Postembryonic maturation and putative modulation of the central pattern generator for flight in *Manduca sexta*

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Chapter 1 – Introduction

The survival of any animal depends on its ability to adapt its behavioral pattern to a continually changing environment. Most of these vital behavioral patterns, such as flying, walking, crawling, swimming, mating, feeding and reproduction consist, to a large extent, of stereotype, rhythmic and repetitive motor patterns. These varying patterns can be generated from central networks, so-called central pattern generators, without rhythmic sensory or central inputs. Based on the internal state of the animal and sensory information from the environment, the appropriate motor programs must be selected and correctly coordinated in order to adapt the behavior of the animal accordingly.

However, the generation and effectuation of motor patterns is not rigid or determined like a reflex, to ensure adaptation to the altering requirements. Such adaptations can be observed on different levels: a) modulation of the central circuits to achieve a behaviorally appropriate selection and adaptation of a motor program, b) adaptation of the effectiveness of neuronal signal transfer to the neuromuscular junction, c) modification of the contraction characteristics of the muscles involved, d) adaptation of the muscular metabolism to optimize the effectiveness of the organ, and e) modulation of the sensitivity of the relevant sensory receptors. It has long been known that these adaptation effects are transmitted by specific neuroactive substances (Marder and Bucher, 2001; Selverston, 1993) and that central pattern generating circuits undergo continual neuromodulation resulting from neuromodulatory substances that are either circulating in the haemolymph/blood or that are released locally (Dickinson, 2006; Grillner, 2003; Harris-Warrick and Marder, 1991; Pearson, 1993).

1.1 Central pattern generators

The generation of rhythmic motor patterns results from the inherent ability of certain circuits in the central nervous system to endogenously produce these patterns without sensory or central inputs. These networks are known as central pattern generators (CPG). The first evidence of the CPG was discovered almost 40 years ago by D.M. Wilson, when, for the first time, it was shown that the isolated central nervous system (CNS) of the desert locust S*chistocerca gregaria* is capable of generating motor patterns without sensory feedback (Wilson, 1961). Wilson showed that these patterns, known as

fictive motor patterns, are very similar to the flight patterns of intact animals. Previous to this, there was no consensus among neuroscientists as to whether central oscillators or reflex chain arrangements form the basis of rhythmic behavioral patterns. Since 1961, the ability to generate central patterns autonomously for a range of greatly differing behavioral patterns has been shown in a variety of species: lamprey (Grillner, 1985); locust: (Ryckebusch and Laurent, 1993; Stevenson and Kutsch, 1987); crustacean: (Marder and Bucher, 2001); stick insects: (Bassler and Buschges, 1998); earthworm: (Mizutani et al., 2002) and lepidoterans as for example *Manduca* (Claassen and Kammer, 1986; Johnston and Levine, 1996).

Even “simple” invertebrate neuronal circuits demonstrate an extraordinary complexity, compared to much larger, more intricate vertebrate circuits, although in both, the function and the dynamics of networks rely on the intrinsic properties and the synaptic wiring of the participating components (Delcomyn, 1980; Marder and Bucher, 2001; Marder and Calabrese, 1996). Numerous analyses of circuit diagrams have shown that the circuit patterns of networks that generate similar patterns of activity may differ greatly (Getting, 1989; Selverston and Moulins, 1985). However, it was also found that varying activity patterns can result from very similar circuit patterns (Getting and Dekin, 1985). The function and the dynamics of neuronal networks are therefore not mainly a result of the synaptic circuits of their components. Furthermore, some neuronal circuits are capable of generating multiple behavioral patterns, rather than producing only one fixed pattern, whereas several networks may contribute to a certain behavioral pattern or single network components (neurons) can be active during multiple behaviors or active during one behaviour and may be inactive during others (Briggman and Kristan, 2008). The stomatogastric nervous system (STNS) of crustaceans is an example of a network in which a very small number of pattern-generating neurons (ca. 26-30) are able to generate two different activity patterns, the fast pyloric rhythm and the slower gastric mill rhythm (Weimann et al., 1991).

Thus, the question arises as to how a network with a fixed number of neurons, with known characteristics and connectivity can produce several different activity patterns and therefore contribute to a flexible and adapted behavioral output. Two common mechanisms are responsible for this: firstly, sensory feedback provides continuous adaptation of centrally generated patterns to behavioral tasks without being involved in the generation of patterns itself; the second possibility, which is the subject of a large part of this study, is

based on the effect of neuromodulatory substances (see below). These substances are not only capable of modulating the complex interactions of intrinsic characteristics and connectivity of central circuits, but also of modulating the sensory input in order to adapt the functional dynamics of the network to behavioral requirements (Harris-Warrick and Marder, 1991).

1.2 Neuromodulation

The difference between neuromodulation and neurotransmission is that neuromodulatory actions are rapid and are not simply confined to excitation or inhibition, but their temporal effect is long-lasting ranging from minutes and hours, or up to several days or months (Katz, 1999). Although neuromodulatory actions are limited to the cellular level, the effects of neuromodulators, such as biogenic amines, amino acids, neuropeptides or gases, have been studied on various levels of the nervous system in various organisms that range from genes and biosynthesis to complex behaviors (Pflüger and Büschges, 2006). In most cases, there is a paracrine release of neuromodulators via varicosities (Consoulas et al., 1999). This means that the target and effect of the modulator is greatly dependent on the localisation of the respective varicosity in the nervous system. The release at central branches of neuromodulatory neurones causes other, central effects, than release at a specialized section of the axon at the terminals, for instance on the neuro-muscular junction, the effect of which is limited to the periphery (Duch and Pflüger, 1999), or the neuromodulator is released unspecifically to the circulatory system (haemolymph), this will cause an effect with a completely different time response at another locality (Orchard, 1982). For instance, the central release of specific neuromodulators or mixtures of different neuromodulators can induce distinct motor behaviors (Nusbaum et al., 2001). Furthermore, monoamines, for example, have been linked to aggression, motivation, and mood in vertebrates as well as in invertebrates (Baier et al., 2002; Kravitz and Huber, 2003; Popova, 2006; Stevenson et al., 2005). It has also been shown that biogenic amines are involved in learning and the formation of memory (Hammer, 1997; Schwaerzel et al., 2003). Their significance for humans usually only becomes discernible when disruptions occur. It is therefore assumed that dysfunctions in monoamine neurotransmission in mammals are linked to neurological disorders, including Parkinson´s disease, schizophrenia, anxiety, and depression (Kobayashi, 2001; Taylor et al., 2005).

Octopamine

Octopamine (OA) is one of the major biogenic amines in insects, and octopamine and its biological precursor tyramine are thought to be the functional homologue of adrenaline and noradrenaline in vertebrates (Roeder, 2005). Octopamine is widely distributed in invertebrate phyla (Pflüger and Stevenson, 2005) and is capable of acting as a neurotransmitter, neurohormone, and neuromodulator (Axelrod and Saavedra, 1977; Orchard, 1982; Roeder, 2005). It mediates diverse cellular and physiological effects by binding to G protein-coupled receptors (GPCRs) (Blenau and Baumann, 2001).

In insect flight, octopamine plays a central role in initiation, maintenance and modification of the flight system. It affects central neuron excitability by modifying neuronal membrane resistance and bursting properties (Ramirez and Pearson, 1991a, b) and also modulates synaptic transmission (Evans and O'Shea, 1977; Leitch et al., 2003) by affecting neurotransmitter release, acting on presynaptic receptors and/ or postsynaptic receptors. Octopamine increases/ decreases sensory sensitivity (Matheson, 1997; Ramirez and Orchard, 1990), acts as neurohormone possessing adipokinetic activity (Orchard et al., 1993) and changes visceral and skeletal muscle properties by modulating pre- and postsynaptic sites, resulting in increased tension and relaxation rates (Bräunig and Pflüger, 2001; Evans and O'Shea, 1978). Finally, it affects the glycolytic activity of muscles during rest, take-off phase and flight (Becker et al., 1996; Mentel et al., 2003; Wegener, 1996). Die octopaminergic neuromodulation of insects show three distinctive features when compared to other modulatory systems: firstly, the central and peripheral release takes place via so-called unpaired median neurons (VUM/ DUM) situated along the ventral (VUM) or dorsal (DUM) ganglion midline. Their unique morphology shows a distinct primary neurite bifurcating into bilaterally axon collaterals. Thus, they are easy to identify and it even allows to discriminate between different subtypes. Unlike fast-transmitting motoneurons, which posses type I terminals, efferent unpaired median neurons release octopamine via type II terminals (Consoulas et al., 1999; Pfluger, 1999). Secondly, despite the relatively low number of octopaminergic neurones in the whole nervous system (between 40 and 100), they supply most of the neuropiles in the brain and large parts of the thoracoabdominal nervous system and also many peripheral effector organs. Thirdly, octopamine is normally synthesized from tyrosine in a two-step synthesis process, whereby tyramine represents the intermediate stage of octopamine. Therefore, all octopaminergic neurones also contain tyramine. Most biogenic amines are represented in non-overlapping

sets of neurones, but octopamine and tyramine can be released from the same neurone. So far, it is suggested that octopamine and tyramine act as antagonistic modulators (Roeder et al., 2003), like adrenaline and nor-adrenaline in vertebrates.

Tyramine

For a long while, tyramine was only considered to be the biochemical precursor molecule of octopamine without playing a physiological role producing distinct effects. To date, we know that various species (primates, fruit flies, molluscs, honeybees, moths and nematodes) have specific tyramine receptors (Blenau et al., 2000; Gerhardt et al., 1997; Miller et al., 2005; Ohta et al., 2003; Rex and Komuniecki, 2002; Saudou et al., 1990) suggesting that tyramine-dependend neuromodulation may be mediated by multiple receptors and more complex than expected. So far, many cellular and physiological effects of tyramine have been described, proving the case for a distinct modulatory role of tyramine: tyramine is involved in cocaine sensitisation (McClung and Hirsh, 1999), in the regulation of hyperactivity in response to ethanol (Schwaerzel et al., 2003), in increased gustatory and visual sensitivity (Scheiner et al., 2002), and in the modulation of chloride conductances in malpighian tubules (Blumenthal, 2003). Similar to octopamine, tyramine is also capable of eliciting fictive flight patterns (Buhl et al., 2008) and of affecting flight motor performance (Brembs et al., 2007). However, so far there is no clear evidence whether tyramine is released independently from octopamine or released concomitantly from the same DUM/VUM neurons (Lange, 2009). Recently, it has been shown in immunohistochemical experiments on locusts (Kononenko et al., 2009) that in the brain, in subesophageal ganglions, and in all fused abdominal ganglia there seem to be et least some purely tyraminergic neurons distinct from octopaminergic neurons.

1.3 Goal of the thesis

In this thesis I aimed to analyze the postembryonic development and putative modulation of the central pattern generator for flight in the tobacco hawkmoth, *Manduca sexta*. The ability of adult flight requires alterations with respect to intrinsic properties and synaptic wiring of neurons to generate the central pattern for flight, as well as different contractile properties of flight muscles, and specialized sensory neurons to adapt the flight rhythm to external fluctuations, which has to take place during pupal development. So,

when does the central pattern generator for flight become functional during pupal development? Is the development of a flight pattern generating network a slow, graduated process during postembryonic develoment which ends in a mature network empowered to produce adult flight patterns after eclosion or even earlier? Or, are the components of the future flight producing network established early during pupal development but only linked to a functional adult circuit at a certain developmental time point? So far, virtually nothing is known about flight interneurons in *Manduca sexta* and the development of the central flight circuitry. Thus, I considered an isolated nerve cord preparation of *Manduca sexta* as particularly suitable in my attempt to answer these questions for the following two reasons: firstly, the central pattern generator for flight can be reliably activated by bath application of the biogenic amine octopamine or its agonist chlordimeform (Claassen and Kammer, 1986; Kinnamon et al., 1984). Second, this preparation allows us to examine the centrally generated flight motor activity unaffected by any sensory feedback in various developmental stages and it widely accepted that these fictive, pharmacologically induced motor patterns closely resemble those generated by behaving animals (Marder and Bucher, 2001; Marder et al., 2005) exept for the slower frequency observed in fictive motor patterns.

First, as a prerequisite for the comparison of fictive motor patterns in different stages, a reference pattern is needed. For this reason quantitative measurements of fictive flight patterns in adult were used as template for comparison of motor pattern features during development. Based on the obtained data maturation (refinement) in pattern features like cycle period, phase relationship and precision are evaluated in the context of published data on structural and physiological development of CNS, especially structural and/or physiological remodeling flight depressor MN5 (Duch and Levine, 2000, 2002; Libersat and Duch, 2002; Meseke et al., 2009), to draw a conclusion about developmental changes of the underlying flight pattern producing network (chapter 3).

Recent work from different areas, as diverse as Parkinson´s disease (Scholtissen et al., 2006) and insect rhythmical motor behavior, indicate that chemical codes inducing specific motor behavioral outputs are bouquets of different biogenic amines rather than single ones (Fox et al., 2006; Fussnecker et al., 2006; Saraswati et al., 2004) contribute to behavioral modulations. With respect to insect flight the central role of octopamine in initiation, maintainance, and modulation of the flight system are well known (see 1.2) and importantly, tyramine has also been shown to affect flight motor behavior in Drosophila

(Brembs et al., 2007) and locusts (Buhl et al., 2008). Do octopamine and tyramine act as antagonistic modulators on the flight system or do they have complemental function during flight? Do octopamine and tyramine affect different aspects of central pattern generating circuits? We therefore continued to make use of pharmacologically induced fictive flight motor output in isolated nerve cord preparations (chapter 2) and tried to elucidate the effects of octopamine and tyramine on the central pattern generation for flight motor output.

Previous work in locusts showed a cholinergic activation of the central flight generating network coupled to aminergic fascilitation (Buhl et al., 2008). Investigations on stationary flight like behavior in Drosophila suggests that octopamine is effective but not required to induce flight (Brembs et al., 2007), we tried to verify a putative cholinergic mechanism for the activation of the flight central pattern generator in *Manduca* (Chapter 5).

Both, tyramine and octopamine are released putatively by the same set of large, efferent unpaired median neurons (VUM/DUM) at peripheral target sites to alter synaptic transmission, muscles properties and muscle metabolism (see 1.2). To fulfil these tasks the release of the neuromodulator has to be adapted spatially and temporally according to the behavioral tasks. Thus, the activity of VUM/DUM neurons have to be coupled differentially to specific motor activities. Earlier investigations described that all larval VUM/DUM neurons are recruited during fictive crawling patterns (Johnston et al., 1999) but it remains still unknown whether adult VUM/DUM neurons form functionally different subpopulations as described for locusts (Baudoux and Burrows, 1998; Baudoux et al., 1998; Burrows and Pfluger, 1995; Duch et al., 1999). Thus we were asking whether and how a homogeneous population of larval neuromodulatory neurons might be divided into different functional subsets during metamorphosis to become activated differentially during different behaviors. As a first step towards this question I examined the activation of these neuromodulatory cells during fictive flight in adults by intracellular recording from mesothoracic VUM neurons before and after pharmacologically induced fictive flight.

The results are presented in two chapters based on two manuscripts; one of which is already published and the other is recently submitted.

Chapter 2:
Vierk R, Pflueger HJ, Duch C (2009) Differential effects of octopamine and tyramine on the central pattern generator for *Manduca* flight. J Comp Physiol A 195:265-277

Chapter 3:
Vierk R, Duch C, Pflueger HJ: Postembryonic maturation and putative modulation of the central pattern generator for flight in *Manduca sexta.* (submitted in J Comp Physiol A)

Two additional chapters show work in progress, which needs further labor after this thesis before being publishable.

Chapter 4:
Activation of ventral unpaired median (VUM) neurons during pharmacologically induced flight motor output in *Manduca sexta*

Chapter 5:
Can cholinergic inputs activate the adult *Manduca* flight network?

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Chapter 2

Postembryonic development of centrally generated flight motor patterns in the hawkmoth, *Manduca sexta*

Postembryonic development of centrally generated flight motor patterns in the hawkmoth, *Manduca sexta*

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Abbreviations:	CDM	chlordimeform
	CPG	central pattern generator
	PTX	picrotoxine
	GABA	g-amino butyric acid
	OAR	octopamine-receptor
	MN1-5	motoneurons 1-5

3.1 Abstract

This study analyses the maturation of centrally generated flight motor patterns during the metamorphosis of *Manduca sexta*. Bath application of the octopamine agonist, chlordimeform (CDM), to the isolated CNS of adult moths reliably induces fictive flight patterns in wing depressor and elevator motoneurons. The maturation of these patterns is investigated by CDM application at different developmental stages. CDM also induces motor patterns in larval ganglia, but these differ markedly from fictive flight, indicating that in larvae and adults, octopamine-receptor activation affects different networks. No motor output is induced at early pupal stages, and first changes in motoneuron activity upon CDM application occur after 50 % of pupal development, at pupal stage P10. Rhythmic motor output is induced in depressor but not in elevator motoneurons by CDM at P12. Adult-like fictive flight activity in motoneurons is observed at P16, two days before adult emergence. Between P16 and adulthood speed and precision of the fictive flight patterns are increased. Pharmacological block of chloride channels with picrotoxin (PTX) also induces fictive flight in adults, but not at pupal stages prior to P16. Our results suggest that the flight pattern generating network becomes gradually established between P12 and P16, and is further refined until adulthood. These findings are discussed in the context of known physiological and structural CNS development during *Manduca* metamorphosis.

3.2 Introduction

The generation of rhythmic motor patterns, such as breathing, walking, swimming, and flying, relies upon activity in central pattern generating (CPG) networks. CPG networks can produce rhythmic motor output in the absence of sensory feedback, and are found at the heart of motor networks in all animals (Grillner et al., 2005; Kiehn and Kullander, 2004; Marder et al., 2005). In many cases both the intrinsic properties of the component neurons as well as network connections within CPGs are altered during development (Fenelon et al., 1998; McLean et al., 2000). An extreme example of CPG remodeling during postembryonic development is the metamorphosis of holometabolous insects, like the tobacco hawkmoth, *Manduca sexta*. During metamorphosis the neural networks and motoneurons responsible for the generation of larval crawling movements are remodeled to generate flight motor patterns in the adult moth (Levine, 1984). Considerable data are available on the remodeling of motoneurons, sensory neurons and muscles during metamorphosis (Consoulas, 2000), but it remains unclear how these developmental events relate to the remodeling and maturation of the flight motor patterns. Towards this question the present study analyses the postembryonic development of centrally generated adult flight motor patterns in *Manduca*.

The larval locomotor behaviors of *Manduca sexta* are burrowing and crawling, which cease at pupation. Metamorphosis ends with eclosion to an adult with a dramatically altered morphology and a completely different behavioral repertoire (walking, flying, reproducing and oviposition). The adult is equipped with a new set of muscles, some of which develop *de novo* during metamorphosis, and others develop from larval templates (Consoulas et al., 1997; Consoulas et al., 2002; Duch et al., 2000). Most larval sensory neurons undergo programmed cell death, and newly born sensory cells form adult sense organs to equip the adult appendages (Consoulas et al., 2000a; Consoulas et al., 2000b; Kent et al., 1996; Lakes-Harlan et al., 1991). Similarly, some motoneurons are used for larval or pupal specific behavior and undergo programmed cell death during pupal life (Fahrbach and Truman, 1987; Taylor and Truman, 1974; Truman, 1983, 1987). By contrast, many larval crawling motoneurons persist metamorphosis and become remodeled to innervate the fast contracting indirect flight power muscles in the adult (Casaday and Camhi, 1976; Consoulas et al., 2000a; Duch et al., 2000; Kammer and Kinnamon, 1976; Rheuben and Kammer, 1980). The transformation of larval crawling into adult flight

motoneurons comprises larval synapse elimination and dendritic retraction (Duch and Mentel, 2004), outgrowth of new adult dendrites (Duch and Levine, 2002; Libersat and Duch, 2004), the formation of new adult synapses (Evers et al., 2006; Meseke et al., 2009) and changes motoneuron active membrane properties (Duch and Levine, 2000, 2002). By contrast, virtually nothing is known about flight interneurons and the development of the central flight circuitry.

However, it is known that in the moth, *Antheraea polyphemus*, an adult like motor pattern can be recorded from pupal muscles 3 days prior to ecdysis (Kammer and Rheuben, 1976), and that in *Manduca sexta* the adult flight CPG can be switched on by bath application of the biogenic amine octopamine (Claassen and Kammer, 1986; Kinnamon et al., 1984; Vierk et al., 2009). Octopamine has widespread modulatory actions on the central and peripheral nervous system of invertebrates (Evans, 1985; Orchard et al., 1993; Roeder, 1999, 2005*)* mediated by pharmacologically distinct subclasses of octopamine receptors (Evans and Maqueira, 2005). In insect flight systems octopamine plays important roles in initiation and maintenance of flight motot output (Brembs et al., 2007) by affecting central neuron excitability (Brembs et al., 2007; Ramirez and Pearson, 1991a, b), synaptic transmission (Evans and O'Shea, 1978; Leitch et al., 2003), sensory sensitivity (Matheson, 1997), hormone release (Orchard et al., 1993), and even muscle metabolism (Mentel et al., 2003). Octopamine acts directly on the flight CPG, so that bath application octopamine agonists can induce fictive flight in isolated insect ventral nerve cords in the absence of sensory feedback (locusts: Duch and Pflueger, 1999; Stevenson and Kutsch, 1987; *Manduca*: Claassen and Kammer, 1986; Kinnamon et al., 1984; Vierk et al., 2009). This study tested when the CPG for flight becomes functional during pupal life and can be switched on by biogenic amines, and whether the flight patterns undergo developmental refinement prior to the animal’s first take-off.

3.3 Methods

3.3.1 Animals and staging

Manduca sexta of either sex were taken from a laboratory culture maintained on artificial diet (Bell and Joachim, 1976) at 27^{o}C under a 16 h light/ 8 h dark period at the Free University of Berlin. L5 represents the fifth larval instar, W0 – W4 refers to the 5 days of the wandering instar (W) before pupation. P0 represents the day of pupal ecdysis. P1 to P18 refer to the 18 stages of pupal life approximately corresponding to the days of pupal development. The exact pupal age was determined by chronological and characteristic morphological changes (Bell and Joachim, 1976; Nijhout and Williams, 1974; Reinecke et al., 1980; Tolbert et al., 1983). Adult animals were subjected to experiments 2 days after eclosion.

3.3.2 Preparation

All recordings were conducted from the isolated (deafferented) central nervous system (CNS), including ventral nerve cord, brain, and subesophageal ganglion. Animals were anesthetized by chilling on ice for 20 to 40 minutes. All stages were dissected along the dorsal midline in cold saline containing (in mM): 140 NaCl; 5 KCl; 28 D-Glucose; 5 Hepes; 1 $CaCl_2$; ph 7,4 (Trimmer and Weeks, 1989). The CNS was removed from the animal, transferred into fresh saline, and pinned ventral side up in a sylgard dish containing saline. The cut ends of lateral nerves were used for pinning, except for the nerves IIN1b and IIN4, which were used for extracellular recordings (see Fig. 1a). Nerve IIN1b contains the axons of the motoneurons, MN1-5. In the larval stage MN1-5 innervate the dorsal external longitudinal (DEL) and dorsal internal oblique 2 (DIO2) body wall muscles (Duch et al., 2000). In the adult stage MN1-5 innervate the dorsal longitudinal flight muscle 1 (DLM1) via nerve IIN1b, which also contains two additional axons of motoneurons innervating the smaller DLM2. Nerve IIN4 contains the axon of a motoneuron innervating the dorsal ventral flight muscle 1 (DVM1).

3.3.3 Intracellular recordings

For intracellular recordings of depressor motoneurons with thin-walled borosilicate glass electrodes with a resistance between 20 - 30 MΩ, the ganglionic sheath was removed mechanically from the prothoracic and mesothoracic neuromeres by using a fine pair of forceps following a short treatment with crystalline Protease (SIGMA P-5147). The electrode tips were filled with a solution of 7% Neurobiotin (Vectorlabs) and Rhodamine Dextran (Molecular Probes, MW 3000) in 2M potassium-acetate. Motoneurons were identified by antidromic stimulation with a suction electrode placed on their axons in nerve IIN1b. Recordings were conducted with an Axoclamp 2A (Molecular Devices) amplifier in bridge mode, digitized with an analogue/digital converter digidata 1200 (Molecular Devices) at a sampling rate of 10 kHz, and viewed and analyzed with PClamp 9 software (Molecular Devices).

3.3.4 Extracellular recordings

Extracellular recordings were made with saline filled suction-electrodes, custom-made from glass microelectrodes and polyethylene tubing. Signals were amplified 100-fold with extracellular amplifiers (Grass P55A). Recordings were always made from mesothoracic nerves (Fig. 1a1) containing the axons of depressor MNs (nerve IIN1b) and one elevator MN (Fig. 1a2, one branch of nerve IIN4) and digitized at a sampling rate of 10 kHz.

3.3.5 Fictive flight

In *Manduca* wing down-strokes (depression) is produced by contraction of the dorso-longitudinal muscles (DLM1) innervated by the depressor motoneurons MN 1-5 (Duch et al., 2000, Fig. 1a1). Up-stroke (elevation) of the wings is produced by dorso-ventral muscles (DVMs). The depressor MNs 1-5 project via nerve IIN1b (Fig. 1a1) and were recorded extracellularly (Fig 1b1, middle trace), whereas elevator MNs to DVM1 (Fig. 1a2) were recorded extracellularly from nerve IIN4 (Fig. 1b1, lower trace) (Nüesch,

1985; Vierk et al., 2009). Extracellular recordings were combined with intracellular recordings from the soma of one prothoracic DLM-motoneuron (MN1-4, Fig. 1b1, upper trace).

Fictive flight motor patterns were induced by bath application of the octopamine receptor agonist chlordimeform CDM (Riedel-de Haen) dissolved in saline (10^{-5} M) in adults, various pupal stages and the larval stage 5. Picrotoxin (PTX), a chloride channel blocker and thus effective g-amino butyric acid $_A$ (GABA $_A$) receptor antagonist (Olsen, 2006; Takeuchi and Takeuchi, 1969), was bath applied at a concentration of 10^{-5} M. All solutions were continuously perfused through a 4-tube gravity perfusion system (ALA BPS-4). Amine injections directly into *Manduca* mesothoracic ganglia have proven to be effective at concentrations as low as 10^{-9}M (Claassen and Kammer, 1986, Kinnamon et al., 1984). However, bath application requires the chemicals to pass the ganglionic sheath, so that higher concentrations have to be applied. We have not tested different concentrations of CDM but instead used concentrations which have previously been reported to successfully induce fictive flight in *Manduca* (Vierk et al., 2009) as well as in other insect preparations (Duch and Pflüger, 1999). Recordings from nerve N1 and nerve N2A in larval preparations were used to analyze the effect of CDM application on larval pattern generating networks.

3.3.6 Data analysis

All parameters of the motor output were analyzed and quantified with respect to depressor activity as recorded intracellularly in all pupal and adult preparations. Data analysis was performed with Clampfit 9.0 and Spike2 from representative bouts of activity over a time period of at least 30 s. *Cycle period* was defined as the duration between the onsets of subsequent depressor bursts as determined by extracellular recordings from nerve IIN1c (Fig. 1b2). *Depressor-elevator interval* (latency) was defined as time between the onset of depressor (dep) activity with respect to the onset of corresponding elevator (elev) activity (Figs. 1b1, b2). *Phase-relationship* (phase) was defined as quotient of depressor-elevator latency over depressor cycle period. The x-axis of *phase histograms* was divided into 100 bins of equal width for better representation of distribution. The spike counts for each bin were divided by the total number of cycles analysed to give a per cycle mean

value. Linear regression analysis was used to determine the relationship between depressor-elevator and elevator-depressor interval and changes in the depressor cycle period. The correlation coefficient (r) was calculated by using the least square error method in MS Excel. *Auto-correlation histograms* were centered around the first spike event on the reference time series (depressor spikes). The occurrences of spikes at different time intervals around the reference spike were counted into 400 bins of 5 ms width, corresponding to a time window of plus and minus 1 second around each depressor spike. Then the histogram was centered around the next depressor spike and counting into bins was repeated. This process was repeated for every depressor spike of the flight bout (30 seconds duration in total). The histograms were not normalized but present total event counts.

Statistics were conducted with the Statistica ´99 (Statsoft, Inc.). One-way ANOVA with Tukey HSD-test (honest significant difference-test) was used to test for statistical differences between multiple groups. We analysed and compared data of five P12, four P14, seven P16, eight P18, and ten adult animals.

4. Results

4.1 Chlordimeform (CDM) induced adult fictive flight motor patterns

Fictive flight motor patterns were elicited in isolated ventral nerve cord preparations (Figs. 1a1, 1a2) by bath application of the octopamine receptor agonist chlordimeform (CDM) as previously described (Vierk et al., 2009). Continuous perfusion with CDM reliably induced a stable and strictly alternating motor pattern which was characterized by 2 phases: the depressor phase was monitored by recordings of the spiking activity of the MNs1-5 which innervate the DLM depressor muscle (Fig. 1b1 top, middle trace; 1b2 top trace). The elevator phase was monitored by recording the spiking activity of one elevator motoneuron (DVM1) (Fig. 1b1 bottom trace; 1b2 bottom trace). Rhythmic, strictly alternating depressor and elevator activity started five to eight minutes after CDM application and lasted for up to 2 hours with stable period and phase relationships (Vierk et al., 2009).

4.2 Fictive flight patterns during different stages of postembryonic development

The first objective was to test at which stages during postembryonic development the nervous system was capable of producing flight like motor patterns. Fictive flight as produced by isolated adult ventral nerve cords (Fig. 2a) served as reference for the comparison of motor patterns induced by CDM application to the ventral nerve cord of earlier developmental stages, P10 to P18. In P18 (Fig. 2b) continuous alternating elevator and depressor spike generation that was qualitatively similar to the adult fictive flight patterns (Fig. 2a) occurred in all preparations. However, in 3 out of 8 experiments some depressor motoneurons did not generate spikes during each cycle. In P16 (Fig. 2c) a rhythmic alternating motor pattern was still observed, although in 3 out of 7 experiments depressor spike failures occurred. Intracellular recordings revealed subthreshold synaptic drive with the fictive flight rhythmicity (Fig. 2c, see asterisk). In only 1 out of 7 preparations rare spike failures were also observed in the elevator motoneuron during the motor bout (Fig. 2c, top right trace, arrow). Unpatterned depressor activity but no elevator activity was induced by CDM application in four P14 (Fig. 2d) and five P12 (Fig. 2e) preparations. In P14 no elevator spikes or subthreshold synaptic activity occurred (Fig. 2d, top trace). In the depressor motoneurons rhythmic spiking activity occurred in 1 out of 4 preparations, but spike failures also occurred (Fig. 2d). However, subthreshold rhythmic drive to the depressor motoneurons occurred in all 4 preparations (Fig. 2d, bottom trace). The results of P12 resembled those of P14 but spike failures in the depressor motoneurons occurred more frequently, and subthreshold synaptic drive was weaker (see below). In P10 no spiking activity was induced by CDM application (Fig. 2f). Although not quantified the frequency of subthreshold synaptic activity seemed to be increased after CDM application, but without any obvious rhythmicity. Similarly, in P8 no effect of CDM on flight motoneuron activity was observed in 1 test recording (data not shown).

To test whether a flight-like motor network responsive to octopamine is present in the larval stage, similar to what is known from the hemimetabolous locust (Stevenson and Kutsch, 1987), CDM was bath applied to the larval nerve cord (Fig. 3). Recordings were made from the main roots of larval nerves, N1 and N2a, which among many others, contain also the axons of the future flight motoneurons (nerve N1). In this developmental stage the future depressor motoneurons 1-5 innervate the larval dorsal body wall muscles DEL and DIO2 (Casaday and Camhi, 1976; Duch et al., 2000) and participate in larval

crawling (Duch and Mentel, 2003). CDM elicited patterned activity that alternated between nerves, N1 and N2a (Fig. 3a and b). However, these motor patterns differ significantly from adult fictive flight or any CDM-elicited motor patterns after P12. In the larval stage motoneurons showed burst of action potentials whereas single spikes occurred during each cycle of fictive flight. Furthermore, larval motor patterns were much slower with median values for cycle periods around 900 ms (Fig. 3c) as compared to approximately 100 ms in the adult. Compared to larval crawling (Johnston and Levine, 1996) the CDM-induced patterns were about 9 times faster.

4.3 Quantitative measurements of fictive flight pattern parameters in different developmental stages

To analyze the maturation of fictive flight patterns, cycle period, intervals (latency) between alternating bursts, number of action potentials per duty cycle, phase relationships, and the precision of rhythmicity were compared quantitatively between different stages.

The median cycle period (Fig. 4a) of motor pattern in adults, pupal stages P16, and P18 were not significantly different. There was, however, a trend to shorter cycle periods from P16 over P18 to adults (Fig. 4a, see inset). The variability of cycle duration was larger in P16 as compared to pharate or emerged adults (Fig. 4a). In earlier pupal stages, cycle periods as measured between postsynaptic potentials (PSPs) or action potentials, were significantly longer and exhibited a much higher variability (Fig. 4a). In adults, single depressor motoneurons fired precisely once per cycle (Fig. 4b). Some failures in the generation of depressor action potentials occurred at P18 (median number of spikes per cycle is 1, see Fig. 4b), more failures occurred at P16 (median number of spikes per cycle is 0.89, see Fig. 4b), and many failures occurred at P14 and P12 (median number of spikes per cycle is 0 in both, see Fig. 4b).

The depressor-elevator interval (latency between the onsets of depressor and elevator bursts) was quantified at the stages P16, P18, and adult because no elevator spikes were induced by CDM at earlier pupal stages (see figure 2). The median depressor-elevator latency was around 50 ms for all three stages with no significant differences between the stages (Fig. 4c). Similarly, the elevator phase was 0.5 for P16, P18, and adult and showed no significant differences between these stages (Fig. 4d), but the data spread was larger at

P16 than at P18 and larger at P18 than at the adult stage. Insets in Fig. 4d illustrate the relative numbers of elevator spikes as phase histograms. This indicated that elevator phase regulation at 0.5 was more variable at late pupal stages as compared to the adult stage.

To further test whether phase constancy existed at all stages we plotted the depressor-elevator and the elevator-depressor latencies over depressor cycle period for contiguous flight bouts from representative recordings (Fig. 5). In the adult recording the cycle period ranged between 78 to 92 ms (Fig. 5a, x-axis) and changes in cycle period correlated with changes in both the depressor-elevator interval (correlation coefficient, 0.53) and the elevator-depressor interval (correlation coefficient, 0.39). This was also the case for pupal stages P18 and P16 (Figs. 5 b, c). Data for all three stages were plotted on the same scales to demonstrate that data spread strongly decreased from P16 over P18 to adult.

Auto-correlation histograms (Fig. 6) were created form extracellular records of the five depressor motoneurons for the stages P16, P18, and adult. These contained 400 bins, each of 5 ms duration, to cover a time period from -1 to +1 seconds around each depressor spike (see “Methods”). The histograms were not normalized but instead presented total event counts (Figs. 6a-c). In all stages, the highest spike counts occurred at 0 seconds and were surrounded by recurrent peaks. Periodic peaks and troughs occurred approximately every 90 ms (Fig. 6a, arrows) demonstrating periodic clustering of spikes at a cycle frequency of roughly 11 Hz in adults. The sharp rises and troughs pointed to precise rhythmicity (Fig. 6a). This precision was lower at P18 (Fig. 6b) than in the adult, and lower at P16 (Fig. 6c) than at P18. In developmental stages P18 (Fig. 6b) and P16 (Fig. 6c) the total counts were decreased, and the cycle period was slowed down as illustrated by longer periods between periodic peaks (110 ms in P18 and 130 ms in P16). However, spike counts in troughs were almost never zero in P18 and never zero in P16. As stated above, stage P14 motor patterns contained many depressor spike failures (Fig. 2d). Therefore, auto-correlation was conducted from subthreshold events recorded intracellularly from one depressor motoneuron. As only single PSPs or spikes occurred the resulting event counts were zero at time 0 seconds. However, periodic peaks and troughs point to some rhythmicity of the subthreshold events in P14. The cycle period was much longer as compared to older stages, and there was less precision of the pattern.

In summary, flight like motor patterns can be elicited from stage P16 on, and become increasingly refined until adulthood. Rhythmical depressor activity can be induced by CDM application already at P14, but not so in elevator motoneurons.

4.4 Is the network inhibited in pupal stages before P16?

The absence of elevator motoneuron spiking or the failures in elevator spikes might either be caused by the absence of excitation or by the presence of active inhibition during earlier stages. To test whether inhibition might have masked the presence of the flight CPG at earlier stages we bath applied the chloride channel blocker picrotoxin (PTX). GABA represents the predominant inhibitory transmitter in insect motor networks, and PTX blocks $GABA_A$ receptor mediated inhibition (Sattelle, 1990; Watson, 1992). PTX application to adult isolated nerve cord preparations (Fig. 7a2) induced a motor pattern similar to that elicited by CDM (Fig. 7a). Both patterns show alternating, rhythmic depressor/elevator activity with similar values of cycle period, frequency, and rhythmicity (quantification not shown). The differences between CDM and PTX induced motor patterns were first, a shift in the elevator phase relationship (Figs. 7a, a2) from 0.5 in CDM induced flight to 0.7 in PTX induced flight (Fig. 7a1), and second, firing of duplet depressor motoneuron spikes in PTX as compared to single spikes per cycle in CDM (Fig. 7a2).

In P16 PTX application released a similar fictive flight like pattern (Fig. 7b), but failures in the generation of depressor and elevator spikes occurred as also described for CDM induced fictive flight at P16 (Figs. 2c, 4b). PTX application at earlier developmental stages, such as P14 and P12 (Figs. 7c, d) also elicited motoneuron spiking, but the pattern did not have any obvious features of fictive flight. First, PTX induced elevator and depressor spikes at P12 and P14, whereas CDM induced only depressor spikes. In PTX elevator and depressor spikes are in phase, which was never observed in CDM induced fictive flight patterns.

5. Discussion

The present study shows that fictive flight like motor patterns can be elicited from isolated *Manduca* ventral nerve cords by bath application of the octopamine agonist, CDM, at pupal stage P16, and then progressively matures in frequency and precision until adulthood. In earlier pupal stages no flight like motor patterns are induced neither by CDM nor by the chloride channel blocker PTX. In our experiments the absence of a rhythmic motor pattern in early pupal stages could potentially be caused by (i) the absence of the pattern generating networks, (ii) the lack of octopamine-receptors (OARs), or (iii) a possible inhibition of existing circuits, or by a combination of the above. We can not conclusively rule out any of these causes, but previously published work on neuromuscular development and the development of the octopaminergic system favor the first hypothesis.

5.1 *Neuromuscular development and the emergence of flight motor patterns*

Adult flight motoneurons are born embryonically, function as fully differentiated crawling motoneurons in the larval stage and become remodeled during metamorphosis to accommodate the requirements of adult flight (Casaday and Camhi, 1976; Kammer and Kinnamon, 1976; Rheuben and Kammer, 1980; Duch et al., 2000; Consoulas et al., 2000). As shown in detail for the depressor flight motoneuron, MN5, this remodeling is accompanied by the retraction of larval dendrites and larval synapse degradation during the dismantling of the larval circuits (Duch and Mentel, 2004), followed by the formation of dendritic filopodia and two distinct phases of dendritic growth (Libersat and Duch, 2002) and synaptogenesis (Evers et al., 2006) during the integration of the motoneurons into the new adult networks. The adult dendritic structure is established at P16, exactly the stage when fictive flight can first be elicited. This indicates that flight motoneurons might not be fully connected to the CPG network prior to P16, although MN5 receives synaptic input during all stages of postembryonic dendritic remodeling (Duch and Levine, 2000). Moreover, calcium and potassium membrane currents are altered in MN5 between the larval stage and pupal stage P12, the first time when CDM evoked spiking in depressor motoneurons. Somatic current injections induce action potentials also during earlier stages (Duch and Levine, 2000), but responsiveness to the octopamine agonist CDM is observed after calcium and potassium currents are adult-like. Therefore, the maturation of

motoneuron electrophysiological properties correlates in time with the first motoneuron responses to CDM application to the isolated ventral nerve cord.

Since interneurons of the central flight pattern generator remain unidentified, it is not clear whether these undergo similar time courses of dendritic growth and electrophysiological remodeling. However, dendritic growth (Matheson and Levine, 1999) and calcium current expression are controlled by general steroid signals which are present at the same time for all neurons located in the pterothoracic ganglia. Therefore, it seems reasonable to assume a comparable time course of developmental events in flight interneurons. The developmental times of in-growth of sensory axons and the formation of their central arbors are not known in the flight system. However, leg sensory neurons grow and arborize in the CNS long before P12 (Consoulas, 2000). Therefore, it seems unlikely that the CPG circuit is fully connected prior to P12 and then triggered functional by the arrival of sensory axons.

5.2 *The development of fictive flight motor patterns*

In this study the development of fictive flight motor patterns was described by recording the activity of elevator and depressor motoneurons after bath application of CDM. A fictive flight pattern was observed in pupal stages later than P16. Therefore, the absence of fictive flight patterns prior to stage P16 could be a consequence of either a lack of OAR expression at earlier stages, or masking of an already present CPG by inhibitory mechanisms (see next chapter). First, CDM did effect the spontaneous activity of depressor motoneurons at P10 (increased number of spontaneous PSPs) and P12 (increased number of spontaneous PSPs and action potentials). Therefore, at least some OARs must be present in the pupal CNS prior to P16. Although no data on expression of OARs are available in *Manduca*, data from *Drosophila* indicate that at least OAR mRNA transcript levels are present at early pupal stages and peak after 50% of pupal development (Hannan and Hall, 1996). Comparable stages in *Manduca* are P8 to P10, but fictive flight can not be induced by CDM until P16. Second, in *Manduca* octopamine levels are regulated during pupal development, and alterations in octopamine levels may cause changes of behavioral activity (Awad et al., 1997; Bodnaryk, 1980). Levels of the enzyme that synthesizes octopamine from tyramine, Tβh (tyramine-ß-hydroxylase), begin to rise from P10 and increase until P18 (Lehman et al., 2000), suggesting the presence of endogenous

octopamine prior to P16. This corresponds to CDM effecting spontaneous depressor MN activity at P10, and eliciting fictive flight at P16. These three findings make it unlikely that the absence of fictive flight motor patterns prior to P16 is caused by the absence of OARs.

However, the antagonistic elevator and depressor systems seem to develop with different time courses. Subthreshold synaptic and the first spiking activities are seen in depressor motoneurons as early as P10 (subthreshold) and P12 (spiking), but elevator motoneurons do not show any responses to CDM application at these stages. Interestingly, in a previous study, the depressor but not the elevator motoneurons were strongly activated by tyramine also suggesting differentiation between these two systems (Vierk et al., 2009).

Our results correspond to earlier findings in the moth, *Anthereae polyphemus*, where a flight-like pattern was detected by electromyogram recordings (EMGs) from flight muscles in pupal stages 3 days prior to ecdysis (Kammer and Rheuben, 1976). In this study it is noted that similar flight-like-patterns could also be recorded from *Manduca* pupal muscles. The development of muscular activity was characterized by four phases: (1) single and small muscle potentials occur irregularly, (2) an increase in activity and amplitude of muscle potentials, (3) more regular and evenly spaced potentials, and (4) appearance of antagonistic units (Kammer and Rheuben, 1976). This agrees with our findings from intracellular recordings where first unpatterned activity is observed in depressor motoneurons, followed by rhythmic activity in depressors motoneurons, followed by coordinated alternating activity of depressor and elevator motoneurons. Differences between the findings from *Anthereae* and *Manduca* may be explained by the two completely different preparations: Kammer and Rheuben (1976) made EMG-recordings from intact pupae with all sensory feedback intact whereas in this preparation isolated ventral nerve cords were used, thus, only revealing the pattern solely produced by the CPG. Interestingly, the muscle potentials recorded from pupal muscle do not lead to contractions that are anywhere as powerful as those occurring during flight. Therefore, the muscle fibers or/and the neuromuscular junctions may still be in a premature state and, perhaps, both motoneuron and muscle firing might even be decoupled (Kammer and Kinnamon, 1979).

5.3 *Does network inhibition occur at early pupal stages?*

GABA is the major inhibitory transmitter in the central and peripheral nervous system of invertebrates (Lunt, 1991). To test the hypothesis that the flight producing network is present and may be potentially capable of generating central flight motor patterns but is inhibited in pupal stages before P16, we blocked important GABA-receptors of invertebrates (Lunt, 1991). Application of PTX, the most specific and effective receptor antagonist for $GABA_A$-receptors (Bloomquist, 2003; Sattelle, 1990), elicited flight-like motor patterns in quiescent isolated nerve cords of adults, of P18, and of P16, but not in earlier pupal stages. This suggests that the flight network does not seem to be present and functionally inhibited prior to P16 by $GABA_A$-receptors. In addition, it seems unlikely that the motoneurons with retracted dendrites are connected to a functional flight network. This finding contrasts those from hemimetabolous insects where many central pattern generating networks seem to be fully developed early during postembryonic life but are suppressed until the final molt, e.g. stridulatory behavior in crickets (Bentley and Hoy, 1970), grasshopper egg laying motor program (Thompson, 1986) and flight motor program in locusts (Stevenson and Kutsch, 1988). The functional connectivity early in the development of hemimetabolous insects differs markedly to holometabolous insects where the peripheral and central nervous systems undergo a substantial postembryonic reorganisation (Levine et al., 1986). However, we can not exclude a putative inhibitory influence of $GABA_B$-receptors, since it has been show that insect MNs also express these receptors (Pinnock et al., 1988; Sattelle et al., 1988), or other inhibitory compounds like glycine. In other systems GABA plays a crucial role in the development of motor patterns. In the stomatogastric ganglion (STG) of crustaceans a single embryonic motor pattern precedes the expression of the adult patterns (Casasnovas and Meyrand, 1995), and neuromodulatory inputs play an important role in this change (Le Feuvre et al., 2001). The expression of the adult motor patterns is dependent on GABA-signaling. If GABA-synthesis is prevented in an adult animal, the subsequent rhythms produced by the STG again resemble those of the embryo (Ducret et al., 2007). In the sea slug *Aplysia*, GABAergic inhibition also modulates the phasing and timing of motor patterns (Jing et al., 2003). We find also some indication that GABA may be involved in modulating the relative timing of depressor and elevator units suggesting rather a functional role in

shaping central motor pattern in *Manduca* like in *Aplysia* than an instructive role for the development of the central flight circuitry like in the STG.

5.4 *Maturation of motor patterns in different organisms*

Amphibians and mammalian neonates possess rudimentary forms of future adult behavior which undergoes a progressive maturation during development. The locomotory rhythms of all vertebrates including mammals start with spontaneous activity in the spinal cord (Hamburger and Balaban, 1963), and basic motor circuits are functioning early in the embryo of chicks (Bekoff, 1992) or mice (Clarac et al., 2004). Progressive development of locomotory patterns are characterized by changes in segmental sensory input, descending neuromodulatory pathways and intrinsic MN properties (Gao and Ziskind-Conhaim, 1998). In larval *Xenopus* a swimming pattern exists early in embryonic life independent from sensory input (Kahn and Roberts, 1982a, b; Sillar et al., 1991), and its maturation to a more precise swimming pattern is caused by descending neuromodulatory, for example serotonergic input (McLean et al., 2000; Sillar et al., 1998; Sillar et al., 1995). Interestingly in further metamorphosis of the motor patterns of larval (tadpoles) to adult frogs, the spinal cord networks undergo profound changes under the control of a variety of neuromodulators among them serotonin, noradrenaline and nitric oxide (Rauscent et al., 2006; Sillar et al., 2008).

In mammals, the respiratory networks develop and mature from birth on (Richter and Spyer, 2001). First, there is a very good correlation between the changing morphological and physiological properties of individual component neurons. Second, network properties also undergo changes such as a reduction in the number of electrical synapses or the availability of voltage-regulated conductances. Therefore, changes in both single neuron and network properties are responsible for the maturation of the mammalian respiratory network (maturational network-burster theory; Richter and Spyer, 2001).

Holometabolous insects also undergo drastic changes in behavior and body plan as they transform from crawling larvae to walking and flying adults which seems to be under control of ecdysteroids (Truman, 1996; Truman and Riddiford, 2007). In contrast, hemimetabolous insects such as locusts demonstrate adult like functional connectivity early in development comparable to vertebrate. Compared to our findings, a “flight-like pattern” was never observed in *Manduca* larvae nor was an adult-like flight pattern masked

in the larva by GABAergic mechanisms. Rather, like in the STG, the adult flight-like pattern appears during metamorphosis, but unlike in the STG (Casasnovas and Meyrand, 1995) a transitional stage between a larval and an adult pattern was never observed suggesting that in *Manduca* the networks for flight are only functional from P16 on. In *Manduca* a progressive refinement of the motor pattern occurs between the pupal stages P16 and the adult with a “spontaneous” start of bouts of activity in depressor motoneurons and then a gradual increase in precision of the motor pattern.

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7. Figures and Figure legends

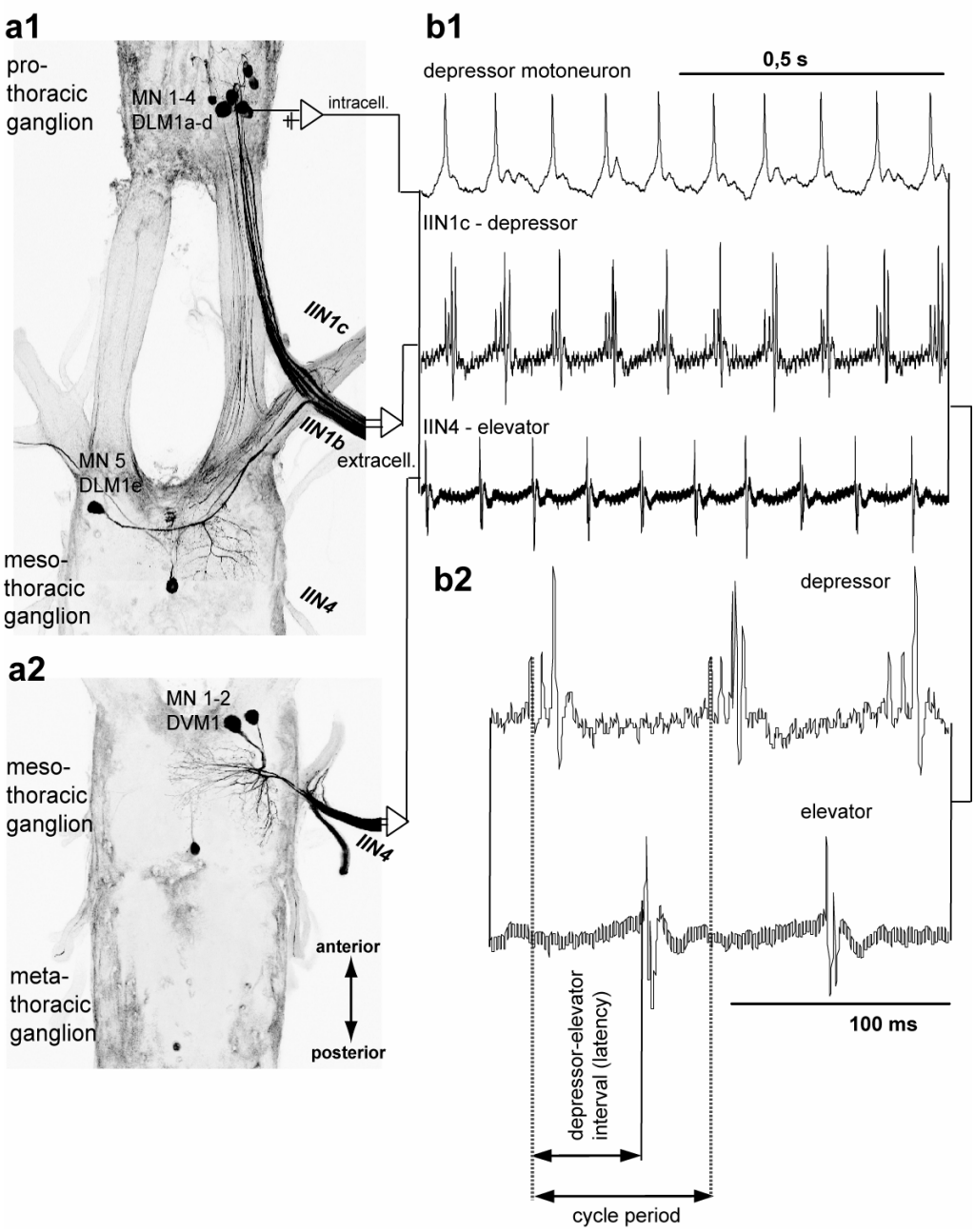

Fig. 1 a Retrograde staining (Neurobiotin and subsequent conj.Cy3-Streptavidin) of the mesothoracic nerve IIN1b (*top*) reveals the location and projection of four prothoracic depressor motoneurons (MN1-4) and one mesothoracic (MN5) depressor motoneuron, two smaller prothoracic motoneurons and one mesothoracic ventral unpaired median neuron. The section below (**a2**) shows a retrograde tracing of nerve IIN4, which contains the axons of two mesothoracic elevator motoneurons and one ventral unpaired median neuron. **b1** Simultaneous intracellular recordings from one prothoracic depressor motoneuron (*upper trace*) and extracellular recordings of five depressor motoneurons from nerve IIN1b (*middle trace*) and from one elevator motoneuron from one branch of nerve IIN4 (*lower trace*) during pharmacologically induced fictive flight. **b2** Expanded time scale from (**b1**). A cycle period is defined from the beginning of one depressor burst to the beginning of the next one (*arrow between grey dotted lines*). Depressor-elevator interval (latency) is indicated by an *arrow from grey dotted to black solid line.*

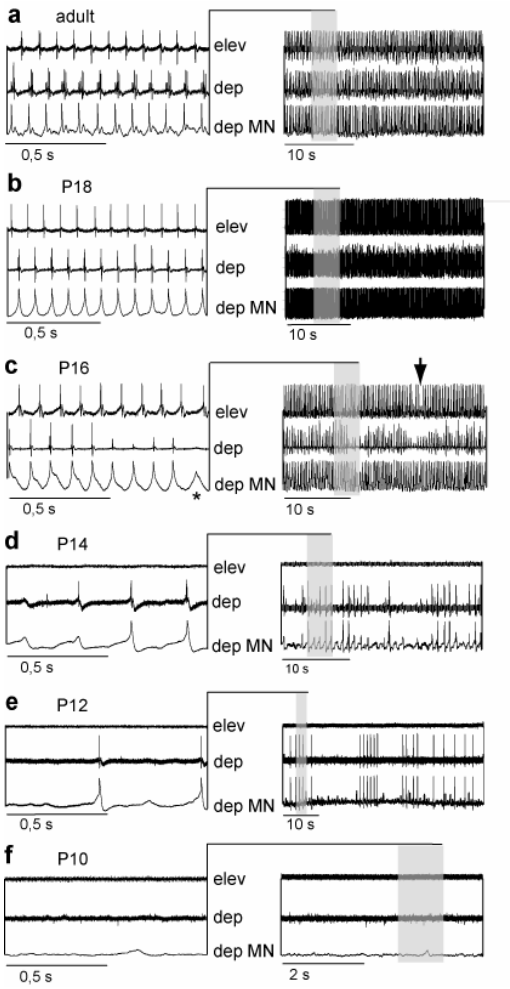

Fig. 2 CDM induced fictive motor patterns in isolated nerve cord preparations from adult (**a**) and different pupal stages (P18, P16, P14, P12, P10) (**b-f**) of *Manduca sexta*. The *top trace* always shows the elevator activity (elev), the *middle trace* shows the depressor activity (dep) and the *bottom trace* displays the activity of one depressor motoneuron (dep MN). *Grey boxes* indicate one second of recorded motor patterns which are enlarged and shown on the *left*.

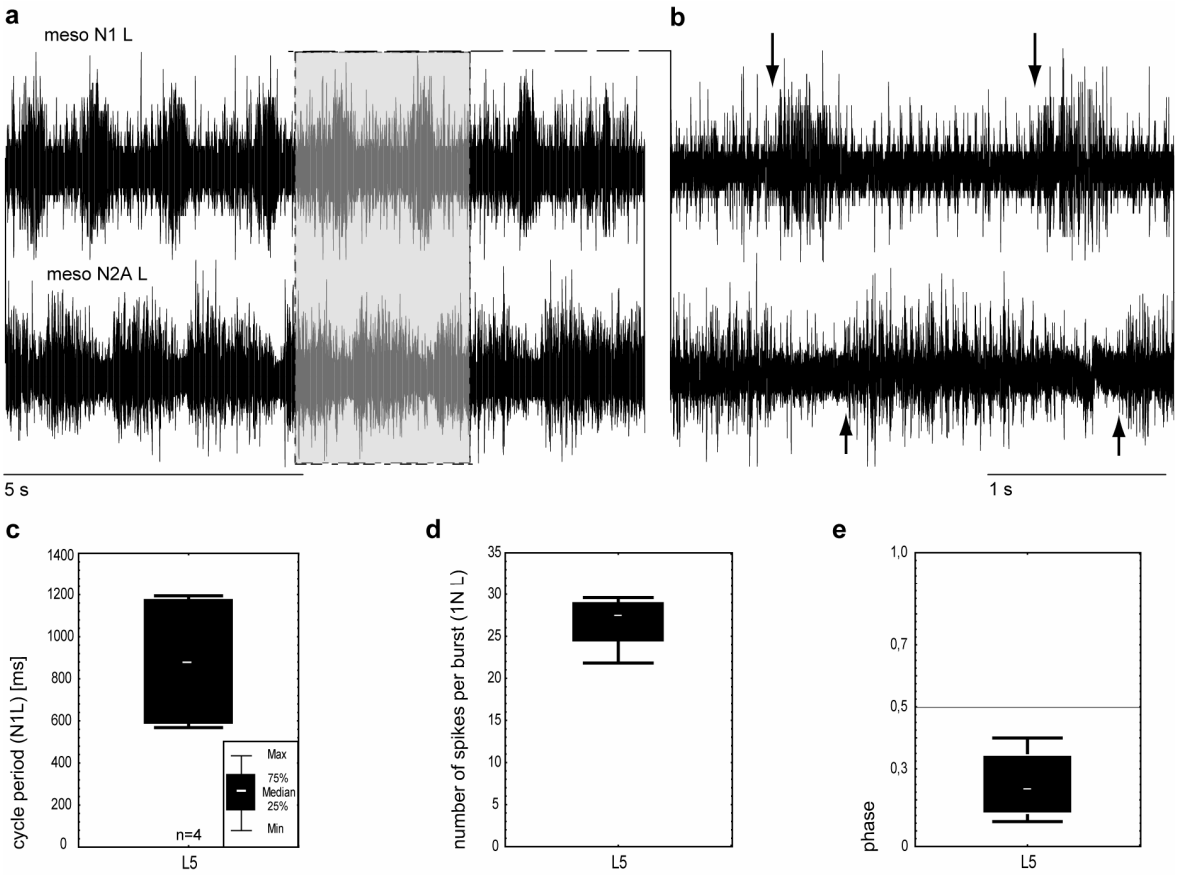

Fig. 3 Effect of CDM application (10^{-5} M) to an isolated nerve cord preparation of larval stage 5 (L5). **a-b** Extracellular recording from mesothoracic nerve 1 (*upper trace*) shows among many other motor units the activity of larval MN1-5 innervating dorsal body wall muscles (dorsal external longitudinal - DEL, dorsal internal oblique 2 - DIO2) and MNs innervating intersegmental body wall muscles. Simultaneous recording from nerve 2A of the same ganglion (*lower trace*) displays the activity of mesothoracic leg depressor (dep) and flexor (flex) MNs. The *grey box* indicates one cycle of alternating activity which is shown enlarged in (**b**). In **c-e** Quantification of cycle period of spiking activity in nerve 1 (**c**), number of spikes per burst of activity in nerve 1 (**d**) and phase of activity in nerve 2A relative to the onset of activity in nerve 1 (onsets marked by *arrows* in **b**) (**e**). Median values were obtained for 65 cycles of 4 preparations during CDM application. Small *white bars* indicate the median values, the *black boxes* represent the 25 and the 75 quartile and the *error bars* indicate the range from minimum to maximum values (see inset in **c**).

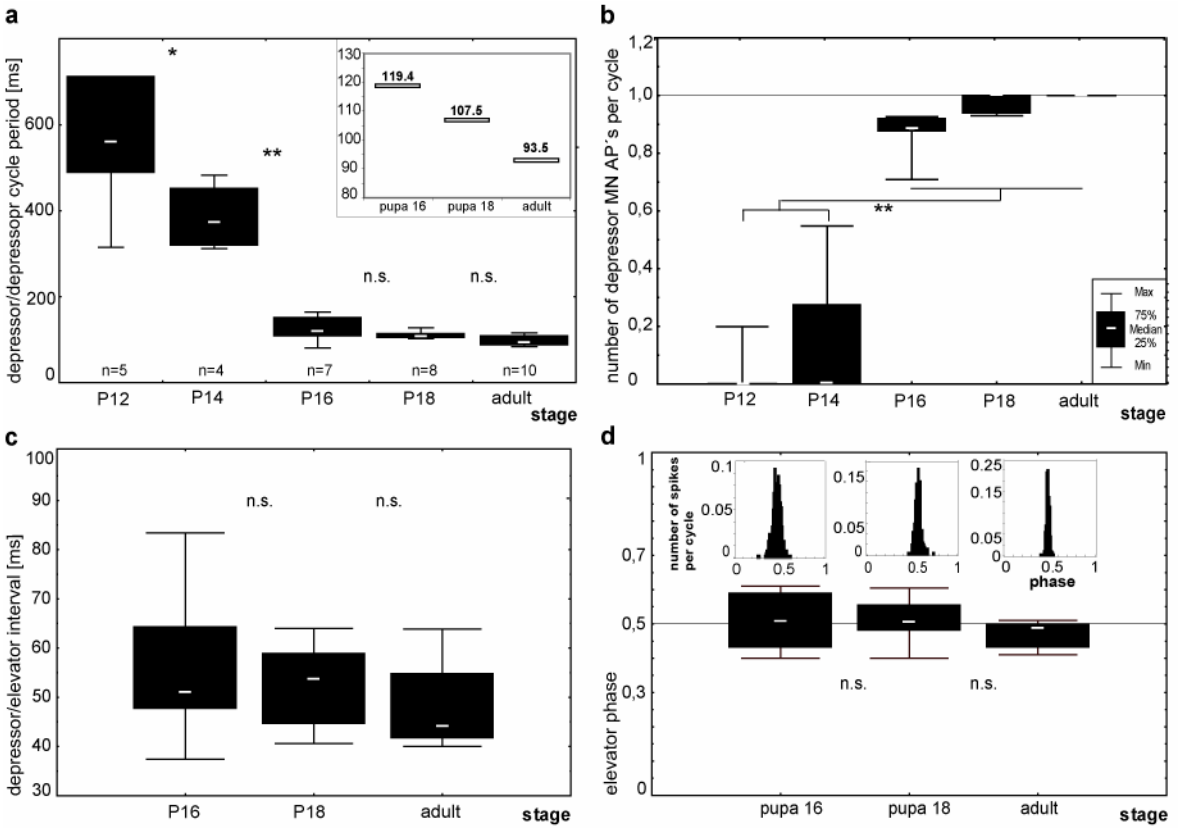

Fig. 4 Quantitative measurements of the following pattern features of CDM induced motor patterns during development. **a** Duration of depressor cycle period in five develpmental stages. The inset depicts the median values of P16, P18 and adult for a better comparison. **b** Comparison of the failures of single depressor motoneuron AP generation of five develpmental stages. **c-d** Depressor/elevator intervals (**c**) and elevator phase (**d**) in the late pupal stages and adults. The insets exemplarily depict phase histograms (data from 200 consecutive cycle periods) of the corresponding stages. **a-d** *White bars* indicate the median values, the *black boxes* represent the 25 and the 75 quartile and the *error bars* indicate the range from minimum - maximum values (see inset **b**). The number (n) of preparations are indiced at the bottom of (*a*). For statistical analysis, average values were calculated for 200 cycles per animal from according developmental stages. Statistical significance is indicated by asterisks, * $p < 0.05$, ** $p < 0.005$, one-way ANOVA, post hoc Tukey HSD-test.

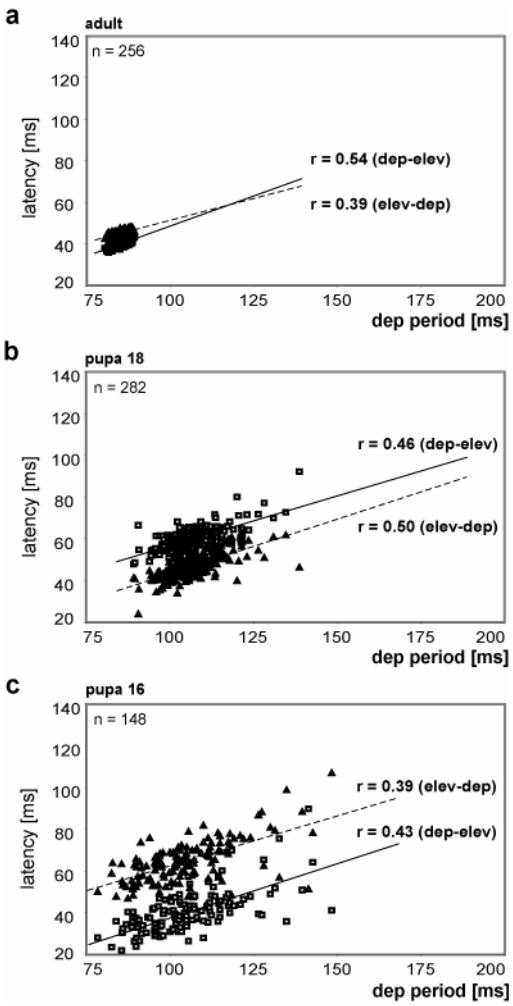

Fig. 5 a-c Depressor-elevator (*open white squares, black line*) and elevator-depressor (*black triangles, dotted line*) latencies plotted as function of the depressor cycle period during CDM induced fictive flight. Data points are values from a single representative preparation (number of cycle periods are indicated in the upper left corner). **a** Depressor-elevator latency and the elevator depressor latency change as the depressor cycle period changes (correlation coefficients, 0.54 and 0.39 respectively; $p < 0,05$) in adults. **b-c** The same analysis during fictive flight in different pupal stages P18 (**b**, $p < 0,05$) and P16 (**c**, $p < 0,05$).

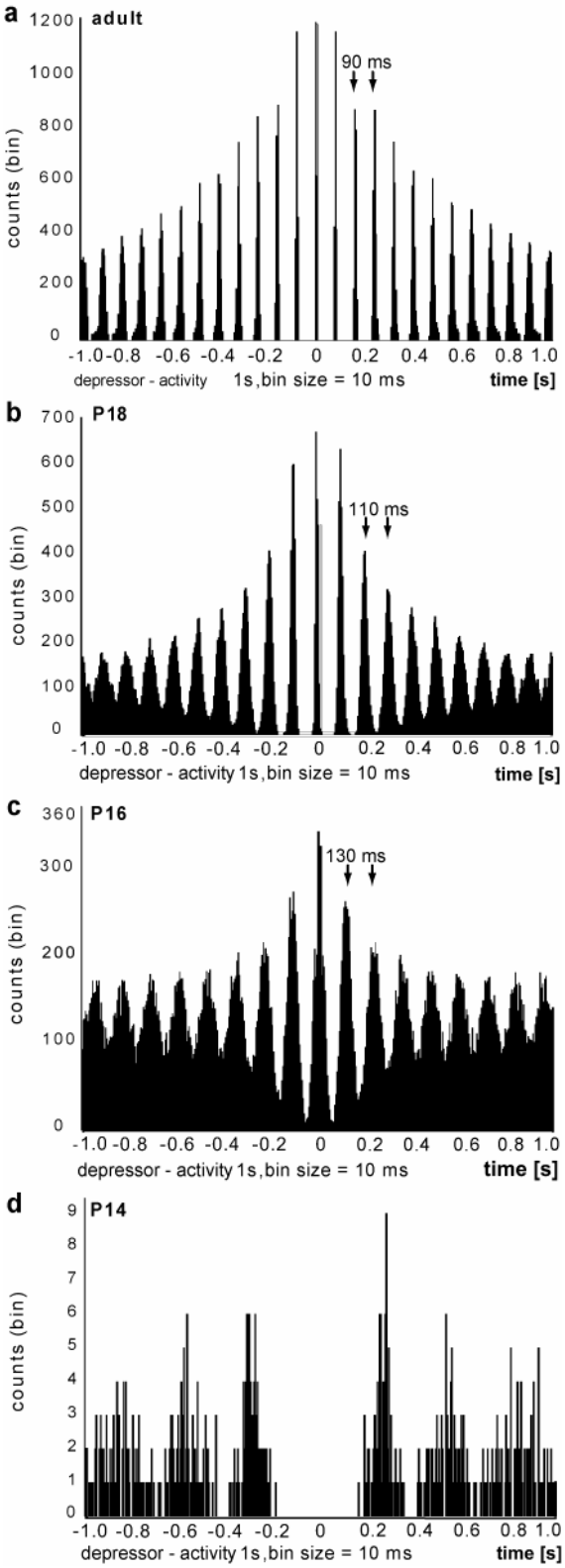

Fig. 6 Rhythmicity and precision of depressor activity in adults and different pupal stages of *Manduca* during CDM induced fictive flight. **a-d** Representative auto-correlation histograms of extracellularly recorded depressor bursting activity (*arrows* denote the cycle period) in adult (**a**), in P18 (**b**), in P16 (**c**) and inP14 (**d**). All auto-correlations of depressor spikes were executed over 30 consecutive seconds (6000 bins total) and 400 bins are shown (bin size = 5 ms, ±1 s displayed in auto-correlation histogram).

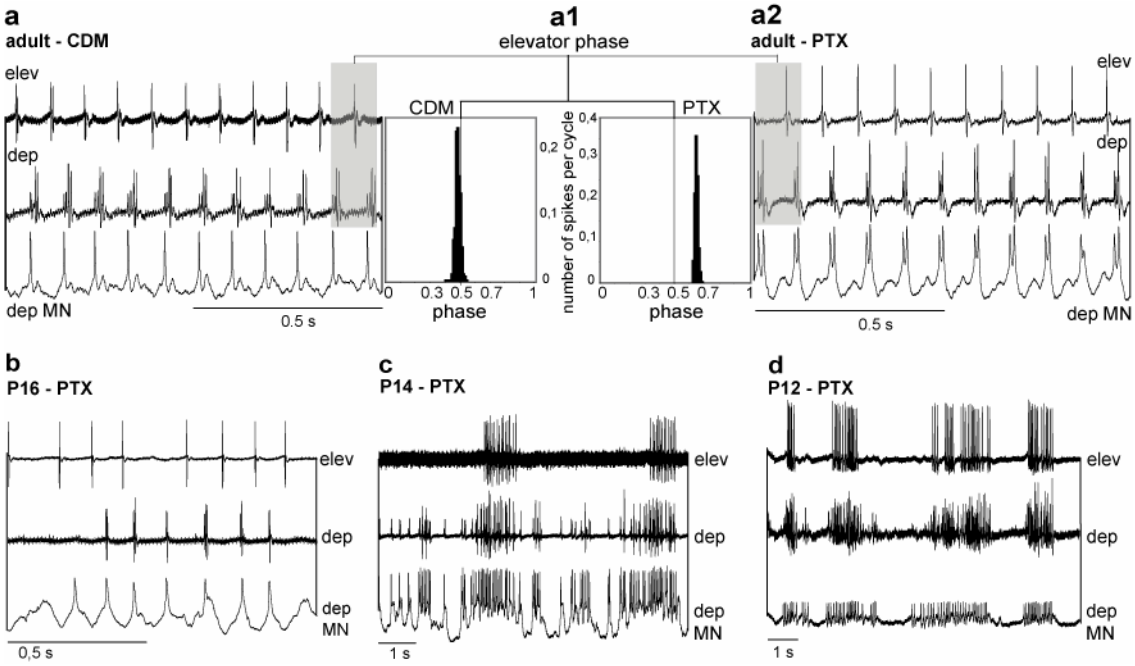

Fig. 7 Removing putative inhibition by bath application of PTX (1 x 10^{-5} M) in different developmental stages. **a-d** Extracellular recordings from elevator (*top trace*) and depressor motoneurons (*middle trace*) and simultaneous intracellular recording (*bottom trace*) of a fore wing depressor motoneuron. **a-a2** Fictive flight motor pattern induced by CDM (**a**) and by PTX application (**a2**) in adult preparations. **a1** Phase histogram of the elevator phase in CDM (data from consecutive 200 wing beat cycles) and PTX (data from 300 consecutive wing beat cycles) induced fictive flight. **b-d** PTX induced motor output in earlier developmental stages P16 (**b**) and P14 (**c**) and P12 (**d**).

Chapter 3

Differential effects of octopamine and tyramine on the central pattern generator for *Manduca sexta*

The original publication is available at
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Differential effects of octopamine and tyramine on the central pattern generator for *Manduca* flight

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Key words: insect, flight, locomotion, modulation, biogenic amine

Running title: tyraminergic modulation of an insect central pattern generating network

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Abbreviations:

CDM, chlordimeform

MN1-5, motoneurons 1-5

Abstract

The biogenic amine octopamine modulates a variety of aspects of insect motor behavior, including direct action on the flight central pattern generator. A number of recent studies demonstrate that tyramine, the biological precursor of octopamine, also affects invertebrate locomotor behaviors, including insect flight. However, it is not clear whether the central pattern generating networks are directly affected by both amines, octopamine and tyramine. In this study, we tested whether tyramine affected the central pattern generator for flight in the moth, *Manduca sexta*. Fictive flight was induced in an isolated ventral nerve cord preparation by bath application of the octopamine agonist, chlordimeform, to test potential effects of tyramine on the flight central pattern generator by pharmacological manipulations. The results demonstrate that octopamine but not tyramine is sufficient to induce fictive flight in the isolated ventral nerve cord. During chlordimeform induced fictive flight, bath application of tyramine selectively increases synaptic drive to depressor motoneurons, increases the number of depressor spikes during each cycle and decreases the depressor phase. Conversely, blocking tyramine receptors selectively reduces depressor motoneuron activity, but does not affect cycle by cycle elevator motoneuron spiking. Therefore, octopamine and tyramine exert distinct effects on the flight central pattern generating network.

Introduction

Rhythmic motor patterns such as breathing, walking, swimming and flying are produced by central circuits that generate rhythmic patterns of activity even in the absence of sensory feedback (Marder and Calabrese 1996; Marder et al. 2005). Pioneering work on locust flight set the stage for today's well accepted concept of central pattern generation by demonstrating that rhythmic motor output could be induced by non-rhythmical stimulation of the nerve cord without sensory feedback (Wilson 1961; 1966; Wilson and Wyman 1965; Edwards 2006). The underlying networks are central pattern generators, which are found at the heart of motor networks in all animals (Kiehn and Kullander 2004; Grillner et al. 2005; Marder et al. 2005) as has been confirmed by a large number of preparations that generate 'fictive motor patterns' when parts of the nervous system are removed from the animal and studied *in vitro*. In the case of many invertebrate preparations, these fictive motor patterns resemble many features of the motor patterns generated by the behaving animal, thus creating confidence that mechanisms studied *in vitro* are relevant to the generation of behavior (Marder et al. 2005), although sensory feedback is also important for the shaping of motor patterns (Pearson 2004).

Neuromodulators play a major role in activating and modifying central pattern generator activity (Marder and Bucher 2001). The central release of specific neuromodulators or mixtures of different modulators can initiate distinct motor patterns (Nusbaum et al. 2001). Early work in locusts has demonstrated that microinjection of the biogenic amine octopamine into distinct neuropil regions elicits either walking or flight motor patterns in isolated ventral nerve cords (Sombati and Hoyle 1984). This has led to the "orchestration hypothesis" (Hoyle 1985), assuming that neuromodulator release into specific neuropils configures distinct neural assemblies to produce coordinated network activity. Monoamines have also been assigned to aggression, motivation, and mood in

vertebrates and invertebrates (Baier et al. 2002; Kravitz and Huber 2003; Stevenson et al. 2005; Popova 2006). Furthermore, specific cognitive functions have been assigned to monoamine codes, such as that in flies octopamine mediates appetitive learning but dopamine mediates aversive learning (Schwaerzel et al. 2003; Riemensperger et al. 2005). In mammals, dysfunctions in monoamine neurotransmission are implicated in neurological disorders, including Parkinson's disease, schizophrenia, anxiety, and depression (Kobayashi 2001; Taylor et al. 2005). However, recent work from areas as diverse as Parkinson's disease (Scholtissen et al. 2006) and invertebrate rhythmical motor behavior suggests that the chemical codes producing specific motor behavior outputs are bouquets of different amines rather than single ones (Saraswati et al. 2004; Fox et al. 2006). In a recent study genetic and pharmacological manipulations in *Drosophila* have demonstrated that flight motor output is affected by concerted interactions of the biogenic amines octopamine and tyramine, (Brembs et al. 2007).

Octopamine and tyramine are decarboxylation products of the amino acid tyrosine, and tyramine is the biological precursor of octopamine, so that all octopaminergic neurons also contain tyramine. Octopamine receptors are abundant throughout all organs in insects (Roeder, 1999). Tyramine receptors have been cloned (Blenau and Baumann 2003) and physiological functions for tyramine have been demonstrated in multiple insect species (McClung and Hirsh 1999; Nagaya et al. 2002). In insect flight systems, octopamine affects central neuron excitability (Ramirez and Pearson 1991a; b), synaptic transmission (Evans and O'Shea 1979; Leitch et al. 2003), sensory sensitivity (Matheson 1997), hormone release (Orchard et al. 1993), and muscle metabolism (Mentel et al. 2003). Octopamine is also known to act directly on the central pattern generator for flight motor output, so that bath application of octopamine, or octopamine agonists can produce fictive flight in isolated insect ventral nerve cords in the absence of sensory feedback (Locust: Stevenson and

Kutsch 1987; Duch and Pflueger 1999; *Manduca*: Claassen and Kammer 1986). Although tyramine has been shown to affect flight motor performance in *Drosophila*, it remains unknown whether octopamine and tyramine affect different aspects of central pattern generation. In *Drosophila* the ability for prolonged flight and the likelihood for take-off are reduced in mutants lacking octopamine. These motor performance deficits can be rescued either by substituting octopamine or by blocking tyramine action, but flies lacking both monoamines also show motor deficits (Brembs et al. 2007). G-protein coupled receptors which are selectively activated by tyramine have recently been cloned in *Drosophila* (Cazzamali et al. 2005), further suggesting tyramine as neuromodulator with a unique function. Here we make use of the ability to initiate fictive flight motor output in the isolated ventral nerve cord of the moth, *Manduca sexta*, by bath application of the octopamine agonist chlordimeform (CDM, Duch and Pflueger 1999) to test pharmacologically whether octopamine and tyramine have differential effects on the central pattern generator for flight motor output. For the first time we show direct evidence for tyramine action on rhythmical motor output generated in the isolated ventral nerve cord. Furthermore, the data indicate that octopamine induces network activity including depressor and elevator activity, whereas tyramine selectively affects depressor activity,

Methods

Animals

All experiments were performed on adult *Manduca sexta* of either sexes taken from a laboratory culture maintained at the Free University, Berlin. Animals were maintained under a 16 h light : 8 h dark period and were used for experiments 2 days after eclosion to avoid age dependent variations.

Preparation

All recordings were made from isolated (deafferented) ventral nerve cords (brain, subesophageal ganglion, pro- and mesothoracic ganglia, see Fig. 1a). Animals were anesthetized by chilling on ice for 20 to 40 minutes before removing the wings and body scales. Thereafter, they were fixed in a sylgard dish, opened along the dorsal midline and superfused with cold saline containing (in mM): 140 NaCl; 5 KCl; 28 D-Glucose; 5 Hepes; 1 $CaCl_2$; ph 7,4 (Trimmer and Weeks 1989). The ventral nerve cord was removed from the animal, transferred into fresh saline and then pinned down ventral side up in a sylgard dish. The cut ends of lateral nerves were used for pinning. Except the nerves IIN1c and IIN4, were used for extracellular recordings (see Fig. 1a). Nerve IIN1c contains the axons of the motoneurons (MN1-5) innervating the dorsal longitudinal flight muscle 1 (DLM1) and two additional axons of motoneurons innervating the smaller DLM2. Nerve IIN4 contains the axon a motoneuron innervating the dorsal ventral flight muscle 1 (DVM1). For intracellular recordings, the ganglionic sheath from the prothoracic and mesothoracic neuromeres was removed mechanically by using a fine pair of forceps following a short treatment with crystalline Protease (SIGMA P-5147).

Intracellular recordings

Intracellular recordings from fore wing DLM motoneurons in the prothoracic ganglion were obtained with thin-walled borosilicate glass electrodes with a resistance between 20 - 30 MΩ. The electrode tips were filled with a solution of 7% Neurobiotin (Vectorlabs) and Rhodamine Dextran (Molecular Probes, MW 3000) in 2M potassium-acetate. Motoneurons were identified by simultaneous suction electrode recordings from their axons in nerve IIN1c. Orthodromic and antidromic stimulation was used for identification. Recordings were conducted with an Axoclamp 2A (Molecular Devices) amplifier in bridge mode, digitized with a digidata 1200 (Molecular Devices) at a sampling rate of 10 kHz, and viewed and analyzed with PClamp 9 software (Molecular Devices).

Extracellular recordings

Extracellular recordings were made with saline filled suction-electrodes (custom-made) made from glass microelectrodes and polyethylene tubing. Signals were amplified 100- fold with extracellular amplifiers (Grass P55A). Recordings were made from mesothoracic nerves (Fig. 1a) containing the axons of depressor motoneurons (nerve IIN1c) and one elevator motoneuron (one branch of nerve IIN4). Recordings were digitized at a sampling rate of 10 kHz.

Fictive flight

Fictive flight motor patterns were induced by bath application of the octopamine receptor agonist chlordimeform (CDM) (Riedel-de Haen) dissolved in saline (10^{-5} M). Flight activity was registered by 2 extracelluar electrode recording from mesothoracic nerves IIN1c (depressor activity) and IIN4 (elevator activity) combined with an intracellular recording from one of the four prothoracic fore wing depressor motoneurons (Fig 1b).

Epinastine, a highly specific and selective antagonist for insect neuronal octopamine receptors (Roeder et al. 1998; Degen et al. 2000), was bath applied at a concentration of 10^{-5} M. Tyramine (Sigma-Aldrich) and yohimbine (Sigma), a tyramine receptor antagonist were bath applied at concentrations of 10^{-5} M in saline. All solutions were continuously perfused by using a 4-tube gravity perfusion system (ALA BPS-4). Amine injections directly into *Manduca* mesothoracic ganglia have proven to be effective at concentrations as low as 10^{-9}M (Kinnamon et al. 1984; Claassen and Kammer 1986). However, bath application requires the chemicals to pass the ganglionic sheath, so that higher concentrations have to be applied. We have not tested different concentrations of CDM, tyramine and yohimbine, but instead used concentrations which have previously been reported to successfully induce fictive flight in other insect preparations (Duch and Pflueger 1999).

Data analysis

Due to the fact that pharmacological manipulations of the tyraminergic system affected mainly depressor motoneuron activity, rhythmical motor pattern analyses were conducted with respect to elevator activity. Therefore, cycle period was defined as the duration between the onsets of subsequent elevator bursts as determined by extracellular recordings from nerve IIN4 (Figs 1a, b). Elevator-depressor interval was defined as the duration between the onset of elevator activity and the onset of the corresponding depressor activity. Phase was defined as quotient of elevator-depressor interval over elevator cycle period. Each fictive flight bout analyzed comprised at least 30 seconds of continuous motor patterns.

Phase histograms (Fig. 4f) were plotted as depressor activity in relation to elevator cycle period. Elevator cycle period was divided into 100 bins of equal durations, and

depressor spikes were counted cumulatively into these bins. Linear regression analysis was used to determine the relationship between elevator-depressor or depressor-elevator interval and the duration of elevator cycle period (Fig. 5). The correlation coefficient (r) was calculated in MS Excel.

Cross correlation histograms were centered around the first spike event on the reference time series (depressor spikes). The occurrences of spikes at different time intervals around the reference spike were counted into 400 bins of 5 ms width, corresponding to a time window of plus and minus 1 second around each depressor spike. Then the histogram was centered around the next depressor spike and counting into bins was repeated. This process was repeated for every depressor spike of the flight bout (30 seconds duration in total). The histograms were not normalized but present total event counts.

Statistics were conducted with the program Statistica ´99 (Statsoft, Inc.). One-way ANOVA with Tukey HSD-test (honest significant difference-test) was used to test for statistical differences between multiple groups.

Results

Fictive flight can be released by chlordimeform and blocked by epinastine

Before bath application of the octopamine agonist CDM to the isolated ventral nerve cord of *Manduca sexta* (Fig. 1) it was kept in saline and did not show any spontaneous activity in the nerves supplying elevator or depressor muscles (Fig. 2a). In addition, very little synaptic activity and no subthreshold membrane oscillations were recorded intracellularly from a depressor motorneuron. However, 5 to 10 minutes after bath application of CDM rhythmical motor output was observed with strictly alternating activity between

motoneurons supplying antagonistic muscles, i.e. elevators and depressors (Fig. 2b). The rhythmicity of alternating depressor and elevator motor activity occurring after CDM application closely resembles that observed during flight motor behavior of the intact animal (Kammer 1967; Claassen and Kammer 1986; Tu and Daniel 2004), and is very different from other centrally generated motor activity such as walking (Johnston and Levine 2002). We, therefore, consider this motor rhythm as fictive flight activity. During normal flight all 5 depressor motoneurons innervating the dorsal longitudinal flight muscle should fire once per wing beat cycle during the depressor phase. We can not unambiguously determine whether all 5 DLM depressor motoneurons are activated during every depressor phase during CDM induced fictive flight. However, the following observations indicate that this may be the case. First, selective enlargements of the depressor spikes recorded extracellularly from nerve 1 during CDM induced fictive flight reveal a compound extracellular spike during each depressor phase which contains several distinct amplitudes which can clearly be distinguished (Fig. 2bi). The smaller amplitudes can be identified form cycle period to cycle period, whereas the larger amplitude spikes vary in amplitude and shape from cycle period to cycle period (Fig. 2bi), indicating the summation of two or more extracellularly recorded DLM motoneuron spikes occurring nearly simultaneously. Second, intracellular recordings from individual DLM flight motoneurons reveal nearly always one spike per depressor phase (see Fig. 4d). However, we have not tested whether some DLM motoneurons may fail to spike during some cycle periods.

Fictive flight activity as induced by bath application of CDM to the deafferented ventral nerve cord can be blocked by bath application of the octopamine receptor antagonist epinastine (Roeder et al. 1998). Figure 2c shows a representative example where the fictive flight rhythm established after CDM bath application (Fig. $2c_i$) is partially

blocked 15 minutes after (Fig. $2c_{ii}$) and then fully blocked 45 minutes (Fig. $2c_{ii}$) after bath application of epinastine. Pharmacological blockade of octopamine receptors causes rhythmical motor output in depressor and in elevator motoneurons to cease.

Both tyramine and yohimbine differentially affect the fictive flight rhythm

To test whether tyramine exerts specific effects on the fictive flight rhythm in an isolated *Manduca* ventral nerve cord, we induced the fictive flight rhythm by bath application of CDM (Fig. 3a) followed by bath application of tyramine (10^{-6} M in saline, Fig. 3b). A profound influence was observed on depressor motoneurons (Fig. 3b). The extracellular record from the respective nerve reveals an increased number of depressor spikes as compared to that observed under CDM, while maintaining the rhythmicity and strict alternation with respect to elevator activity. During CDM induced fictive flight several small and a few large spike amplitudes were observed in the extracellular recordings suggesting that, most likely, all 5 depressor motoneurons innervating the DLM flight muscle are active with some of them more or less simultaneously (Figs. 2bi, 3a). In contrast, during tyramine application large amplitudes of depressor action potentials were predominant in the extracellulr recording. It was difficult, however, to decide from extracellular recordings whether individual depressor motoneurons fired more action potentials per cycle in the presence of tyramine, or whether spike time precision of depressor motoneurons was reduced, so that the duration of the depressor burst was increased without increasing the numbers of action potentials produced by a single depressor neuron. In this case, different depressor motoneuron action potentials would become separated in the extracellular recording, and would not be partially superimposed on each other in a compound spike, as observed in CDM induced fictive flight. However, some extracellularly recorded depressor bursts clearly show more than 5 DLM motoneuron

spikes per depressor burst, indicating that at least one DLM motoneuron fired more than one action potential following bath application of tyramine. Simultaneous intracellular recordings of depressor motoneurons reveal that under CDM an individual depressor motoneuron receives an oscillatory synaptic drive that leads to a sharp rise of the membrane potential and a spike (Fig. 3a, lower trace). In the presence of tyramine, however, we observed an increase in the amplitude of the synaptic oscillatory drive which may cause a decrease in spike time precision (Fig. 3b, lower trace). In addition, dual spikes were riding on top of the large membrane oscillations. By contrast, elevator motoneuron activity was not affected by bath application of tyramine, because the elevator motoneuron continued to fire rhythmically with one spike per cycle although with a slightly longer cycle period. This indicated that tyramine selectively increased the amplitude and also the duration of the synaptic drive to depressor motoneurons specifically, leaving the elevator motoneurons more or less unaffected (Figs. 3a and b, upper traces).

In a complementary series of experiments the tyramine-receptor blocker yohimbine (10^{-6} M in saline) was applied to CDM induced fictive flight preparations. Again the elevator motoneurons were hardly affected and continued to fire rhythmically (Fig. 3c, upper trace). In contrast, rhythmicity in the depressor motoneurons was lost and many depressor motoneurons did not fire at all (Fig. 3c, middle and lower trace). Intracellular recordings from a depressor motoneuron revealed that a subthreshold oscillatory drive was still present, but depressor spike failures occurred during multiple elevator periods, and single depressor spikes occurred only every second or third cycle period. The sporadic spiking of depressor motoneurons was less tightly coupled to the elevator rhythm (Fig. 3c) than in CDM-induced fictive flight coupled to the elevator rhythm (Fig. 3a, lower trace).

Quantitative analysis of the effects of tyramine and yohimbine on fictive flight

Figures 4 to 6 provide quantitative measurements and analyses of the recorded fictive flight patterns. Figure 4a shows that CDM reliably induced fictive flight patterns with a constant average period over 200 cycles. In any given CDM induced fictive flight bout that consisted of more than 200 consecutive cycle periods, the average period of the first 100 cycles was not statistically different from the average period of the last 100 cycles. Therefore, the effects of bath application of either tyramine or yohimbine on CDM induced fictive flight patterns can be evaluated by comparing 100 cycle periods in the presence of CDM alone with subsequent 100 cycle periods following the additional bath application of tyramine or yohimbine. Elevator cycle period, measured from the onset of one elevator burst to the next, is similar in CDM induced fictive flight and in CDM induced fictive flight with subsequent bath application of tyramine (Fig. 4b). By contrast, bath application of yohimbine elongated the average elevator cycle period and increased the cycle to cycle variability (Fig. 4b). Median cycle period is significantly increased by yohimbine but not altered by tyramine (Fig. 4c). However, both tyramine and yohimbine treatment caused an increase in the variability of the elevator cycle period (Fig. 4c).

During CDM induced fictive flight, depressor motoneurons fire precisely once per cycle period (Fig. 4d). Yohimbine reduces this number significantly (to approximately 0.5) which accounts for the fact that frequent spike failures are observed (Fig. 4d, compare also Fig. 3c). In contrast, a highly significant increase in the number of depressor spikes was induced by the application of tyramine (to approx. 1.5, Fig. 4d). Calculating the occurrence of the depressor spikes in relation to the cycle period reveals no difference between CDM-induced fictive flight and that under yohimbine influence, although in the latter the variability is increased (Fig. 4e). By contrast, the application of tyramine significantly reduced the phase of the depressor motoneurons (Fig. 4e). Plotting a total of 200 elevator

depressor spikes from one representative fictive flight bout as a function of phase confirms the tight regulation of the phase around 0.5 in CMD induced fictive flight (Fig. $4f_1$). Most depressor spikes occur precisely at phase 0.5 of the elevator cycle period. In 12 CDM treated animals mean depressor phases, as measured for 200 cycles in each animal, ranged between 0.48 ± 0.02 and 0.57 ± 0.03. The average depressor phase between these 12 animals was 0.518 ± 0.046. After addition of the tyramine receptor blocker yohimbine to the bath the depressor phase is slightly longer in this animal (Fig. $4f_2$). The peak occurrence of depressor spike lies at phase 0.6 (Fig. $4f_2$) and is similarly tightly regulated as in CDM induced fictive flight (Fig. $4f_1$). In 6 yohimbine treated animals mean depressor phases, as measured for 200 cycles in each animal, ranged between 0.36 ± 0.1 and 0.76 ± 0.13. The average depressor phase between these 6 animals was 0.552 ± 0.163. The mean depressor phase is not statistically different in CDM induced fictive flight and in CDM induced fictive flight with subsequent yohimbine treatment, but the variation of depressor phases within each animal and between animals is larger in the presence of yohimbine. By contrast, depressor phases are significantly shorter and less tightly regulated after tyramine bath application (Fig. $4f_3$). In 6 tyramine treated animals mean depressor phases, as measured for 200 cycles in each animal, ranged between 0.11 ± 0.4 and 0.38 ± 0.07. The average depressor phase between these 6 animals was 0.298 ± 0.1.

To test whether alterations in cycle period duration were caused by changes in the elevator/depressor interval, by changes in the depressor/elevator interval, or by both, the intervals were plotted over elevator cycle period for randomly selected flight bouts from representative animals (Fig. 5). In CDM-induced fictive flight (Fig. 5a) the cycle period ranged between 75 and 90 ms (Fig 5a, x-axis). Changes in period correlated with changes in both the elevator/depressor interval (correlation coefficient, 0.63) and the depressor/elevator interval (correlation coefficient, 0.60). After bath application of the

tyramine receptor blocker, yohimbine, cycle period durations increased to range from 100 to 140 ms (Fig. 5b). However, changes in cycle duration correlated with changes in the depressor/elevator interval ($r = 0.54$) and with the elevator/depressor interval ($r = 0.32$; Fig. 5b). After bath application of tyramine the duration of elevator cycle periods ranged from 85 to 105 ms (Fig. 5c), and correlated with changes in the elevator/depressor interval ($r = 0.53$, whereas the duration of the depressor/elevator interval remained largely unaffected ($r = 0.04$).

Cross-correlations between depressor and elevator spikes were used to compare the precision of rhythmicity in CDM induced fictive flight motor output before and after bath application of either yohimbine of tyramine (Fig. 6). Representative simultaneous extracellular recordings of the elevator and depressor motoneurons show regular rhythmical motor output with alternating elevator and depressor activity and a cross-correlation analysis confirming this alternating rhythmical activity over 244 cycles. Cross correlation histograms contained 200 bins, each bin 10 ms wide, to cover time lags from -1 to +1 seconds around each depressor spike (see methods). In the cross correlograms (Fig. 6), the lack of spike counts around 0 seconds demonstrated strict alternation of depressor and elevator spikes. The occurrence of periodic peaks and troughs occurring approximately every 80 ms demonstrated periodic clustering of spikes at a cycle frequency of roughly 12 Hz in CDM induced fictive flight (Figs. 6a, b). After bath application of the tyramine receptor blocker yohimbine to the same preparation, depressor spiking activity was markedly reduced, and elevator cycle period was slowed down (Fig. 6ai). Cross-correlation analysis demonstrated that the remaining depressor spikes still occurred in strict alternation to elevator spikes (Fig. 6ai, no counts around time 0). Periodic peaks and troughs occurring approximately every 125 ms demonstrated periodic clustering of spikes at a cycle frequency of roughly 8 Hz, indicating that rhythmical motor output remained after

yohimbine addition for a large number of cycles (n = 149). However, spike counts in troughs were never 0, demonstrating that periodicity of motor output was less organized after bath application of yohimbine (Fig. 6ai) as compared to CDM (Fig. 6a). If period remained constant over multiple cycle periods of rhythmical motor output, and strict alternation occurred between peaks and troughs, counts for elevator spikes would be zero in multiple troughs around each depressor spike. A similar representative experiment and cross correlation analysis is shown for bath application of tyramine to a preparation in which fictive flight motor output was induced by CDM (Fig. 6b). Cross-correlation demonstrated the same strict alternation of depressor and elevator spikes and periodicity for CDM induced fictive flight as shown in figure 6a. Bath application of tyramine caused the occurrence of additional depressor spikes, but cross-correlation analysis indicated that antagonist alternation was not affected and periodicity was affected only mildly (Fig. 6bi). Periodic peaks and troughs occurring approximately every 100 ms demonstrated periodic clustering of spikes at a cycle frequency of roughly 10 Hz. The contrast between peaks and troughs was slightly lower after bath application of tyramine (Fig. 6bi) as compared to CDM induced fictive flight (Fig. 6b).

Discussion

A number of studies have demonstrated that tyramine has pronounced effects on invertebrate locomotor behavior. For example, *Drosophila* crawling (Saraswati et al. 2004; Fox et al. 2006) and flight motor performance (Brembs et al. 2007) are affected by octopamine and tyramine. Furthermore, a study using tyrosine decarboxylase and tyramine *β*-hydroxylase mutations in *C. elegans* has provided evidence that octopamine and tyramine play distinct roles in egg laying and locomotor behaviors (Alkema et al. 2005). In

honey bees, changes in octopamine and tyramine levels correlate with shifts in cast related motor behaviors, such as nurse bees taking care of the brood, and foraging bees (Harris and Woodring 1992; Bozic and Woodring 1998; Wagener-Hulme et al. 1999; Schulz and Robinson 2001). Specific receptors for either octopamine or tyramine have been cloned from *Drosophila* (Cazzamali et al. 2005) and from the honey bee (Blenau and Baumann 2001; Blenau and Baumann 2003), further suggesting distinct functions of both amines for behavior. However, to the best of our knowledge the effects of tyramine on motor output from a central pattern generator have not been investigated.

The results of this study confirm the finding that octopamine acts on the central pattern generator producing flight motor output in the ventral ganglia of *Manduca sexta* (Claassen and Kammer 1986). This is in accordance with earlier studies in multiple insect species which have demonstrated (Sombati and Hoyle 1984; Stevenson and Kutsch 1987; Duch and Pflueger 1999) that octopamine is sufficient to induce fictive flight motor output in the isolated ventral nerve cord. However, a recent study shows that acetylcholine rather than octopamine may be the natural transmitter to induce flight motor output in the locust, and that octopamine plays a solely modulatory role (Buhl et al. 2008). This agrees with data in *Drosophila* demonstrating that flies lacking tyramine beta hydroxylase and thus octopamine, as well as flies with targeted genetic ablation of all octopaminergic neurons can still fly but show flight performance deficits (Brembs et al. 2007).

However, our data demonstrate for the first time that tyramine exerts also specific effects on the flight central pattern generator. The octopamine agonist CDM is sufficient to induce fictive flight motor output in the isolated ventral nerve cord in the absence of sensory feedback, and tyramine selectively affects motor output from the wing depressor motoneurons without affecting output from the elevator motoneurons. Activation of tyramine receptors by tyramine bath application increases depressor spiking output,

whereas tyramine receptor blockade by bath application of yohimbine inhibits depressor motoneuron spiking output. In addition to depressor spike failures, tyramine receptor blockade slows down the cycle period of fictive flight and reduces accuracy of the rhythmicity. This suggests that tyramine receptors might support the amplitude and the precision of synaptic drive to depressor motoneurons. The specificity of yohimbine as an antagonist at tyramine receptors has previously been demonstrated (Saudou et al. 1990; Vanden Broeck et al. 1995; Ohta et al. 2003). However, bath application of tyramine alone to the isolated ventral nerve cord was not sufficient to induce spiking output from depressor motoneurons, but tyramine affected depressor motor output only during ongoing CDM induced fictive flight. This is in contrast to locusts, where tyramine was even more effective in releasing a fictive flight pattern than octopamine (Buhl et al. 2008). Therefore, we conclude that in *Manduca* tyramine modulates depressor network output only during ongoing flight motor patterns. However, we have not tested whether tyramine acted directly on the excitability of depressor motoneurons in the absence of CDM.

Possible sites of tyramine action during fictive flight

Octopamine and tyramine act via G-protein coupled receptors expressed on the surface of the responsive cells (for recent reviews see: Blenau and Baumann 2001; Blenau and Baumann 2003; Evans and Maqueira 2005). To the best of our knowledge direct actions of tyramine on any of the components of insect central pattern generators have not been demonstrated so far. By contrast, direct actions of octopamine on the membrane properties of flight interneurons have been demonstrated in locusts (Ramirez and Pearson 1991a). There, octopamine increases the excitatory response of elevator motoneurons to stimulation of the hindwing tegula afferents. This is caused in part by the occurrence of octopamine-induced plateau potentials in elevator interneurons in response to synaptic input from the

hindwing tegula or in response to current injections into elevator interneurons (Ramirez and Pearson 1991b). Interestingly, we find that in *Manduca* tyramine acts primarily on depressor motor output, suggesting that each, the elevator and the depressor circuitry of the insect flight central pattern generator, are selectively affected by one of both amines. Intracellular recordings from a depressor motoneuron suggest that bath application of tyramine during ongoing CDM induced fictive flight enhances excitatory synaptic drive, because in general, cycle by cycle depolarizations show larger amplitudes and durations in the presence of tyramine (see Fig. 3). This results in an increased number of depressor motoneuron spikes per cycle as revealed by extracellular recordings form nerve 1 (Figs. 3b, 4a-c, 6bi) which contains the axons of all 5 depressor motoneurons innervating the dorsal longitudinal flight muscle (DLM). By contrast, bath application of the tyramine receptor antagonist, yohimbine, indicate slight decreases in the amplitudes of cycle by cycle depolarizations and causes depressor motoneuron spike failures during ongoing CDM induced fictive flight bouts.

Increased excitatory drive to depressor motoneurons by tyramine during fictive flight could be caused by two mechanisms. First, tyramine might act directly on the membrane properties of depressor interneurons. Second, tyramine might increase the efficacy of synaptic transmission between depressor interneurons and depressor motoneurons. Both, aminergic modulation of the membrane properties of flight interneurons (Ramirez and Pearson 1991a; b) and also of synaptic transmission in the flight circuitry (Leitch et al. 2003) have been demonstrated in locust. However, this study lacks a rigorous quantitative analysis of the effects of tyramine on the magnitude of excitatory synaptic drive to depressor motoneurons or on depressor motoneuron excitability. Therefore, we can not exclude direct effects of tyramine on the membrane properties of depressor motoneurons. In addition, we can not conclude where in the central flight

circuitry tyramine might exert its effects, but depressor synapses between pre-motor depressor interneurons and depressor motoneurons seem good candidates for future studies. However, flight interneurons have not been identified in *Manduca* so far, and thus, the locust might be a better suited model for further studies on the sites of tyramine action.

Possible mechanisms of tyramine action during fictive flight

Tyramine receptor has been cloned in *Drosophila* and characterized in two heterologous expression systems, CHO cells and *Xenopus* oocytes (Cazzamali et al. 2005). These data showed that tyramine activation caused calcium release from internal stores, which in turn activated calcium dependent chloride channels in the cell membranes of oocytes. These data indicate that tyramine receptorss are coupled to a Gq protein (Cazzamali et al. 2005). However, other studies suggested that tyramine was more potent than octopamine at mediating inhibition of adenylyl cyclase (Chatwin et al. 2003; Mustard et al. 2005). In *Bombyx mori*, a *lepidopteran* species closely related to *Manduca sexta*, a cDNA was isolated encoding a putative tyramine receptor that was also negatively coupled to adenylyl cyclase when expressed in HEK-293 cells (Ohta et al. 2003). Although this makes cAMP levels a likely target for bath application of tyramine during fictive flight, activation of a Gq protein is an alternative possibility. Either way, it remains unclear what the resulting actions on neuronal excitability might be, as both pathways could potentially modulate a number of ionic currents or receptors. Therefore, we can not make conclusions about the intracellular mechanisms by which tyramine might affect fictive flight.

Possible source of tyramine release during real flight

Despite the clear effects of pharmacological activation or blockade of tyramine receptors during CDM induced fictive flight, it remains an open question whether tyramine is used as

an endogenous modulator of motor output during real flight. The most prominent population of octopaminergic neurons in insect pterothoracic ganglia are dorsal or ventral unpaired median (DUM or VUM) neurons (Pflueger et al. 1993; Pflueger 1999). Since tyramine is the biological precursor of octopamine, octopaminergic unpaired median (UM) neurons also contain tyramine (Monastrioti et al. 1995; Nagaya 2002; Kononenko et al. 2008; Lange 2008). Therefore, one source of tyramine may actually be release from "octopaminergic" UM-cells. Despite this possibility ultrastructural studies on the dendrites of UM neurons have shown predominantly input synapses but no clear output synapses except a few vesicles in the state of exocytosis (Pflueger and Watson 1995). However, in contrast to locusts where the UM-neurons supplying wing elevator and depressor muscles are inhibited during flight (Duch and Pflueger 1999), some of the UM-neurons supplying similar muscles in *Manduca* are active during flight and, thus, could theoretically be a source of both octopamine and/or tyramine (Vierk, unpublished observations). In addition, in the locust cells and fibers within the connectives and within each segmental ganglion have been identified that only label by a tyramine-antibody and, therefore, have to be regarded as "pure" tyraminergic. Similar cells in *Manduca* could be another source of tyramine.

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Figure legends

Figure 1. (a) Schematic ventral view of the isolated adult ventral nerve cord preparation, consisting of the brain, subesophageal ganglion (SEG), prothoracic, mesothoracic, metathoracic ganglion and fused first 3 abdominal ganglia. The locations and axonal projections of the depressor motoneurons, MN1-5, the elevator motoneurons, MN1-2, and a ventral unpaired median neuron are indicated. Elevator motoneurons are recorded extracellularly from the root of nerve IIN4, and depressor motoneurons are recorded either extracellularly from nerve II1c or intracellularly with a sharp electrode. (b1) Simultaneous extracellular recordings from elevator motoneurons from mesothoracic nerve IIN4 (upper trace), depressor motoneurons from nerve IIN1c (middle trace), and intracellular recording from a fore wing depressor motoneuron (lower trace) during pharmacologically induced fictive flight. (b2) Expanded time scale from (b1). A cycle period is defined from the beginning of one elevator burst to the beginning of the next one (see arrow between dotted lines). Elevator-depressor interval is indicated by dotted lines and arrow. (c) Schematic longitudinal section through the head and thorax illustrates the location of relevant mesothoracic indirect flight muscles. The ventral nerve cord is indicated in grey (modified after Eaton, 1988).

Figure 2. CDM-induced fictive flight motor patterns in the isolated nerve cord preparation of *Manduca sexta*. (a) Extracellular recordings from elevator (from IIN4, top trace) and depressor motoneurons (from IIN1c, middle trace) and intracellular recording (bottom trace) of a fore wing depressor motoneuron in saline. (b) Same recordings as in (a) 20 minutes after bath application of CDM (10^{-5} M). The grey box indicates two cycles which are selectively enlarged in (bi). (c) Representative example of CDM induced fictive flight

patterns recorded extracellularly from elevator (top trace) and depressor motoneurons (middle trace), and intracellularly from a depressor motoneuron (bottom trace). CDM induced fictive after 15 minutes (ci) and after 45 (cii) minutes of bath application of the octopamine receptor blocker epinastine.

Figure 3. Effect of tyramine on CDM induced fictive flight. (a) Extracellular recordings from elevator (from IIN4, top trace) and depressor motoneurons (from IIN1c, middle trace) and intracellular recording of a fore wing depressor motoneuron (bottom trace) after bath application of CDM (10^{-5} M). (b) Same recording as in (a) after additional bath application of tyramine (10^{-5} M). (c) Different preparation with recordings from the same identified neurons as in (a) and (b). Fictive flight was induced by bath application of CDM and subsequently, preparations were superfused with the tyramine receptor antagonist yohimbine (10^{-5} M). Grey boxes depict one cycle period during each representative flight bout.

Figure 4. Quantitative parameters of CDM induced fictive flight before and after manipulations of the tyraminergic system. (a) Elevator cycle period plotted over 200 consecutive cycles of representative CDM induced fictive flight bouts from 12 different animals (grey lines). The black line shows the average cycle periods of these 12 animals. Error bars represent standard deviation. (b) Comparison of average elevator cycle period over 100 consecutive cycle periods from CDM induced fictive flight bouts of 12 different animals (black line), from CDM induced fictive flight bouts with subsequent addition of tyramine of 6 different animals (dark grey line), and from CDM induced fictive flight bouts with subsequent addition of yohimbine from 6 different animals (light grey line). In (c) to (e) median values were obtained for 100 cycles periods of 12 preparations with bath

application of CDM, and 6 preparations each with subsequent bath application of either yohimbine or tyramine. White lines indicate the medians, black boxes represent the 25 and the 75 quartiles, and error bars indicate the minimum and maximum values (see inset a). For statistical analysis, average values were calculated for 200 cycles per animal within each group. Statistical significance is indicated by asterisks, * $p < 0.05$, ** $p < 0.005$, one-way ANOVA, posthoc Tukey HSD-test. (c) Duration of elevator cycle period in the three groups. (d) Comparison of the number of depressor spikes per cycle period in the three groups. (e) Comparison of elevator-depressor phase relationship between the three groups. (f1-f3) Phase histograms of representative recordings after CDM application (f1, data from consecutive 300 cycle periods), CDM application with subsequent yohimbine application (f2, data from 200 consecutive cycle periods), or with subsequent tyramine application (f3, data from 200 consecutive cycle periods). Numbers of depressor spikes with relation to the elevator cycle (x – axis) are counted (numbers on y – axis) into bins of 0.01.

Figure 5. Elevator/depressor and depressor/elevator latencies plotted as function of elevator cycle period. Data points are values from single representative preparations (number of cycle periods are indicated in the upper right corners). (a) Depressor/elevator (dep-elev, open white squares, black line) and elevator/depressor interval (elev-dep, black triangles, dotted line) are plotted over elevator cycle period in CDM induced fictive flight (correlation coefficients, 0.63 and 0.60 respectively; $p < 0.05$). (b) Same analysis after bath application of the tyramine receptor blocker yohimbine. Alterations in cycle period correlated with changes in the depressor/elevator interval (open white squares, black line, correlation coefficient, 0.54; $p < 0.05$) and with changes in the elevator/depressor interval (filled black triangles, dotted line, correlation coefficient, 0.32; $p < 0.05$). (c) After bath application of tyramine elevator changes in cycle period correlated with changes in the

elevator depressor interval (correlation coefficient, 0.53; $p < 0.05$), but not with changes in the depressor elevator interval (correlation coefficient, 0.04; $p < 0.05$).

Figure 6. Rhythmicity and precision of alternating elevator and depressor activity. (a) Representative example of alternating elevator and depressor motoneuron activity during CDM induced fictive flight. Cross correlation between elevator and depressor spikes from 30 consecutive seconds of this flight bout is shown below (bin size = 10ms, 200 bins, +/- 1 second displayed in cross-correlation diagram). (ai) Bath application of yohimbine to the same preparation. (b) Alternating elevator and depressor spiking activity (extracellular recordings) after application of CDM (b) and after subsequent application of tyramine (bi).

Figures

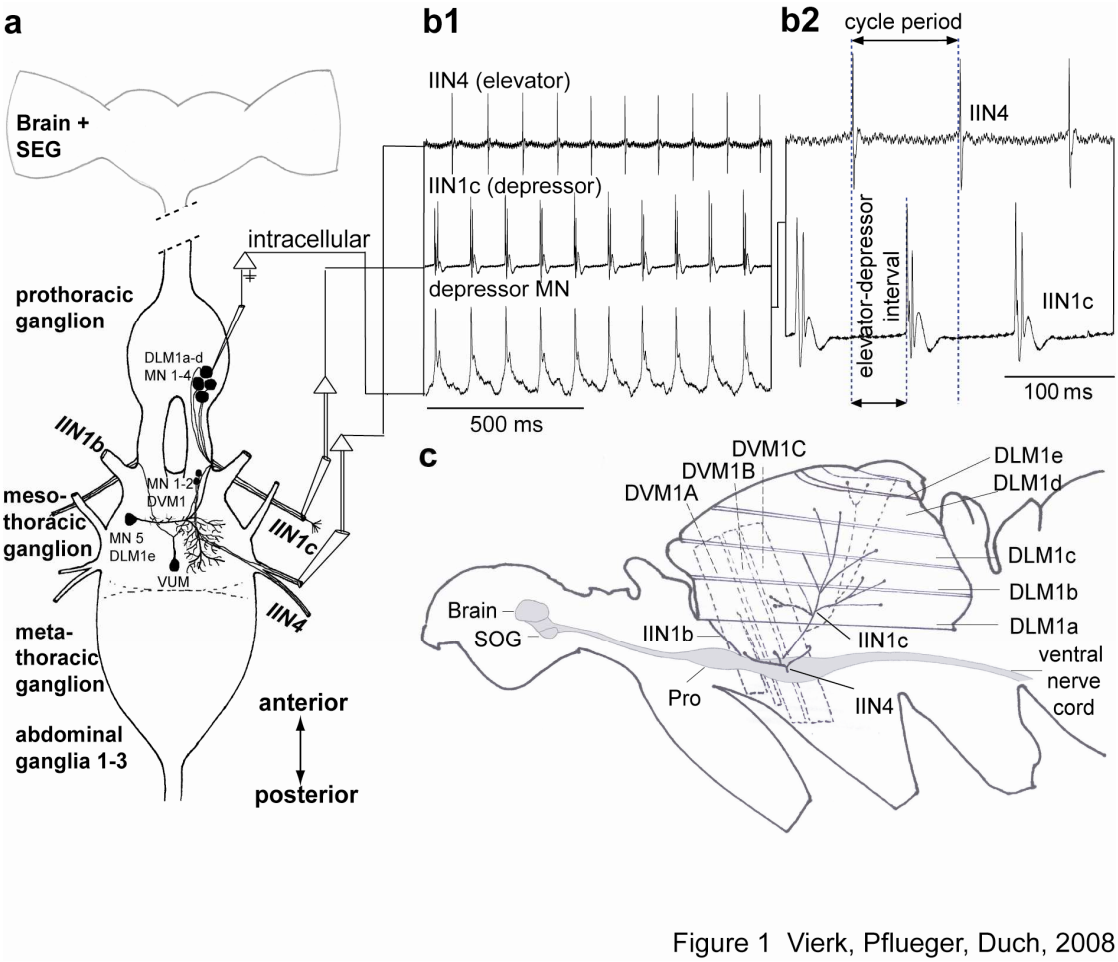


Figure 1 Vierk, Pflueger, Duch, 2008

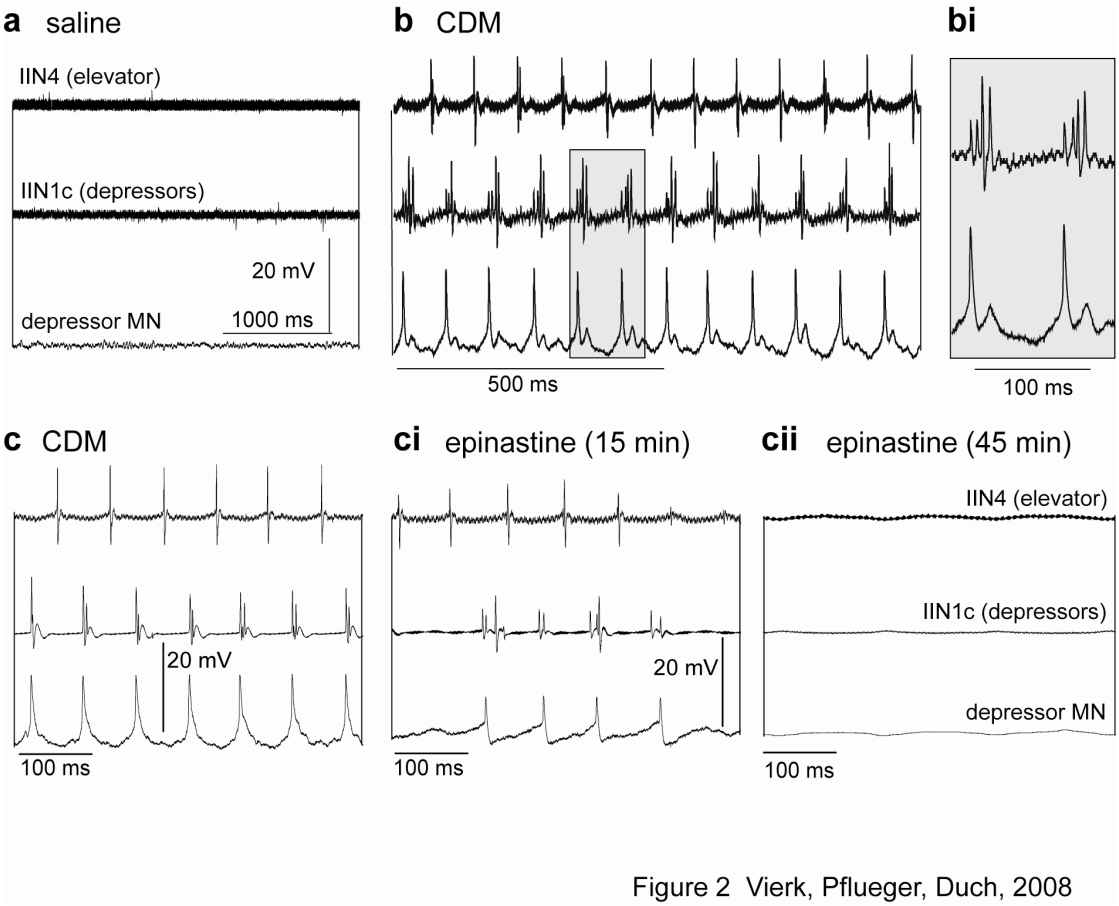
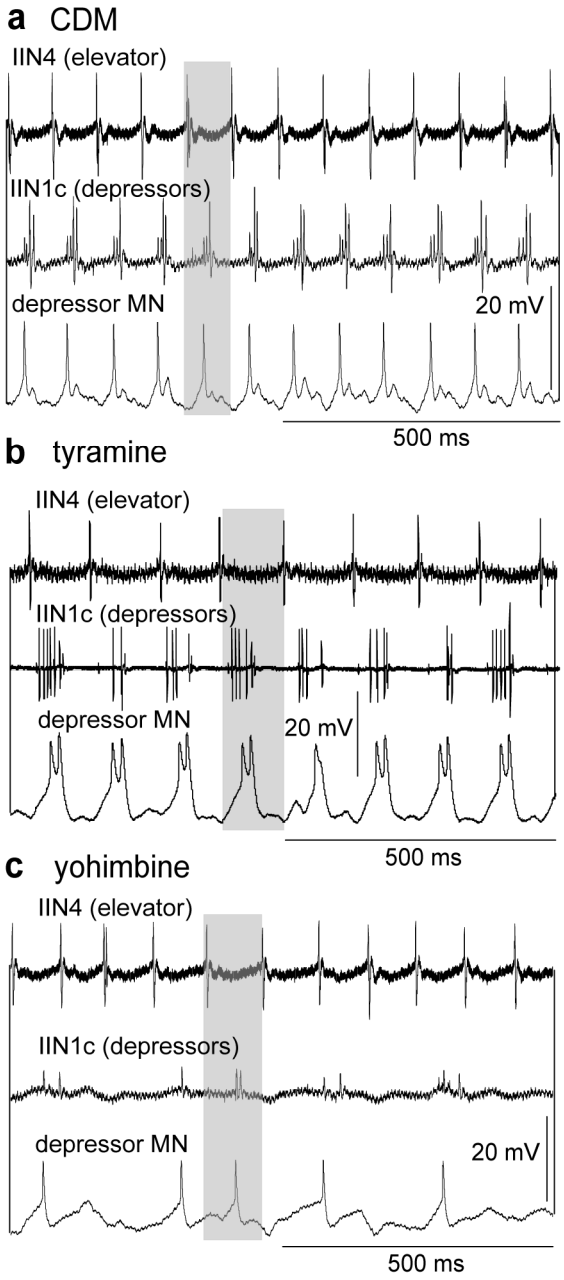


Figure 2 Vierk, Pflueger, Duch, 2008



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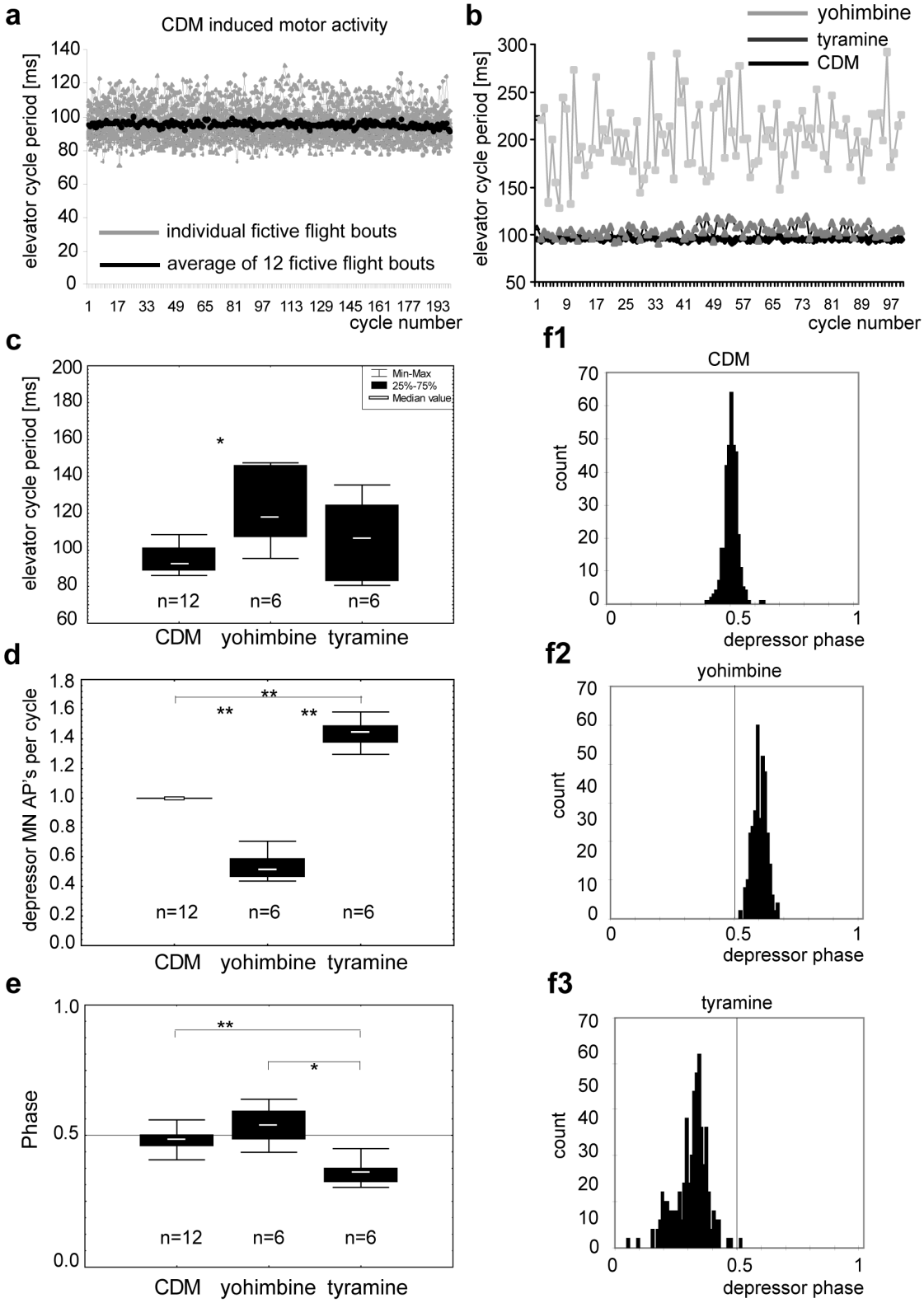

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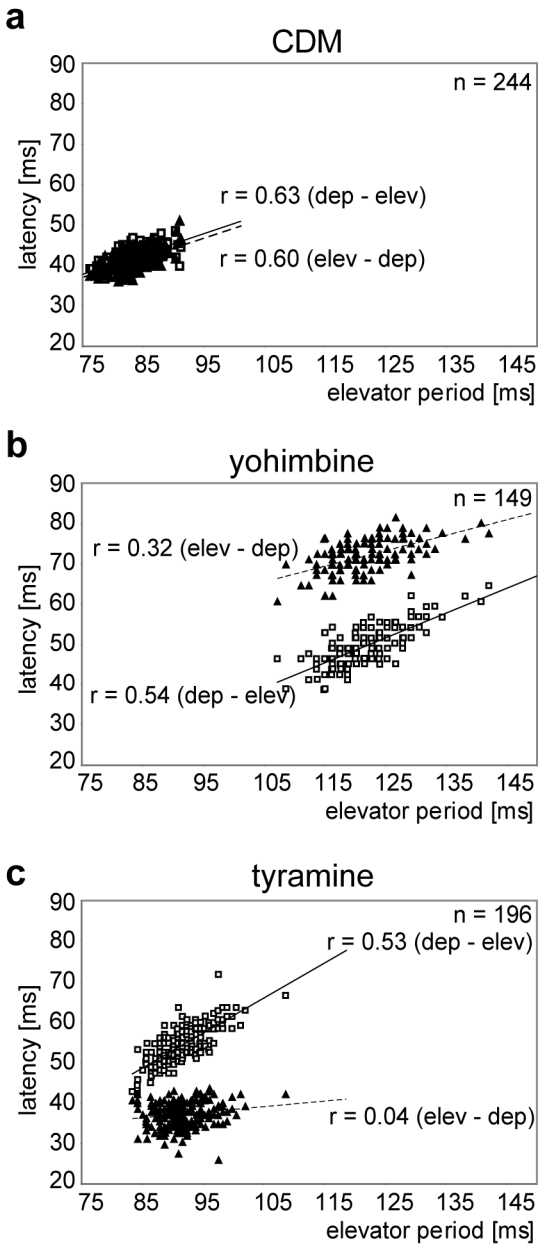

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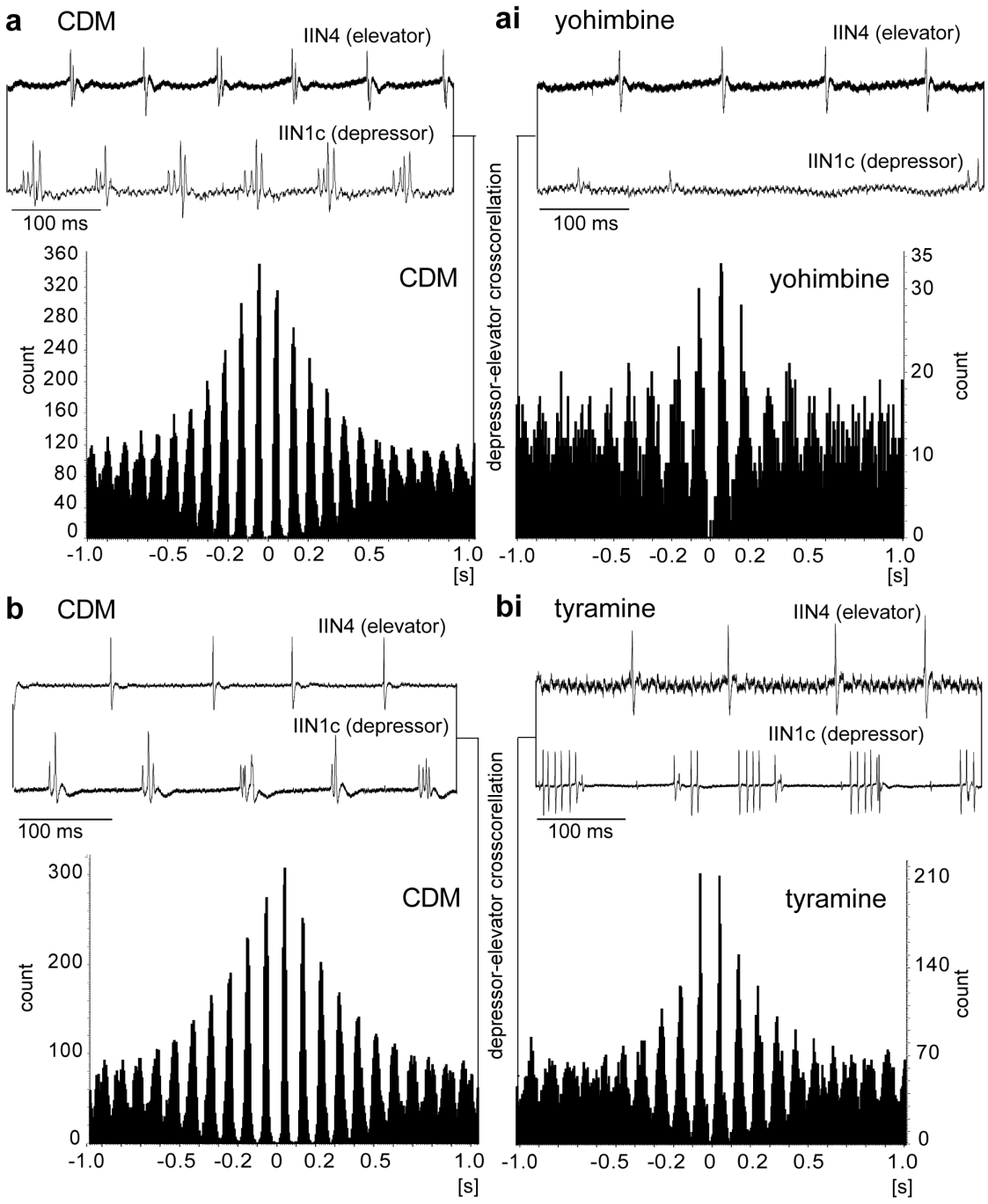

Figure 6, Vierk, Pflueger, Duch, 2008

Chapter 4

Activation of ventral unpaired median (VUM) neurons during pharmacologically induced flight motor output in *Manduca sexta*

A direct effect of octopamine (OA) on the CPG for flight is to induce plateau potentials and bursting in flight related interneurons , similar to what has been shown in the locust (Ramirez and Pearson, 1991a, b). This is already described in chapter 2 and 3. Chlordimeform (CDM) as OA receptor agonist reliably induces fictive flight motor patterns in isolated *Manduca* ventral nerve cords in the absence of any sensory feedback. In addition, our results suggest that tyramine, the precursor of OA, has a differential effect on central pattern generation compared to OA, indicated by effects on the depressor systems but not the elevator systems (Vierk et al. 2009, chapter 3).

Octopamine and tyramine are released peripherally from large, efferent unpaired median (UM) neurons possessing unique morphological characteristics. These types of neurons are situated ventrally (VUM neuron) or dorsally (DUM neuron) along the ganglionic midline and their distinct primary neurite bifurcates into two bilaterally symmetrical axons making these neurons easily identifiable. In locusts, unpaired median neurons form groups of anatomically defined sub-types according through which peripheral nerves they send their axons to their peripheral targets (Duch et al., 1999; Stevenson and Sporhase-Eichmann, 1995; Watson, 1984). Additionally, a recruitment/inhibition of specific subpopulations of these neurons during specific motor patterns (Baudoux and Burrows, 1998; Baudoux et al., 1998; Burrows and Pfluger, 1995; Duch et al., 1999) was described. This indicates that these neurons are coupled to different functional tasks. For example, during locust jumping only those DUM neurons are activated which innervate leg muscles, in parallel to the activity of leg muscle motoneurons whereas others such as DUM neurons to wing muscles are either inhibited or unaffected (Burrows and Pfluger, 1995). During locust flight DUM neurons which innervate wing muscles are inhibited and those innervating leg muscles are activated (Duch and Pflüger, 1999), reflecting such a differential inhibition/ activation. These results suggest to appropriately adapt muscle contractile properties to specific behavioral tasks (Burrows and Pfluger, 1995; Evans and O'Shea, 1977; O´Shea and Evans, 1979) and to provide a potent link between neuronal activity and muscle metabolism (Mentel et al., 2003).

Most results on DUM neuron activity were obtained during locust kicking and flight, or stick insect walking (Mentel et al. 2008), but much less is known for holometabolous insects such as *Manduca*. They possess similar unpaired median neurons which are activated during fictive crawling (Johnston et al., 1999) and receive common synaptic inputs descending from SEG (subesophageal ganglion) and persist during

metamorphosis (Pflüger et al., 1993). Since a subpopulation-specific recruitment during specific motor patterns was described for DUM neurons in adult locusts, we asked whether the general activation of larval unpaired median neurons during crawling is reconfigured during metamorphosis to a sub-type specific activation in adult *Manduca*?

4.1 Basic differences between larval and adult VUM neurons?

The first objective was to compare the number of VUM neurons before and after metamorphosis. The second objective was to record from VUM neurons the axons of which run in the nerve innervating wing muscles and, thus, study their activation during pharmacologically induced fictive flight rhythms. Immunocytochemical staining against OA (Fig. 1a) revealed 2-3 octopaminergic neurons along the midline of the mesothoracic ganglion in *Manduca* larval stage 5 (L5). Moreover, intracellular staining by using sharp glass electrodes filled with Neurobiotin and later visualization by conjugated Cy3-streptavidin, labeled two ventrally situated somata showing the specific bifurcation point of their primary neurites (Fig. 1c) thereby identifying (Duch et al., 1999; Stevenson and Sporhase-Eichmann, 1995; Watson, 1984) them as VUM neurons. The more anterior neuron sends bilaterally projecting axons through nerve N1, therefore termed VUM1, and consequently, the more posterior localized neuron sends its axons through nerve N2 and was named VUM2. The neuromodulatory neuron VUM1 project to larval body wall muscles (DEL, DIO2) which are also innervated by the larval MNs1-5 (Consoulas et al., 1999). Interestingly, like motoneurons MN1-5 larval VUM neurons persist during metamorphosis and undergo a similar postembryonic remodeling (Pfluger et al., 1993). Both types, motor and neuromodulatory neurons acquire new target muscles in the adult animal. Immunocytochemical staining against OA in the adult labeled a cluster of 6-8 octopaminergic cell bodies (Fig. 1b) in the mesothoracic ganglion which demonstrates an enormous increase in the number of octopaminergic neurons during metamorphosis in line with earlier results (Lehman et al. 2000).

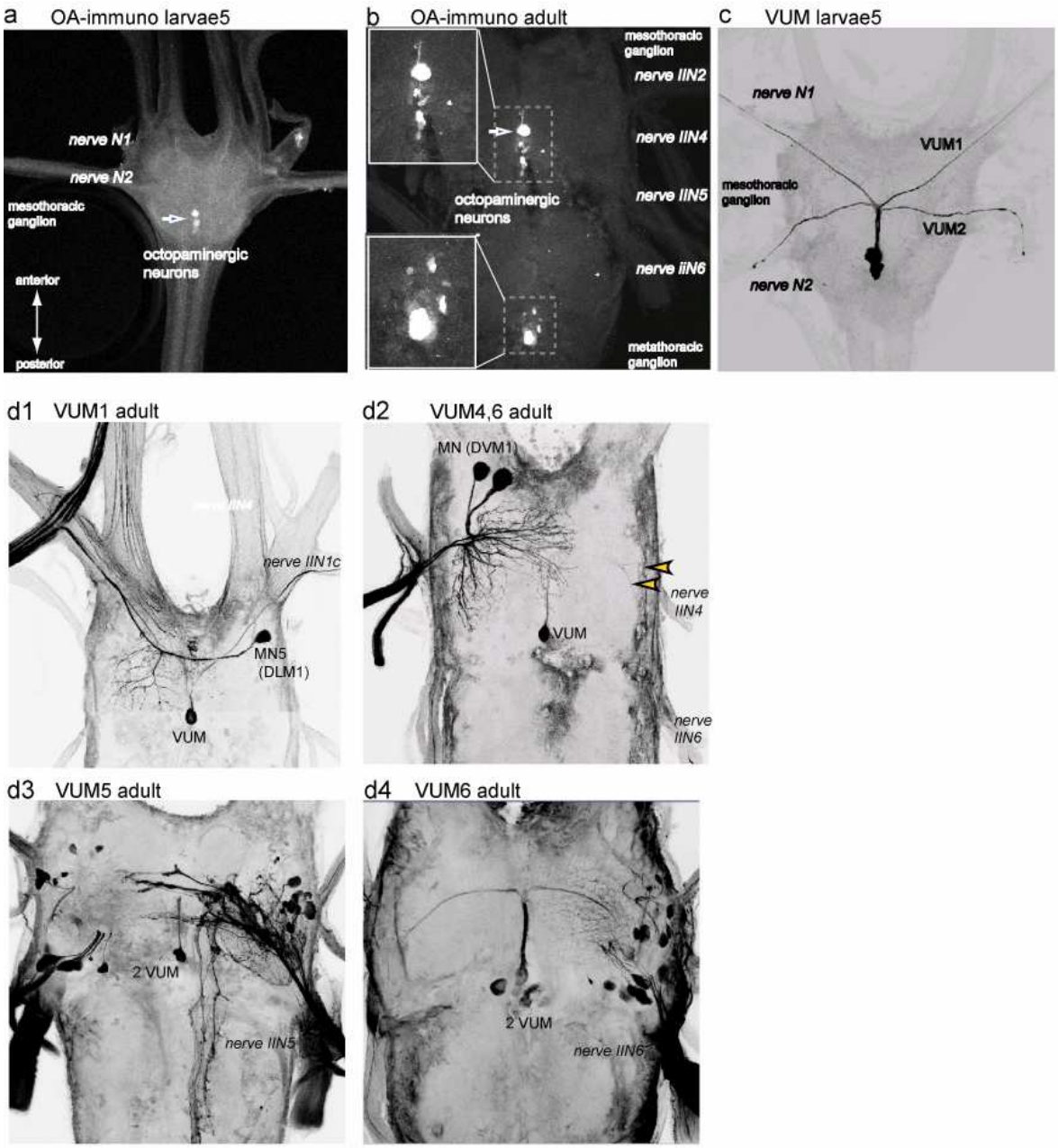

Fig. 1: Number and sub-types of larval and adult mesothoracic VUM neurons. **a, b** Immunocytochemistry against octopamine in mesothoracic ganglia of L5 (**a**) and adult (**b**). Somata of octopaminergic neurons are indicated by an white arrow and selective enlargements of these somata are shown as insets (*top*-mesothoracic octopaminergic, *bottom*-metathoracic octopaminergic neurons) **c** Intracellular staining of two VUM neurons (VUM1 and VUM2) in larval mesothoracic ganglia. **b1 - b4** Retrograde tracing of different mesothoracic nerves, reveals one VUM1 neuron projecting via nerve IIN1c (**b1**), (**b2**) shows one VUM4,6 neuron sending axons through nerve IIN4 and nerve IIN6 (*arrows*), IIN5 show two VUM5 neuron somata (**b3**) and backfilling nerve IIN6 demonstrate two VUM6 neurons (**b4**).
(Immunocytochemical stainings a, b are courtesy of H. Wolfenberg, Prof. H.-J. Pflüger)

Using the retrograde axonal tracing technique ("backfill") of various mesothoracic nerves enabled us to identify four antomically different sub-populations of mesothoracic VUM neurons in adults (Fig. 1d1-d4). With respect to the axonal projection patterns within the lateral nerves we found the following four groups: (I). one VUM1 sends its axons bilaterally through nerve IIN1c (Fig. 1d1), (II). one VUM4,6 projects through nerve IIN4 and IIN 6 (Fig. 1d2), (III). two VUM5 send their axons through nerve IIN5 (Fig. 1d3) and (IV). two VUM6 project through nerve IIN6 (Fig. 1d4). Thus, in adult *Manduca* we found anatomically different sub-types of VUM neurons similar to what is known from locusts (Duch et al., 1999). One type of VUM neuron, VUM1, and MNs1-5 shared the same innervation pattern with respect to the target muscles in larval stages (DEL, DIO2) and adults (DLM1).

Another remarkable feature of large efferent unpaired median neurons is their somatic excitability (Grolleau and Lapied, 2000). Unlike invertebrate motoneurons or interneurons, unpaired median neurons generate somatic large overshooting action potentials with a prominent after-hyperpolarisation in both larvae and adults (Fig. 2a) To compare the excitability of VUM neurons in different developmental stages we analyzed basic properties like input resistance, resting membrane potential, frequency- current relationship and current-voltage relationship in seven larval and six adult isolated nerve cord preparations (Fig. 2). A significant difference was observed in the input resistance between larval (32.6 MΩ) and adult (17.7 MΩ) stages (Fig. 2b), whereas no difference was found in the resting potential (~ -42.2 mV, Fig. 2c). Interestingly, the reduced input resistance was not indicative of a reduced frequency-current relationship (Fig. 2d) and current-voltage relationship (Fig. 2e), as was described for the postembryonic remodeling of the MN5 (Duch and Levine, 2000). Larval and adult VUM spiking frequency increased with larger amounts of currents injected, and resulted in a similar mean frequency of about 3 Hz when depolarized by 2 nA (Fig. 2d) with injection of more depolarizing currents not further increasing the spiking frequency. Here, no further analysis of the different morphological sub-types of VUM neurons (Fig. 1b1-4) with respect to their ion channel composition like in the locust, was carried out (see Heidel and Pflüger, 2006).

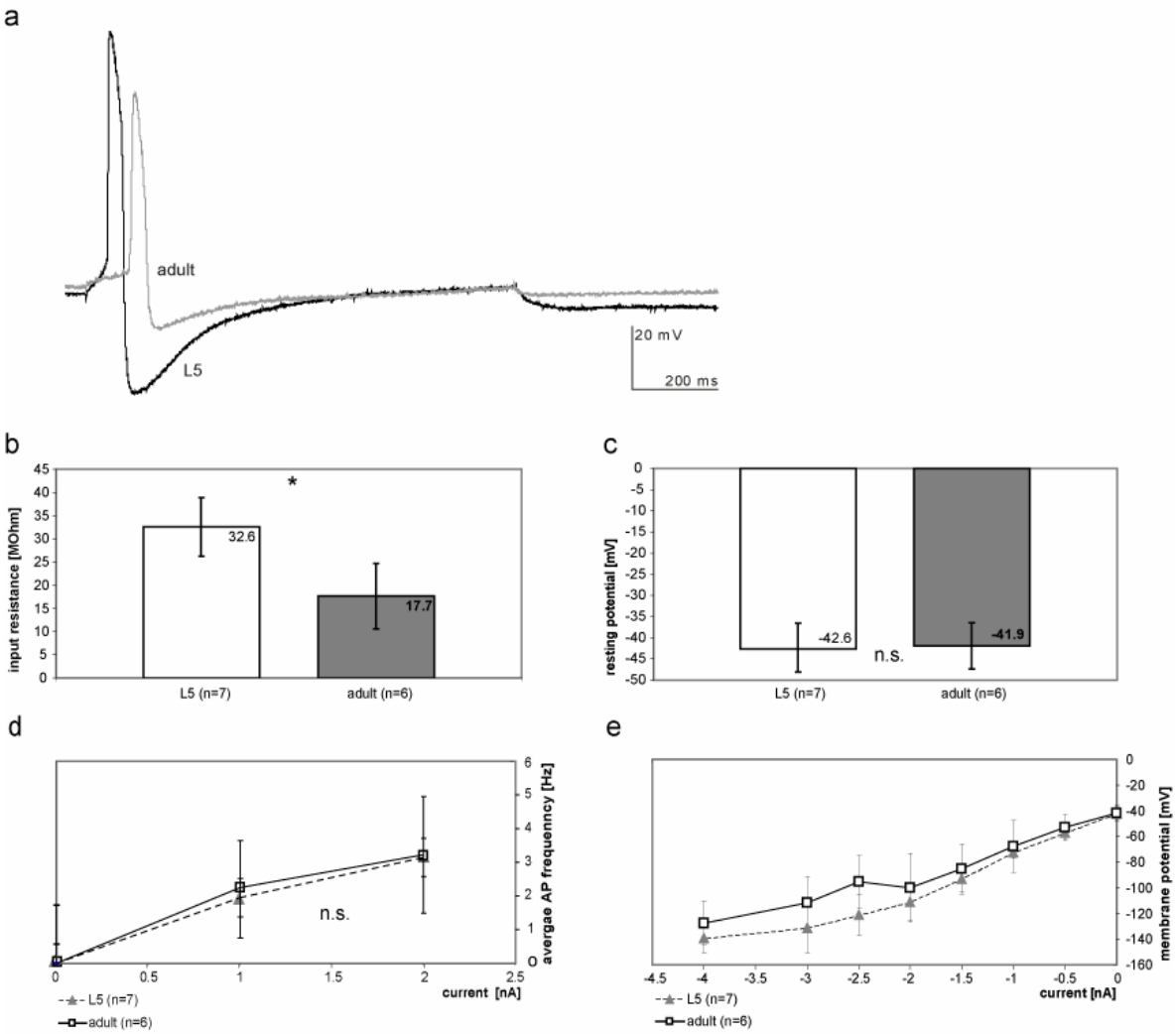

Fig. 2: **a** Action potentials elicited by injection of 0.2 nA depolarising current in larvae (*black*) and in adult (*grey*) VUM neurons. **b-e** Measurement of different intrinsic properties of VUM neurons in Manduca stages L5 (*white*) and adult (*grey*). (*p < 0.05, n.s. - not significant, students t-test, number of cells = *n*) **b** VUM neuron input resistance under physiological conditions. **c** Comparision of VUM resting potential in L5 and adults. **d** L5 (*white box*) and adult (*grey triangle*) VUM neuron frequency-current (f-I) relationship. **e** Differences current-voltage (I-V) relationship during hyperpolarising current injection between larval (*white*) and adult (*grey*) VUM neurons.

4.2 Activity of VUM neurons during CDM induced fictive flight

The next objective was to compare the activation of larval and adult mesothoracic VUM neurons during pharmacologically eleicited fictive motor patterns. Based on the fact that DUM/VUM neurons in mature locusts (Baudoux and Burrows, 1998; Burrows and Pfluger, 1995; Duch et al., 1999) form morphologically and functionally different

subpopulations, we were wondering whether the unpaired median neurons in *Manduca* which fire synchronously during larval fictive crawling (Johnston et al., 1999), will start to fire differentially and to form functionally different subpopulations in adults? To examine the activation patterns of mesothoracic VUM neurons during CDM induced fictive flight in adults we used the same preparation as described before (see chapter 2 and 3). Both, nerve IIN1c (Fig. 1b1) and IIN4 (Fig. 1b2) contain at least one VUM neuron projecting to the flight depressor (DLM1) and elevator (DVM1) muscles. The activity of this VUM1 neuron was recorded intracellularly (Fig. 3b-c, bottom trace) in parallel to extracellular recordings from elevator nerve IIN4 (Fig. 3b-c2, top trace) and depressor nerve IIN1c (Fig. 3b-c2, middle trace) before (Fig. 3b) and after (Fig. 3c) inducing fictive flight motor activity. Under continuous saline perfusion neither depressor motoneurons (Fig. 3c, middle trace) nor elevator motoneurons (Fig. 3c, upper trace) showed spontaneous activity, whereas VUM neurons spiked spontaneously (Fig. 3c, lower trace) with very low frequencies (0.01-0.1 Hz). When a fictive flight rhythm was induced by CDM application the spiking frequency of the VUM neuron increased (1Hz). However, the VUM spiking activity showed no direct cycle to cycle coupling to the depressor or elevator activity and only a few postsynaptic potentials were detectable in the intracellular recording from the VUM1 neuron even in higher resolution (see PSPs in bottom trace of Fig. 3c1). The synaptic drive to VUM neurons also did not reveal any coupling to flight motoneuron activity. However, no clear coupling in a cycle-to-cycle fashion could be observed in fourteen out of fifteen preparations, as it was observed for locust kicking, walking and flight (Baudoux and Burrows, 1998; Baudoux et al., 1998; Burrows and Pfluger, 1995; Duch et al., 1999). Unfortunately, intracellular staining of a previously recorded neuron proved difficult in adult *Manduca* ganglia, and therefore the exact type of VUM neuron could not be determined. However, one recording from a subsequently identified VUM1 neuron demonstrated clear, contigous EPSPs (Fig. 3d, bottom trace). If the neuron was hyperpolarized the underlying synaptic drive was revealed (Fig. 3d1). In the enlarged selection of Fig. 3d gray boxes indicate one wing cycle from one depressor motoneuron activity to the following. Clearly, each EPSP in the VUM neuron follows depressor motoneuron activity (Fig. 3d, lower trace).

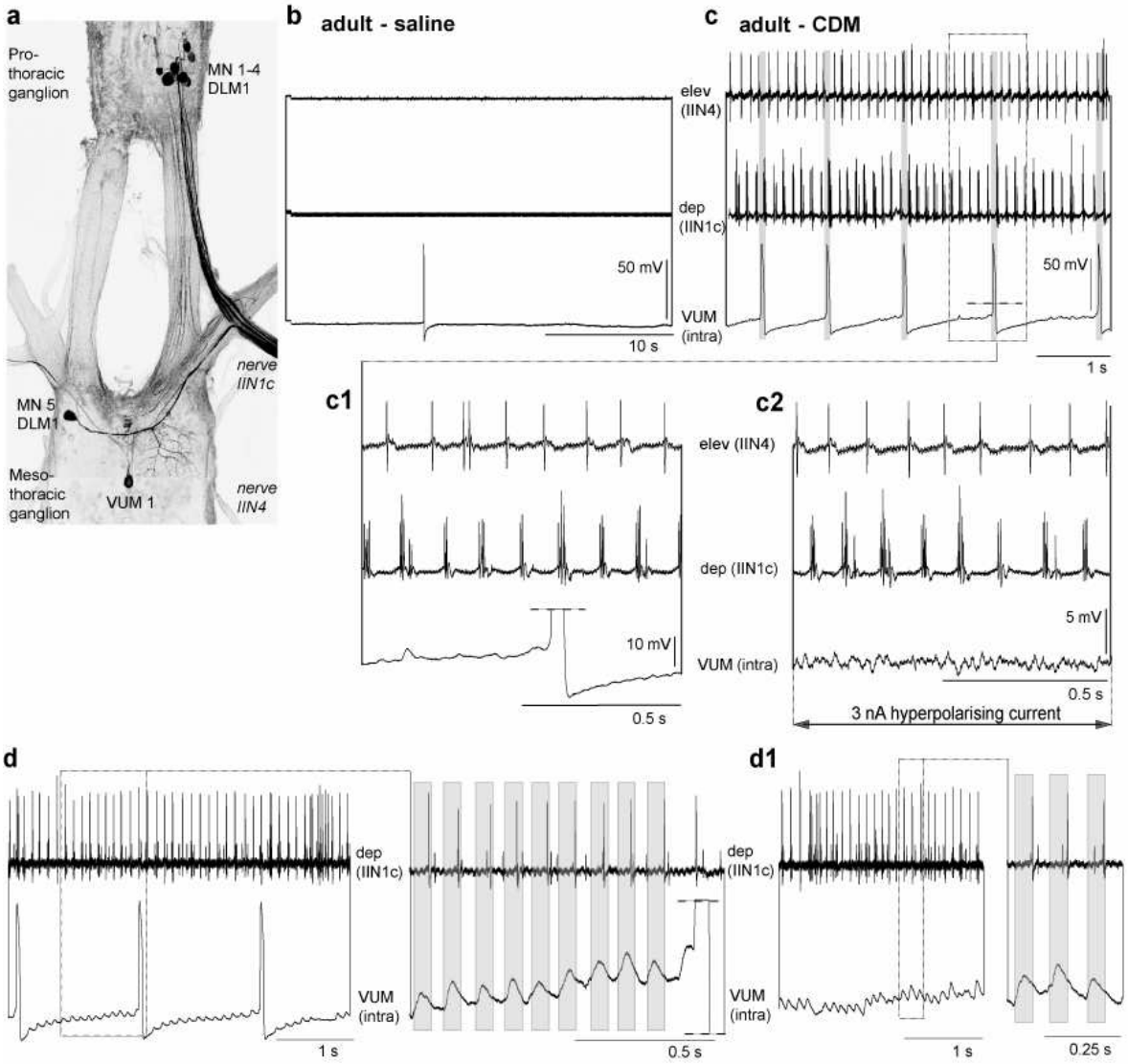

Fig. 3: **a** Retrograde axonal staining (Neurobiotin and subsequent conj.Cy3-streptavidin) of the mesothoracic nerve IIN1c reveals the location and axonal projection of depressor MN1-5 and one ventral unpaired median (VUM1) neuron. **b-c** Extracellular recording from one elevator motoneuron (*top trace*), depressor MN1-5 (*middle trace*) and an intracellular recording from a ventral unpaired median neuron (*bottom trace*). **b** Recording during saline application (control). **c** VUM activity during CDM induced fictive flight motor pattern (representative for 14 from 15 preparations). **c1** Expanded time scale from (c, dotted frame) illustrating 8 rhythmic flight bouts and VUM1 activity including synaptic inputs. **c2** Injection of 3 nA hyperpolarising currents during CDM application. **d** VUM1 activity during CDM induced fictive flight motor pattern of depressor MN1-5 in a particular preparation (1 from 15 preparations). The dotted frame is selectively enlargemed and grey boxes highlight coincidences of EPSPs in VUM neurons and depressor spikes. **d1** Injection of 2.5 nA hyperpolarising current reveal the underlying synaptic drive in the VUM1 neuron.

In summary, we found that different mesothoracic VUM neurons receive no patterned synaptic inputs during CDM induced fictive flight in 14 out of 15 recordings which demonstrates cycle-to-cycle coupling. Such a coupling was, however, revealed in a recording from one identified VUM1 neuron showing a synaptic drive coupled to the depressor motoneurons. This can not be explained by just systematic, experimental failures, but may be implicated to other experiments we did (see chapter 5). The application of pilocarpine, a muscarinic receptor agonist which reliably induces fictive walking in *Manduca* (Johnston and Levine, 2002), elicited a fictive flight like alternating, rhythmic depressor-elevator activity. This could indicate a putative cholinergic activation of the flight circuitry (see chapter 5). However, we observed that in 3 out of 3 recordings mesothoracic VUM neurons received synaptic input which was coupled to the depressor-elevator activity (Fig. 4) during pilocarpine induced motor patterns. Thus, our "contradictory" results during CDM induced fictive flight seem not to be due to a systematic or experimental failure, except the small number of experiments. It seems to be more likely that differences exist between the activation of mesothoracic VUM neurons during CDM induced fictive flight patterns and pilocarpine induced fictive flight like patterns. This observation may point to cholinergic, sensory influence on the activation pattern of VUM neurons.

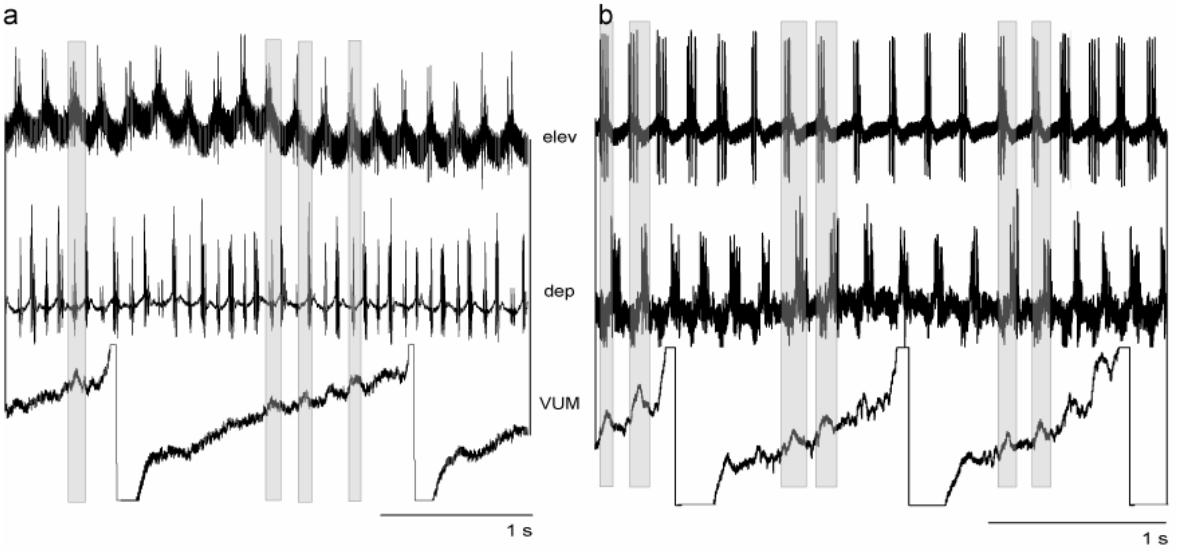

Fig.4: Activition of mesothoracic VUM neurons during pilocarpine induced depressor/elevator activity. **a-b** Extracellular recording from one elevator motoneuron (*top trace*), depressor MN1-5 (*middle trace*) and an intracellular recording from a ventral unpaired median neuron (bottom trace). **a** Activity during pilocarpine application after onset of fictive motor patterns. *Grey boxes* indicate synaptic input of the VUM neuron simultaneously coupled to depressor and elevator activity. **b** A different preparation during pilacarpine induced fictive motor patterns showing the same effect.

Further characterization of this putative coupling is needed and using the same experimental setup in semi-intact preparations leaving the wing sensory feedback functional but eliminating additional visual or acoustic influences could clarify if the activation of octopaminergic neurons during specific motor patterns depends on additional sensory informations.

We find much less (6-8) efferent unpaired median neurons in *Manduca* compared to 19-21 cells in locusts which anatomically and functionally form a heterogeneous group of modulatory neurons (Duch et al., 1999; Heidel and Pfluger, 2006). Compared to the number of larval octopaminergic, unpaired median neurons in the mesothoracic ganglion we verified an increase in number of adult neurons in the same ganglion after metamorphosis, which may be an explanation for the developmental increase in level of octopamine coincidencing with increases in the level of the enzyme tyramine-beta-hydroxylase available for OA synthesis (Lehman et al., 2000).

We also find anatomically different subpopulations of VUM neurons in *Manduca* but could not prove functional differences between these sub-types relating to their recruitment during fictive flight. All 15 recordings showed the same increase in VUM spiking activity from less than 0.1 Hz in saline rising up to a maximum of 1Hz during CDM application. The activity of different VUM sub-types during fictive flight always demonstrates a similar regular and tonic spiking. We never observed bursting activity of VUMs or unaffected slow tonic spiking activity like the one observed before induction of fictive flight. This may perhaps suppose that other than in flying locusts, for *Manduca* flight a cycle-to-cycle coupling is not required. In addition, in *Manduca* the smaller number of VUMs do not reach high spiking frequencies (1 Hz) if compared to locusts 10 Hz and more. This may suggest that the peripheral tasks of the *Manduca* VUM cells are slightly different to those from locusts. This is supported by the fact that we never observed a VUM neuron that was inhibited during fictive flight in contrast to what is known from locusts (Duch and Pflüger, 1999).

Chapter 5

Can cholinergic inputs activate the adult *Manduca* flight network?

Chapter 5

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Central pattern generators (CPGs) for several motor behaviors such as fictive walking, fictive flight and fictive crawling can be activated pharmacologically in isolated nerve cord preparations of various insect species (Büschges et al., 1995; Claassen and Kammer, 1986; Johnston and Levine, 1996; Ryckebusch and Laurent, 1993; Stevenson and Kutsch, 1988) by either the muscarinic agonist pilocarpine or the octopamine-receptor agonist chlordimeform. For insect flight it remains still unclear whether the activation of OA-receptors is sufficient and necessary to elicit flight patterns in locusts and moths. Mutant *Drosophila* lacking OA show normal behavior (Monastirioti et al., 1996) although a more detailed recent behavioral analysis of *Drosophila* flight suggests that OA is not necessary for flight initiation but for flight maintenance in this species (Brembs et al., 2007). In the locust fictive flight could also be elicited by cholinergic drugs (Buhl et al., 2008) demonstrating that octopamine is not necessary to initiate flight behavior. This is supported by results from the cockroach where flight could be induced by pilocarpine (Ridgel and Ritzmann, 2005). Therefore, we wondered whether the CPG for flight in *Manduca* could also be activated by putative muscarinic or cholinergic mechanisms. So far, it was described for *Manduca* that the muscarinic receptor agonist pilocarpine induces fictive crawling in larvae (Johnston and Levine, 1996) and fictive walking in adults (Johnston and Levine, 2002) like in other insect species (Büschges et al., 1995; Ryckebusch and Laurent, 1993).

Thus, to test whether cholinergic drugs also lead to induction of a fictive flight motor pattern, pilocarpine hydrochlorid (10^{-5} M, dissolved in saline, Fluka) and actylcholine hydrochloride (10^{-5} M, dissolved in saline, Riedel de Haen) were bath applied to isolated adult nerve cord preparations.

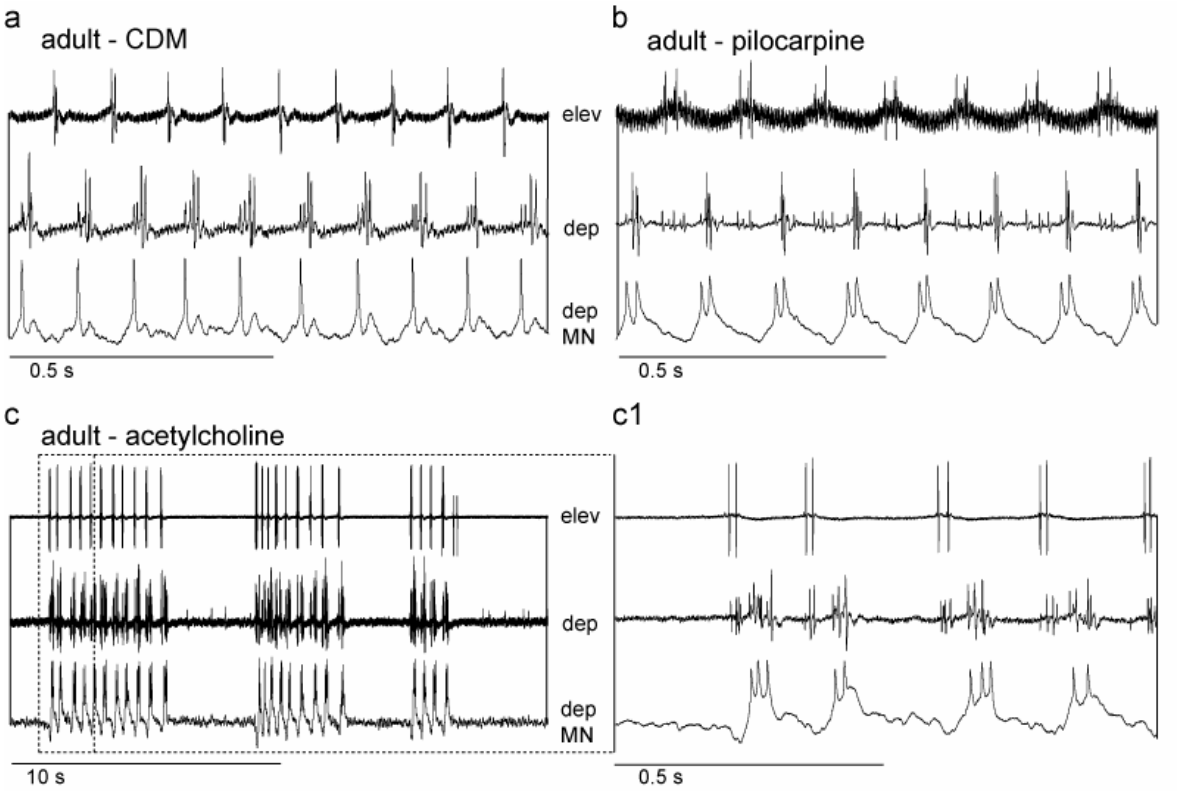

Fig. 5: Pharmacologically-induced motor patterns in isolated nerve cord preparations of adult Manduca sexta. **a-c** Extracellular recordings from mesothoracic nerve IIN4 (elevator, *upper trace*) and mesothoracic nerve IIN1c (depressor, *middle trace*) combined with an intracellular recording (*bottom trace*) of one of the prothoracic MN´s innervating the DLM1 flight muscle. **a-b** Continous fictive flight motor output after bath application of 10^{-5} M CDM (**a**) and 10^{-5} M pilocarpine (**b**). **c** Sporadic bouts of motor activity after bath application of 10^{-6} M acetylcholine. **c1** A selective enlagement of (*dotted frame*, **c**) during one bout of activity with higher x-axis resolution (1 s), identical to **a**, **b**.

Representative recordings of pharmacologically elicited motor patterns 10 minutes after application are shown in figure 5: CDM (Fig. 5a), pilocarpine (Fig. 5b) and acetylcholine (Fig. 5c, c1). Pilocarpine (Fig. 5b) elicited continuous motor activity with an alternating depressor/ elevator activation similar to CDM induced fictive flight seen in Fig. 5a. However, bath application of acetylcholine (Fig. 5c, c1) induced short bouts of alternating depressor and elevator bursts rather than continuous rhythmic motor patterns over the whole range of tested concentrations (not shown). Although this acetylcholine elicited motor pattern does not resemble a fictive flight or walking pattern it demonstrates a rhythmic motor activity which, perhaps, represents an ecdysis related behavior (Kim et al., 2006). One obvious difference between the pilocarpine- and CDM-induced pattern is that depressor and elevator motoneurons generate one spike per cycle period during CDM induced fictive flight and more than one spike during pilocarpine induced motor activity.

This is demonstrated by extracellular recordings from one elevator motoneuron (Fig. 5b, upper trace) and an intracellular recording from one depressor motoneuron (Fig. 5b, bottom trace) showing that two spikes settle upon the depolarisation during pilocapine induced depressor activity. To examine whether the pilocarpine elicited patterns resemble the CDM induced fictive flight patterns, we measured and compared different motr pattern parameters. Compared to CDM induced fictive flight, the depressor cycle period during pilocarpine elicited motor patterns (Fig. 6a) was significantly longer than that of CDM induced fictive flight. The larger spread of the 25% and 75% quartile demonstrates a higher variability in the depressor cycle period for pilocarpine induced motor patterns. Pilocarpine elicited depressor-elevator latency (Fig. 6b) is also significantly longer and shows a higher variability. The depressor cycle period and the depressor-elevator interval are increased during pilocarpine application but both parameters are shifted by nearly the same factor, so that the phase of the elevator activity during the depressor cycle (Fig. 6c) is about 0.55 compared to 0.5 during fictive flight. Auto-correlations of recurrent depressor burst were used to compare the precision of rhythmicity in CDM and pilocarpine induced motor patterns (Fig. 6d). Representative extracellular recordings of depressor activity during CDM and pilocarpine elicited motor patterns show regular rhythmic spiking patterns and auto-correlation analysis confirms this repetitive rhythmical activity over 244 cycles. Periodic peaks and throughs occuring approximately every 80 ms during CDM application and every 145 ms during pilocarpine application demonstrate periodic clustering of spikes at a cycle frequency of around 11 Hz or 7 Hz respectively. The clustering of spikes during pilocarpine application forms much broader peaks with smaller counts indicating less precision in depressor bursting. Our results demonstrate that pilocarpine is sufficient to elicit alternating rhythmic depressor-elevator motor activity which resembles CDM induced fictive flight in major pattern features but is somewhat slower and with less precision. This may actually be the result of increased excitability, which results somehow in a situation of overexcitation and thereby involve two elevator spikes per cycle instead of one during CDM induced fictive flight and two spikes in depressor motoneurons instead of one. Additionally, longer cycle periods and latencies support this assumption. Two mechanisms could cause this increased excitatory drive to flight motoneurons: first, pilocarpine directly affects the membrane properties of pre-motor flight interneurons or the motoneurons itself and second, it may alter the efficacy of synaptic transmission in the CPG for flight. Both, the cholinergic modulation of

conductances in cockroach motoneurons (David and Pitman, 1995) and modulation of presynaptic muscarinic receptors (Judge and Leitch, 1999; Trimmer, 1994; Trimmer and Weeks, 1993) have been demonstrated in *Manduca* and locusts. Recent work showed a muscarinic cholinergic activation of flight CPG in locusts which may be subject to aminergic modulation (Buhl et al., 2008).

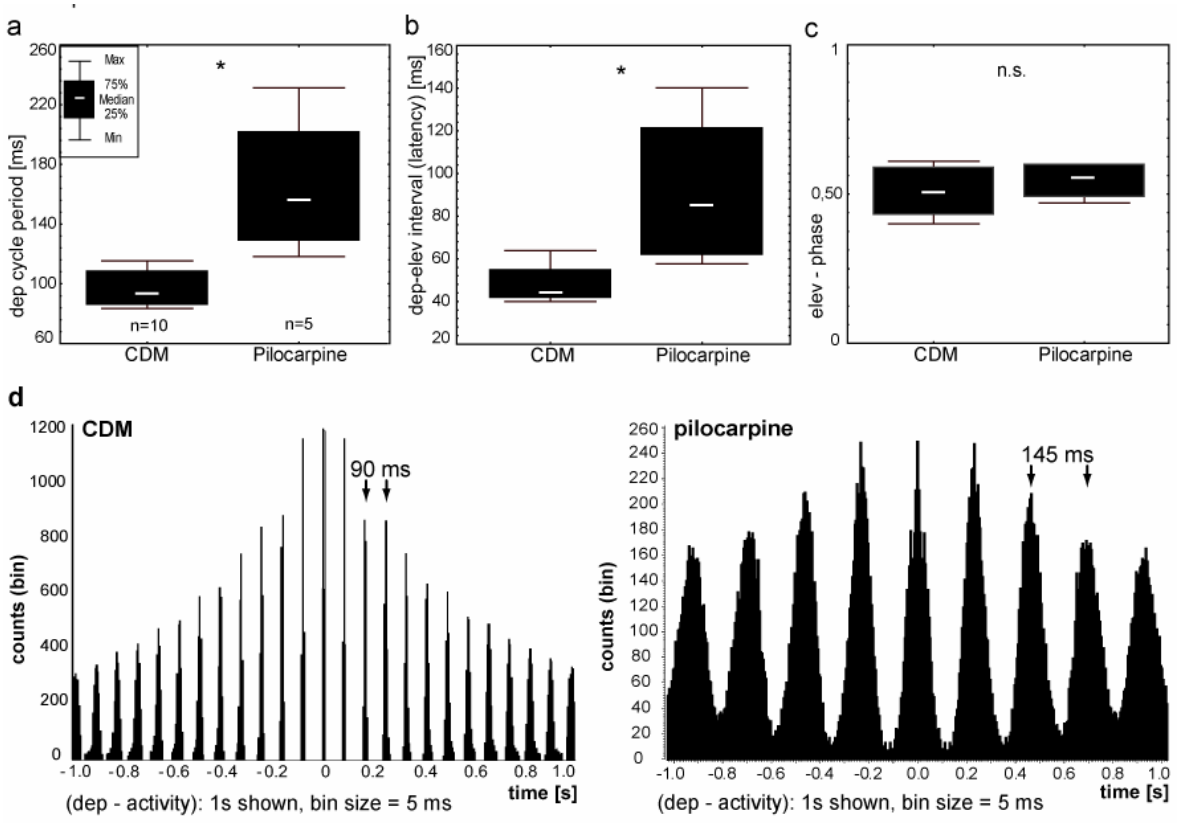

Fig. 6: Quantitative measurements of motor activity induced by application of CDM (10^{-5} M) and pilocarpine (10^{-5} M) in adult preparations. **a** Duration of depressor cycle period, **b** depressor-elevator intervals and **c** elevator phase. **d** Representative auto-correlation histogram of extracellularly recorded depressor activity (244 cycles) during CDM induced ficitive flight (*left*) and pilocarpine (*right*) elicited motor activity. (*arrows* denote one depressor bursting period)
a-c *White lines* indicate the median, the *black boxes* represent the 25 and the 75 quartile and the *error bars* indicate the range (see inset **a**). Number (*n*) of analysed preparations for CDM = 10, piloracpine = 5. Statistical significance is indicated by asterisks: *p < 0.05, **p < 0.005, students t-test.

They showed that pilocarpine (5 mmol l^{-1}) and CDM (500 mmol l^{-1}) induced fictive flight patterns are very similar to those induced by wind stimuli, but CDM had to be applied in 100 times higher concentrations. Both drugs induced fictive motor patterns lasting only a few minutes. In all our experiments we reliably induced fictive motor patterns with much less drug concentrations (0.1 µmol l^{-1}) and lasting for 30 min and longer. On the contrary to our results, a significant difference between the elevator phase

during pilocarpine (0.52) and CDM (0.76) induced motor patterns were found (Buhl et al., 2008). In contrast to the findings in the locust fictive flight patterns could not be induced by tyramine in *Manduca* (Vierk et al., 2009).

To study the differences between locusts and *Manduca*, more detailed studies are required of tyraminergic and cholinergic effects on excitability of flight motoneurons or on modulation of synaptic inputs to flight motoneurons.

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Chapter 6 – Summary and outlook

Centrally generated rhythmic motor patterns are the result of the complex arrangement, synaptic wiring and communication a multiplicity of neurons. For anyone trying to understand such complex neuronal procedures, model systems that are simple in structure and nevertheless complex in their generated behavioral patterns, provide the opportunity to do so as in such systems the movement is measurable and quantifiable. This thesis describes and interprets the postembryonic development of central pattern generation for flight during complete metamorphosis, which is a unique feature of holometabolous insects, such as the hawkmoth, *Manduca sexta*. In particular, the possible influences of the biogenic amine tyramine on the central generation for the motor flight patterns are examined, a topic very much under discussion within vertebrate as well as invertebrate neurobiology.

Even if the connectivity of the neuronal circuit not yet has been described, the flight motor system in the isolated nervous system of *Manduca sexta* offers the possibility of inducing model, fictive motor patterns pharmacologically. Thus it is possible to describe its functionality, possible changes of the flight pattern and modulatory effects on the motor output of the central pattern generator for flight, and to discuss and draw appropriate conclusions in connection with the structural and physiological changes in the central nervous system during metamorphosis, which have already been studied.

The first manuscript (Chapter 2) examines the starting point of network activity in the central pattern generator and whether during the process of development further adjustments to the pattern develop, or whether the network is able from a certain point of time to produce mature, adult flight patterns, since the central network already is established postembryonically. The investigations show that it is not possible to release fictive flight pattern in development stages prior to the pupal stage P16 (from a whole of 20 days of the development). During remaining pupal development, the central generated flight pattern matures progressively, the frequency and the precision increase, probably in absence of sensory influences, to become the underlying motor pattern of the mature, adult flight. The results suggest that the changes in the physiological properties of the flight motoneuron MN5, one of the five depressor motoneurons for flight, correlate temporally with the first occurrence of EPSPs in depressor motoneurons, which are pharmacologically inducible by CDM. Furthermore, the structural development of the MN5 and the functional integration into the flight pattern generating network coincidences with the occurrence of pre-mature, fictive flight patterns in pupal stage P16. The observation that no flight-like motor patterns are generated by the central pattern generator prior to P16 is not a result of $GABA_A$-dependent inhibition. Indirect references point to different courses in the development of elevator motoneurons compared to the course of depressor motoneurons.

In the second manuscript (Chapter 3) it was examined, whether the biogenic amines octopamine and tyramine have different effects on the central pattern generator for flight in *Manduca*, or how so far accepted, cause antagonistic effects. In this work it could be shown for the first time that tyramine has a direct influence on the generation of the central flight pattern and contrary to octopamine selectively affects

only the activity of the depressors. This result may contribute to carrying for the future acceptance of tyramine as independent neuromodulator with distinct modulatory effects.

This chapter (Chapter 4) examines whether a functionally homogeneous population of larval octopaminergic, unpaired median neurons (VUM/DUM) differentiates into functionally different subpopulations during metamorphosis. Firstly, it is shown that the number of this type of neurons increase during postembryonic development. This could explain the increase of the octopamine level during developement, which has already been described and also correlates with changes in the level of the enzyme tyramine-beta-hydroxylase, which is available for octopamine synthesis. Secondly, even if anatomically different subpopulations of unpaired median neurons, as seen in locusts, are also shown for *Manduca*, no functional differences regarding the recruitment during the fictive flight motor pattern were found. The number of neurons differs clearly in locusts, approx. 19-21 neurons, whereas only 6-8 are in *Manduca*. The results suggest that the function of the mesothoracic VUM neurons is different in *Manduca* another to than in locusts.

In the last chapter of the thesis (Chapter 5) I pose the question as to whether there is a cholinergic mechanism for the activation of the central pattern generator in *Manduca sexta*, as described in locusts. Muscarinic stimulation of the central flight circuitry showed a motor pattern comparable the CDM induced fictive flight motor pattern, which was however slower and less precise. This seems to be caused by a strong overexcitation of the central pattern generating network or of the flight motoneurons, which however could not be verified so far.

Further I would like briefly, outgoing from the results represented here, possible, future experiments suggest, which could contribute to further understanding of the octopaminergic and tyraminergic modulation of the central pattern generator for flight in *Manduca*. In order to verify the selective effect of tyramine on the depressor activity, exact quantitative analyses must examine the influence of tyramine, on the one hand on the magnitude of the excitatory, synaptic drive to depressor motoneurons and on the other hand on the excitability of depressor Motoneurons themselves. The cholinergic modulation on the synaptic drive and the excitability of the flight motoneurons should be also examined. Taken together, this could contribute to compare the octopaminergic and tyraminergic influences on the central pattern generation of locusta and *Manduca* and to serve for a better understanding of the modulation of central networks. Since DUM/VUM Neurone can release putatively octopamine and tyramine, some of these neurons project to flight muscles and this work also shows, that this neurones show continuous, tonic activity during fictive flight motor patterns, possible modulatory effects of tyramine on the synaptic transmission at the neuromuscular junction and physiological influences on flight muscles properties and metabolism should be examined in both organisms. Additionally, knowledge of the distribution of the tyramine receptors in different tissues would contribute to a better understanding of the anatomy and function of the tyraminergic system in insects.

Chapter 7 – Zusammenfassung

Zentral generierte rhythmische Bewegungsmuster sind die Konsequenz einer komplexen Anordnung, Verschaltung und Kommunikation zwischen einer Vielzahl von Neuronen. Für diejenigen die versuchen solche komplexen neuronalen Vorgänge zu verstehen bieten sich Modellsysteme an die simpel im Aufbau und dennoch komplex in dem generierten Bewegungsmuster sind, besonders deshalb an, weil in solchen Systemen die Bewegung messbar und quantifizierbar ist. Die hier vorliegende Dissertation beschreibt und interpretiert die postembryonale Entwicklung der zentralen Mustergeneration für Flug während der – für holometabole Insekten typischen – Metamorphose des Tabakschwärmers, *Manduca sexta*. Im Besonderen wurden die möglichen Einflüsse des biogenen Amines Tyramine auf die zentrale Generation der motorischen Flugmuster untersucht, ein im Bereich der Vertebraten, sowie invertebraten Neurobiologie, aktuell diskutiertes Thema.

Wenn auch die Verschaltung des Neuronalen Neztwerkes nicht beschrieben ist, so bietet das gut untersuchte motorische Flugsystems von *Manduca sexta* die Möglichkeit im isolierten Nervensystem des Tabakschwärmers, modellhaft fiktive motorische Muster pharmakologisch zu induzieren. Somit ist es möglich die Funktionalität, mögliche Veränderungen des Flugmusters und modulatorische Effekte auf den motorischen Ausgang des zentralen Mustergenerators für Flug zu beschreiben, und entsprechende Rückschlüsse im Zusammenhang mit den bereits publizierten strukturellen und physiologischen Veränderungen im zentralen Nervensystem zu diskutieren.

Im ersten Manuskript (Chapter 2) wurde untersucht ab wann das Netzwerk für die Generation der zentralen Flugmusters in der Lage ist, flugähnliche Muster zu generieren und ob es im Verlauf der weiteren Entwicklung zu Anpassungen des Musters kommt, oder ob das Netzwerk ab einem bestimmten Zeitpunkt in der Lage ist, das reife, adulte Flugmuster zu produzieren, da das neuronale Netzwerk bereits postembryonal angelegt war. Die Untersuchungen zeigen, dass es nicht möglich ist fiktive Flugmusters in jüngeren Entwicklungsstadien als das Puppenstadium 16 auszulösen (aus einer Gesamtheit von 20 Tagen der Entwicklung). Während der verbleibenden Zeit der Puppenentwicklung reift das zentral generierte Flugmuster, die Frequenz und die Präzision nehmen zu, wahrscheinlich in Abwesenheit sensorischer Einflüsse, progressiv zum fertigen, adulten Flug zugrunde liegendem Muster. Die Ergebnisse deuten darauf hin, dass die Veränderungen in den physiologischen Eigenschaften des Flugmotoneurons MN5, als eines der fünf Depressor Motoneurone für Flug, zeitlich mit dem ersten Auftreten von EPSPs, nach pharmakologischer Induktion des Fluges, in den Depressor-Motoneuronen korrelieren. Weiterhin koinzidieren die strukturelle Entwicklung des MN5 und somit wahrscheinlich die funktionelle Integration in das Flugnetzwerk mit dem Auftreten prä-maturer, fiktiver Flugmuster im Puppenstadium 16. Die Tatsache, dass flugähnliche Muster nicht vom zentralen Mustergenerator für Flug in jüngeren Puppenstadien als P16 generiert werden, ist nicht auf eine $GABA_A$-vermittelte Inhibition des Netzwerkes zurückzuführen. Zudem finden sich indirekte Hinweise darauf, dass sich die Elevator Motoneurone mit einem anderen zeitlichen Verlauf als die Depressor Motoneurone entwickeln.

Im zweiten Manuskript (Chapter 3) wurde untersucht, ob die biogenen Amine Oktopamine und Tyramine unterschiedliche Effekte auf den zentralen Mustergenerator für Flug in *Manduca* haben, oder wie bisher angenommen, antagonistische Wirkungen hervorrufen. In dieser Arbeit konnte zum ersten Mal gezeigt werden, dass Tyramin einen direkten Einfluss auf die Generierung des zentralen Flugmusters hat und im Gegensatz zu Oktopamin selektiv nur auf die Aktivität der Depressoren wirkt. Dieses Ergebnis trägt vielleicht dazu bei, der zukünftigen Akzeptanz von Tyramin als eigenständiger Neuromodulator mit abgrenzbaren Wirkungen Rechnung zu tragen.

Dieses Kapitel (Chapter 4) untersucht, ob sich aus der funktionell homogenen Population larvaler octopaminerger, unpaariger Neurone während der Metamorphose funktionell unterschiedliche Subpopulationen differenzieren. Es konnte gezeigt werden, dass die Zahl dieser Neurone im Verlauf der postembryonalen Entwicklung zunimmt. Dies könnte die bekannte Zunahme des Oktopamin Levels während der Entwicklung erklären und korreliert zudem mit dem Level der für die Oktopamin-Synthese verfügbaren Enzyms Tyramine-beta-hydroxylase. Wenn auch anatomisch unterschiedliche Subpopulationen der unpaarigen Neurone, wie in Locusten beschrieben, ebenfalls für *Manduca* gezeigt werden konnten, wurden keine funktionellen Unterschiede hinsichtlich der Rekrutierung während des fiktiven Flugs gefunden. Die Zahl der Neurone unterscheidet sich deutlich in Locusten, ca. 19-21 Neuronen, gegenüber nur 6-8 in *Manduca*. Die Ergebnisse deuten darauf hin, dass die Funktion der mesothorakalen VUM Neurone in *Manduca* eine andere ist, als in Locusta beschrieben.

In dem letztem Kapitel der hier vorliegenden Dissertation (Chapter 5) möchte ich die Frage stellen, ob es wie in Locusta einen cholinergen Mechanismus für die Aktivierung des zentralen Mustergenerators in *Manduca sexta* gibt. Muskarinische Stimulation des Flugnetzwerks zeigten ein dem fiktiven Flug ähnliches, aber langsameres, rhythmisches Muster, welches durch eine starke Übererregung im Flugnetzwerk oder der Motoneurone bewirkt wird, was aber nicht weiter verifiziert wurde.

Weiterhin möchte ich kurz, von den hier dargestellten Ergebnissen ausgehend, mögliche, zukünftige Experimente vorschlagen, welche dem weiteren Verständnis der oktopaminergen und tyraminergen Modulation des zentralen Mustergenerators für Flug in *Manduca* beitragen könnten. Um der selektiven Wirkung von Tyramin auf die Depressor Aktivität weiter zu verifizieren, müssen genaue quantitative Analysen den Einfluss von Tyramin, zum einen auf die Größe der exzitatorischen, synaptischen Eingänge der Depressor Motoneurone und zum anderen auf die Erregbarkeit der Depressor Motoneurone selbst aufklären. Ergänzend sollte die Analyse der cholinergen Modulation der synaptischen Eingänge und die Erregbarkeit der Flug-Motoneurone ebenfalls untersucht werden. Zusammenfassend könnten bereits gesammelte und weitere Ergebnisse dem Vergleich der oktopaminergen und tyraminergen Einflüsse auf die zentrale Mustergeneration von Locusta und *Manduca* dienen und zu einem besseren Verständnis der Modulation zentraler Netzwerke beitragen. Da DUM/VUM Neurone theoretisch Oktopamin und Tyramin freisetzen können, einige dieser Neurone auf wichtige Flugmuskeln projezieren und in dieser Arbeit auch gezeigt werden konnte, dass diese Neurone während des fiktiven Flugs kontinuierlich, tonisch aktiv sind, sollten mögliche modulatorische Effekte von Tyramin auf die synaptische Übertragung an der motorischen

Endplatte und physiologische Einflüsse auf die Flugmuskeln in beiden Organismen genau untersucht werden. Kenntnis über die Verteilung der Tyramin-Rezeptoren in verschiedenen Geweben würde helfen, die Anatomie und Funktion des tyraminergen Systems, besser zu verstehen.

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Eidesstattliche Versicherung

Ich versichere von Eides statt, dass ich die Arbeit selbstständig angefertigt und die wörtlich oder inhaltlich aus anderen Quellen übernommenen Stellen als solche kenntlich gemacht habe. Die Inanspruchnahme fremder Hilfen wurde namentlich aufgeführt.

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Hamburg , den 14.07.2009