic synaptic terminal varicosities in the ventral nerve cord (VNC) neuropil. Moreover, manipulation of a putatively octopaminergic and/or tyraminergic subpopulation of interneurons, located in anterior brain regions, is sufficient to induce a similar, albeit smaller, larval crawling deficit. This indicates that the effects of locomotion may be induced in the central nervous system. This is confirmed in identified motor neurons as chronic changes in octopaminergic and/or tyraminergic signalling increase the frequency of bursting of action potential firing. In addition, the synaptic current amplitudes are substantially reduced in both ventral and dorsal muscle-innervating motor neurons, indicative of an effect to presynaptic excitation. In contrast, the function of neuromuscular junction remains largely unchanged.

Taken together, this data shows that neuromodulation is sufficient to alter the output of a relatively small group of neurons, that comprise the locomotor CPG. The site of action of these modulators is, however, likely to be diverse.

DECLARATION AND DISCLAIMER

I, Waldemar Ockert, declare that no portion of the work referred to in this dissertation has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

All work and experiments conducted and described in this thesis are my own except for PCR experiments described in Chapter 4, which have been conducted by Dr. Wei-Hsiang Lin.

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ACKNOWLEDGEMENTS

First of all, I would like to thank Prof. Richard Baines for his help, advice and support throughout the last 4 years. It has been a great challenge, opportunity and privilege to work on this project in his lab. I would also like to thank all members of the lab including Dr. Wei-Hsiang Lin, Dr. Nara Muraro, Dr. Verena Wolfram, Dr. Richard Marley, Dr. Graham Coutts, Fiona He, Dr. Heather Brooke and Holly Pay for their advice and help in carrying out this work.

Mein Dank gebührt meinen Eltern, Alexander und Theresa Ockert, deren Liebe und Unterstützung mir ein Leben ermöglicht haben, das ich mir zu erträumen nicht hätte wagen können. Ich liebe und danke Euch! Ich möchte mich vor allem bei Rachel bedanken. Ich kann meine Liebe für Dich, meinen Dank an Dich und meine Freude an Dir nicht in Worte fassen.

ABBREVIATIONS

AB	anterior burster neuron
AC	adenylate cyclase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	cyclic 3', 5'-adenosine monophosphate
CCIN	contralateral and caudal interneurons
cDNA	complementary DNA
CNS	Central Nervous System
cGMP	cyclic guanosine monophosphate
CPG	Central Pattern Generator
DNA	Deoxyribonucleic acid
Dnc	*dunce*
dNTPs	deoxyribonucleotide
DUM	dorsal unpaired median
EC	edge cells
EIN	excitatory interneurons
eIN	excitatory interneurons
EJP	excitatory junctional potential
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
FasII	Fasciclin II
FRET	fluorescence resonance energy transfer
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GI	giant interneurons
GPCR	G-protein coupled receptor
I_A	transient K^+ currents
IC	inferior cardiac neuron
I_{Ca}	Ca^{2+} currents
iIN	inhibitory interneurons
I_K	persistent K^+ currents
I_{KCa}	Ca^{2+}-dependent K^+ currents
IP_3	inositol 1,4,5-triphosphate
LIN	lateral interneurons
LP	lateral pyloric neuron
mAChR	muscarinic acetylcholine receptor
mEJP	miniature excitatory junctional potential

mEPSC	miniature excitatory postsynaptic current
MN	myotomal motor neuron
mRNA	messenger RNA
mV	millivolt
NMDA	N-methyl-D-aspartate
NMJ	neuromuscular junction
NSF	N-ethylmaleimide-sensitive fusion protein
pA	picoAmpere
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	pyloric dilator neuron
PIR	Post-inhibitory Rebound
PKA	Protein Kinase A
PKC	protein kinase C
PLC	phospholipase C
PM	paramedial
PY	pyloric neuron
RNAi	RNA-mediated gene interference
RPCH	red pigment concentrating hormone
SNARE	Soluble NSF Attachment Protein receptor
STG	Stomatogastric Ganglion
STNS	Stomatogastric Nervous System
Tdc2-CD8.GFP	Abbreviated form of Tdc2-GAL4/UAS-CD8.GFP
Tdc2-control	Abbreviated form of Tdc2-GAL4/UAS-TNT VIF (inactive isoform)
Tdc2-Kir2.1	Abbreviated form of Tdc2-GAL4/UAS-Kir2.1
Tdc2-NaChBac	Abbreviated form of Tdc2-GAL4/UAS-NaChBac
Tdc2-nsyb.eGFP	Abbreviated form of Tdc2-GAL4/UAS-nsyb.eGFP
Tdc2-syt.eGFP	Abbreviated form of Tdc2-GAL4/UAS-syt.eGFP
Tdc2-TeTx*	Abbreviated form of Tdc2-GAL4/UAS-TNT G (active isoform)
Tdc2-TβH	Abbreviated form of Tdc2-GAL4/UAS-TβH
UAS	Upstream activator sequence
VD	ventricular dilator neurons
VNC	ventral nerve cord
VUM	ventral unpaired median

Chapter 1

General Introduction

Research over the last decades has shown that, across the animal kingdom, many aspects of animal behaviour including walking, swimming, flying and breathing are enabled by small neuronal networks, which in turn control specified muscle groups. These networks, called central pattern generators (CPGs), are intrinsically capable of reproducing a specified output in isolation and often multiple CPG networks have to be coordinated to enable complex behaviours. Whilst the output of CPGs needs to be robustly reliable in order to enable an appropriate behavioural output, these networks also need to be flexible so that animals can adapt their behaviour according to external and internal factors. There are, however, substantial challenges on the organisation and function of networks at the systemic, cellular and molecular levels in order to achieve this. Studies in several model systems including crustaceans, molluscs, leech (*Hirudo medicinalis*) and lamprey (*Petromyzon marinus*) have shown that the function and output of CPGs and ultimately animal behaviour can be flexibly adapted by neuromodulators. This is achieved by numerous mechanisms that enable neuromodulators to specifically target voltage- and/or ligand-gated ion channels in constituent single neurons within a network and thus to transform the output of CPGs. However, whilst the intrinsic currents and synaptic physiology of single neurons display a remarkable degree of plasticity, many studies have also identified potent homeostatic mechanisms, which tightly regulate both the intrinsic membrane excitability as well as the synaptic efficacy. As a result, neurons and their constituent compartments consistently balance the requirements for both functional robustness and their ability to be modulated. Whilst many mechanisms of functional plasticity and homeostasis in neurons have been identified, the ability as to whether entire networks can balance the need for functional robustness and plasticity and how this is achieved remains unresolved. Considering the degree of plasticity in constituent neurons in a network, it remains unclear, how neuronal network outputs can remain functionally robust. Moreover, how chronic changes in neuromodulatory signalling affect the development and function of CPG networks and, particularly, if and how such changes are homeostatically compensated for on different timescales is currently unresolved.

This thesis aims to address the effects of chronic perturbations in the signalling of the neuromodulators octopamine and tyramine on locomotor CPG networks and larval crawling behaviour in *Drosophila* larvae, which have remained largely unaddressed. The *Drosophila* model system is genetically highly amenable,

which is a key advantage when studying effects of chronic and specific perturbations of neuromodulatory signalling. In addition to available mutant genotypes in this model system, the GAL4/UAS system enables a spatially and temporally controlled expression of specific proteins in target tissues (Figure 1.1) (Brand and Perrimon, 1993; Duffy, 2002; McGuire et al., 2004; Nicholson et al., 2008).

The effects of specific genetic and/or pharmacological manipulations of neuromodulatory signalling on neurons and networks can be studied by a combination of well established techniques in this model system including locomotion assays, imaging techniques as well as electrophysiologically accessible identified muscles and motor neurons (Jan and Jan, 1976; Wang et al., 1997; Baines and Bate, 1998; Vömel and Wegener, 2008). A combination of these techniques can potentially provide a systemic perspective of the effects of chronically altered neuromodulatory signalling at a molecular, cellular, network and ultimately behavioural level. Moreover, a combination of chronic and acute manipulations of neuromodulatory signalling can potentially provide insights into the regulation of plasticity and homeostasis in locomotor CPG networks. The high degree of conserved sequence homology in ligand- as well as voltage-gated ion channels, G-protein coupled receptors (GPCRs), structural proteins and synaptic organisation suggests a high degree of functional conservation across the animal kingdom, which validates the relevance of findings in this model system (Littleton and Ganetzky, 2000). *Drosophila* larvae may thus serve as a suitable model system to study the plasticity and homeostasis of neuronal networks.

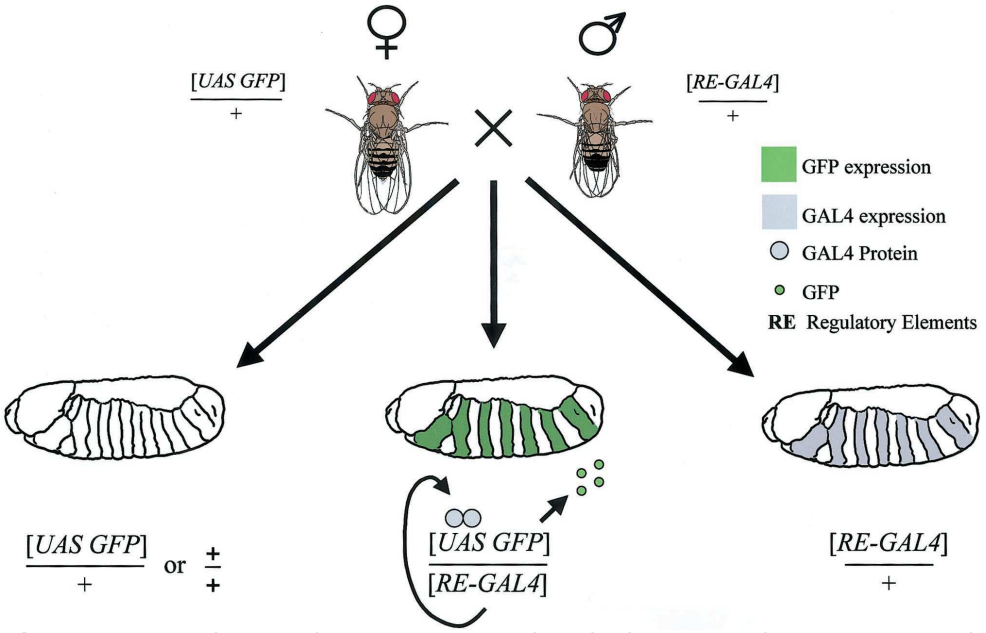

Figure 1.1 The UAS/GAL4 system. When both UAS and GAL4 transgenic chromosomes are present in one organism as a result of a genetic cross, GAL4 initiates gene expression controlled by UAS responder elements in specified regions resulting in a spatially controlled gene expression. Taken from (Duffy, 2002).

This chapter will introduce organisational and functional principles of single and multiple inter-connected CPG networks. It also aims to address how the output of CPG networks can be modulated as well as homeostatically maintained. Finally, it will address what is known about the effects of octopamine and tyramine on various cells and tissues across several species.

1.1 CENTRAL PATTERN GENERATOR NETWORKS

Animal behaviour is generated by coordinated activity patterns of small neuronal networks, called central pattern generators (CPGs), which in turn activate specific muscle groups in a coordinated temporal sequence (Grillner, 2006). CPG networks can be defined as "[…] an assembly of neurons which, by virtue of their intrinsic properties and synaptic interactions, is capable of generating and controlling the spatial and temporal activity of motor neurons"[1]. The activity of single or multiple CPG networks enables specified behavioural outputs such as walking, swimming, flying, breathing and eating. CPG networks are intrinsically capable of producing a specified motor pattern without external or sensory input, but they are usually regulated and modulated by higher command centres and sensory information (Alford et al., 2003; Selverston, 2005; Grillner, 2006). Although the specific wiring and neuronal activity patterns in CPGs can differ, most CPG networks have common principles that govern their function. For example, most CPG networks include a dominance of inhibitory synapses and specific activity patterns (Selverston et al., 1997; Nusbaum and Beenhakker, 2002). Also, whilst some behaviours only require a single CPG network for their control, such as the crustacean pyloric function, others, such as swimming in lamprey, require a coordinated activity of multiple unit CPG networks controlling their respective muscle groups (Grillner, 2006).

1.1.1 Organisation and Function of a Central Pattern Generator Network

In *Drosophila*, CPG networks that regulate behavioural outputs remain largely unresolved at both the cellular and molecular level. In contrast, the CPG networks in the stomatogastric ganglion (STG) in decapod crustacean models such as lobster, crab, shrimp and crayfish are well known and have established many of the fundamental principles about the organisation and function of CPG networks (Nusbaum and Beenhakker, 2002). The function of these CPG networks has been

[1] Getting, PA (1988), Chapter 4: Comparative Analysis of Invertebrate Central Pattern Generators. In Cohen, AH, Rossignol, S, and Grillner, S eds. Neural Control of Rhythmic Movements in Vertebrates. John Wiley & Sons, page 102

elucidated at single neuron and molecular levels. The crustacean stomach is categorised into four compartments, namely the oesophageal, cardiac sac, gastric mill and the pyloric regions, which food has to pass through sequentially before entering the digestive system (Hooper and DiCaprio, 2004). In adults, each of these compartments in the crustacean stomach is controlled by a specific CPG network. These networks emerge as independent functional entities from a single functional CPG network throughout development in the stomatogastric nervous system (STNS) (Casasnovas and Meyrand, 1995). However, the total number of neurons in the STNS remains unchanged. Neurons in separate CPG networks are electrically connected, which potentially allows networks to switch into synchronicity. For example, application of the red pigment concentrating hormone (RPCH) modulates the cardiac sac and gastric mill CPG rhythmicity and synchronises the output of these networks (Dickinson et al., 1990).

The STG comprises 25-30 neurons and contains the two CPGs regulating the function of the gastric mill and the pylorus responsible for chewing and food filtering in the stomach, respectively (Nusbaum and Beenhakker, 2002). Individual neurons or groups of neurons can be an integral part of more than one CPG enabling different behavioural outputs (Marder and Bucher, 2001). For example, several neurons in the STG are defined as gastro-pyloric, because of their flexibility to participate in either of the stomatogastric CPGs or both (Nusbaum and Beenhakker, 2002). Thus, the organisation and synaptic connectivity between CPGs further increases the functional diversity of these networks in addition to their ability to be modulated. The neurotransmitters released from the synapses in these networks are known (Nusbaum and Beenhakker, 2002). Although the STG can function in isolation, it also receives input from other ganglia in the crustacean central nervous system (CNS) (Nusbaum and Beenhakker, 2002). These networks are two of the best studied pattern generator networks. In order to identify some of the principles governing CPGs, the pyloric CPG will be described in more detail.

The pyloric CPG network contains 14 neurons made up of 6 classes, namely the anterior burster (AB), pyloric dilator (PD), lateral pyloric (LP), inferior cardiac (IC), pyloric (PY) and the ventricular dilator (VD) neurons (Figure 1.2) (Harris-Warrick, 1988). In principle, a CPG motor pattern can be triggered by oscillation of either pacemaker cells or by reciprocal excitation and/or inhibition between neuronal clusters within a network via chemical transmission or gap junctions (Marder and Bucher, 2001; Grillner, 2006). The pyloric CPG is driven by a pacemaker cell group consisting of an anterior burster (AB) neuron and two pyloric dilator (PD) neurons, which are electrically coupled via gap junctions (Eisen and Marder, 1982). The synaptic connections within the STG are exclusively inhibitory and hyperpolarise the postsynaptic membrane, inactivating their target neurons (Baro and Harris-Warrick, 1998). The pacemaker group is connected

synaptically to other neurons in the pyloric CPG network and inhibits them by glutamatergic and cholinergic neurotransmission. The respective receptors mediate chloride currents and thereby hyperpolarise the postsynaptic membrane potential (Cleland and Selverston, 1995). Cholinergic innervation has a slow onset and a long effect of the inhibition, whereas glutamate has a fast onset and fast offset. This results in a differential temporal integration of inhibition on the target neurons in the network.

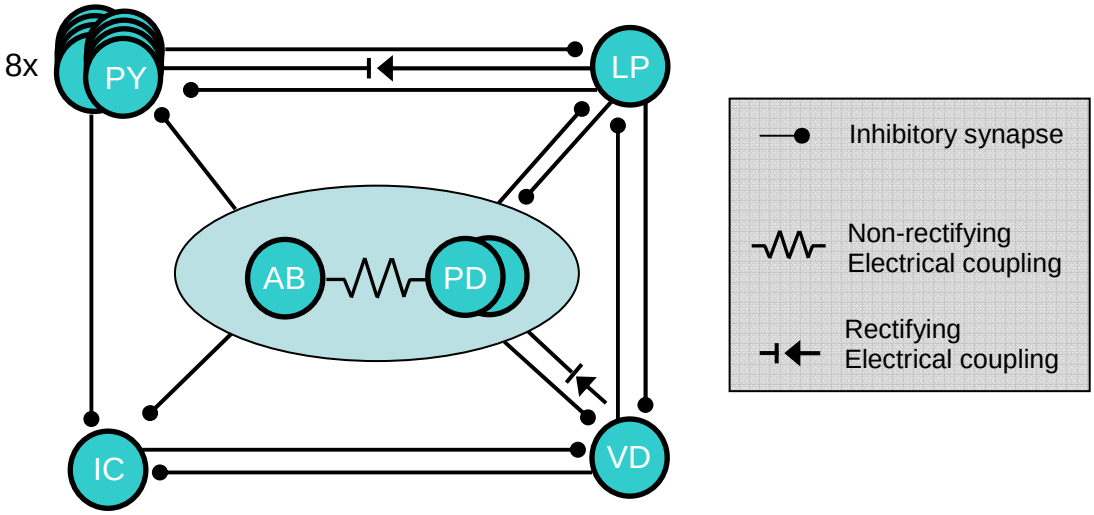

Figure 1.2 The crustacean pyloric CPG network. Schematic diagrams of the pyloric CPG network architecture with inhibitory synaptic connection and electrical coupling (non-rectifying: resistor symbol between AB and PD neurons. Rectifying: diode symbol between LP and PY as well as VD and PD neurons). The pacemaker group consisting of one AB and two PD neurons project inhibitory synapses to all neuronal classes in the network with additional, often reciprocal, inhibitory synaptic projections from target neurons. Reproduced from (Johnson et al., 2011).

Each of the neurons in the network has specific intrinsic electrical characteristics and activity patterns. The membrane properties are determined by the localisation and composition of ion channels in these neurons, which are transcriptionally and translationally regulated in each of the neuronal classes (Nusbaum and Beenhakker, 2002). The differential membrane properties of single neurons in the network determine the timing of post-inhibitory rebound (PIR) after inhibition and the subsequent neuronal activity patterns. The onset of activity after a period of quiescence as a result of inhibition depends on hyperpolarisation-activated inward currents that slowly depolarise the membrane potential towards the threshold for action potential firing (Hooper and Moulins, 1989). The time delay of PIR also largely depends on the transient K^+ currents (I_A), of which several types are present in the STG. These channels are activated in the subthreshold range of action potentials and open transiently after a hyperpolarisation (Baro and Harris-

Warrick, 1998). The maximal conductance as well as the activation and inactivation rates of I_A is cell-type specific and determine the specific PIR dynamics, which in turn determines the temporal sequence of activation of the respective neuronal classes after inhibition from the pacemaker neurons. The six different cell types in the STG express different amounts of I_A Shal or Shaker channels, which correlate to the specific amplitude and kinetic features of I_A currents (Baro et al., 2000). Furthermore, identified neurons within the network display a remarkable degree of variability in their current amplitude and their messenger ribonucleic acid (mRNA) levels, which can vary two- to four-fold (Schulz et al., 2006).

The network function is therefore determined by the specific synaptic connections and the differential intrinsic membrane properties of neurons, which in turn determine the temporal sequence of activity and output from the network. As a result of different membrane properties the lateral pyloric (LP) and inferior cardiac (IC) neurons fire first after the hyperpolarisation from the pacemaker neurons. Only after that the pyloric (PY) and ventricular dilator (VD) neurons return to activity. The order of firing in the pyloric CPG is invariant, but the timing relationship for these neurons can vary and changes with different rhythmic output requirements (Baro and Harris-Warrick, 1998).

Another well resolved single CPG is that controlling the leech heart. This network shares similar features to the pyloric CPG including pace setting neurons and exclusively inhibitory synaptic connections. The leech heart consists of two muscular lateral heart tubes across the length of the animal. At any one time, one tube contracts in peristaltic waves from the posterior to anterior end of the animal, whilst the other contracts synchronously along the entire length of the longitudinal heart tube with regular switches in the activity pattern on each side (Wenning et al., 2004). The muscles of the heart vessels are innervated by segmental ganglia from the CNS and are activated by HE motor neurons, which do not form an integral part of the CPG networks and follow the rhythm determined by the heart CPG (Marder and Calabrese, 1996). The motor neuronal activity is rhythmic due to inhibitory inputs from HN interneurons. Without the inhibitory inputs, motor neurons are tonically active (Calabrese and Arbas, 1989). The rhythm is determined by bilateral pairs of interneurons (HN) in the first 7 ganglia of the nerve cord, which form the heart CPG (Figure 1.3). Within this group, pairs of interneurons in the first 4 ganglia determine the rhythmic activity, whereas the more posterior interneurons in the network are follower interneurons. The rhythm is set by the HN3 and HN4 bilateral and reciprocally inhibitory pairs of interneurons, which can oscillate endogenously (Peterson, 1983b; Arbas and Calabrese, 1987; Cymbalyuk et al., 2002). Between these two ganglia, however, there are no direct synaptic connections. Both HN3 and HN4 interneurons form inhibitory synapses with HN1 and HN2 interneurons, which are functionally indistinguishable. As a result, HN1

and HN2 interneurons temporally integrate the inhibitory signals from HN3 and HN4 interneurons and in turn inhibit HN3 and HN4 (Peterson, 1983a). All synaptic connections between these interneurons as well as their innervation onto motor neurons are inhibitory cholinergic synapses, which increase Cl^- conductance (Schmidt and Calabrese, 1992).

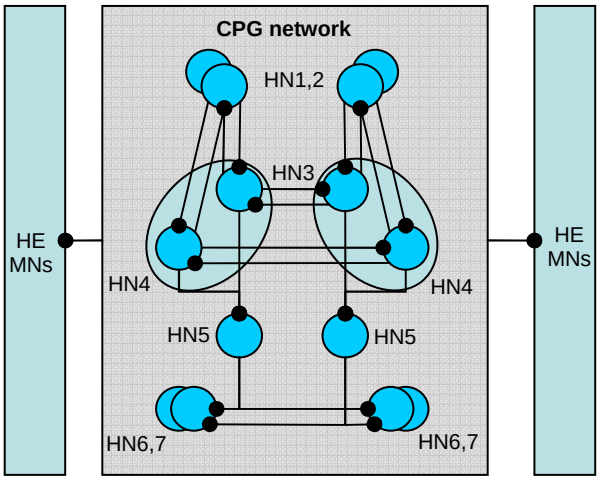

Figure 1.3 The leech heart CPG. Schematic diagram of the inhibitory synaptic connections (filled circles) between the HN interneurons in the first 7 segmental ganglia in the leech ventral cord (HN1-7) comprising the heart CPG. The inhibitory synaptic output of the heart CPG interneurons onto HE motor neurons are simplified in this diagram. Adapted from (Marder and Calabrese, 1996).

The crustacean pyloric and the leech heart CPGs exemplify how organisation and connectivity can generate a temporal sequence of neuronal activity within a network, which eventually drives motor neurons producing behaviour. These networks primarily produce a temporal sequence of neuronal activity by predominantly inhibitory synapses within the networks. However, these networks are functionally specialised as they require mostly persistent activity. Thus, they may not be representative of CPG networks involved in locomotion. Nevertheless, these networks provide valuable insights into the organisation and function of CPGs.

1.1.2 Organisation and Coordination of Multiple CPG Networks in Segmental Locomotion

Animal behaviour is often complex and requires the coordinated output of multiple CPG networks, which control only specific segmental and/or limb muscle groups. Locomotion, for example, is enabled by unit CPG networks located in the CNS, which control their respective muscle groups and provide a specific spatial and temporal sequence of neuronal and muscle activity (Grillner, 2006). The

principle of CPG networks controlling only specific muscles and aspects of behaviour has been established across several vertebrate as well as invertebrate species including leech, *Xenopus* tadpoles, mollusc species, hawkmoths (*Lepidoptera*), locusts (*Locusta migratoria*), stick insects (*Carausius morosus*), lobsters, lampreys and cats (*Felis catus*) (Harris-Warrick, 1988; Johnston and Levine, 1996; Orlovsky et al., 1999b; Büschges et al., 2008). The locomotor CPG networks in *Drosophila* are currently unresolved. Although the modes of locomotion can differ substantially, the basic modular organisation of CPG networks controlling only a small muscle group is a common feature. Isolated CPG networks can reproduce a specified output in the absence of central command centre inputs upon mechanical or pharmacological stimulation and are often coordinated across several CPG networks to produce specified behavioural outputs (Teravainen and Rovainen, 1971; Matsushima and Grillner, 1992; Cattaert and Birman, 2001). However, as exemplified in lamprey, the specific motor programme that is active at any one time is determined by the brainstem, in particular the basal ganglia and reticulospinal neurons (Grillner et al., 2008). For example, the start and stop signals, speed and timing as well as postural adjustments for swimming CPGs are determined by the descending higher command centres located in the mesencephalic reticular nucleus (MRN) and three rhombencephalic nuclei in lamprey (Grillner and Matsushima, 1991; Buchanan and Einum, 2008). Similarly, locomotion is triggered and modulated by neurons located in the suboesophageal ganglion in leeches and has been suggested to provide suitable and correct coordination and general arousal (Brodfuehrer and Friesen, 1986a; Cornford et al., 2006). Thus, based on studies in various species across the animal kingdom, a model has been proposed which suggests that, in locomotion, higher command centres in the brain coordinate and control the activation of spinal or ventral cord unit CPG networks, which in turn activate their respective motor neurons in order to activate muscles and enable behaviour (Figures 1.4) (Selverston et al., 1997). Sensory neurons provide feedback directly to spinal unit CPG networks or higher command centres or both. Locomotion therefore requires the coordinated and tuned activity of multiple specialised networks controlling several aspects of behaviour.

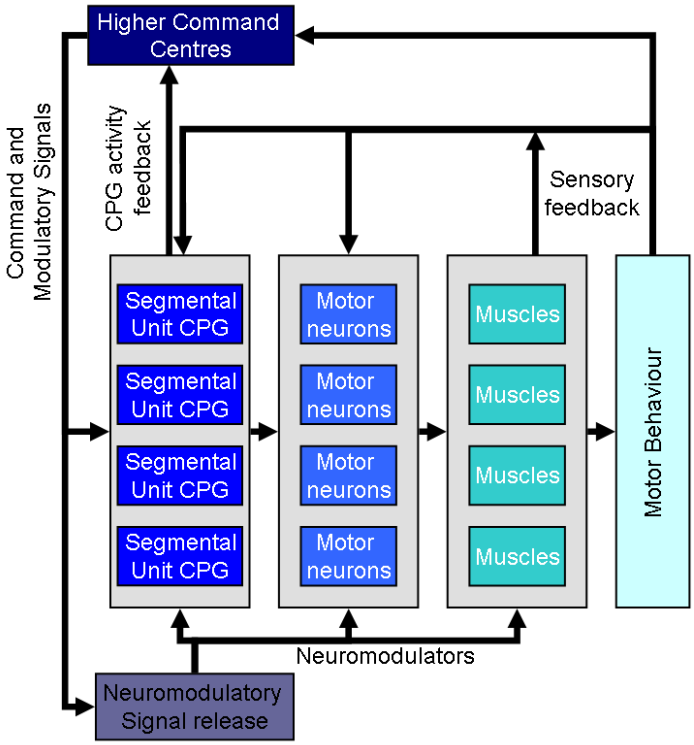

Figure 1.4 The hierarchical organisation of the CNS and the role of unit CPG networks in generating locomotion behaviour. Adapted from (Selverston et al., 1997).

Locomotion of *Drosophila* larvae, the model system in this study, requires the coordinated activity of multiple abdominal muscle segments (Fox et al., 2006). Although the organisation of locomotor CPGs in *Drosophila* is unresolved, evidence suggests that these networks are located in the ventral nerve cord (VNC) (Cattaert and Birman, 2001; Fox et al., 2006), which is consistent with findings in both vertebrates and invertebrates (Orlovsky et al., 1999b). Studies in both vertebrate and invertebrate species that require the activity of multiple repeated muscle segments for locomotion, similar to *Drosophila* larvae, elucidated common features in the organisation and function of CPG networks. In such modes of locomotion, segmentally organised multiple unit CPG networks are connected as coupled oscillators, which control their respective segmental muscle groups. Three of the best described systems include swimming in lamprey, *Xenopus* tadpole and leech.

Swimming in lamprey requires a coordinated activity of multiple repeated CPG networks. In this system, the fastest segmental network sets the oscillation frequency (Matsushima and Grillner, 1992). Excitatory interneurons and additional propriospinal interneurons provide excitation and appropriate activity coupling to the next or numerous segmental CPG networks, respectively (Matsushima and

Grillner, 1992; Hill et al., 2003a). The temporal coordination of multiple CPG networks is flexible and the overall frequency and phasing of activity can be modulated by altering sensory feedback as well as central command centre control (Matsushima and Grillner, 1992). Furthermore, the correct timing and phase activities of multiple segmental unit CPGs is sufficiently coordinated by intersegmental interneurons in the absence of central input (Rovainen, 1985; Cohen, 1987). However, locomotion is initiated and regulated by higher command centres.

In lamprey, locomotion is generated by alternating activity of hemi-segmental unit CPG clusters on opposite body sides, which in turn activate their respective myotomal segments (Orlovsky et al., 1999b; Grillner, 2006). These hemi-segmental unit CPG networks are intrinsically capable of oscillation (Wallen and Grillner, 1987). Identified interneurons as well as motor neurons in the segmental unit CPG networks have been shown to oscillate when all neuronal activity is blocked by tetrodotoxin (Wallen and Grillner, 1987). The individual hemi-segmental CPG units are inter-connected via excitatory and inhibitory interneurons. Activity of hemi-segmental CPGs and muscles on one side are concomitant with inactivity on the contralateral side, which enables alternating contractions from side to side within a single segment (Grillner, 2006). These contralateral contractions propagate like waves with a small phase delay across the length of the animal and thereby enable swimming.

The lamprey spinal cord contains approximately 500 neurons per hemi-segmental unit CPG, which consist of six classes of neurons, namely lateral interneurons (LIN), excitatory interneurons (EIN), edge cells (EC), giant interneurons (GI), contralateral and caudal interneurons (CCIN) and myotomal motor neurons (MN). The following three classes form the core unit CPG networks: LIN, CCIN, and EIN (Figure 1.5) (Grillner and Matsushima, 1991; Buchanan, 1993). The segmental movements in lamprey are mainly due to excitatory glutamatergic premotor EINs forming the kernel of hemi-segmental CPG networks, which mediate their effects via both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors (Grillner et al., 2008). The EINs in turn are controlled by the level of activity of excitatory descending reticulospinal neurons, located in the brainstem (Grillner et al., 2008). The EINs within a network excite each other as well as provide ipsilateral excitatory inputs onto motor neurons as well as inhibitory glycinergic CCINs and LINs (Buchanan and Grillner, 1987). The ipsilateral activation of motor neurons leads to contraction and requires inhibition on the contralateral side. This is achieved by activation of CCINs. The inhibitory CCINs project to and inhibit the contralateral MNs, EINs, LINs and CCINs thereby inhibiting activity of the contralateral hemi-segmental unit CPG, although excitatory CCIN have also been

identified (Buchanan, 1982; Buchanan, 1993). Activation of LINs by EIN results in a temporally delayed inhibitory effect on CCINs and thereby regulates the termination of ipsilateral unit CPG bursts of activity and enables the contralateral to side to initiate activity (Buchanan, 1993; Grillner et al., 2008). Nevertheless, the activity of hemi-segmental unit CPG networks requires inputs from higher command centres for activation, interaction with other CPG networks and sensory inputs from ipsilateral and contralateral sensory neurons, which primarily provide excitatory ipsilateral feedback and inhibitory contralateral inputs (Rovainen, 1974; Viana Di Prisco et al., 1990; Grillner and Jessell, 2009). This sensory feedback is provided by giant interneurons (GI) and edge cells (EC), which are intraspinal stretch receptors (Viana Di Prisco et al., 1990; Buchanan, 1993).

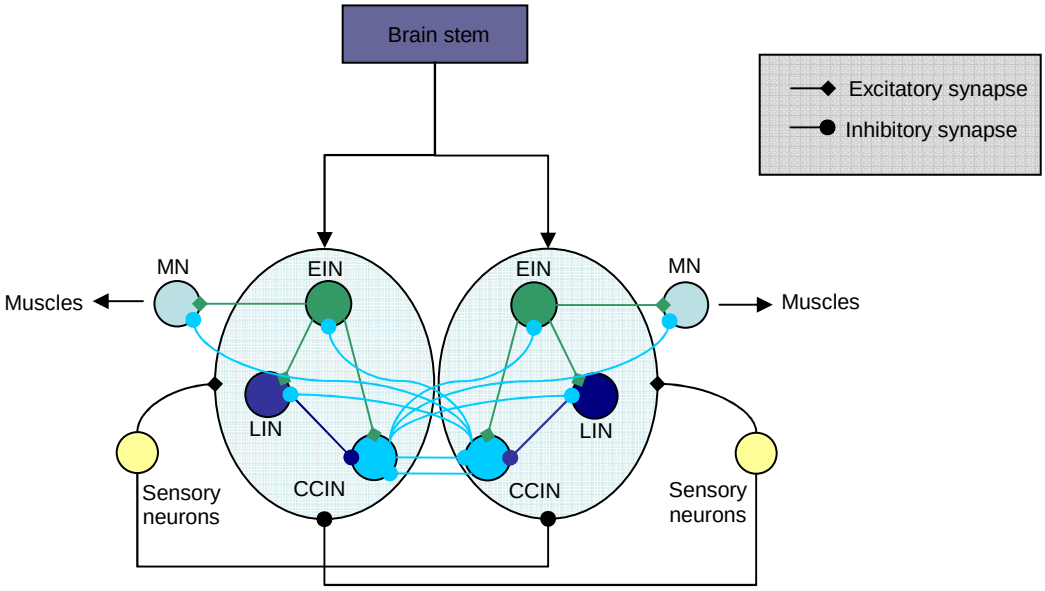

Figure 1.5 Organisation and structure of lamprey segmental unit CPG networks. Inhibitory glycinergic and excitatory glutamatergic synapses are represented by circles and rhomboids, respectively. Abbreviations: myotomal motor neurons (MN), contralateral and caudal interneurons (CCIN), lateral interneurons (LIN), excitatory interneurons (EIN). Sensory neuronal feedback by edge and giant cells is schematically simplified. Adapted from (Grillner and Jessell, 2009) and (Grillner and Matsushima, 1991).

As a result of the spatial and temporal activity patterns of core unit CPGs and sensory neurons, myotomal motor neurons (MNs) periodically receive both excitatory inputs to initiate muscle contraction as well as inhibitory inputs depending on the hemi-segmental CPG network activity during contralaterally reciprocal activity cycles (Kahn, 1982). The reciprocal inhibition is not essential for coordination of CPGs to produce swimming as despite surgical separation of the hemicord, hemi-segmental unit CPGs are still able to oscillate in their activity levels, albeit the frequency is altered (Cangiano and Grillner, 2003, 2005).

Many organisational features of the lamprey hemi-segmental unit CPGs are shared by other model systems including *Xenopus* tadpole and leech swimming CPGs. For example, the segmental CPGs in *Xenopus* tadpoles swim by similar lateral alternating contraction waves moving from the anterior to posterior end of the animal. In the spinal cord, 8 classes of interneurons have been identified. However, only 2 classes are considered essential for the rhythm generation in segmental CPG networks, namely the inhibitory interneurons (iINs) and the excitatory interneurons (eINs) (Figure 1.6) (Marder and Calabrese, 1996). The eINs provide excitatory inputs to ipsilateral motor neurons, iINs as well as other ipsilateral eINs within a hemi-segmental unit CPG, but probably also project more posteriorly and provide excitatory inputs by NMDA and AMPA glutamate receptor mediated excitatory synaptic transmission (Dale and Roberts, 1985). The iINs provide strong glycinergic inhibition to eINs, iINs and motor neurons on the contralateral side as well as weak inhibition on the ipsilateral side on all three cell types (Dale, 1985). This enables reciprocally alternating motor neuronal activity on contralateral sides.

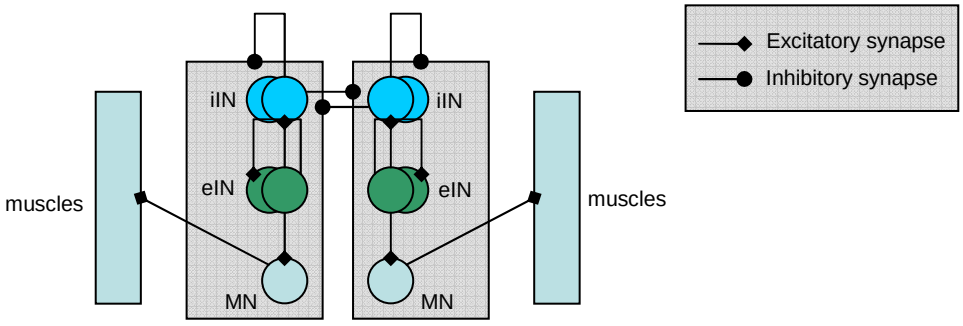

Figure 1.6 Segmental locomotion CPGs in the *Xenopus* tadpole spinal cord. Hemi-segmental units consist of excitatory interneurons (eINs), which form excitatory synaptic connections (rhomboids) with other eINs, inhibitory interneurons (iINs) and motor neuron. iINs form inhibitory synapses (circles) with all neuronal in the contralateral CPG networks and weak inhibitory synapses on the ipsilateral side. Reproduced from (Marder and Calabrese, 1996).

By comparison, the hemi-segmental oscillator networks mediating swimming behaviour in leech are relatively complex (Kristan et al., 2005). Across 21 segmental ganglia in leech, 10 pairs of interneurons and motor neurons have been identified, which are involved in swimming behaviour (Friesen, 1989b). The neurons within these hemi-segmental oscillator networks form predominantly inhibitory synapses as well as some excitatory synapses and are active at different phases in the activity cycle (Orlovsky et al., 1999a). In this system, numerous inhibitory as well as excitatory synaptic connections between adjacent hemi-

segmental units along the dorso-caudal axis regulating intersegmental oscillator activity have been identified (Nusbaum et al., 1987). Another striking feature of this system are 11 segmental pairs of both excitatory and inhibitory motor neurons, which can be electrically coupled and, additionally, form synapses with other motor neurons (Stuart, 1970; Friesen, 1989a).

In summary, both vertebrate and invertebrate model systems have a hemi-segmental organisation of oscillator networks, which control their respective hemi-segmental muscle groups. In these examples, hemi-segmental unit CPGs are reciprocally inhibited by the contralateral network. The interneurons within the networks form both excitatory and inhibitory synaptic connections both with interneurons within networks and/or other networks. Motor neurons receive either both excitatory and inhibitory inputs, such as in lamprey, or exclusively excitatory inputs, such as in *Xenopus*. In *Drosophila* 1st instar larval motor neurons only excitatory inputs have been identified (Baines et al., 2001). In contrast, motor neurons in 3rd instar larvae have been shown to be responsive to hyperpolarising γ-aminobutyric acid (GABA)-ergic and glutamatergic inputs, mediated by increased Cl^- currents (Rohrbough and Broadie, 2002). However, their functional relevant is currently unclear. Moreover, whilst motor neurons in the leech are interconnected and therefore play an important role in generating a sequence of activity, motor neurons in *Xenopus* and lamprey do not form an integral part in generating activity and are thus follower neurons. By comparison, CPG networks in insects involved in locomotion are not as well resolved. However, there is considerable evidence that behaviours such as walking in stick insect, cockroach and locust as well as flying in locust are coordinated by respective CPGs (Orlovsky et al., 1999b). In *Drosophila* larvae, a putative CPG controlling pharyngeal muscles has been shown to be activated in isolation (Gorcryca et al., 1991). Moreover, locomotor CPGs in the VNC can be pharmacologically activated when anterior brain regions are sectioned and activity falls silent (Cattaert and Birman, 2001). This suggests that the CPGs controlling the abdominal segments in larval crawling are located in the VNC. Although the mode of locomotion in *Drosophila* larvae does not require contralateral alteration in muscle activity and thereby differs from the systems described above, it is very similar in other respects as it requires coordinated activity of segmentally organised muscle groups. Considering that the segmental organisation of CPG networks has been identified in both vertebrate and invertebrate systems, it is likely that the CPG networks generating larval crawling in *Drosophila* are similarly segmentally organised.

1.1.3 Modulation of Central Pattern Generator Networks

Neuronal networks are not static in their function and output. The capacity for modulation in CPG networks by specific modulatory signals enables animals to adapt to internal and external factors and thus to exhibit a remarkable degree of behavioural diversity. The following section reviews some of the identified principles in the modulation of CPG network function and output.

Many networks are known to be modulated by a number of signalling molecules. For example, the CPG networks in the STG are modulated by several axonal projections from sensory neurons and neurons located in descending ganglia, with a remarkable array of neuromodulatory signals and effects (Nusbaum and Beenhakker, 2002). Fifteen neuromodulators including octopamine, dopamine, serotonin, histamine, GABA and acetylcholine have been identified to act on the CPGs in the STG and produce several different forms of acceleration or inhibition of the rhythmic outputs (Harris-Warrick, 1988; Harris-Warrick et al., 1997; Nusbaum and Beenhakker, 2002; Dickinson, 2006). The result of the modulatory effects of a multitude of often simultaneously released signals and their differential effects on specific cellular and molecular targets is a remarkable diversity of network outputs and thus behavioural patterns. Neuromodulators alter CPG networks by a number of mechanisms, regulating intrinsic membrane excitability as well as synaptic transmission and selectively affect one or multiple neurons within a network (Cantrell et al., 1996; Cantrell et al., 1997; Alshuaib et al., 2003; Yuan and Lee, 2007; Faber et al., 2008; Higley et al., 2009; Varela et al., 2009; Giessel and Sabatini, 2010).

The biogenic amines octopamine, dopamine and serotonin all increase the cycle frequency of the pyloric CPG rhythmicity, although the resultant CPG outputs are characteristically different as these neuromodulators exert very specific and qualitatively different changes on targeted neurons (Figure 1.7) (Flamm and Harris-Warrick, 1986b). These effects may be mediated by cyclic 3', 5'-adenosine monophosphate (cAMP)-dependent signalling pathways (Flamm et al., 1987). On a cellular level, modulation of neuronal activity patterns can be achieved by targeting voltage- as well as ligand-gated ion channels (Getting, 1988). A neuron's uniquely characteristic properties of excitability and activity patterns, referred to as intrinsic excitability, are determined by the localisation, composition and conductance of diverse voltage-gated ion channels (Zhao and Wu, 1997; Marder and Goaillard, 2006). Targeted modulation of voltage-gated ion channels can therefore transform the activity patterns of targeted neurons and ultimately networks. For example, dopamine has been shown to exert its effects by modulating a multitude of target molecules in specific cells. It has been shown to increase the transient K^+ (I_A) and

Ca^{2+}-dependent K^+ (I_{KCa}) currents in PD neurons and thereby modulates the resting potential, hyperpolarisation and post-inhibitory rebound rates as well as spike duration, which together modulate the overall activity pattern of this neuronal class (Harris-Warrick et al., 1998; Kloppenburg et al., 1999). In PY and LP neurons, dopamine increases input resistance, reduces the I_A current and additionally increases the inwardly rectifying K^+ current in the PY neuron (Harris-Warrick et al., 1995; Gruhn et al., 2005). The effects exemplify how dopamine differentially either increases or decreases I_A currents in specific neurons and thereby modifies the rhythmicity and output of the pyloric CPG (Harris-Warrick et al., 1998). Dopamine also differentially modulates Ca^{2+} currents (I_{Ca}) in multiple neurons in the pyloric CPG. It has been shown to increase I_{Ca} in PY, LP and IC neurons, but to reduce this current in VD, PD and AB neurons (Johnson et al., 2003). This highlights that neuromodulators can have antagonistic effects on the same ionic current, but this depends on the neuronal identity. Dopamine also increases the hyperpolarisation-activated inward current (I_h) in VD and LP neurons (Harris-Warrick et al., 1998). This shows how diverse the effects of a single neuromodulator can be as multiple currents are specifically modulated in multiple neurons within the pyloric CPG network. It suggests that the modulation of network output is enabled by the functional diversity of receptor subtypes located in specific neurons. As a result, the patterns of activity are differentially altered and qualitatively transform the network rhythmicity and output by various neuromodulators (Flamm and Harris-Warrick, 1986a; Flamm and Harris-Warrick, 1986b).

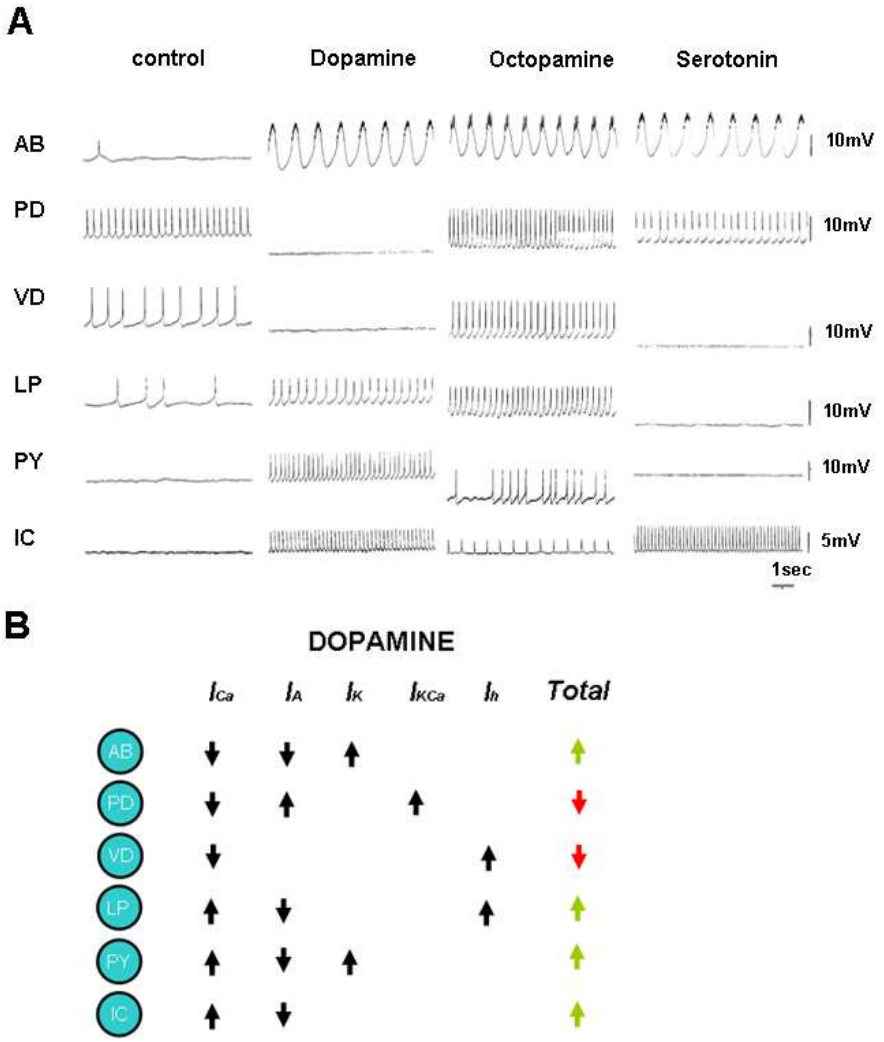

Figure 1.7 Modulation of the pyloric CPG network. **A**. Effects of application of dopamine, octopamine and serotonin on the activity patterns in identified neurons in the pyloric CPG. Taken from (Flamm and Harris-Warrick, 1986a). **B**. Identified effects of dopamine on specific currents in identified neurons in the pyloric CPG. Adapted from (Harris-Warrick et al., 1998; Nusbaum and Beenhakker, 2002; Johnson et al., 2003; Gruhn et al., 2005).

In addition to modulating the intrinsic activity pattern of constituent neurons within a network, CPG output can also be modulated by targeting the synaptic transmission between neurons. Biogenic amine neuromodulators have also been shown to differentially regulate the electrical coupling between the pacemaker group of AB and PD neurons and additional coupling to VD neurons and thereby putatively affect the pacemaker output as well as the entire pyloric CPG output (Johnson et al., 1993). Dopamine and octopamine increase, but serotonin reduces the synaptic current from LP to PD neurons, which form the only direct inhibitory synaptic connection with the pacemaker groups, and thereby shift the phasing of rhythmicity (Johnson et al., 2011). Biogenic amines also differentially modulate the

synaptic strength of graded transmission of PD neurons on three postsynaptic targets, LP, PY and IC neurons. Dopamine reduces the synaptic strength in all of these synapses, serotonin differentially down- or up-regulates synaptic strength, whereas octopamine increases the synaptic strength in all synapses (Johnson and Harris-Warrick, 1990). Moreover, dopamine has been shown to antagonistically regulate the amplitude of graded and spike-evoked neurotransmission in the opposite directions and alters the target neuronal input resistance in LP neurons forming synapses onto PD, VD and PY neurons (Ayali et al., 1998). The described effects exemplify how the CPG network output can be modulated by targeting specific neurons and molecular targets that ultimately transform network activity. These examples in the well established pyloric CPG network show how specific and diverse modulatory effects on the intrinsic excitability of synaptic transmission in constituent neurons can transform the CPG network output.

Similar modulatory effects have also been identified in multiple coordinated CPG networks that control locomotion. The lamprey has 100 segments and the phase delay in the activation of EINs per hemisegment is approximately 1% of the cycle phase, independent of the cycle frequency (Grillner et al., 2007). However, the real time delay can vary substantially (Matsushima and Grillner, 1992). Segmental unit CPG network output and phasing across multiple unit CPGs can be modulated by numerous neuromodulatory signals acting via various molecular and cellular targets. This enables a high degree of specified modulation of locomotion. For example, the frequency of bursts in the unit CPGs in swimming is slowed by serotonin from descending neurons (Grillner and Matsushima, 1991; Biro et al., 2006). Serotonin acts on Ca^{2+}-activated K^{+} channels, which decreases the slow afterhyperpolarisation phase in CCIN and LIN and thereby reduces the burst rate in the CPGs (Wallen et al., 1989; Biro et al., 2006). Serotonin has also been shown to reduce currents in high-voltage activated Ca^{2+} channel in dissociated spinal cord neurons (Hill et al., 2003b). Similarly, in the *Xenopus* tadpole, serotonin and noradrenaline decrease or increase inhibitory glycinergic synaptic current amplitude from inhibitory interneurons, respectively, which modulates the time delay of rostra-caudal propagation waves during swimming (McLean et al., 2000). As a result, serotonin accelerates swimming, whereas noradrenaline slows it. Across several locomotion model systems with multiple coordinated CPGs, numerous neuromodulatory pathways have been identified, which regulate synaptic transmission, burst frequency, slow afterhyperpolarisation of excitatory and inhibitory motor neurons as well as sensory neurons by releasing substances including dopamine, serotonin, GABA, glutamate, acetylcholine and approximately 20 neuropeptides (Grillner and Matsushima, 1991; Grillner and Jessell, 2009).

The effects of modulating a specific current may or may not alter the intrinsic excitability of a single neuron, depending on the density and composition of all

channel proteins (Goldman et al., 2001). The effect on a specific current can thus transform the neuronal and network function or be negligible due to compensatory co-variation of other currents, which sustains an identical neuronal and network output (Grashow et al., 2010). As such, neuromodulators can modulate specific currents without identifiable effects on the network output, yet simultaneously prime neurons to respond to additional neuromodulators targeting specific currents such that both effects may transform neuronal and network output (Nusbaum and Beenhakker, 2002). Nevertheless, the output and responsiveness of neuronal networks to modulators has been shown to be largely reliable, despite disparate underlying parameters in neuronal and network function (Prinz et al., 2004; Grashow et al., 2009). Additionally, the output of a neuronal network can remain unchanged despite major variability in both synaptic and intrinsic voltage-gated currents (Grashow et al., 2010). For example, it has been shown in the leech heart CPG that the coordination of two physiological modes of heartbeat synchronicity is reliably constant despite considerable variations in both strength and phase timing of multiple synaptic inputs from several neurons in the CPG (Norris et al., 2011).

The potent and specific effects of neuromodulators on multiple ionic currents, often within a single neuron, as well as synaptic transmission on multiple neurons exemplify the complexity as well as diversity of modulatory effects on a small network outputs. Given that multiple neuromodulators dynamically interact with the neuronal network further adds to the complexity of network function. However, as a result of neuromodulation the functional diversity of neuronal networks is dramatically increased and therefore enables highly diverse behaviours.

1.1.4 Network Homeostasis

Major advances have been achieved in the understanding of homeostasis and plasticity of intrinsic excitability (Golowasch et al., 1999; Klein et al., 2003; MacLean et al., 2003; Mee et al., 2004; Swensen and Bean, 2005; Marder and Goaillard, 2006; Muraro et al., 2008) as well as synaptic physiology in single neurons (O'Brien et al., 1998; Turrigiano and Nelson, 2004; Thiagarajan et al., 2005; Sutton et al., 2006). However, ultimately organisms need to primarily have a homeostatic regulation of entire neuronal networks and therefore appropriate behavioural outputs. Over the last two decades, fascinating studies have emerged, which suggest that neuronal network output itself may be under homeostatic control (Thoby-Brisson and Simmers, 1998; Maffei and Fontanini, 2009). It is unclear if neuromodulatory signalling such as octopamine and tyramine in *Drosophila* play a role in the homeostasis of locomotor CPGs. It is therefore important to investigate this further.

Several studies have shown that neuronal networks are able to maintain an appropriate output throughout developmental changes, despite unfavourable conditions or are able to recover with time after perturbation. For example, although the CNS and its components undergo substantial growth related changes during developing, the pyloric STG network output and phase relationship of its constituent neurons remain constant throughout development (Bucher et al., 2005). Networks are also able to provide near constant outputs despite great variations in external and internal conditions. This is seen when a crustacean pyloric CPG changes its output properties in response to differing temperatures. Whilst the frequency of network activity is substantially increased in warmer temperatures, the phase relationship of neuronal firing in the network remains constant as the amplitude of I_h and I_A currents change with varying temperatures and thereby alter the cycle frequency (Tang et al., 2010). Lastly, network activity can recover with acute and profound changes to network function. For example, blocking cholinergic and glutamatergic neurotransmitter release in the developing chick spinal cord blocks spontaneous network activity. However, the network activity recovers to near physiological levels after a delay (Chub and Oâ€™Donovan, 1998).

The pyloric CPG in the STG requires modulatory inputs to the network to maintain its correct rhythmicity. When this network is decentralised from external modulatory inputs, its output falls silent. However, this CPG network can recover its rhythmic output after several days as specific conductances including I_h, I_A, and I_{KCa} are homeostatically altered (Thoby-Brisson and Simmers, 1998; Haedo and Golowasch, 2006; Khorkova and Golowasch, 2007). The frequency and rhythmicity of the pyloric CPG network can also fully recover after several days when the intrinsic excitability of neurons within the network is unfavourably modified after decentralisation by application of tetrodotoxin, which reduces activity, or by elevated external K^+ levels, which is excitatory (Thoby-Brisson and Simmers, 1998). These findings suggest that networks are intrinsically capable of homeostatically maintaining their output, which requires adaptive changes in specific ion channel conductances in constituent neurons. The time to recover can be shortened or prolonged by neuromodulators, which have been suggested to mediate an appropriate co-regulation of specific currents (Khorkova and Golowasch, 2007; Zhang et al., 2009). This suggests that both intrinsic characteristics as well as additional modulatory effects are important in the recovery process. These findings are unsurprising in light of studies that suggest a crucial role of modulatory inputs into a CPG for appropriate circuit development to occur (Fenelon et al., 1998). Neuromodulators are associated with both maintenance and tuning functions of CPG circuitry by a postulated regulation of receptor and neurotransmitter synthesis, recycling and activity, which in turn regulates target cell excitability (Fenelon et al., 1998). These findings show that

neuromodulators play an important role in the maintenance and robustness throughout development as well as acute functional modulation in the pyloric CPG (Khorkova and Golowasch, 2007). It is unclear if these effects are common in functionally different CPG networks. More specifically, it is unclear if neuromodulators play a similarly important role in the maintenance of CPG network output in locomotion, particularly in the maintenance of multiple coordinated CPG networks such as *Drosophila* larvae.

In summary, evidence suggests that neuromodulators play an important role in the maintenance of network output and activity. The effects of neuromodulators are therefore likely to be of far greater importance than the exclusive acute functional modulation of single neurons and networks. They are likely to be involved in the functional homeostasis of neuronal network outputs throughout development. However, whether neuromodulators, such as octopamine and tyramine, are important in the homeostatic regulation of locomotor CPGs, such as in *Drosophila* larvae, or whether these networks can regulate themselves when neuromodulatory signalling is chronically altered is currently unknown and is investigated in this study.

1.2 OCTOPAMINE AND TYRAMINE

Octopamine and tyramine are potent neuromodulators (Figure 1.8), which are homologous to adrenaline and noradrenaline in mammals and have been shown to exert modulatory effects on diverse signalling pathways, cells and tissues in both invertebrates and some vertebrates (Axelrod and Saavedra, 1977). Octopaminergic effects have been shown in a number of species including nematode worm (*Caenorhabditis elegans*) (Suo et al., 2006), snail (*lymnaea stagnalis*) (Vehovszky et al., 2004), locust (Evans, 1981), cricket (*Gryllus bimaculatus*) (Spörhase-Eichmann et al., 1992), cockroach (*Periplaneta Americana*) (Sinakevitch et al., 1994), honeybee (*Apis mellifera*) (Kreissl et al., 1994; Grohmann et al., 2003) lobster (Flamm and Harris-Warrick, 1986a) and rat (*rattus*) (Frascarelli et al., 2008). Tyramine has long been assumed to be simply the biochemical precursor in the synthesis of octopamine, but has since been established as a potent modulatory signal with identified receptors and physiological effects in a number of species (Saudou et al., 1990; Vanden Broeck et al., 1995; Blenau and Baumann, 2001; Blumenthal, 2003; Alkema et al., 2005). The effects of these compounds on behaviour, with a focus on insect physiology, will be introduced in the following section.

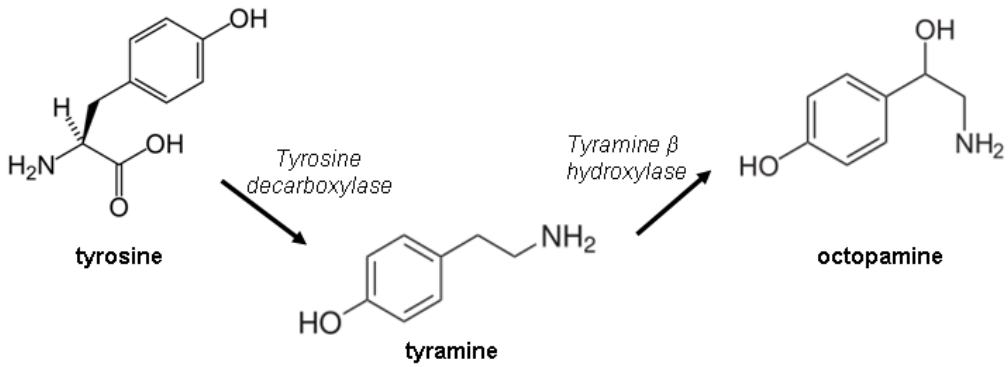

Figure 1.8 The structure and synthetic pathway of tyramine and octopamine (Livingstone and Tempel, 1983).

1.2.1 The Effects of Octopamine and Tyramine in Invertebrates

Octopamine and/or tyramine are widely expressed throughout the CNS and peripheral tissues and have been shown to have effects in many different tissues and cell types across multiple model systems. In the case of octopamine it is known to be released both as a neurotransmitter exerting its effects directly at the synapse as well as a neurohormonal signal thereby modulating diverse tissues by circulating in the hemolymph (Belanger and Orchard, 1988; Roeder, 1999). Synaptic terminals from octopaminergic neurons in the locust CNS have been shown to contain both small clear vesicles as well as large-dense core vesicles, which often contain neuropeptides (Hoyle et al., 1980). It is not clear if the large-dense core vesicles also contain octopamine (Hoyle et al., 1980). The diversity of octopaminergic/tyraminergic GPCRs and their coupling to several signalling pathways confers a considerable degree of functional versatility. Some of the known effects of these neuromodulators on the physiology in various cells, primarily in insects, are summarised below.

Octopamine has been shown to be expressed in the CNS in several species, including *Drosophila*, cockroach, locust, honeybee and *C.elegans* and often in not dissimilar neuronal structures (Sinakevitch et al., 1994; Monastirioti et al., 1995; Sinakevitch et al., 2005; Suo et al., 2006). It can be released as a neurohormone, paracrine or neurotransmitter signal (Roeder, 1999). Similarly, tyraminergic neurons have also been identified in the CNS in both *Drosophila* and *C.elegans* using immunoreactivity against tyramine itself or against the enzymatic pathway in its synthesis, respectively (Nagaya et al., 2002; Alkema et al., 2005). These tyraminergic neurons putatively do not contain octopamine. However, in light of the structural similarities of octopamine and tyramine and the limited data

on the specificity of the antibody against tyramine in *Drosophila*, it is inconclusive if the identified neurons are exclusively tyraminergic. In insects, the only neurons identified as octopaminergic in the VNC are dorsal or ventral unpaired median neurons (DUM or VUM) (Duch et al., 1999). In locusts, DUM neurons can be distinguished by different axonal projections through peripheral nerves and do not form synapses, suggesting that their neuromodulators are released as paracrine signals (Hoyle et al., 1980; Watson, 1984). The octopaminergic DUM neurons have different neuronal excitability profiles as they differ in their composition of I_A, I_{KCa}, I_{Na}, I_h currents, but not in their I_{Ca} currents (Heidel and Pflüger, 2006). The DUM neurons in locusts have been shown to share some common interneuronal inputs, but also receive differential and specific interneuronal inputs in response to sensory stimuli including auditory, visual and mechanosensory inputs from both ascending and descending interneurons (Duch et al., 1999). The DUM neurons themselves are not electrically connected (Duch et al., 1999). These subsets of octopaminergic DUM or VUM neurons are differentially activated, often simultaneously with motor neuronal activation and target different muscle groups and thereby modulate different behaviours (Burrows and Pfluger, 1995; Baudoux et al., 1998). Similarly, in *Manduca* hawkmoth motor neurons and octopaminergic UM neurons are active in phase during fictive crawling activity cycles (Johnston et al., 1999). Octopaminergic neurons have also been described in anterior brain regions. Octopaminergic neurons in the suboesophageal ganglia project to posterior ganglia in the VNC in locusts without peripheral projections suggesting that these neurons exert their effects exclusively in the CNS (Bräunig and Burrows, 2004). Lesions of the connections between the brain and the thoracic ganglia abolishes the responsiveness of DUM neurons to mechanosensory stimuli suggesting that the suboesophageal ganglion plays a central role in the integration of sensory information (Duch et al., 1999). These findings indicate that octopaminergic and/or tyraminergic neurons in the CNS are likely to be involved in the integration of sensory information. Moreover, considering that octopaminergic and/or tyraminergic neurons in the VNC are active during motor activity in locusts and hawkmoth suggests that these neuromodulators are likely to play an important role in the modulation and/or coordination of motor behaviour. Thus, chronic changes in the signalling of these neuromodulators in *Drosophila* larvae may serve as a suitable model to study the long-term effects on locomotor CPG networks.

Many studies have identified a number of effects of these neuromodulators in muscle and the CNS. Octopamine and, to some extent, tyramine are known modulators of multiple aspect of muscular physiology. In locusts, octopamine increases the contraction force of flight muscles (Malamud et al., 1988). In addition, octopamine increases the contraction and relaxation rates in locust flight and hindleg muscles (Evans and Myers, 1986; Whim and Evans, 1988) mediated by

increases in cAMP (Whim and Evans, 1991). In the tibial leg muscle, octopamine reduces the myogenic rhythm and increases muscle tone (Evans and O'Shea, 1977; Evans and O'Shea, 1978), both mediated by increases in cAMP and induced by octopaminergic neurons in the CNS (Evans and Siegler, 1982; Evans, 1984). Octopamine increases hyperpolarisation-activated conductances of Cl^- and reduces resting K^+ currents in locust hindleg muscles, which are again both mediated by cAMP (Walther and Zittlau, 1998). Octopamine has also been shown to increase the basal muscle tone in spermathecal tissue (Clark and Lange, 2003). In *Drosophila,* octopamine has been shown to increase muscle contraction strength in oviduct peritoneal sheath muscles (Middleton et al., 2006). In contrast, tyramine has also been shown to decrease the locust oviduct muscle tone and muscle membrane potential by decreasing cAMP signalling in locust (Donini and Lange, 2004). Furthermore, mutant flies with genetically elevated tyramine levels and no octopamine exert a weaker muscle contraction for jumping (Zumstein et al., 2004). These examples show that behavioural output can be modified by direct modulatory effects on muscular physiology.

Octopamine and tyramine also modulate the synaptic and intrinsic neuronal physiology in the periphery and CNS. Octopamine has been shown to increase the excitatory junction potential (EJP) in the neuromuscular junction physiology in locust (Evans and O'Shea, 1977), crayfish (Breen and Atwood, 1983), *Drosophila* (Nishikawa and Kidokoro, 1999; Nagaya et al., 2002; Koon et al., 2011) as well as the miniature EJP (mEJP) in adult moth (Fitch and Kammer, 1986; Klaassen et al., 1986). In *Drosophila*, tyramine decreases EJP amplitude (Nagaya et al., 2002).

Octopamine has also been shown to modulate both the output of the CNS onto the musculature as well as the sensory neuronal feedback from the periphery. For example, DUM neurons directly innervate and increase proprioceptor responsiveness to mechanical stimulation in the locust hindlegs (Bräunig and Eder, 1998). In proprioceptive neurons in the locust leg, octopamine increase the spike frequency pattern in response to flexed but not extended leg positions (Matheson, 1997). Similarly, octopamine and tyramine have also been shown to increase spike frequency of leg mechanosensory neurons in spiders (Widmer et al., 2005).

In the CNS, octopamine has also been shown to modulate both synaptic transmission and neuronal intrinsic excitability. It reduces the postsynaptic ESPC amplitude from identified sensory neurons onto flight motor neurons in locust (Leitch et al., 2003). The excitability of motor neurons and the frequency of synaptic inputs onto motor neurons in response to octopamine applications is increased as the afterhyperpolarisation is reduced and the action potential width is broadened (Parker, 1996). Octopamine application results in reciprocal rhythmic activity in antagonistic tibial muscle groups in the absence of sensory feedback,

which is indicative that octopamine can directly drive central pattern generators (Parker, 1996). Octopamine also modulates flight interneuronal intrinsic excitability and activity patterns (Ramirez and Pearson, 1991a; Ramirez and Pearson, 1991b). Applications of octopamine on locust flight interneurons induces burst like depolarisations in response to brief pulses that last longer than the pulse of drug application and elicit endogenous bursting activity (Ramirez and Pearson, 1991a; Ramirez and Pearson, 1991b).

The effects of tyramine are in the CNS are not well resolved. In the adult moth, tyramine has been shown to specifically suppress spiking of the wing depressor motor neurons (Vierk et al., 2009). In locust, tyramine has also been shown to modulate flight pattern generators (Buhl et al., 2008). Despite the potent and diverse effects of octopamine and tyramine, their effects in the CNS have been suggested to be modulatory only and are probably not sufficient to drive and initiate activity (Buhl et al., 2008).

These studies exemplify the functional diversity of these neuromodulators as they have been shown to modulate muscle physiology, synaptic transmission in the neuromuscular junction (NMJ), sensory neuronal feedback and the function of motor neurons in the CNS. Octopamine and tyramine may act antagonistically as exemplified by their effects on muscles and cAMP levels. However, this antagonistic relationship is currently inconclusive. Considering that many of the modulatory functions are likely to be exerted simultaneously, such as in locust behaviour, suggests that octopamine and/or tyramine may have a fundamental role in the modulation of behaviour.

1.2.2 Octopamine and Tyramine in *Drosophila*

In *Drosophila*, octopamine has been shown to be involved in the regulation of aggression (Hoyer et al., 2008; Zhou et al., 2008), egg-laying (Monastirioti et al., 1996; Lee et al., 2003), starvation stress responsiveness (Koon et al., 2011) decision-making (Certel et al., 2010), sleep (Crocker and Sehgal, 2008; Crocker et al., 2010) learning and memory (Schwaerzel et al., 2003; Unoki et al., 2005; Honjo and Furukubo-Tokunaga, 2009) as well as flight initiation (Brembs et al., 2007). Similarly, the *inactive (iav)* mutation, a fly genotype with low octopamine levels, has been shown to cause locomotor deficits, low mating success and a reduction in longevity (O'Dell, 1993). However, it is unclear, if these effects are caused directly by a reduction in octopamine levels. Tyramine has been suggested to be involved in olfaction as tyramine receptor mutants are deficient in avoiding repellent odours (Kutsukake et al., 2000). In larvae, octopamine and tyramine modulate crawling behaviour. A mutation of the tyramine β hydroxylase gene results in drastically reduced octopamine levels and approximately 7-8-fold increased tyramine levels

and profoundly reduces crawling speed in larvae (Monastirioti et al., 1996; Saraswati et al., 2004; Fox et al., 2006). The deficit in crawling speed can be rescued by either feeding TβH^{nM18} larvae octopamine or the tyramine receptor antagonist yohimbine, which increases larval crawling speed by approximately 50% in both cases (Saraswati et al., 2004). These results imply an antagonistic relationship for octopamine and tyramine in *Drosophila* larval locomotion, although the sites and mechanisms of action are unknown.

Octopamine and/or tyramine have been shown to exert effect on the muscle contraction force in the oviduct and leg muscles (Zumstein et al., 2004; Middleton et al., 2006). In addition to muscular effects, octopamine and/or tyramine also modulate NMJ physiology and as well as neuronal function in the CNS. Octopamine has been shown to increase the amplitude of excitatory junction potentials (EJPs) in muscles M1 and M2 as well as M12 and 13, which are directly innervated by octopaminergic synaptic terminals (Monastirioti et al., 1995; Nagaya et al., 2002; Koon et al., 2011). Conversely, in muscle M6 and M7, which are not innervated by octopaminergic synaptic terminals, octopamine has been shown to decrease EJPs (Nishikawa and Kidokoro, 1999). In contrast, tyramine decreases the EJP amplitude in muscles M12 and M13 (Nagaya et al., 2002). Notably, the octopaminergic effect could be reproduced in a tyramine receptor mutant, whereas the tyramine response disappeared, suggesting that distinctive tyramine and octopamine receptors are located and have a function at the NMJ (Kutsukake et al., 2000; Nagaya et al., 2002). In addition to the effects in the NMJ, central effects in the adult brain have also been identified. The octopaminergic GPCR subtype OAMB has been suggested to putatively reduce the Slowpoke Ca^{2+}-activated K^{+} current (Crocker et al., 2010).

The expression patterns of octopaminergic and/or tyraminergic neurons and their putative projections have been described in detail. Using immunocytochemical and genetic marker proteins, a number of octopaminergic and potentially tyraminergic neurons have been identified in the larval CNS (Monastirioti et al., 1995; Vömel and Wegener, 2008). Octopamine immunoreactivity and genetic labelling in the larval CNS identified single pairs or clusters of neuronal cell bodies along the midline in the ventro-medial parts of the ventral nerve cord (VNC), particularly in the abdominal and thoracic segments (Monastirioti et al., 1995; Vömel and Wegener, 2008). Moreover, tyramine immunoreactivity has also been demonstrated in the larval CNS with a remarkably similar staining pattern to octopamine immunoreactivity (Nagaya et al., 2002). However, this study provided little evidence for the specificity of the antibody against tyramine. Immunoreactivity and genetic labelling indicate that octopamine and/or tyraminergic neurons putatively project synaptic terminals into the neuropil regions, suggesting that these neuromodulators may be released in the CNS

(Monastirioti et al., 1995; Vömel and Wegener, 2008). In addition, most body wall muscles have also been shown to be innervated by octopaminergic type II synaptic terminals (Monastirioti et al., 1995). The localisation and projection pattern of octopaminergic and/or tyraminergic neurons will be described in more detail in chapter 4. These studies suggest a widespread innervation of muscles, NMJs and within the CNS with a number of putative release sites, which may be indicative of a global modulatory role for these signalling molecules in tissue function and eventually larval behaviour.

In addition to the widespread putative innervation and release, the receptor molecules and signalling pathways for these neuromodulators have also been described in a number of studies. Octopamine and tyramine, and the respective enzymes required for their synthesis, have been characterised in the insect CNS (Livingstone and Tempel, 1983). Both neuromodulators are derived from tyrosine, which is converted into tyramine by the enzyme tyrosine decarboxylase. Tyramine in turn is converted into octopamine by tyramine β hydroxylase. *Drosophila* expresses two isoforms of the enzyme tyrosine decarboxylase, one of which, Tdc2, is expressed in the CNS and female reproductive tract, whereas Tdc1 is expressed in non-neuronal tissues with largely unidentified functions (Cole et al., 2005). Octopamine and tyramine exert their effect via G-protein-coupled receptors (Roeder, 1999, 2005). GPCR signalling pathways typically increase or decrease the activity of adenylate cyclase (AC) via stimulatory or inhibitory G-proteins (Blenau and Baumann, 2001). Adenylate cyclase activity converts adenosine triphosphate (ATP) to cAMP, which is a potent signalling molecule (Blenau and Baumann, 2001). Alternatively, GPCRs may activate the phospholipase C (PLC) signalling pathway via another subtype of G-proteins, which releases inositol 1,4,5-triphosphate (IP_3) and diaceylglycerol. In turn, IP_3 activates receptors on intracellular Ca^{2+} stores and releases Ca^{2+} into the intracellular space. Diacylglycerol activates protein kinase C (PKC) (Blenau and Baumann, 2001). The *Drosophila* genome contains four octopamine receptor subtypes, one tyramine/octopamine receptor, and one tyramine receptor based on sequence homology and pharmacological profiles (Evans and Robb, 1993; Cazzamali et al., 2005; Evans and Maqueira, 2005). The octopamine receptor subtype in mushroom bodies (OAMB), which is classified as an α-adrenergic type GPCR, preferentially binds octopamine and modestly increases cAMP, but primarily increases Ca^{2+} levels (Han et al., 1998) (Maqueira et al., 2005). The *Drosophila* genome contains three β-adrenergic-like receptors, classified as Octβ1R, Octβ2R and Octβ3R that do not alter Ca^{2+} levels, but increase cAMP (Balfanz et al., 2005; Maqueira et al., 2005). The genome also contains one tyramine receptor with a higher affinity for tyramine than for octopamine, which reduces cAMP levels upon activation (Arakawa et al., 1990; Saudou et al., 1990). Finally, the octopamine/tyramine

receptor differentially couples both neurotransmitters to different signalling pathways as tyramine reduces adenylate cyclase activity whereas octopamine increases Ca^{2+} levels (Robb et al., 1994). These studies have been largely conducted by expression of these receptor subtypes in mammalian cell lines. The effects exerted by the receptors on the intracellular signalling pathways are likely to be representative of their effects in *Drosophila,* however, in light of the specificity of secondary signalling pathways, there is also a need for caution.

As described above, octopamine and/or tyramine are involved in the modulation of locomotor CPGs in *Drosophila* larvae, which suggests that this is a suitable model to study the effects of chronically altered neuromodulatory signalling on network activity and output. Moreover, octopamine and/or tyramine have identified effects on muscles, NMJ neurotransmission and on specific currents in neurons in the CNS and exert their effects by a number of receptor subtypes and signalling pathways. These findings suggest that these neuromodulators are likely to exert their effects on multiple cellular and molecular targets in order to modulate crawling behaviour.

1.3 OUTLINE OF THE THESIS

This project addresses the effects of chronically altered octopaminergic and/or tyraminergic neuromodulatory signalling on multiple coordinated segmental CPG networks. In order to study this, I aimed to characterise the role of octopamine and tyramine in the modulation of *Drosophila* larval crawling behaviour. A previous study has identified an important role for these neurotransmitters in modulating crawling speed (Saraswati et al., 2004). However, it is currently unresolved where these neuromodulators exert their effects and what mechanisms of actions they employ. A combination of genetic, behavioural, imaging and electrophysiological techniques was applied to address the effects of these signalling molecules on segmental unit CPGs in locomotion on a systemic and cellular level.

The behavioural effects of octopamine and/or tyramine on larval locomotion are described in Chapter 3. This chapter identifies the role of these neuromodulators in the modulation of stride length, stride duration and resultant crawling speed. Further analysis identified the effects of octopaminergic and/or tyraminergic modulation on different phases of the peristaltic contraction cycle during forward locomotion in this model system as well as their effects on the dynamics of segmental muscle contractions.

Chapter 4 describes the localisation and synaptic terminal projection patterns of octopaminergic and/or tyraminergic neurons throughout the larval CNS

in relation to identified motor neurons. In addition, the chapter describes the synaptic terminals projections of a subpopulation of putatively octopaminergic and/or tyraminergic neurons in anterior parts of the brain in relation to identified motor neurons as well as their role in the modulation of crawling behavioural.

Chapter 5 addresses the effects of these neurotransmitters on the NMJ physiology as well as their effects on the synaptic physiology and intrinsic excitability of identified motor neurons in an attempt to identify some of the target tissues and cells in the modulation of crawling behaviour.

The significance and implications of the findings of this study in light of previous publications as well as the outstanding questions and future research prospects are discussed in chapter 6.

Chapter 2

Materials and Methods

2.1 FLY FOOD

Fly stocks were maintained at 18°C on standard fly food in vials or bottles on a standard 12 hour dark-light cycle. For experiments, larvae were reared at 25°C throughout development. The fly food was prepared at the Faculty of Life Sciences at the following composition: 360g organic maize, 250g autolysed yeast powder, 396g glucose, 50g agar were to 5L of water and the mixture was heated for 10min to 98°C. After cooling, 135ml of nipagen and 15ml of propionic acid were added.

2.2 FLY STOCKS

The fly lines used in this study are listed below. Homozygous GAL4 driver lines and UAS-reporter lines were crossed to obtain heterozygote larval progeny, which will be referred to in an abbreviated form as, for example, Tdc2-Kir2.1 instead of Tdc2-GAL4/UAS-Kir2.1. Furthermore, to distinguish this more clearly, the UAS responder line with the active isoform of tetanus toxin light chain, UAS-TNT G, will be referred to as, for example, Tdc2-TeTx* whereas the inactive isoform, UAS-TNT VIF, will be referred to as, for example, Tdc2-control. Balancer and marker chromosomes are not included in the abbreviated form for lines such as Pin/cyo; UAS-CD8.GFP (green fluorescent protein).

Table 2.1 Fly stocks used in this study.

Genetic background	Relevance	Experimental Use	Obtained from	ID
CS	wild-type	behavioural	Prof. Baines (University of Manchester)	
w^{1118}	wild-type	behavioural	Prof. Cobb (University of Manchester)	
w^-; Tdc2-GAL4	Targeted protein expression in octopaminergic/tyraminergic neurons	multiple	Bloomington Stock Center	9313
w^-; UAS-nsyb.eGFP	fluorescent synaptic terminal marker	imaging	Bloomington Stock Center	6921
w^-; UAS-syt.eGFP	fluorescent synaptic terminal marker		Bloomington Stock Center	6925
w^-; pin/cyo; UAS-CD8::eGFP	fluorescent cell membrane marker		Bloomington Stock Center	6925
w^-; UAS-TNT VIF (TeTx)	inactive tetanus toxin light chain isoform	behavioural and electro-physiological	Prof. Baines (University of Manchester)	
w^-; UAS-TNT G (TeTx*)	active tetanus toxin light chain isoform		Prof. Baines (University of Manchester)	
w^-; UAS-Kir2.1	inwardly rectifying voltage-gated potassium channel	behavioural	Prof. Baines (University of Manchester)	
w^-; UAS-TßH	tyramine β hydroxylase		Dr. Zhou Chuan (Peking University)	
w^-; +; UAS-NaChBac	bacterial voltage-gated sodium channel		Bloomington Stock Center	9469
$TßH^{nM18}$/FM6.GFP	tyramine β hydroxylase mutant	behavioural and electro-physiological	Dr. Elliott (Univeristy of York)	
w^-; $TßH^{rM6}$	revertant tyramine β hydroxylase mutant	behavioural	Dr. Zhou Chuan (Peking University)	
y^-w^-; tsh-GAL80 (y^+)/cyo	enhancer trap suppressing GAL4 activity	behavioural and imaging	Dr. Landgraf (Univeristy of Cambridge)	

2.3 BEHAVIOURAL ASSAYS

Third instar (L3) wall climbing larvae were picked from standard 2cm diameter food vials within a 5mm perimeter of the food surface, washed 3-4 times and allowed to crawl at room temperature (20-22°C) on an agarose gel (SeaKem LE Agarose, Lonza, USA) in 140mm Petri dishes (UKGE, UK) for approximately 2 minutes. The Petri dishes containing agarose were prepared by placing 1% of

agarose w/w in deionised water. The solution was heated in a microwave with frequent swirling to avoid lumps and was then left to set at room temperature. To ensure that possible variations in the quality of agarose gels did not compromise the results, experiments were conducted by testing the respective larvae at the same time on agarose gels prepared from one batch.

Low resolution locomotion assay: To record locomotion behaviour, larvae were placed on Petri dishes on a light panel (model LP812 Jessop, UK) to illuminate the visual field. The behaviour was recorded with a USB uEye camera, (model UI-2230SE-C-HQ,UDS, Germany), at a frame rate of 4 frames/sec. The locomotion was analysed using Image-Pro Plus (version 6.3, MediaCybernetics, USA) tracking software. The '*track objects*' option was used to track larval movement based on larval centroid body measurements, which are automatically generated by the software. The centroid body measurement is based on light intensity range on a scale from 0-255. The intensity range used by the software to identify objects of interest was adjusted manually for every larva to achieve optimal contrast as well as optimal outlining of the larval body. The raw centroid data was transferred and analysed using Microsoft Excel. Based on the centroid measurement data of locomotion, the following parameters were defined and measured: 1) overall speed as a measure of velocity over the entire time of the recording; 2) peak speed is defined as a measure of velocity during locomotion without obstruction or turning behaviour. The raw data from a period of peak speed crawling was used to calculate both stride length and stride duration. Stride duration was determined by dividing the time required to traverse the entire peak speed distance by the number of strides taken, which were determined visually. The stride length was determined by dividing the distance traversed during peak speed crawling by the number of strides taken. Thus, stride duration and stride length are defined as the time taken to complete a single complete peristaltic contraction cycle for forward locomotion and the distance traversed with each peristaltic cycle, respectively (Figure 2.1). The spatial calibration in the Petri dishes was determined by placing an object of known length, which was used to determine the proportion of pixels per cm. For the analysis of the locomotion parameters the time and scale were always adjusted to provide the output in cm, mm, min and sec.

Drug feeding experiments were conducted by adding 500µl of a 10% (wt/wt) dried yeast extract (Melford, UK) solution containing pharmacological agents at the referenced concentrations to vials containing 3rd instar larvae on standard food. Larvae were allowed to feed for 2 hours prior to recording and were recorded within 1 hour of doing so. Thus, the overall drug feeding time was 2-3hours. The compounds used in this study include DL- tyramine, mianserine,

yohimbine (Sigma-Aldrich, UK) and octopamine (Fluka, UK) at concentrations as stated.

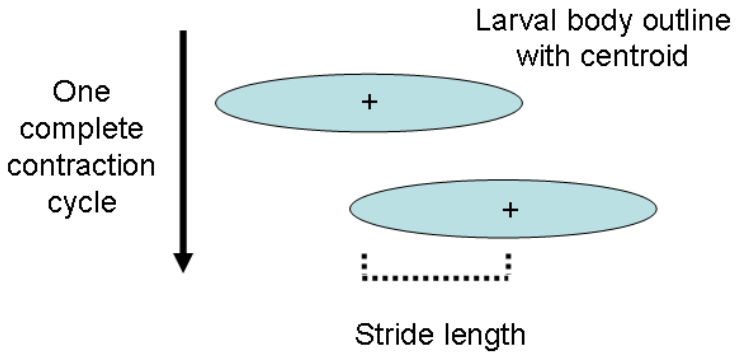

Figure 2.1 Schematic diagram of larval body centroid measurements used to determine the larval stride length.

High resolution locomotion assay: In order to analyse the duration of peristaltic contraction waves across all segments, as well as segmental contraction length and timing parameters in 3rd instar larvae, crawling behaviour was recorded by inverting 5cm diameter Petri dishes. The Petri dishes contained a fine and transparent film of agarose so that denticle bands could be visualised. The camera was mounted on a microscope (MZ6, Leica, Germany) and the behaviour was recorded at 20x magnification at a frame rate of or 20frames/sec using a USB uEye camera (model UI-2230SE-C-HQ,UDS, Germany). Stride length and stride duration were determined by measuring the distance traversed by a single forward peristaltic contraction wave from the posterior ends of denticle band D7. The duration of the forward peristaltic wave is defined as the time difference of the most posterior denticle band, D8, in abdominal segment A8 lifting off the surface until denticle band D2 in abdominal segment A2 lifting off the ground (Figure 3.6, Chapter 3). Abdominal segment A1 is frequently affected by head movements and leads to denticle band D1 often being lifted as a result of head swings and was therefore not used for this analysis.

This setup was used to determine the segmental length at relaxed and contracted states and the resultant segmental operational range (Figure 3.12, Chapter 3). These are defined as the distance of the posterior ends of the enclosing denticle bands when segments were fully relaxed and at peak contractions during forward peristaltic waves. The operational range is defined as the length difference between the relaxed and the most contracted state of a segment. The segmental contraction rate is defined as the time when the posterior enclosing denticle band lifts off the ground until the most contracted state of the segment is reached, whereas the segmental relaxation rate is defined as the time

difference from the most contracted state until both the posterior and anterior denticles rest on the surface again.

2.4 DISSECTIONS

First and second instar larval dissections were carried out in external saline on coverslips covered with cured Sylgard Elastomer (Dow Corning, USA). The composition of the external saline was as follows (in mM): NaCl (135), KCl (5), $MgCl_2$*6H2O (4), $CaCl_2$ (2), N-Tris[hydroxymethyl]methyl-2-amonoethanesulfonic acid (TES; 5), and sucrose (36), at pH 7.15 (Table 2.2). First instar larvae (L1, 1-4 hr after hatching) were glued down with Histoacryl glue (Braun, Germany) at the anterior and posterior ends. Subsequently, the larvae were opened dorsally using sharpened tungsten wires, the gut and trachea were removed, and the body walls were glued down on either side to expose the CNS.

Third larval instar dissections for evoked NMJ electrophysiology and intact animal NMJ recordings were carried out using trimmed stainless steel pins (Minutiens 0.1mm Insect Pins, Austerlitz) in standard HL3 solution for EJP recordings (Table 2.2) (Jan and Jan, 1976; Stewart et al., 1994) or modified HL3 solution for recordings in intact larvae during fictive locomotion (Table 2.2) (Barclay et al., 2002). The anterior and posterior ends of the larvae were pinned down first. The larvae were opened dorsally with fine scissors; the trachea and gut were removed. The body walls were gently stretched and pinned down peripherally to expose the CNS and enable NMJ recordings.

2.5 IMMUNOCYTOCHEMISTRY AND DYE LABELLING

For confocal microscopy imaging, larvae were dissected as outlined above in external saline, which was subsequently replaced by 4% paraformaldehyde in phosphate buffered saline (PBS, pH7.4) and left to incubate for 30-40min at room temperature (Table 2.2). Subsequently, the preparation was washed several times in PBS and left to incubate in PBS containing 0.3% TritonX-100 (PBT) for approximately 30min. In experiments using antibody staining, this was followed by a primary antibody incubation with a mouse-anti-Fas2 antibody (Developmental Studies Hybridoma Bank, USA) at a 1:10 dilution in PBT overnight at 4°C for preparations in 1^{st} instar larvae. After removal of the primary antibody, the preparation was washed several times and left to incubate in PBT for approximately 30min. Subsequently, the preparation was incubated with a fluorescent secondary goat-anti-mouse antibody (Alexa Fluor 633, Invitrogen, UK)

for 2hr at room temperature in the dark. This was followed by several washes and incubation steps with PBT for approximately 30min. The PBT was subsequently replaced by Mowiol 4-88 (Calbiochem, Merck, UK) containing 1,4-Diazabicyclooctane (DAPCO, Sigma-Aldrich, UK). Confocal microscopy imaging carried out in 2nd and 3rd instar larvae did not include antibody staining procedures.

Motor neuronal cell bodies and projections were visualised using the lipid soluble 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI) dye (Invitrogen, UK) at a 4mg/ml concentration dissolved in soya-bean oil (Sigma-Aldrich, UK). The dye was initially dissolved in ethanol for storage. Before use, soya-bean oil was added to the dye in ethanol. This was heated at 50-60°C for 5-10min, placed briefly in a vortex machine and spun down prior to placing it in an oven at 35-40°C for 20-30min to evaporate the ethanol. Sharpened glass electrodes were backfilled with the dye dissolved in oil overnight prior to experiments. To expose the NMJ, larvae were dissected as described above. The preparations were subsequently fixed for 20-30min in 4% paraformaldehyde in PBS (Table 2.2) solution, followed by several washes in PBS. Small droplets of dye were place on the NMJs in muscles M6 and M7 in dissected and pinned 2nd or 3rd instar larvae in a PBS solution using air filled 10ml single use syringes via (BD Plastipak) tubing under a microscope to visualise the NMJ (BX51WI, Olympus, UK). The preparations were incubated at 4°C overnight in order to allow the dye to diffuse. The dye droplets were removed prior to imaging and the preparations were washed several times in PBS.

2.6 CONFOCAL MICROSCOPY

Confocal images were obtained on a Leica TCS SP5 (Leica Microsystems) system at 512x512 or 1024x1024 pixel resolution in variable steps ranging from 0.1-1µm on the z-axis. Brightness, contrast and laser intensity were adjusted for each preparation. 3D volume-rendering image stacks were obtained using Leica image or McMasters Biophotonics Facility (MBF) Image J (McMaster University, Canada) software. The lateral view of 3D images was obtained by a 90° x-axis rotation with the Leica LAS AF Lite or MBF Image J software. The 1st instar confocal microscopy images were processed with Adobe Photoshop 9.0 (Adobe Systems), whereas data obtained from larvae in later developmental stages were processed by using MBF Image J. Red secondary antibody and DiI fluorescence were rendered magenta using Adobe Photoshop 9.0 or MBF Image J.

2.7 PCR

Isolating aCC neurons using a fluorescence activated cell sorter (FACS): Two hundred isolated CNSs were dissociated by incubating in 300µl of 1X PBS containing 1 mg/ml Collagenase/Dispase II (Roche, Germany) for 2 hours at room temperature. Following a wash in 500 µl of 1X PBS, cells were triturated in 200 µl of Schneider's *Drosophila* medium (Gibco, UK) and strained through a 35 µm nylon mesh filter (BD Biosciences, USA) to remove larger chunks of material. Green fluorescence positive cells were sorted and collected using a BD FACSAria^{TM} cell sorter (BD Biosciences).

Octβ2R RT-PCR: RNA was extracted from whole embryos, 3rd instar larvae CNSs or FACS-sorted aCC cells using the RNeasy Micro kit (Qiagen). cDNA synthesis was carried out in a total volume of 20µl. The primers, oligo(dT) (0.5µg) and random hexamer (0.2µg), were mixed with RNA and made up to 12µl with RNase-free water. The primer sequences were based on a previous study (Koon et al., 2011). Forward primer sequence: CATGCTGATGCACCGACCATC. Reverse primer sequence: CACTCCTCGCAGGTCATGGAG. The mix was incubated at 65°C for 5 min to denature RNA followed by incubation on ice for 2 min. 4µl of reaction buffer (in mM: 250 Tris-HCl, 250 KCl, 20 $MgCl_2$, 50 DTT), 2 µl of 10 mM dNTPs, 1µl of RNase inhibitor and 1 µl of RevertAid M-MuLV (monkey murine leukemia virus) reverse transcriptase (RevertAid First Strand cDNA Synthesis kit Fermentas, UK). The reaction was incubated at 25°C for 10 min, 42°C for 60 min and followed by 70°C for 10 min. One µl of cDNA was amplified in a 20µl reaction mixture containing 20 pmol each of gene-specific primer pairs, 200 µM each of the four dNTPs, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$ and 2.5 U of BIOTAQ^{TM} DNA polymerase (Bioline, UK). Reactions were run for 35 cycles of amplification (10 sec at 94^{o}C, 20 sec at 58^{o}C and 30 sec at 72^{o}C) and followed by a 10-min extension at 72^{o}C. The PCR products were resolved by 2 % TAE-agarose gel electrophoresis. Primer sequences were: forward primer, 5'-catgctgatgcaccgaccatc -3' and reverse primer, 5'- cactcctcgcaggtcatggag -3'. The PCR experiments were conducted by Dr. Wei-Hsiang Lin.

2.8 ELECTROPHYSIOLOGY

Whole-cell patch clamp recordings were performed at room temperature (20-22°C) in the wall-climbing 3rd instar larval CNS once removed from the larval body after dissection (Baines and Bate, 1998). The CNS was placed and glued (Histoacryl) onto coverslips covered with cured Sylgard Elastomer (Dow Corning, USA). The glial sheath was disrupted in focal patches on the ventral nerve cord

(VNC) by applying pressure through 10-20µm diameter borosilicate glass pipettes (GC100TF-10, Harvard Apparatus, USA) filled with 10µg/ml bacterial protease in external saline (Type XIV Streptomyces griseus, Sigma-Aldrich, UK). For patch clamp recordings, borosilicate glass electrodes (GC100F-10; Harvard Apparatus, USA) were used within a resistance range of 15-20 MΩ after heat polishing. Recordings were performed using an Axopatch-1D amplifier (Axon Instruments, USA) controlled by pClamp 8.2 and a Digidata 1322A (Axon Instruments,USA). Neurons were identified based on location in the ventral nerve cord, size and neurite projections determined by labelling neurons (0.22 mM fluorophore filled electrodes, Alexa Fluor 488, Invitrogen,UK). aCC and RP2 motor neurons were reliably labelled and ventral muscles innervating RP1, RP3, RP4 and RP5 were identified based on their localisation along the midline between aCC and RP2 motor neurons. After training, patch clamp recordings were obtained without dye labelling. Only cells (L3) with an input resistance >250MΩ were used for analysis. The cell capacitance was calculated in Clampfit 8.2 or 10.2 by integration of the area of transient currents averaged from 50 voltage steps from -60mV to -90mV. Endogenously occurring synaptic currents and mEPSCs were recorded in voltage clamp mode at 100kHz sampling frequency and a holding potential of -60mV. Recordings were filtered at 0.2kHz lowpass. Current clamp recordings were conducted at 5kHz sampling frequency. Amplitude and frequency of miniature excitatory postsynaptic currents (mEPSC) was determined using the Minianalysis software (Synaptosoft,UK). The amplitude, frequency and kinetic parameters of excitatory postsynaptic currents (EPSC) were determined using Clampfit 10.2 after adjustment of the baseline. The rise time is defined as time difference between the 10% and 90% of the synaptic currents amplitude, whereas the decay time is defined as the time difference from 90% to 30% of the synaptic current amplitude. The synaptic current amplitude was corrected for the capacitance of the recorded cells. The intrinsic membrane excitability was recorded in current clamp mode for neurons with a resting membrane potential of <-55mV at 5kHz sampling frequency. Currents were injected for 1sec in 4pA steps. The recordings were carried out using solutions at the following compositions and concentrations. External saline (in mM): NaCl (135), KCl (5), $MgCl_2$*6H2O (4), $CaCl_2$ (2), N-Tris[hydroxymethyl]methyl-2-amonoethanesulfonic acid (TES; 5), and sucrose (36), at pH 7.15. Internal patch solution consisted of (in mM): K^+ methylsulfonate (KCH_3SO_3, 140), $MgCl_2$.6H2O (2), Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, 2), KCl (5), and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES, 20), at pH 7.4. Chemicals were obtained from the following manufacturers: NaCl, KCl, $NaHCO_3$, and Sucrose (Fisher Scientific, UK), $CaCl_2$, HEPES (VWR International, UK), $MgCl_2$ *6H2O (Merck, Germany), KCH_3SO_3,

EGTA , TES (Sigma-Aldrich, UK). Saline compositions are also summarised in Table 2.2.

Evoked NMJ electrophysiology in muscles 6 and 12 in abdominal segment A3-A5 were made with intracellular borosilicate glass electrodes (CG100F-10, Harvard Apparatus, USA) at a resistance of 15-25 MΩ, filled with 3M KCl using an Axoclamp-2B amplifier (Axon Instruments, USA) controlled by pClamp 8.2 via a Digidata 1322A (Axon Instruments, USA). Recordings were obtained in a standard HL3 solution at a 1.5mM $CaCl_2$ concentration at pH 7.2. Only muscles with a resting membrane potential of -55mV or lower were used for analysis. Severed segmental nerves were inserted into a polished stimulating electrode filled with HL3 solution by suction and evoked excitatory junction potentials (EJPs) were stimulated by applying pulses of 600-800μs and 20-40V using a Constant Voltage Isolated Stimulator (model DS2A-MkII, Digitimer) attached to the Axoclamp-2B amplifier to sufficiently stimulate both Ib and Is excitatory junction potentials in muscle M6 for recordings. Recordings were filtered at 10KHz and sampled at 5kHz . Miniature excitatory junction potentials (mEJPs) were measured in current clamp mode for approximately 2min to determine the frequency and amplitude. Excitatory junction potential (EJP) amplitude and kinetic parameters were measured using Clampfit 10.2. The rise time and decay time are defined as 10-90% and 90-30% of the EJP voltage amplitude, respectively. The input resistance of muscles was determined by applying 5 current steps of 500ms and 1mV onto the muscle to measure input resistance, which were measured in Clampfit 10.2.

NMJ electrophysiology in intact third instar larvae were carried out as previously reported (Cattaert and Birman, 2001; Barclay et al., 2002). Third instars larvae were dissected as described above but with the exception that the CNS and segmental nerves were left intact. Thus these larvae display fictive forward locomotion peristaltic contraction waves and responded to tactile stimuli. Intracellular recordings in longitudinal body wall muscles M6, in two adjacent segments between abdominal segments A2 and A4, were made with borosilicate glass electrodes (CG100F-10, Harvard Apparatus, USA) at a resistance of 15-25MΩ, filled with 3M KCl using an Axoclamp-2B amplifier (Axon Instruments, USA) controlled by pClamp 8.2 via a Digidata 1322A (Axon Instruments,USA) in a current clamp setup at a 5kHz sampling rate filtered at 10kHz. The recordings were analysed in Clampfit8.2 and 10.2. In each preparation, one recording in the anterior segmental muscle was used to manually analyse spiking frequency of excitatory postsynaptic potentials (EPSPs) and the duration of bursts from the first to the last spike. A burst is defined as an identifiable block of high frequency spiking activity correlated to posterior to anterior contraction waves and does not include sporadically occurring spikes that did not correlate to coordinated muscle movements. The time delay between bursts of activity, in adjacent segments, is

defined as the time difference between the last spikes in two adjacent segments, respectively. External saline is based on a previously used modified HL3 solution for two simultaneous NMJ recordings in intact third instar larvae (in mM) (Barclay et al., 2002) : NaCl (70), KCl (5), $MgCl_2$*6H2O (20), $NaHCO_3$ (10) , Sucrose (120), HEPES (5), $CaCl_2$ (0.8) at pH 7.2. Composition is summarised in Table 2.2. Chemicals were obtained from the following suppliers: NaCl, KCl, $NaHCO_3$, and Sucrose (Fisher Scientific, UK), $CaCl_2$, (VWR International, UK), $MgCl_2$*6H2O (Merck, Germany), HEPES (Sigma-Aldrich, UK).

Table 2.2 Summary of the compositions of solutions used in this study (in mM). The external solution was used for dissections and for patch-clamp electrophysiological experiments. The internal patch clamp solution was used as the solution in patch clamp electrodes. The standard and modified HL3 solutions were used for NMJ electrophysiological experiments and the phosphate buffered saline (PBS) was used in imaging experiments.

Compound	External solution	Internal patch clamp solution	Standard and Modified HL3	PBS
NaCl	135		70	137
KCl	5	5	5	2.7
$MgCl_2$*6H2O	4	2	20	
$CaCl_2$	2		1.5 or 0.8	
TES	5			
Sucrose	36		120	
KCH_3SO_3		140		
EGTA		2		
HEPES		20	5	
$NaHCO_3$			10	
Na_2HPO_4				10
KH_2PO_4				2

2.9 STATISTICAL ANALYSIS

Data was statistically analysed using ANOVA in the *Origin 8* software package (Originlab, USA) to determine the statistical significance, which is defined as the following p values: 0.05 (*), 0.01 (**), and 0.001 (***) in this study. As the sample sizes were not always equal, the Schefffe's post hoc test was applied for all ANOVA tests. Unless otherwise stated, statistical significance using ANOVA has been confirmed by the Scheffe's test.

Chapter 3

The Role of Octopamine and Tyramine in Modulating Larval Crawling Behaviour

3.1 INTRODUCTION

The analysis of intact animal behaviour is increasingly regarded as a complementary method to the reductionist approach of *in vitro* as well as *in vivo* experimentation under non-physiological or semi-physiological conditions. Behavioural analyses have increasingly been applied to study neuroscience-related question in *Drosophila* larvae (Suster and Bate, 2002; Suster et al., 2003; Saraswati et al., 2004). Animal behaviour can potentially provide a systemic view and understanding of an entire organism and the interactions of the respective components within it at the molecular, cellular and/or tissue level within a whole organism, particularly when combined with other methods. This behavioural approach has been developed and used increasingly over the last few years. The development has been facilitated by technological advances that allow for fast and high-throughput computation of large amounts of data and by the development of software that enables automated analysis of behavioural parameters.

Drosophila larval behavioural has been systematically studied and described since the 1980's (Green et al., 1983). Since then, arguably, one of the most powerful demonstrations of this approach was the elucidation of the effect of a single gene polymorphism, a cyclic guanosine monophosphate (cGMP)-dependent protein kinase, on the foraging behaviour of *Drosophila* larvae (Osborne et al., 1997; Engel et al., 2000). Even earlier studies reported anomalous locomotion behaviour in Na^+ and K^+ channel mutants in *Drosophila* (Wang et al., 1997). Both studies exemplified how modifications of single genes can impact on the behaviour of an organism and provided insights on the molecular, cellular and system level effects of small genetic changes. Behavioural assays in *Drosophila* have been used in very diverse fields studying the mechanisms of courtship (Certel et al., 2007; Liu et al., 2008), decision-making (Certel et al., 2010), aggression (Hoyer et al., 2008; Zhou et al., 2008), stress responsiveness (Koon et al., 2011), olfaction (Kutsukake et al., 2000; Schwaerzel et al., 2003; Selcho et al., 2009), sleep (Crocker and Sehgal, 2008), learning (Unoki et al., 2005; Honjo and

Furukubo-Tokunaga, 2009), sensory neurophysiology (Hughes and Thomas, 2007; Song et al., 2007) and locomotion (Caldwell et al., 2003; Hardie et al., 2007).

In this study, a behavioural approach has been used to investigate the regulation of crawling behaviour by neuromodulators. As described in the Introduction, it is known that across the animal kingdom central pattern generator (CPG) networks controlling segmental muscle groups are themselves segmentally organised, often referred to as unit CPGs, which can reproduce their physiological output when stimulated in isolation (Cattaert and Birman, 2001; Grillner, 2006; Puhl and Mesce, 2008; Grillner and Jessell, 2009). Animals that require a multi-segmental organisation to enable movement, such as *Drosophila* larvae (Figure 3.1), therefore offer a potent model system to study the effects of neuromodulators on locomotor CPG networks (Cattaert and Birman, 2001). When combined with the powerful genetic tools available in *Drosophila*, this model system has the potential to provide great insights into the mechanisms of acute functional modulation and homeostasis of multiple CPG networks. Although neither the CPG network architecture nor the functional output of locomotor CPGs are resolved in *Drosophila* larvae, a detailed description of locomotor behaviour on a segmental level could potentially become a starting point for a top-down approach to resolve both the network architecture and function. This chapter describes studies aimed to address the questions of chronically altered neuromodulatory signalling on putatively multiple coordinated CPGs networks in *Drosophila*.

Fig. 3.1

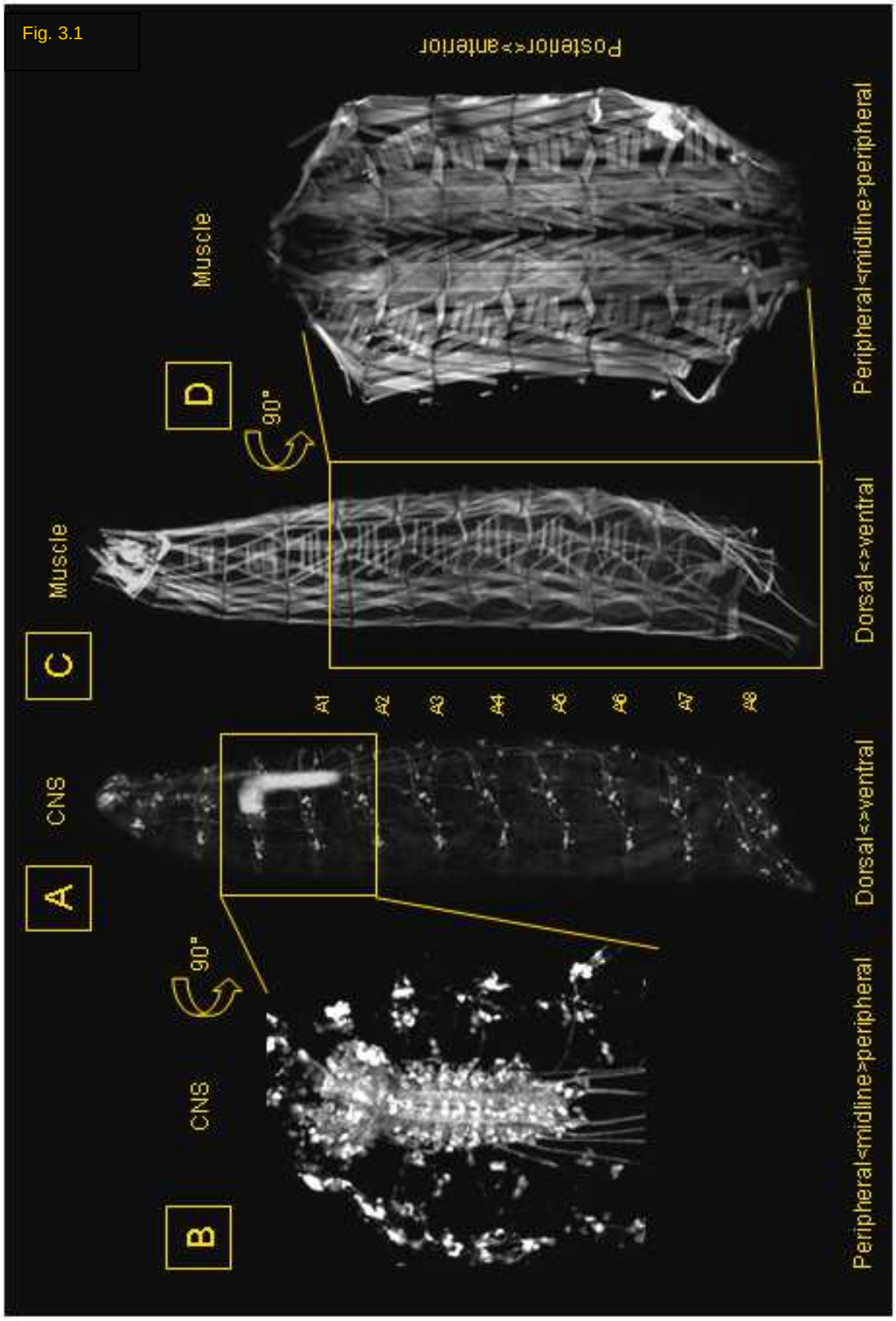
A
CNS
B
CNS
90°
C
Muscle
D
Muscle
90°
A1
A2
A3
A4
A5
A6
A7
A8
Peripheral<midline>peripheral
Dorsal<>ventral
Dorsal<>ventral
Peripheral<midline>peripheral
Posterior<>anterior

Figure 3.1 Organisation of the body wall musculature as well as the CNS and sensory neurons in *Drosophila* larvae. **A**. Lateral view of pan-neuronal cell labelling elucidating the CNS and peripheral clusters of sensory neurons in *Drosophila* larvae. Reproduced from (Hughes and Thomas, 2007). **B**. Dorsal view of CNS and labelled sensory neurons in dissected larvae. **C**. Lateral view of segmental organisation of the larval body wall musculature. Reproduced from (Hughes and Thomas, 2007). **D**. Dorsal view of musculature in dissected larvae. Reproduced from (hoylab.cornell.edu).

Octopamine and tyramine are well known neuromodulators in *Drosophila* (Roeder, 1999, 2005) as well as other insects and have been shown to be involved in the modulation of highly diverse behaviours including locomotion, olfaction, decision making, flight initiation, and aggression (Monastirioti et al., 1996; Nagaya et al., 2002; Saraswati et al., 2004; Brembs et al., 2007; Certel et al., 2007; Zhou et al., 2008). These neuromodulators are widely expressed in the CNS and to a large extent in the periphery in both larval (Monastirioti et al., 1995; Nagaya et al., 2002; Vömel and Wegener, 2008) and adult stages (Cole et al., 2005; Busch et al., 2009; Busch and Tanimoto, 2010). Octopamine and tyramine exert their effects via G-protein coupled receptors (GPCRs). In *Drosophila*, four octopamine receptor subtypes, classified into three β-adrenergic-like receptors and one α-adrenergic-like receptor (Balfanz et al., 2005; Maqueira et al., 2005), one tyramine/octopamine receptor, and one tyramine receptor have been identified based on sequence homology and pharmacological profiles (Evans and Robb, 1993; Cazzamali et al., 2005; Evans and Maqueira, 2005).

Considering the highly diverse effects on behaviour, the wide expression patterns, the diverse receptor subtype and signalling physiology, octopamine and tyramine are thus likely to be among the primary molecular, cellular and/or tissue level modulators of *Drosophila* behaviour and may provide great opportunities to study multi-unit CPG modulation.

2.1.1 Hypotheses

- Octopamine and tyramine antagonistically regulate crawling speed by modulating the stride duration without affecting stride length. Octopamine increases speed by reducing the stride duration and Tyramine reduces speed by increasing the stride duration.
- In order to modulate the stride duration, octopamine and/or tyramine modulate both the duration of the anterior to posterior segmental peristaltic contraction wave as well as the time delay between contraction waves during forward locomotion.
- The stride length remains largely constant as each of the contractile segments used in forward locomotion maintains a constant segmental operational range between a fully relaxed and fully contracted segment.

- Octopamine and tyramine affect the rate of contraction as well as the rate of relaxation of abdominal segments during forward locomotion, which is indicative of a modulatory role in muscle contractility.

3.2 RESULTS

The effects of neuromodulators on larval locomotion were initially measured in a low resolution locomotion assay as described in Chapter 2. Crawling behaviour in 3rd instar *Drosophila* larvae is characterised by bouts of linear forward peristaltic waves, head swings, direction changing and turning behaviour as well as infrequent and short periods of reverse peristaltic waves. Several kinematic parameters such as speed, stride length and stride duration were measured (Berrigan and Pepin, 1995; Wang et al., 1997; Belanger and Trimmer, 2000). These parameters have been reported to some extent in previous studies, but the understanding of the effects of neuromodulators on these parameters of crawling behaviour is limited. The effects of octopamine and tyramine on these behavioural parameters were studied using mutant and transgenic *Drosophila* larvae in order to assess their effect on the larval locomotor CPGs.

3.2.1 Reliability of the Locomotion Behaviour Assay

Third instar wall climbing *Drosophila* larvae represent a developmental stage with a considerable degree of variability. When flies are allowed to lay eggs for 30min, resultant larvae enter their pupal stage on a timescale of approximately ±6 hours, which makes the absolute age of larvae a poor indicator of the developmental stage, particularly as some genotypes have a prolonged development (Ashburner et al., 2005). In order to reduce the variability only larvae crawling within a narrow perimeter of approximately 5mm from the food surface were picked for crawling experiments. Larvae in the mid-late 3rd instar developmental stages often migrate in and out of the food. Later developmental stages of wall climbing larvae crawl further away from the food surface in order to find a suitable and dry location for pupation. Picking larvae close to the food surface therefore limits the variability in the larval developmental stages. Pilot experiments testing stride duration, stride length and speed show that results are reproducible when different batches of larvae are recorded on 3 consecutive days (Figure 3.2). There are no statistically significant changes within the same genotypes recorded from different batches on three consecutive days for either the stride length, stride duration or speed. However, there are marked and statistically significant changes in stride duration and crawling speed between the two tested genotypes. Moreover, the changes are reproducible and comparable in the degree of change on each of the consecutive days. These data confirm a remarkable degree of reliability of the locomotion assays used in this study to measure the

stride length, stride duration and crawling speed of larvae and thereby validate the application of this behavioural assay.

Fig. 3.2

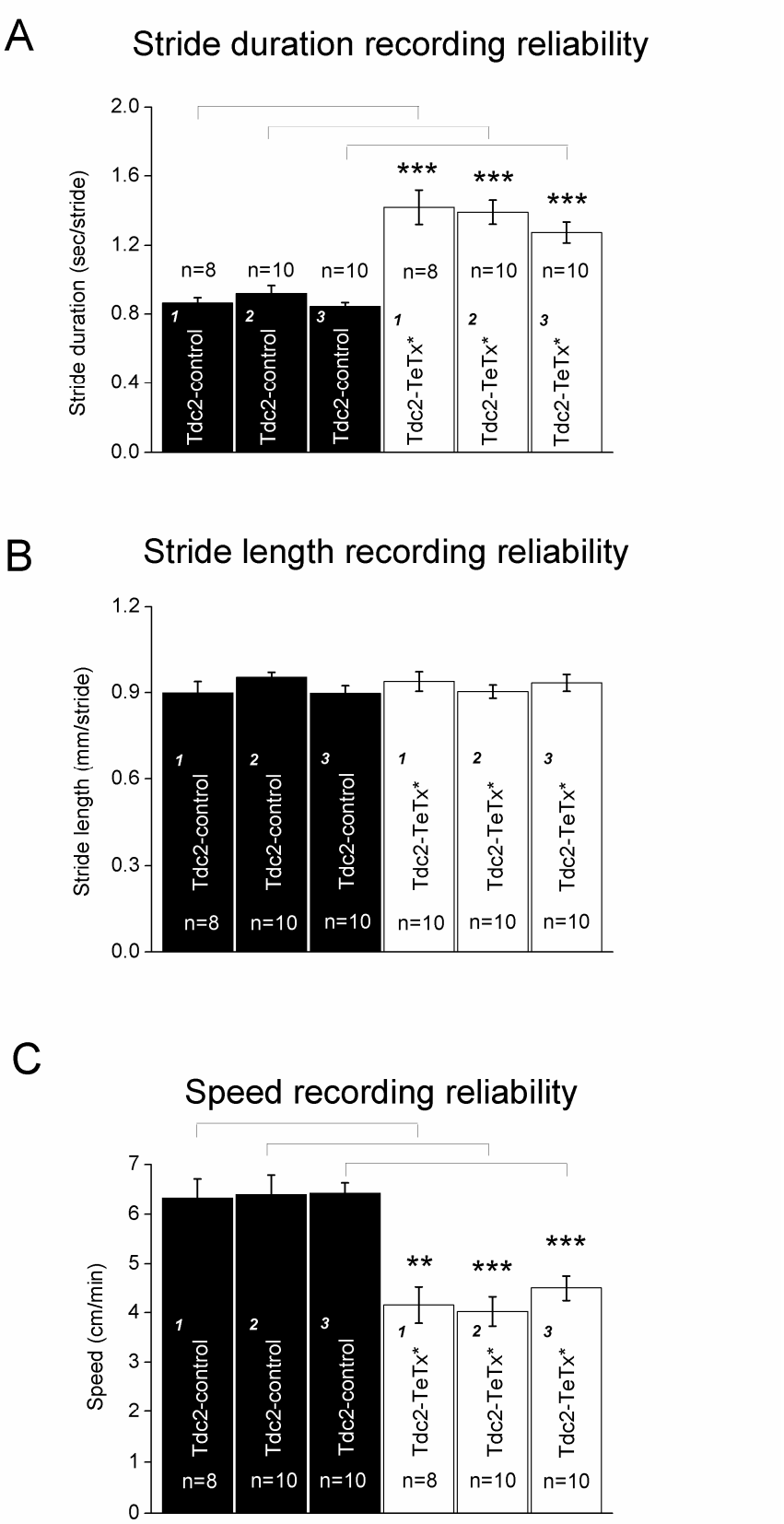

Figure 3.2 Analysis of the reliability and validity of the behavioural assay using Tdc2-control and Tdc2-TeTx* crawling data from 3 consecutive days. **A.** The stride duration is unchanged on consecutive days within the same genotypes, but is significantly increased in Tdc2-TeTx* larvae compared to Tdc2-control larvae on all of the consecutive days. **B.** The stride length remains constant on consecutive days both within the same genotypes and between different genotypes. **C.** The crawling speed is also unchanged on consecutive days within the same genotype, but is significantly decreased on consecutive days between the genotypes. Statistical data within the same genotype is presented in the following order (day): 1-2, 1-3, 2-3. Genotypes were compared for data gathered on the same day.

3.2.2 The Impact of the *white* Gene on Larval Locomotion

Drosophila has traditionally been used as a genetic model system. Numerous mutant and transgenic lines have been generated from different original strains, which are now being used as genetic tools. However, this raises questions about the use of different genetic strains and their comparability as the transgenic insertions are superimposed on pre-existing differences. In this study, octopaminergic and tyraminergic signalling was manipulated by using various transgenic constructs to specifically manipulate neurons that express these neuromodulators. The GAL4-UAS expression system allows spatially controlled expression of proteins driven by the yeast transcription regulating protein GAL4, which initiates transcription from Upstream Activating Sequences (UAS) (Brand and Perrimon, 1993; Duffy, 2002). In this study, the neurotoxin tetanus toxin light chain (TeTxLC) and other proteins such as the bacterial voltage-gated sodium channel (NaChBac), and the mammalian inwardly rectifying potassium channel (Kir2.1), have been expressed in octopaminergic/tyraminergic neurons to determine their effects on locomotion. In order to determine, if the original genetic background and the transgenic constructs, *per se,* affect locomotion, homozygous transgenic larvae as well as F1 generation crosses were tested.

The w^{1118} mutant has traditionally been used to generate transgenic flies. The white eye phenotype is due to a mutation in the *white* gene, which transforms the red eye phenotype of wild-type flies to white. This phenotype has been useful as a powerful marker as the insertion of transgenes can be verified by fusing the mini*white* gene with a gene of interest for insertion in a plasmid. Insertion of the transgenes into the genome of *Drosophila* transforms white eyes to red and thereby indicates a successful insertion of the construct into the genome. In order to determine, if the w^{1118} mutation has an effect on larval behaviour, these larvae were compared to CS wild-type larvae as shown in (Table 3.1). The data indicate that strains, which are widely regarded as wild-type strains such as Canton S and w^{1118}, differ substantially in basic parameters of behaviour such as crawling speed, stride length and stride duration ($p<0.001$ in all cases). The peak and overall

crawling speed is substantially reduced when comparing w^{1118} to Canton S wild-type flies ($p<0.001$ in both cases). Furthermore, the stride length is reduced ($p<0.01$), while stride duration is substantially increased ($p<0.001$).

The UAS transgenic constructs were statistically tested to determine underlying differences. None of the behavioural parameters are changed when comparing the homozygous inactive isoform of TeTx with the active isoform, UAS-control and UAS-TeTx* respectively, or the voltage-gated sodium channel construct UAS-NaChBac ($p>0.05$ in all cases). However, when comparing the inactive isoform of TeTxLC with the mammalian inwardly rectifying potassium channel UAS-Kir2.1, there is no difference in the peak and overall speed or the stride duration ($p>0.05$), but the stride length is significantly reduced in homozygous UAS-Kir2.1 larvae ($p<0.05$).

Table 3.1 Crawling parameter data of wild-type as well as homozygous GAL4 and UAS transgenic 3rd instar larvae in this study. Stride length and stride durations were calculated as averages from a stretch of crawling without stopping and turning behaviour used to determine the peak speed. Stride length=distance traversed/number of strides taken. Stride duration=duration of crawling/number of strides taken.

Genotype	***n***	***Peak speed (cm/min)***	***Overall speed (cm/min)***	***Stride length (mm/stride)***	***Stride duration (sec/stride)***
+;CS/CS	27	5.927 ± 0.17	5.034 ± 0.17	1.057 ± 0.02	1.094 ± 0 .03
+/w$^-$; CS/UAS-control (UAS-TeTxLC, inactive)	10	5.98 ± 0.28	5.31 ± 0.3	0.92 ± 0.04	0.93 ± 0.04
w$^-$; w^{1118}/UAS-TeTx* (TeTxLC, active)	14	3.6 ± 0.17	3.12 ± 0.22	0.95 ± 0.02	1.63 ± 0.09
w^{1118}	23	3.3 ± 0.11	3.02 ± 0.14	0.98 ± 0.02	1.82 ± 0.07
w$^-$; Tdc2-GAL4	9	4.11 ± 0.29	3.38 ± 0.29	0.9 ± 0.03	1.37 ± 0.09
w$^-$; UAS-control (TeTxLC, inactive)	8	2.82 ± 0.24	2.64 ± 0.24	0.87 ± 0.03	1.98 ± 0.23
w$^-$; UAS-TeTx*	11	2.96 ± 0.15	2.47 ± 0.34	0.88 ± 0.02	1.82 ± 0.08
w$^-$; UAS-Kir2.1	14	3.03 ± 0.18	2.7 ± 0.15	0.78 ± 0.03	1.62 ± 0.12
w$^-$; +;UAS-NaChBac	7	2.7 ± 0.21	2.66 ± 0.14	0.96 ± 0.03	2.45 ± 0.34

In summary, as the presence of the *white* gene as well as underlying differences in transgenic lines affect the measured behavioural parameters, further experimental design ensured that, as far as possible, an appropriate genetic control background for experimental genotypes was chosen. The appropriate w^{1118} genetic backgrounds as well as identical numbers of mini*white* insertions were compared with one another, respectively.

3.2.3 The Role of Octopamine and Tyramine in Larval Locomotion

Neuromodulators are potent modulators of metabolic, muscular and neuronal function and ultimately behaviour. In order to start addressing how octopamine and tyramine affect behaviour, their role in larval locomotion was tested by using mutants with affected octopamine and tyramine levels as well as various transgenic construct to specifically manipulate octopaminergic/tyraminergic signalling.

The role of octopamine and tyramine in larval locomotion was first tested by using a tyramine-ß-hydroxylase, TßH^{nM18}, null mutant line. This enzyme converts tyramine into octopamine (Figure 3.3). The TßH^{nM18} mutant has been shown to have no octopamine and a 7-8-fold increase in tyramine levels in the adult *Drosophila* brain (Monastirioti et al., 1996; McClung and Hirsh, 1999). The control genetic background is TßH^{rM6}, a revertant excision of a hypomorphic mutation, which restores wild-type genetic function and phenotype (Monastirioti et al., 1996). TßH^{rM6} does not show any significant changes in the levels of either tyramine, octopamine or its metabolites when compared to wild-type flies (Monastirioti et al., 1996).

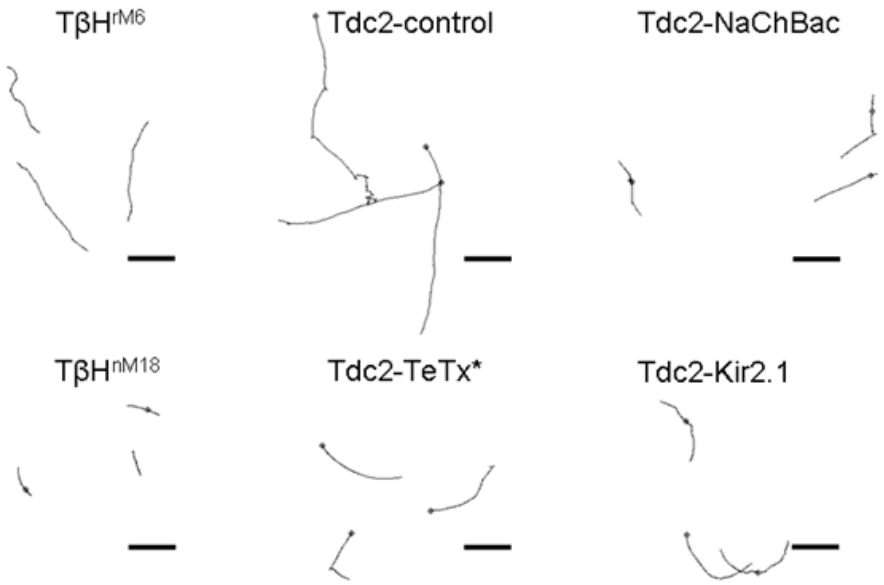

Figure 3.3 Sample locomotion traces represent distance traversed by three larvae in respective genotypes each within 1min. Scale bars: 1cm.

Both the peak as well as the overall speed is drastically reduced in TßH^{nM18} larvae compared TßH^{rM6}, which has been reported in a previous study and

validates the reliability of this assay (Figure 3.4) (Saraswati et al., 2004). Stride duration is also substantially increased with a concomitant increase in stride length. A lack of octopamine and/or an increase in the tyramine levels therefore substantially slows down larval crawling speed by greatly increasing the stride duration and by also increasing stride length.

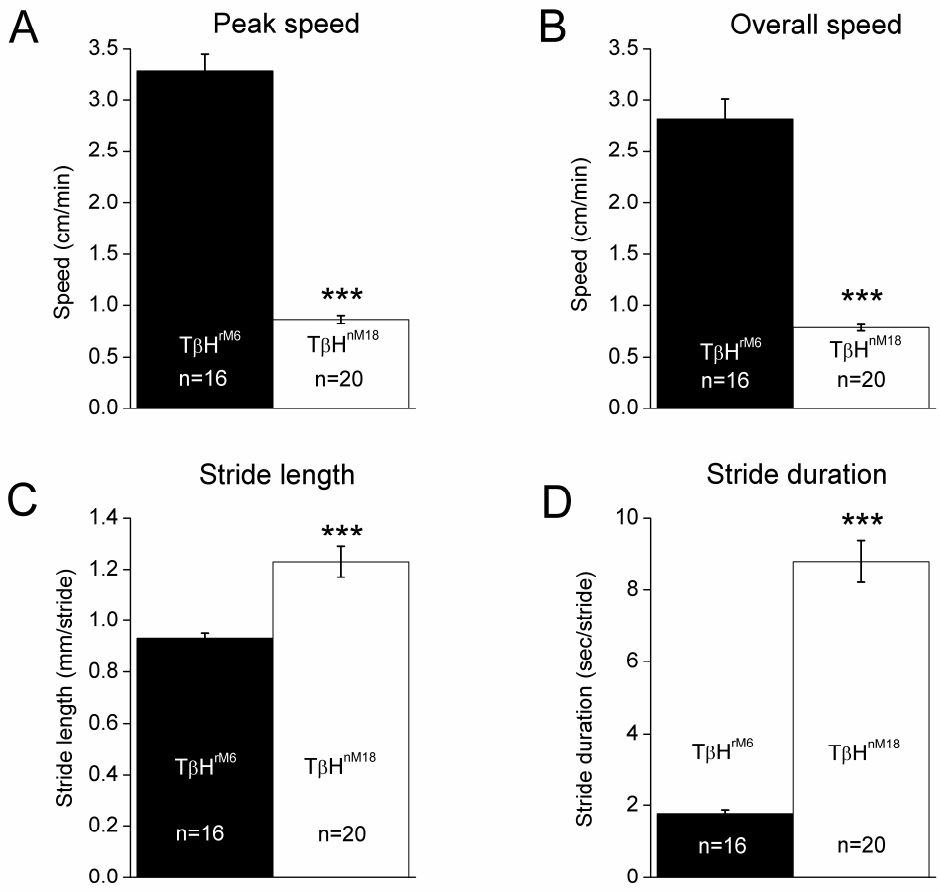

Figure 3.4 Crawling analysis of TßH^{nM18} larvae lacking octopamine and elevated tyramine levels. **A** and **B.** The peak speed as well as the overall is significantly reduced when no octopamine is present and tyramine levels are increased. **C** and **D.** The stride length and stride duration are significantly increased in TßH^{nM18} larvae.

Octopaminergic/tyraminergic neurons were also manipulated by using the CNS-specific tyrosine decarboxylase construct, Tdc2-GAL4, which is an enzyme upstream of both tyramine and octopamine in the synthetic pathway (Figure. 3.3). This transgenic construct allows a spatially controlled expression of proteins in putatively octopaminergic/tyraminergic neurons alone. This construct was crossed

to transgenic lines expressing tetanus toxin light chain (TeTxLC), a bacterial voltage-gated sodium channel or an inwardly rectifying potassium channel to determine their effects on locomotion.

TeTxLC cleaves the Soluble N-ethylmaleimide-sensitive fusion protein (NSF) Attachment Protein receptor (SNARE) complex protein, synaptobrevin, thereby blocking evoked, but not spontaneous, synaptic vesicle release (Sweeney et al., 1995). When the active isoform of the TeTx construct is expressed in octopaminergic and/or tyraminergic neurons, it results in both a reduced peak speed and overall speed (Figure. 3.5). The stride duration is increased proportionately to the decrease in speed, whilst the stride length remains constant. These results suggest that putatively blocking evoked neurotransmitter release substantially reduces crawling speed, which is achieved by inversely increasing the stride duration.

In order to test, if other genetically induced perturbations in the function of octopaminergic/tyraminergic neurons also exert an effect on crawling behaviour, I expressed specific voltage-gated ion channels in Tdc2-GAL4 driven neurons. Expression of the bacterial voltage-gated sodium channel, NaChBac, putatively alters the intrinsic excitability and the action potential firing patterns in octopaminergic/tyraminergic neurons (Joiner et al., 2006). The resulting behavioural effects were almost identical when compared to TeTx expression. The peak and the overall speed are substantially reduced with a concomitant increase in stride duration. However, the stride length again remained constant.

In order to silence octopaminergic and/or tyraminergic neurons, the UAS-Kir2.1 construct was used. Kir2.1 is a mammalian inwardly rectifying channels potassium channel, which putatively silences neurons by strong outward hyperpolarising K^+ currents (Baines et al., 2001; Burrone et al., 2002). When this construct is expressed in putatively octopaminergic/tyraminergic neurons, it largely reproduces the behavioural effects of the other genetic manipulations described above. The peak and overall speed reductions are comparable to other manipulations. The stride duration is also increased. However, unlike in Tdc2-TeTx* and Tdc2-NaChBac larvae, the stride length is increased and does not remain constant. The reason for the discrepancy in stride length compared to other genotypes is unclear, however, considering that the homozygous UAS-Kir2.1 genotypes displayed a statistically different stride length, the underlying genetic difference may account for this result.

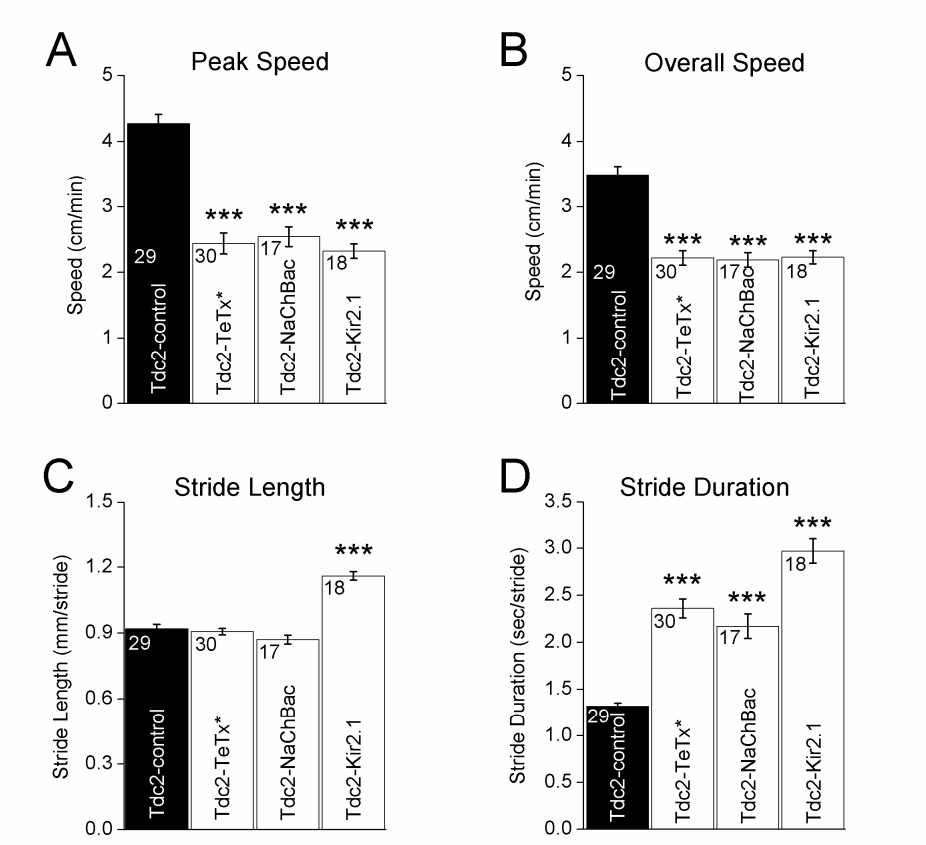

Figure 3.5 Crawling analysis in larvae with modulated tyraminergic and octopaminergic signalling. **A** and **B.** The peak speed as well as the overall speed are significantly reduced when octopaminergic/tyraminergic neuronal function is modulated in Tdc2-TeTx*, Tdc2-NaChBac and Tdc2-Kir2.1 larvae. **C.** The stride length is unchanged in unchanged in Tdc2-control, Tdc2-TeTx* and Tdc2-NaChBac larvae, but is increased in Tdc2-Kir2.1 larvae. **D.** The stride duration is significantly increased in Tdc2-TeTx*, Tdc2-NaChBac and Tdc2-Kir2.1 larvae.

In summary, a reduction in octopaminergic and/or tyraminergic signalling has a profound impact on crawling behaviour in 3rd instar larvae. In the TßH^{nM18} mutant, crawling speed is profoundly reduced by a lack of octopamine and/or increased tyramine levels. Furthermore, manipulating the function of octopaminergic/tyraminergic neurons by either blocking neurotransmitter release, silencing or modulating activity patterns induces a very similar locomotion deficit. As a result, the peak and overall crawling speed are reduced by the same degree in all instances. Strikingly, when either neurotransmitter release is blocked by TeTx* or the neuronal activity is modulated by NaChBac, stride length is maintained constant. In contrast, the constant stride length was not maintained when octopaminergic and tyraminergic neurons were silenced by Kir2.1 or in the TßH^{nM18} mutant. These experiments may therefore suggest that chronic manipulations of octopaminergic/tyraminergic signalling primarily reduce crawling speed by increasing the stride duration, but by and large maintain a constant stride length.

3.2.4 Effects of Octopamine and Tyramine on Crawling Kinematics in Larvae

Forward locomotion in *Drosophila* larvae is characterised by a cycle of peristaltic contraction waves from the posterior to anterior end until the next contraction is initiated. A change in crawling speed as a result of altered octopaminergic/tyraminergic signalling could potentially affect different phases of the locomotion cycle. In order to analyse this in more detail, crawling behaviour was recorded on Petri dishes with a very thin and transparent film of agar. Larvae were placed on the agar and the dish was then inverted to visualise the crawling behaviour at a higher resolution, using the denticle bands as markers.

Drosophila larvae move forward starting with a contraction of the most posterior segment initiating a peristaltic contraction wave moving towards the anterior segments (Figure 3.6). As each segment contracts, the denticle bands are lifted off the surface and are thrust forward by a telescopic-like projection to a more anterior position and are lowered until they touch the surface again. *Drosophila* larvae primarily use their eight abdominal segments, which are contracted in a progressive peristaltic wave to move forward in single strides until the next peristaltic wave is initiated. A prolongation of the stride duration in larvae with altered octopaminergic/tyraminergic signalling could therefore be due to either a prolonged forward peristaltic wave from the posterior to anterior segments or a longer time delay for the re-initiation of the next peristaltic wave or both.

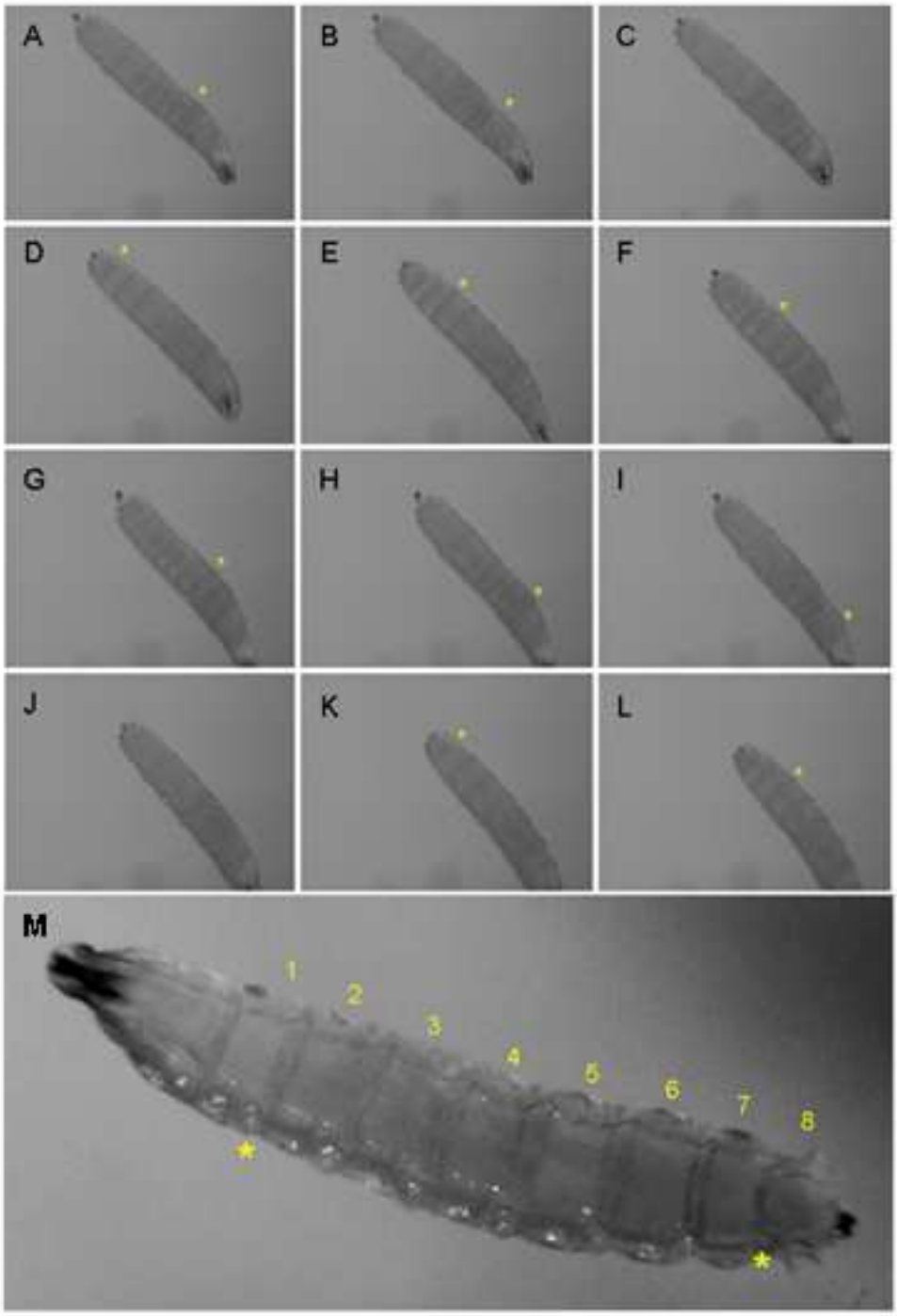

Figure 3.6 **L**arval crawling from a ventral perspective. The video recording for this montage was obtained from a Tdc2-control 3rd instar larva at 20x magnification. The time interval between each of the images shown is 100msec. Asterisks indicate the most anterior denticle band, which has lifted off the surface. **A-C**. Contraction wave from the posterior to the anterior end. **D-I**. A new peristaltic contraction wave for forward locomotion is initiated at the abdominal segment (A8) and progressively leads to contractions of segments along the animal until the anterior abdominal segments are reached. **M.** Ventral view of larval body indicating the abdominal segments A1-A8 and the respective denticle bands, D1-D8. Asterisks indicate the denticle bands used for peristaltic wave analysis.

The crawling behaviour of TßH^{nM18} larvae was shown to be substantially compromised using the low resolution locomotion assay. Similarly, using the high resolution locomotion assay, the crawling speed for TßH^{rM6} and TßH^{nM18} larvae was calculated at 5.44cm/min and 0.54cm/min, respectively (Figure 3.7), which represents a larger reduction in speed than determined by low resolution locomotion assay. In contrast to the low resolution assay data (Figure 3.4), the stride length is reduced in TßH^{nM18} compared to TßH^{rM6} larvae (Figure. 3.7). The stride duration is drastically increased. A further breakdown of the different phases of forward locomotion shows that the duration of the forward peristaltic wave (Chapter 2), across the abdominal segments is increased in TßH^{nM18} compared to TßH^{rM6} larvae. More specifically, the increase in the duration of progression of the peristaltic contraction wave across the segments is largely proportional. The time delay for contraction between denticle bands D8-D6, D8-D4 and D8-D2 is increased by 105%, 104%, and 133% in TßH^{nM18} larvae, respectively. The time delay to the onset of the next peristaltic wave is more substantially increased in TßH^{nM18} compared to TßH^{rM6} larvae. It is therefore apparent that the forward peristaltic contraction wave in forward locomotion is more than doubled without octopamine and increased tyramine levels. The delay in the peristaltic wave is largely proportional across all the abdominal segments and therefore indicates that all segments are equally affected. However, the prolongation of the forward peristaltic wave is far less substantial than the time delay for the initiation of the next peristaltic wave in forward locomotion. The dramatic decrease in crawling speed in TßH^{nM18} mutants is therefore largely due to increased pausing between peristaltic contraction waves during forward locomotion.

In Tdc2-control and Tdc2-TeTx* larvae, the high resolution crawling analysis of the different phases of peristaltic movement confirms the constant stride length (Figure 3.7) as established in previous experiments (Figure 3.5). The stride duration is also prolonged. The forward peristaltic wave in the locomotion cycle is increased in Tdc2-TeTx* compared to Tdc2-control. The time delay until re-initiation of the peristaltic cycle is also prolonged. The increased stride duration is therefore due to both increased duration of the forward peristaltic wave as well as a longer delay until the initiation of the next peristaltic wave. In contrast to TßH^{nM18} mutants the delay until the initiation of the next contraction wave is far shorter and therefore accounts for the relatively smaller speed reduction compared to control genotypes.

Fig. 3.7

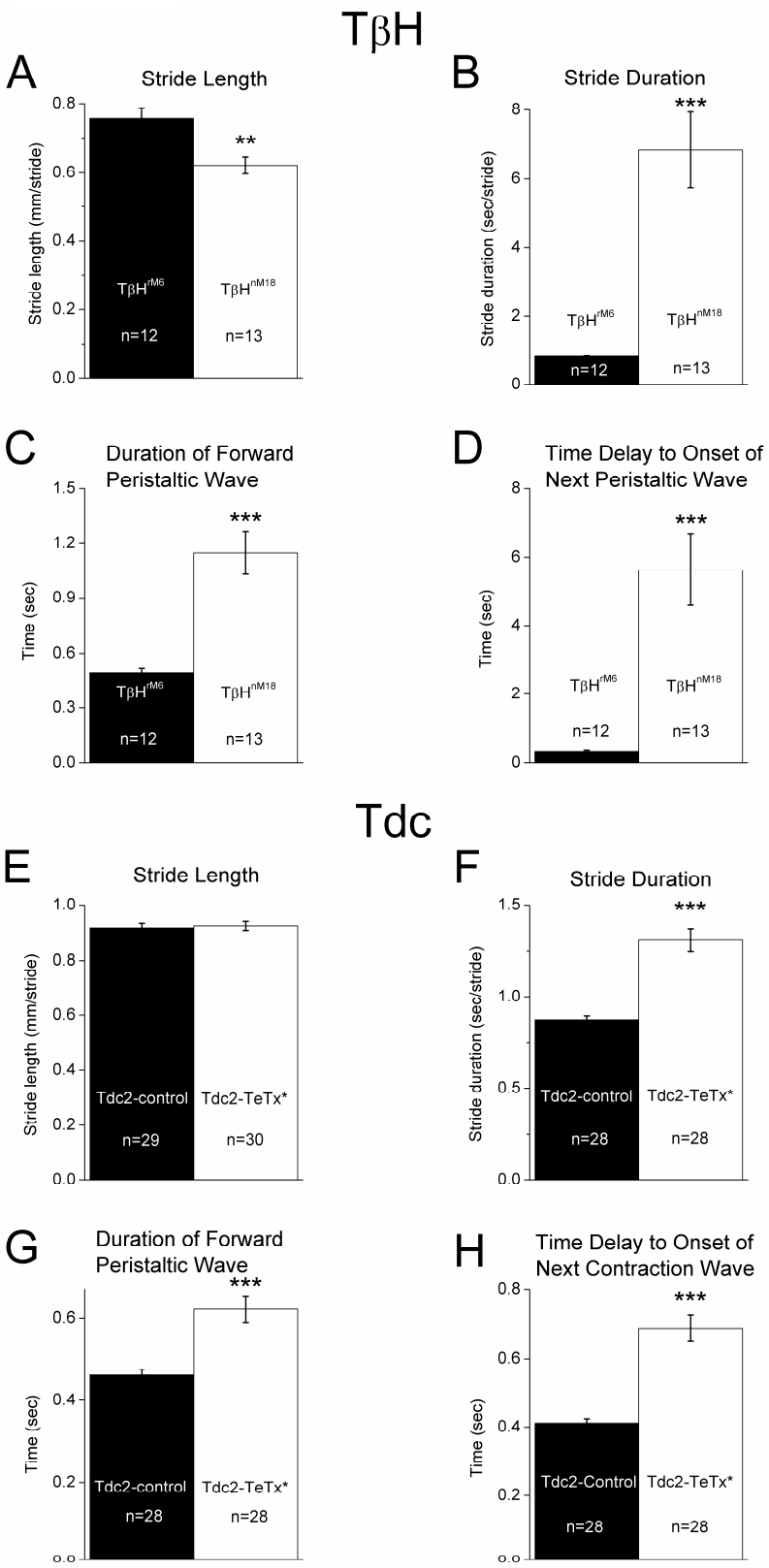
TβH
A
Stride Length
Strice length (mm/stride)
0.8
0.6
0.4
0.2
0.0
**
TβH^rM6
n=12
TβH^nM18
n=13
B
Stride Duration
Stride duration (sec/stride)
8
6
4
2
0
***
TβH^rM6
n=12
TβH^nM18
n=13
C
Duration of Forward Peristaltic Wave
Time (sec)
1.5
1.2
0.9
0.6
0.3
0.0
***
TβH^rM6
n=12
TβH^nM18
n=13
D
Time Delay to Onset of Next Peristaltic Wave
Time (sec)
8
6
4
2
0
***
TβH^rM6
n=12
TβH^nM18
n=13
Tdc
E
Stride Length
Stride length (mm/stride)
1.0
0.8
0.6
0.4
0.2
0.0
Tdc2-control
n=29
Tdc2-TeTx*
n=30
F
Stride Duration
Stride duration (sec/stride)
1.5
1.0
0.5
0.0
***
Tdc2-control
n=28
Tdc2-TeTx*
n=28
G
Duration of Forward Peristaltic Wave
Time (sec)
0.6
0.4
0.2
***
Tdc2-control
n=28
Tdc2-TeTx*
n=28
H
Time Delay to Onset of Next Contraction Wave
Time (sec)
0.8
0.6
0.4
0.2
***
Tdc2-Control
n=28
Tdc2-TeTx*
n=28

Figure 3.7 Analysis of peristaltic contraction waves in forward locomotion in larvae. Tested genotypes include TßH^{rM6}, TßH^{nM18} (A-D) Tdc2-control and Tdc2-TeTx* (E-H) 3rd instar larvae. **A**. The stride length is reduced in TßH^{nM18} compared to TßH^{rM6} larvae. **B**. The stride duration is significantly increased. **C.** The duration of the forward peristaltic wave is significantly increased. **D**. The time delay to the onset of the next peristaltic wave is also significantly increased. **E**. In Tdc2-control and Tdc2-TeTx* larvae, the stride length is unchanged. **F.** The stride duration is significantly increased. **G.** The duration of the forward peristaltic wave is significantly increased. **H.** The time delay to the onset of the next peristaltic wave is also significantly increased.

In summary, these data show that chronic changes in octopaminergic/tyraminergic signalling affect both the duration of the forward peristaltic contraction wave and, in addition, the delay until the onset of the next contraction wave. This suggests a prolonged inter-segmental time delay in the initiation of segmental unit CPG activity during forward locomotion contraction waves as a result of altered octopaminergic/tyraminergic signalling.

3.2.5 The Specific Effects of Octopamine and Tyramine on Locomotion

Octopamine and tyramine have been shown to be potent modulators of crawling behaviour, however, the genetic manipulations and available mutants used in this study affect both neurotransmitters. These neuromodulators cannot be manipulated individually using genetic tools. In order to determine the specific effects of each of these neuromodulators on locomotion, further genetic and pharmacological experiments were carried out. Using pharmacology in one genotype also has the additional benefit of potentially validating the effects observed with chronic genetic perturbations without potentially compromising effects of underlying differences in different genotypes.

In order to genetically modify the balance of synthesis of tyramine and octopamine, the UAS-TβH (Zhou et al., 2008) effector line was used. Over-expression of tyramine β hydroxylase in octopaminergic/tyraminergic neurons putatively shifts the balance towards an increased synthesis of octopamine at the expense of tyramine (Figure 3.3). The behavioural assay of Tdc2-control and Tdc2-TβH larvae shows that shifting the neurotransmitter balance towards octopamine does not change the crawling speed, stride duration or stride length as all parameters remain unchanged (Figure 3.8). This suggests that increased octopamine levels and reduced tyramine levels do not have any effect on crawling speed in larvae. Conversely, reduced octopamine and elevated tyramine levels have a dramatic effect on crawling as shown in the TßH^{nM18} mutant.

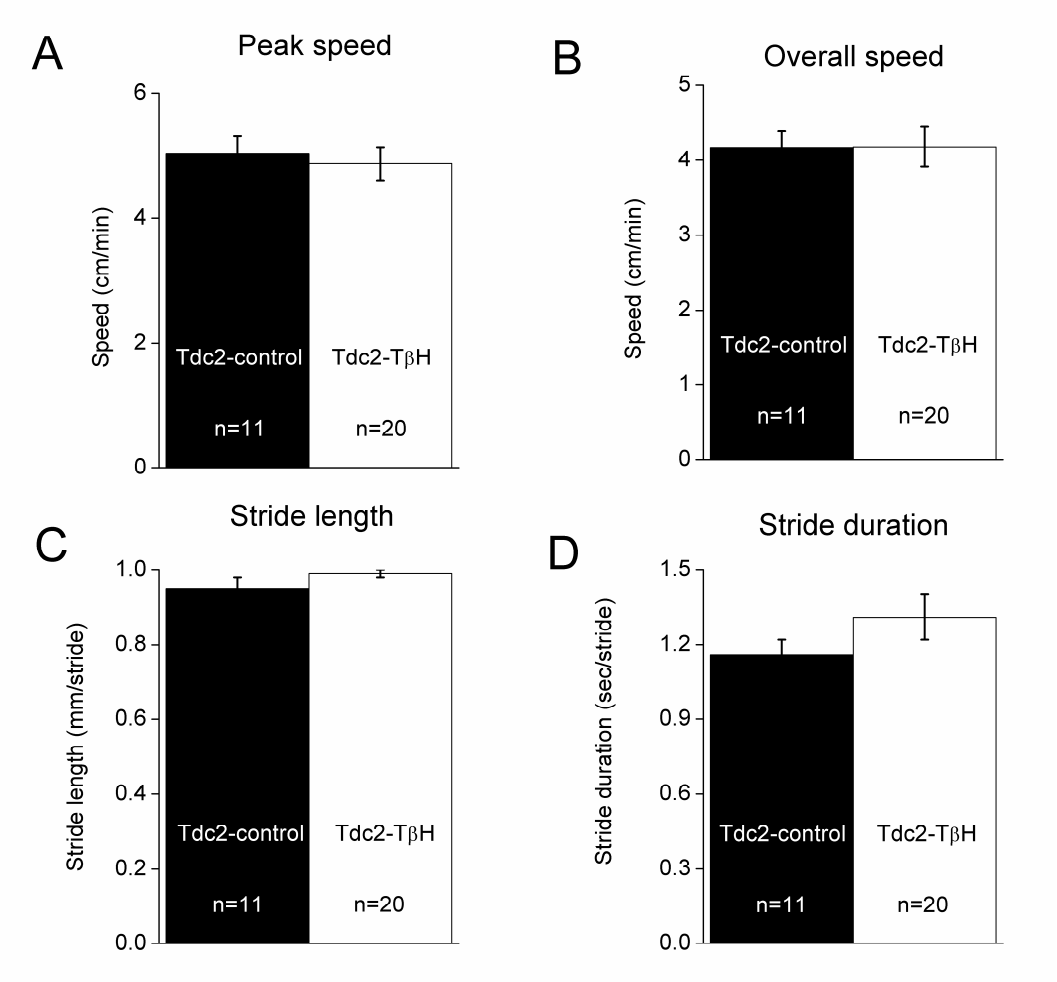

Figure 3.8 Crawling analysis in larvae over-expressing tyramine ß hydroxylase in octopaminergic/tyraminergic neurons. **A** and **B**. The peak and overall speed are unchanged. **C** and **D**. The stride length and stride duration are also unchanged in both groups.

The specific effects of octopamine and tyramine on the crawling speed were tested pharmacologically by feeding these neuromodulators at comparable concentrations used in a previous study to wall climbing 3rd instar larvae (Saraswati et al., 2004). First, the effects of octopamine on crawling behaviour were tested by feeding this compound to Tdc2-control, Tdc2-TeTx*, TßH^{rM6} and TßH^{nM18} larvae for 2 hours prior to experimentation. Feeding octopamine to Tdc2-TeTx* larvae shows significant effects on crawling behaviour. The peak and overall speed is gradually increased in response to feeding increasing concentrations of octopamine at 5mM, 50mM and 150mM concentrations (Figure 3.9). The stride length remains unchanged, whilst the stride duration is gradually decreased in response to increasing octopamine concentrations. Conversely, feeding octopamine to Tdc2-control larvae shows no statistically significant effects on crawling behaviour. The peak and overall speed as well as the stride length and stride duration remain unchanged in response to feeding increasing concentrations of octopamine (Figure

3.9). Increased levels octopamine therefore increase crawling speed and partially rescue the behavioural deficit with blocked octopaminergic/tyraminergic neurotransmission, but do not affect larvae with unaffected octopamine/tyramine signalling. It implies that additional octopamine cannot speed up larvae with physiologically uncompromised octopamine levels.

Similarly, feeding 150mM octopamine to 3rd instar TßH^{nM18} larvae, which are compromised in their behaviour, partially, but significantly, rescues the crawling deficit. As a result of the feeding, both the peak and overall speeds are increased in octopamine fed larvae (Figure 3.10). In addition, both the stride length and stride duration are reduced in octopamine fed larvae. In contrast, feeding of 150mM octopamine to 3rd instar in the control genotype, TßH^{rM6} larvae, did not show any effects on the crawling behaviour for the parameters measured.

Therefore, increasing the availability of octopamine in wall climbing 3rd instar larvae increases the crawling speed and thereby rescues the behavioural deficit in larvae with blocked octopaminergic/tyraminergic neurotransmission or genetically altered neuromodulator levels. However, in animals where octopaminergic levels and neuronal function are unaffected, octopamine does not affect crawling behaviour for the parameters measured. This therefore suggests that octopamine can increase crawling speed up to a maximal physiological level, which is primarily achieved by reducing the stride duration and maintaining a constant stride length.

Fig. 3.9

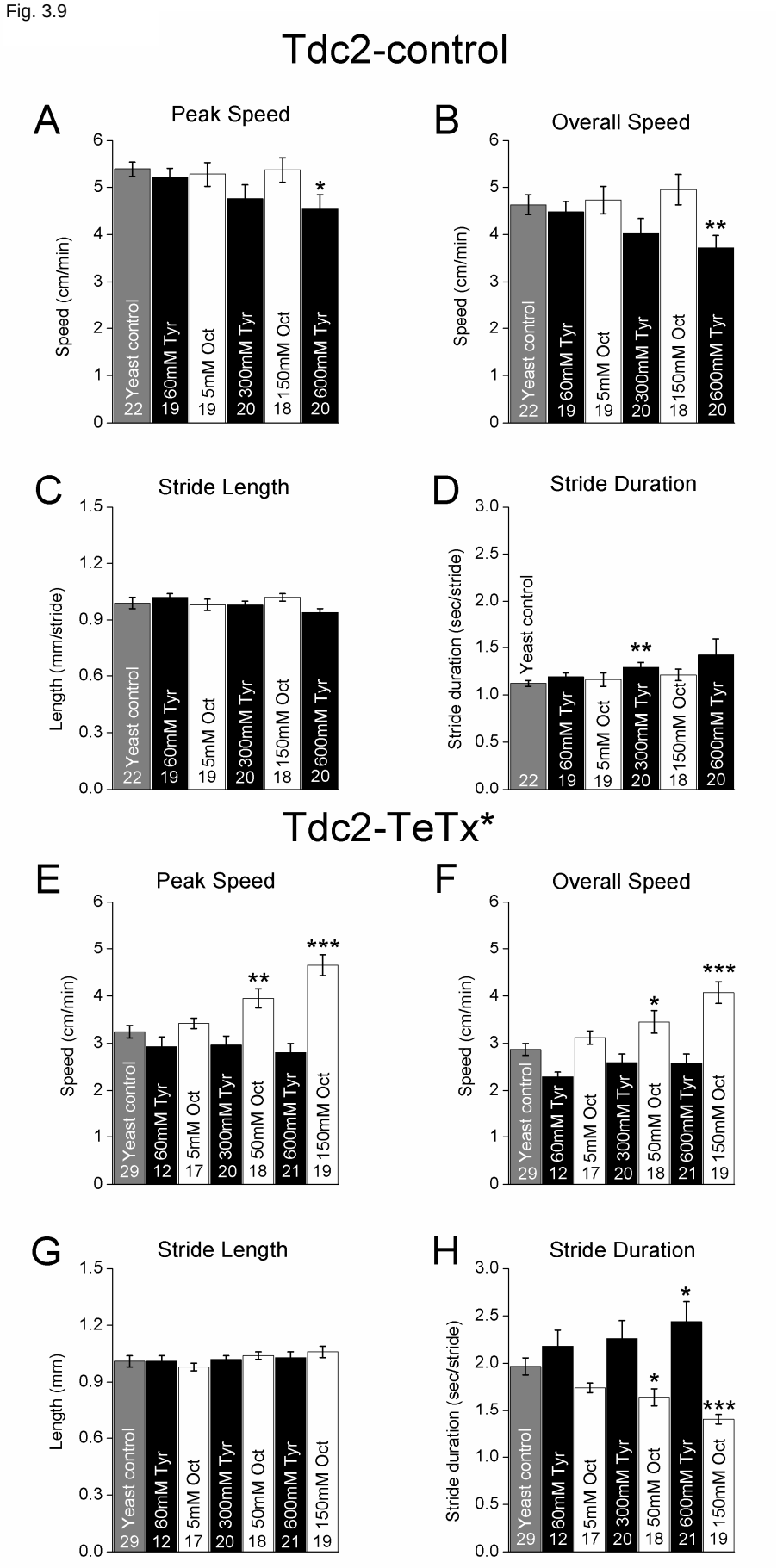
Tdc2-control
A
Peak Speed
Speed (cm/min)
B
Overall Speed
Speed (cm/min)
C
Stride Length
Length (mm/stride)
D
Stride Duration
Stride duration (sec/stride)
Tdc2-TeTx*
E
Peak Speed
Speed (cm/min)
F
Overall Speed
Speed (cm/min)
G
Stride Length
Length (mm)
H
Stride Duration
Stride duration (sec/stride)
Yeast control
60mM Tyr
5mM Oct
300mM Tyr
50mM Oct
600mM Tyr
150mM Oct

Figure 3.9 Effects of feeding octopamine or tyramine to Tdc2-control and Tdc2-TeTx* larvae. Crawling analysis of Tdc2-control larvae in response to feeding of increasing octopamine (5mM, 150mM) and tyramine (60mM, 300mM, 600mM) concentrations. **A.** The peak speed is unchanged with increasing octopamine concentrations, but is reduced with increasing tyramine concentrations **B**. The overall speed is also unchanged in response to octopamine feeding, but is decreasing in response to tyramine feeding. **C.** The stride length is unchanged in both octopamine as well as tyramine fed larvae. **D**. The stride duration remains unchanged in response to increasing octopamine concentrations, but is increased in response to increasing tyramine concentrations
Crawling analysis of Tdc2-TeTx* larvae in response to feeding of increasing octopamine (5mM, 50mM, and 150mM) and tyramine (60mM, 300mM, 600mM) concentrations. **E**. The peak speed is increased in response to increasing octopamine concentrations. The peak speed remains at lower, but comparable levels with increasing tyramine concentrations. **F**. The overall speed is also increasing in response to octopamine feeding. The overall speed is decreasing in response to feeding increasing tyramine concentrations. **G**. The stride length is unchanged in both octopamine and tyramine fed larvae. **H**. The stride duration is decreasing in larvae fed increasing octopamine concentrations. The stride duration is slightly increasing with feeding increasing tyramine concentrations.

Fig. 3.10

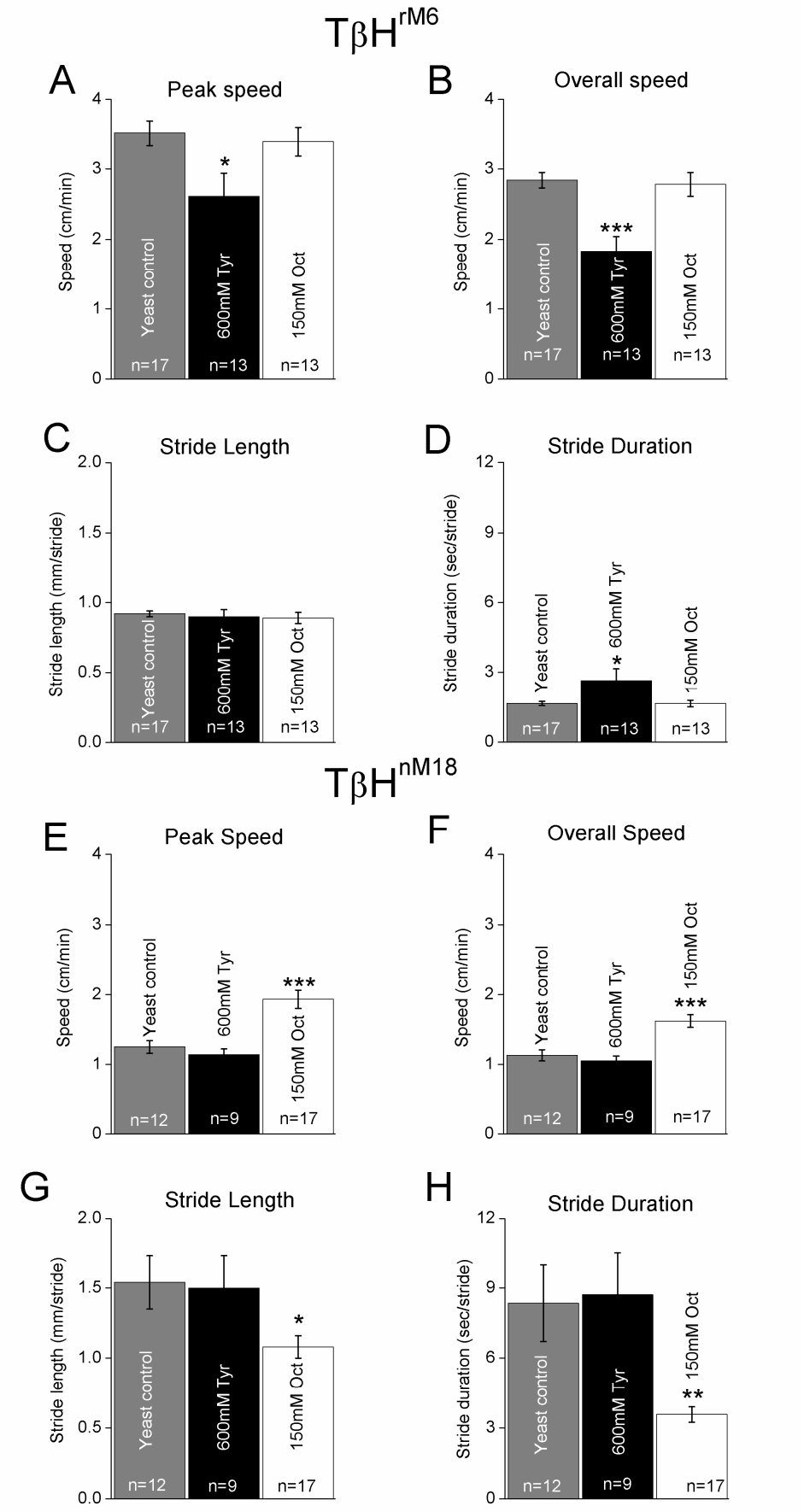
TβH^rM6
A
Peak speed
Speed (cm/min)
Yeast control
600mM Tyr
150mM Oct
n=17
n=13
n=13
*
B
Overall speed
***
C
Stride Length
Stride length (mm/stride)
D
Stride Duration
Stride duration (sec/stride)
TβH^nM18
E
Peak Speed
F
Overall Speed
n=12
n=9
n=17
G
Stride Length
H
Stride Duration
**

Figure 3.10 Effects of feeding octopamine or tyramine to TßH^{rM6} and TßH^{nM18} larvae. Neuromodulators were fed at the following concentrations: 600mM (tyramine) and 150mM (octopamine). **A** and **B.** In TßH^{rM6} larvae, the peak and overall speed is reduced in tyramine fed larvae and is unchanged in octopamine fed larvae. **C.** The stride length is unchanged in both tyramine and octopamine fed larvae. **D.** The stride duration is increased in tyramine fed larvae and is unchanged in octopamine fed larvae. **E** and **F.** In TßH^{nM18} larvae, the peak and overall speed is unchanged tyramine fed larvae and is increased in octopamine fed larvae. **G**. The stride length is unchanged in tyramine fed larvae and is decreased in octopamine fed larvae. **H**. The stride duration is unchanged in tyramine fed larvae and is reduced in response to octopamine feeding.

In order to determine the effects of tyramine, increasing concentrations of this neuromodulator were fed to 3rd instar larvae in the same genotypes. Feeding tyramine to Tdc2-control larvae expressing the inactive form of tetanus toxin light chain shows significant effects on crawling behaviour. The peak and overall speed is gradually decreased in response to feeding increasing concentrations of tyramine at 60mM, 300mM and 600mM (Figure 3.9). The stride length remains unchanged. The changes in crawling speed result in a modification of the stride duration, which is gradually and proportionately increasing in response to increasing tyramine concentrations.

Feeding tyramine to Tdc2-TeTx* larvae shows limited effects on crawling behaviour. The peak speed remains at comparable levels (Figure 3.9) and the overall speed is slightly, but significantly decreased in response to the lowest fed tyramine concentration. The stride length remains constant across all concentrations, whilst the stride duration is slightly and gradually increased with higher tyramine concentrations.

In the mutant background, feeding 600mM tyramine to 3rd instar TßH^{rM6} larvae induces a behavioural phenotype resembling the genetically induced changes in octopaminergic and tyraminergic signalling. The peak and overall speed is reduced (Figure 3.10). The stride length is unchanged, whilst the stride duration is increased. Feeding tyramine to TßH^{nM18} mutant larvae, which already have genetically induced 7-fold elevated tyramine levels, does not alter crawling behaviour as all parameters remain unchanged (Figure 3.10) (Monastirioti et al., 1996).

These results suggest that increased tyramine levels decrease crawling speed. Increasing the availability of tyramine in larvae with unaffected tyraminergic and octopaminergic signalling results in a reduced speed and an increased stride duration, which is the exact opposite effects of octopamine. Strikingly, stride length again remains constant. In contrast, increased tyramine in larvae with compromised tyraminergic and octopaminergic signalling does not substantially affect the behavioural deficit.

In order to validate if octopamine and/or tyramine modulate crawling behaviour, octopamine and/or tyramine receptor antagonists were fed to 3rd instar larvae. The first pharmacological agent used was the β-adrenergic-like octopamine receptor subtype antagonist mianserine, which is one of the most potent insect octopamine receptor antagonist, to CS wild-type larvae for 2 hours prior to experimentation (Evans and Robb, 1993; Maqueira et al., 2005). As a result, the peak and overall speed are reduced by a similar proportion to genetic manipulations (Tdc2-TeTx*) and increases the stride duration, whilst the stride length again remains constant (Figure. 3.11). Conversely, feeding the tyramine receptor antagonist yohimbine, a compound that has been shown to rescue TßH^{nM18} larvae in a previous study, to Tdc2-control larvae did not affect crawling behaviour (Arakawa et al., 1990; Saudou et al., 1990; Saraswati et al., 2004).

Fig. 3.11

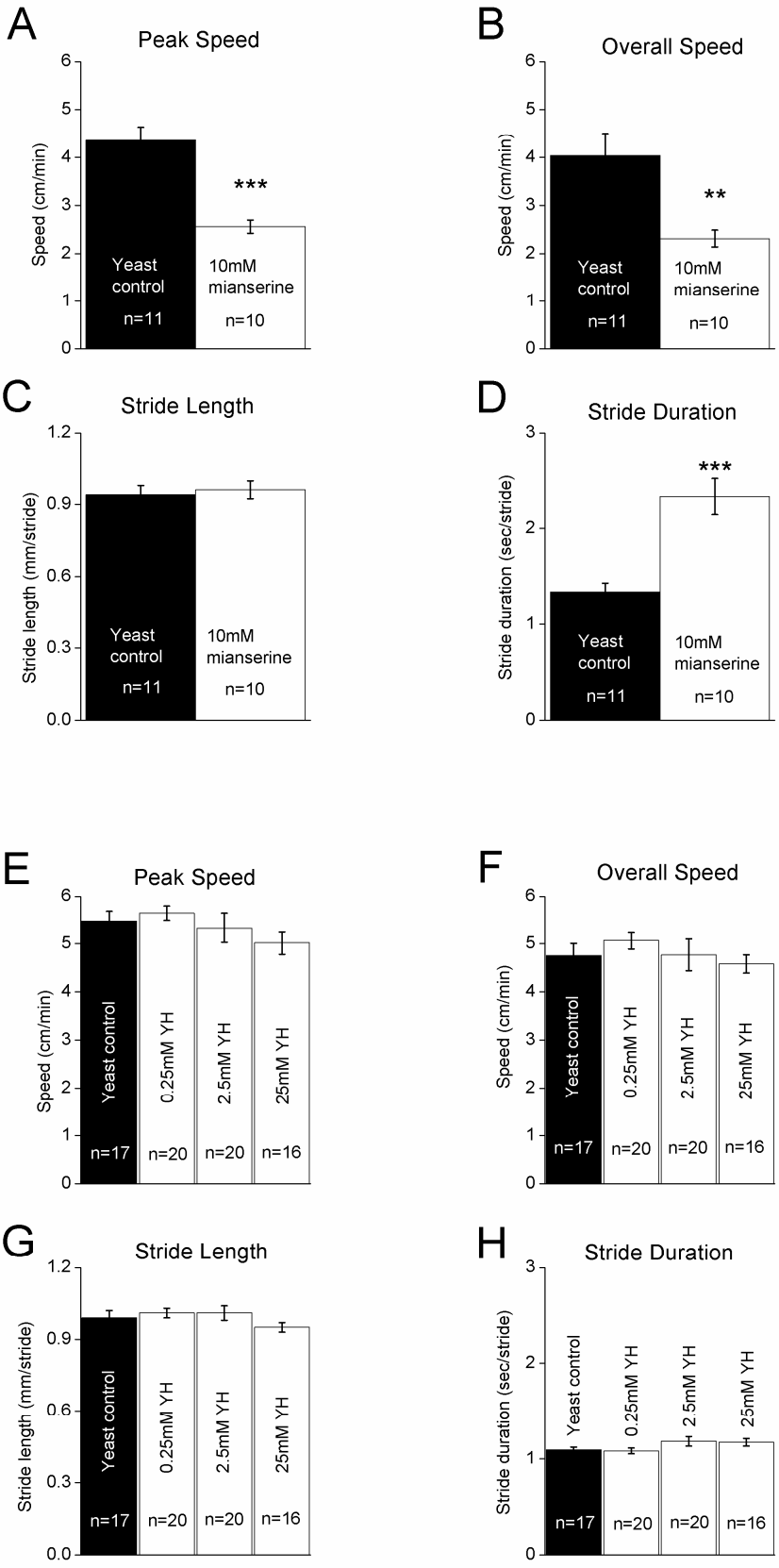
A
Peak Speed
Speed (cm/min)
***
Yeast control
n=11
10mM mianserine
n=10
B
Overall Speed
Speed (cm/min)
**
Yeast control
n=11
10mM mianserine
n=10
C
Stride Length
Stride length (mm/stride)
Yeast control
n=11
10mM mianserine
n=10
D
Stride Duration
Stride duration (sec/stride)
***
Yeast control
n=11
10mM mianserine
n=10
E
Peak Speed
Speed (cm/min)
Yeast control
0.25mM YH
2.5mM YH
25mM YH
n=17
n=20
n=20
n=16
F
Overall Speed
Speed (cm/min)
Yeast control
0.25mM YH
2.5mM YH
25mM YH
n=17
n=20
n=20
n=16
G
Stride Length
Stride length (mm/stride)
Yeast control
0.25mM YH
2.5mM YH
25mM YH
n=17
n=20
n=20
n=16
H
Stride Duration
Stride duration (sec/stride)
Yeast control
0.25mM YH
2.5mM YH
25mM YH
n=17
n=20
n=20
n=16

Figure 3.11 Crawling behaviour of larvae fed mianserine or yohimbine. **A-D:** CS wild-type larvae fed 10mM mianserine. **A** and **B.** The peak and overall speed is significantly reduced in response to feeding of 10mM mianserine . **C.** The stride length is unchanged in response to mianserine feeding. **D.** The stride duration is significantly increased. **E-H:** Crawling analysis of Tdc2-control larvae fed yohimbine at 0.25mM, 2.5mM and 25mM concentrations. **E** and **F.** The peak and overall speed is unchanged in larvae fed increasing concentrations of yohimbine. **G** and **H.** Both the stride length and stride duration remain unchanged in larvae fed increasing yohimbine concentrations.

In summary, the data on feeding octopamine and tyramine as well as their receptor antagonists suggests that tyramine reduces crawling speed by proportionately increasing the stride duration, whilst maintaining a constant stride length. In contrast, octopamine increases speed by proportional decreases in stride duration. Octopamine and tyramine therefore seemingly have opposite effects on crawling speed by regulating stride duration without affecting the stride length. These data also suggest that the neuromodulators modulate speed within a physiological range as octopamine increases crawling speed up to a maximal speed, whilst similarly, tyramine cannot further reduce the crawling speed in already compromised larvae.

3.2.6 Segmental Timing and Length Analysis of Octopaminergic and Tyraminergic Modulation

Octopamine and tyramine are both widely expressed throughout the larval organism. Moreover, these neuromodulators innervate peripheral body wall muscles and octopamine is a known modulator of muscle physiology in *Drosophila* and other model systems. For example, octopamine modifies the contraction force as well as the contraction and relaxation rates in locust muscles (Evans and Siegler, 1982; Malamud et al., 1988; Whim and Evans, 1988). In *Drosophila*, octopamine has been reported to increase the muscle contractions in the oviduct peritoneal sheath muscles and a reduced jumping force has been shown in $T\beta H^{nM18}$ mutant adult flies with reduced octopamine and increased tyramine levels (Middleton et al., 2006). In light of these studies and considering that octopaminergic and/or tyraminergic synaptic terminals are found on almost all peripheral muscles (Monastirioti et al., 1995; Nagaya et al., 2002), the crawling deficit may be induced by octopaminergic and/or tyraminergic modulation of the body wall muscle physiology. However, effects of octopamine and/or tyramine on the contraction and relaxation rates of peripheral body wall muscles in *Drosophila* larvae have not been reported.

In order to address potential effects on muscles, the high resolution behavioural data was used to develop a novel assay and to test the effects of

altered octopaminergic/tyraminergic signalling on the segmental muscle contraction length and dynamics. The length of an identified segment was measured in a fully relaxed and peak contracted state at the posterior ends of the enclosing denticle bands (Figure 3.12). The length difference between both states is defined as the segmental operational range. These parameters provide data on the effects of octopamine and/or tyramine on the muscle contractility. In addition, the time to reach a peak contracted state and to return to a fully relaxed state were also determined and are defined as the segmental contraction rate and the segmental relaxation rate, respectively. This provides information on the contraction and relaxation rates in response to chronically altered octopaminergic/tyraminergic signalling.

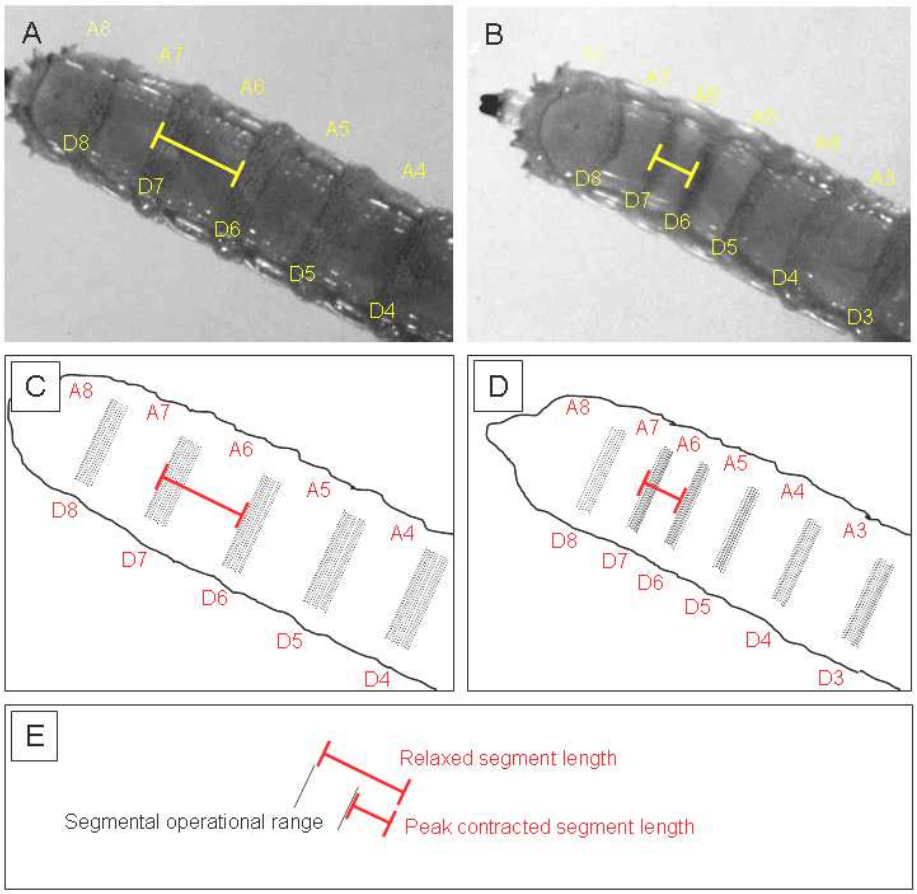

Figure 3.12 The length of abdominal segments in a relaxed state at rest and at peak contraction during forward locomotion. Abbreviations: Abdominal segment (A). denticle band (D). **A** and **B**. Larval abdominal segments in a relaxed and peak contracted state, respectively. **C** and **D**. Schematic outline of larval abdominal segments in a relaxed and peak contracted state, respectively. **E**. The segmental operational range is defined as the difference between the relaxed and peak contracted length of abdominal segments.

These effects were measured in Tdc2-control, Tdc2-TeTx*, TßH^{rM6} and TßH^{nM18} larval genotypes. The segmental contraction rate in abdominal segment A6 is increased in Tdc2-TeTx* larvae compared to Tdc2-control larvae (Figure 3.13). The segmental relaxation rate also increased when octopamine and tyramine release is blocked. These findings indicate that altered octopaminergic/tyraminergic signalling affects the contraction and relaxation rates of individual muscles in the segment. Moreover, the segment lengths in the relaxed and peak contracted state are both decreased in response to blocking tyramine and octopamine release. However, the segmental operational range of abdominal segment A6 is virtually identical in both genotypes, which reflects the constant stride length in these two genotypes. It suggests that although the muscle tone may be increased with altered octopaminergic/tyraminergic signalling, these muscles can nevertheless contract sufficiently during peristaltic contraction waves and traverse the same distance as their control genotype.

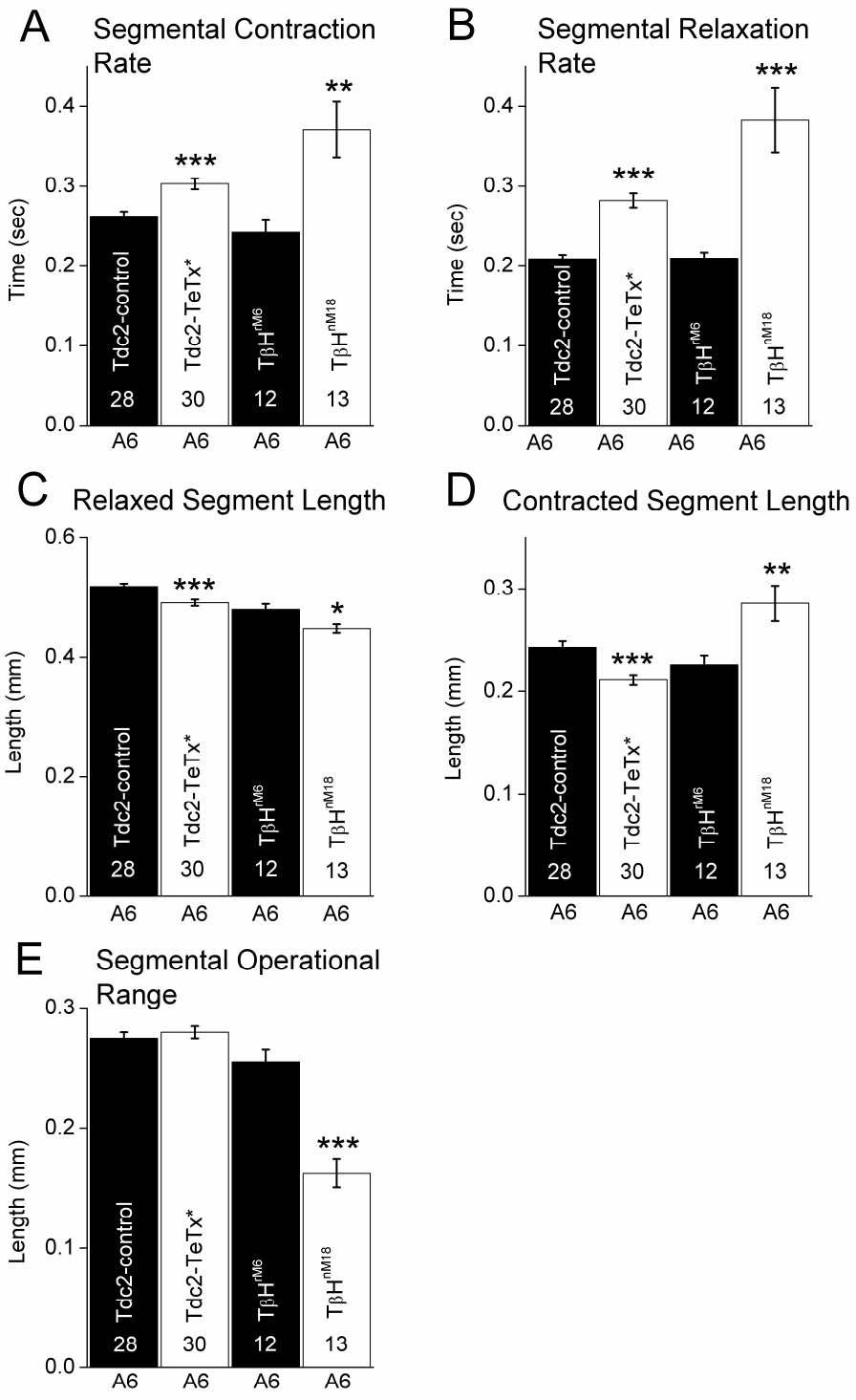

Figure 3.13 Timing and length analysis of contraction in the abdominal segment A6 in Tdc2-control, Tdc2-TeTx*, TßH^{nM18} and TßH^{rM6} larvae. **A.** The segmental contraction rates are significantly increased in both Tdc2-TeTx* and TßH^{nM18}larvae compared to their respective control genotypes. **B.** The segmental relaxation rate are also significantly increased in both Tdc2-TeTx* and TßH^{nM18}larvae. **C.** The length of the relaxed abdominal segment A6 is slightly reduced in both Tdc2-TeTx* and TßH^{nM18}larvae . **D.** The length of the abdominal segment A6 in the peak contracted state is significantly reduced in Tdc2-TeTx*, but is increased in TßH^{nM18}larvae. **E**. The operational range in abdominal segment A6 is unchanged.in Tdc2-TeTx*, but is decreased in TßH^{nM18}larvae.

In TßH^{nM18} larvae, the segmental contraction rate in abdominal segment A6 is increased compared to TßH^{rM6} animals (Figure 3.13). In addition, the segmental relaxation rate is also increased suggesting that both the contraction and the relaxation rates are considerably increased in TßH^{nM18} larvae. The segmental length is defined as the distance between posterior end of two adjacent denticle bands. The length of the segment in a relaxed state is slightly decreased, whereas the contracted segmental length is increased, respectively. As a result, the segmental operational range is considerably reduced in TßH^{nM18} larvae. This suggests that the contractility of the body wall muscles in abdominal segment A6 is reduced in this genotype and may account for the reduced stride length as a smaller distance can be traversed with smaller contractions.

However, the peristaltic waves were notably different in TßH^{nM18} larvae compared to TßH^{rM6} as well as Tdc2-control and Tdc2-TeTx*. TßH^{nM18} larvae display weak segmental contractions in the most posterior segments, whereas the more anterior segments were largely comparable to the other tested genotypes. Further analysis of segmental contraction in the more anterior abdominal segment A3 showed a considerable difference to abdominal segment A6. The segmental contraction rate is increased from 0.22 ± 0.01 (n=9) to 0.37 ± 0.04sec (n=10) ($p<0.01$). The segmental relaxation rates is also increased from 0.19 ± 0.004 to 0.34 ± 0.03sec ($p<0.001$). This suggests that the contraction and relaxation rates are similarly affected in both segments. However, the changes in the segmental length during contraction and relaxation are altered substantially. The length of the relaxed abdominal segment A3 is unchanged at 0.44 ± 0.01 and 0.46mm ± 0.01, ($p>0.05$). Moreover, the length of the abdominal segment A3 at the most contracted state also unchanged at 0.19 ± 0.01 to 0.18 ± 0.01mm ($p>0.05$). As a results, the operational range also remains unchanged at 0.25 ± 0.01 and 0.27mm ± 0.01 ($p>0.05$). These findings suggest that the contractility of muscles in abdominal segment A3 in TßH^{nM18} larvae remains unchanged. The distance traversed by a single contraction cycle in multi-segmentally contracting animals is limited by the distance traversed by each of the segments. These data suggest that the short stride length in TßH^{nM18} larvae is due to rigid posterior segments, whereas the anterior segments are apparently unaffected by altered octopaminergic/tyraminergic signalling.

In summary, these data strongly suggest that the contraction and relaxation rates of peripheral body wall muscles in the abdominal segments are prolonged when either octopaminergic/tyraminergic transmitter release is blocked or when tyramine levels are increased and no octopamine is present. Muscles within single segments take longer to both contract and to relax after contraction when octopaminergic and tyraminergic signalling is manipulated. It is not unlikely that one or both of these neuromodulators may be involved in modulating the

muscle tone and the muscle physiology. Although the segmental length in a contracted and relaxed state may be altered as a result of modulated neuromodulatory signalling, the operational range per segment appears unchanged with the exception of the particularly rigid posterior abdominal segments in TßH^{nM18} mutants. This suggests that octopamine and/or tyramine play an important role in the modulation of muscle physiology in order to regulate muscle tone as well as contraction and relaxation rates.

3.3 DISCUSSION

3.3.1 Octopamine and Tyramine Antagonistically Modulate Crawling Speed

Octopamine plays a role in the modulation of locomotion related behaviours in numerous model systems such as leech (Mesce et al., 2001; Crisp and Mesce, 2003), earthworm (Mizutani et al., 2002), locust (Ramirez and Pearson, 1991a; Parker, 1996), crayfish (Gill and Skorupski, 1999), and *Drosophila* (Saraswati et al., 2004; Hardie et al., 2007). Similarly, tyramine has been shown to cause behavioural effects in *C.elegans* (Alkema et al., 2005) as well as crawling behaviour in *Drosophila* (Saraswati et al., 2004). This study aimed to address how octopamine and tyramine modulate the multi-segmentally coordinated crawling behaviour in *Drosophila* larvae and where they exert their effects. Elucidating the effects of neuromodulators on the coordination and output of multiple CPG networks in locomotion may provide the opportunity to study the underlying structural and functional effects on a neuronal level.

It has been reported previously that a lack of octopamine and increased levels of tyramine in *Drosophila* TßH^{nM18} mutant larvae results in a drastic reduction in crawling speed (Saraswati et al., 2004), which has been confirmed in this study (Figure 3.4). In addition, this study utilised the GAL4/UAS system to manipulate putatively octopaminergic and/or tyraminergic neurons, which has not been reported before in the larval CNS. This study shows that blocking octopaminergic and tyraminergic neurotransmitter release by expressing TeTxLC has a profound effect on crawling behaviour in comparison to Tdc2-control larvae (Figure 3.5). The peak as well as the overall speed are substantially reduced when both tyraminergic and octopaminergic neurotransmission is blocked by TeTx expression. These changes in crawling speed are reproducible by suppressing octopaminergic/tyraminergic neuronal activity as a result of over-expressing the inwardly rectifying potassium channel Kir2.1 (Baines et al., 2001) or modulating neuronal activity by expressing the voltage-gated sodium channel NaChBac (Joiner et al., 2006; Lin et al., 2010).

The precise effects of the transgenic constructs used in this study on the octopaminergic/tyraminergic neuronal function are unknown without direct electrophysiological investigation. However, these constructs have been well characterised and used extensively in previous studies. TeTxLC is a toxin cleaving synaptobrevin and thereby inhibits evoked, but not spontaneous neurotransmitter release in various neurotransmitter systems (Sweeney et al., 1995; Baines et al., 1999; Li et al., 2000; Baines et al., 2001; Baines et al., 2002; Rodriguez Moncalvo and Campos, 2009). Over-expression of the inwardly rectifying potassium channel

Kir2.1 has been shown to increase leak currents and thereby reduces the membrane potential towards the K^+ reversal potential (Burrone et al., 2002). In addition, it increases K^+ conductance and thus opposes both synaptic and intrinsic depolarising currents (Burrone et al., 2002; Lin et al., 2010). Expression of this channel effectively blocks action potential firing and thereby silences neurons (Baines et al., 2001). Against expectation, over-expression of the NaChBac construct does not always increase neuronal excitability and action potential frequency, but can reduce frequency whilst drastically increasing action potential amplitude (Sheeba et al., 2008). As a result, neuronal firing patterns are altered, but are not necessarily over-excited. The precise effects of these channels on neuronal physiology cannot be extrapolated without direct electrophysiological analysis as different neuronal subtypes have different characteristics and specific homeostatic mechanisms to respond to changes. Nevertheless, in conclusion, these findings suggest that any chronic perturbation of octopaminergic/tyraminergic neuronal function or signalling capability causes an identical physiological effect on crawling speed independent of the genetic background.

By comparison, the peak and overall speed is reduced more dramatically in $TßH^{nM18}$ mutants in proportion to its respective control genotype in the low resolution behavioural assay, similar to previously reported results (Saraswati et al., 2004). However, the $TßH^{nM18}$ mutant contains an almost 7-8-fold increased level of tyramine compared to $TßH^{rM6}$ control larvae, whereas virtually no octopamine can be detected in these animals (Monastirioti et al., 1996; McClung and Hirsh, 1999). The balance of these neuromodulators is thus substantially shifted with a considerable oversupply of tyramine. This may decrease crawling speed further than other genetically induced changes used to manipulate both octopaminergic and tyraminergic signalling. Using the high resolution behavioural assay, an even bigger speed reduction of 90% is calculated. The discrepancy is probably due to the technical limitations of the behavioural analysis using a semi-automated software such as Image Pro. The software measures speed by calculating the distances traversed by a centroid in the outline of the larval body. However, this approach induces a degree of ‘noise’ into the data as centroid translocation may be due to a variability of larval body outline from frame to frame. As crawling speed is reduced, the noise in the recordings becomes proportionally bigger and thus overestimates the distance traversed by a larva. This confounds the true value of speed and its determinants of stride length and stride duration. The high resolution recordings should therefore be considered more accurate in this instance.

The underlying factors causing the differences in the effects on locomotion between the mutant and the GAL4/UAS transgenically modulated larvae are currently unclear. However, a number of potential factors may account for these

differences. The TßH^{nM18} mutant larvae have highly elevated tyramine levels and have no detectable levels of octopamine, which may have very substantial effects on either the neuronal and muscular physiology or both. This severe imbalance in the neuromodulator levels is unlikely to be reproduced in the GAL4/UAS modulated larvae and may account for the differences in locomotion. Whilst the GAL4/UAS transgenic larvae have a targeted functional modulation of putatively octopaminergic and/or tyraminergic neurons only, the induced effects in the neuromodulator levels in the mutant larvae are not spatially confined. The imbalance in the neuromodulator levels is therefore likely to have a potentially global effect in the mutant larvae in tissues where the respective receptors are expressed. Lastly, a potential difference in the temporal onset and duration of modulatory effects in neuromodulator signalling in the mutant and transgenicially modified larvae may contribute to this difference, but cannot be fully resolved at this stage. However, further work would be required to fully resolve the underlying factor causing the difference between these genotypes.

In addition to genetic experiments, a pharmacological approach was used to determine the specific effects of each of these neuromodulators. The feeding experiments indicate an antagonistic relationship between octopamine and tyramine in modulating crawling speed as shown by the feeding experiments in TßH^{nM18}, TßH^{rM6}, Tdc2-control and Tdc2-TeTx* larvae. The speed deficit in larvae with blocked octopaminergic/tyraminergic neurotransmission or a lack of octopamine in the mutant genotype can be partially rescued by feeding increasing concentrations of octopamine to these animals (Figure 3.9 & 3.10). This partial rescue has been reported previously and was attributed to an insufficient spatial and temporal assimilation of physiological neuromodulator signalling in order to fully rescue the behavioural deficit (Saraswati et al., 2004). However, notably there is no increase in the crawling speed in the Tdc2-control or TßH^{rM6} larvae in response to feeding increasing concentrations of octopamine. Similarly, an over-expression of the tyramine β hydroxylase enzyme in octopaminergic and tyraminergic neurons (Zhou et al., 2008), which converts tyramine into octopamine and thereby putatively shifts the balance towards increased octopamine levels at the expense of tyramine levels, shows no increase in crawling speed (Figure 3.8). It suggests that octopamine increases crawling speed. However, as crawling speed cannot be increased further in larvae with physiological levels of octopamine suggests that it cannot increase speed beyond an upper physiological limit. In contrast, increasing the availability of tyramine in TßH^{nM18} and Tdc2-TeTx* larvae does not significantly decrease crawling speed. Conversely, tyramine feeding in TßH^{rM6} and Tdc2-control larvae reduces crawling speed and thereby reproduces the genetically induced effects (Figure 3.9 & 3.10). These findings are consistent

with previous studies showing that octopamine increases speed, while tyramine decreases crawling speed (Saraswati et al., 2004).

Similarly, feeding the β-adrenergic-like octopamine receptor antagonist mianserine at a 10mM concentration also reduces crawling speed by a comparable proportion to the genetically induced changes in octopaminergic/tyraminergic signalling properties (Figure 3.11). Mianserine is one of the most potent insect octopamine receptor antagonists (Evans and Robb, 1993; Evans and Maqueira, 2005). In a range of antagonists, it has been shown to be the most potent inhibitor of β-adrenergic-like octopamine receptor induced increases in cAMP (Maqueira et al., 2005). However, this antagonist has a very similar receptor affinity for the three β-adrenergic-like octopamine and the tyramine receptor in *Drosophila* (Saudou et al., 1990; Maqueira et al., 2005). Nevertheless, although the receptor affinity is comparable, the potency of mianserine to modulate tyramine receptor signalling is unclear. It is thus possible that both octopamine as well as tyramine receptor signalling is blocked. Feeding yohimbine, a tyramine receptor antagonist with an approximately 100-fold higher affinity for tyramine compared to octopamine receptors (Arakawa et al., 1990; Saudou et al., 1990) and a limited ability to block octopamine receptor signalling (Maqueira et al., 2005) did not have any effect on locomotion (Figure 3.11). Taken together, these findings may suggest that the reduction in crawling speed by feeding mianserine is likely to be due to a block of octopamine receptor signalling.

Although the pharmacological feeding experiments exhibit a largely consistent and, where applicable, dose-dependent effects, a degree of caution in the interpretation of these results is required. In these experiments, the plasma osmolality of the hemolymph after feeding of pharmacological agents was not determined. Moreover, the hemolymph concentrations of octopamine and/or tyramine were not determined before or after feeding. As a result of the comparatively high concentrations of the respective agonists or antagonists, potential effects on the hemolymph osmolality cannot be excluded. Significant changes in the hemolymph osmolality could result in altered intracellular and extracellular electrolyte concentrations and/or movement of water across cellular membranes in larvae. The effects of osmolality on the cellular function in muscles and the CNS are currently poorly resolved, but in light of the functional importance of ions such as Na^+, K^+ and Ca^{2+}, significant functional effect are possible (Manley et al., 2004). However, identical concentrations fed to Tdc2-control and $TßH^{rM6}$ did not cause any obvious behavioural effects and did not affect locomotion, which argues against a potential impact of an altered osmolality on larval behavioural.

In summary, both genetic and pharmacological experiments suggest that octopamine and tyramine antagonistically modulate crawling speed in *Drosophila* larvae. These data provide evidence that these neuromodulators exerts a

functional effect on the locomotor CPGs in *Drosophila* larvae. Octopamine increases crawling speed, whilst tyramine decreases crawling speed. However, these neuromodulators are likely to operate within a physiological range as octopamine cannot increase speed beyond an upper limit, whereas tyramine does not decrease speed any further in larvae with compromised octopaminergic/tyraminergic signalling.

3.3.2 The Stride Length Remains Constant when Crawling Speed is Modulated

Speed is determined by both the stride length as well as the stride duration. Interestingly, while the stride duration is apparently inversely correlated with crawling speed, the stride length remains constant across several genetic and pharmacological experiments that change octopaminergic/tyraminergic signalling. These results have not been previously reported.

The data on stride length shows a remarkable degree of consistency across different genotypes. Modulating the function of octopaminergic and/or tyraminergic neurons by expressing TeTxLC and NaChBac shows that, in both cases, the speed is reduced considerably and that the stride duration is increased proportionately, but stride length is constant (Figure 3.5). One transgenic cross, Tdc2-Kir2.1, did not exhibit a constant stride length. However, homozygous UAS-TeTx and UAS-Kir2.1 larvae are different in their crawling parameters from the outset. The genetic crosses may thus exacerbate these underlying differences (Table 3.1). As no other suitable control line is available, the UAS-Kir2.1 construct was not used in further experiments.

The constant stride length was also observed in a number of pharmacological experiments. This excludes the possibility a purely genetic effect. The crawling speed can be increased or decreased with feeding of octopamine and tyramine, respectively. Whilst the stride duration is modified as a result of changes in speed, the stride length again remains constant (Figure 3.9 & 3.10). Similarly, feeding of 10mM mianserine to CS wild-type flies can reproduce the behavioural deficit induced by expressing TeTxLC in octopaminergic/tyraminergic neurons in the proportion of speed reduction and the increase in stride duration (Figure 3.11). However, in this experiment, the stride length again remains constant. Interestingly, a constant stride length was also observed in preliminary studies that manipulated dopaminergic and/or serotonergic signalling. Modulating dopaminergic and/or serotonergic neurotransmission also reduces crawling speed, but the stride length remains constant, which has been shown in two independent genetic experiments (Appendix A1).

TßH^{nM18} mutant larvae did not exhibit a constant stride length as it was either shown to be increased (Figure 3.4) or decreased (Figure 3.7) compared to its control genotype, depending on the behavioural assay and measurement methods used. Some factors need to be considered in this context. As discussed above, the difference is most likely due to the higher proportion of noise to locomotion in the calculations for speed, stride length and stride duration for slow larvae using the low resolution behavioural assay. The high resolution behavioural analysis can be considered more accurate. It is also important to consider the segmental lengths during contraction and relaxation as well as the resulting segmental operational ranges in abdominal segments A6 and A3. As shown (Figure 3.13), the posterior segments in TßH^{nM18} larvae are largely compromised in their ability to contract and relax and differ in their operational ranges when compared to the more anterior segment, A3, which shows a virtually identical operational range to its control genotype. The segmental length analysis suggests that the reduction in stride length is due to a compromised ability in these larvae to sufficiently contract the posterior segments. The reasons for this striking difference in the two described segments are currently unclear. TßH^{nM18} mutant larvae have highly elevated levels of tyramine, which has been shown to modulate muscle tone in locust (Donini and Lange, 2004). Tyraminergic effects on the muscle physiology in larvae may account for the weak contractility of segment A6. However, this cannot account for the fact that segment A3 seems unaffected. A differential distribution of these neuromodulators between anterior and posterior segments could nevertheless account for these differences. Alternatively, these results may suggest a differential effect on the neuronal control of locomotion in different segments or on the muscle modulation by highly elevated tyramine level and a lack of octopamine, but this cannot be resolved at this stage.

Stride length is determined by a sufficient contraction of each of the segments to propagate the larva to a more anterior position. The stride length is therefore limited by the segments with smaller operational ranges. The rigid posterior segments are thus likely to reduce the stride length in TßH^{nM18} mutant larvae. Conversely, the segmental operational ranges in abdominal segment A6 in Tdc2-control and Tdc2-TeTx* larvae are identical and confirm the findings on constant stride length.

In conclusion, several lines of evidence suggest that the changes in speed are caused by a variable stride duration whilst the stride length remains constant in response to chronically altered neuromodulator signalling. The constant stride length is apparently due to a constant operational range in the abdominal segments. A constant operational range implies that the output from each segmental unit CPG network onto its respective segmental muscle groups remains constant in order to achieve the same level of contraction.

3.3.3 Octopamine and Tyramine Affect Speed by Modulating the Stride Duration

As described above, the speed of locomotion is determined by both stride length as well as stride duration. While the stride length remains constant in several experiments that chronically modulate octopaminergic/tyraminergic signalling, the stride duration parameter changes across all these experiments. These results have not been reported previously, although prior publications noted the reduced frequency of peristaltic bouts in TβH^{nM18} larvae (Saraswati et al., 2004; Fox et al., 2006). Moreover, the change in stride duration is inversely proportional to the change in crawling speed. This correlation has been identified upon expression of NaChBac and TeTxLC in comparison to Tdc2-control larvae (Figure 3.5). In contrast, expressing the mammalian inwardly rectifying potassium channel Kir2.1 increases both the stride duration as well as stride length (Figure 3.5). Similarly, feeding octopamine or tyramine to different genetic strains showed that speed can be increased or decreased in response to altered octopamine/tyramine levels by changing the stride duration, whilst the stride length remained constant (Figure 3.9 & 3.10). However, it could not be reliably measured in TßH^{nM18} larvae using the low resolution behavioural assay due to technical limitations as discussed above. The β-adrenergic like octopamine receptor antagonist mianserine also displayed a slow crawling speed and an inversely proportionately increased stride duration, whilst the stride length remained constant.

The increase in stride duration has been shown to be due to both a slower progression of the peristaltic contraction wave from the posterior to anterior segments as well as a longer delay in the re-initiation of the subsequent peristaltic contraction in forward locomotion. This finding is consistent in both TßH^{nM18} as well as Tdc2-TeTx* larvae compared to their control groups, respectively (Figure 3.7). This data suggests that, with altered octopaminergic/tyraminergic signalling, there is a longer time delay in the onset of contraction from one segment to the next as the increase in duration of the peristaltic waves is largely proportional across several segmental measurements. The time it takes to initiate the next peristaltic wave in forward locomotion is also delayed when octopaminergic/tyraminergic signalling is altered. Particularly in TßH^{nM18} larvae the drastic reduction in crawling speed is largely determined by a disproportionately prolonged period of quiescence between individual peristaltic waves. By comparison, the changes induced by blocking the release of octopamine and/or tyramine in Tdc2-TeTx* larvae are milder. The data on the onset of the next peristaltic wave needs to be interpreted with some caution as it includes the time of the forward peristaltic wave to proceed from abdominal segment A2 to A1, but since the time delay of contraction onset

from one segment to the next is comparable across the entire peristaltic wave, the contribution to the overall time is comparatively small.

In summary, octopamine and tyramine modulate crawling speed by primarily regulating the stride duration. As octopamine increases crawling speed, it reduces stride duration, whilst tyramine increases stride duration as it reduces the crawling speed. Moreover, the speed is modulated by regulating the duration of both the peristaltic propagation and the delay to re-initiation of contraction. This suggests the inter-segmental time delay in the onset and offset of segmental unit CPG networks is variable, while the output of single segmental unit CPGs onto their respective muscle groups may remain constant as indicated by the constant stride length.

3.3.4 Single Segment Contraction Length and Time

Octopamine and tyramine are known modulators of muscle physiology. Octopamine has been shown to modulate muscle contraction and relaxation rates (Evans and Siegler, 1982; Evans, 1984; Whim and Evans, 1988) as well as the basal muscle tone and contraction force in various muscle types in locusts (Malamud et al., 1988; Clark and Lange, 2003), mediated by increases in cAMP (Evans and Myers, 1986; Whim and Evans, 1991). Furthermore, octopamine modulates conductances of Cl^- and resting K^+ currents in locust muscles, which are both mediated by cAMP (Walther and Zittlau, 1998). In *Drosophila,* octopamine increases muscle contraction strength in oviduct peritoneal sheath muscles (Middleton et al., 2006). Furthermore, the TßH^{nM18} mutant flies have been shown to exert a substantially reduced muscle force for jumping (Zumstein et al., 2004). In contrast, tyramine has been shown to decrease the muscle tone in oviduct muscles in locusts (Donini and Lange, 2004). In order to determine potential effects of altered octopaminergic/tyraminergic signalling on muscle physiology, and therefore crawling behaviour, segmental muscle contraction parameters were tested. The data on segment length and timing of contraction in the single abdominal segment A6 in Tdc2-control and Tdc2-TeTx* and abdominal segments A6 and A3 in TßH^{nM18} and TßH^{rM6} larvae indicate that the muscle length as well as the muscle contraction and relaxation rates are modulated by octopamine and tyramine (Figure 3.13).

Tdc2-control larvae have a longer segmental length both in the contracted and relaxed state compared to Tdc2-TeTx*, which implies that blocking octopaminergic/tyraminergic signalling may affect the basal muscle tone in *Drosophila*. Nevertheless, the segmental operational range shows no difference between these genotypes. This accounts for the constant stride length as an identical distance is traversed by this segment during forward peristaltic locomotion

in both genotypes. Similarly, the segmental operational range in TßH^{nM18} and TßH^{rM6} larvae in abdominal segment A3 is also unchanged. However, in abdominal segment A6, the segmental operational range is markedly reduced, primarily due to a very limited ability of TßH^{nM18} larvae to sufficiently contract this segment. Thus, TßH^{nM18} larvae exhibit a rigidity of the posterior segments with a smaller operational range than more anterior segments, whereas TßH^{rM6} operational ranges are identical in both segments measured. The cause for the rigidity is unclear. However, the most striking difference to other tested genotypes is the lack of octopamine and the increased tyramine levels. The elevated tyramine levels could therefore account for the rigidity due to effects on the NMJ or muscle physiology (Nagaya et al., 2002; Donini and Lange, 2004). The shorter posterior segmental operational range is likely to account for a shorter stride length in TßH^{nM18} larvae as it is determined by the smallest segmental operational ranges in a multi-segmental peristaltic contraction wave. Collectively, these findings imply that octopamine and/or tyramine may modulate the basal tone as well as the contraction force of peripheral body wall muscles.

These neuromodulators are also involved in the both the rate of muscle contraction and relaxation. The rate of contraction in abdominal segment A6 in Tdc2-TeTx* larvae is prolonged compared to Tdc2-control larvae (Figure 3.13). In addition to that, the rate of segmental relaxation is more noticeably prolonged in Tdc2-TeTx* compared to Tdc2-control larvae. Furthermore, these increases are more profound in TßH^{rM6} and TßH^{nM18} larvae. A substantial difference between all these genotypes is the elevated tyramine concentration in TßH^{nM18} larvae, which may again indicate an effect on muscle physiology by this neuromodulator and account for the noticeably longer segmental contraction and relaxation rates. These findings have not been reported in *Drosophila* before, but as described above and in the Introduction, they are consistent with reported changes in the muscle contraction and relaxation rates in locust (Whim and Evans, 1988). As octopamine is known to increase the rate of contraction and relaxation, the genetically induced reductions in octopaminergic signalling could therefore account for the reduced contraction and relaxation rates across all genotypes. It is not clear, if tyramine could potentially act antagonistically to octopamine by decreasing the rate of contraction and relaxation. Considering that tyramine acts antagonistically to reduce the crawling speed, this deserves a serious consideration, although it is currently unclear where tyramine exerts its effect to reduce crawling speed. In view of the fact that muscle contraction and relaxation rates as well as the basal muscle tone is modulated by octopamine and tyramine in locust, their effects on the muscle physiology in *Drosophila* ideally need to be addressed experimentally, which has now become possible (Paterson et al., 2010).

In summary, these previously unreported findings suggest that the decreased crawling speed in these animals can be accounted for by the altered segmental muscle contraction and relaxation rates. It is not unlikely that a slowed contraction and relaxation rate alters the timing of sensory signalling, which in *Drosophila* feeds back directly into the CNS via the multi-dendritic neurons (Hughes and Thomas, 2007; Song et al., 2007). Delays in the sensory neuronal signalling to trigger the onset of contraction of anterior segments could be sufficient to prolong the duration of the forward peristaltic contraction wave and thereby slow the crawling speed of the animals. Without evidence that octopamine and tyramine have release sites and alter the neurophysiology directly in the CNS, the muscle property modifications and/or resulting changes to sensory neuronal feedback could be a sufficient explanation for the slowed crawling speed.

3.3.5 The Impact of Genetics on Crawling Behaviour

Differences in crawling behaviour parameters in wild-type and mutant flies suggest that a mutation in the *white* gene alone is sufficient to induce a substantial behavioural phenotype and requires caution in the experimental setup and the comparison of different genetic strains. The *white* gene is an ABC transporter protein, which determines the eye colour (Sullivan and Sullivan, 1975; Campbell and Nash, 2001). Dimerisation with the brown protein enables transport of guanine across the plasma membrane, whereas dimerisation with scarlet enables transport of tryptophan into cells (Campbell and Nash, 2001). Both guanine and tryptophan are essential precursors for pigments that determine eye colour, but have been shown to be important for other functions as well. For example, tryptophan is a precursor for serotonin. Guanine is a precursor for cGMP-dependent functions of G-protein coupled receptors, which are linked to very important neurobiological functions (Gudermann et al., 1997). Furthermore, GMP is converted into dihydrobiopterin, a precursor of drosopterin in pigment cells, which is one of the pigments that turns the *Drosophila* eye red (Kumer and Vrana, 1996). In neurons, however, dihydrobiopterin is converted into tetrahydrobiopterin, which is an essential substrate in the synthesis of dopamine (Kumer and Vrana, 1996). The impact of white protein is therefore likely to extend beyond the changes of eye colour as the white protein is expressed in the CNS and has been strongly implicated in neurobiological functions. For example, the w^{1118} strain has been shown to be less sensitive to volatile anaesthetics, an effect attributed to CNS functions (Campbell and Nash, 2001). It has also been established that mutations in *brown*, *white* and *scarlet* genes lead to reduced bioamine levels in adult heads in Drosophila and to reduced neurotransmitter content in synaptic vesicles, whereas

extracellular levels of these bioamines are elevated compared to wild-type flies (Borycz et al., 2008). In addition to that, mis-expression of mini*white* has been shown to induce male-male courtship behaviour (Zhang and Odenwald, 1995), which is associated with dopaminergic signalling in the CNS (Liu et al., 2008). Furthermore, the effects of the mutation of *white* on the behaviour in Drosophila have not been studied extensively, but have been suggested to cause a deficiency in spatial learning (Diegelmann et al., 2006).

These findings suggest that the *white* gene mutation is very likely to have substantial effects on neuronal functions due to changes in GPCR signalling pathways as well as neuromodulator synthesis and precursor transport. The substantial neurophysiological changes are thus likely to account for the considerable differences in crawling behaviour between the homozygous control CS and w^{1118} larvae. For the purposes of this study, it sufficed to determine that the presence of either the wild-type or mutant *white* gene has a substantial effect on locomotor behaviour. Thus, wherever possible, experiments were designed to use a comparable genetic background and using the same number of copies of mini*white* in transgenic lines.

3.3.6 Conclusion

This study aimed to address some of the outstanding questions of acute and long-term effects neuromodulatory signalling in multiple segmental unit CPGs and, in particular, their effects on target tissues and mechanisms of action. These experiments have confirmed the potent modulatory effects of octopamine and tyramine on crawling behaviour in *Drosophila* larvae. Whilst octopamine has been shown to increase crawling speed up to a maximal physiological limit, tyramine acts antagonistically and reduces crawling speed to a minimal physiological limit, which is in line with a previous publication and validates the results from these assays (Saraswati et al., 2004). In this study, the modulatory effects of octopamine and tyramine on locomotion were analysed in more detail. I have shown that larvae change their crawling speed primarily by inversely and proportionately changing the stride duration. The stride length has been shown to remain constant when the crawling speed and stride duration are altered in both genetic and pharmacological experiments. This affects both the duration of the peristaltic contraction wave as well as the time delay to the onset of the subsequent peristaltic wave in forward locomotion. A constant stride length and segmental operational range suggests that the output of single segmental unit CPGs onto their respective muscle groups remains constant, whereas the time delay to activate the next anterior unit CPG is increased, which slows the forward peristaltic wave and thus crawling speed (Figure 3.14).

As octopamine and tyramine are widely expressed throughout the CNS (Monastirioti et al., 1995; Vömel and Wegener, 2008) and the periphery with highly diverse effects and numerous receptor subtypes and signalling pathways, it is unclear, where these neuromodulators exert their effects to affect crawling behaviour and what mechanisms of action they employ. In this chapter, I have shown that octopamine and tyramine are involved in the modulation of segmental muscle contraction and relaxation rates, which may account for the slowed crawling behaviour. The results presented in this chapter are summarised in Table 3.2. The following chapters will try to elucidate, if octopamine and/or tyramine, additionally, exert modulatory effects in the CNS as previous studies identified diverse putative synaptic release sites in the peripheral musculature as well as in the neuropilar and suboesophageal regions in the CNS (Vömel and Wegener, 2008).

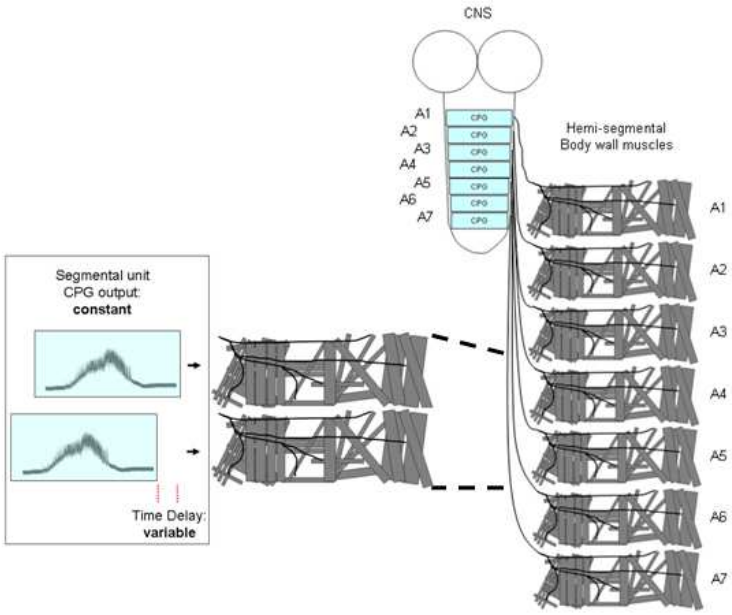

Figure 3.14 Model for the modulation of crawling speed. The larval CNS is organised in segmental unit CPG networks, which innervate their respective segmental muscle groups. Forward locomotion is initiated from the posterior segments and progressively activates anterior segments in a peristaltic contraction wave. As crawling speed is modulated by chronic changes in neuromodulator signalling, the output from segmental unit CPG networks remains constant, whereas the time delay of activation between individual CPG networks is variable. As a result, contraction strength in segmental muscle groups and therefore stride length remains constant, whereas the stride duration is variable. Muscle diagram adapted from (Bate, 1993).

Table 3.2 A summary of the experiments and results presented in Chapter 3.

Parameter	*Experimental Category*	*Genotypes*	*Additional experimental protocol*	*Result*
Peak speed	genetic	Tdc2-TeTx*		reduced
		Tdc2-NaChBac		reduced
		Tdc2-Kir2.1		reduced
		$T\beta H^{nM18}$		reduced
		Tdc2-TβH		constant
	pharmacological	Tdc2-control	Feeding of octopamine	constant
		Tdc2-control	Feeding of tyramine	reduced
		Tdc2-TeTx*	Feeding of octopamine	increased
		Tdc2-TeTx*	Feeding of tyramine	constant
		$T\beta Hr^{M6}$	Feeding of octopamine	constant
		$T\beta Hr^{M6}$	Feeding of tyramine	reduced
		$T\beta H^{nM18}$	Feeding of octopamine	increased
		$T\beta H^{nM18}$	Feeding of tyramine	constant
		CS	Feeding of mianserine	decreased
		Tdc2-control	Feeding of yohimbine	constant
Stride length	genetic	Tdc2-TeTx*		constant
		Tdc2-NaChBac		constant
		Tdc2-Kir2.1		increased
		$T\beta H^{nM18}$		increased
		Tdc2-TβH		constant
	pharmacological	Tdc2-control	Feeding of octopamine	constant
		Tdc2-control	Feeding of tyramine	constant
		Tdc2-TeTx*	Feeding of octopamine	constant
		Tdc2-TeTx*	Feeding of tyramine	constant
		$T\beta Hr^{M6}$	Feeding of octopamine	constant
		$T\beta Hr^{M6}$	Feeding of tyramine	constant
		$T\beta H^{nM18}$	Feeding of octopamine	constant
		$T\beta H^{nM18}$	Feeding of tyramine	constant
		CS	Feeding of mianserine	constant
		Tdc2-control	Feeding of yohimbine	constant
Stride duration	genetic	Tdc2-TeTx*		increased
		Tdc2-NaChBac		increased
		Tdc2-Kir2.1		increased
		$T\beta H^{nM18}$		increased
		Tdc2-TβH		constant

	pharmacological	Tdc2-control	Feeding of octopamine	constant
		Tdc2-control	Feeding of tyramine	increasing trend
		Tdc2-TeTx*	Feeding of octopamine	decreased
		Tdc2-TeTx*	Feeding of tyramine	increasing trend
		$T\beta Hr^{M6}$	Feeding of octopamine	constant
		$T\beta Hr^{M6}$	Feeding of tyramine	increased
		$T\beta H^{nM18}$	Feeding of octopamine	constant
		$T\beta H^{nM18}$	Feeding of tyramine	decreased
		CS	Feeding of mianserine	increased
		Tdc2-control	Feeding of yohimbine	constant
Duration of forward peristaltic wave	genetic	Tdc2-TeTx*		increased in A6
		$T\beta H^{nM18}$		increased in A6
		$T\beta H^{nM18}$		increased in A3
Delay of onset to the next peristaltic wave	genetic	Tdc2-TeTx*		increased in A6
		$T\beta H^{nM18}$		increased in A6
		$T\beta H^{nM18}$		increased in A3
Segmental contraction rate	genetic	Tdc2-TeTx*		increased in A6
		$T\beta H^{nM18}$		increased in A6
		$T\beta H^{nM18}$		increased in A3
Segmental relaxation rate	genetic	Tdc2-TeTx*		increased in A6
		$T\beta H^{nM18}$		increased in A6
		$T\beta H^{nM18}$		increased in A3
Relaxed segment length	genetic	Tdc2-TeTx*		decreased in A6
		$T\beta H^{nM18}$		decreased in A6
		$T\beta H^{nM18}$		constant in A3
Contracted segment length	genetic	Tdc2-TeTx*		decreased in A6
		$T\beta H^{nM18}$		increased in A6
		$T\beta H^{nM18}$		constant in A3
Segmental operational range	genetic	Tdc2-TeTx*		constant in A6
		$T\beta H^{nM18}$		increased in A6
		$T\beta H^{nM18}$		constant in A3

Chapter 4

The Synaptic Projections of Octopaminergic/Tyraminergic Neurons in the Larval CNS

4.1 INTRODUCTION

Both octopamine and tyramine have been shown by previous studies and in the preceding chapter to play an important functional role in the modulation of *Drosophila* larval crawling behaviour (Saraswati et al., 2004). However, it is currently unclear, where these neuromodulators exerts their effects and what mechanisms of action they employ. It is therefore unresolved, whether these neuromodulators have a direct modulatory role in locomotor CPGs in the CNS or whether they exert their effects in the periphery and thereby indirectly affect the function of segmental unit CPGs.

As described previously, octopamine and tyramine have both been identified as important and potent modulators of animal behaviour in several invertebrate model systems such as fruit fly, honey bee, cockroach and locust (Roeder, 1999). Octopamine has been shown to act in the periphery as it modulates the NMJ transmission in locust, crayfish, moth and *Drosophila* (Evans and O'Shea, 1977; Breen and Atwood, 1983; Fitch and Kammer, 1986; Kutsukake et al., 2000; Nagaya et al., 2002; Koon et al., 2011) as well as muscle contraction and relaxation rates (Malamud et al., 1988; Whim and Evans, 1988). In addition, octopaminergic modulatory effects have been identified in the CNS. Both synaptic amplitude and proprioceptive neuronal intrinsic activity patterns have been shown to be modulated by octopamine in separate sensory neuronal pathways in locust and spider (Ramirez and Pearson, 1991b; Parker, 1996; Matheson, 1997; Baudoux et al., 1998; Leitch et al., 2003; Widmer et al., 2005). In contrast, however, the central effects of octopaminergic modulation in the larval and largely the adult *Drosophila* CNS are unknown. Nevertheless, effects on K^+ currents and numerous behavioural effects attributable to CNS function have been reported in the adult brain (Crocker et al., 2010).

Octopamine and tyramine are widely expressed throughout both the larval as well as the adult stages in *Drosophila*. Octopaminergic and tyraminergic axonal projections and synapses have been shown in the peripheral body wall musculature originating from octopaminergic VUM neuron in the larval CNS (Monastirioti et al.,

1995; Nagaya et al., 2002; Vömel and Wegener, 2008). In the adults CNS, an extensive array of putatively octopaminergic/tyraminergic neuronal cell bodies (Busch et al., 2009; Busch and Tanimoto, 2010) and an innervation of the ovary has been shown (Lee et al., 2003; Middleton et al., 2006). In addition to that, extensive dendritic and axonal branching in the neuropile, the cell body free space where many synaptic connections are formed, has been shown in both adults and larvae (Monastirioti et al., 1995; Vömel and Wegener, 2008; Busch et al., 2009). There is thus a discrepancy between the known projections of octopaminergic/tyraminergic neurons in the CNS and their functional effects on behavioural and neuronal physiology in *Drosophila*.

In the larval CNS, there are repeatable and identifiable expression patterns of small, putatively octopaminergic and/or tyraminergic, neuronal clusters across the thoracic and abdominal segments as well as expression in the anterior parts of the brain, particularly in the suboesophageal ganglia (Dr. Andreas Thum, unpublished data) (Vömel and Wegener, 2008). A summary of the number and localisation of the putatively octopaminergic and/or tyraminergic neurons is provided in Figure 4.1 and Table 4.1. A repetitive pattern of small clusters of neuronal cell bodies are seen in the thoracic and abdominal segments in the VNC, which are located in the ventral median position and are referred to as ventral unpaired median (VUM) neurons (Landgraf et al., 2003). It was previously reported that the abdominal segment A1 contains 5 neurons, segments A2-A6 were reported to have 3 labelled VUM neurons each, whereas A7 was reported to contain 2 VUM neurons (Vömel and Wegener, 2008), however, a recent study identified 3 neurons in A7 as well (Dr. Andreas Thum, unpublished data). The thoracic ganglia in previous studies have also been shown to contain 3 octopaminergic/tyraminergic as well as a pair of paramedial (pm) octopaminergic/tyraminergic neurons each as in the abdominal segment A1 (Vömel and Wegener, 2008). In addition, approximately 39 neurons were identified in the brain lobes as well as the suboesophageal ganglia, which comprise approximately 24 putatively octopaminergic/tyraminergic neurons in total across three suboesophageal clusters (Dr. Andreas Thum, unpublished data). Overall, approximately 82 putatively octopaminergic/tyraminergic neurons have been identified throughout the larval CNS in a previous study, approximately 39 neurons in the brain lobes as well as the suboesophageal ganglia and approximately 43 in the VNC with some level of variation between larvae have previously been reported (Dr. Andreas Thum, unpublished data).

The synaptic projections of these clusters of octopaminergic/tyraminergic neurons and a potential spatial proximity to motor neuronal or interneuronal dendrites could provide for widespread and potent modulatory effects. However, the functional relevance of octopaminergic/tyraminergic axonal projections and release sites across the larval CNS and neuropile are currently unclear. It is also unknown,

which neurons are targeted and what mechanisms of actions are employed by the octopaminergic/tyraminergic neurons. In this chapter, some of the questions relating to the localisation of octopaminergic/tyraminergic neuronal cell bodies, axonal projections and synaptic terminal release sites in relation to motor neuronal dendritic branching will be addressed. This approach may provide further insights whether neurons that are likely to form a part of segmental unit CPG networks may be targeted by octopaminergic and/or tyraminergic modulatory inputs.

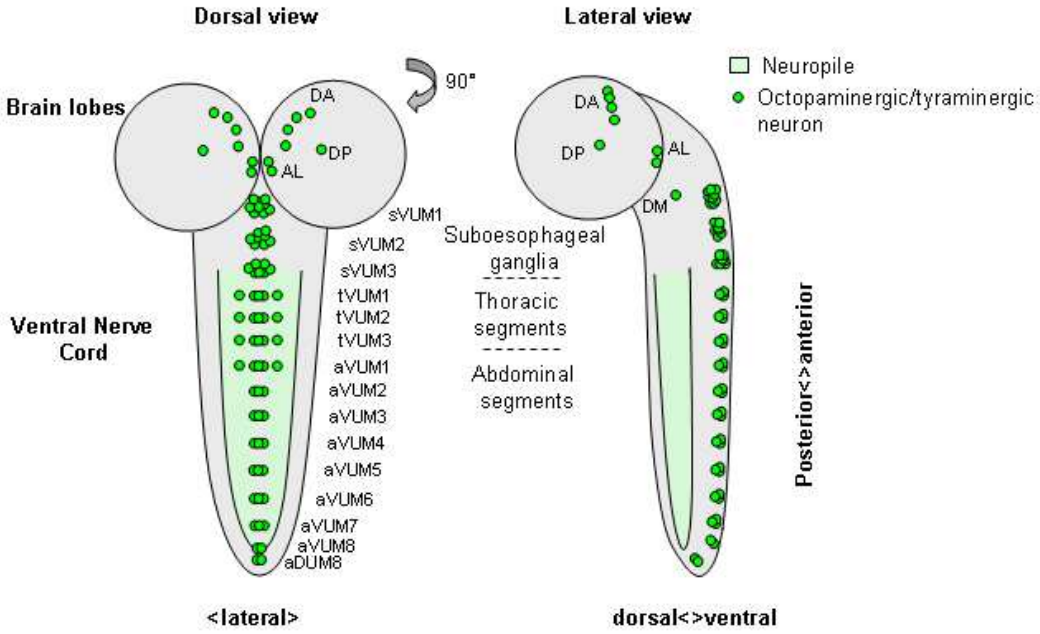

Figure 4.1 Schematic diagram of octopaminergic/tyraminergic neurons in the *Drosophila* larval CNS. The diagram shows the localisation of neuronal cluster in the brain lobes (DP, DA, AL), suboesophageal ganglia (sVUM1-sVUM3), thoracic (tVUM1-tVUM3) and abdominal segments (aVUM1-aDUM8). Abbreviations: ventral unpaired median (VUM), dorsal unpaired median (DUM).

Table 4.1 The number of putatively octopaminergic/tyraminergic neurons in the brain lobes as well as suboesophageal, thoracic and abdominal ganglia. Data based on: (Dr. Andreas Thum, unpublished data) and (Vömel and Wegener, 2008).

Anatomical structure	*Cell Cluster*	*Number of cells*
Brain lobes	DP	1
	DA	4
	AL	2
Suboesophageal Ganglia	DM	1
	sVUM1	9
	sVUM2	8
	sVUM3	7
	Brain lobes+ suboesophageal ganglia subtotal	**39**
Thoracic Ganglia	tVUM1	5
	tVUM2	5
	tVUM4	5
Abdominal Ganglia	aVUM1	5
	aVUM2	3
	aVUM3	3
	aVUM4	3
	aVUM5	3
	aVUM6	3
	aVUM7	3
	aVUM8	2
	aDUM8	2-3
	VNC (Thoracic and abdominal ganglia) subtotal	**42-43**
	CNS total	**82**

2.1.2 Hypotheses

- Octopamine and/or tyramine are expressed throughout the larval developmental stages and may therefore have a developmental role in the larval CNS as well as act as acute neuromodulatory signals.
- Octopaminergic and/or tyraminergic synaptic terminals are localised in vicinity of identified motor neuronal dendritic branching, potentially enabling them to exert direct neuromodulatory effects on the output neurons of locomotor unit CPG networks.
- Functional modulation of a subset of octopaminergic and/or tyraminergic neurons in the suboesophageal ganglion with putative release sites in larval CNS, but without identified peripheral target innervation relevant to larval locomotion, is sufficient to induce a crawling deficit.

- Octopamine and/or tyramine receptors are expressed in the larval CNS and, more specifically, in identifiable motor neurons and are therefore potential targets of octopaminergic and/or tyraminergic modulation.

4.2 RESULTS

4.2.1 Octopaminergic/tyraminergic neurons in the larval CNS

The widespread expression of putatively octopaminergic/tyraminergic neurons throughout the larval CNS brain lobes and in the VNC has been reported in several studies, using different techniques, to elucidate the expression pattern and projections of these neurons (Monastirioti et al., 1995; Python and Stocker, 2002; Vömel and Wegener, 2008). However, the genetic and immunocytochemical techniques used provide only indirect evidence for the presence and potential release of octopamine from the labelled neurons. The labelled neurons can therefore only be regarded as putatively octopaminergic/tyraminergic and henceforth need to be understood as that. Most of the previous studies were carried out in 3rd instar larvae and allowed a detailed elucidation of the neuroanatomy and projection patterns of putatively octopaminergic/tyraminergic neurons (Figure 4.1 and Table 4.1). However, the localisation and expression of octopaminergic/tyraminergic neurons in the early larval stages is unclear.

Considering that octopamine and tyramine have been shown to be potent modulators of crawling behaviour in the preceding chapter, the putative presence and expression of these neurotransmitters and the functionality of the Tdc2-GAL4 driver line in earlier larval stages needed to be determined. The genetic tools to label putatively octopaminergic/tyraminergic neurons in previous studies were used to determine, if putatively octopaminergic/tyraminergic neurons are being labelled and manipulated at least throughout the larval stages in this study. In order to address that, putatively octopaminergic and/or tyraminergic neurons were labelled and visualised in 1st instar larvae after hatching. *Drosophila* larvae undergo three developmental stages as 1st, 2nd and 3rd instar larvae prior to pupation. The expression pattern (Figure 4.2) in 1st instar larvae strongly resembles the expression pattern and localisation of putatively octopaminergic/tyraminergic neurons previously reported in 3rd instar larvae labelled with the same driver and marker lines (Vömel and Wegener, 2008), Tdc2-GAL4 and UAS-CD8::eGFP (Cole et al., 2005). In this qualitative analysis, the number of octopaminergic/tyraminergic neurons per segment was not determined due to the small sample size. However, most abdominal segments contain two to three labelled neurons corresponding to similar numbers reported in 3rd instar larvae (Vömel and Wegener, 2008 In this study, the paramedial neurons have not been visualised in 1st instar larvae (Figure 4.2), which may be due to a differential strength of Tdc2-GAL4 driven protein expression or a difference in the CNS developmental anatomy, organisation and

neurotransmitter synthesis {Vömel, 2008 #150). In addition to the neurons located in the VNC, a dense cluster of octopaminergic/tyraminergic neurons is identified in the suboesophageal region, located ventrally in the space between brain lobes and the VNC. These subesophageal neurons correspond to the localisation of putatively octopaminergic and/or tyraminergic neurons in 3rd instar larvae.

The same VUM neuronal subpopulations as well as neurons in the suboesophageal areas and in the brains lobes of the larval CNS, however, have also been shown to be immunoreactive against tyramine in a previous study (Nagaya et al., 2002). In addition to that, dorsolateral neurons are stained with a tyramine antibody, which are not labelled genetically or immunologically when targeting octopaminergic neurons (Nagaya et al., 2002). This suggests that some neurons in the larval CNS only synthesise tyramine, but not octopamine. In addition to the labelling of neuronal cell bodies, the membrane localisation of the CD8::eGFP construct also visualises neurite projections throughout the CNS in this study. Similar to the previously reported neuroarchitecture in 3rd instar larvae, 1st instar octopaminergic/tyraminergic neurons in the VNC also project dorsally, where they bifurcate and innervate areas of the neuropile regions before they eventually leave the CNS via the segmental nerve to innervate the peripheral body wall musculature (Vömel and Wegener, 2008).

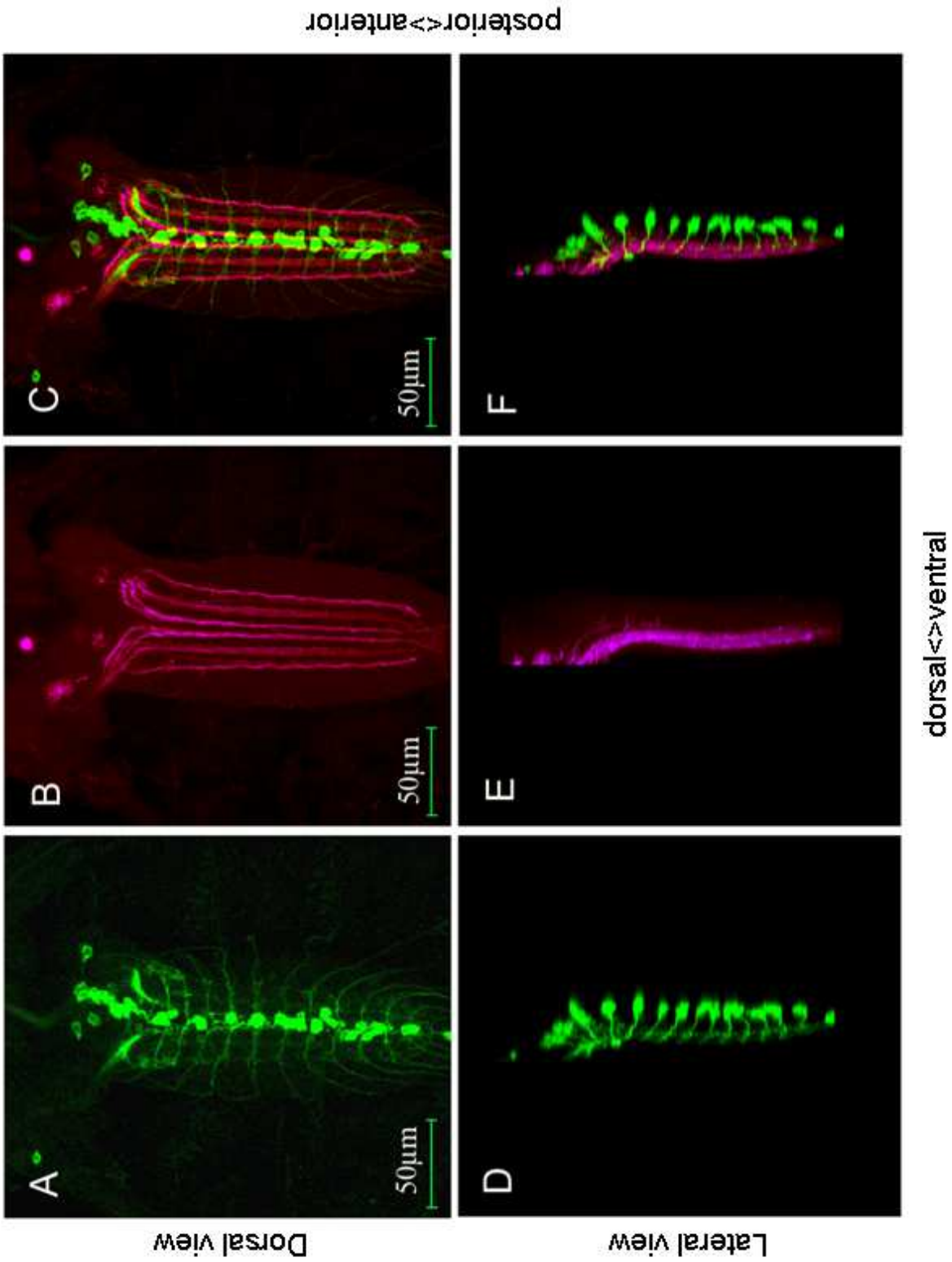

Figure 4.2 Localisation and architecture of octopaminergic/tyraminergic neurons in the larval CNS. Preparations were labelled with GFP and the Fasciclin II (FasII) landmark system (Landgraf et al., 2003) **A-C.** Dorsal view of the ventral nerve cord of 1st instar larvae. **A.** Tdc2-CD8::eGFP expression in larvae in the CNS. **B.** FasII antibody staining in magenta shows landmark fascicles in the VNC. **C.** Merged image of Tdc2-CD8::eGFP expression and FasII antibody staining in larvae. **D-E.** Lateral view of the CNS of 1st instar larvae. **D.** Tdc2-CD8::eGFP expression in the VNC. **E.** Lateral view of FasII antibody staining in the ventral nerve cord. **F.** Merged image of GFP expression and FasII antibody staining.

In summary, this data provides evidence showing that octopamine/tyramine are synthesised at least as early as after hatching. This shows that genetic perturbations using the Tdc2-GAL4 construct affect octopaminergic/tyraminergic neuronal function throughout the larval developmental stages.

4.2.2 Octopaminergic/tyraminergic synaptic terminals are present in the VNC neuropile and are in close proximity to motor neuronal dendrites

Several studies have shown putatively octopaminergic/tyraminergic synaptic terminal varicosities in the neuropile regions in the larval VNC as well as widespread innervations throughout the brain lobes (Monastirioti et al., 1995; Monastirioti et al., 1996; Vömel and Wegener, 2008). However, although these putative octopamine/tyramine release sites have been identified using immunological and genetic techniques to label the presence of the neurotransmitter and putatively functional release sites, their functional relevance remains unclear. Octopamine/tyramine have been shown to be involved in regulating larval crawling behaviour in this as well as previous studies (Saraswati et al., 2004; Fox et al., 2006). However, it is unclear, where these neuromodulators are released and which tissue functions are targeted in order to modulate larval crawling. Octopamine and/or tyramine may modulate behaviour by targeting neuronal function in the CNS, however, possible effects on motor neurons by neurotransmitter or paracrine release mechanisms have so far not been investigated. To identify potentially direct effects of octopaminergic/tyraminergic neurons on motor neurons, octopaminergic/tyraminergic neuronal membranes or putative release sites and dye backfilled motor neurons innervating muscles M6/M7 were simultaneously labelling (Figure 4.3). Two motor neurons, RP3, also referred to as MN6/7-1b, and RP5, also referred to as MNSNb/d-Is, can be labelled by backfilling from muscle M6 and M7 NMJs (Broadie and Bate, 1993; Landgraf et al., 1997; Choi et al., 2004). The images shown have morphologically identified late 2nd instar RP3 motor neurons, based on the dorsal localisation along the midline and their characteristic contralateral projections (Choi et al., 2004). It is possible that, in principle, in addition to RP3 and RP5 motor neuron innervating muscles M6 and M7, additional motor neurons and, at times, octopaminergic/tyraminergic VUM neurons could labelled due to the close proximity of external muscle layers, such as muscles M15 and M14.1 and M14.2 (Landgraf et al., 1997).

The putatively octopaminergic/tyraminergic synaptic terminals were labelled by over-expression of fluorescent synaptic terminal marker proteins. Expression of GFP- labelled synaptotagmin (UAS-syt.eGFP) and neuronal synaptobrevin (UAS-nsyb.eGFP) (Zhang et al., 2002), both synaptic vesicle proteins, consistently label

the neuropilar regions of the larval CNS. These synaptic terminal markers labelled extensive regions of varicosities clusters throughout the VNC neuropile as well as throughout the brain lobes, suggesting that octopamine/tyramine are released in the larval CNS. Over-expression of these GFP labelled proteins can lead to dislocalisation from the synaptic terminals and eventually lead to filling of dendrites, axons and even cell bodies. In these experiments, however, there is no evidence of dislocalisation, indicating that the varicosities are indeed most likely to be neuromodulator release sites. The motor neuronal dendrites are in very close proximity to these putative GFP-labelled neuromodulator release sites. These data thus suggest that octopamine/tyramine are putatively released in close proximity to motor neuronal membrane. A direct effect on motor neuronal function is therefore not unlikely.

Fig. 4.3

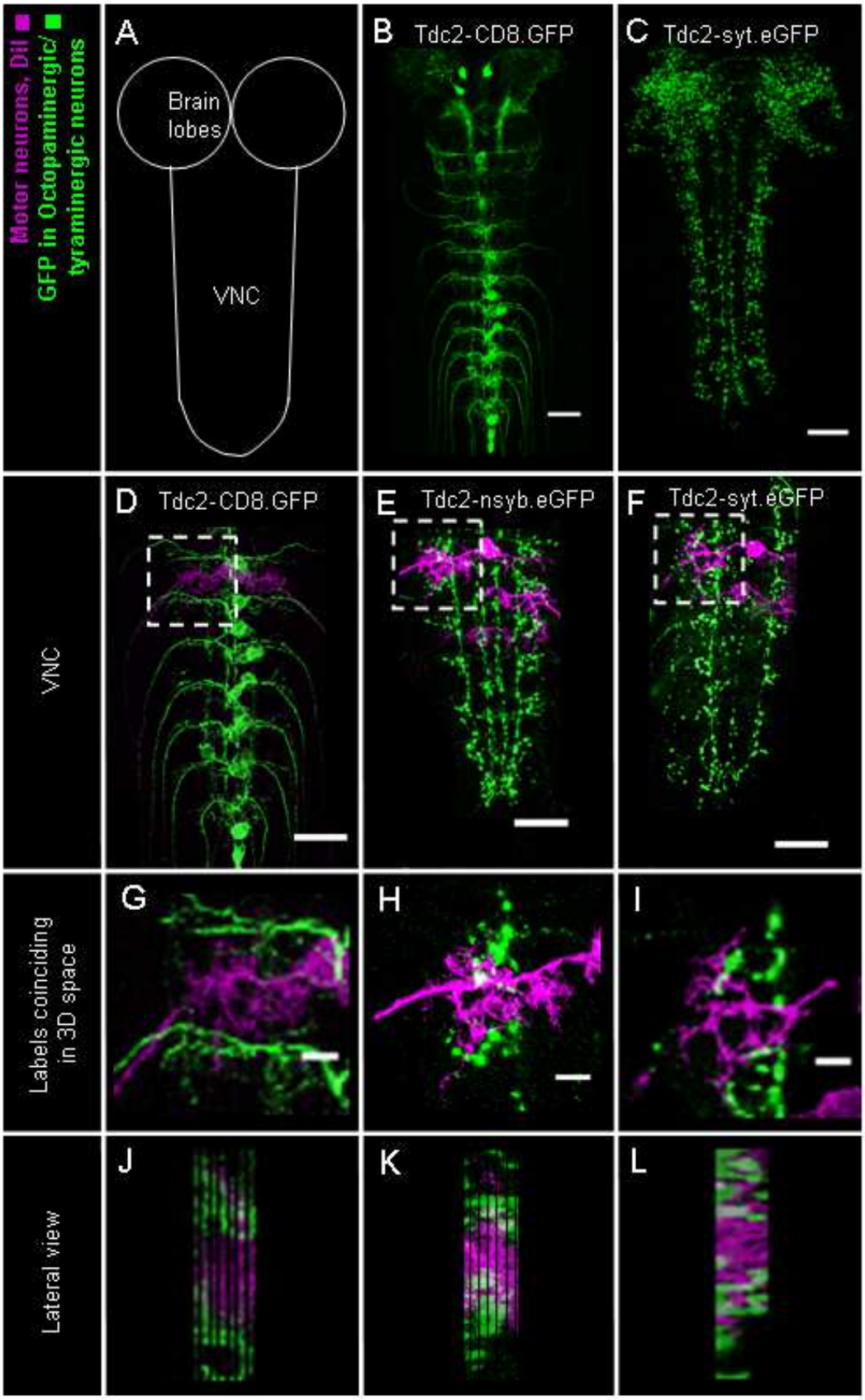
Motor neurons, DiI
GFP in Octopaminergic/
tyraminergic neurons
A
Brain
lobes
VNC
B Tdc2-CD8.GFP
C Tdc2-syt.eGFP
VNC
D Tdc2-CD8.GFP
E Tdc2-nsyb.eGFP
F Tdc2-syt.eGFP
Labels coinciding
in 3D space
G
H
I
Lateral view
J
K
L

Figure 4.3 Expression pattern and localisation of octopaminergic/tyraminergic neuronal cell bodies and putative release sites in relation to dendrites of identified motor neuron in the larval CNS. Octopaminergic/tyraminergic cell bodies, dendritic branches and synaptic terminal varicosities: green (GFP). Motor neuronal cell bodies, dendritic branches and axons: magenta (DiI dye). **A**. Schematic outline and orientation of larval CNS, the VNC and brain lobes. **B**. Expression pattern of octopaminergic/tyraminergic cell bodies and projections in the brain lobes as well as into the VNC neuropile regions. Cell bodies are found in the brain lobes and in a regular pattern in a ventral median position in each of the thoracic and abdominal segments in the VNC. **C**. Extensive localisation of labelled octopaminergic/tyraminergic synaptic terminal varicosities in the VNC and the brain lobes. **D.** Abdominal localisation of octopaminergic/tyraminergic cell bodies and projections. **E** and **F**. Localisation of labelled octopaminergic/tyraminergic synaptic terminal varicosities in the VNC and dye filled projections of motor neurons innervating muscles M6/M7. **G, H, I**. Coincident localisation of octopaminergic/tyraminergic synaptic terminals and motor neuronal dendrites simultaneously found in close proximity in a confined 3D space within the VNC. **J, K, L**. Lateral view of coinciding motor neuronal dye labels and synaptic terminal markers in confined 3D space in the VNC. Scale bars: 20µm (**B-F**), 5µm (**G-I**).

In summary, these data provide circumstantial evidence that putatively octopaminergic/tyraminergic neurons expressed throughout the larval CNS project their synaptic terminal varicosities throughout the larval CNS and the neuropil regions, the primary space of synapse formation in the CNS. In addition, labelling motor neurons dendrites shows that the octopaminergic/tyraminergic release sites are in very close proximity. Therefore, direct modulatory effects of neuronal function in the CNS are possible.

4.2.3 A subset of octopaminergic/tyraminergic without peripheral targets have release sites close to motor neuron dendrites and exert behavioural effects

An additional attempt was made to determine the localisation, projections and potentially the function of a subpopulation of octopaminergic/tyraminergic neurons. In light of research in other model systems suggesting an important modulatory role for the suboesophageal ganglia in behaviour (Brodfuehrer and Friesen, 1986b; Cornford et al., 2006), I attempted to genetically sub-fractionate the putatively octopaminergic/tyraminergic neuronal population by using the selective GAL4 suppressor protein, tshGAL80. GAL80 is an enhancer trap, which exerts its effects by binding to the GAL4 transcriptional activator domain and thereby suppresses GAL4 activity (Suster et al., 2004). When GAL4 and GAL80 are used in combination, Upstream Activator Sequence (UAS) controlled gene expression is suppressed in cells where both GAL4 and GAL80 are present. The tshGAL80 construct selectively suppresses GAL4 driven expression of the transgenic UAS construct in thoracic and abdominal segments in the VNC. Using this genetic tool

only enables neurons located in the brain lobes, the suboesophageal ganglia and the abdominal apex of the VNC to express the UAS transgenic construct (Figure 4.4). Expression of the UAS construct is therefore suppressed in the thoracic and most of the abdominal segments. Strikingly, very extensive projections throughout the brain lobes as well as throughout the VNC neuropilar regions were identified, although these were characteristically weaker in their intensity compared to unsuppressed Tdc2-GAL4 driven expression. The weaker intensity probably reflects on a loss of GFP expression in these regions as a result of blocked GFP expression from VUM neurons in the VNC. Furthermore, extensive putatively octopaminergic/tyraminergic synaptic varicosities were identified throughout the brain lobes as well as the neuropile in the VNC, thereby largely reproducing the projection patterns and putative release sites when all putatively octopaminergic/tyraminergic neurons are labelled. Moreover, synaptic varicosities were again identified in very close proximity to motor neuronal dendritic branches. However, in contrast to the unsuppressed Tdc2-driven GFP expression, there are far fewer synaptic varicosities in the dorsal areas of the VNC neuropile where most motor neurons project their dendrites and establish synaptic connections (Mauss et al., 2009). Also, labelling the putatively octopaminergic/tyraminergic neuronal membrane by CD8::eGFP did not visualise axonal projections to the peripheral body wall musculature as only internal projections throughout the CNS were identified, with the exception of the neurons labelled in the apical posterior segment A8. This is congruent with currently unpublished data in another study (Dr. Andreas Thum, unpublished data). Putative octopaminergic/tyraminergic neurons located in the anterior parts of the larval CNS have extensive projections in the brain lobes and the VNC, thus putatively releasing these neurotransmitters in the CNS. The lack of peripheral projection from anteriorly located octopaminergic/tyraminergic neurons suggests that the reported effects on the structure and morphology of modulatory octopaminergic type II and excitatory glutamatergic type I synaptic boutons in the peripheral musculature are likely to be exerted by VUM octopaminergic/tyraminergic neurons in the abdominal segments of the larval VNC (Landgraf and Thor, 2006; Vömel and Wegener, 2008; Koon et al., 2011).

Fig. 4.4

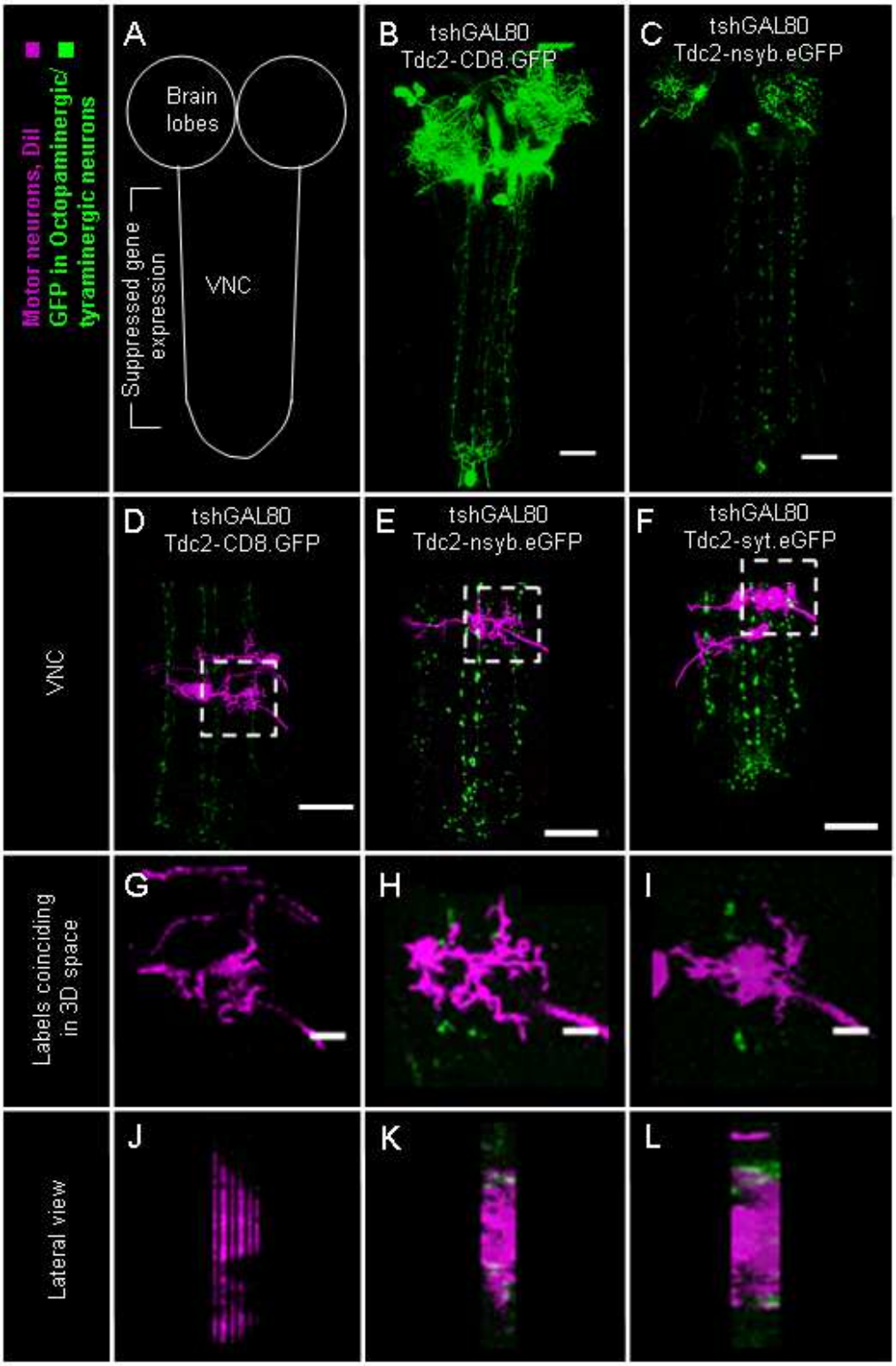
Motor neurons, DiI
GFP in Octopaminergic/ tyraminergic neurons
A
Brain lobes
VNC
Suppressed gene expression
B tshGAL80 Tdc2-CD8.GFP
C tshGAL80 Tdc2-nsyb.eGFP
VNC
D tshGAL80 Tdc2-CD8.GFP
E tshGAL80 Tdc2-nsyb.eGFP
F tshGAL80 Tdc2-syt.eGFP
Labels coinciding in 3D space
G
H
I
Lateral view
J
K
L

Figure 4.4 Expression pattern and localisation of a subpopulation of octopaminergic/tyraminergic neuronal cell bodies and putative release sites in relation to dendrites of identified motor neurons in the larval CNS. Octopaminergic/tyraminergic neuronal cell bodies, dendritic branches and synaptic terminal varicosities: green (GFP). Their spatial proximity to muscle M6/M7 innervating motor neuronal cell bodies, dendritic branches and axons: magenta (DiI dye). **A.** Schematic outline and orientation of larval CNS, the VNC and brain lobes outlining the extensive suppression of gene transcription in neurons localised in the VNC. **B.** Expression pattern of octopaminergic/tyraminergic cell bodies and projections in the brain lobes as well as into the VNC neuropil regions. Cell bodies are also labelled at the apex of the VNC, probably labelling neurons in abdominal segment A8. **C.** Localisation of labelled octopaminergic/tyraminergic synaptic terminal varicosities in the VNC and the brain lobes. **D.** Abdominal localisation of octopaminergic/tyraminergic projections. **E** and **F.** Localisation of labelled octopaminergic/tyraminergic synaptic terminal varicosities in the VNC and dye filled projections of motor neurons innervating muscles M6/M7. **G, H, I.** Coinciding localisation of octopaminergic/tyraminergic synaptic terminals and motor neuronal dendrites simultaneously found in close proximity in a confined 3D space within the VNC. **J, K, L.** Lateral view of coinciding motor neuronal dye labels and synaptic terminal markers in confined 3D space in the VNC. Scale bars: 20µm (B-F), 5µm (G-I).

The role of this putatively octopaminergic/tyraminergic neuronal subpopulation in crawling behaviour was tested by using the low resolution behavioural assay. Interestingly, expression of the TeTxLC in this subpopulation of octopaminergic/tyraminergic neurons indeed affects crawling speed in 3rd instar larvae (Figure 4.5). The crawling speed is reduced by almost 20% upon expression of TeTx* in these neurons. The overall speed is similarly reduced. The stride length again remains constant, whilst the stride duration is increased in proportion to the reduction in crawling speed. This data suggests that a putatively octopaminergic/tyraminergic subpopulation located in the anterior brain regions modulates behaviour by neurotransmitter release in the CNS alone as no peripheral projections from these neurons were identified. The reduction in crawling speed is proportionately smaller compared to blocking all octopaminergic/tyraminergic neurons in the CNS (Chapter 3). It implies that the effects of blocked octopaminergic/tyraminergic neurotransmission in this neuronal subpopulation noticeably contributes to the speed reduction, but is not solely responsible for it. Conversely, this also argues against a modulation of solely muscular or NMJ functions to affect segmental muscular contraction and relaxation rates and thereby reducing the crawling speed.

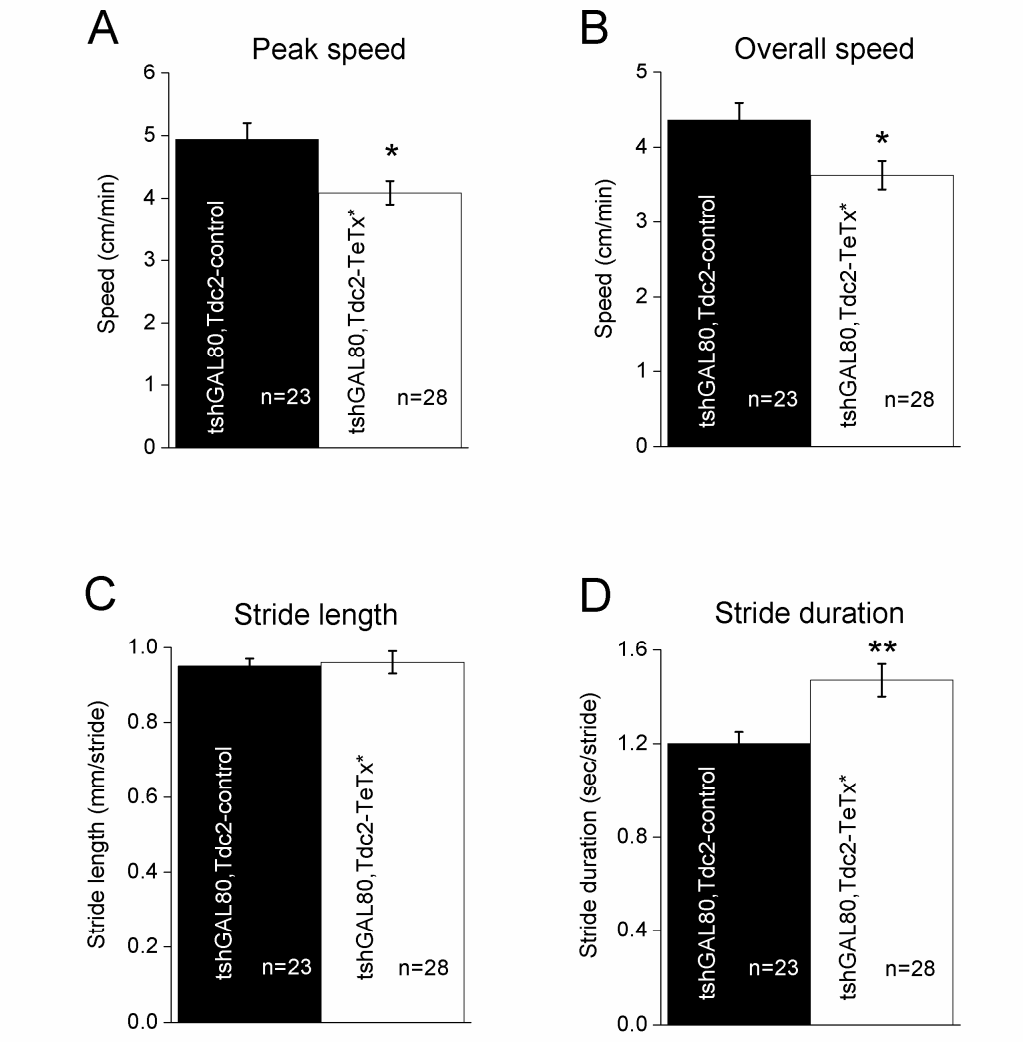

Figure 4.5 Impact of blocked neurotransmitter release from a subset of octopaminergic/tyraminergic neurons on larval crawling behaviour. **A** and **B.** The peak and overall speed is reduced in tshGAL80,Tdc2-TeTx* larvae compared to the tshGAL80,Tdc2-control genotype. **C**. The stride lengths are identical in both genotypes. **D**. The stride duration is increased in tshGAL80,Tdc2-TeTx* larvae.

In summary, these data therefore provide the first line of evidence that an octopaminergic/tyraminergic neuronal subpopulation projects its axons and synaptic terminals into the VNC neuropile into close proximity to motor neuronal dendrites. In addition, blocking neurotransmitter release from this octopaminergic/tyraminergic neuronal subpopulation alone can induce a locomotion deficit. This suggests an important functional role for these specific octopaminergic/tyraminergic neurons in locomotion.

4.2.4 Octopamine receptor Octβ2R is expressed in the larval and adult CNS as well as in aCC and RP2 motor neurons

The close proximity of octopaminergic/tyraminergic synaptic terminals to the dendritic branching of motor neurons suggests a potentially direct modulation of motor neuronal function by neurotransmitter or paracrine release. In order to test this hypothesis, the expression of the octopamine receptor subtype Octβ2R in larval and adult CNS was tested in a preliminary pilot study (Figure 4.6) (Evans and Maqueira, 2005). This receptor subtype has been shown to regulate morphology and function of excitatory glutamatergic type I and modulatory octopaminergic type II NMJs (Landgraf and Thor, 2006; Koon et al., 2011). The expression of this receptor was also tested specifically in aCC and RP2 motor neurons, which innervate the dorsal body wall muscles M1 and M2, respectively.

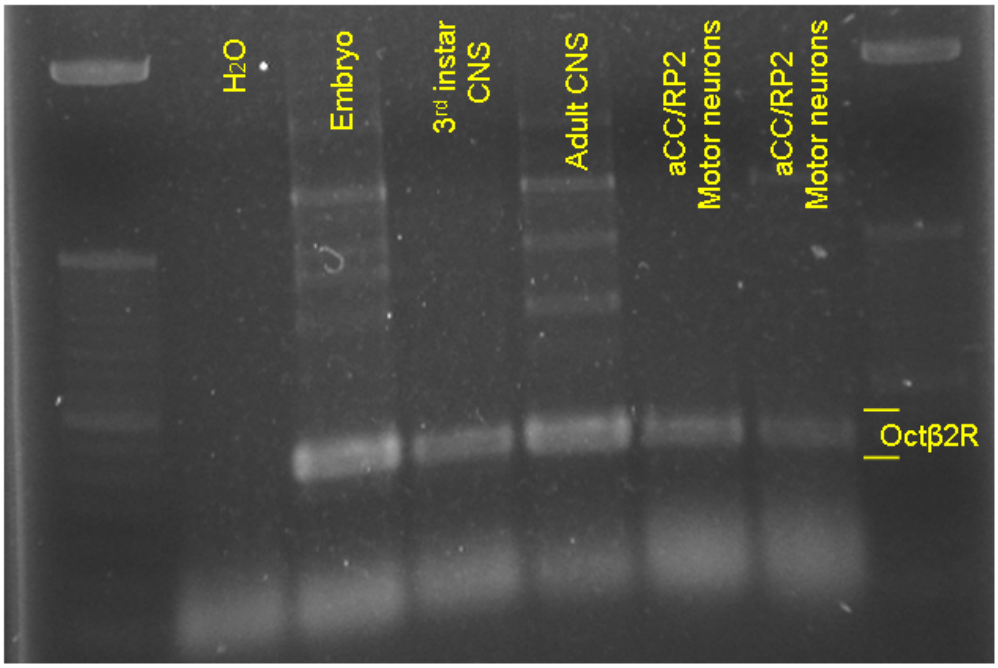

Figure 4.6 Expression of the Octβ2R receptor subtype in the adult and larval CNS as well as in aCC and RP2 motor neurons. RT-PCR reactions show Octβ2R cDNA products of the Octβ2R receptor in the embryo, larval CNS, adult CNS and in aCC/RP motor neurons (Work completed by Dr. Wei-Hsiang Lin).

The data in (Figure 4.6) indicate that this receptor subtype is likely to be expressed in embryonic tissue, the larval as well as the adult CNS, and more importantly, in identified motor neurons in the larval CNS. This suggests that, in addition to a close spatial proximity to octopaminergic/tyraminergic release sites, larval motor neurons express a receptor required to receive and be modulated by octopaminergic signalling.

4.3 DISCUSSION

4.3.1 Identified motor neuronal dendrites are in close proximity to octopaminergic/tyraminergic varicosities and express the Octβ2R receptor

Octopamine and tyramine are established neuromodulators in crawling behaviour (Chapter 3) (Saraswati et al., 2004). However, although their effects on the structure and function of the larval NMJ have been well established (Nishikawa and Kidokoro, 1999; Nagaya et al., 2002; Koon et al., 2011), modulation of other potential target tissues and organs have remained largely unaddressed. Several previous studies have identified the putative presence and localisation of octopaminergic/tyraminergic neurons as well as their axonal and putative synaptic projections throughout the larval CNS (Monastirioti et al., 1995; Monastirioti et al., 1996; Vömel and Wegener, 2008). However, it remains unclear if and where these neurons release octopamine/tyramine in the CNS and, more specifically, in segmental locomotor unit CPGs as well as what effects they exert.

This study established a close spatial proximity of extensive octopaminergic/tyraminergic synaptic terminal varicosities and dendritic branches of two motor neurons innervating muscles M6 and M7 (Figure 4.3). This implies a potentially direct modulatory effect by synaptic or paracrine octopamine/tyramine release on the function of interneurons and motor neurons in the direct vicinity of these release sites. Synaptic terminals are frequently found in varicosity clusters along axonal projections and may indicate multiple synapses at one varicose bouton (Shepherd et al., 2002). However, varicosities may or may not contain synapses and may release neuromodulators as a paracrine signal into their vicinity. For example, studies in rats have shown that serotonergic varicosities in both the Substantia Nigra and Raphe nucleus are non-junctional and allow serotonin diffusion of approximately 5µm, acting paracrinally on other neurons (Bunin and Wightman, 1998). Similarly, serotonergic varicosities in the locust CNS have been suggested to be largely non-junctional (Peters and Tyrer, 1987). Also, in locusts, dorsal unpaired median neurons do not form synapses on muscles (Hoyle et al., 1980), whereas in the CNS octopaminergic neurons have been implied to form synapses (Watson, 1984). In *Drosophila*, serotonergic neurons display regular and patterned distribution of putatively non-junctional paracrine release similar to octopaminergic/tyraminergic distribution (Chen and Condron, 2008; Vömel and Wegener, 2008; Chen and Condron, 2009). Despite the similarities in biogenic amine synthesis, recycling and degradation pathways, it cannot be determined at the moment, if octopaminergic/tyraminergic varicosities contain synapses in the

Drosophila larval CNS. Nevertheless, the reported diffusion distance for paracrine signals to remain effective, substantially exceeds the distance observed between putative octopaminergic/tyraminergic release sites and motor neuronal dendrites in this study. It is therefore not unlikely that a direct paracrine modulatory effect is exerted by octopamine/tyramine on motor neuronal function.

However, it remains unclear, if octopamine and/or tyramine are putatively released in synapses or as paracrine signals into the neuropil. Further experiments using fluorescent dyes as well as genetic tools including fluorescent dendritic marker proteins and GFP Reconstitution Across Synaptic Partners (GRASP) would be able to identify potential direct synaptic connections between motor neurons and putatively octopaminergic/tyraminergic neurons (Feinberg et al., 2008; Tripodi et al., 2008; Nicolai et al., 2011).

A direct modulatory effect of octopamine/tyramine on motor neuronal function is further supported by the finding that at least one of the octopamine receptor subtypes is expressed in the larval CNS and, more specifically, in aCC/RP2 motor neurons, which innervate dorsal muscles (Figure 4.6) (data: Dr. Wei-Hsiang Lin). This is a very important finding showing that octopamine released in the CNS can act upon specific receptors in motor neurons and potentially additional neurons in the CNS. In conclusion, both these findings strongly imply that octopamine released in the VNC neuropile can act upon motor neurons and modulate their function. This suggests that octopamine and/or tyramine in the *Drosophila* larval CNS are likely to act directly on motor neurons, which may either form an integral part of segmental unit CPGs or receive direct CPG inputs. However, the potentially paracrine release of these neuromodulators into the neuropil could potentially affect a substantial number of interneurons. This could at least partially account for the substantial functional effects of octopamine and/or tyramine in the modulation of locomotor CPGs.

4.3.2 An anterior subpopulation of octopaminergic/tyraminergic neurons in the CNS with release sites in the VNC neuropile exerts behavioural effects

Octopaminergic/tyraminergic neurons are extensively expressed throughout the larval CNS. Numerous octopaminergic neurons are found in the brain lobes, the suboesophageal ganglia as well as in a repetitive pattern across the thoracic and abdominal segments (Dr. Andreas Thum, unpublished) (Vömel and Wegener, 2008). In this study, a genetic tool enabled a selective and extensive suppression of marker protein expression in putatively octopaminergic/tyraminergic neurons in the thoracic and abdominal segments, with the exception of abdominal segment A8

(Figure 4.4). Neurons located in either the brain lobes or the suboesophageal ganglia project into the posterior abdominal segments of the larval VNC. With the exception of abdominal segment A8, no projections to peripheral body wall muscles in the abdominal segments have been identified in these larvae as seemingly all axonal and synaptic terminal projections are contained within the CNS. Similar suboesophageal octopaminergic neuronal clusters are also seen in other insects including the honey bee, cockroach, locust and *Drosophila* adult brains (Bräunig and Burrows, 2004; Sinakevitch et al., 2005; Busch et al., 2009) and have been shown to be relevant in the integration of sensory information resulting in direct effects in neurons located in the VNC (Duch et al., 1999). In locust, the suboesophageal neurons have also been shown to exclusively project into the VNC and not to project into the periphery (Bräunig and Burrows, 2004). These neurons also provide many of the extensive octopaminergic/tyraminergic synaptic terminal varicosities in the VNC neuropil. Moreover, the putative synaptic release sites from these anterior octopaminergic/tyraminergic neurons are also in close proximity to dendritic branches of motor neurons innervating muscles M6 and M7. This suggests that the anterior clusters of octopaminergic/tyraminergic neurons may have a role in modulating interneurons and motor neurons in the VNC.

Blocking neurotransmitter release from neurons located only in the anterior parts of the larval CNS also resulted in a behavioural deficit. The peak and overall speed are reduced by approximately 20% each (Figure 4.5). Strikingly, again, the stride length remains constant, whilst the stride duration is increased inversely proportionately to the decreased crawling speed. However, the reduction in crawling speed is not as severe as blocking octopaminergic/tyraminergic neurotransmission throughout the CNS. Furthermore, a regulatory or modulatory role for neurons located in anterior regions of the CNS and more specifically, suboesophageal ganglia, have been shown for other model systems including locust, leech and lamprey (Brodfuehrer and Friesen, 1986b, a; Duch et al., 1999; Grillner, 2006). These neuronal clusters are thus likely to have an important role in the modulation of crawling behaviour by targeting neurons in the VNC.

In summary, octopaminergic/tyraminergic neurons located in the suboesophageal ganglia and brain lobes in the larval CNS project their axons and synaptic terminals into the VNC. The putative neuromodulator release sites are in close spatial proximity to identified motor neurons and may imply a functional role in the modulation of locomotor CPG networks. This study provides evidence that octopamine and/or tyramine modulate crawling behaviour, which is, at least partially, likely to be due to modulation of neuronal function in the CNS.

4.3.3 Conclusions

In conclusion, this work has shown that octopaminergic/tyraminergic neurons are fully developed and presumably synthesise these neuromodulators throughout the larval developmental stages. The octopaminergic/tyraminergic neurons project their axons and synaptic terminal varicosities throughout the brain lobes and VNC neuropile and are in close proximity to dendritic branching of identified motor neurons. Many of these projections and synaptic terminals in the VNC originate from the brain lobes and suboesophageal ganglia, which also have putative release sites in the vicinity of motor neuron dendrites. This subpopulation of octopaminergic/tyraminergic neurons exerts a modulatory effect on larval crawling behaviour. The larval CNS and more specifically, aCC/RP2 motor neurons, express the Octβ2R receptor subtype, which may be the primary receptor for octopaminergic modulation of neuronal physiology in the CNS and potentially of locomotor CPG function.

Chapter 5

Octopamine and Tyramine in the Modulation of Neuronal Function and Synaptic Physiology

5.1 INTRODUCTION

Locomotion requires a finely tuned coordination of different tissues and individual cells. The remarkable diversity of different methods of locomotion across the animal kingdom and great variability of locomotive modes within a species offer a glimpse of the complexity and the physiological challenges posed by locomotion. Moreover, the organisation and architecture of an organism requires both a remarkable degree of functional robustness of the entire organism and its individual components as well as flexibility to successfully adapt to changes and to enable highly diverse behaviours. This challenge can be exemplified by the spinal cord CPG networks in bipedal and quadrupedal mammals, which can provide different outputs to enable diverse behaviours including walking, running, scratching, jumping, reverse walking, and swimming. The flexibility of individual neuronal circuits and the coordination of multiple networks to enable diverse behaviours is a remarkable feature and requires a substantial degree of modulation by various signalling molecules. By comparison, the diversity of behaviour in *Drosophila* larvae is relatively limited as it is usually confined to bouts of forward locomotion, turning behaviour, directional changes as well as occasional reverse crawling. Furthermore, in contrast to pedal locomotion, the architecture in larvae is far simpler with virtually identical abdominal segmental motor neuronal and muscular organisation across the larval body. However, the challenges and principles that enable flexible modulation of CPG networks whilst maintaining robustness and stability in a network remain the same.

The preceding chapters have identified the modulatory effects of octopamine and tyramine on crawling behaviour in larvae as well as muscle physiology. However, previous studies have shown that, in addition to identified octopaminergic effects in modulating muscle physiology in *Drosophila* and other insects (Evans and Siegler, 1982; Evans, 1984; Zumstein et al., 2004), these signalling molecules also modulate neuronal and synaptic physiology. For example, octopamine has been shown to increase the EJP amplitude in several model systems, including locust, crayfish, moth and *Drosophila* (Evans and O'Shea, 1977; Breen and Atwood, 1983; Nagaya et al., 2002) and has been shown to modulate NMJ synapse formation (Koon et al., 2011). In contrast, tyramine decreases the EJP amplitude in *Drosophila* (Nagaya et al., 2002). Octopamine has also been shown to increase both graded and evoked synaptic transmission in the lobster pyloric CPG (Johnson and Harris-Warrick, 1990; Johnson et al., 2011). In locust, octopamine has been shown to reduce the ESPC

amplitude and to increase the afterhyperpolarisation in sensory neuronal to motor neuronal synaptic transmission (Parker, 1996; Leitch et al., 2003). Moreover, octopamine has also been shown to transform tonic activity patterns into bursting activity in locust flight interneurons (Ramirez and Pearson, 1991a; Ramirez and Pearson, 1991b). In both locust and spider, octopamine increases the spike frequency of sensory neurons (Matheson, 1997; Widmer et al., 2005). In *Drosophila*, activation of the OAMB octopamine receptor has been suggested to reduce the Slowpoke Ca^{2+}-activated K^{+} current (Crocker et al., 2010). These studies exemplify the effects of octopamine and, to some extent, tyramine in modulating synaptic physiology as well as the intrinsic neuronal excitability. Such effects can transform the output of a neuronal network, as shown in the crustacean pyloric CPG (Flamm and Harris-Warrick, 1986b; Flamm and Harris-Warrick, 1986a).

In light of these studies, the potent modulatory effects of octopamine and tyramine on larval crawling behaviour and the widespread octopaminergic/tyraminergic synaptic terminal projection in the CNS, the effects of these neurotransmitters on the total CPG network output, the NMJ and motor neuronal synaptic transmission and the intrinsic motor neuronal excitability were investigated. The model proposed in Chapter 3 suggests that octopamine and tyramine modulate crawling speed by primarily modulating the time delay of inter-segmental unit CPG activation during forward contraction waves, whilst the individual segmental unit CPG outputs on their respective muscle groups remain constant. This flexibly modulates the stride duration, whilst the stride length and the segmental operational range remain constant. This chapter attempts to address, which target tissues are affected by octopaminergic/tyraminergic signalling and what mechanisms of action they employ when modulating crawling behaviour.

2.1.3 Hypotheses

- The duration and frequency of bursts of EJP activity patterns from segmental unit CPGs onto their respective muscle groups during fictive forward locomotion remains constant, which may account for the constant segmental operational range.
- The time delay between the ending of bursting EJPs in two adjacent segments during fictive forward locomotion is prolonged in larvae with compromised octopaminergic and/or tyraminergic signalling and may account for the reduced speed.
- Functional modulation of octopaminergic and/or tyraminergic signalling induces bursting activity patterns in identified motor neurons.
- Functional modulation of octopaminergic and/or tyraminergic signalling reduces evoked synaptic current amplitudes in identified motor neurons.
- Developmental modulation of octopaminergic and/or tyraminergic signalling does not affect the amplitude of evoked or miniature excitatory junction potentials at the neuromuscular junction.

5.2 RESULTS

5.2.1 The Effects of Octopaminergic/Tyraminergic Modulation on the Timing and Output of Adjacent Segmental Unit CPG Networks

The constant stride length in many tested genotypes with chronically altered neuromodulator signalling as well as more detailed data on segmental lengths and operational ranges in the previous chapters suggest that the output of individual segmental CPG networks onto their respective muscle groups remains constant. Instead, it suggests that the time delay of bursting activity in the next anterior segmental unit CPG network during forward locomotion is altered. This hypothesis was further investigated in third instar larvae with an exposed intact CNS and peripheral muscles that enable intracellular recordings in muscles M6 in adjacent abdominal segments, ranging from abdominal segments A2-A4, during fictive forward locomotion bouts of the larvae (Cattaert and Birman, 2001; Barclay et al., 2002). This enabled an analysis of the duration of bursts onto a single muscle, the frequency of spiking as well as the time delay of the termination of bursts in two adjacent segments as the onset of bursting in two adjacent segments is more variable. Compared to other methods of measuring segmental unit CPG output during fictive locomotion, this technique offers a higher resolution of the activity of only 2 neurons (Cattaert and Birman, 2001; Barclay et al., 2002), RP3 and an RP5, innervating muscle M6 (Broadie and Bate, 1993; Landgraf et al., 1997; Choi et al., 2004). By comparison, *en passant* suction electrodes record the simultaneous activity of segmental and intersegmental nerves containing all 30 efferent motor neurons as well as afferent sensory neurons per hemisegment and thereby limit the spatial and temporal resolution of neuronal activity (Sink and Whitington, 1991; Fox et al., 2006). These experiments are technically difficult because of strong muscle movements during fictive locomotion, which make stable muscle impalements by the recording electrodes very challenging. I therefore chose to pursue this work by comparing Tdc2-control with $T\beta H^{nM18}$ larvae. These two genotypes have displayed the biggest difference in their locomotion speed with the exception of CS wild-type larvae, as shown in Chapter 3. Providing these two genotypes displayed the same segmental operational ranges in the segments used for electrophysiological analysis limited the required sample size to determine, if the differences in the intersegmental time delay were significant. As suggested in Chapter 3, the shorter stride length of $T\beta H^{nM18}$ larvae is due to a shorter segmental operational range in the posterior abdominal segments, whereas the anterior abdominal segments are largely comparable.

In order to verify if segmental unit CPG networks are likely to be comparable, the segmental lengths in a relaxed state at rest, at peak contraction and the resulting segmental operational ranges were determined for the anterior abdominal segments A2, A3 and A4 in

Tdc2-control and TβH^{nM18} larvae (Figure 5.1). The analysis shows that the relaxed segmental length in all measured segments is shorter in TβH^{nM18} compared to Tdc2-control larvae, whilst differences were also seen between segments within the genotypes. In a peak contracted state, segments are also shorter in TβH^{nM18} compared to Tdc2-control and length variations are also apparent within the same genotypes. Despite the segmental lengths being different in relaxed as well as peak contracted states, all the segmental operational ranges both within and across different genotypes are virtually identical (Figure 5.1). These results provide further circumstantial evidence that the segmental operational range is largely constant across all the segments in larvae. All parameters shown in Figure 5.1 are virtually identical in abdominal segment A6 in Tdc2-control larvae as presented in Chapter 3 compared to abdominal segments A2-A4 ($p>0.05$ in all cases). In TβH^{nM18} larvae, however, the lengths of the abdominal segments A2-A4 are smaller compared to A6 and the segmental operational ranges in these segments are larger in the same segments, respectively ($p<0.001$ in all cases). In contrast, the relaxed length of the respective segments is comparable and only shows a marginal difference between segments A2 and A6 ($p<0.05$).

A constant segmental operational range indicates that the output of the segmental unit CPG onto the respective segmental muscle group is comparable in order to achieve the same level of muscle contraction. Considering that the segmental operational ranges are constant across several abdominal segments in larvae that substantially differ in their crawling speed suggests that the segmental unit CPG output is constant, whilst the time delay of inter-segmental CPG activation is increased in slower larvae.

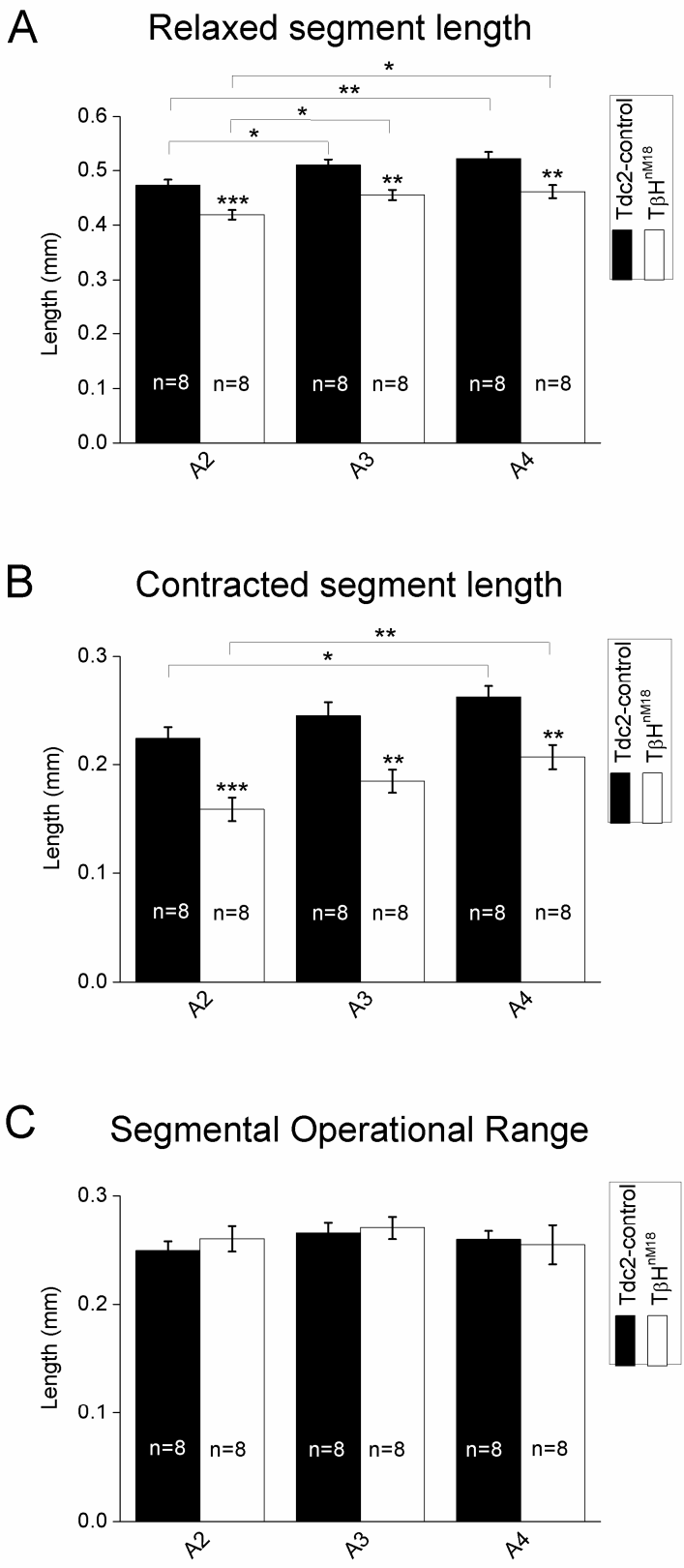

Figure 5.1 Segmental lengths in relaxed and contracted states as well as segmental operational ranges in abdominal segments A2, A3, and A4 in Tdc2-control and TβH^{nM18} larvae. **A.** Segmental length in relaxed resting state in abdominal segments A2-A4. In each segment, the segmental length is shorter in TβH^{nM18} larvae compared to Tdc2-control larvae. **B.** Segmental length in peak contracted state in abdominal segments A2-A4. In each segments, the segmental length is again shorter in TβH^{nM18} larvae compared to Tdc2-control. **C.** The segmental operational ranges are unchanged comparing TβH^{nM18} to Tdc2-control.

In order to test this hypothesis, CPG output onto muscles M6 in two adjacent segments was measured as described above in segments that displayed an identical segmental operational range. This approach enabled an electrophysiological comparison of the output and timing of segmental unit CPGs in both genotypes. The time delay of inter-segmental activation was measured from when the respective posterior segmental burst finished until the end of the anterior segmental burst. For unknown reasons, the time delay in the onset of bursting activity in adjacent segments is more variable. The inter-segmental burst delay is considerably prolonged in TβH^{nM18} compared to Tdc2-control larvae (Figure 5.2). This indicates a slower propagation of activity from posterior to anterior segmental unit CPG networks and accounts for a longer forward peristaltic wave in TβH^{nM18} larvae. In contrast, both the duration and spiking frequency of bursts from an individual segmental unit CPG onto muscle M6 are unchanged, which suggests that the output of individual CPG networks is indeed unchanged when comparing TβH^{nM18} to Tdc2-control larvae (Figure 5.2).

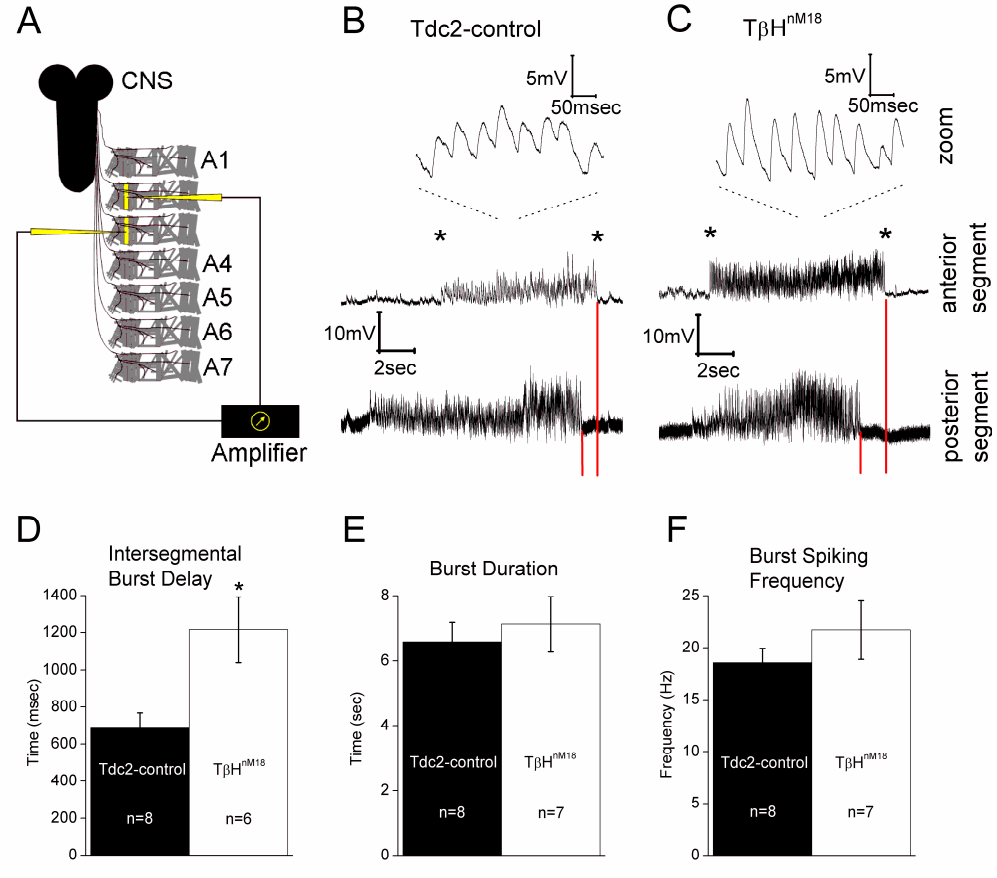

Figure 5.2 Simultaneous intracellular recordings in muscles M6 in two adjacent abdominal segments (A2-A4) recorded during fictive forward locomotion **A**. Schematic diagrams of recordings in muscles M6 in two adjacent segments in intact dissected 3rd instar larvae during fictive locomotion. **B** and **C**. Recordings of bursting EJPs after baseline adjustments onto muscles M6 in two adjacent abdominal segments in Tdc2-control larvae and TβH^{nM18} larvae during fictive forward locomotion. Burst durations in the anterior segments are indicated by asterisks. Time delay between the ends of bursting in two adjacent segments in both genotypes is indicated by red bars. **D**. Time delay of ending of bursting EJPs onto M6 in adjacent abdominal segments is increased in TβH^{nM18} compared to Tdc2-control larvae. **E.** Duration of bursting EJPs onto M6 in the anterior segment in each recording is unchanged. **F**. EJP frequency within a burst onto M6 in the respective anterior segments is also unchanged.

In summary, these data provide evidence that the segmental unit CPG network output onto their muscle groups are constant and account for the virtually identical segmental operational ranges. As a result, segmental muscle groups contract by the same length and traverse the same distance when activated during forward locomotion. This accounts for the constant stride length. Slower animals with chronically altered octopaminergic/tyraminergic signalling have slower anterior to posterior peristaltic contraction waves, which is due to an increased time delay of inter-segmental unit CPG burst activation.

5.2.2 Effects of Octopamine/Tyramine on the Intrinsic Membrane Excitability of Motor Neurons in the Ventral Nerve Cord

The results presented in this study show that octopamine and tyramine exert potent modulatory effects on larval locomotion, which affects the time delay of inter-segmental unit CPG activation. Data presented in Chapter 3 suggests that these neuromodulatory signals modulate segmental muscle contraction and relaxation rates, which could account for the slower peristaltic contraction waves and thus slower crawling. However, as shown in the preceding chapter and in previous studies (Monastirioti et al., 1996; Vömel and Wegener, 2008), octopamine is synthesised in numerous neurons in the larval VNC, the suboesophageal ganglion and to some extent in the larval brain lobes. Octopamine and probably tyramine are found in the VNC neuropil, but their functional relevance has so far not been elucidated in the larval brain, although numerous functions in adults for octopamine including the regulation of aggression (Hoyer et al., 2008; Zhou et al., 2008), learning (Unoki et al., 2005) and sleep (Crocker and Sehgal, 2008) are known. In the adult CNS, octopamine has been shown to putatively reduce the Ca^{2+}-dependent K^{+} currents by cAMP-dependent OAMB receptor subtype signalling (Crocker et al., 2010). Therefore, considering that putative octopaminergic/tyraminergic synaptic terminals are localised in close proximity to identified motor neurons and as a subpopulation has an identified effect on locomotion suggests that modulatory effects on synaptic physiology and/or intrinsic membrane excitability in the CNS are likely. In light of that, the role of octopamine and/or tyramine in the modulation of neuronal function was investigated. Based on the proposed model, the output of single segmental unit CPGs remains constant in response to chronically altered octopaminergic/tyraminergic signalling. It is therefore predicted that these neuromodulators affect the time delay of activity in interneurons involved in the temporal inter-segmental coordination or motor neurons or both whilst maintaining their characteristic activity patterns. Both a modulation of synaptic physiology as well as intrinsic membrane excitability in motor neurons delaying their activation could account for the increased inter-segmental burst delay. Based on previous publications, particularly the Shal and Shaker I_A currents were predicted as likely potential targets due to their ability to delay the onset of action potential firing (Choi et al., 2004; Schaefer et al., 2010; Ping et al., 2011).

The role of octopamine and/or tyramine in modulating the intrinsic membrane excitability in identified motor neurons was investigated. The intrinsic membrane excitability of

identified motor neurons was tested in current clamp mode, which allows the membrane potential to freely oscillate while successive current steps,increasing by 4pA, are injected into the neuron. Neurons display characteristic activity patterns in response to the stimuli and enable an assessment of the effects of neuromodulators on the intrinsic membrane excitability. The neuronal activity patterns were qualitatively classified into tonic action potential firing, mild bursting phenotypes with mildly burst-like action potential intervals, medium bursting phenotypes with variations in both basal voltages after action potential firing and irregular action potential interval as well as severe bursting phenotypes with major fluctuations in the membrane potential within and in between bursts of action potentials (Figure 5.3).

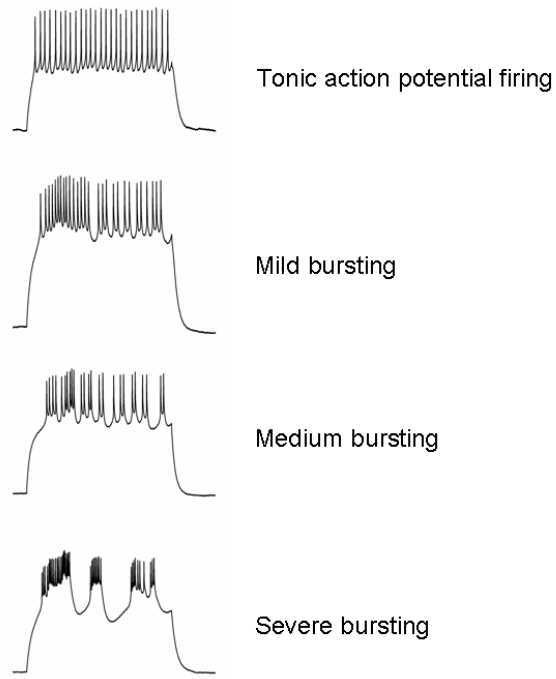

Figure 5.3 Examples of qualitative classification of bursting activity patterns in the motor neuronal intrinsic excitability.

The effects of blocking octopaminergic/tyraminergic neurotransmission were assessed in aCC motor neurons (Figure 5.4), which innervate dorsal body wall muscle M1, in both Tdc2-control and Tdc2-TeTx* larvae. These motor displayed a higher prevalence of bursting activity patterns in Tdc2-TeTx* larvae aCC compared to the Tdc2-control genotype (Figure 5.5). The higher prevalence of bursting phenotypes was seen in response to rheobase stimuli, which is defined as the first stimulus to elicit intrinsic activity patterns, as well as to subsequent higher current steps. For the sake of clarity only the rheobase and next subsequent stimuli are shown by the red and black traces in Figure 5.5, respectively. The qualitative assessment of bursting phenotypes suggests that bursting activity patterns are more frequent across all levels of phenotypic severity in Tdc2-TeTx* compared to Tdc2-control larvae. In addition, analysis of the relative proportions of each of the classes of

severity suggests that aCC motor neurons in Tdc2-TeTx* larvae tend to exhibit more severe bursting phenotypes. Both findings indicate that aCC motor neurons have both more frequent and severe bursting phenotypes in response to a chronically blocked release of octopamine and/or tyramine in Tdc2-TeTx* compared to Tdc2-control larval motor neurons. However, despite the strong qualitative differences between Tdc2-control and Tdc2-TeTx* larval motor neurons, a quantitative analysis of functionally relevant parameters did not exhibit any differences.

The time delay to the initial action potential being fired in response to the stimulus required to trigger action potentials, also referred to as rheobase stimulus, as well as to the ∆4pA and ∆8pA stimuli, which represent two subsequent current steps that are 4pA and 8pA higher than the rheobase stimulus, show no difference between these two genotypes. These results indicate that the onset of intrinsic activity patterns does not seem to be delayed in aCC motor neurons in Tdc2-TeTx* larvae to account for a contribution to the delayed inter-segmental activation in these larvae. In addition, the number of action potentials fired in response to 1sec stimuli in rheobase, ∆4pA and ∆8pA stimuli is also unchanged. Furthermore, the action potential amplitude and time interval between the action potentials is unaltered. Lastly, the threshold in the membrane potential to elicit action potentials is also unchanged. The qualitative differences in intrinsic activity pattern therefore seem to results in a similar functional output with unchanged action potential intervals, although the spatial integration of this activity across dendritic and axonal compartments cannot be extrapolated at this stage. To test, if the bursting is induced by the UAS-TeTx* transgenic construct itself, intrinsic excitability recordings were obtained from homozygous larvae, however, none of the recorded neurons displayed any bursting activity patterns (n=5 aCC and RPs).

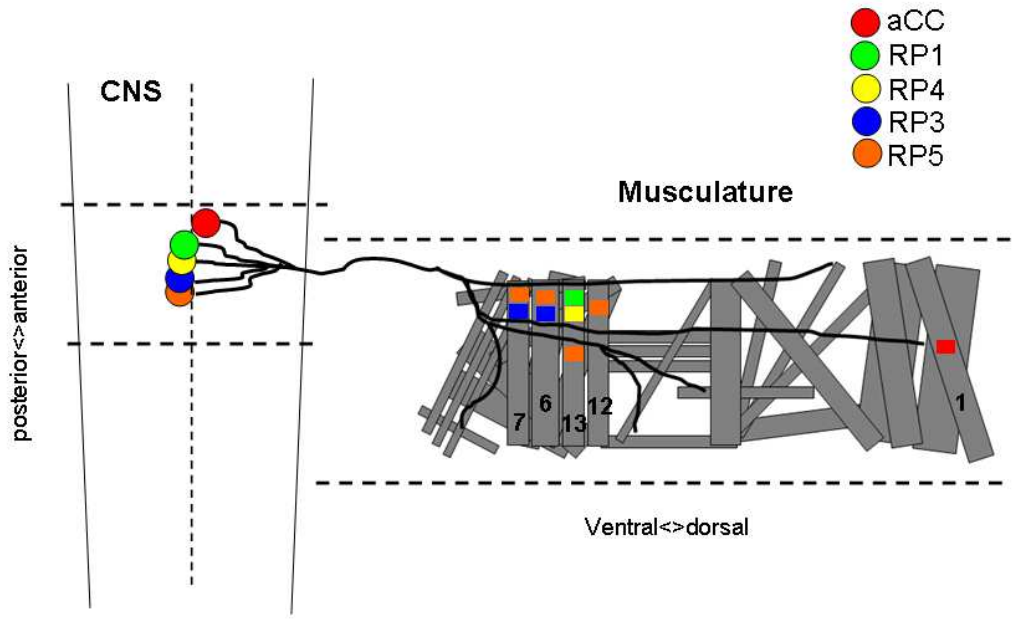

Figure 5.4 Schematic diagram of a group of motor neurons and the respective muscular targets in a single segment. The aCC motor neuron innervates the dorsal muscle M1. RP3 motor neurons innervate ventral muscle M6 and M7. RP1 and RP4 motor neurons innervate the ventral muscle M13 and RP5 innervates muscles M12, M13, M6, M7 (shown) as well as M30, M14, M15 and M16 in more external muscles layers (not shown) (Landgraf et al., 1997; Choi et al., 2004).

Fig. 5.5

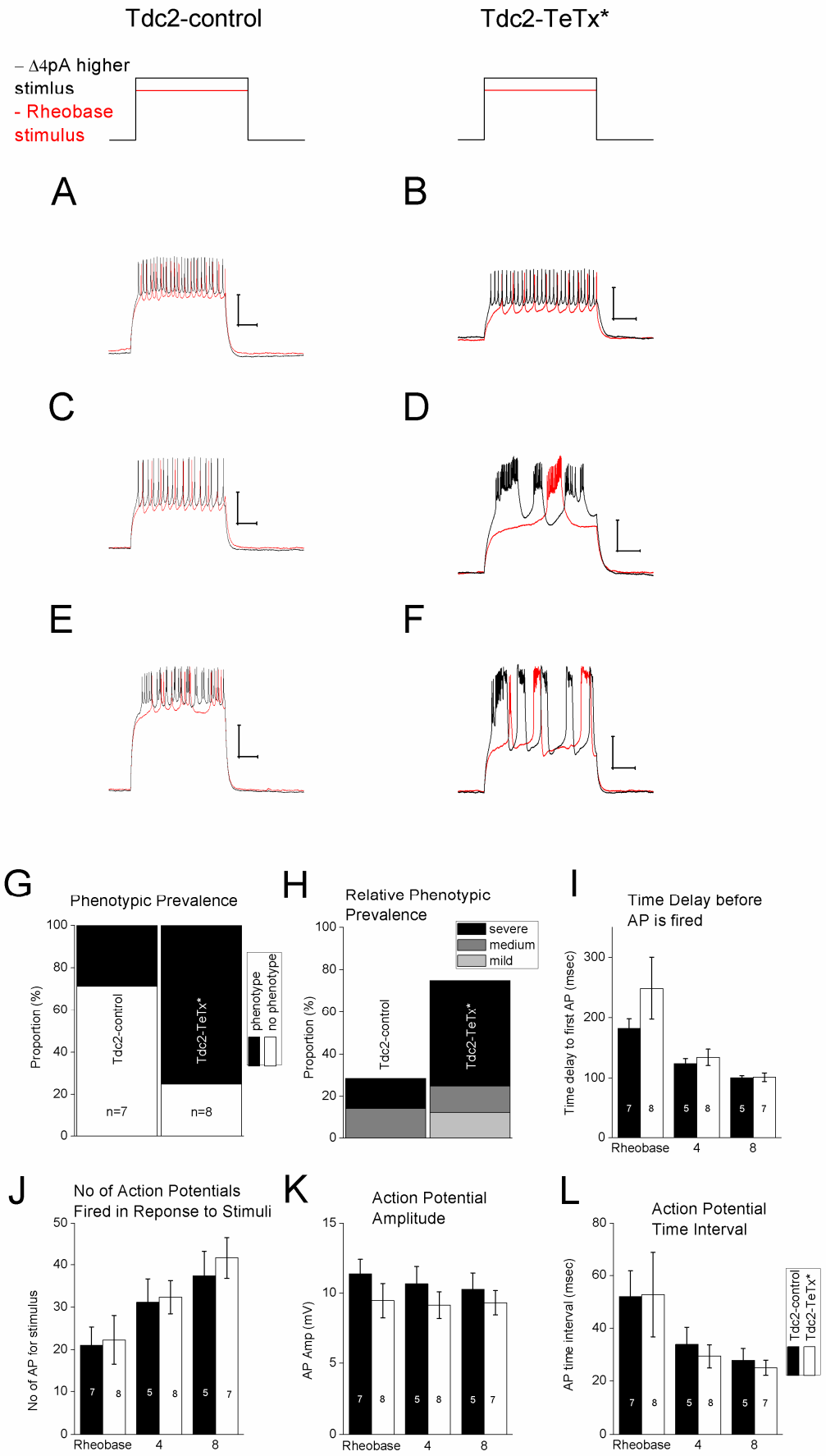
Tdc2-control
Tdc2-TeTx*
– Δ4pA higher stimlus
- Rheobase stimulus
A
B
C
D
E
F
G
Phenotypic Prevalence
Proportion (%)
Tdc2-control
Tdc2-TeTx*
n=7
n=8
phenotype
no phenotype
H
Relative Phenotypic Prevalence
severe
medium
mild
I
Time Delay before AP is fired
Time delay to first AP (msec)
Rheobase
4
8
J
No of Action Potentials Fired in Reponse to Stimuli
No of AP for stimulus
K
Action Potential Amplitude
AP Amp (mV)
L
Action Potential Time Interval
AP time interval (msec)

Figure 5.5 Intrinsic membrane excitability profiles of Tdc2-control and Tdc2-TeTx* aCC motor neurons. Neuronal activity patterns are triggered by injected current steps. The red traces represent the rheobase stimulus elicited intrinsic activity patterns, whilst black represent the activity in response to a 4pA higher stimulus. **A, C, E.** Tdc2-control aCC motor neuron excitability profiles. **B, D, F.** Tdc2-TeTx* aCC motor neuron excitability profiles. **G**. Proportional prevalence of bursting action potential firing in aCC motor neurons. **H**. Relative proportion of mild, medium and severe phenotypes of bursting in aCC motor neurons. **I**. The time delay to the initial action potential being fired is comparable for the rheobase stimulus in both genotypes as well as for the next two subsequent current steps, increasing by ∆4pA and ∆8pA compared to the rheobase stimulus, respectively. **J**. The number of action potentials fired in response to the rheobase, ∆4pA and ∆8pA current steps is also comparable. **K**. The action potential amplitude is also unchanged for the rheobase, ∆4pA and ∆8pA current steps. **L**. Lastly, the time interval between the action potentials fired in response to the rheobase, ∆4pA and ∆8pA current steps is also comparable. Scale bars (A-F): 200msec and 10mV.

The bursting phenotype is also sporadically observed in CS wild-type aCC motor neurons, similar to Tdc2-control larvae (Figure 5.6). In order to test, if the prevalence and severity of bursting activity patterns is due to altered octopaminergic/tyraminergic signalling, CS wild-type larvae were fed with a pharmacological agent, mianserine, to inhibit octopaminergic and/or tyraminergic signalling. As shown in Chapter 3, feeding mianserine, one of the most potent insect octopamine receptor antagonists (Evans and Robb, 1993; Evans and Maqueira, 2005), at a 10mM concentration to CS wild-type larvae for 2-3 hours prior to recording reproduces the locomotion deficit induced by the TeTxLC expression in octopaminergic/tyraminergic neurons to a similar degree. For the electrophysiological experiments, larvae were fed at the same concentration that was shown to exert behavioural effects in CS wild-type larvae. As a result of feeding mianserine, the prevalence and severity of bursting is markedly increased in aCC motor neurons (Figure 5.6). A block of octopaminergic/tyraminergic signalling thus induces bursting activity patterns in the intrinsic membrane excitability. In order to test, if the bursting phenotype in larvae with compromised octopaminergic/tyraminergic signalling can be rescued, Tdc2-TeTx* larvae were fed octopamine at a 150mM concentration prior to electrophysiological recordings. This concentration has been shown to partially rescue the locomotion deficit (Chapter 3). In aCC motor neurons a milder prevalence and proportionate severity of bursting phenotypes was observed. This result requires some caution as this is a qualitative observation and cannot be verified statistically with the available sample size of aCC motor neuronal recording in octopamine fed larvae. Thus, a larger data set is required to determine statistically, if feeding of octopamine can reduce the bursting phenotype.

Further experiments were conducted in an attempt to start to identify the likely molecular targets for the modulation of intrinsic activity patterns in motor neurons. Considering that no time delay in the activation of action potential firing was identified in Tdc2-TeTx* larval motor neurons, octopaminergic and/or tyraminergic effects on the I_A currents are unlikely. Previous studies in *Drosophila* muscles and neuronal cultures showed that a lack of the Ca^{2+}-dependent K^+ current in Slowpoke channel mutants displayed similarly bursting activity patterns (Saito and Wu, 1991; Brenner et al., 2000) This channel subtype was

therefore investigated as a potential target for octopaminergic/tyraminergic modulation. Similarly bursting activity patterns were identified in aCC motor neurons in *slowpoke* Ca^{2+}-dependent K^+-channel mutant slo^4 larvae, an inversion of the *slowpoke* gene, which eliminates Slowpoke currents (Atkinson et al., 1991; Brenner et al., 2000). The prevalence and severity of bursting activity in these larvae is comparable to aCC motor neurons in Tdc2-TeTx* larvae and therefore implies the Slowpoke channel as the molecular target for octopaminergic/tyraminergic modulation of intrinsic membrane excitability.

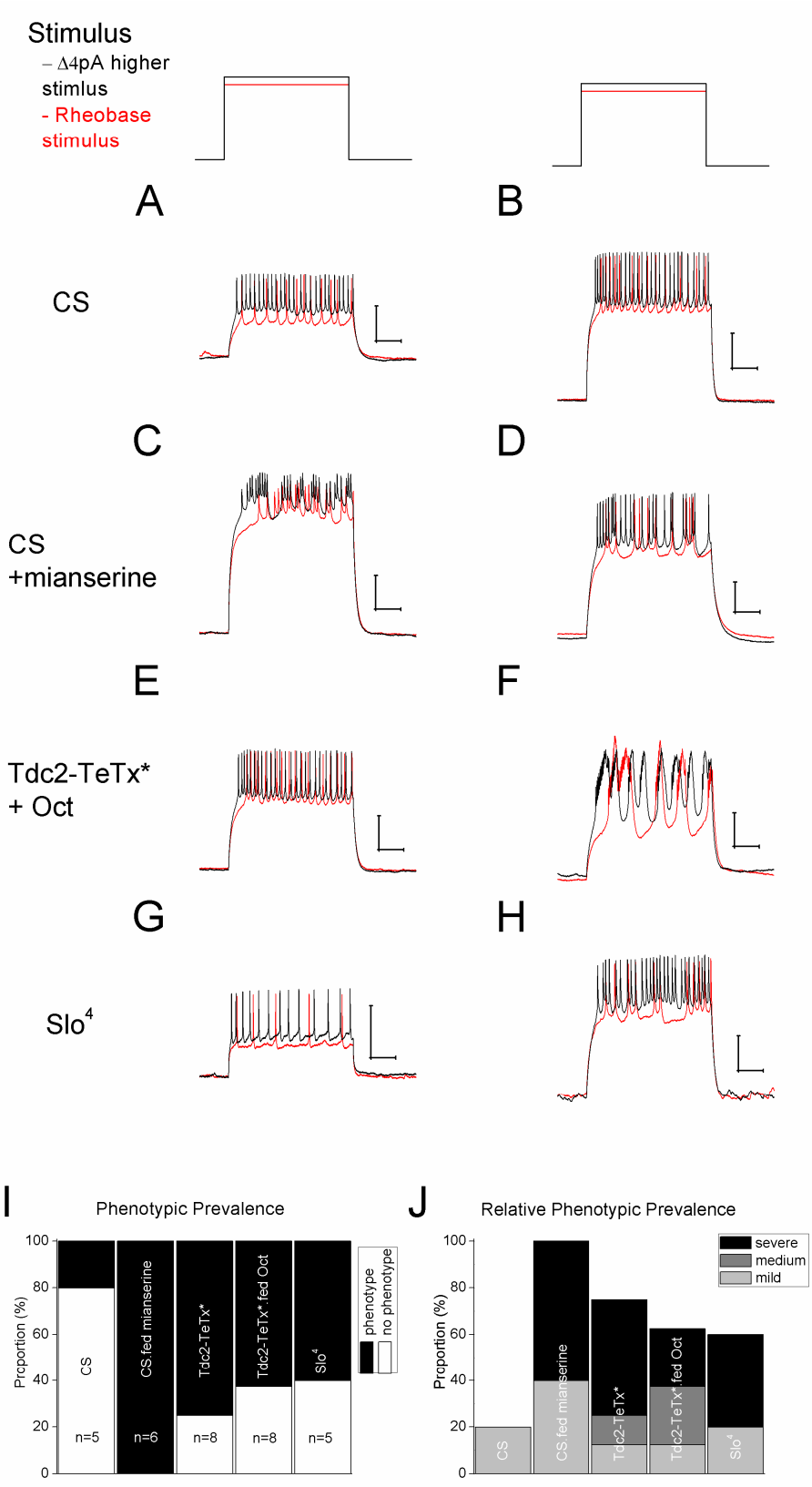

Figure 5.6 Phenotypic analysis of intrinsic neuronal excitability in aCC motor neurons in genetically or pharmacologically induced bursting. **A** and **B**. CS wild-type larvae. **C** and **D**. CS wild-type larvae fed 10mM mianserine for 2hours prior to recording. **E** and **F**. Tdc2-TeTx* larvae fed 150mM Oct for 2hours prior to recording. **G** and **H**. *Slowpoke* K^+-channel mutant slo^4. **I**. Proportion of neurons in CS, CS+mianserine, Tdc2-TeTx*, Tdc2-TeTx*+Oct, and slo^4 recordings with and without bursting phenotype. **J**. Relative proportion of mild, medium and severe bursting phenotypes in each of the phenotypes present. Scale bars: 10mV,200msec.

In summary, these results suggest that a lack of octopaminergic/tyraminergic neuromodulation leads to bursting activity patterns in response to depolarising stimuli in aCC motor neurons. An increased prevalence and severity of bursting activity patterns can be induced genetically and pharmacologically. In addition to identified effects in Tdc2-TeTx* larvae, feeding the octopamine and tyramine receptor antagonist mianserine induces bursting activity phenotypes in both ventral and dorsal muscles innervating motor neurons in CS wild-type larvae. Both findings substantiate the evidence that reduced octopaminergic/tyramine signalling is responsible for these effects. Feeding octopamine to Tdc2-TeTx* larvae at a concentration that shows a partial behavioural rescue results in a mildly decreased prevalence and severity of bursting activity patterns in aCC motor neurons. However, due to the comparatively low sample sizes in these experiments, results need to be considered with caution. Lastly, the bursting activity is also observed in slo^4 larvae, a mutant *slowpoke* Ca^{2+}-dependent K^+-channel, which largely reproduce the prevalence and severity of bursting activity when compared to Tdc2-TeTx* larvae in aCC motor neurons.

5.2.3 Octopaminergic/Tyraminergic Effects on the Synaptic Physiology in Motor Neurons

In addition to the experiments on the role of octopamine and/or tyramine in the modulation of the intrinsic membrane excitability of motor neurons in the larval CNS, their effects on the synaptic physiology in identified motor neurons were tested in Tdc2-control and Tdc2-TeTx* larvae. It is known that octopamine exerts substantial effects on the synaptic physiology in the locust CNS. For example, octopamine has been shown to reduce the EPSC amplitude and to reduce afterhyperpolarisation in sensory neuron to flight motor neuron synapses (Parker, 1996; Leitch et al., 2003). It has also been shown to depolarise and induce plateau bursting potential in flight interneurons (Ramirez and Pearson, 1991a; Ramirez and Pearson, 1991b). Functional effects of octopamine and/or tyramine on the synaptic physiology in the Drosophila larval CNS are therefore not unlikely. These experiments aimed to identify their effects on the synaptic current amplitude as well as on the frequency and amplitude of spontaneous neurotransmitter release, which can indicate effects on the presynaptic vesicle release probability and/or postsynaptic effects including changes in the receptor subtype density, composition or functional modulation.

The experiments were conducted in the current clamp mode at a constant membrane voltage, which enables measurements of the synaptic inputs onto identified motor neurons. The evoked and spontaneous neurotransmitter release in both dorsal and ventral muscles innervating motor neurons was tested (Figure 5.4). The synaptic currents in motor neurons in the *Drosophila* larval CNS are mediated by acetylcholine release, which is the primary excitatory neurotransmitter in the insect CNS (Baines and Bate, 1998; Baines et al., 2001). The results show that chronically blocking neurotransmission from octopaminergic/tyraminergic neurons by TeTxLC in the larval brain has profound effects on

the synaptic input onto motor neurons innervating both ventral and dorsal muscles (Figure 5.7 & 5.8). A lack of octopaminergic/tyraminergic neurotransmission almost halves the synaptic current amplitude in the ventral muscles M6, M7, M12 and M13 innervating motor neuron pool (Figure 5.7). This group consists of RP1 and RP4 motor neurons innervating the ventral muscle M13, motor neuron RP3, which innervates muscles M6 and M7 as well as potentially RP5, which is located below the other RP motor neurons along the VNC midline and innervates a group of muscles including M6, M7, M12, M13, M14, M15, M16, M30 and is therefore likely unrepresented in this pool (Sink and Whitington, 1991; Van Vactor et al., 1993; Landgraf et al., 1997; Choi et al., 2004). However, the synaptic current half-width, rise time and decay are unchanged as is the synaptic current frequency in these isolated CNS preparations. Furthermore, the mEPSC amplitude and frequency are unchanged. The unchanged mESPC amplitude suggests that the postsynaptic membrane responsiveness is unaltered in response to chronically altered octopaminergic/tyraminergic signalling. In addition, the unaltered mEPSC frequency indicates that the presynaptic vesicle release probability is also unaffected.

Fig. 5.7

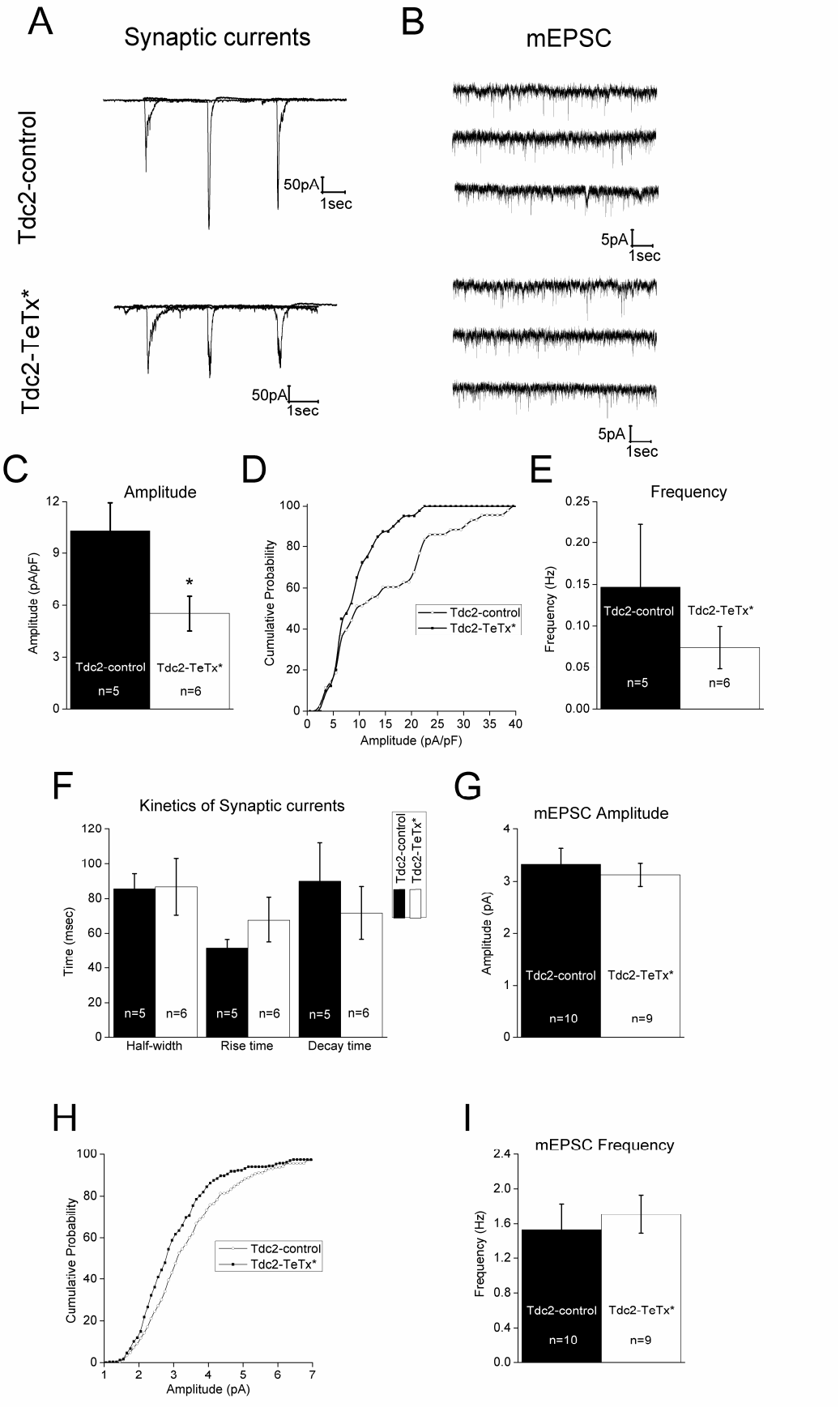
A
Synaptic currents
Tdc2-control
50pA
1sec
Tdc2-TeTx*
50pA
1sec
B
mEPSC
5pA
1sec
5pA
1sec
C
Amplitude
Amplitude (pA/pF)
Tdc2-control
n=5
Tdc2-TeTx*
n=6
*
D
Cumulative Probability
Amplitude (pA/pF)
Tdc2-control
Tdc2-TeTx*
E
Frequency
Frequency (Hz)
Tdc2-control
n=5
Tdc2-TeTx*
n=6
F
Kinetics of Synaptic currents
Time (msec)
Tdc2-control
Tdc2-TeTx*
n=5
n=6
n=5
n=6
n=5
n=6
Half-width
Rise time
Decay time
G
mEPSC Amplitude
Amplitude (pA)
Tdc2-control
n=10
Tdc2-TeTx*
n=9
H
Cumulative Probability
Amplitude (pA)
Tdc2-control
Tdc2-TeTx*
I
mEPSC Frequency
Frequency (Hz)
Tdc2-control
n=10
Tdc2-TeTx*
n=9

Figure 5.7 Effects of blocked octopaminergic and tyraminergic neurotransmission on synaptic physiology in RP1, RP3, RP4 and RP5 motor neurons innervating ventral body wall muscles. **A**. Overlaid traces of synaptic currents onto RP motor neurons from three neurons prior to capacitance adjustment in Tdc2-control and Tdc2-TeTx* larvae, respectively. **B**. mESPCs in Tdc2-control and Tdc2-TeTx* larvae in three neurons, respectively. **C**. The synaptic current amplitude is reduced in in Tdc2-TnTx* compared to Tdc2-control larvae. **D**. Cumulative probability of synaptic current amplitudes in Tdc2-control and Tdc2-TeTx* RP motor neurons. **E**. The synaptic current frequency reduction is statistically insignificant.. **F**. The differences in synaptic current half-width, rise time or decay time are statistically insignificant.. **G**. The mESPC amplitude is unchanged in Tdc2-control and Tdc2-TnTx* larvae. **H**. Cumulative probability of mESPC amplitudes in Tdc2-control and Tdc2-TeTx* RP motor neurons. **I**. The mESPC frequency is unchanged.

A similar result is found in dorsal muscle M1 innervating aCC motor neurons (Landgraf et al., 1997). The synaptic current amplitude recorded in aCC motor neurons is more profoundly reduced by approximately 67% (Figure 5.8). The half width is also reduced, whilst the rise and decay time are not statistically different. The synaptic current frequency is unchanged in these genotypes. However, in contrast to the RP motor neurons, the mEPSC amplitude is reduced as well, whereas the mEPSC frequency is unchanged. The reduced mEPSC amplitude indicates a reduced postsynaptic membrane responsiveness to the content of a single vesicle and the reduction is consistent across a random distribution as shown in the cumulative frequency of amplitudes. This suggests that this is not due to a small population of small conductance receptor subtypes, which skew the overall result, but that the reduction is consistent across all recorded amplitudes. This reduction could be due to several effects including an altered ligand-gated receptor subunit composition, which often differ in their conductance and kinetic parameters (DiAntonio et al., 1999; Thiagarajan et al., 2002). It could also indicate alterations in the receptor density on the postsynaptic membrane as a result of receptor trafficking or structural changes. Lastly, it can indicate a lower conductance due to functional channel modulation such as phosphorylation or other modifications (Varela et al., 2009). However, it remains unresolved, which effects are induced by a chronically blocked octopaminergic/tyraminergic neurotransmission and further electrophysiological and imaging studies would be required to determine these conclusively.

Octopamine/tyramine therefore have a profound effect on the synaptic physiology in *Drosophila* larval motor neurons. A lack of octopamine/tyramine reduces the synaptic current amplitude onto both dorsal muscle M1 innervating aCC motor neurons as well as ventral muscles M6, M7, M12 and M13 innervating motor neurons RP1, RP3, RP4 and RP5.

Fig. 5.8

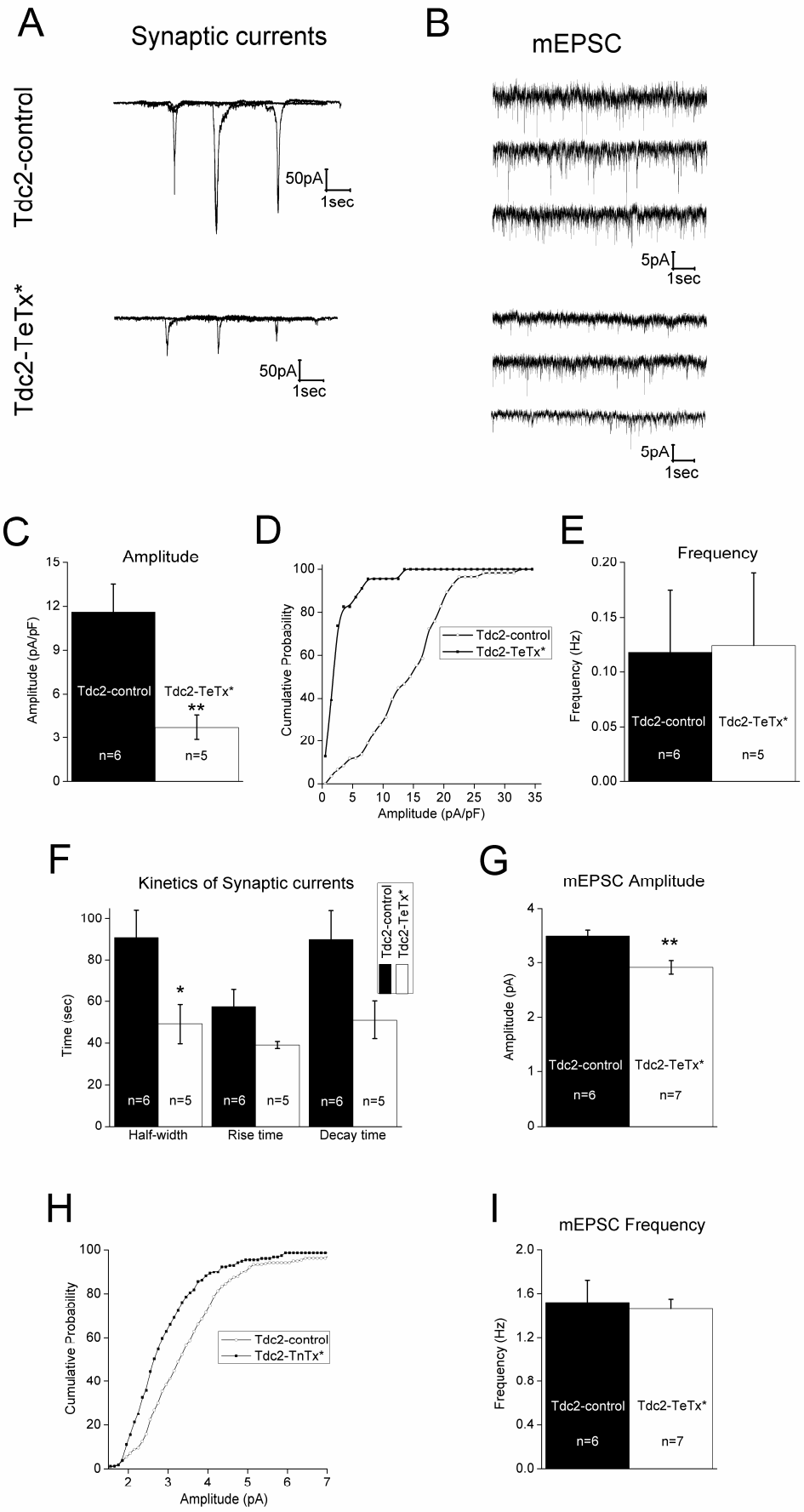
A
Synaptic currents
Tdc2-control
50pA
1sec
Tdc2-TeTx*
50pA
1sec
B
mEPSC
5pA
1sec
5pA
1sec
C
Amplitude
Amplitude (pA/pF)
Tdc2-control
Tdc2-TeTx*
**
n=6
n=5
D
Cumulative Probability
Amplitude (pA/pF)
Tdc2-control
Tdc2-TeTx*
E
Frequency
Frequency (Hz)
Tdc2-control
Tdc2-TeTx*
n=6
n=5
F
Kinetics of Synaptic currents
Time (sec)
Tdc2-control
Tdc2-TeTx*
*
n=6
n=5
Half-width
Rise time
Decay time
G
mEPSC Amplitude
Amplitude (pA)
**
Tdc2-control
Tdc2-TeTx*
n=6
n=7
H
Cumulative Probability
Amplitude (pA)
Tdc2-control
Tdc2-TnTx*
I
mEPSC Frequency
Frequency (Hz)
Tdc2-control
Tdc2-TeTx*
n=6
n=7

Figure 5.8 Effects of blocked octopaminergic and tyraminergic neurotransmission on synaptic physiology in aCC motor neurons innervating dorsal body wall muscle M1. **A**. Overlaid traces of synaptic currents onto aCC motor neurons in Tdc2-control and Tdc2-TeTx* larvae in three neurons before capacitance adjustments each, respectively. **B**. mESPC recordings in three aCC motor neurons in Tdc2-control and Tdc2-TeTx* larvae, respectively. **C**. The synaptic current amplitude is reduced in Tdc2-TnTx* compared to Tdc2-control to larvae. **D**. Cumulative probability of synaptic current amplitudes in Tdc2-control and Tdc2-TeTx* aCC motor neurons. **E**. The synaptic current frequency is unchanged. **F.** The synaptic current half-width is reduced, where the the synaptic current rise time and decay time remain unchanged. **G**. The mESPC amplitude is reduced. **H**. Cumulative probability of mESPC amplitudes in Tdc2-control and Tdc2-TeTx* aCC motor neurons. **I.** The mEPSC remains unchanged.

However, despite the substantial changes in the synaptic physiology, current clamp recordings, which allow the membrane potential to freely oscillate, show that synaptic currents in Tdc2-TeTx* in aCC motor neurons excite the motor neurons sufficiently to initiate action potentials and appear qualitatively very similar to Tdc2-control aCC motor neuronal current clamp recordings (Figure 5.9). Five current clamp recordings were obtained after the voltage clamp measurements in the aCC motor neurons data pool, which all displayed synaptic currents that elicited action potentials. This result implies that the reduction in synaptic current amplitude may be a homeostatic response as a smaller current may be required to depolarise the postsynaptic membrane to the threshold of action potential firing. The reasons for this observation are unclear, but this may be due to an increased input resistance, which is indicative of the number of ion channel expressed in the membrane. Stochastic openings of channel pores cause small conductances, often referred to as leak current, which have to be overcome by synaptic inputs in order to reach the action potential firing membrane potential threshold. A smaller number of ion channels would hence result in smaller leak currents that would need to be overcome to trigger action potentials by synaptic currents. Similarly, structural changes in the dendritic branching due to altered octopaminergic/tyraminergic signalling could affect the resistance in these fine projections, which also affect the total amplitude of current required to elicit the same depolarisation level. However, at this stage, the reasons for this observation remain inconclusive and further experiments would be required to elucidate the effects.

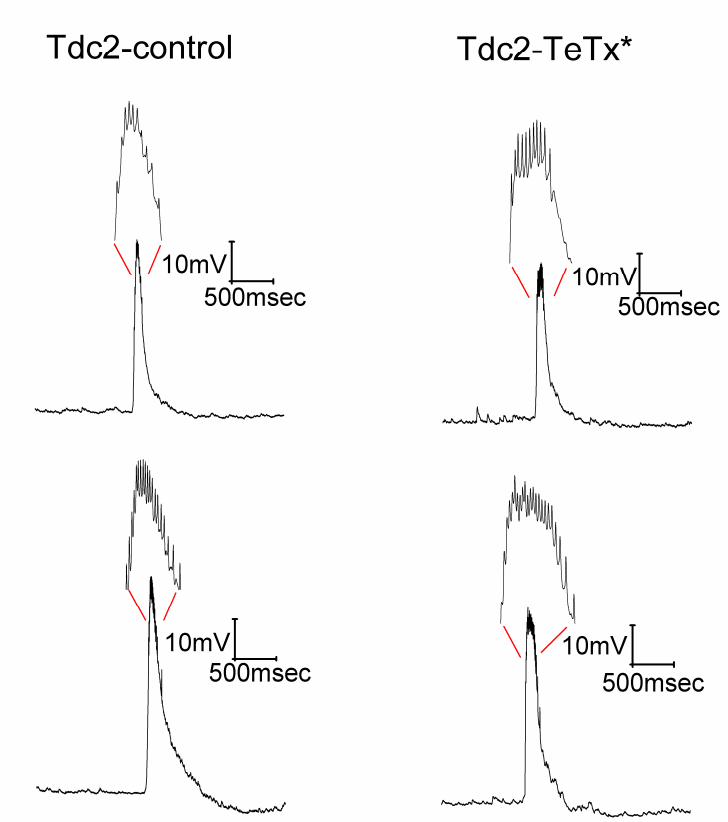

Figure 5.9 Synaptic currents in Tdc2-control and Tdc2-TeTx* aCC neurons recorded in current clamp mode.

5.2.4 Developmental Effects of Altered Octopaminergic/Tyraminergic Signalling on the Neuromuscular Junction Physiology

Considering that the output of segmental unit CPGs is likely to remain constant when octopaminergic/tyraminergic signalling is chronically modulated, it is important to determine where octopamine/tyramine exert their effects. Neurons containing octopamine innervate most peripheral body wall muscles in type II boutons (Monastirioti et al., 1995). In addition, neurons containing tyramine innervating peripheral muscles have also been identified (Nagaya et al., 2002). Although recent findings have shed some light on the effects of octopamine on synapse formation and morphology of both glutamatergic type I and octopaminergic type II synaptic terminals as well as its role in starvation stress responsiveness in *Drosophila* (Koon et al., 2011) and, similarly, in *C.elegans* (Suo et al., 2006), its long-term functional effects are largely unknown. Furthermore, the modulatory effects of octopamine and tyramine on neuromuscular junction (NMJ) physiology functions have largely been shown in response to *ad hoc* changes in their signalling functions. For example, the acute application of octopamine or tyramine increases and decreases the excitatory junction potential (EJP) amplitude in muscles M1 and M2, respectively (Nagaya et

al., 2002). Similarly, octopamine also acutely increases the EJP amplitude in muscles M12 and M13 (Koon et al., 2011). Conversely, in muscle M6 and M7, octopamine has been shown to decrease EJC amplitude (Nishikawa and Kidokoro, 1999). However, long-term effects are mostly unknown due to a lack of genetic tools in other model systems and remain unresolved in *Drosophila*. It is thus not clear, if a chronic lack of octopaminergic and/or tyraminergic modulation is homeostatically compensated or dramatically impacts the NMJ physiology and animal behaviour. As segmental contraction and relaxation rates are likely to be slowed in larvae with altered octopaminergic/tyraminergic signalling, changes to the NMJ physiology could account for the changes to muscle excitation and thereby modify the force and rates of muscle contraction. This hypothesis was investigated in 3rd instar larvae from genotypes used in the behavioural assays, TβH^{rM6} and TβH^{nM18} mutants as well as in Tdc2-control and Tdc2-TeTx* larvae, to test the effects of chronic changes in octopaminergic/tyraminergic signalling on the NMJ physiology. The NMJ electrophysiological experiments were carried out in larvae with severed nerves projecting from the CNS, which were stimulated, whilst the respective muscle was impaled to measure the EJP parameters. This technique provides a read-out of the NMJ synaptic efficacy and can indicate changes to NMJ physiology. Evoked neurotransmission as well as mEJP release was tested in muscles M6 and M12. In contrast to muscle M12, muscle M6 does not contain type II octopaminergic synaptic terminals and thereby does not receive direct octopaminergic synaptic innervation (Monastirioti et al., 1995). This experimental setup therefore enables a differentiated analysis of the NMJ physiology with and without direct octopaminergic innervation (Figure 5.10).

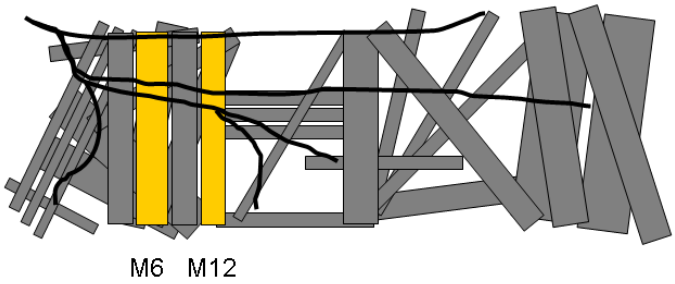

Figure 5.10 Schematic diagram of the localisation of peripheral body wall muscles M6 and M12.

The NMJ physiology was tested in TβH^{rM6} and TβH^{nM18} mutant genotypes, which display a severe locomotion deficit, to measure the amplitude and kinetic parameters half-width, rise time and decay time of EJPs (Figure 5.11). The EJP amplitude, half-width, rise time or decay time are unchanged when comparing these genotypes in both muscles M6 and M12. In addition, the mEJP amplitude and the quantal content, which reflects number of vesicles being released to excite the postsynaptic membrane, are unchanged in both muscles. This suggests that the synaptic efficacy and the postsynaptic muscle membrane responsiveness are unaffected by altered octopaminergic/tyraminergic signalling. However, in muscle M12, but not in muscle M6, the mEJP frequency is increased. This may indicate an

increase in the vesicle release probability from the presynaptic membrane and could be the result of a homeostatic response. In addition, the input resistance in muscle M6 is increased, but remains unchanged in muscle M12. These findings suggest that the number of ion permeable channels may be decreased in muscle M6.

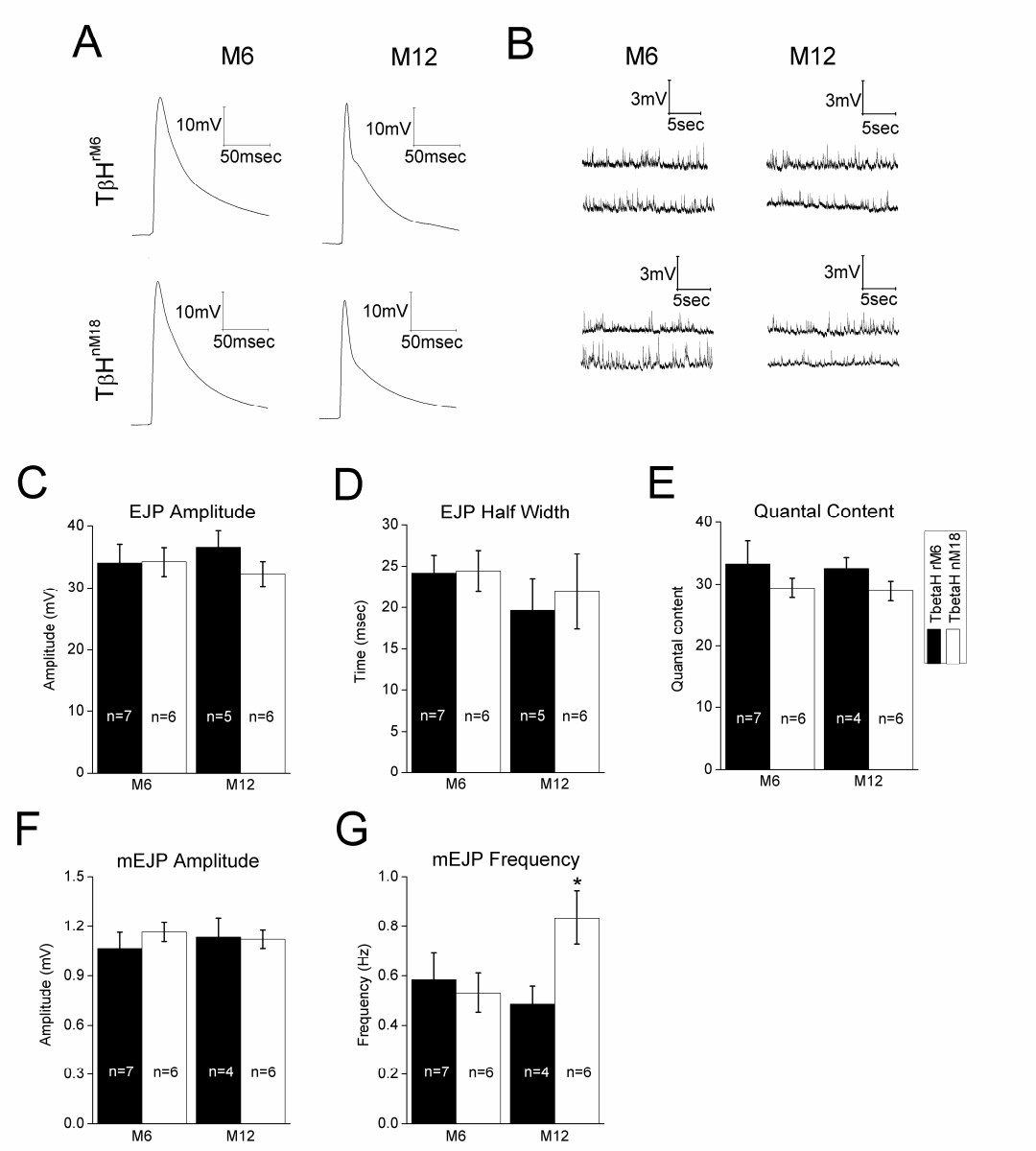

Figure 5.11 The function of the neuromuscular junctions in muscles M6 and M12 in TβH^{rM6} and TβH^{nM18} larvae. **A**. Sample traces from ten averaged EJPs in a single NMJ of TβH^{rM6} and TβH^{nM18} larvae in M6 and M12. **B**. mEJP recordings in respective genotypes and muscles. **C**. The EJP amplitudes in muscles M6 and M12 are unchanged in TβH^{rM6} and TβH^{nM18} larvae.. **D**. There are no changes in the EJP half-width in either M6 or M12 in TβH^{rM6} and TβH^{nM18} larvae. **E**. The differences in quantal content in M6 and M12 are statistically insignificant. **F**. The mEJP amplitude in M6 and M12 in both genotypes also remains unchanged..**G**. The mEJP frequency in M6 is comparable in both genotypes, but is increased in M12 TβH^{nM18} larvae.

A comparison of Tdc2-control and Tdc2-TeTx* larvae shows that there are minor changes to the NMJ physiology in these two genotypes (Figure 5.12). In muscle M12, no changes to any of the measured parameters have been identified and the input resistance also remains unchanged. No changes in EJP amplitude, half-width, rise or decay time, quantal content or mEJP amplitude and frequency are apparent. In contrast, whilst almost all parameters measured remained unchanged, the half-width in muscle M6 is increased in Tdc2-TeTx* larvae. The repolarisation of the muscle membrane in response to excitation is primarily due to K^+ channels. A change in the half-width could thus imply a change in the K^+ currents on the postsynaptic membrane.

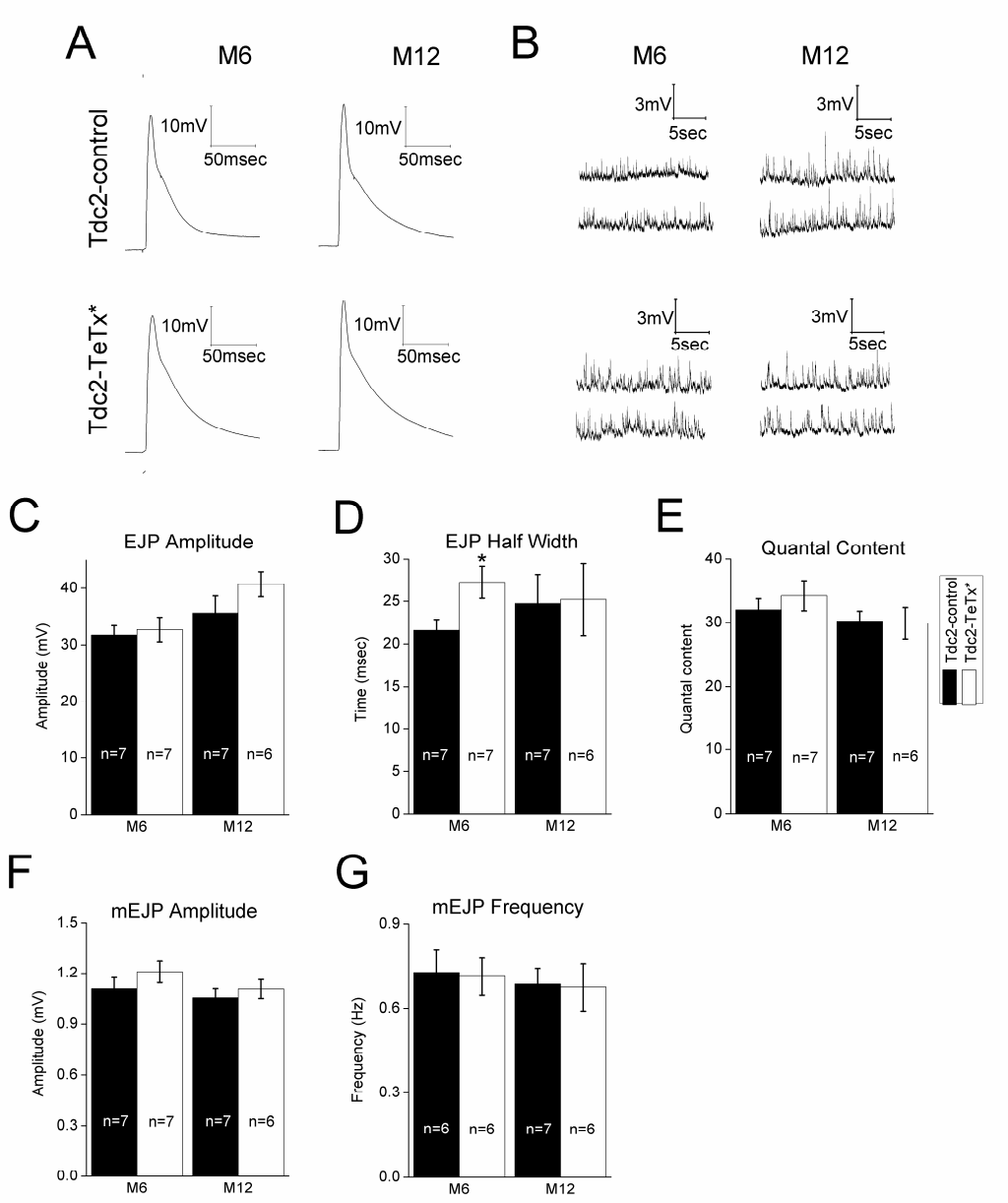

Figure 5.12 The function of the neuromuscular junctions in muscles M6 and M12 in Tdc2-TeTx* and Tdc2-control larvae. **A**. Sample traces from ten averaged EJPs in a single NMJ of Tdc2-control and Tdc2-TeTx* larvae in M6 and M12. **B**. mEJP recordings in respective genotypes and muscles. **C**. The EJP amplitude in muscles M6 and M12 remains unchanged in Tdc2-TeTx* larvae compared to the Tdc2-control genotype. **D**. The EJP half-width is increased in muscle M6, but remains unchanged in M12. **E.** The quantal content in M6 and M12 is unchanged in both genotypes. **F**. The mEJP amplitude in M6 and M12 also remains unchanged. **G**. The mEJP frequency also remains unchanged in both genotypes in muscles M6 and M12.

In summary, chronic changes in octopaminergic/tyraminergic signalling do not reliably affect the NMJ physiology. Minor changes in mEJP frequency, input resistance or EJP half-width have been detected, however, these changes are not consistent in both muscles or across the genotypes tested. The detected changes may indicate potential differences in the postsynaptic membrane as the half-width is increased in EJP half width in muscle M6 in Tdc2-

TeTx* larvae, which is most likely to be due to postsynaptic membrane currents repolarising the membrane potential. Similarly, the change in input resistance may indicate a change in the number of ion channels inserted into the muscle membrane. In addition, the increased mEJP frequency may indicate an increased vesicle release probability in $T\beta H^{nM18}$ mutant in muscle M12. However, the EJP and mEJP amplitude as well as the quantal content remain unchanged and therefore suggest that the overall synaptic efficacy is largely unaffected by altered octopaminergic/tyraminergic signalling. It is thus unlikely that the identified effects could account for the profound locomotion deficit in the tested genotypes. This is particularly striking in muscle M12, which receives direct octopaminergic innervation.

5.3 DISCUSSION

5.3.1 Octopamine/Tyramine Affect the Intrinsic Neuronal Excitability

Chronically blocking octopaminergic/tyraminergic signalling induces a higher prevalence and severity of bursting action potential firing patterns in dorsal muscle M1 innervating aCC motor neurons (Figure 5.5). The bursting activity is characterised by highly variable intervals between individual action potentials as well as between bursts of action potentials. Interestingly, apart from the bursting phenotype, neither the time delay to the first action potential being fired nor the number of action potentials in response to the stimulus is altered. In addition, the action potential frequency and amplitude are unchanged. Lastly, the membrane potential threshold for action potentials being fired is also unchanged. Feeding mianserine to 3rd instar CS wild-type larvae both reproduces the locomotion phenotype and induces an increased prevalence and severity of bursting activity patterns similar to Tdc2-TeTx* larvae (Figure 5.6). This finding suggests that the bursting motor neuronal activity patterns are due to a block of octopaminergic/tyraminergic signalling in the larval CNS. Mianserine is one of the most potent insect octopamine receptor antagonists (Evans and Robb, 1993; Evans and Maqueira, 2005) and is the most potent inhibitor of *Drosophila* β-adrenergic-like octopamine receptor induced increases in cAMP (Maqueira et al., 2005). However, the receptor affinity of this antagonist for the tyramine receptor and β-adrenergic-like octopamine receptors in *Drosophila* is very similar (Saudou et al., 1990; Maqueira et al., 2005), although its potency in inhibiting tyramine receptor function is currently unclear. Considering that feeding of yohimbine to Tdc2-control larvae did not induce a locomotion deficit at similar concentrations that have been shown to partially rescue the TβH^{nM18} locomotion deficit argues against a role of the tyramine receptor in modulating behaviour and possibly motor neurons. However, no data is available on the motor neuronal membrane excitability in yohimbine fed larvae to pharmacologically distinguish the role of octopamine and tyramine receptors. Conversely, feeding of 150mM octopamine to Tdc2-TeTx* larvae only marginally reduces the prevalence and severity of the bursting phenotype in aCC motor neurons. However, this cannot be verified statistically at this stage as the data pool is too small to detect small changes. It is currently inconclusive, if octopamine feeding can rescue the bursting activity pattern phenotype, but the data imply that this is a possibility. Octopamine has been shown to be involved in regulating bursting and tonic action potential firing patterns in previous studies. A transition from tonic action potential firing to bursts of activity has been shown in response to octopamine applications in locust flight interneurons (Ramirez and Pearson, 1991a; Ramirez and Pearson, 1991b). In contrast, this study suggests that either a lack of octopamine or tyramine induces bursting action potential firing patterns in motor

neurons. This indicates that octopamine and/or tyramine have opposite effects in the modulation of activity in functionally diverse neuronal populations in *Drosophila* and locusts.

Moreover, both the prevalence and severity of bursting activity patterns in the *slowpoke* mutant *slo*^{4} are strikingly similar to Tdc2-TeTx* motor neurons. It suggests that reduced Slowpoke Ca^{2+}-dependent K^{+}-channel currents alter the intrinsic membrane excitability and induce bursting activity patterns in aCC motor neurons. In previous studies, *slowpoke* channel mutants have also been shown to induce bursting activity patterns in both adult muscles in response to injected currents (Elkins et al., 1986; Elkins and Ganetzky, 1988) and neuronal cell cultures in *Drosophila* (Saito and Wu, 1991). The bursting action potential firing pattern is consistent with the role of Ca^{2+}-dependent K^{+}-channels in determining the frequency and rhythmicity of neuronal activity patterns (Hille, 2001). Slowpoke currents have been shown to regulate the delay of excitation, reducing the spike amplitude as well as increasing the spike duration and the interspike interval (Elkins and Ganetzky, 1988; Shao et al., 1999; Shahidullah et al., 2009). Furthermore, Slowpoke currents regulate the repolarisation rate of action potentials (Elkins et al., 1986; Saito and Wu, 1991; Brenner et al., 2000), particularly during sustained activity when action potentials gradually become longer over time due to an onsetting inactivation of I_A, whereas I_{Kca} currents inactivate slowly (Saito and Wu, 1991; Brenner et al., 2000). These findings are thus congruent with the known effects of Ca^{2+}-dependent K^{+}-currents on the intrinsic membrane excitability, however, further work is required to verify, if this channel is the primary target of octopaminergic/tyraminergic modulation in motor neurons.

In summary, this work suggests that chronically altered octopaminergic/tyraminergic signalling induces a higher prevalence and severity of bursting activity patterns in motor neurons innervating dorsal muscle M1. These findings provide evidence that motor neurons, which either form an integral part of segmental unit CPG networks or directly receive inputs from CPGs in *Drosophila* larvae, are functionally modulated by octopamine and/or tyramine. It therefore indicates that the motor neurons are likely cellular targets for the modulation of single or multiple unit CPG network in order to modulate crawling behaviour, although an indirect effect may account for this. Acute application of octopamine or tyramine as well as octopamine/tyramine receptor antagonists could determine if the bursting phenotype is indeed due to octopaminergic and/or tyraminergic signalling pathways. Furthermore, as the prevalence and severity of bursting activity is reproduced in the *slowpoke* mutant, circumstantial evidence implies this channel as the potential target of octopaminergic/tyraminergic modulation of motor neuronal function. However, further work is required to determine if a modulation of Slowpoke currents is necessary and sufficient to induce bursting activity patterns. This could be established by using Ca^{2+}-dependent K^{+}-channel antagonists such as charybdotoxin, which has limitations in the specificity, its inability to reproduce the mutant phenotype and its very narrow concentration range without affecting Ca^{2+} currents (Elkins et al., 1986). In addition, TEA has been suggested to be selective for Slowpoke currents in *Drosophila* and could be used to provide further evidence (Shahidullah

et al., 2009). The Slowpoke channel has been shown to be modulated by various kinases, cAMP, Calcium/calmodulin-dependent protein kinase II (CaMKII) and binding of proteins such as the Slo-channel binding protein (Slob) in *Drosophila* (Schopperle et al., 1998; Wang et al., 1999; Shahidullah et al., 2009). The cAMP signalling pathway is primarily utilised by β-adrenergic like octopamine receptors in the *Drosophila* genome (Balfanz et al., 2005; Koon et al., 2011) and the Octβ2R receptor has been suggested in this study to be expressed in aCC motor neurons (Chapter 4). Moreover, the cAMP signalling pathways has been shown to affect K^+ currents in *Drosophila* (Wright and Zhong, 1995; Zhao and Wu, 1997; Delgado et al., 1998), and, in some cases, has been attributed more specifically to octopamine receptors in identified neurons in adult brains, such as the OAMB receptor putatively reducing the Slowpoke Ca^{2+}-activated K^+ current (Crocker et al., 2010). Providing both the neurotransmitters and the Slowpoke channel can be confirmed as the primary mediators and target involved in the bursting activity of motor neurons, further work can establish if these effects are mediated by the Octβ2R receptor and if the cAMP signalling pathway is utilised to modulate Slowpoke currents. The relevant receptor and signalling pathway mutants and transgenic constructs, in addition to pharmacological tools, are available to modulate cAMP signalling and determine their effects on the intrinsic motor neuron membrane excitability.

5.3.2 Octopamine and/or Tyramine Modulate Synaptic Physiology in aCC and RP Motor Neurons

In addition to modulating the intrinsic membrane excitability, octopamine and/or tyramine also profoundly affect the motor neuronal synaptic physiology. The synaptic current amplitude is substantially decreased in both the ventral muscles innervating RP motor neuron pool as well as in dorsal muscle innervating aCC motor neurons (Figure 5.7 & 5.8). The aCC motor neurons are more dramatically affected than RP motor neurons as the synaptic current amplitude is reduced by 67% and 46%, respectively. The ventral and dorsal muscle innervating motor neurons receive different cholinergic synaptic inputs from putatively different interneurons, which may account for the disparity in the severity of the effect (Baines et al., 2002). Previous studies in locusts have identified similar octopaminergic effects on the synaptic physiology in locusts. Octopamine reduces the synaptic current repolarisation rates, afterhyperpolarisation and amplitude onto locust leg motor neurons (Parker, 1996). Octopamine, as well as serotonin and dopamine, have also been shown to reduce the EPSP amplitude from cholinergic sensory afferent neurons onto flight motor neurons in locusts (Leitch et al., 2003).

Changes in synaptic physiology can indicate a number of possible effects. A reduction in the synaptic current amplitude may suggest either a reduction in the number of synapses, active zones, modifications to the synaptic vesicle release probability or the reserve vesicle pool size affecting the sum total of neurotransmitters released from the presynaptic membrane (Murthy et al., 2001; Turrigiano and Nelson, 2004). Moreover, a

reduction in the density, composition or the sensitivity of receptors on the postsynaptic membrane could equally account for the changes (DiAntonio et al., 1999). Considering that the synaptic current amplitude in ventral and dorsal muscle innervating motor neurons and, additionally, the mESPC amplitude are reduced in aCC motor neurons, both pre- as well as postsynaptic membrane effects, at least in aCC motor neurons, are likely. The reduced mESPC amplitude in aCC motor neurons suggests postsynaptic effects in these neurons, which could be due to altered receptor subunit density, composition or sensitivity (Petersen et al., 1997; Thiagarajan et al., 2002). However, there are no changes in RP motor neurons in either the mEPSC amplitude or frequency, which suggests that the postsynaptic membrane responsiveness is unaltered in these neurons. The vesicle release probability and the size of the readily releasable vesicle pool are correlated to the size of the synapse, but the density of docked vesicles remains unchanged when synaptic strength is altered (Murthy et al., 2001). Thus, changes in synaptic size and morphology may provide a feasible explanation for the profound changes in synaptic physiology. Further work is required to elucidate the effects of altered octopaminergic/tyraminergic signalling on the number and size of synaptic connections as well as on the morphology of the dendritic branching. In addition, further work is required to verify if octopaminergic/tyraminergic signalling affects the presynaptic or postsynaptic membrane or both.

It is striking that both the intrinsic membrane excitability as well as the synaptic physiology in motor neurons are altered in response to changed octopamine/tyramine signalling. As the mechanisms of action that cause these effects are unclear, it is not possible to say if both of these effects are the result of chronic changes in neuromodulatory signalling. Alternatively, chronically altered modulation of either the synaptic physiology or the intrinsic membrane excitability could lead to secondary homeostatic responses considering that the synaptic physiology and the intrinsic membrane excitability are interdependently and tightly regulated (Baines et al., 2001; Baines, 2003; Mee et al., 2004; Muraro et al., 2008). For example, blocking action potential firing affects mEPSC amplitude, but not the mEPSC frequency and exemplifies the homeostatic and inter-dependent regulation of synaptic and intrinsic membrane excitability functions (Sutton et al., 2006). Conversely, the postsynaptic membrane excitability adapts to the amplitude of synaptic inputs as, for example, Na^+ and K^+ currents increase proportionately in response to a lack of synaptic inputs in *Drosophila* motor neurons (Baines et al., 2001).

Several K^+ channels such as Shaker, Shab, and Slowpoke have been shown to regulate synaptic plasticity (Gho and Ganetzky, 1992; Ueda and Wu, 2006; Lee et al., 2008). They regulate presynaptic neurotransmitter release by negatively regulating extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels and repolarisation rates in vertebrate and invertebrate synapses (Gho and Ganetzky, 1992; Yazejian et al., 1997; Raffaelli et al., 2004; Salkoff et al., 2006; Ueda and Wu, 2006). Mutations in *shaker*, for example, markedly increase the duration of synaptic currents and decrease the repolarisation rates (Warbington et al., 1996) as well as decreasing the EJP amplitude (Ueda and Wu, 2006). Similarly, in

addition to the modulation of intrinsic membrane excitability, *slowpoke* has been implicated in the regulation of synaptic physiology regulating plasticity and homeostasis (Lee et al., 2008). *Slowpoke* reduces the EJP amplitude at the NMJ as a result of reduced neurotransmitter release without exerting effects on the frequency of mEJPs or the structure and number of synaptic boutons (Warbington et al., 1996). There are differing findings as to whether the mEJP amplitude is reduced or unaffected in *slowpoke* mutant larval NMJs in *Drosophila* (Warbington et al., 1996; Lee et al., 2008). Similarly, large conductance Ca^{2+}-dependent K^{+}-channels have also been shown to regulate synaptic physiology in mammals (Hu et al., 2001). Moreover, the synaptic physiology can be specifically modulated by targeting voltage-gated ion channels. In mouse hippocampal neurons, the muscarinic acetylcholine receptor M1 (mAChR) has been shown to inhibit small conductance (SK) Ca^{2+}-dependent K^{+}-channels and thereby increase the amplitude and duration of synaptic potentials without affecting ionotropic glutamate receptors (Giessel and Sabatini, 2010). In the rat amygdala, β-adrenoceptor have been shown to remove SK Ca^{2+}-dependent K^{+}-channels from the postsynaptic membrane by protein kinase A (PKA)-dependent signalling thereby enhance synaptic transmission (Faber et al., 2008). As Slowpoke currents have been identified as a possible target in the octopaminergic modulation of intrinsic membrane excitability and an effect on synaptic physiology has been established, this channel could account for the synaptic effects as well, providing both presynaptic interneurons and motor neurons are modulated by this mechanism. The presynaptic interneurons are unidentified, however, considering the widespread distribution of and potentially paracrine release from octopaminergic/tyraminergic synaptic terminals in the neuropil (Chapter 4), modulation of interneurons by these neurotransmitters deserves serious consideration.

It is currently unclear, which receptor subtype, what signalling pathway and which molecular targets are responsible for the synaptic effects. This study has already identified a putative expression of β-adrenergic like Octβ2R (Chapter 4) in the larval as well as the adult CNS, and specifically in aCC/RP2 motor neurons. It is therefore feasible that the changes to synaptic and intrinsic excitability are induced by this receptor subtype. Octopamine and tyramine receptors in *Drosophila* either increase Ca^{2+} or cAMP levels to exert their effects (Arakawa et al., 1990; Saudou et al., 1990; Robb et al., 1994; Han et al., 1998). The Octβ2R receptor subtype has already been shown to regulate synapse formation in starvation via the cAMP-signalling in octopaminergic type II boutons and glutamatergic type I boutons (Koon et al., 2011). In addition, there is evidence that cAMP is involved in the regulation of K^{+} currents, which in turn regulate synaptic plasticity (Delgado et al., 1992; Crocker et al., 2010). A combination of pharmacological and genetic experiments could determine if Octβ2R receptor-dependent signalling via cAMP modulates the synaptic physiology in motor neurons.

Lastly, it is striking that the vast majority of motor neurons recorded in current clamp mode were capable of eliciting action potentials (Figure 5.9). This suggests that the synaptic current amplitude may be homeostatically reduced in larvae with compromised octopaminergic/tyraminergic signalling as only smaller synaptic currents amplitudes are

required for the same functional effects. This observation may suggest changes in the dendritic branching and/or alterations in the density of ion channels expressed on the membrane surface. One possible mechanism would be a change in the number of expressed ion channels on the membrane surface, which affect current leaks and therefore input resistance. A higher input resistance would make small synaptic current inputs more effective and thereby elicit a similar impact. Although space clamp problems are unlikely in the *Drosophila* larval CNS as the distance of the dendritic branching from the soma, where currents are recorded, is relatively small (Chapter 4) (Bar-Yehuda and Korngreen, 2008), problems with the reliably controlling the membrane potential in distal parts of the neurons remain a possibility. However, at this stage, the reasons for this observation remain elusive.

In summary, a block of octopaminergic/tyraminergic neurotransmitter release dramatically reduces synaptic current amplitude in RP and aCC motor neurons as well as the half-width in aCC motor neurons. In addition, the amplitude of spontaneous neurotransmitter release onto aCC motor neurons is reduced. Further work is required to establish, if these effects are due to octopamine, tyramine or both. In addition, since Octβ2R receptors are putatively expressed in at least some of the motor neurons described in this study, it would be interesting to determine the functional relevance of this receptor in modulating synaptic physiology. Furthermore, in light of previous studies, a possible role for the Ca^{2+}-dependent K^{+}-channel Slowpoke as the target to modulate synaptic physiology deserves a serious consideration. These results imply that octopamine and tyramine affect crawling behaviour by modulating both motor neuronal intrinsic activity patterns as well as the synaptic transmission. The effects on the motor neuronal physiology may at least partially account for the modulatory effects on locomotor CPG networks.

5.3.3 The Limited Developmental Effects of Octopamine/Tyramine on the Neuromuscular Junction Physiology

The synaptic physiology of the NMJ is largely unchanged in response to chronically altered octopamine and tyramine neurotransmitter levels or blocked release (Figure 5.11 & 5.12). Only minor effects in evoked or spontaneous EJPs in two muscles with and without direct octopaminergic innervation in four different genotypes have been identified. Since both the segmental unit CPG output and NMJ physiology remain constant, this provides indirect evidence that altered contraction and relaxation rates of muscles are due to altered muscle physiology. The amplitude or the kinetic parameters remain unchanged in all genotypes in muscle M12, which receives direct innervation from octopaminergic synaptic terminals. However, the mEJP frequency is increased in the $T\beta H^{nM18}$ mutant and suggests a possibly increased vesicle release probability or an increased number of synapses. In muscle M6, the input resistance is reduced in the $T\beta H^{nM18}$ mutant, which may reflect upon an increased expression of channel proteins on the postsynaptic muscle membrane. In Tdc2-TeTx* larvae, the evoked EJP half-width is increased in muscle M6. Strikingly, this muscle is not innervated

directly by octopaminergic neurons. The prolonged EJP half-width is most likely attributable to the density and composition of repolarising voltage-gated ion channel currents on postsynaptic muscle membrane (Salkoff and Wyman, 1983). *Drosophila* larval muscles express Shaker and Slowpoke channels. As Slowpoke currents have been implied in this study as a potential target of octopaminergic/tyraminergic modulation in motor neurons, this signalling pathway could also potentially account for the identified effects on EJP half-width. Overall, the data suggest that the NMJ physiology remains largely unaltered, although minor changes have been observed.

These data, therefore, are in contrast to reports showing that acute application of octopamine increases the EJP amplitude in muscle M12 and 13 (Koon et al., 2011). A similar effects of octopamine has been shown in muscles M1 and M2 in response to acute octopamine applications, but a decrease was reported in M6 and M7 (Nishikawa and Kidokoro, 1999; Nagaya et al., 2002). It suggests that octopamine exerts differential effects on the EJP amplitude depending on direct innervation by octopaminergic synaptic terminals and may indicate target specificity. In contrast, tyramine decreases the EJP amplitude in M1 and M2 (Nagaya et al., 2002). The results in this study suggest that chronic effects induced by genetically changing octopaminergic/tyraminergic signalling are likely to be homeostatically regulated to maintain the synaptic strength at the NMJ. Numerous studies have shown potent homeostatic mechanisms of synaptic physiology (Turrigiano et al., 1998; Thiagarajan et al., 2002) including presynaptic regulation of the number and size of synapses as well as regulation of the active zone (Schuster et al., 1996a, b; Murthy et al., 2001), the release probability by retrograde signals (Petersen et al., 1997; Davis et al., 1998; Paradis et al., 2001), as well as postsynaptic regulation of receptor subtype density, composition and sensitivity (DiAntonio et al., 1999; Davis, 2006).

In conclusion, these data suggest that although octopamine/tyramine acutely affect EJP amplitude and starvation stress responsiveness (Nagaya et al., 2002; Koon et al., 2011), chronic changes in octopaminergic/tyraminergic signalling do not alter NMJ physiology. It is therefore likely that the changes induced genetically in this study have no effects on NMJ physiology as they are sufficiently compensated for by homeostatic mechanisms. Nevertheless, several issues would need to be addressed to understand the effects of chronic changes in octopaminergic/tyraminergic signalling at the NMJ. It is not clear if these neurotransmitters are involved in the modulation of EJP amplitude and kinetic parameters in high frequency stimulation occurring physiologically in response to segmental unit CPG network activity (Dasari and Cooper, 2004). Furthermore, it is known that a lack of Slowpoke currents as well as octopaminergic signalling both increase satellite synaptic structures (Lee and Wu, 2010). The effects of chronically changed octopaminergic/tyraminergic signalling on the morphology and size of both type I and type II synaptic terminals therefore need to be elucidated.

5.3.4 The Constant Segmental Unit CPG Output and Variable Inter-Segmental Timing with Altered Octopaminergic/Tyraminergic Signalling

In the preceding chapters, experiments in several genetic lines, manipulating four different neurotransmitter systems as well as feeding of pharmacological agents did not affect the stride length, whilst crawling speed and stride duration were inversely altered. In addition, both Chapter 3 and data presented above show that the segmental operational range, which determines the stride length, remains largely unchanged when octopaminergic/tyraminergic signalling is chronically manipulated, although the segmental lengths at rest and at peak contraction may be substantially altered (Figure 5.1). This suggests that the time delay of activation of the next anterior segmental unit CPG during forward locomotion is variable as crawling speed is modulated whilst the segmental unit CPG output onto the respective muscle groups remains constants. This proposed model is supported by electrophysiological data measuring the output of RP3 and RP5 motor neurons onto muscles M6 in two adjacent segments during fictive forward locomotion (Figure 5.2). The segmental unit CPG bursts onto muscles M6 in two adjacent segments are significantly delayed in $T\beta H^{nM18}$ larvae when compared to Tdc2-control larvae when measuring the ends of bursting in adjacent segments. This data needs to be interpreted with caution as the onset of bursting during fictive forward locomotion in adjacent segments is variable and may suggest that, in order to elicited the same degree of contraction of segmental muscles, the output of CPG networks may be segment specific. This increased time delay is likely to account for the prolonged peristaltic propagation wave in slower larvae. In contrast, both the spiking frequency and burst duration from single segmental unit CPGs remain unchanged in Tdc2-control and $T\beta H^{nM18}$ mutant larvae. This suggests that the segmental unit CPG output onto their respective muscle groups indeed remains constant.

Overall, as described in Chapter 3 (Figure 3.14), speed is modulated by regulating the duration of the forward peristaltic propagation wave and the timing of initiation of the next peristaltic wave, whilst the stride length remains largely constant. In view of the profound effects on both the intrinsic excitability and synaptic physiology in identified motor neurons, these data suggest that modulatory neurotransmitters affect the physiology and function of motor and interneurons within these networks, which may be accompanied by appropriate homeostatic mechanisms to keep the sum total network output constant.

5.3.5 Conclusions

In summary, several lines of evidence confirm a constant segmental unit CPG output in crawling third instar larvae. High resolution imaging during crawling show that the segmental operational range in Tdc2-control, $T\beta H^{nM18}$ as well as $T\beta H^{rM6}$ and Tdc2-TeTx* larvae (Chapter 3) remain constant. In addition, electrophysiological recordings in two

adjacent segments during fictive locomotion provide evidence that the time delay of activation is increased between two adjacent segments, but that the output from a single segmental unit CPG network remains constant in animals with a drastically compromised crawling speed.

This chapter also provides evidence to suggest that the NMJ physiology remains largely unchanged comparing four different genotypes in two body wall muscles, with and without octopaminergic/tyraminergic innervation when octopaminergic/tyraminergic signalling is chronically affected. It suggests that the NMJ physiology is, therefore, unlikely to account for the dramatic speed reduction, although the changes in segmental muscle contraction and relaxation rates could substantially contribute the variability in crawling speed.

However, against all expectation, the synaptic physiology as well as the intrinsic membrane excitability of motor neurons innervating both ventral as well as dorsal muscles is profoundly altered when octopaminergic/tyraminergic neurotransmission is chronically blocked. There is thus a striking discrepancy between the constant total output from a segmental unit CPG network and the profound reduction in synaptic input and altered intrinsic excitability in individual motor neurons that receive inputs from the segmental unit CPG or form an integral part of the entire network. How precisely network function and output can remain constant despite profound changes in the motor neuronal physiology remains unclear.

Chapter 6

General Discussion

6.1 CONCLUSIONS

Appropriate animal behaviour depends on a finely tuned balance between reliability and flexibility of multiple aspects of molecular, cellular and tissue function. Whilst they need to be robust and reliable, animals also require the flexibility to adapt to environmental changes, integrating external stimuli and internal factors, which govern and modulate their behaviour. How these principles are applied in the central nervous and entire CPG networks is not resolved, whereas significant progress has been achieved in the understanding of homeostasis in individual neurons. This study focused on the functional effects of altered neuromodulatory signalling on the locomotor segmental unit CPGs. I aimed to investigate how *Drosophila* larval crawling behaviour is modulated by two established neuromodulators in insects, octopamine and tyramine and the effects of chronic changes in the signalling of these two neuromodulators. I confirmed previous findings that these biogenic amines modulate crawling speed antagonistically within a physiological range (Saraswati et al., 2004). Octopamine increases crawling up to a maximum, whilst tyramine reduces crawling speed to a lower limit. However, this study provides a more detailed analysis of larval crawling, in particular on the stride length and stride duration, the two determinants of crawling speed. I have shown that the changes in crawling speed primarily result from inversely proportional changes in stride duration, whilst stride length remains largely constant when octopaminergic/tyraminergic signalling is chronically altered. These findings have been validated by both chronic genetic and pharmacological manipulations of octopaminergic and/or tyraminergic signalling. Strikingly, similar results were obtained in experiments that chronically blocked dopaminergic and/or serotonergic neurotransmission as the stride length also remained constant, despite variable crawling speed and stride duration (Appendix). The effects on crawling speed are therefore mediated by a variable time delay in the activation of segmental unit CPG networks and thus stride duration in forward locomotion, a hypothesis supported by both electrophysiological and behavioural imaging data. Moreover, several lines of evidence from behavioural and electrophysiological experiments suggest that the output of segmental unit CPG networks onto their respective muscle groups remain constant and therefore enable a constant stride length. These findings therefore imply that, at least in *Drosophila* larval locomotion, appropriate neuromodulatory signalling throughout development is not essential to enable correct segmental unit CPG network function and output.

Interestingly, preliminary studies suggest that both stride length and stride duration are flexibly adjusted in response to acutely modulated octopaminergic/tyraminergic neuronal function in temperature sensitive transgenic larvae, although further work would be required to validate these findings. These results are in contrast to the constant stride length in response to chronic neuromodulatory perturbations. This may suggest underlying mechanisms, which homeostatically restore and/or maintain the segmental unit CPG output and thus stride length in response to chronically altered neurotransmitter signalling. In legged vertebrate locomotion, both stride length and stride duration are flexibly adjusted to regulate speed, which optimises energy expenditure (Heglund et al., 1974). At this stage, one can only speculate why the stride length is kept constant in larval crawling. However, a constant stride length may provide a suitable strategy to balance locomotion speed and energy expenditure. Although the muscle tension itself is apparently linearly correlated with energy expenditure (Baguet and Gillis, 1968), organisms require an optimisation between the efficiency of movement and the mechanical force on a systemic scale (Umberger and Martin, 2007).

This study also provides indirect evidence from crawling imaging analysis for a role of octopaminergic/tyraminergic signalling in the modulation of muscle contraction and relaxation rates in segmental body wall muscles, in support of data published on locust musculature (Evans and Siegler, 1982; Evans and Myers, 1986; Malamud et al., 1988; Whim and Evans, 1988) and several lines of evidence in adult *Drosophila* that octopamine increases muscle contraction force (Zumstein et al., 2004; Middleton et al., 2006). Moreover, the constant stride length depends on the segmental operational range to maintain the distance traversed by a single stride in multi-segmental crawling. Although the muscle tone may differ as a result of altered octopaminergic/tyraminergic signalling, the operational range in segments remains constant.

This study also provides new lines of evidence for both the putative localisation and release of octopamine/tyramine in the CNS as well as their effect on the intrinsic membrane excitability and synaptic physiology in identified motor neurons in the ventral nerve cord in *Drosophila* larvae. I have also been able to reproduce previous findings, which show that, putatively, octopamine/tyramine are found in synaptic terminal varicosities. In addition, this study shows that synaptic terminal varicosities are in close vicinity to motor neuronal dendrites. It is unclear if octopamine/tyramine are putatively released as neurotransmitters into a synaptic cleft or whether they act as paracrine signals released into the neuropilar hemolymph. These experiments do not provide direct evidence for the presence and release of these neuromodulators in the CNS. In order to determine the localisation, projections and functional effects of neuronal subpopulations in the brain, I used tshGAL80 to sub-fractionate the octopaminergic/tyraminergic populations in the larval CNS. These neurons, located in the brain lobes and suboesophageal ganglia, also project synaptic terminal varicosities into the neuropil in close proximity to motor neuronal dendrites without identified projections to peripheral tissues. I have shown that these neurons play a role in modulating crawling speed, which suggests that octopamine/tyramine exert at least part of their behavioural effects by

modulating neuronal physiology in larvae. This finding is consistent with the proposed role of command structures in the anterior brain regions, a common feature of hierarchical CNS organisation across the animal kingdom, which integrate sensory information as well as coordinate and modulate segmental unit CPG output (Selverston et al., 1997). More specifically, the leech suboesophageal ganglion has been shown to play an important role in the integration of sensory information as well as in the modulation and correct coordination of segmental unit CPG function (Brodfuehrer and Friesen, 1986b; Cornford et al., 2006).

Electrophysiological analysis shows that chronically altered octopaminergic/tyraminergic signalling reduces excitatory synaptic current amplitude in both ventral and dorsal muscles innervating motor neurons in the CNS as well as the mEPSC amplitude in dorsal muscle innervating motor neurons. In view of multiple potent homeostatic mechanisms that are able to adapt to changes and to maintain synaptic excitability within a physiological range (Turrigiano et al., 1998; Paradis et al., 2001; Burrone et al., 2002; Thiagarajan et al., 2002) as well as the comparable synaptic excitation levels to elicit action potentials in both tested genotypes may suggest that the synaptic amplitude is homeostatically reduced. In addition, chronically altered octopaminergic/tyraminergic signalling also results in an increased prevalence and severity of bursting activity patterns in response to stimulation. This observation is likely to be due to reduced Slowpoke Ca^{2+}-dependent K^+ channel currents when octopaminergic/tyraminergic signalling is chronically altered, similar to previously shown effects on bursting activity patterns in *Drosophila* neuronal cultures and muscles (Elkins et al., 1986; Elkins and Ganetzky, 1988; Saito and Wu, 1991). I propose a model, in line with previous studies, that octopamine modulates Slowpoke channel function by activating Octβ2R receptors, which in turn exert their effects via the cAMP signalling pathway (Figure 6.1) (Wang et al., 1999). Interestingly, the reduced synaptic currents are still capable to sufficiently depolarise the neuronal membrane and to elicit action potentials firing. The synaptic current amplitude may thus be reduced due to a smaller excitatory current being required to reach the same level of excitation and may thus represent a homeostatic response mechanism. The functional relevance of increased prevalence and severity of bursting is unclear. It is therefore not impossible that these changes are an adaptation to maintain the output of the segmental unit CPG network constant at the expense of temporal inter-segmental coordination. In addition, both the changes in the intrinsic membrane excitability and the altered synaptic physiology may be the result of altered activity of cAMP due to reduced octopamine receptor signalling, which could potentially provide a convergent target to affect both.

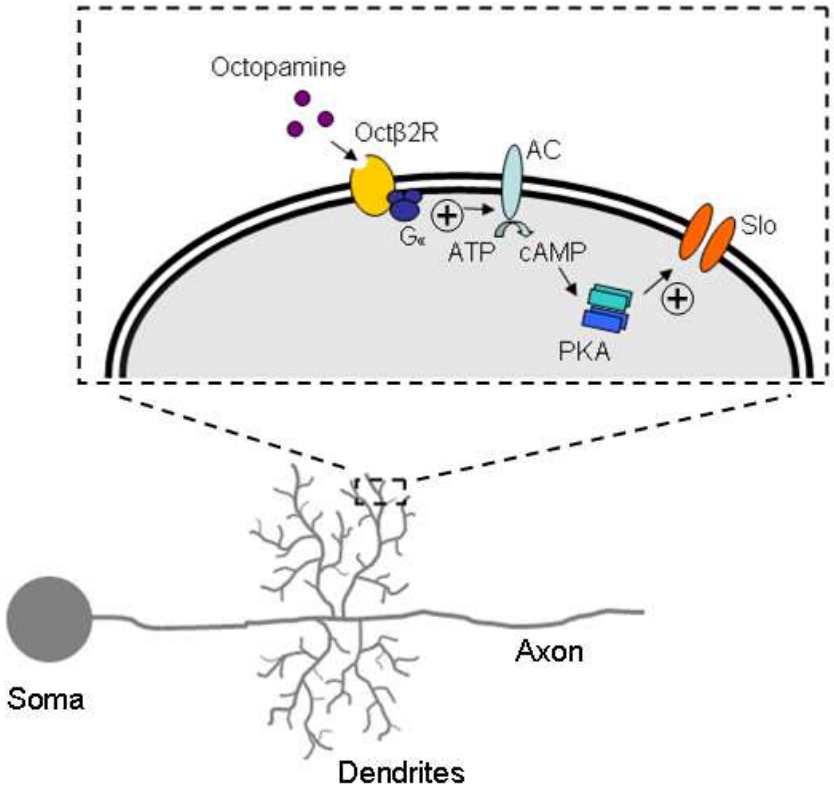

Figure 6.1 Model of motor neuronal modulation by octopamine. Octopamine binds and activates the Octβ2R receptor, which in turn leads to increased activity of adenylate cyclase (AC) via the G_α protein. Adenylate cyclase converts ATP to cAMP, which in turn binds and activates the catalytic subunits of protein kinase A (PKA). PKA then binds and modulates the *Drosophila* Slowpoke (Slo) Ca^{2+}-dependent K^+ channel. Signalling pathway adapted from (Blenau and Baumann, 2001).

Over the last two decades, studies have started to emerge showing that neuronal networks and their constituent neurons display a remarkable ability for homeostatic regulation of network function (Thoby-Brisson and Simmers, 1998; Maffei and Fontanini, 2009), at times even indirectly and across multiple networks (Maffei et al., 2004; Maffei et al., 2006; Maffei and Turrigiano, 2008). In this context, this study suggests that, at least under some conditions, segmental unit CPG networks in *Drosophila* are homeostatically regulated and maintain a constant output. To the best of my knowledge, this study is the first to provide several lines of evidence that multiple repeated neuronal networks are simultaneously regulated by homeostatic mechanisms to keep their output constant when neuromodulatory signalling is chronically altered. Whilst several studies suggest that network stability and output can be maintained despite highly variable intrinsic properties in individual neurons within networks, it is currently unclear how it can be maintained within a physiological target range (Prinz et al., 2004; Maffei and Fontanini, 2009; Grashow et al., 2010; Norris et al., 2011). It is not known, if the sum total of the plethora of intrinsic homeostatic mechanisms regulating the various neuronal compartments can collectively and independently maintain network function. Alternatively, intrinsic feedback loops within neuronal networks, or sensory feedback may be necessary and/or sufficient to homeostatically maintain network function.

For example, an interesting feature of the rat visual cortex are recurrent inhibitory feedback circuits as well as inhibitory feed-forward neurons receiving thalamic inputs and provide inputs to layer 4 pyramidal neurons, which are selectively up- and down-regulated, respectively, in visual deprivation and largely keep network output constant (Maffei et al., 2004). This suggests that, at least in the mammalian visual cortex, networks are wired and connected in a way that enables them to intrinsically monitor their activity and output. Although the role of sensory feedback in larval crawling is important in this context, it is very difficult to assess experimentally. Studies show that sensory neuronal feedback is essential for crawling as sensory neuronal inhibition leads to cessation of crawling (Hughes and Thomas, 2007; Song et al., 2007). Nevertheless, this is an important question that remains unaddressed.

Interestingly, the effects of chronic perturbation of octopaminergic/tyraminergic signalling on the overall crawling behaviour as well as the constant segmental unit CPG output and variable intersegmental time delay are difficult to reconcile with the effect on the motor neuronal physiology. The data suggests that the segmental unit CPG output can remain constant, despite profound changes in the physiology of motor neurons, which form an integral part of the network output (Figure 6.2). Considering that motor neurons, an integral part of the segmental unit CPG network output, are profoundly affected in response to chronic, but potentially not acute, changes in octopaminergic/tyraminergic signalling may suggest that the constant network output may be prioritised over a maintained physiology of individual neurons within a network. However, further work is required to investigate this hypothesis. Interestingly, studies show that a large number of different combinations of various currents can produce an identical CPG network output (Prinz et al., 2004). This suggests that synaptic strength or intrinsic excitability can be highly variable yet the network output can be maintained constant. On a cellular level, it has also been established that disparate combinations and densities of various currents can have identical activity patterns. Thus, the functional relevance of a single variable in the pool of properties that determine the excitability of single neurons may be very profound and transform the behaviour or have almost no significance due to co-variation of other variables compensating for the change to maintain the output constant (Goldman et al., 2001; Goaillard et al., 2009). Moreover, research shows that spike threshold levels, input resistance or intrinsic firing frequency can be insufficient to accurately predict the behaviour of individual neurons or whole networks (Grashow et al., 2010). Further work is therefore required to fully establish the effects of chronically altered octopaminergic/tyraminergic signalling on specific currents and, ultimately, the functional effects on network output and behaviour.

The apparent discrepancy between constant segmental unit CPG output and profoundly altered motor neuronal intrinsic and synaptic physiology raises substantial questions about the precise mechanisms for this observation on the molecular, cellular and circuit level. Based on the constant CPG output and variable inter-segmental time delay, an effect of altered octopaminergic/tyraminergic signalling on interneurons regulating the timing in inter-segmental coordination or a delay in the activation of motor neurons had been

predicted. Considering that the segmental unit CPG output remains constant, but the time delay of activation between individual segments is increasing with reduced crawling speed, a modulatory effect of octopamine/tyramine on the I_A current appeared more likely. I_A currents, mediated by *shaker* and *shal*, are known to regulate the rate of depolarisation rate and therefore the time delay to the onset of action potential firing (Schaefer et al., 2010; Ping et al., 2011). Thus, an increase in I_A currents in response to modulated octopaminergic/tyraminergic signalling may have sufficed in delaying the inter-segmental unit CPG communication delaying the onset of activation of the next adjacent segments (Choi et al., 2004). Particularly Shal currents have been shown to regulate the delay of spiking onset, which are predominantly expressed in somatodendritic areas and thereby play a crucial role in the temporal integration of synaptic input and intrinsic activity patterns (Choi et al., 2004; Ping et al., 2011). However, no delay in the onset of action potential firing in motor neurons has been identified. In light of the observed behavioural effects on crawling speed, it is thus counterintuitive for octopamine/tyramine signalling pathways to primarily target the Slowpoke Ca^{2+}-dependent K^+-channel in *Drosophila* larvae with profound effects on intrinsic and synaptic physiology in motor neurons. Moreover, the effects on motor neuronal physiology alone are probably insufficient to account for the time delay in the activation of segmental unit CPGs as no time delay to the first action potential in response to stimulation was detected. This is indicative of changes in additional neurons within the segmental CPG networks or interneurons stimulating individual CPG networks. A multi-neuronal modulatory role for octopamine/tyramine in segmental unit CPG networks regulating crawling speed is thus likely, particularly in view of a potentially paracrine release into the VNC neuropil where numerous interneurons and motor neurons form their synaptic connections. Hence, octopaminergic/tyraminergic effects on I_A currents in interneurons upstream of motors neuron remain a possibility.

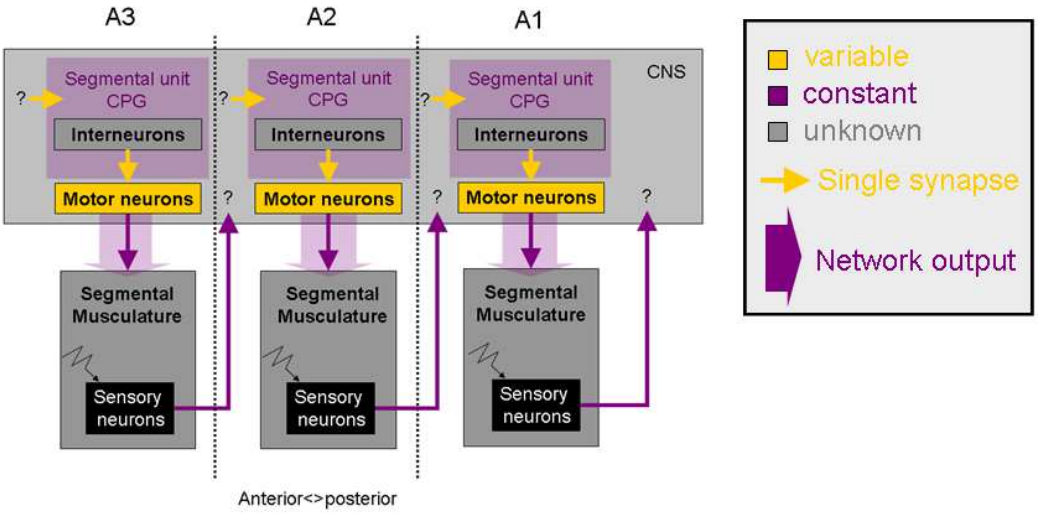

Figure 6.2 Model diagram of octopaminergic/tyraminergic modulatory effects on crawling speed. Whilst the motor neuronal intrinsic membrane excitability and the synaptic inputs onto the motor neurons are profoundly altered (yellow), the sum total segmental unit CPG (A1-A3) output onto their respective segmental muscle groups remains constant (purple). Furthermore, the input of individual motor neurons onto their respective muscles also remains unchanged (purple). The time delay in the activation of the next posterior unit CPG is flexible, which determines the stride duration. The effects on the interneuronal and muscle physiology are currently not clear.

My results suggest that *Drosophila* larvae may serve as a suitable and interesting model system for the study of simultaneous homeostasis of multiple segmental unit CPG networks. This system provides key advantages including its genetic amenability, electrophysiologically accessible and identifiable NMJs and motor neurons as well as a behavioural readout of single segmental contractions parameters and systemic effects on behaviour.

6.2 OUTLOOK

The following areas require further elucidation in order to start to address some of the outstanding questions and to further test the hypothesis of network homeostasis in *Drosophila* segmental unit CPGs on a cellular and molecular level.

This study provides evidence for a modulatory role of octopamine and/or tyramine in motor neuronal function. Slowpoke has been identified as a potential target of these modulatory effects in the CNS, causing bursting activity pattern and potentially altered synaptic physiology. Further experiments are required in order to validate if a reduction of the Slowpoke current is induced by a lack of octopamine and whether it is necessary and sufficient to increase the prevalence and severity of bursting intrinsic membrane excitability. Pharmacological experiments using compounds such charybdotoxin and tetraethylammonium could determine the acute effects of altered octopaminergic/tyraminergic signalling specifically

on Slowpoke currents and on the overall intrinsic membrane excitability as well as the potential effects on synaptic current amplitude (Elkins et al., 1986; Shahidullah et al., 2009). This would avoid potential homeostatic changes in other currents. Also, using a motor neuron specific GAL4 driver line (RRA-GAL4) to block *slowpoke* channel gene function by expressing Slowpoke channel specific RNA-mediated gene interference (RNAi) (UAS-slo-RNAi) (Dietzl et al., 2007), would elucidate the necessity and sufficiency of motor neuronal Ca^{2+}-dependent K^+-current modulation for induction of bursting activity and, moreover, in modulating crawling behaviour (Lee and Wu, 2010). A direct modulatory effect on Slowpoke currents could be established by measuring Ca^{2+}-dependent K^+ currents in identified motor neurons in response to acute applications of octopamine and/or tyramine. Furthermore, the likely signalling pathway of octopaminergic/tyraminergic modulation via cAMP could be established by genetic and pharmacological experiments. A direct effect of octopaminergic/tyraminergic signalling on cAMP signalling could be established by fluorescence resonance energy transfer (FRET) analysis, which has been shown to putatively modulate Slowpoke currents in *Drosophila* adults by OAMB receptors (Crocker et al., 2010). Moreover, using the phosphodiesterease mutant *dunce* (Dnc), which fails to degrade cAMP and thus results in abnormally high cAMP signalling (Dudai et al., 1976), and the adenylate cyclase mutant *rutabaga,* which decreases cAMP signalling (Livingstone et al., 1984), would indicate if this signalling pathway has an effect on Slowpoke currents in these motor neurons. Further validation of the results could be attained by testing the effects of PKA, which acts downstream of both GPCR signalling and adenylate cyclase activity. This could be achieved by over-expression of a constitutively active isoform of PKA (UAS-PKAact) or a mutant regulatory PKA subunit with decreased cAMP sensitivity and decreased PKA activity (UAS-PKAinh) in aCC/RP2 motor neurons (Davis et al., 1998). These experiments would establish, whether octopamine and/or tyramine affect Slowpoke currents and, if so, whether the cAMP signalling pathway is employed.

Further work would also be required to determine the effects of acutely and chronically altered octopaminergic/tyraminergic signalling on transient and persistent Na^+-, Ca^{2+} and particularly K^+ currents. Provided that octopamine and/or tyramine have modulatory effects on Slowpoke currents, a long-term absence of this modulation would be expected to result in concomitant changes in other currents. In this context, it would be particularly important to determine, if potential effects on these ionic currents are the result of direct modulation or secondary homeostatic responses, which could be addressed by genetic and pharmacological experiments on different timescales. In addition, the effects on the amount of channel specific mRNAs could be determined by qPCR. These experiments would start to address potentially secondary homeostatic effects on the motor neuronal excitability in response to altered octopaminergic/tyraminergic signalling.

The effects of octopamine/tyramine on the structural morphology of motor neurons and the synaptic connectivity with interneurons have remained unaddressed in this study. Octopamine has recently been shown to regulate acute NMJ synapse formation in glutamatergic type I and octopaminergic/tyraminergic type II boutons in the peripheral body

wall musculature (Koon et al., 2011). In addition, studies show that synaptic input is an important regulator of dendritic arborisation by PKA-dependent signalling pathways (Tripodi et al., 2008). In light of these findings and the reduced synaptic current amplitudes in motor neurons in larvae with compromised octopaminergic/tyraminergic signalling, further work is required to elucidate the structural effects in motor neurons and their synaptic connectivity to interneurons. Neuronal dyes as well as genetic fluorescent synaptic and dendritic markers enable a 3D reconstruction of motor neuronal arborisation and putative synaptic contacts with interneurons (Mauss et al., 2009; Nicolai et al., 2011). The density of direct synaptic connections of cholinergic interneurons, and also possibly octopaminergic/tyraminergic neurons, with identified motor neurons could be further tested by genetic labelling using the GFP Reconstitution Across Synaptic Partners (GRASP) technology (Feinberg et al., 2008).

The results from these experiments would provide valuable information about the functional and structural effects of octopamine and/or tyramine in the *Drosophila* larvael CNS and could provide a platform for future experiments to reconcile and integrate the results of locomotor CPG networks on a systemic, network and cellular level by further experimentation.

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Appendix

Figure A.1 Analysis of crawling behaviour in larvae with modulated dopaminergic and serotonergic neurotransmission. **A**. *TH:* The peak speed is significantly reduced when dopamine release is. *Ddc:* The peak speed is significantly reduced when dopamine and serotonin release is blocked. **B**. *TH:* The overall speed is also significantly reduced. *Ddc:* The overall speed is also significantly reduced when both dopamine and serotonin release is blocked. **C**. *TH:* The stride length is unchanged in both groups. *Ddc:* The stride length is unchanged in both groups. **D**. *TH:* The stride duration is significantly increased. *Ddc:* The stride duration is significantly increased. Abbreviations: TH: tyrosine hydroxylase. Ddc: Dopa decarboxylase.