Properties of Cholinergic Currents in Identified Leg Motoneurons of the Stick Insect, *Carausius morosus*

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Encare seus medos e viva seus sonhos

Face your fears and live out your dreams

Stelle dich deinen Ängsten und verwirkliche deine Träume

(unknown author)

Abstract

To better understand how animals control their motor output for walking and other locomotor behaviors, it is necessary to elucidate the cellular and network properties of the neurons involved that underlie these behaviors. Neuroactive substances available in the bloodstream or released locally by neurons modify the network activity altering the membrane and synaptic properties of network neurons. This dissertation was conducted attempting to better understand the synaptic and integrative properties of the component neurons within the stick insect leg sensory-motor system. Patch-clamp recordings and fast optical Ca^{2+} imaging were performed to characterize acetylcholine-induced currents (I_{ACh}) in leg motoneurons of the stick insect *Carausius morosus*. The experiments were performed under biophysically controlled conditions on freshly dissociated leg motoneurons to avoid secondary effects from the network. To allow for unequivocal identification, the leg motoneurons were backfilled with a fluorescent dye through the main leg nerve prior to cell dissociation. In 87 % of the motoneurons, I_{ACh} consisted of a fast-desensitizing (I_{ACh1}) and a slow-desensitizing component (I_{ACh2}), with EC_{50} values of 3.7 x 10^{-5} M and 2.0 x 10^{-5} M, respectively. Ca^{2+} imaging revealed that a considerable portion of I_{ACh} (~ 18 %) is carried by Ca^{2+}, suggesting that I_{ACh}, besides mediating fast synaptic transmission,

could also induce Ca^{2+}-dependent processes. Using specific nicotinic and muscarinic acetylcholine receptor ligands, it was observed that I_{ACh} was exclusively mediated by nicotinic acetylcholine receptors. Distinct concentration-response relations of I_{ACh1} and I_{ACh2} for these ligands indicated that they are mediated by different types of nicotinic acetylcholine receptors. The biogenic amines octopamine and serotonin consistently reduced the I_{ACh} but showed different modulatory efficacies. Recently discovered neonicotinoids abolished both I_{ACh1} and I_{ACh2} with high potencies.

Zusammenfassung

Um das Laufen und andere lokomotorische Prozesse von Tieren besser verstehen zu können, ist es notwendig, die zugrundeliegenden zellulären Mechanismen und Netzwerkeigenschaften der involvierte Neurone zu klären. Neuroaktive Substanzen im Blut oder lokal, durch Neurone freigesetzt modifizieren die Netzwerkaktivität durch Veränderung von Membranleitfähigkeiten und synaptischer Eigentschaften der Neurone im Netzwerk. Diese Dissertation soll dazu beitragen, synaptische und integrative Eigenschaften der Neurone des senso-motorischen Netzwerks zur Steuerung eines Beins der Stabheuschrecke besser zu verstehen. Versuche mit der *"patch-clamp"* Technik in Verbindung mit schnellem, optischen Ca^{2+} Imaging wurden durchgeführt um Acetylcholin-induzierte Ströme (I_{ACh}) in Beinmotoneuronen der Stabheuschrecke *Carausius morosus* zu charakterisieren. Die Experimente wurden unter biophysikalisch kontrollierten Bedingungen an frisch dissoziierten Beinmotoneuronen durchgeführt um sekundäre Netzwerkeffekte auszuschließen. Um eine eindeutige Zuordnung zu ermöglichen, wurden die Beinmotoneurone vor der Dissoziation mit einem Fluoreszenzfarbstoff retrograd über den Hauptnerv des Beins gefärbt. In 87 % der Motoneurone bestand I_{ACh} aus einer schnell desensitisierenden (I_{ACh1}) und einer langsam desensitisierenden Komponente (I_{ACh2}). Beide Komponenten waren konzentrations-

abhängig mit einem EC_{50} von jeweils 3.7 x 10^{-5} M und 2.0 x 10^{-5} M. Es konnte mit Ca^{2+}Imaging Versuchen gezeigt werden, dass ein relativ großer Anteil von I_{ACh} von Ca^{2+} getragen wird (~18 %), was vermuten lässt, dass I_{ACh} zusätzlich zur Vermittlung der schnellen synaptischen Übertragung, auch Ca^{2+} abhängige Prozesse induzieren könnte. Durch die Nutzung von spezifischen, nikotinischen und muskarinischen Acetylcholinrezeptorliganden konnte gezeigt werden, dass I_{ACh} ausschließlich durch nikotinische Acetylcholinrezeptoren ausgelöst wird. Unterschiede in den Beziehungen der Konzentrationsantworten zwischen I_{ACh1} und I_{ACh2} deuten darauf hin, dass diese Ströme von verschiedenen nikotinischen Acetylcholinrezeptortypen ausgelöst werden. Die biogenen Amine Oktopamin und Serotonin reduzierten beide einheitlich I_{ACh} aber zeigten unterschiedliche modulatorische Wirksamkeit. Kürzlich entdeckte Neonikotinoide waren in der Lage I_{ACh1} und I_{ACh2} mit hoher Wirksamkeit zu eliminieren.

Contents

A. Introduction

The ability to efficiently move in complex environments is a key property of animals. The nature of their walking systems has certainly contributed to their success in such important behaviors as predator avoidance and searching for food and mates for reproduction (Ijspeert 2008; Orlovsky 1999; Ritzmann and Büschges 2007). To accomplish neural control of locomotion with remarkable efficiency, animals have evolutionary developed complex neural circuits. The neural networks underlying locomotory actions adapt their output to the environmental and behavioral requirements by integrating centrally generated activity with local afferent, intersegmental coordinating and neuromodulatory inputs. In the end, the motor pattern is shaped by a) the intrinsic electrical properties, and b) the synaptic properties and connectivity of the component neurons. Insect motor systems have served as very successful models for the investigation of basic principles of walking and locomotion in, e.g., cockroaches, locusts and stick insects (Burrows 1996; Orlovsky 1999; Ritzmann and Büschges 2007). Across insect sensory-motor pathways, the endogenous transmitter acetylcholine (ACh) mediates cholinergic neurotransmission through interaction with two types of receptors, nicotinic (nAChRs) and muscarinic (mAChRs) (Burrows 1996; Parker and Newland 1995; Trimmer and Weeks 1989; Trimmer 1995). To better comprehend the synaptic and integrative properties of the neurons within the insect locomotor networks, pharmacological characterizations of cholinergic currents induced in identified leg motoneurons of the stick insect *Carausius morosus* were performed. The possible regulation of these currents by actions of biogenic amines and insecticides were investigated.

1 Cholinergic neuronal signalling

Nervous system functions depend on interneuronal communication that involves transmitter–receptor interactions. Although the discovery of different neurotransmit-

ters has expanded our knowledge, the general mechanisms of cholinergic synaptic transmission (depicted in Figure A.1) provide a foundation for modern concepts of chemical neurotransmission. Shortly, sequential actions of intracellular key enzymes result in the biosynthesis of the acetylcholine (ACh), which is stored in vesicles and delivered for release into the synaptic cleft. The ACh release is normally elicited by cell depolarization and is calcium-dependent. Once in the synaptic cleft, ACh will interact with its own postsynaptic receptors or with presynaptic release-regulating autoreceptors. The inactivation of ACh is mainly due the actions of acetylcholine esterase (AChE), resulting in choline and acetic acid. ACh is a classical excitatory neurotransmitter, without any participation in insect neuromuscular transmission (Sattelle 1980; Usherwood 1994) and has been implicated in locomotor network functions in many vertebrates (Huang et al. 2000; Panchin et al. 1991; Quinlan et al. 2004; Quinlan and Buchanan 2008) and invertebrates (Hue et al. 2007; Le Bon-Jego et al. 2006; Miller et al. 1992; Parker and Newland 1995; Trimmer and Weeks 1989; 1993; Widmer et al. 2006; Westmark et al. 2009).

1.1 Variety of acetylcholine receptors (AChRs)

The acetylcholine receptors (AChRs) were originally categorized in the vertebrate peripheral nervous system as either muscarinic or nicotinic (Dale, 1914), on the basis of the resemblance of muscarine and nicotine effects with those of ACh. Nicotinic acetylcholine receptors (nAChRs) are members of the super-family of ligand-gated ion channels, whereas muscarinic acetylcholine receptors (mAChRs) are G-protein coupled receptors (Figure A.2). mAChRs and nAChRs can be localized post-synaptically as well as pre-synaptically (Grilli et al. 2008; Jones and Wonnacott 2004; Mrzljak et al. 1996; 1998) and these receptors are coexpressed in many network component neurons involved in the control of behavioral processes (Bany et al. 2003; Huang et al. 2000; Miller et al. 1992; Panchin et al. 1991; Raizen et al. 1995; Steger

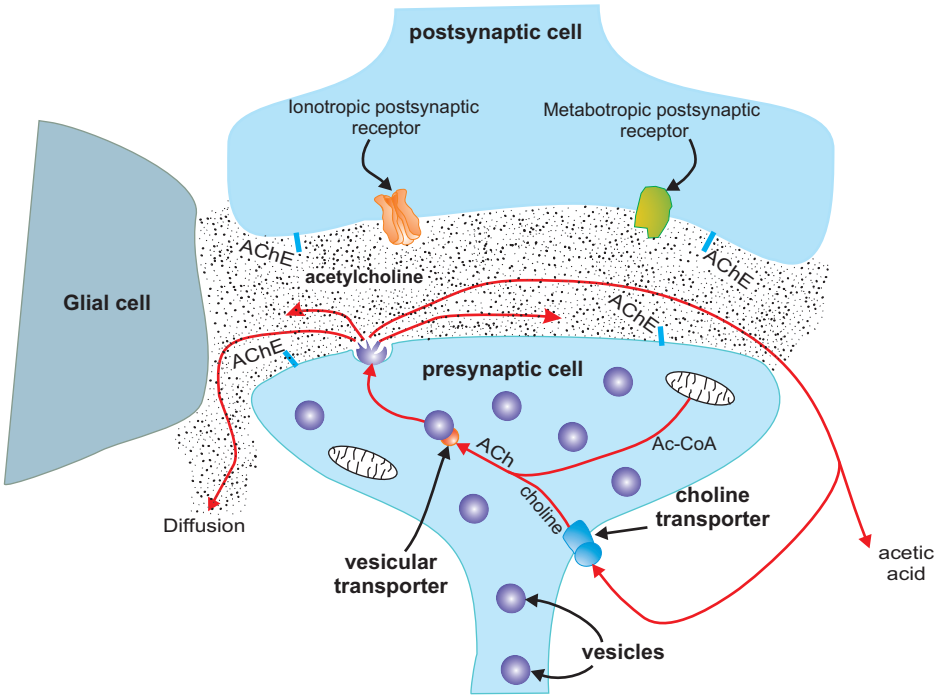

Figure A.1: *Schematic representation for neurotransmission through cholinergic synapses. The enzyme choline acetyltransferase (ChAT) acetylates the choline using acetyl-CoA (Ac-CoA) to form the transmitter acetylcholine (ACh), which is accumulated into vesicles by the vesicular transporter. The released ACh may interact with postsynaptic muscarinic or nicotinic cholinergic receptors, or can be taken up into the neuron by a choline transporter. Acetylcholine can be degraded after release by the enzyme acetylcholine esterase (AChE), resulting in choline and acetic acid. Adapted from Deutch and Roth (2004).*

and Avery 2004; Weeks et al. 1997). Both cholinergic receptor types exhibit structural and pharmacological heterogeneity (see below) and AChRs with both nicotinic and muscarinic sensitivities have also been described (David and Pitman 1993; Lapied et al. 1990; Parker et al. 2003; Verbitsky et al. 2000; Zwart and Vijverberg 1997).

Nicotinic acetylcholine receptors (nAChRs)

nAChRs mediate fast cholinergic synaptic transmission and are among the most thoroughly studied molecules in nervous systems. In vertebrates, the neuronal nAChRs consist of pentameric ligand-gated cation channels, formed by the assembly of multiple α (α2–α10) and β (β2–β4) subunits. The α subunits are defined by a characteristic

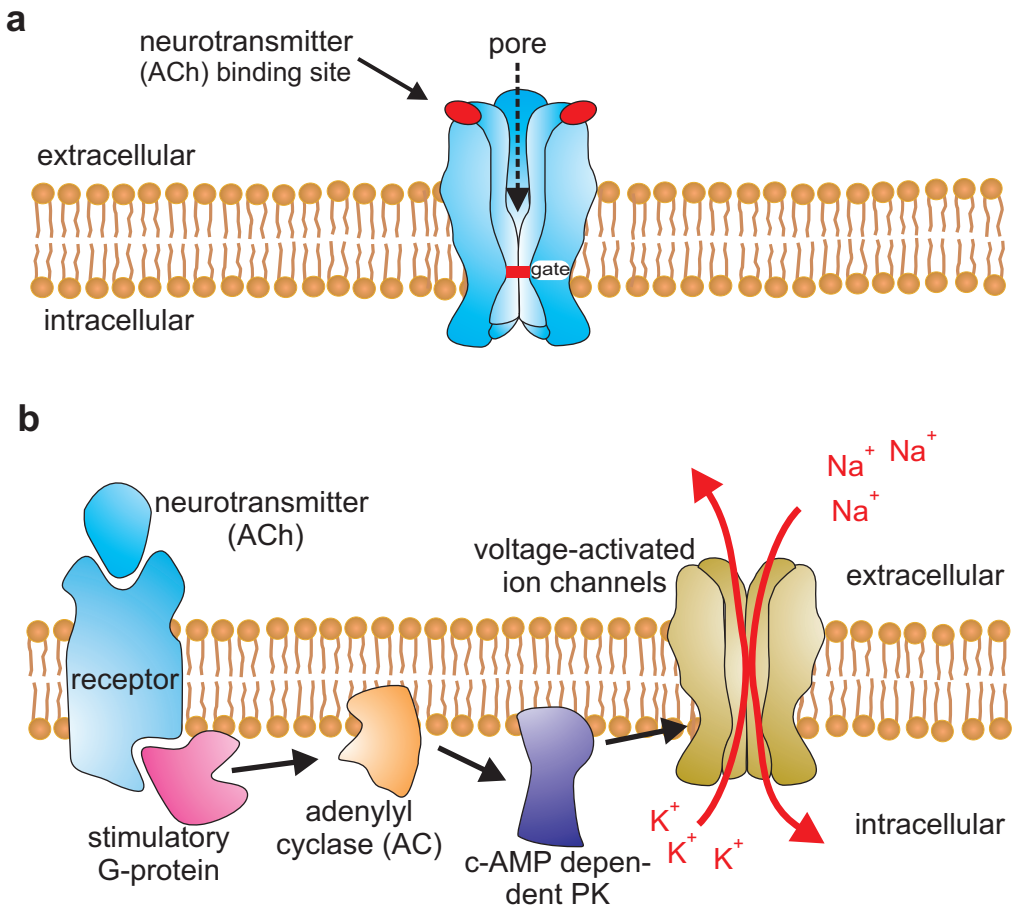

Figure A.2: *Schematic comparison of ionotropic and metabotropic receptor structures.* ***a*** *Ionotropic receptors are pentameric macromolecules composed of five identical subunits (homomeric receptors) or different subunits (heteromeric receptors) arranged around a central pore that is selective for cations (i.e., Na^+, $K^+$and Ca^{2+}).* ***b*** *Metabotropic receptors are G-protein coupled receptors, which contain seven putative transmembrane domains and do not exert their effects through the direct opening of an ion channel. Once the G-protein coupled receptor is stimulated, G proteins will activate enzymes such as adenylyl cyclase (AC) to produce second messenger such as cAMP. Through the activation of cAMP-dependent protein kinase (PK), ion channels become phosphorylated, which affects their gating properties. Adapted from Hille (2001) and Kandel et al. (1991).*

YxCC motif in the ACh-binding extracellular loop C, whereas β subunits lack this feature (Karlin 2002). Each receptor subunit consist of four transmembrane segments (TM1–4) and an extracellular ligand binding domain (loops A–F) at the N-terminus (Karlin 2002). Different subunit combinations result in distinct receptor subtypes with distinctive pharmacological profiles (Gotti et al. 2009; Karlin 2002; Le Nòvere et al. 2002; Tomizawa and Casida 2001; 2003).

In insects, more than 10 nAChR genes have been cloned, and post-translational modification of subunit mRNAs has been demonstrated (Grauso et al. 2002; Lansdell and Millar 2002; Jones et al. 2005; Rinkevich and Scott 2009; Sattelle et al. 2005), suggesting the existence of many nAChR subtypes. While the identification and pharmacological profiling of nAChR subtypes is limited, many studies have reported fast, nAChR-mediated inward currents in various insect neurons (Albert and Lingle 1993; Barbara et al. 2005; 2008; Beadle et al. 1989; Campusano et al. 2007; Cayre et al. 1999; Courjaret and Lapied 2001; Courjaret et al. 2003; David and Pitman 1993; Déglise et al. 2002; Goldberg et al. 1999; Grolleau et al. 1996; Gu and O'Dowd 2006; Hermsen et al. 1998; Jepson et al. 2006; Lapied et al. 1990; Nauen et al. 2001; Salgado and Saar 2004; Su and O'Dowd 2003; Suter and Usherwood 1985; van den Beukel et al. 1998; Van Eyseren et al. 1998; Wüstenberg and Grünewald 2004).

While nAChRs are associated with fast synaptic transmission, it has been shown in vertebrates that a fraction of the current through nAChRs can be carried by Ca^{2+} (Burnashev 1996; Dani 2001; Fucile 2004; Rathouz et al. 1996; Vernino et al. 1994). This is of interest because synaptic activity could generate intracellular signals that act as a second messenger (Berridge et al. 2000; Bootman et al. 2002) and might, for example, activate Ca^{2+}-dependent ion channels that induce sustained changes in membrane potential and/or excitability.

Muscarinic acetylcholine receptors (mAChRs)

In addition to nAChRs, acetylcholine can also stimulate muscarinic acetylcholine receptors (mAChRs). In vertebrates, mAChRs are G-protein coupled receptors which contain seven putative transmembrane domains. mAChRs exist as five subtypes, termed M1 to M5, where M1, M3 and M5 mAChR subtypes are positively coupled to the phosphatidylinositol pathway while M2 and M4 are negatively coupled to adeny-

late cyclase (Caulfield 1993; Caulfield and Birdsall 1998). mAChRs have been shown to activate various second messenger pathways (Caulfield 1993; Caulfield and Birdsall 1998; Felder 1995; Gregory et al. 2007; Lanzafame et al. 2003), which can modulate ion channels or receptors in the membrane, regulating the membrane properties (Hasselmo 2006; Jones 1993; Klink and Alonso 1997; Lanzafame et al. 2003). While many binding (Abdallah et al. 1991; Honda et al. 2007; Liu and Casida 1993; Onai et al. 1989; Orr et al. 1991; Qazi et al. 1996; Shapiro et al. 1989), immunohistochemical (Blake et al. 1993; Cui-ping et al. 2008; Harrison et al. 1995; Heck et al. 2009; Shirai et al. 2001) and systemic electrophysiological (Bai and Sattelle 1994; Buhl et al. 2008; Büschges 1998; Johnston and Levine 2002; Trimmer 1995; Trimmer and Weeks 1989; Westmark et al. 2009) studies on insect neurons suggested the expression of mAChRs, only very few studies have reported direct mAChR-mediated effects on membrane conductance in isolated neurons (Benson 1992; Lapied et al. 1992; Van Eyseren et al. 1998). This might be due to the fact that nicotinic receptors predominate over muscarinic ones in the insect nervous system (Orr et al. 1991; Trimmer 1995).

1.2 Modulation of cholinergic receptors by biogenic amines and neurotransmitters

Throughout the animal kingdom, functional properties of cholinergic currents can be altered by actions of other neurotransmitters and neuromodulators. In insects, modulation of cholinergic responses by actions of octopamine and serotonin have been described (Butt and Pitman 2002, 2005; Casagrand and Ritzmann 1992 a; Hill and Blagburn 2001; Leitch et al. 2003; Oleskevich 1999; Westmark et al. 2009). It has been reported that while octopamine increased the ACh responsiveness of motoneurons, the serotonin actions upon these neurons were responsible for down-regulating their sensitivity to ACh (Casagrand and Ritzmann 1992 b; Hill and Blagburn 2001). In

other insect neurons, however, octopamine and serotonin similarly suppressed the ACh responsiveness (Butt and Pitman 2002; Leitch et al. 1993). This lead more evidence to the assumption that the modulation of cholinergic responses by actions of octopamine and serotonin might vary according to the type of neuron and synapse (i.e., sensory – interneuron, interneuron–interneuron, sensory–motoneuron and interneuron–motoneuron). Regulation of nAChR-mediated responses by activation of mAChRs has been observed in vertebrates (Brown and Galligan 2003; Shen et al. 2009; Verbitsky et al. 2000; Zwart and Vijverberg 1997) as well as in invertebrates (Courjaret et al. 2003; David and Pitman et al. 1996; Liu et al. 2007). Although direct modulations of mAChRs by actions of nAChRs has been less studied, it has been demonstrated that activation of α7 nAChRs increases the intracellular Ca^{2+} concentrations, which can trigger intracellular signalling cascades involved in the regulation of mAChRs.

2 Cholinergic receptors: targets for commercially important insecticides

Insect cholinergic neurotransmission is the major target for current insecticides. Effective control of insect pests and helminth parasites has been achieved by targeting invertebrate cholinergic receptors (Jones and Sattelle 2008; Matsuda et al. 2001, 2005; Tomizawa and Casida 2003). Although insect mAChRs are potential targets for insecticide actions (Benting et al. 2005; Dick et al. 1997; Honda et al. 2007), the importance of nAChRs in the fast excitatory neurotransmission in the insect nervous system underly the nAChR usefulness as selective targets for neurotoxic insecticides (Casida and Quistad 1998; Jeschke 2007; Jeschke and Nauen 2008; Matsuda et al. 2001; Nauen and Bretschneider 2002; Tomizawa and Casida 2003).

Knowledge of nAChR structure and species-insecticide specificity has potential

immediate implications for improving the effectiveness and safety of pest control (Jeschke and Nauen 2008; Matsuda 2009 a, b; Tomizawa and Casida 2001). Nereistoxin analogues (Lee et al. 2003; Delpech et al. 2003), spinosyns (Orr et al. 2009; Salgado 1998; Salgado et al. 1998; Salgado and Saar 2004) and neonicotinoids (see Ihara et al. 2006; Jeschke and Nauen 2008; Matsuda 2009 a, b; Tomizawa and Casida 2001) are insecticide classes known to selectvitely target insect nAChRs, with perhaps the most notable being the neonicotinoid imidacloprid (Jeschke and Nauen 2008; Matsuda 2009 a, b; Millar and Denholm 2007). Due to a high efficacy in the control of invertebrate pests in numerous crops, imidacloprid was the first commercially successful neonicotinoid on the global insecticide market (Mencke and Jeschke 2002; Nauen and Bretschneider 2002).

3 Pharmacological regulations of stick insect locomotory processes

The stick insect is a well established animal system used in studies for slow locomotion. These insects have a simply organized, easily accessible nervous system and show a relatively simple behavioral repertoire. Due to these experimental properties, sensory-motor pathways related to the motor control in the stick insect have been investigated using systemic electrophysiological studies (for reviews: Bässler 1983; Bässler and Büschges 1998; Büschges 2005; Büschges and Gruhn 2008; Ritzmann and Büschges 2007).

Evidences for the participation of γ-aminobutyric acid (GABA) (Sauer et al. 1997; Stein et al. 2006), ACh (Büschges et al. 1995; Büschges 1998; Ludwar et al. 2005; Westmark et al. 2009) and biogenic amines (Büschges et al. 1993; Ramirez et al. 1993; Westmark et al. 2009) in the activation and/or regulation of physiological processes in-

volved in the neuronal control of locomotion in stick insects have been collected. Inhibition of GABAergic pathways enhanced the intrasegmental transmission of sensory information from the femoral chordotonal organ (fCO) to the non-spiking interneurons (NSIs) and motoneurons (Sauer et al. 1997). Stein et al. (2006) demonstrated that the application of picrotoxin (PTX, a well known blocker of GABAergic responses) enables intersegmental transmission of specific fCO signals to inter- and motoneurons of neighboring legs although the animals remain in an inactive state and do not perform active leg movements. Cholinergic pathways are believed to be involved in the activation of the rhythmic activity patterns observed in stick insect middle leg motoneurons when the muscarinic agonist pilocarpine was applied to the thoracic ganglion (Büschges et al. 1995; Büschges 1998). These rhythmic activity patterns are based on a tonic depolarization and alternating phasic excitatory and inhibitory inputs (Büschges 1998; Gabriel 2005; Ludwar et al. 2005; Schmidt et al. 2001). In a recent investigation, Westmark et al. (2009) showed that the tonic depolarization evoked during single leg walking and a cholinergic current in dissociated motoneurons were reversibly abolished by atropine (a well known cholinergic blocker). It is unclear, however, how the findings observed in Westmark et al. (2009) relates to the the findings described in Büschges (1998).

The involvement of the biogenic amines octopamine and serotonin in the neuronal control of locomotory processes in stick insects has been demonstrated in previous studies. In intact and inactive stick insects, injections of octopamine into the hemolymph caused an activation of the animal and suppressed pathways involved in the resistance reflex (Büschges et al. 1993; Ramirez et al. 1993). In a semi-intact single-foreleg preparation, tonic depolarizations recorded in middle leg motoneurons and elicited by activation of foreleg stepping sequences were up-regulated by application of octopamine (Westmark et al. 2009). In contrast to the results obtained to octopamine, serotonin either increased or decreased the tonic depolarizations observed in these motoneurons (Westmark 2007), which can suggest that these biogenic

amines might trigger different responses among neurons or even in the same neurons. The participation of Ca^{2+}- and cAMP-dependent intracellular pathways in the modulation of motoneuron activities has also been described (Westmark et al. 2009), indicating that not only the aminergic actions but all neurotransmitter-mediated synaptic responses might be regulated by alteration of the availability of these intracellular effectors.

4 Objectives of this thesis

In stick insects, evidence for a potential role of ACh (as well other neuroactive substances) in the control of the leg motoneuron activitities has been collected in semi-intact preparations (see preceding text). However, it is unclear whether ACh modifies the motoneuron properties directly or indirectly (*via* presynaptic neurons). To avoid secondary effects caused by interaction with other network participating neurons, the experiments conducted in this dissertation were performed under biophysically controlled conditions on freshly dissociated, unequivocally identified leg motoneurons.

This dissertation was conducted with the following specific goals:

- Pharmacological characterization of cholinergic currents induced in identified leg motoneurons isolated from the stick insect *Carausius morosus.*
- Estimation of the relative calcium contribution for the acetylcholine-induced currents (I_{ACh})
- Characterization of biogenic amine (octopamine and serotonin) and insecticide actions on these cholinergic currents

B. Materials & Methods

1 Experimental Animal and Material

Carausius morosus were obtained from breeding colonies at the University of Cologne. Animals were reared in crowded colonies at a temperature of 20 ± 2 °C under an artificial 12:12 h light/dark photoperiod regimen and 70 ± 5 % relative humidity on a diet of blackberry leaves (*ad libitum*) and water. Only adult females (see Figure B.1) were used in this study.

Figure B.1: *Stick insect C. morosus female on a blackberry leaf.*

C. morosus belong to the phylum: arthropoda, class: insecta, subclass: pterygota, superorder: orthopteroidea, order: phasmida, family: phasmatidae, subfamily: phasmatinae. It was originally domiciled in India and is also known under the trivial name Indian stick insect. They are wingless nocturnal animals. Adults grow up to 8 cm and the reproduction is generally parthenogenetic.

All chemicals, unless stated otherwise, were obtained from Applichem (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany) with a purity grade of p.a. (per analysis).

2 Cell identification

Under cold-induced anesthesia, the animal's legs were amputated distad of the coxa and the animals were mounted ventral side up in a plastic foam platform. The thorax was opened in the regions of the thoracic ganglia with scalpel and forceps. Fat and connective tissue were removed from the nervus cruris manually and the nerve's cut end was placed in a Vaseline trough (PZN2761298, Medical Pharma, Bremerhaven, Germany) filled with tetramethylrhodamine-dextran (0.5-1.0 % in distilled H20, MW 3000, D3308, Invitrogen). To prevent evaporation, the dye solution was covered with Vaseline. For backfilling the somata of the motoneurons the preparation was kept in the dark for 48 h at ~ 4 °C.

The number of labeled neurons was checked with an inverted microscope (Olympus IX71 microscope, Olympus Deutschland, Hamburg, Germany) equipped with fluorescence optics (excitation: BP 530-550 nm; emission: LP 590 nm; beam splitter: DCLP 570 nm).

3 Confocal microscopy

The fluorescence images of the wholemount and sections were captured with an LSM 510 and LSM 510 Meta scanner/detector system (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) mounted on a fixed stage inverse microscope (Zeiss Axiovert 100M with PlanApochromat 20x/0.75 NA objective). Tetramethylrhodamine-dextran was excited with a He-Ne laser at 543 nm excitation, and emission was collected through a 560 nm LP filter. Scaling, contrast and z-projections were performed using ImageJ v1.35d with the WCIF plugin bundle (www.uhnresearch.ca/facilities/wcif/). The final figure was prepared with Photoshop and Illustrator CS2 (Adobe Systems Incorporated, San Jose, CA).

4 Cell culture

4.1 Thoracic ganglion dissection

After backfilling, the thoracic ganglia were dissociated at room temperature (RT) and neurons were cultured according to modified protocols that were described previously (Alix et al. 2002; Salgado and Saar 2004; Husch et al. 2008; Zhao et al. 2003). Three thoracic ganglia with labeled motoneurons were removed and immersed in cold, sterile 'normal' extracellular saline (modified from Schmidt et al. 2001 and Husch et al. 2008), containing in (mM): 180 NaCl; 4 KCl; 5 $CaCl_2$; 1 $MgCl_2$; 10 HEPES and 48 sucrose, adjusted to pH 7.2 (with NaOH) and to 430 mOsm (with sucrose). First, the outer sheath was manually removed. Before manual removal of the inner sheath the ganglia were enzyme treated (10 min, RT) with a mixture of collagenase (253 units • ml^{-1}, C0130, Sigma-Aldrich) and trypsin (8550 units • ml^{-1}, T8003, Sigma-Aldrich) in Hanks's Ca^{2+}- and Mg^{2+}-free buffered salt solution (GIBCO 14170-070), containing (in mM): 10 HEPES and 135, adjusted to pH 7.2 (with NaOH). The ganglia were transferred to normal extracellular solution and the underlying perineurium was manually removed. To facilitate dissociation of the cell bodies, the ganglia were enzyme treated for a second time (20 min, RT). To abort the enzyme treatment, the ganglia were rinsed at least five times with normal extracellular saline (RT).

4.2 Dissociation of identified motoneurons

The ganglia were dissociated by gentle trituration through a series of pipettes of decreasing tip diameter. The neurons of one ganglion were plated in 1-2 culture dishes. Dishes with glass bottoms were custom-made according to modified protocols previously described (Hayashi and Hildebrand 1990; Hayashi and Levine 1992). A 20 mm diameter hole was drilled in the bottom of a 35 mm culture dish (627160, Greiner Bio-

one Inc., Frikenhausen, Germany). A glass coverslip (BB022022A1, Thermo Fisher Scientific Inc, Portsmouth, New Hampshire, USA) was sealed with Sylgard (Dow Corning Corp., Midland, Michigan, USA) to the bottom of the culture dish. The dishes were sterilized with UV radiation for 2 h and the glass bottom was coated with concanavalin A (C-2010, Sigma, 0.7 mg • ml^{-1} dissolved in distilled H_2O). Neurons were allowed to settle for at least 1 h to adhere to the glass surface and used for electrophysiological experiments within 8 h.

For recordings, the cells were visualized with an inverted microscope (IX71, Olympus, Hamburg, Germany) using a 40x water objective (UAPO 40x, 1.15 NA, 0.25 mm WD, Olympus) with phase contrast and fluorescence optics. Motoneurons could be unequivocally identified by their fluorescence and diameter.

5 Electrophysiological recordings

5.1 Whole-cell patch-clamp recordings

Whole-cell recordings were performed at 24 °C following the methods described by Hamill et al. (1981). Electrodes with tip resistances between 3-5 MΩ were fashioned from borosilicate glass (0.86 mm inner diameter [ID], 1.5 mm outer diamenter [OD], GB150-8P, Science Products, Hofheim, Germany) with a temperature-controlled pipette puller (PIP5, HEKA-Elektronik, Lambrecht, Germany), and filled with a solution containing (in mM): 190 K-aspartate, 10 NaCl, 1$CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 EGTA, 3 ATP and 3 GTP adjusted to pH 7.2 (with KOH), resulting in an osmolarity of ~ 415 mOsm. During the experiments, if not stated otherwise, the cells were superfused constantly with normal extracellular saline solution.

5.2 Data aquisition

Whole-cell voltage-clamp recordings were made with an EPC9 patch-clamp amplifier (HEKA-Elektronik) that was controlled by the program Pulse (version 8.63, HEKA-Elektronik) running under Windows. The electrophysiological data were sampled at intervals of 100 µs (10 kHz). The recordings were low pass filtered at 2 kHz with a 4-pole Bessel-Filter. Compensation of the offset potential and capacitance were performed using the 'automatic mode' of the EPC9 amplifier. The liquid junction potential between intracellular and extracellular solution of 15.8 mV (calculated with Patcher's-Power-Tools plug-in from http://www.mpibpc.gwdg.de/abteilung/140/-software/index.html for Igor Pro 6 Wavemetrics, Portland, Oregon, USA]) was also compensated. Voltage errors due to series resistance (RS) were minimized using the RS-compensation of the EPC9. RS was compensated between 30% and 70% with a time constant (τ) of 2 µs. If not stated otherwise the cells were held at -60 mV, which closely resembles their normal resting potential (Schmidt et al. 2001).

6 Ligand application

The neurons were continuously superfused with normal extracellular saline (0.5 ml min^{-1}) through a teflon tube (483 µm ID) placed 600 µm from the cell. Ligands were applied with a U-tube system (Figure B.2) as described previously (Westmark et al. 2009; Wu et al. 2004; Zhao et al. 2003). The U-tube consisted of a teflon tube (254 µm ID, 508 µm OD). Two synchronized computer-controlled pinch valves activated the U-tube and simultaneously stopped the superfusion with the normal extracellular solution. The U-tube hole (50 µm diameter) was positioned near the cell (~ 500 µm), with its flow direction approximately perpendicular to the flow direction of the bath (Figure B.2 *b*). If not stated otherwise the ligands were applied for 1 s.

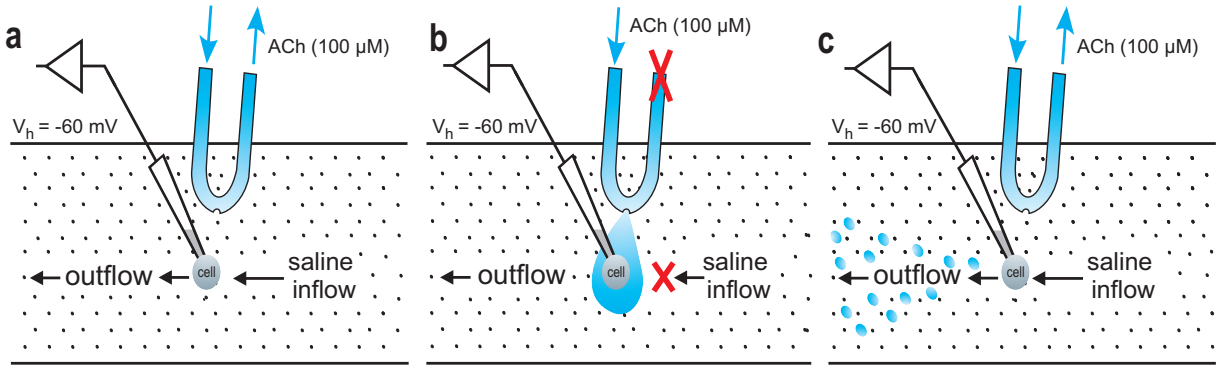

Figure B.2: *U-tube application system. Sketches indicate the situation before (**a**), during (**b**) and after (**c**) the ligand application. Adapted from Westmark et al. 2009.*

7 Ligands

Except α-bungarotoxin (α-BGTX, 630-075-M001, Alexis Biochemicals, Lausen, Switzerland) and the novel neonicotinoids (see RESULTS, section 5), all ligands were purchased from Sigma-Aldrich (Taufkirchen, Germany): Acetylcholine (ACh, A2661), α-BTX, atropine (ATRO, A0257), 8-bromoadenosine 3,5-cyclic monophosphate sodium salt (8-Br-cAMP, B7880), imidacloprid (IMI, 37894), (±)-muscarine (MUSC, M104), (-)-nicotine (NIC, N3876), (±)-octopamine (OA, O0250), oxotremorine (OXO, O100), pilocarpine (PILO, P6503) and 5-hydroxytryptamine hydrochloride (5-HT, serotonin, H9523). IMI and the novel neonicotinoids were dissolved in dimethyl sulfoxide (DMSO, D8418) with a maximum final DMSO concentration of 0.1% (v/v). All other ligands were dissolved in normal extracellular saline.

8 Concentration-response relation

To determine concentration-response relations, each concentration was applied at least three times. Ligand applications arrived at least 2 min apart.

If appropriate, concentration-response relations for each cell were fit with the Hill

equation:

$$\frac{I}{I_{max}} = \frac{[ligand]^{n_H}}{K^{n_H} + [ligand]^{n_H}} \tag{B.1}$$

for agonists, or

$$\frac{I}{I_{max}} = 1 - \frac{[ligand]^{n_H}}{K^{n_H} + [ligand]^{n_H}} \tag{B.2}$$

for antagonists, where $I_{\max}$ is the maximum current amplitude, K the EC_{50} (concentration that activates the half-maximal current) or IC_{50} (concentration that blocks half of the maximal current) and n_H the Hill-coefficient. Data were scaled as a fraction of the calculated maximal current and fit again.

9 Fluorimetric Ca^{2+} measurements

The Ca^{2+} imaging experiments were performed in cooperation with and under the guidance of Andreas Pippow.

Intracellular Ca^{2+} concentrations were measured with fura-2, a ratiometric Ca^{2+} indicator suitable for determination of absolute intracellular Ca^{2+} concentration once calibrated (Grynkiewicz et al. 1985; Poenie 1990). The imaging setup for fluorimetric measurements consisted of an Imago/SensiCam CCD camera with a 640x480 chip (Till Photonics, Gräfelfing, Germany) and a Polychromator IV (Till Photonics) that was coupled via an optical fiber into an inverted microscope (IX71, Olympus, Hamburg, Germany) equipped with a 40x water objective (objective: UAPO 40x, 1.15 numerical aperture, 0.25 mm working distance, Olympus). The camera and the polychromator were controlled by the software Vision (version 4.0, Till Photonics) run on a Windows PC. The neurons were loaded with fura-2 via the patch pipette (0.2 mM in the pipette) and illuminated during data collection with 340 nm and 380 nm

light from the polychromator that was reflected onto the cells with a 410 nm dichroic mirror (DCLP410, Chroma, Gräfeling, Germany). Emitted fluorescence was detected through a 440 nm long-pass filter (LP440). Data were acquired as 80x60 frames using 4x4 on-chip binning for fast kinetic measurements. Images were recorded in analog-to-digital units (ADUs) and stored and analyzed as 12 bit grayscale images. For all calculations of kinetics, the mean value of ADU within regions of interest (ROIs) from the center of the cell bodies were used. ROIs were adjusted to each cell.

9.1 Calibration

The free intracellular Ca^{2+}concentrations were determined as in Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_{d,Fura} \frac{f_{380,min}}{f_{380,max}} \frac{(R - R_{min})}{(R_{max} - R)} \tag{B.3}$$

$[Ca^{2+}]_i$ is the free intracellular Ca^{2+} concentration for the background subtracted fluorescence ratio R from 340 nm and 380 nm excitation. $R_{\min}$ and $R_{\max}$ are the ratios at a Ca^{2+} concentration of virtually 0 M (i.e. ideally no fura-2 molecules are bound to Ca^{2+}) and at saturating Ca^{2+} concentrations (i.e. ideally all fura-2 molecules are saturated with Ca^{2+}), respectively. $K_{\text{d, Fura}}$ is the dissociation constant of fura-2. $f_{380,\min}/f_{380,\max}$ the ratio between the emitted fluorescence of Ca^{2+} free dye and the emitted fluorescence of Ca^{2+} saturated dye at 380 nm excitation, reflecting the dynamic range of the indicator.

The term $K_{d,Fura}$ $(f_{380,\min}/f_{380,\max})$ in Eq. B.3 is dependent on the dye concentration and is substituted with the effective dissociation constant $K_{d,Furaeff}$, which is independent of the dye concentration and specific for each experimental setup (Neher 1989):

$$K_{d,Fura,eff} = [Ca^{2+}]_i \frac{(R_{max} - R)}{(R - R_{min})} \tag{B.4}$$

We used in vitro calibration (in solution). For calibration $K_{d,Fura,eff}$ was determined by measuring fura-2 fluorescence ratios for$R_{\max}$, $R_{\min}$ and $R = R_{def}$. R_{def} is the ratio for a defined $[Ca^{2+}]_i$, which was set to 0.22 µM (see below for the preparation of the solutions). $K_{d,Fura,eff}$ was then calculated from Eq. B.4. To account for environmental differences between the cytoplasmic milieu and *in vitro* conditions, we used a correction factor (P) for $R_{\max}$ and $R_{\min}$, as as suggested by Poenie (1990; see also Pippow et al. 2009):

$$K_{d,Fura,eff} = [Ca^{2+}]_i \frac{(R_{max}P - R)}{(R - R_{min}P)} \tag{B.5}$$

P was determined as described by (Poenie 1990). First the fluorescence (peak) deflection at 340 nm was divided by that at 380 nm excitation from voltage-induced intracellular calcium transients ($R_{d,cell}$). Second, the ratio ($R_{d,vitro}$) from pairs of calibration solutions ($R_{\max}$, $R_{\min}$) was determined by dividing ($f_{340,\max}$- $f_{340,\min}$)/($f_{380,\min}$- $f_{380,\max}$). The correction factor P is the fraction of $R_{d,cell}/R_{d,vitro}$.

The fluorescence ratio R of an intracellular transient can then be converted to $[Ca^{2+}]_i$ using:

$$[Ca^{2+}]_i = K_{d,Fura,eff} \frac{(R - R_{min}P)}{(R_{max}P - R)} \tag{B.6}$$

9.2 Calibration solutions

The free Ca^{2+} concentrations of the calibration solutions were adjusted by using appropriate proportions of Ca^{2+} and the Ca^{2+} chelator EGTA. The ability of EGTA to bind calcium is highly dependent on the environmental conditions such as temperature, ionic strength, pH and the concentrations of other metals that compete for binding (Harrison and Bers 1989, 1987). In theory, the necessary amount of Ca^{2+} and EGTA to set the free Ca^{2+} concentration for the experimental conditions can be computed (Patton et al. 2004). However, small variations in the parameters such as pH,

temperature, impurities of chemicals, pipetting or weighing errors can lead to considerable errors in the estimate of the free Ca^{2+} in EGTA-buffered Ca^{2+} solutions with computer programs (McGuigan et al. 2007). To account for such variations, the free Ca^{2+} concentrations were determined in the calibration solutions by using a Ca^{2+} selective electrode following the guide from (McGuigan et al. 1991), as described in the supplemental methods (appendix 1).

9.3 Analysis of relative Ca^{2+} influx

After establishing the whole-cell configuration, neurons were voltage-clamped at their resting potentials (~ -60 mV) and dye was loaded into cells for at least 10 minutes. Imaging was not started until the fluorescence reached a constant level. After loading, the cells were first superfused with normal extracellular saline and stimulated with 10^{-4}M ACh (1s). Then the cells were superfused with a solution containing specific ion channel blockers (10^{-7} M TTX, 4 x 10^{-3} M 4-AP and 20 x 10^{-3} M TEA) to isolate voltage activated calcium currents (I_{Ca}). I_{Ca} was induced by stepping the membrane potential for 1s to between -30 and -20 mV. The voltage steps were adjusted that the resulting I_{Ca} was in the same range of I_{ACh}. To monitor the induced increases of intracellular Ca^{2+} concentrations ratiometrically, pairs of images at 340 nm (4 ms exposure time) and 380 nm (2 ms exposure time) excitation were acquired at 10 Hz for 20 s. Before the whole-cell configuration was established (break in), an image was obtained in on-cell mode for each excitation wavelength. This 'background fluorescence' was subtracted from each image of the time series.

9.4 Estimating Ca^{2+} fluxes

The net charge influx through ACh activated channels was given by $\int dI_{ACh}/d_t = Q_{ACh}$ and the charge influx through voltage-activated Ca^{2+} channels during a voltage pulse was given by $\int dI_{Ca}/d_t = Q_{Ca}$, respectively. The quantities Q_{ACh} and Q_{Ca} were determined by integrating the current traces. From the amplitudes of the Ca^{2+} transients ($\Delta[Ca^{2+}]_i$) and the net charge influxes (Q), the fraction f, which has been defined by (Neher 1995), was determined.

$$f_{ACh} \equiv \frac{\Delta\left[Ca^{2+}\right]_{i,ACh}}{Q_{ACh}} \tag{B.7}$$

and

$$f_{Ca} \equiv \frac{\Delta\left[Ca^{2+}\right]_{i,Ca}}{Q_{Ca}} \tag{B.8}$$

When the Ca^{2+} binding ratio of fura 2 (exogeneous buffer) outcompetes the endogenous buffers, the fraction of Ca^{2+} contributing to the ACh induced ion flux can be estimated with:

$$P_f \equiv \frac{f_{ACh}}{f_{Ca}} \tag{B.9}$$

The fractions f_{Ca} and f_{ACh} are the slopes from linear fits (Y = β0 + β1 x using the 'R function' lm (R Development Core Team [2007]) of their $\Delta[Ca^{2+}]_i$ / Q relations (Eqs. B.7 and B.8). To estimate the variance of the slope (fraction f) we used the bootstrap method (Efron 1979) implemented in the boot library in R (fixed-x resampling, 1000 bootstrap samples, boot: Bootstrap R Functions, R package version 1.2-27). This resulted in bootstrap distributions (1000) for f_{Ca} and f_{ACh}. The distributions were log-transformed to make them closer to a Gaussian. To determine differences in means between the different cell types, ANOVA was performed; post hoc pairwise compar-

isons were performed using t- tests with the Holm method for p- value adjustment. A significance level of 0.05 was accepted for all tests. The ratio f_{Ca}/f_{ACh} (P_f) provided an estimate of the proportion of Ca^{2+} contributing to an ACh induced current (Vernino et al. 1994; Zhou and Neher 1993). To ensure that the exogenous Ca^{2+} buffer outcompetes the endogenous Ca^{2+} buffer usually high concentrations of Ca^{2+} indicator are used (Fucile et al. 2000; 2006; Ohyama et al. 2000; Tempia et al. 1996; Vernino et al. 1994; Zhou and Neher 1993). However, high indicator (exogenous Ca^{2+} buffer) concentrations reduce the amplitude of the free Ca^{2+} concentration. For small Ca^{2+} influxes the signal can be reduced to the range of the signal noise. In this study we used 0.2 mM fura-2 and I_{ACh} (10^{-4} M) induced a clearly detectable free Ca^{2+} signal. The linear relationship between the amplitudes of the Ca^{2+} transients ($\Delta[Ca^{2+}]_i$) and the charge influxes (Q) confirmed that fura-2 was the main buffer (Vernino et al. 1994).

10 Data analysis

Electrophysiological and imaging data were analyzed with the software Pulse (version 8.63; HEKA), Igor Pro 6 (Wavemetrics; including the Patcher's Power Tools plug-in, http://www.mpibpc.gwdg.de/abteilungen/140/software/index.html) and Sigma Stat (Systat Software, INC., San Diego, USA) for analysis of electrophysiological data. If not stated otherwise, all calculated values are expressed as mean ± standard deviation. All calculations for the determination of EGTA purity, its dissociation constant and the free Ca^{2+} concentrations in the calibration solutions were performed in R (R Development Core Team [2008], http://www.R-project.org). To determine differences in means unpaired t-tests were performed. A significance level of 0.05 was accepted for all tests.

C. Results

This thesis was carried out attempting to describe and characterize the biophysical and physiological properties of the acetylcholine-induced currents (I_{ACh}) in identified leg motoneurons of the stick insect, *Carausius morosus*. In order to assure an unequivocal identification of these motoneuron cell bodies in cell culture, *in vivo* retrograde tracing (*via* main leg nerve, *nervus cruris*) and ganglion dissociation procedures were established. An accurate description of the acetylcholine-induced current properties was achieved by means of whole-cell voltage-clamp recordings in freshly dissociated motoneuron cell bodies.

The results are structured in several sections. After a short description of the recorded neurons and of their responsiveness to acetylcholine, the calcium contribution to ACh-induced currents (I_{ACh}), pharmacological properties of I_{ACh} and modulatory action of biogenic amines on the I_{ACh} will be described. In the last section, the desensitizing effects of imidacloprid (a well established neonicotinoid insecticide, well known to target insect acetylcholine-activated receptors) and recently discovered neonicotinoids on the acetylcholine-induced currents are described.

1 Description of acetylcholine-induced current (I_{ACh})

1.1 Identification of leg motoneurons

An important prerequisite for this study was an unequivocal identification of the motoneurons, which was achieved by retrograde tracing with tetramethylrhodamine-dextran *via* the main leg nerve (*nervus cruris*). In the thoracic ganglion, retrograde tracing *via* the *nervus cruris* for 48 h labeled up to 25 flexor tibiae motoneurons (Goldammer 2008; Storrer et al. 1986), 8 motoneurons of the tarsal muscle and 4 motoneurons of the retractor unguis muscle (Godden 1972) (Figure C.1, *a* and *b*).

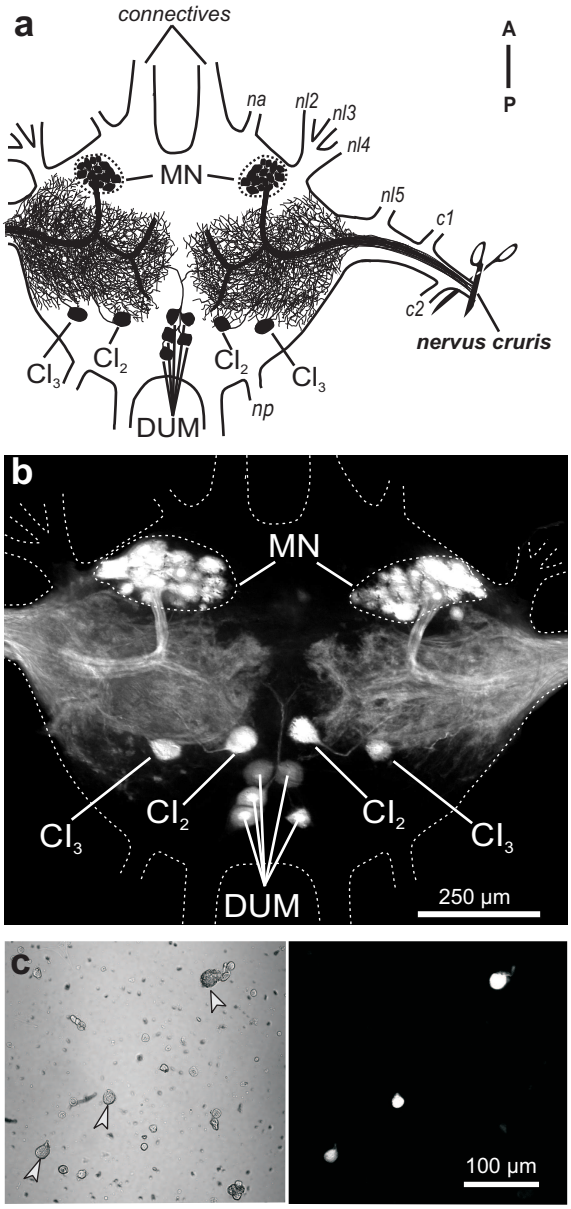

Figure C.1: *Leg motoneurons of C. morosus.* ***a*** *Scheme of a mesothoracic ganglion with the neurons that send their axons along the main leg nerve (nervus cruris), including motoneurons (MN), common inhibitors (CI), dorsal median neurons (DUM) and sensory afferences. The motoneuron cell bodies were located in the anterior-lateral region (most of them are ventrally located) of the thoracic ganglion, while the common inhibitors were situated in the middle of the posterior region of each thoracic hemiganglion. The DUM neuron cell bodies formed a cluster in the posterior crotch of the thoracic ganglia, where the posterior connectives originated. Nerves are labeled according to the nomenclature of Marquardt (1940); Bässler (1977) and Graham (1985). na: nervus anterior; nl2-5: nervi laterali 2-5; c1-2: coxa branches 1 and 2; np: nervus posterior.* ***b*** *Whole-mount preparation of a mesothoracic ganglion showing neurons as revealed by backfilling the nervus cruris with tetramethylrhodamine-dextran and viewed from the ventral aspect with a confocal microscope.* ***c*** *After dissociation, motoneuron cell bodies (arrowhead) were unequivocally identified by their fluorescence and size.*

All of these somata were located in the anterior-lateral region (most of them are ventrally located) of the thoracic-ganglion and had their dendritic fields in the dorsal-intermediary part of the thoracic ganglion. The soma size of these motoneurons is 40 μm or less. In addition, the staining revealed up to 2 common inhibitor motoneurons (CI) and up to 6 dorsal unpaired median (DUM) neurons (Goldammer 2008; Mentel et al. 2008) (Figure C.1, *a* and *b*). Both neuron types had soma diameters of 50 μm or more (Debrodt and Bässler 1989; Goldammer 2008; Storrer et al. 1986). This project is based on 208 whole-cell patch-clamp recordings from acutely dissociated neurons of the thoracic ganglion that were identified as leg motoneurons in culture by their fluorescence and soma size (Figure C.1 *c*).

1.2 Characterization of I_{ACh}

To characterize I_{ACh} here, the neurons were superfused with normal extracellular saline and clamped at -60 mV. The resting membrane potential measured immediately after establishing the whole-cell configuration was -63.3 ± 2.49 mV (n = 208). ACh was applied for 1 s *via* a U-tube system (see Material and Methods). Over 90 % (197 / 208) of the investigated neurons generated an inward current upon application of a 1 s ACh puff (10^{-7}M - 10^{-3}M, Figure C.2 *a*).

In agreement with previous work (Heidel and Pflüger 2006; Kloppenburg and Hörner 1998; Kloppenburg et al. 1999; Westmark et al. 2009) the labeling procedure used in this study did not cause obvious changes in the electrophysiological membrane properties (Figure C.3). Whole-cell patch-clamp recordings were performed in 72 freshly dissociated neurons that were not labeled by tetramethylrhodamine-dextran. In these cells, ACh-induced current properties (Figure C.3, *a* and *b*) and resting potentials (Figure. C.3 *c*) were similar to those obtained in dissociated labeled neurons.

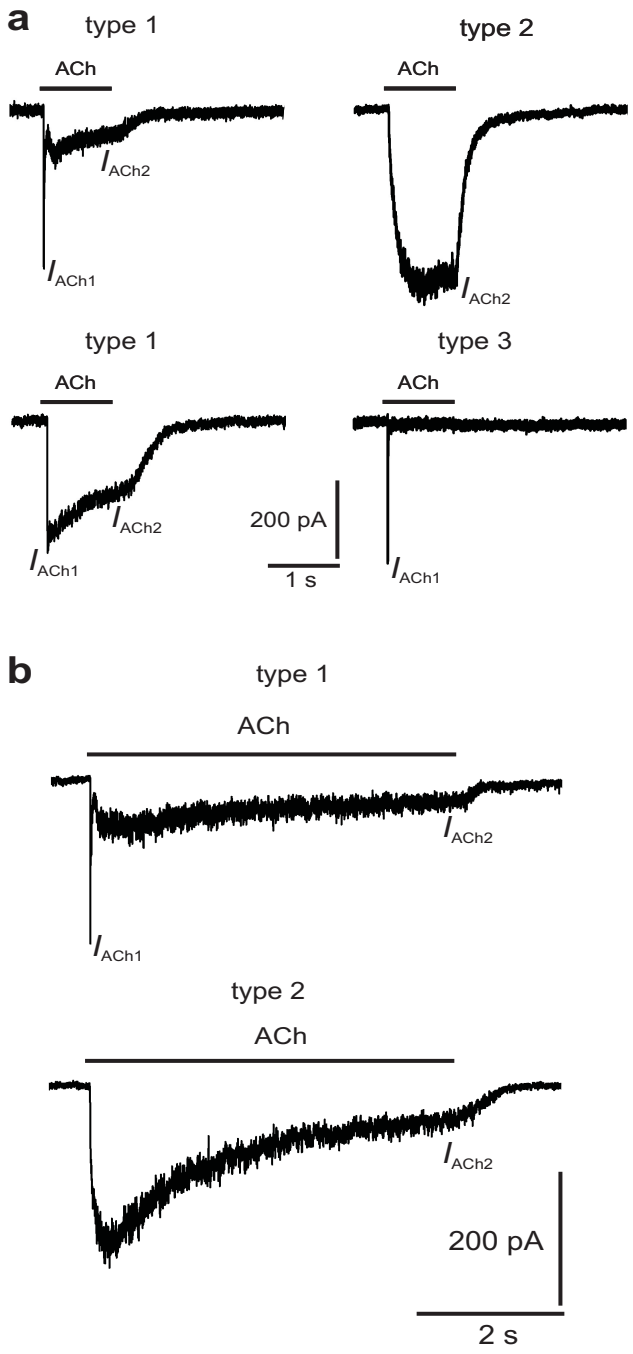

Figure C.2: *Acetylcholine-induced current (I_{ACh}).* ***a*** *ACh (10^{-4} M) elicited a fast-desensitizing (I_{ACh1}) and/or slow-desensitizing current component (I_{ACh2}). Three principle response types were observed: Type 1: I_{ACh1} and I_{ACh2}. The ratio between both components was variable. Type 2: I_{ACh2} only. Type 3: I_{ACh1} only. Type 1 was the most frequent response (173 / 197) cells). For the data analysis the peak of the transient component (I_{ACh1}) and the mean amplitude between 950 ms and 1000 ms (I_{ACh2}) was determined.* ***b*** *I_{ACh1} and I_{ACh2} desensitized differentially under prolonged ACh application. Note the differences in ACh application time between* ***a*** *and* ***b****.*

The membrane resting potentials were in the range of those obtained in recordings from leg motoneurons in the intact ganglion of the stick insect (compare with Schmidt et al. 2001).

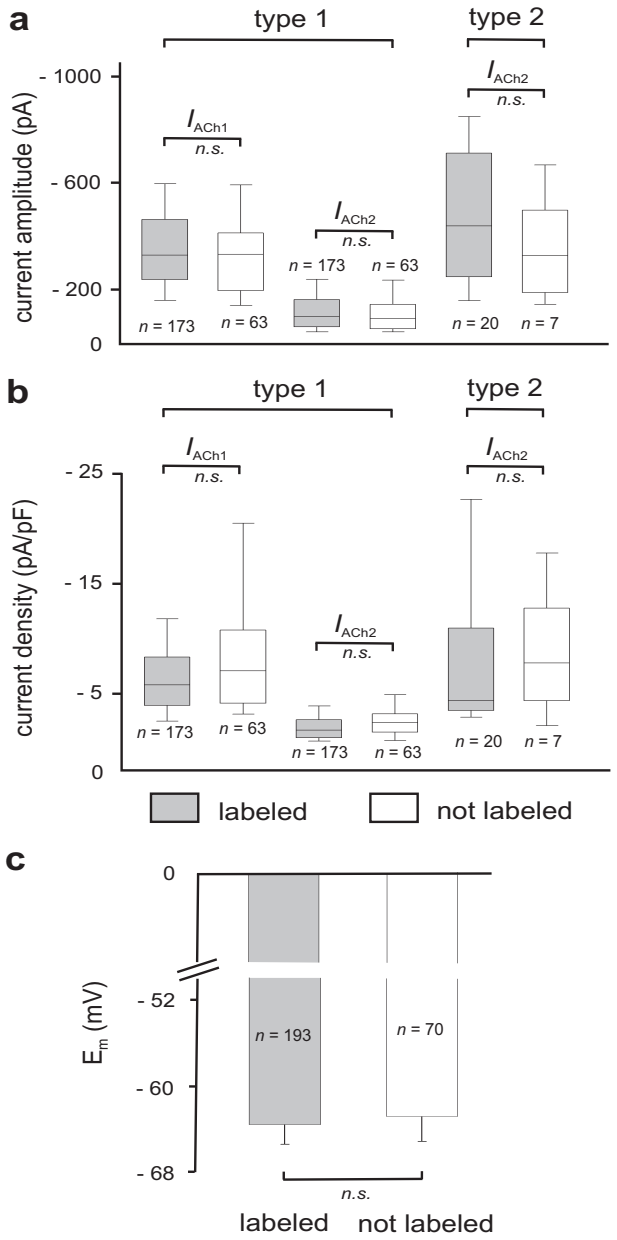

Figure C.3: *Comparisons among electrophysiological membrane properties of labeled and not labeled motoneurons. Labeled and not labeled motoneurons showing the same ACh-response type (type 1 or type 2) had similar distribution of current amplitudes (**a**), current densities (**b**) and resting potentials (**c**). **a-b** The horizontal lines represent median values. The rectangular boxes indicate the 25% and 75% interquartiles; the error bars indicate the 5% and 95% ranges.*

1.3 Subtypes of I_{ACh}

ACh induced a fast-desensitizing and a slow-desensitizing component (I_{ACh1} and I_{ACh2} respectively; Figure C.2, *a* and *b*). In 88 % (173 / 197) of the ACh responsive neurons both components were apparent (type 1; Figure C.2 *a*). A comparison of the current profiles, however, revealed that the ratio between the components was highly variable. In 10 % (20 / 197) of the neurons I_{ACh} consisted of I_{ACh2} only (type 2; Figure C.2 *a*). Neurons showing only I_{ACh1} were observed in 2 % (4 of 197) of the recorded neurons (type 3; Figure C.2 *a*). Since type 2 and type 3 responses were rare, only type 1 responses were analyzed in detail during this study.

Both I_{ACh1} and I_{ACh2} were clearly concentration-dependent. The concentration-current relation of I_{ACh} was determined with increasing concentration steps between 10^{-7} M and 10^{-3} M (Figure C.4). The concentration-current relations of I_{ACh1} (peak current) and I_{ACh2} (current amplitude averaged between 950 ms and 1000 ms of ACh application) were fit with the Hill relation (Eq. B.1; Figure C.4). Both components started to activate at ACh concentrations above 3 x 10^{-6} M and their concentration-current curves had similar EC_{50} and Hill coefficients (I_{ACh1}: EC_{50} = 3.7 x 10^{-5} ± 1.9 x 10^{-6} M, n_H = 1.20 ± 0.09, n = 14; I_{ACh2}: EC_{50} = 2.0 x 10^{-5} ± 1.5 x 10^{-6} M; n_H = 1.10 ± 0.08, n = 14; P = 0.09). The maximum amplitude of I_{ACh1} determined from Hill fits was 6.17 x 10^{-1} ± 2.82 x 10^{-1} nA (n = 14). Given a mean whole-cell capacitance (C_M) of 89.4 ± 49.7 pF (n = 14), this corresponded to a current density ($I_{ACh1} \bullet C_M^{-1}$, $I_{ACh1} \bullet A_M^{-1}$) of 8.6 ± 5.7 pA • pF^{-1} (8.6 x 10^{-2} ± 5.7 x 10^{-2} pA • μm^{-2}). For I_{ACh2} the Hill fits yielded a maximal amplitude of 3.51 x 10^{-1}± 2.87 x 10^{-1} nA (n = 14), which corresponded to a current density ($I_{ACh2} \bullet C_M^{-1}$, $I_{ACh2} \bullet A_M^{-1}$) of 4.5 ± 3.9 pA • pF^{-1} (4.5 x 10^{-2} ± 3.9 x 10^{-2} pA • μm^{-2}). The absolute amplitude and the ratio between I_{ACh1} and I_{ACh2} were variable between different motoneurons. I_{ACh} was reproducible during repeated ACh applications in a given neuron for more that 2 h (Figure C.5).

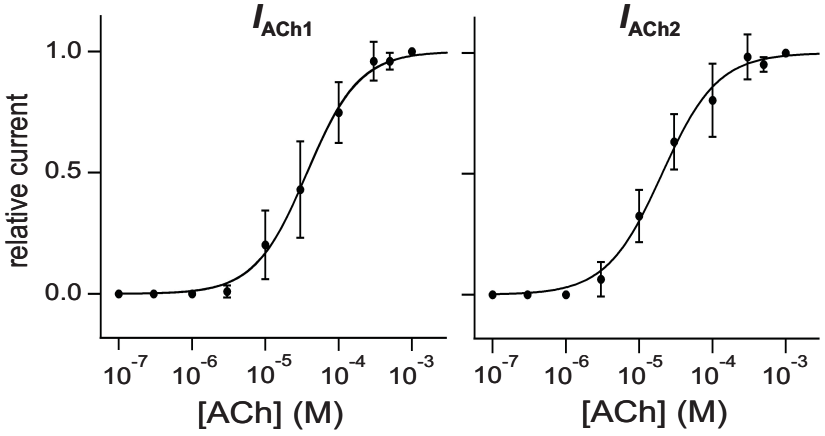

Figure C.4: *Concentration-response curves for I_{ACh1} and I_{ACh2}. Data were calculated as fractions of the calculated maximal current. The curves are fits to a Hill relation (Eq. B.1) with the following parameters: I_{ACh1}: EC_{50}= 36.7 ± 1.9 µM; n_H = 1.20 ± 0.09; n = 14 . I_{ACh2}: EC_{50} = 20.5 ± 1.5 µM; n_H = 1.10 ± 0.08; n = 14.*

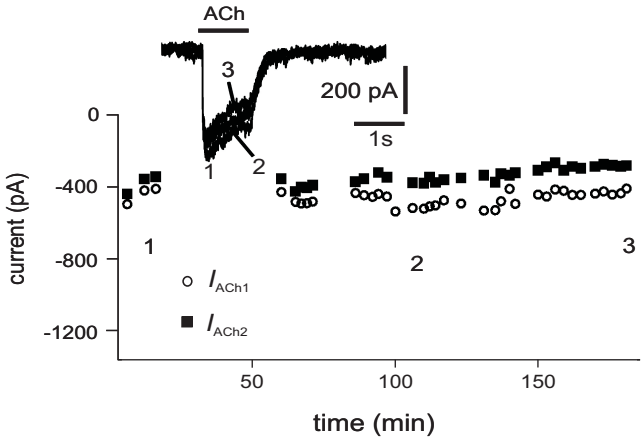

Figure C.5: *During repeated ACh applications, I_{ACh} was stable for more than 2 h. ACh applications arrived 1 - 2 min apart. The inset shows three traces of I_{ACh} that were recorded at the indicated times. Open circles represent I_{ACh1}, and filled squares represent I_{ACh2}*

2 Contribution of calcium ions to I_{ACh}

The Ca^{2+} imaging experiments were performed in cooperation with and under the guidance of Andreas Pippow.

The fura-2 loaded (0.2 mM) neurons were voltage-clamped at their resting potential (~ -60 mV) in normal extracellular saline (Figure C.6). First, I_{ACh} was induced by 1 s ACh puffs (10^{-4} M). I_{ACh} had a net charge influx of Q_{ACh} = 127.3 ± 95.0 pC (n = 8)

while the free intracellular Ca^{2+} concentration increased by $\Delta[Ca^{2+}]_i$ = 2.8 ± 1.3 nM. Second, the voltage-activated Ca^{2+} current (I_{Ca}) was isolated pharmacologically (10^{-7} M TTX, 4 x 10^{-3} M 4-AP and 20 x 10^{-3} M TEA) and the amplitude of a 1 s depolarizing voltage pulse was adjusted to set the magnitude of I_{Ca} to the range of I_{ACh} .

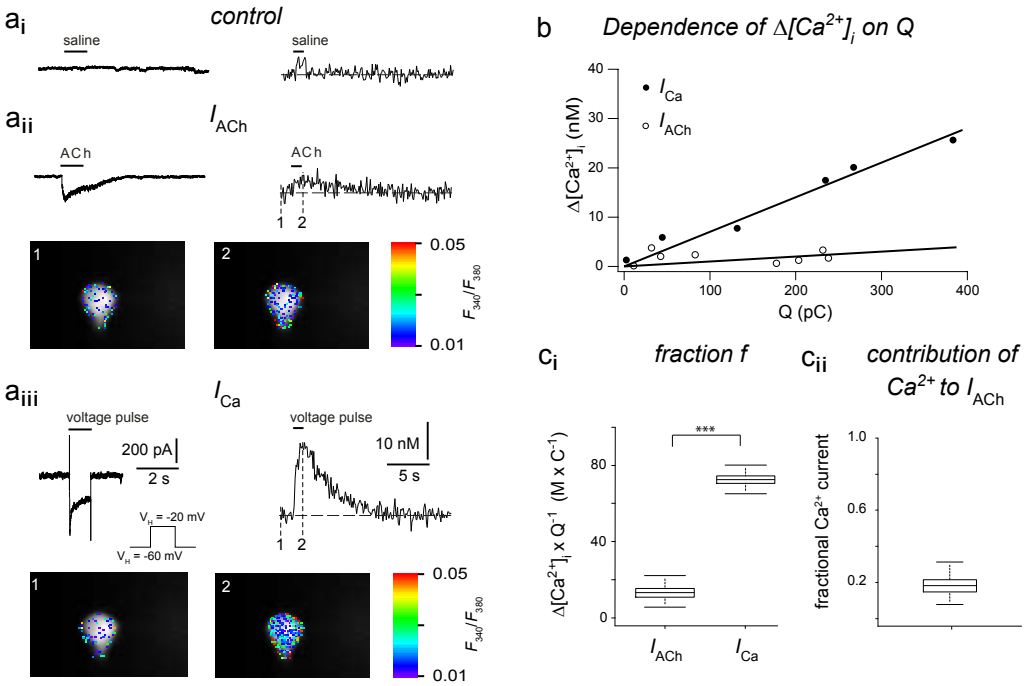

Figure C.6: *Calcium influx through AChRs.* ***a*** *Voltage-clamp (left) combined with optical recordings of the intracellular Ca^{2+} concentration (right) in normal extracellular saline (**a**$_i$, **a**$_{ii}$) and in a solution to isolate I_{Ca} (10^{-7} M TTX, 4 x 10^{-3} M 4-AP and 20 x 10^{-3} M TEA). The images show superimposed tetramethylrhodamine-dextran and fura-2 fluorescence before (1) and during (2) stimulation.* **a**$_i$*: U-tube application of normal extracellular saline did not induce any current.* **a**$_{ii}$*: I_{ACh} was induced by 10^{-4} M acetylcholine.* **a**$_{iii}$*: I_{Ca} was induced by a voltage pulse from -60 mV to -20 mV.* ***b*** *Linear relation between the net charge influx (Q) and the increase in free intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) for I_{Ca} (●) and I_{ACh} (○). I_{Ca} is carried by Ca^{2+}. As indicated by the differences in slope, I_{ACh} is only carried in part by Ca^{2+} (slopes: I_{Ca}: 72.4 ± 2.8 M x C^{-1} and I_{ACh}: 13.3 ± 3.3 M x C^{-1}).* ***c*** *Contribution of Ca^{2+} to I_{ACh}.* **c**$_i$*: Fraction f ($\Delta[Ca^{2+}]_i$ x Q^{-1}) for I_{Ca} and I_{ACh} as determined from the slopes of the linear fit in* ***b***. *To estimate the variance of the slopes (fraction f) the bootstrap method (1000 bootstrap samples) was used. *** $P < 0.001$, ANOVA with post hoc pairwise t-test.* **c**$_{ii}$*: Contribution of Ca^{2+} to I_{ACh}. The ratio f_{Ca}/f_{ACh} provides an estimate of the proportion of Ca^{2+} contributing to I_{ACh} (18.4 ± 4.7 %)*

The adjusted I_{Ca} induced a charge influx of Q_{Ca} = 177.4 ± 144.4 pC (n = 6) and increased the free Ca^{2+} concentration by $\Delta[Ca^{2+}]_i$ = 15.2 ± 10.8 nM.

The fraction f_{ACh} = 13.3 ± 3.3 M x C^{-1} (n = 8, 1000 bootstrap samples, Eq. B.7), which was determined from the slope of the linear fit in Figure C.6 *b*, indicated how much the intracellular Ca^{2+} concentration increases per Coulomb of I_{ACh}, while the fraction f_{Ca} = 72.4 ± 2.8 M x C^{-1} (n = 6, 1000 bootstrap samples, Eq. 7) indicated how much the intracellular Ca^{2+} concentration increases per Coulomb of I_{Ca} (Figure C.6 c_i). The ratiof_{Ca}/f_{ACh} = 18.4 ± 4.7 % (Eq. B.9) is an estimate of the proportion of I_{ACh} that is carried by Ca^{2+} (Figure C.6 c_{ii}). These results show that a considerable portion of I_{ACh} is carried by Ca^{2+}.

3 Pharmacological properties of I_{ACh}

Using whole-cell voltage-clamp recordings, the ionic currents that were induced by exogenously applied ACh (I_{ACh}) and related ligands were investigated on isolated, identified leg motoneurons of the adult stick insect (*C. morosus*) in culture. The pharmacological properties of I_{ACh} are summarized in Table C.1.

3.1 Nicotinic ligands

Nicotine, like ACh, induced an inward current consisting of a fast-desensitizing (I_{NIC1}) and a slow-desensitizing (I_{NIC2}) component (Figure C.7 *a*). A quantitative comparison with I_{ACh}, however, revealed significant differences in some physiological important parameters (Table C.1). At the same concentration (10^{-4} M) the amplitude of I_{NIC1} was similar to I_{ACh1} (P = 0.14; n = 13; paired t-test; Figure C.7*b*), however, I_{NIC2} was 50.3 % smaller compared to I_{ACh2} (P = 0.017; n = 13; paired t-test; Figure C.7*b*). While the nicotine concentration-response curve for I_{NIC1} showed a similar EC_{50} (EC_{50} = 3.5

x 10^{-5}± 1.6 x 10^{-6} M) and Hill's coefficient (n_H = 1.50 ± 0.10) compared to I_{ACh1}, the concentration-response curve for I_{NIC2} was inverted-U shaped with a similar response threshold (3 x 10^{-6} M), EC_{50} (~ 10^{-5} M) and a maximum at 10^{-4} M (Figure C.7*c*). These results suggested the presence of at least 2 nAChR in stick insect leg motoneurons.

Lending more evidences to this hypothesis, applications of imidacloprid, a neonicotinoid insecticide known to target insect nAChRs (Barbara et al. 2008; Brown et al. 2006; Buckingham et al. 1997; Courjaret and Lapied 2001; Déglise et al. 2002; Ihara

Table C.1: *Properties of cholinergic currents in stick insect motoneurons.*

Agonists		**EC_{50} (µM)**	**n_H**
Acetylcholine (n = 14)			
I_{ACh1}		37.0 ± 1.9	1.2 ± 0.09
I_{ACh2}		20.5 ± 1.5	1.1 ± 0.08
Nicotine (n = 5)			
I_{NIC1}		35.0 ± 1.6	1.5 ± 0.10
I_{NIC2}*		~ 10.0	---
Imidacloprid (n = 4)			
I_{IMI1}		49.7 ± 3.2	1.4 ± 0.10
I_{IMI2}*		~ 10.0	---
Antagonists of I_{ACh}		**IC_{50} (µM)**	**n_H**
α-bungarotoxin (n = 6)			
I_{ACh1}	*Hill model*	2.3 ± 1.0	0.40 ± 0.05
	linear fit	3.3 ± 1.1	---
I_{ACh2}	*Hill model*	0.31 ± 0.08	0.44 ± 0.06
	linear fit	0.25 ± 0.01	---
Atropine (n = 5)			
I_{ACh1}	*linear fit*	1.70 ± 0.52	---
I_{ACh2}	*linear fit*	0.76 ± 0.25	---

Asterisks indicate the occurrence of inverted-U shaped concentration-response relation. EC_{50} is the concentration that activates the half-maximal current. IC_{50} is the concentration that blocks half of the maximal current. n_H means the Hill-coefficient.

et al. 2006; Jepson et al. 2006; Nauen et al. 2001; Salgado and Saar 2004), elicited inward currents (I_{IMI}) consisting of a fast-desensitizing (I_{IMI1}) and slow-desensitizing (I_{IMI2}) components (Figure C.8 *a*) and showed a concentration-response relation very similar to the I_{NIC1} (Figure C.8 *b*, Table C.1). I_{IMI1} showed a similar EC_{50} (EC_{50} = 4.9 x 10^{-5} ± 3.2 x 10^{-6} M) and Hill's coefficient (n_H = 1.40 ± 0.10) compared to I_{ACh1} (Table C.1). The concentration-response curve for I_{IMI2} was also inverted-U shaped with a response threshold at ~ 3 x 10^{-6} M, EC_{50} ~ 10^{-5} M and a maximum at 10^{-4} M (Figure C.8, Table C.1).

α-bungarotoxin (α-BGTX) is a specific nicotinic antagonist in many insect systems (Albert and Lingle 1993; Campusano et al. 2007; Cayre et al. 1999; Salgado and Saar 2004). In agreement with the findings that nicotine induced an I_{ACh}-like current in the leg motoneurons (see preceding text), α-BGTX blocked both I_{ACh1} and I_{ACh2} with similar concentration-response patterns (Figure C.9). The block starts at concentrations around 10^{-11} M and I_{ACh} is completely blocked at concentrations of 10^{-6} and higher. As shown in Figure C.9 the choice for the model to fit the α-BGTX concentration-response relation was not obvious. The Hill model and a linear relation were used. The IC_{50} estimated from the Hill model for α-BGTX was 3.3 x 10^{-9} ± 1.1 x 10^{-9} M (n_H = 0.40 ± 0.05) for I_{ACh1} and 2.5 x 10^{-10} ± 0.1 x 10^{-10} M (n_H = 0.44 ± 0.06) for I_{ACh2}. With the linear fit the IC_{50} was 2.3 x 10^{-9} ± 1.0 x 10^{-9} M for I_{ACh1} and 3.1 x 10^{-10} ± 0.8 10^{-10} M for I_{ACh2}. The finding that α-BGTX was more potent against I_{ACh2} (Figure C.9), suggests the existence of different subtypes of nAChR. The α-BGTX block is relatively difficult to reverse (< 50.0 % reversibility after 45 min wash).

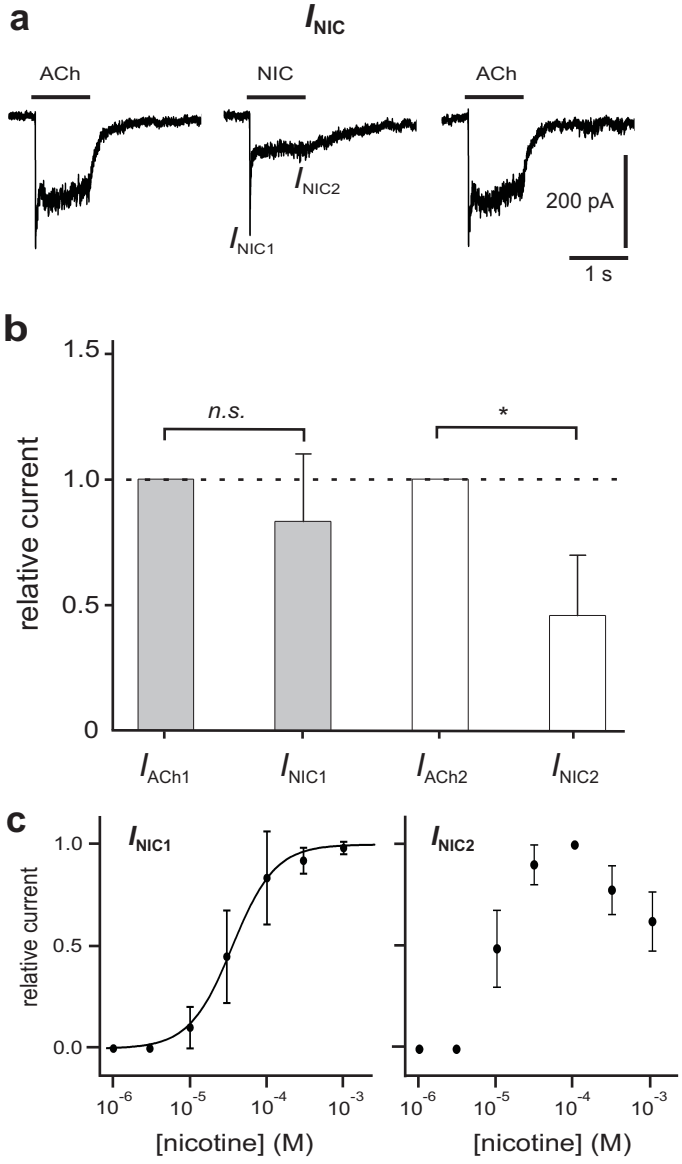

Figure C.7: *Nicotine induced current (I_{NIC}). The holding potential was -60 mV.* ***a*** *Nicotine, like ACh, induced an inward current consisting of a fast-desensitizingt (I_{NIC1}) and a slow-desensitizing (I_{NIC2}) component.* ***a, b*** *At the same concentration (10^{-4} M) the amplitude of I_{NIC1} was similar to I_{ACh1} ($P = 0.14$; $n = 13$; paired t-test). However, I_{NIC2} (current amplitude averaged between 950 ms and 1000 ms of NIC application) was 50.3 % smaller compared to I_{ACh2} ($P = 0.017$; $n = 13$; paired t-test).* ***c*** *Concentration-response relations for I_{NIC1} and I_{NIC2}. The data for I_{NIC1} were well fit with a Hill relation ($EC_{50} = 3.5 \times 10^{-5} \pm 1.6 \times 10^{-6}$ M; $n_H = 1.50 \pm 0.10$). The concentration-response curve for I_{NIC2} component was also inverted-U shaped with a response threshold at ~ 3×10^{-6} M, $EC_{50} \sim 10^{-5}$ M and a maximum at 10^{-4} M.*

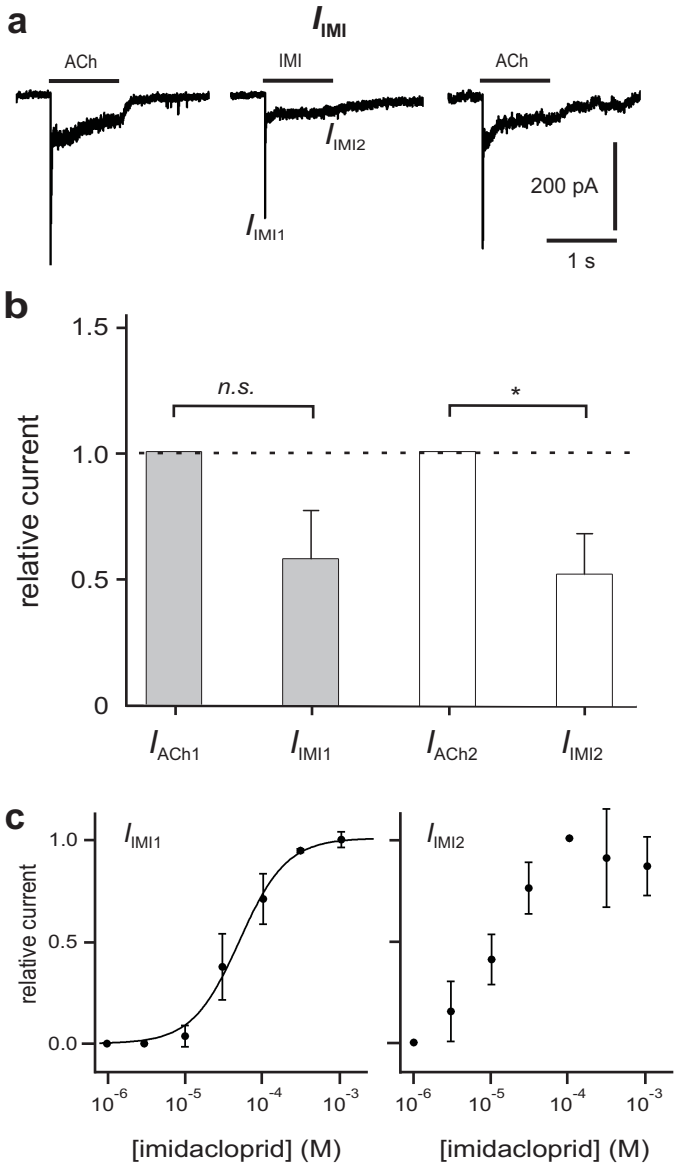

Figure C.8: *Imidacloprid induced current (I_{IMI}). The holding potential was -60 mV.* ***a*** *Imidacloprid, like ACh, induced an inward current consisting of a fast-desensitizing (I_{IMI1}) and a slow-desensitizing (I_{IMI2}) component.* ***a, b*** *At the same concentration (10^{-4} M) the amplitude of I_{IMI1} was similar to I_{ACh1} ($P = 0.06$; $n = 4$; paired t-test). However, I_{IMI2} (current amplitude averaged between 950 ms and 1000 ms of NIC application) is 52.4 % smaller compared to I_{ACh2} ($P = 0.014$; $n = 4$; paired t-test).* ***c*** *Concentration-response relations for I_{IMI1} and I_{IMI2}. The data for I_{IMI1} were well fit with a Hill relation ($EC_{50} = 4.9 \times 10^{-5} \pm 3.2 \times 10^{-6}$ M, $n_H = 1.40 \pm 0.10$). The concentration-response curve for I_{IMI2} was also inverted-U shaped with a response threshold at ~ 3×10^{-6} M, $EC_{50} \sim 10^{-5}$ M and a maximum at 10^{-4} M.*

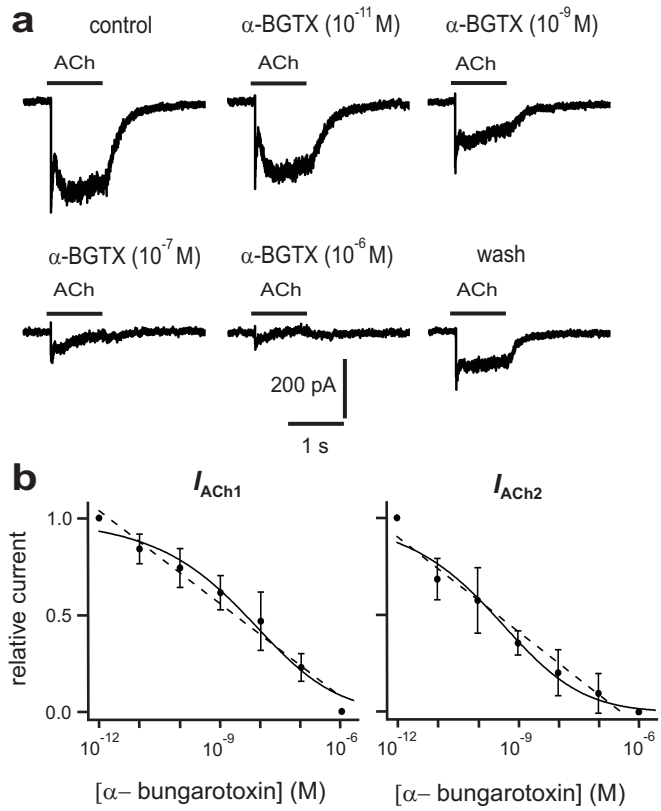

Figure C.9: *Block of I_{ACh} by α-bungarotoxin. I_{ACh} was induced by application of 10^{-4} M ACh. α-BGTX was bath-applied in the indicated concentrations. Holding potential was -60 mV.* ***a*** *Both components of I_{ACh} were α-BGTX sensitive. The α-BGTX effects reversed slowly and only in part (< 50.0 % recovery in 45 min).* ***b*** *Concentration-response relations of the α-BGTX block for both components of I_{ACh}. Current amplitudes were scaled as a fraction of I_{ACh1} and I_{ACh2} evoked by 10-4 M ACh without α-BGTX. The model for the concentration-response relation was not obvious. From linear fits, it was estimated an IC_{50} for I_{ACh1} of 2.3 x 10^{-9} ± 1.0 x 10^{-9} M and 3.1 x 10^{-10} ± 0.8 x 10^{-10} M for I_{ACh2}. From fits with a Hill relation, it was estimated an IC_{50} for I_{ACh1} of 3.3 x 10^{-9} ± 1.1 x 10^{-9} M (h = 0.40 ± 0.05) and 2.5 x 10^{-10} ± 0.1 x 10^{-10} (n_H= 0.44 ± 0.06) for I_{ACh2}.*

3.2 Muscarinic ligands

To test if muscarinic receptors contribute to I_{ACh}, muscarine and two 'classical' muscarinic agonists (oxotremorine and pilocarpine) that have been shown to be effective in insects (Buhl et al. 2008; Corronc and Hue 1993; Okada et al. 2009; Parker and Newland 1995; Ryckebusch and Laurent 1993; Trimmer 1995; Trimmer and Weeks 1989) were applied. At concentrations up to 10^{-3} M none of these muscarinic agonists elicited any current, even when applied for 20 s (Figure C.10). These experiments show that I_{ACh} in the leg motoneurons, as recorded in the experimental conditions used in this dissertation, does not contain a muscarinic component.

Despite the lack of a muscarinic component in I_{ACh}, it was observed that the muscarinic receptor antagonist atropine blocked both components of I_{ACh} (Figure C.11), however, at relatively high concentrations. Atropine had an effect at concentrations of 10^{-8} M and blocked I_{ACh} at 10^{-4} M nearly completely. The IC_{50}, estimated from linear fits of the concentration-current relation was $1.7 \times 10^{-6} \pm 0.5 \times 10^{-6}$ M for I_{ACh1} and $7.6 \times 10^{-7} \pm 2.5 \times 10^{-7}$ M for I_{ACh2}. Note that atropine is several orders of magnitude less potent than the nicotinic antagonist α-BGTX (Table C.1). The atropine block is relatively difficult to reverse (< 70 % reversibility after 30 min wash).

4 Neuromodulation of cholinergic currents

Based on the results described in the preceding sections of this dissertation, it is completely acceptable to assume that cholinergic pathways participate in the neural control of locomotion in the stick insects. Applications of ACh on isolated motoneurons elicited inwards currents (I_{ACh}), which seemed to be evoked by actions of two nicotinic acetylcholine receptors (nAChRs). In other animal systems, it has been shown that neuromodulators (and neurotransmitters) can trigger intracellular pathways that regulate the functional properties of cholinergic currents (Dani and Bertrand 2007;

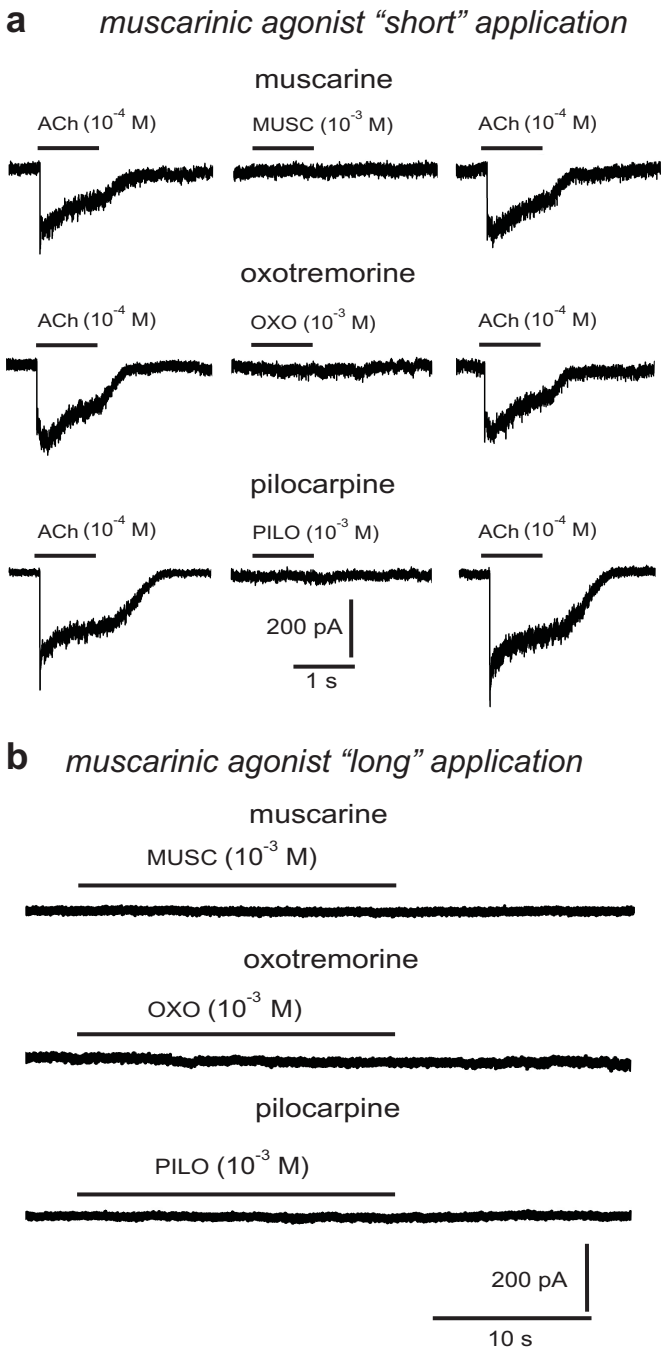

Figure C.10: *Effects of muscarinic agonists. The holding potential was -60 mV.* ***a***, ***b*** *Short (1s) and long (20s) application of muscarine (MUSC), oxotremorine (OXO) and pilocarpine (PILO) at* 10^{-3} *did not induce any currents*

Lee and O'Dowd 2000; McCamphill et al. 2008; Paterson and Nordberg 2000; Thany et al. 2007). Modulations of cholinergic currents in isolated stick insect motoneurons

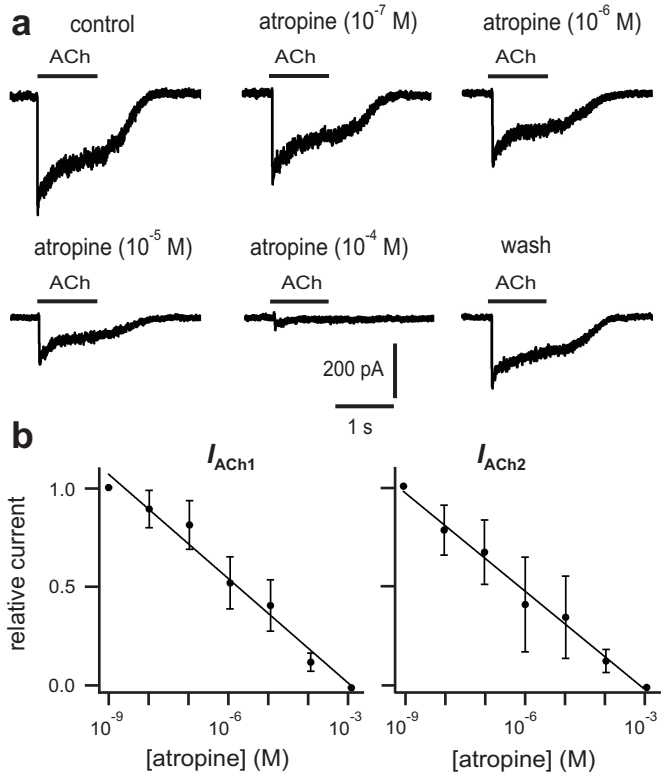

Figure C.11: *Block of I_{ACh} by atropine. I_{ACh} was induced by application 10^{-4} M ACh. Atropine was bath-applied in the indicated concentrations. Holding potential was -60 mV.* ***a*** *Both components of I_{ACh} were atropine sensitive. The atropine effects reversed slowly and only in part (< 70.0 % recovery in 30 min).* ***b*** *Concentration-response relations of the atropine block for both components of I_{ACh}. Current amplitudes were scaled as a fraction of I_{ACh1} and I_{ACh2} evoked by 10^{-4} M ACh without atropine. From linear fits, it was estimated an IC_{50} for I_{ACh1} of 1.7 x 10^{-6} ± 0.5 x 10^{-6} and 7.6 x 10^{-7} ± 2.5 x 10^{-7} M for I_{ACh2}.*

were studied using co-applications of ACh and biogenic amines or co-application of cholinergic agonists. The direct effects of octopamine and 8-Br-cAMP on isolated stick insect motoneurons, obtained in this dissertation, were already described in a recent investigation (Westmark et al. 2009), where I appear as a contributing author.

4.1 Neuromodulation of I_{ACh} by biogenic amines

Application of biogenic amines has been shown to modulate neural activities associated with locomotor behaviors in stick insects (Büschges et al. 1993; Ramirez et al.

1993; Westmark 2007; Westmark et al. 2009). Attempting to verify the direct actions of octopamine and serotonin on stick insect motoneurons, whole-cell voltage-clamp recordings in freshly dissociated motoneuron cell bodies were performed. The neuromodulatory actions of octopamine and serotonin on I_{ACh} are summarized in Table C.2.

Table C.2: *Neuromodulatory effects of biogenic amines on I_{ACh}.*

Biogenic amines	**IC_{50} (µM)**	**n_H**
Octopamine (n = 5)		
I_{ACh1}	158.7 ± 34.8	0.88 ± 0.16
I_{ACh2}*	---	---
Serotonin (n = 6)		
I_{ACh1}	20.7 ± 3.8	0.78 ± 0.09
I_{ACh2}	22.1 ± 5.3	0.74 ± 0.11

Asterisk indicates that the concentration-current relation could not be fitted by the Hill's model. IC_{50} is the concentration that blocks half of the maximal current. n_H is the Hill-coefficient.

4.1.1 Suppression of I_{ACh} by octopamine

Octopamine, the invertebrate analogue of noradrenaline (Roeder 1999), can act as a neurotransmitter, neurohormone and neuromodulator (Orchard 1982). In insects, the regulation of many physiological processes is considerably dependent on octopaminergic pathways, which are usually mediated by metabotropic receptors (Roeder 2002). In intact and inactive stick insects, injections of octopamine into the hemolymph caused an activation of the animal and suppressed pathways involved in the resistance reflex (Büschges et al. 1993; Ramirez et al. 1993). In a stick insect semi-intact single-foreleg preparation, tonic depolarizations recorded in middle leg motoneurons and elicited during foreleg stepping sequences were up-regulated by application of octopamine (Westmark et al. 2009).

Here, direct effects of octopamine on motoneurons were tested. Application of ACh (10^{-4}M) on isolated motoneurons induced inward currents, which were suppressed by bath application of increasing concentrations of octopamine (Figure C.12, Table C.2). In all five motoneurons studied, bath application of octopamine at concentrations between 10^{-6}M and 10^{-3}M did not evoke membrane currents (holding potential = -60 mV) but reduced the fast-desensitizing current (I_{ACh1}) in a concentration-dependent manner (Figure C.12, *a* and *b*). The slow-desensitizing ACh-induced current (I_{ACh2}) was not significantly reduced up to an octopamine concentration of 10^{-4}M but was completely blocked at a concentration of 10^{-3}M octopamine (Fig. C.12 *a*). The concentration-current relation for I_{ACh1} produced an octopamine half-maximal inhibitory concentration (IC_{50}) of 1.6 x 10^{-4}± 0.3 x 10^{-4}M and Hill's coefficient (n_H) of 0.88 ± 0.16 (Figure C.12 *b*).

These results indicate that the increase in tonic depolarization observed in stick insect motoneurons in the presence of octopamine (Westmark et al. 2009) was not due to an up-regulation of the motoneurons' response to ACh.

4.1.2 Suppression of I_{ACh} by serotonin

Serotonin (5-HT) acts through multiple receptor subtypes to modulate numerous physiological and behavior-related processes in both vertebrates and invertebrates (Baier et al. 2002; Hill and Blagburn 2001; Jacklet et al. 2006; de Miguel and Trueta 2005; Kravitz and Huber 2003; Popova 2006; Schlenstedt et al. 2006; Stevenson et al. 2005). Serotonergic actions are usually transmitted by a set of metabotropic receptors, which can be found at the peripheral as well at the central nervous system, modulating the behavioral state of the animal at different levels (Roeder 2002). In stick insects, serotonergic actions were described to the control of salivary glands secretion (Ali 1997) and to the regulation of tonic depolarizations observed in middle motoneurons, which were elicited by activation of foreleg stepping sequences (Westmark 2007). In

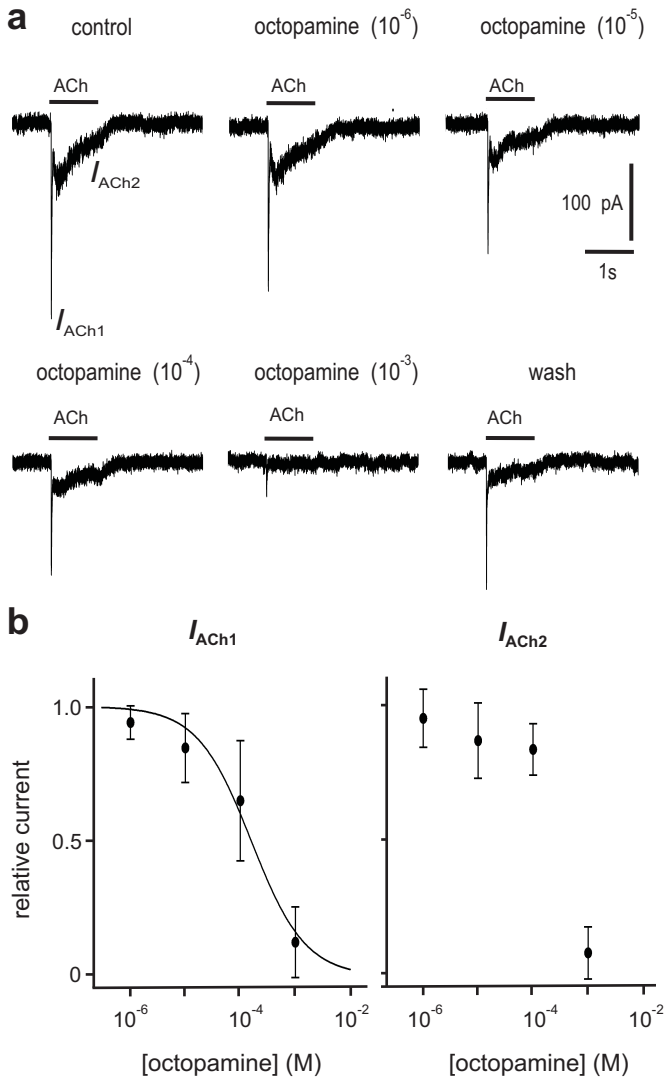

Figure C.12: *Block of I_{ACh} by octopamine. I_{ACh} was induced by application 10^{-4}M ACh. Octopamine was bath-applied in the indicated concentrations. Holding potential was -60 mV.* ***a*** *During a whole-cell patch-clamp recording of a labelled motoneuron cell body acetylcholine (ACh) was applied by means of a fast U-tube application system. Octopamine reversibly inhibited the fast-desensitizing component of the ACh-induced current (I_{ACh1}) in a concentration dependent manner. A significant block of the slow-desensitizing component (I_{ACh2}) was detected at an octopamine concentration of 10^{-3}M.* ***b*** *Concentration-response relations of the octopamine block for both components of I_{ACh} (n = 5). Current amplitudes were scaled as a fraction of I_{ACh1} and I_{ACh2} evoked by 10^{-4} M ACh without octopamine. Estimation of octopamine IC_{50} (using a Hill relation, eq. B.2) for I_{ACh1} was of 1.6 x 10^{-4}± 0.3 x 10^{-4}M (n_H= 0.88 ± 0.16).*

this last investigation, serotonin had an opposing effect on the regulation of the tonic depolarization amplitude. It was shown that serotonin increased, but also decreased

the tonic depolarization amplitude in different experiments (Westmark 2007).

Here, direct effects of serotonin on motoneurons were tested. Application of ACh (10^{-4} M) on isolated motoneurons induced inward currents, which were suppressed by bath application of increasing concentrations of serotonin (Figure C.13). In all five motoneurons studied, bath application of serotonin at concentrations between 10^{-6} M and 10^{-3} M did not evoke membrane currents (holding potential = -60 mV). Serotonin, however, reduced the amplitude of both components of I_{ACh} more efficiently than the octopamine (Table C.2). Unlike octopamine, serotonin acted similarly on both components of I_{ACh} (C.13 *a* and *b*). Concentration-response parameteres were estimated from fits with a Hill relation (eq. B.2). Serotononin blocked acetylcholine fast-desensitizing current (I_{ACh1}) with an IC_{50} of 2.0 x 10^{-5} ± 3.8 x 10^{-6} M (n_H = 0.78 ± 0.09). The acetylcholine slow-desensitizing current (I_{ACh2}) was blocked by serotononin with an IC_{50} 2.2 x 10^{-5} ± 5.3 x 10^{-6} (n_H= 0.74 ± 0.11).

Since bath application of serotonin did not alter the holding current (V_h= -60 mV) in the absence of ACh but reduced the cellular ACh responsiveness in all six isolated motoneurons studied, served to comprove that serotonin act rather modulating the excitatory transmission in stick insects. In semi-intact preparation, serotonin might modulate the activity in premotor neurons (e.g. non-spiking interneurons), which could be an explanation for the the up- or downregulation of tonic depolarizations observed in stick insect motoneurons *in situ* (Westmark 2007).

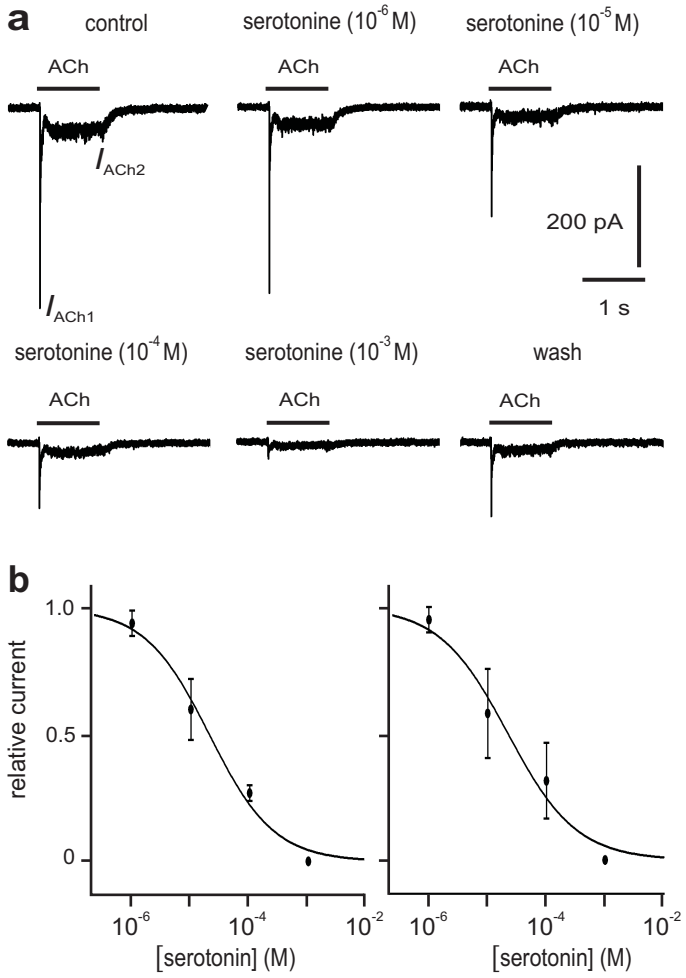

Figure C.13: *Block of I_{ACh} by serotonin. I_{ACh} was induced by application 10^{-4}M ACh. Serotonin was bath-applied in the indicated concentrations. Holding potential was -60 mV.* ***a*** *During a whole-cell patch-clamp recording of a labelled motoneuron cell body acetylcholine (ACh) was applied by means of a fast U-tube application system. Serotonin reversibly inhibited the fast-desensitizing and the slow-desensitizing components of the ACh-induced current (I_{ACh1} and I_{ACh2}, respectively) in a concentration dependent manner.* ***b*** *Concentration-response relations of the serotonin block for both components of I_{ACh}(n = 6). Current amplitudes were scaled as a fraction of I_{ACh1} and I_{ACh2} evoked by 10^{-4} M ACh without serotonin. Serotononin blocked I_{ACh1} with an IC_{50} of 2.0 x 10^{-5} ± 3.8 x 10^{-6} M (n_H = 0.78 ± 0.09). The I_{ACh2} was blocked by serotononin with an IC_{50} of 2.2 x 10^{-5} ± 5.3 x 10^{-6} M (n_H= 0.74 ± 0.11).*

4.1.3 Suppression of I_{ACh} by 8-Br-cAMP

Across insect species, responses regulated by serotonin (Baines et al. 1990; Blenau and Baumann 2001; Parker 1995; Tierney 2001) or octopamine (Achenbach et al. 1997; Balfanz et al. 2005; Bischof and Enan 2004; Han et al. 1998; Parker 1996; Walther and Zittlau 1998) depend on the participation of cAMP-pathways. 8-Br-cAMP is an effective membrane-permeable cAMP analog in many insect systems (Grasshoopers: Lundquist and Nässel 1997; Wenzel et al. 2002. Fruit flies: Scheiner et al. 2004. Cockroaches: Wicher 2001; Wicher et al. 2004. Honey bees: Fiala et al. 1999. Tobacco horn worms: Dedos and Birkenbeil 2003; Zayas and Trimmer 2007.Crickets: Kosakai et al. 2008). In stick insects, the involvement of cAMP pathways on an up-regulation of tonic depolarizations in leg motoneurons was demonstrated by bath-applying 8-Br-cAMP (Westmark et al. 2009).

Attempting to discover whether the applied 8-Br-cAMP affect the stick insect motoneurons directly, or indirectly *via* premotor neurons, the ACh-induced currents on isolated motoneurons in the presence and absence of 8-Br-cAMP (2.0 x 10^{-6} M) were recorded. Direct applications of ACh (1.0 x 10^{-4} M) elicited inward currents consisting of a fast-desensitizing (I_{ACh1}) and a slow-desensitizing component (I_{ACh2}) (Figure C.14). In all three motoneurons recorded, 8-Br-cAMP reversibly reduced the fast-desensitizing current amplitude by 46.9 ± 4.2% but did not significantly affect the slow-desensitizing current (FigureC.14). Effects of 8-Br-cAMP (2.0 x 10^{-6} M) on holding current (V_h: -60 mV) in the absence of ACh were not observed.

These experiments indicate that the up-regulation of tonic excitatory inputs observed in leg motoneurons in the semi-intact preparations (Westmark 2007) was not based on 8-bromo-cAMP direct up-regulation of motoneuron ACh responsiveness.

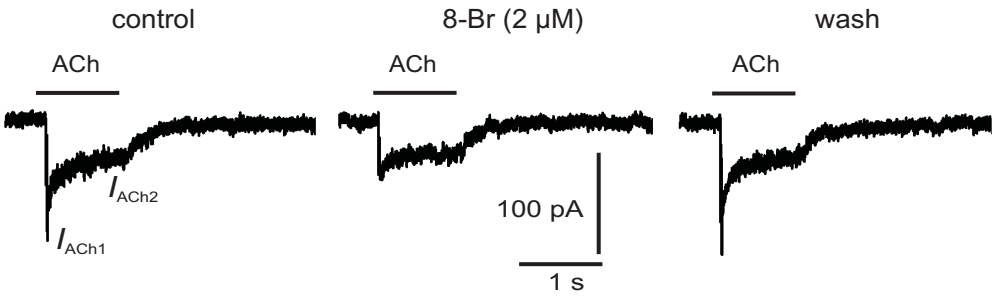

Figure C.14: *Block of I_{ACh} by 8-Br-cAMP. Bath application of 8-Br-cAMP (2.0 x 10^{-6} M) reversibly blocked the fast-desensitizing component of inward current evoked by applications of ACh (1.0 x 10^{-4} M). During a whole cell patch-clamp recording of a motoneuron cell body ACh was applied by means of a fast U-tube application system. Holding potential was -60 mV. 8-Br-cAMP was bath applied 2 min before ACh application. The slow-desensitizing component of I_{ACh} was not affected by applications of 8-Br-cAMP.*

4.2 Neuromodulation of I_{ACh} by cholinergic agonists

Functional interactions between actions of nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) have been shown in vertebrates (Brown and Galligan 2003; Shen et al. 2009; Verbitsky et al. 2000; Zwart and Vijverberg 1997)as well in insects (Courjaret et al. 2001; David and Pitmann 1996; Liu et al. 2007). In stick insects, although no inward currents were elicited by applications of muscarinic agonists (see RESULTS, section 3.2), the possible regulation of nAChR functional properties by mAChR-mediated processes can not be ruled out, since the acetylcholine-induced currents were sensible to bath application of the muscarinic antagonist atropine (see RESULTS, section 3.2).

To test if the activation of mAChRs had any effect on the nAChR-activated currents, two sets of experiments were performed. Firstly, the responses elicited by applications of acetylcholine (ACh) or nicotine (NIC) alone were measured and compared with the responses resulted from co-applications of these agonists with muscarine (MUSC). In this experimental set, all cholinergic ligands were co-applied (1 s) *via* an U-tube application system and had the same concentration (1.0 x 10^{-4} M). Currents elicited by simultaneous application of acetylcholine and muscarine ($I_{(ACh+MUSC)}$) were quite

similar to the currents induced by ACh alone ($I_{(ACh+MUSC)1}$: P =0.38. $I_{(ACh+MUSC)2}$: P =0.07; n= 4; paired t-test; Figure C.15, Table C.3). Amplitude of currents induced by nicotine alone (I_{NIC1}) did not differ from the amplitude of currents elicited by co-applications of nicotine and muscarine ($I_{(NIC+MUSC)}$) ($I_{(NIC+MUSC)1}$: P =0.49. $I_{(NIC+MUSC)2}$: P =0.40; n= 3; paired t-test;Figure C.16, Table C.3).

Table C.3: *Properties of currents elicited by co-application of cholinergic ligands*

Agonist co-applications	**Relative current**
Acetylcholine (ACh) + muscarine (MUSC) (n = 4) [a]	
$I_{(ACh+MUSC)1}$	0.92 ± 0.07
$I_{(ACh+MUSC)2}$	0.86 ± 0.16
Nicotine (NIC) + muscarine (n = 3) [b]	
$I_{(NIC+MUSC)1}$	0.98 ± 0.02
$I_{(NIC+MUSC)2}$	1.00 ± 0.06
Acetylcholine + nicotine (n = 4) [a]	
$I_{(NIC+ACh)1}$	1.25 ± 0.42
$I_{(NIC+ACh)2}$	0.67 ± 0.18
Muscarinic block actions	**Relative current**
Acetylcholine (n = 3) [a]	
I_{ACh1}*	0.62 ± 0.05
I_{ACh2}*	0.52 ± 0.09
Nicotine (n = 3) [b]	
I_{NIC1}	0.90 ± 0.14
I_{NIC2}	0.92 ± 0.13

a *Current amplitudes of the acetylcholine-induced currents were used to normalize the current amplitudes under co-application circunstances.* ***b*** *Indicate where the nicotine-induced current amplitude were used to normalize the amplitude of ligand co-applied-induced currents. Asterisks indicates statistical significance for $P < 0:05$.*

Similarly to the current elicited by ACh alone (I_{ACh}), co-applications of ACh and NIC induced inward currents consisting of a fast-desensitizing ($I_{(ACh+NIC)1}$) and a

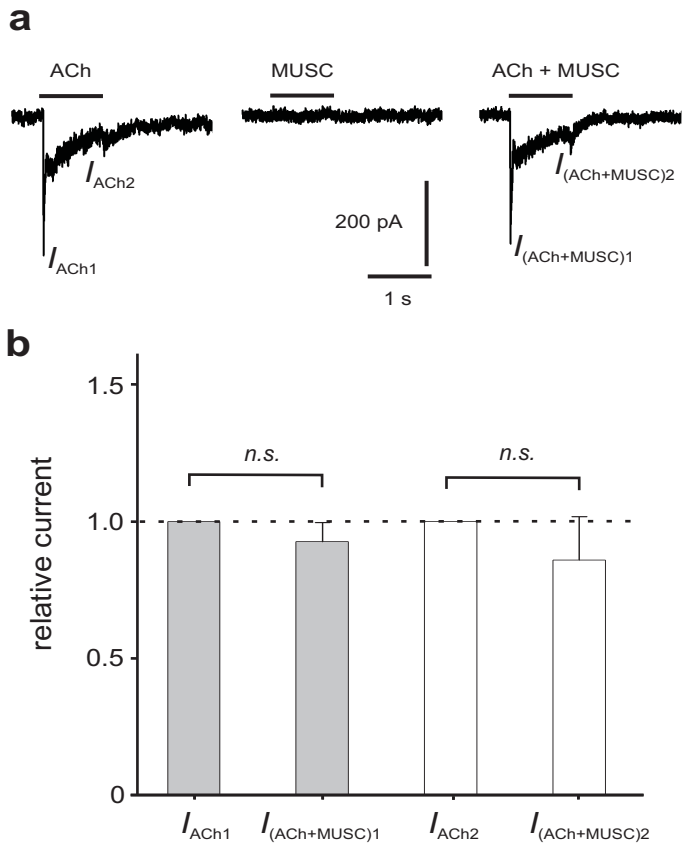

Figure C.15: *Inward currents elicited by co-applications of acetylcholine (ACh, 1.0 x 10^{-4}M) and muscarine (MUSC, 1.0 x 10^{-4}M).* ***a*** *Traces from inward currents elicited in a single neuron by application of acetylcholine and muscarine alone as well their co-application. Applications arrived 2 min apart.* ***b*** *The current amplitudes resulted of simultaneous acetylcholine and muscarine co-application were quite similar of the current amplitude of acetylcholine alone application ($I_{(ACh+MUSC)1}$: P =0.38. $I_{(ACh+MUSC)2}$: P =0.07; n= 4; paired t-test).*

slow-desensitizing component ($I_{(ACh+NIC)2}$), with current amplitudes that did not differ ($I_{(ACh+NIC)1}$: P =0.21. $I_{(ACh+NIC)2}$: P =0.06; n= 4; paired t-test) from the I_{ACh} amplitudes (Figure C.17,Table C.3). However, while the amplitude of $I_{(ACh+NIC)1}$ increased by ~ 25 %, the amplitude of $I_{(ACh+NIC)2}$ decreased by 34 %. These results indicate that the ACh and nicotine agonistic actions occur primarily at the same site of action and thereby a competitive interaction exists between them.

In a second set of experiments, recordings of responses evoked by acetylcholine or nicotine were preceeded by bath applications of muscarine (1.0 x 10^{-4} M; 4 min). When

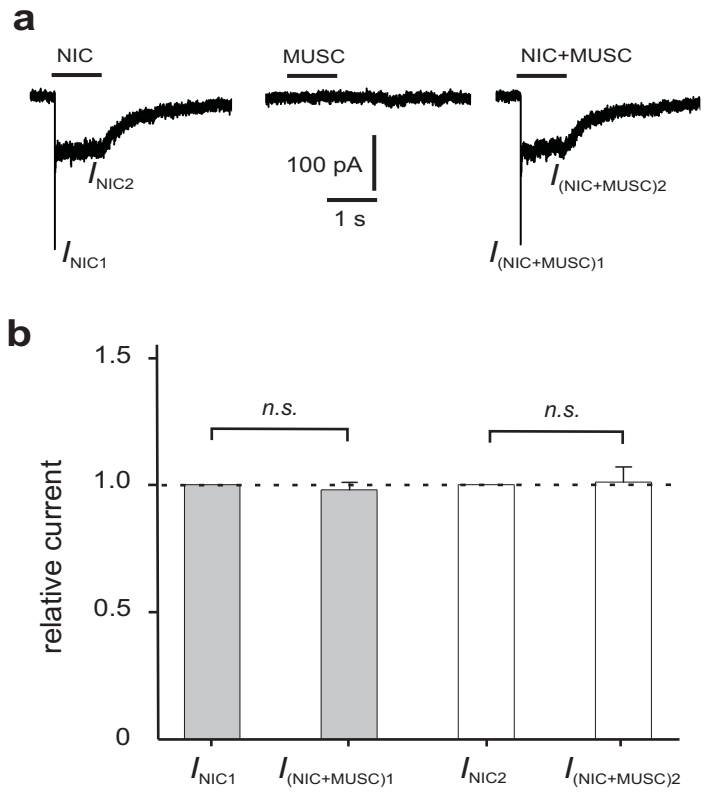

Figure C.16: *Inward currents elicited by co-applications of nicotine (NIC, 1.0 x 10^{-4}M) and muscarine (MUSC, 1.0 x 10^{-4}M).* ***a*** *Traces from inward currents elicited in a single neuron by application of nicotine and muscarine alone as well their co-application. Applications arrived 2 min apart.* ***b*** *The current amplitudes resulted of simultaneous nicotine and muscarine co-application were quite similar of the current amplitude of nicotine alone application ($I_{(NIC+MUSC)1}$: P =0.49. $I_{(NIC+MUSC)2}$: P =0.40; n= 3; paired t-test).*

muscarine was bath applied, the acetylcholine fast- and slow-desensitizing currents ($I_{ACh1}$and I_{ACh2}, respectively) were significantly smaller (I_{ACh1} : P =0.011. I_{ACh2} : $P <$ 0.001, n= 3; paired t-test) than the currents elicited before the muscarine application (Figure C.18,Table C.3), indicating that muscarine might trigger intracellular signaling pathways capable of modulating the nAChRs activity in stick insect motoneurons. However, bath applications of muscarine, did not significantly change (I_{NIC1} : P =0.41. I_{NIC2}: P =0.49; n= 3; paired t-test) the properties of nicotine-induced currents (Figure C.19, Table C.3).

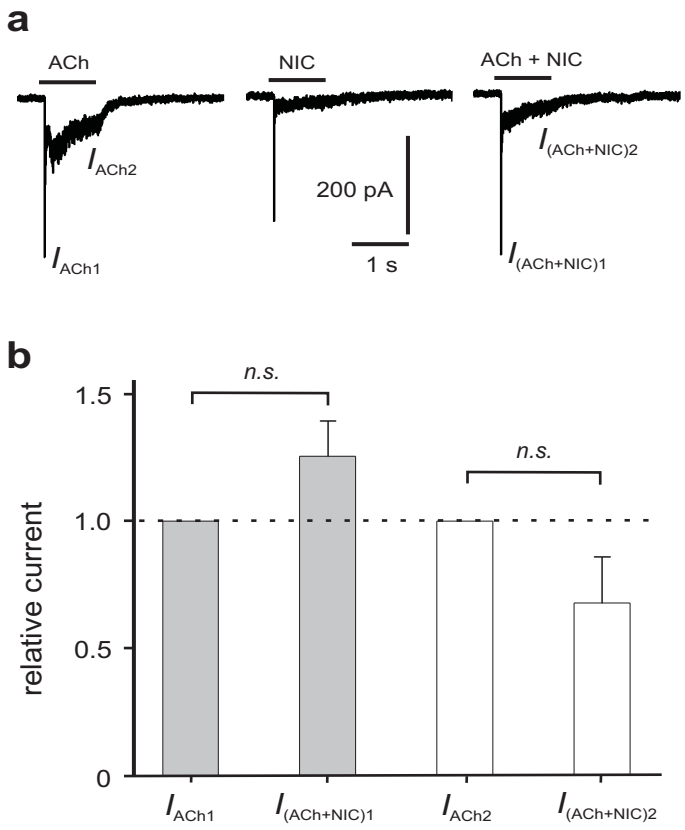

Figure C.17: *Inward currents elicited by co-applications of acetylcholine (ACh, 1.0 x 10^{-4}M) and nicotine (NIC, 1.0 x 10^{-4}M).* ***a*** *Traces from inward currents elicited in a single neuron by application of acetylcholine and nicotine alone as well their co-application. Applications arrived 2 min apart.* ***b*** *The current amplitudes resulted of simultaneous acetylcholine and nicotine co-application were quite similar of the current amplitude of acetylcholine alone application ($I_{(ACh+NIC)1}$: P =0.21. $I_{(ACh+NIC)2}$: P =0.06; n= 4; paired t-test).*

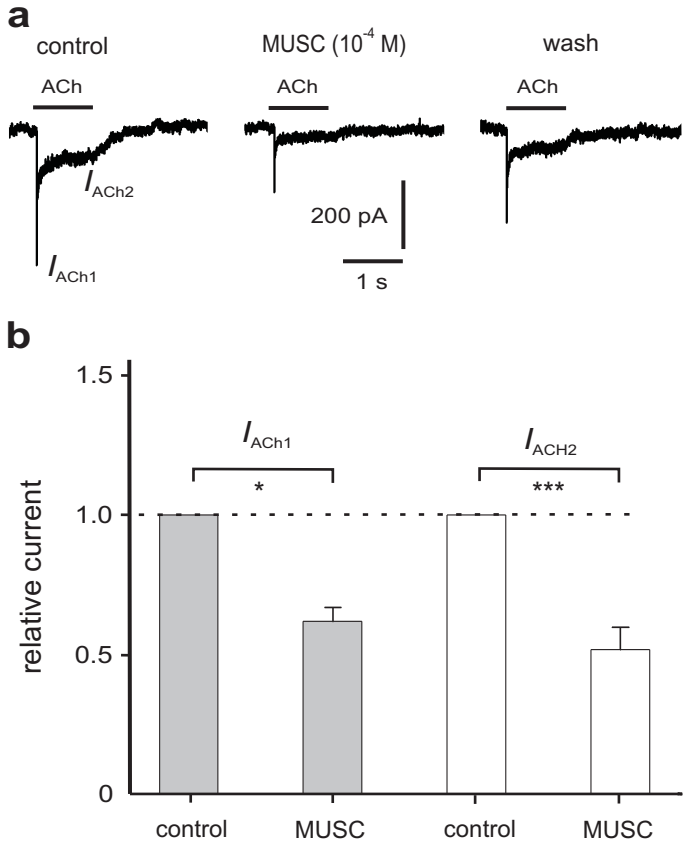

Figure C.18: *Block of I_{ACh} by muscarine.* ***a*** *Preincubation of the neurons with muscarine (MUSC, 1.0 x 10^{-4} M, 4 min) blocked both components of inward currents evoked by applications of 1.0 x 10^{-4} M acetylcholine (ACh).* ***b*** *In the presence of muscarine, the acetylcholine fast- and slow-desensitizing currents ($I_{ACh1}$and I_{ACh2}) were 38 ±5 % and 48 ±9 % respectively smaller than the currents elicited before the muscarine application(I_{ACh1} : P =0.011. I_{ACh2} : $P < 0.001$, n= 3; paired t-test).*

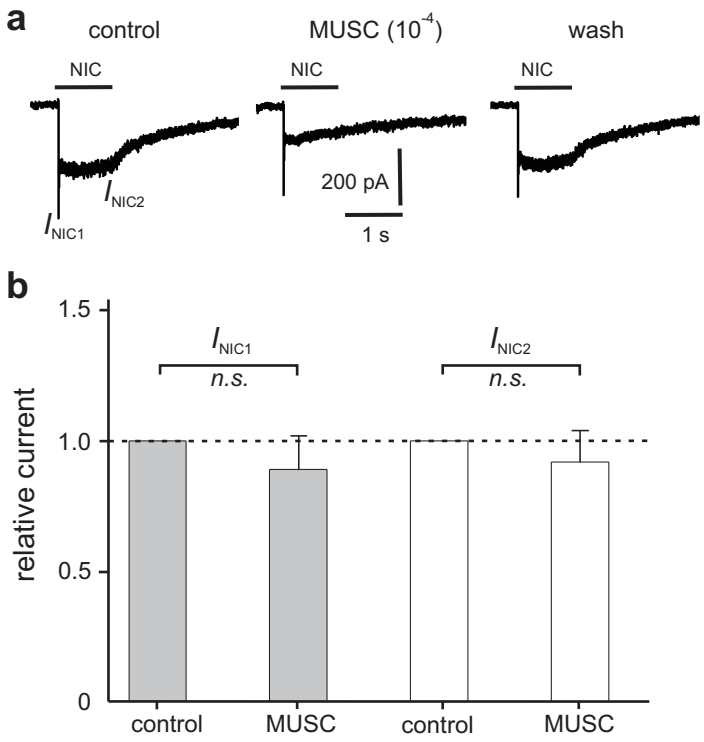

Figure C.19: *Block of I_{NIC} by muscarine.* ***a*** *Previous bath application of muscarine (1.0 x 10^{-4} M, 4 min) did not change the properties of inward currents evoked by applications of 1.0 x 10^{-4} M nicotine (NIC).* ***b*** *In the presence of muscarine, the nicotine fast- and slow-desensitizing currents (I_{NIC1} and I_{NIC2}) were only 10 ± 14 % and 8 ± 13 % respectively smaller than the currents elicited before the muscarine application (I_{NIC1} : P =0.41. I_{NIC2}: P =0.49; n= 3; paired t-test)*

5 Actions of neonicotinoids on the I_{ACh}

Neonicotinoids are insecticides known to act selectively on insect nicotinic acetylcholine receptors (nAChRs), with a widespread use against a broad spectrum of sucking and certain chewing insect-pests (Bai et al. 1991; Jeschke and Nauen 2008; Matsuda et al. 2001; Nauen and Bretschneider 2002; Zhang et al. 2000). Neonicotinoids have been used as effective probes for structural (Matsuda et al. 2001; Nishiwaki et al. 2003; Tomizawa and Casida 2003; Zhang et al. 2000) and functional investigations of insect nAChRs (Bai et al. 1991; Brown et al. 2006; Ihara et al. 2006; Salgado and Saar 2004). In cockroach thoracic neurons, imidacloprid had a nanomolar affinity for a desensitizing nicotinic receptor (nAChD) and activated a nicotinic non-desensitizing receptor (nAChN) only with micromolar potencies (Salgado and Saar 2004), allowing a clear pharmacological distinction of the two nAChRs. In stick insect motoneurons, the acetylcholine-induced currents (I_{ACh}) were conducted by at least two nAChR subtypes (see RESULTS, section 3.1). Attempting to achieve a clear pharmacological distinction of these nAChR subtypes, effects of imidacloprid on cholinergic currents were recorded.

Despite of high variability among the five neurons recorded, imidacloprid reduced I_{ACh} amplitude similarly to the nicotinic antagonist α-bungarotoxin (see RESULTS, section 3.1). The imidacloprid actions started at concentrations around 10^{-13} M and I_{ACh} was completely inhibited at concentrations of 10^{-6} M (Figure C.20). Both components of I_{ACh} (I_{ACh1} and I_{ACh2}) were blocked by imidacloprid with similar concentration-response patterns (Figure C.20 *b*).

Since the results obtained with imidacloprid applications did not allow a clear classification of the nAChRs subtypes observed in the stick insect motoneurons, new sets of experiments using novel neonicotinoids (here generically named as *compound A*, *compound B* and *compound C*) were performed. The concentration-response parame-

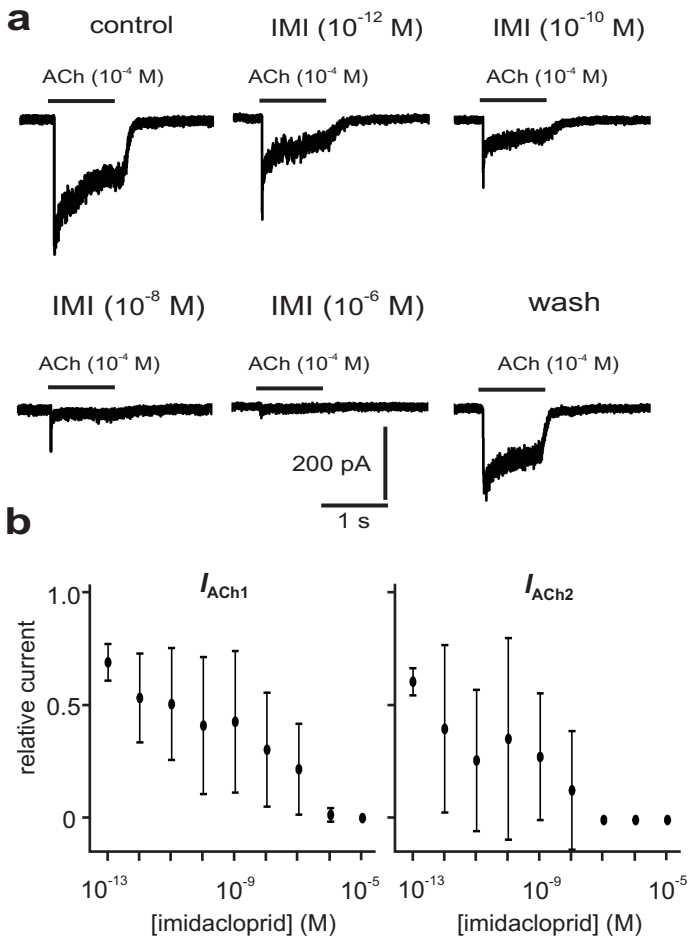

Figure C.20: *Block of I_{ACh} by imidacloprid. I_{ACh} was induced by application 10^{-4} M ACh. Imidacloprid was bath-applied in the indicated concentrations. Holding potential was -60 mV.* ***a*** *Both components of I_{ACh} were imidacloprid sensitive. The imidacloprid effects reversed slowly and only in part (< 80.0 % recovery in 25 min).* ***b*** *Concentration-response relations of the imidacloprid block for both components of I_{ACh}. Current amplitudes were scaled as a fraction of I_{ACh1} and I_{ACh2} evoked by 10^{-4} M ACh without imidacloprid. Due the high variability on the imidacloprid actions among the five neurons recorded, no fit could be used to deteremine any IC_{50} value. However, imidacloprid reduced the I_{ACh} in a concentration dependent manner. The imidacloprid actions started at concentrations around 10^{-13} M and I_{ACh} was completely inhibited at concentrations of 10^{-6} M.*

ters for all neonicotinoids are summarized in Table C.4. All neonicotinoids used in this dissertation blocked both components of I_{ACh} with similar concentration-response relations (Figures C.21-C.23).

The neonicotinoids reduced the acetylcholine slow-desensitizing current (I_{ACh2}) with IC_{50} values no higher than 3.0 x 10^{-9} M (*compound A*: IC_{50}= 2.6 x 10^{-9} ± 0.8 x

Table C.4: *Block actions of neonicotinoids on I_{ACh}.*

neonicotenoids	IC_{50} (nM)	n_H
compound A (n = 4)		
I_{ACh1}	108.9 ± 74.0	0.3 ± 0.06
I_{ACh2}	2.6 ± 0.8	0.7 ± 0.12
compound B (n = 6)		
I_{ACh1}	45.9 ± 27.8	0.4 ± 0.07
I_{ACh2}	2.4 ± 0.5	0.8 ± 0.12
compound C (n = 4)		
I_{ACh1}	22.3 ± 13.8	0.3 ± 0.08
I_{ACh2}	2.8 ± 0.6	0.7 ± 0.10

IC_{50} is the concentration that activates the half-maximal current. n_H is the Hill-coefficient.

10^{-9} M; n_H = 0.70 ± 0.12. *Compound B*: IC_{50}= 2.4 x 10^{-9} ± 0.5 x 10^{-9} M; n_H = 0.80 ± 0.12. *Compound C*: IC_{50}= 2.8 x 10^{-9} ± 0.6 x 10^{-9} M; n_H = 0.70 ± 0.10). They were not always, however, capable of completely block the fast-desensitizing current (I_{ACh1}). The *compound A* blocked the I_{ACh1} with the smallest efficacy, reducing only 67 % of I_{ACh1} when applied at 1.0 x 10^{-5} (Figure C.21) and showed an IC_{50} of 108.0 x 10^{-9} ± 74.0 x 10^{-9} M (n_H = 0.30 ± 0.06). Both *compound B* and *compound C* reduced around of 85 % of I_{ACh1} and presented IC_{50} values of 45.9 x 10^{-9} ± 27.8 x 10^{-9} M (n_H = 0.40 ± 0.07) and 22.3 x 10^{-9} ± 13.8 x 10^{-9} M (n_H = 0.30 ± 0.08), respectively (Figures C.22 and C.23). Although the results with the neonicotinoids used here did not always allow a clear pharmacological separations of the acetylcholine-induced currents, the finding that all compounds, like α-bungarotoxin, were a few orders more potent against I_{ACh2} (Figures C.21-C.23), suggests the existence of different subtypes of nAChR.

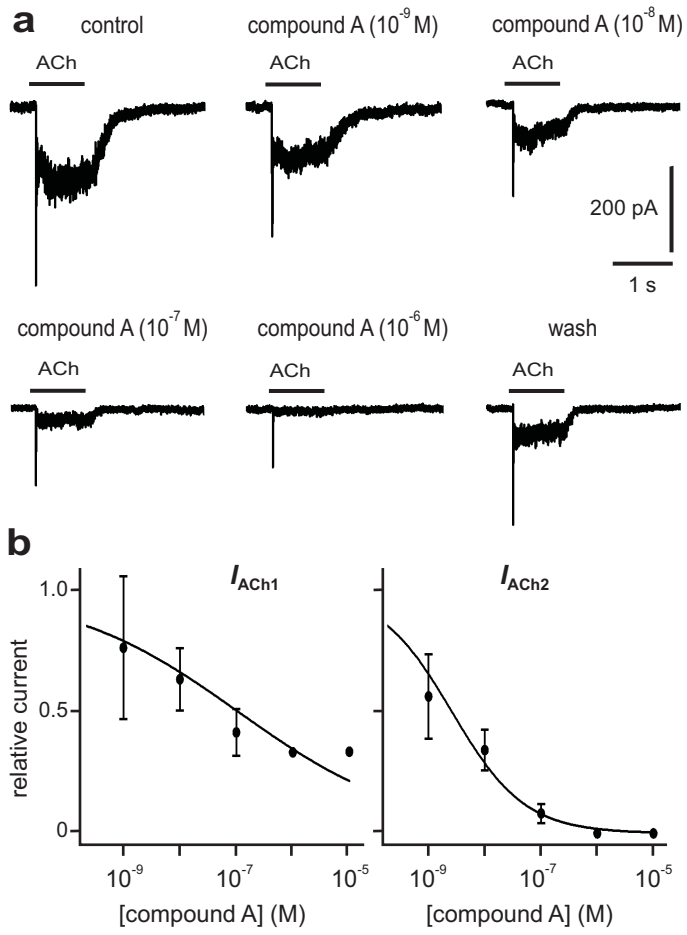

Figure C.21: *Block of I_{ACh} by compound A. I_{ACh} was induced by application of 10^{-4} M ACh. Compound A was bath-applied in the indicated concentrations. Holding potential was -60 mV.* ***a*** *Both components of I_{ACh} were compound A sensitive. The compound A effects reversed slowly and only in part (75.0 % recovery in 25 min).* ***b*** *Concentration-response relations of the compound A block for both components of I_{ACh} (n = 4). Current amplitudes were scaled as a fraction of I_{ACh1} and I_{ACh2} evoked by 10^{-4} M ACh without compound A. Compound A blocked I_{ACh1} with an IC_{50} of 108.9 x 10^{-9} ± 74.0 x 10^{-9} M (n_H = 0.30 ± 0.06). The I_{ACh2} were blocked by compound A with an IC_{50} of 2.6 x 10^{-9} ± 0.8 x 10^{-9} M (n_H= 0.70 ± 0.12).*

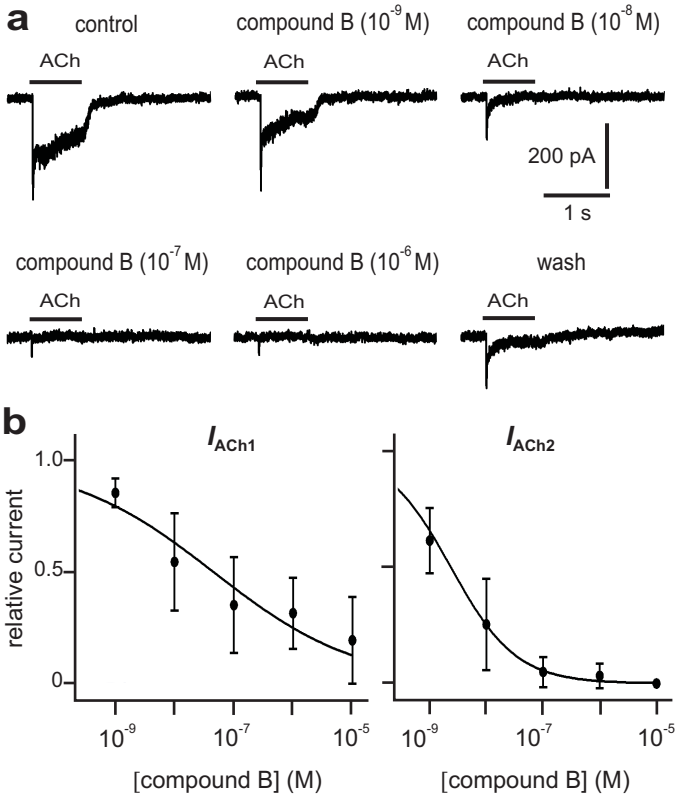

Figure C.22: *Block of I_{ACh} by compound B. I_{ACh} was induced by application of 10^{-4} M ACh. Compound B was bath-applied in the indicated concentrations. Holding potential was -60 mV.* ***a*** *Both components of I_{ACh} were compound B sensitive. The compound B effects reversed slowly and only in part (65.0 % recovery in 20 min).* ***b*** *Concentration-response relations of the compound B block for both components of I_{ACh} (n = 6). Current amplitudes were scaled as a fraction of I_{ACh1} and I_{ACh2} evoked by 10^{-4} M ACh without compound B. Compound B blocked I_{ACh1} with an IC_{50} of 45.9 x 10^{-9} ± 27.8 x 10^{-9} M (n_H = 0.40 ± 0.07). The I_{ACh2} were blocked by compound B with an IC_{50} of 2.4 x 10^{-9} ± 0.5 x 10^{-9} M (n_H= 0.80 ± 0.12).*

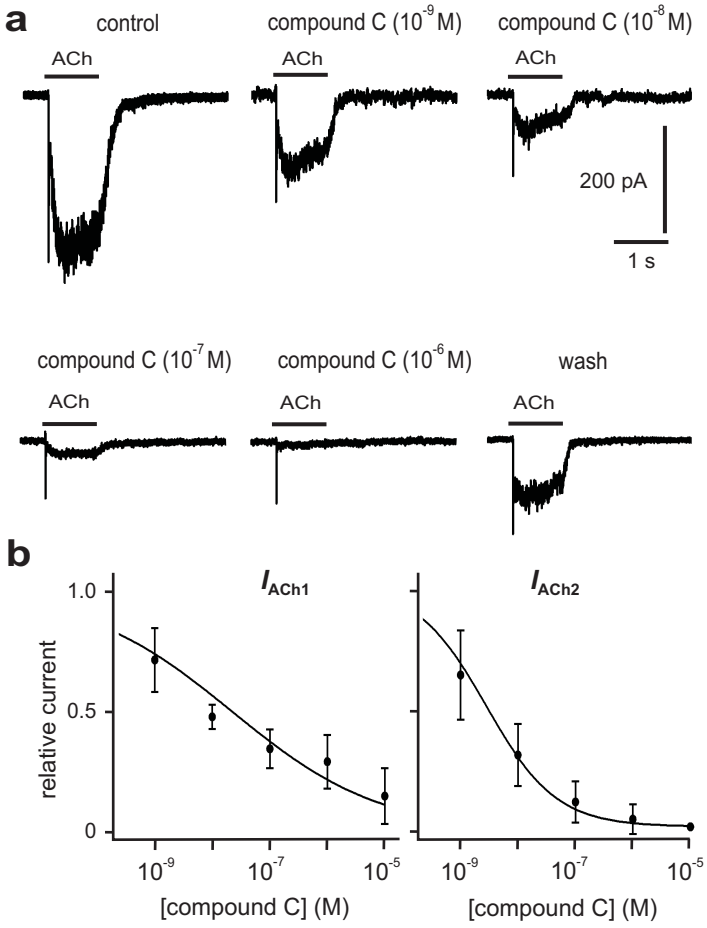

Figure C.23: *Block of I_{ACh} by compound C. I_{ACh} was induced by application of 10^{-4} M ACh. Compound C was bath-applied in the indicated concentrations. Holding potential was -60 mV.* ***a*** *Both components of I_{ACh} were compound C sensitive. The compound C effects reversed slowly and only in part (75.0 % recovery in 20 min).* ***b*** *Concentration-response relations of the compound C block for both components of I_{ACh} (n = 4). Current amplitudes were scaled as a fraction of I_{ACh1} and I_{ACh2} evoked by 10^{-4} M ACh without compound C. Compound C blocked I_{ACh1} with an IC_{50} 22.3 x 10^{-9} ± 13.8 x 10^{-9} M (n_H = 0.30 ± 0.08). The I_{ACh2} were blocked by compound C with an IC_{50} of 2.8 x 10^{-9} ± 0.6 x 10^{-9} M (n_H= 0.70 ± 0.10).*

D. Discussion

In this dissertation, whole-cell patch-clamp recordings were used to examine the acetylcholine-activated ionic currents (I_{ACh}) in acutely dissociated leg motoneurons of the stick insect *Carausius morosus*. To ensure an unequivocal identification of the motoneurons, their somata were backfilled with a fluorescent dye prior to dissociation. After a pharmacological characterization of ionic currents induced by exogenous application of acetylcholine (ACh) and related ligands, estimations of the relative calcium contribution for I_{ACh} were determined by using optical imaging techniques. Finally, the possible functional interactions of insecticides or biogenic amines with these cholinergic currents were investigated. Taking all this together, the results described in this dissertation provide important insights into the ACh signaling of the stick insect's leg sensory-motor system, that has served as a very successful model to investigate basic principles of walking on the network level.

1 Identification of leg motoneurons in cell culture

Dextrans conjugated with a fluorescent label have been used successfully as dyes for retro- and anterograde labeling of living neurons (Heidel and Pflüger 2006; Kloppenburg and Hörner 1998; Lakes-Harlan et al. 1998; Mentel et al. 2008; Schmued et al. 1990; Westmark et al. 2009). Here tetramethlyrodamine-dextran was used to label leg motoneurons by conventional backfilling *via* the *nervus cruris*. The motoneurons' cell bodies and their major neurites were easily detected in the intact ganglion, revealing all the previously described major morphological features of these neurons (Debrodt and Bässler 1989; Goldammer 2008; Storrer et al. 1986). DUM neurons and inhibitory motoneurons that were also labeled *via* the *nervus cruris* could be easily differentiated from the motoneurons by their soma size. The dye did not leak out of the neurons nor did it show any significant bleaching or phototoxicity during the brief exposure to UV light for cell identification. In agreement with previous works (Heidel and

Pflüger 2006; Kloppenburg and Hörner 1998; Kloppenburg et al. 1999; Westmark et al. 2009) the labeling procedure did not cause obvious changes in the electrophysiological membrane properties. Current-clamp recordings from tetramethylrhodamine-dextran-labeled motoneurons had similar resting potentials and ACh-induced currents as unlabeled cultured neurons (Westmark et al. 2009) and they had similar resting potentials as leg motoneurons in the intact ganglion (compare with Schmidt et al. 2001).

2 Characteristics of ACh-induced currents

In more than 90% of the investigated motoneurons, ACh induced an inward current. The concentration-response parameters of I_{ACh} were well within the range reported for other insect neurons (Locust: Hermsen et al. 1998; van den Beukel et al. 1998. Honey bee: Barbara et al. 2005, 2008; Goldberg et al. 1999; Wüstenberg and Grünewald 2004. Cockroach: David & Sattelle 1984. Fly: Albert and Lingle 1993; Brown et al. 2006; Vömel and Wegener 2007; Wegener et al. 2004). Patch-clamp recordings combined with optical Ca^{2+} imaging revealed that a considerable fraction of I_{ACh} is carried by Ca^{2+} (~ 18 %). This Ca^{2+} influx is of great interest because Ca^{2+} can act as a second messenger (Berridge et al. 2000; Bootman et al. 2002) and could, for example, influence the cellular excitability by activation of Ca^{2+}-dependent ion channels (Catterall 1998; Klink and Alonso 1997; Lanzafame et al. 2003; Taylor and Peers 2000; Vernino et al. 1992; Vernino et al. 1994). Such a mechanism might mediate the sustained depolarization of leg motoneurons during step sequences of foreleg as reported by Ludwar et al. (2005) and Westmark et al. (2009). The Ca^{2+} permeability observed in this study (~ 18%) was well within the range reported in vertebrate nAChRs (Delbono et al. 1997; Fucile et al. 2006) and agreed with the high calcium permeability suggested in Barbara et. al. (2008) of honey bee nAChRs.

I_{ACh} consisted of fast-desensitizing and/or slow-desensitizing inward currents. 80% of the neurons generated both the fast-desensitizing and slow-desensitizing components, clearly demonstrating that leg motoneurons possess functional ACh receptors. Components of I_{ACh} with marked differences in desensitization time constants have been observed in other insect neurons (Honey bee: Barbara et al. 2008. Cockroach: Salgado and Saar 2004. Locust: Hermsen et al. 1998, van der Beukel et al. 1998) and in the single channel current conductance (Fly: Albert and Lingle 1993. Cockroach: Beadle et al. 1989).

While the fast-desensitizing component was usually larger compared to the slow-desensitizing component, their ratios were variable between cells. Although not addressed in detail in this study, the findings obtained here suggest that there are sub-types of leg motoneurons with distinct ACh receptor compositions, possibly resulting in distinct synaptic properties. From insect motor systems, it is known that within any one, there are usually different classes of motoneurons (fast, slow, and semi fast motoneurons that innervate different muscle fiber types) that differ in their physiological properties (Burrows 1996; Gabriel et al. 2003; Parker 1996; Schmidt et al. 2001). Neuronal properties are largely determined by the types of ion-channels and receptors expressed, and by their rate of expression. It will be interesting in the future to determine with recordings from identified motoneurons in the intact ganglion, whether different ratios of receptors can be attributed to different sub-types of leg motoneurons, possibly reflecting their different physiological properties and function.

Nicotinic ligands

The findings that nicotine and imidacloprid induced I_{ACh}-like currents and that α-bungarotoxin (α-BGTX) blocked I_{ACh}, suggest that I_{ACh} is mediated by nicotinic acetylcholine receptors (nAChRs). The nicotine and imidacloprid concentration-response

relations were well within the range reported for many other insect neurons (Barbara et al. 2008; Campusano et al. 2007; Cayre et al. 1999; Déglise et al. 2002; Ihara et al. 2006; Vömel and Wegener 2007; Wegener et al. 2004; Wüstenberg and Grünewald 2004). The following results suggest that the two components, which desensitize with different time constants, are mediated by 2 different nAChRs: a) the amplitude ratios of fast-desensitizing (I_{ACh1}) and slow-desensitizing (I_{ACh2}) ACh-induced currents were variable between different motoneurons, suggesting independent variability in channel densities. b) The form and EC_{50} values of the concentration-response curves for several nAChR ligands were different between I_{ACh1} and I_{ACh2}.

At equal ligand concentrations, similar amplitudes for I_{ACh1} and fast-desensitizing nicotine-induced current (I_{NIC1}) were found. The slow-desensitizing nicotine-induced current (I_{NIC2}), however, had a smaller amplitude than the I_{ACh2}. These results suggest a differential affinity of nicotine to the receptor subtypes, with nicotine being a full ACh agonist for the receptors mediating I_{ACh1}, but only a partial agonist for the receptors mediating I_{ACh2}. Differential effects of nicotine on receptor subtypes have been described previously in several insect neuron types, in which nicotine can act as a full or better ACh agonist (Cayre et al. 1999; Courjaret and Lapied 2001; David and Pitman 1993; Lapied et al. 1990; Trimmer and Weeks 1989) or as a partial ACh agonist (Albert and Lingle 1993; Barbara et al. 2005; Beadle et al. 1989; Déglise et al. 2002; Van Eyseren et al. 1998). In some studies, it has been observed that nicotine acted as a partial ACh agonist, but with equal or even lower potency than ACh (Honey bee: Barbara et al. 2008; Wüstenberg and Grünewald 2004. Drosophila: Brown et al. 2006; Vömel and Wegener 2007; Wegener et al. 2004). While α-BGTX blocked both components of I_{ACh} completely, it had a higher affinity to the nAChR that generated I_{ACh2}. Differential affinity of α-BGTX to nAChR sub-types has been reported by Salgado and Saar (2004) in cockroach thoracic neurons, in which α-BGTX had a higher affinity to the nAChR sub-type that generated a non-desensitizing I_{ACh}.

Muscarinic ligands

Even high concentrations of muscarine and 'classical' muscarinic agonists (oxotremorine and pilocarpine) did not induce any current under the experimental conditions used in this study, showing that mAChRs did not contribute to I_{ACh}. While many binding, immunohistochemical and systemic electrophysiological studies on insect neurons suggested the expression of mAChR (Binding studies: Abdallah et al. 1991; Honda et al. 2007; Liu and Casida 1993; Onai et al. 1989; Orr et al. 1991; Qazi et al. 1996; Shapiro et al. 1989. Immunolabeling studies: Blake et al. 1993; Cui-ping et al. 2008; Harrison et al. 1995; Shirai et al. 2001. Systemic electrophysiological studies: Büschges et al. 1995; Corronc and Hue 1993; Johnston and Levine 2002; Trimmer and Weeks 1989; Trimmer 1995), the results obtained in this dissertation are in agreement with many previous studies on dissociated insect neurons, in which 'classical' mAChR agonists induce only very small or no ionic currents (Albert & Lingle 1993; Cayre et al. 1999; Hermsen et al. 1998; van Eyseren et al. 1998, Wüstenberg & Grünewald 2004).

Provided that the cholinergic receptor on the motoneuron cell bodies and on the dendritic regions have similar pharmacological properties (e.g., Buckingham et al. 1994; Fickbohm and Trimmer 2003; Harrow and Sattelle 1983), the finding that stick insect motoneurons do not appear to be equipped with muscarinic receptors that induce ionic currents indicates that neither the tonic depolarization observed in stick insect motoneurons during pilocarpine-induced rhythmic activity (Büschges 1998) nor the tonic depolarization observed in motoneurons during walking (Ludwar et al. 2005; Westmark et al. 2009) are related to ACh binding to motoneuron mAChRs. This would suggest that muscarinic action for generation of rhythmic activity in the stick insect locomotor system, and perhaps also in locomotor systems of other insects, is confined to premotor neurons.

However, the lack of muscarinic-induced currents might also be caused by the ex-

perimental conditions used in this investigation. If the mAChRs were exclusively expressed on the neurites, their currents would not be measured in their soma preparation used here. Because mAChRs are known to activate second messenger pathways (Caulfield 1993; Caulfield and Birdsall 1998; Felder 1995; Gregory et al. 2007; Lanzafame et al. 2003), it could be that much longer application times are necessary to activate mAChR-mediated ionic currents, or that mAChRs evoke no currents but instead modulate them, as suggested in Courjaret et al. (2003).

At 10^{-4} M, the mAChR antagonist atropine blocked I_{ACh} almost completely. Given this high concentration to achieve block, and the finding that muscarinic agonists did not induce any ionic current, the blocking effect appears to be not specifically muscarinic. In addition, no evidence for 'mixed' receptors as reported in cockroach DUM neurons (Lapied et al. 1990) or motoneurons (David and Pitman 1993) was observed in the results obtained in this dissertation. However, note that the 'mixed' receptors, observed by David and Pitman (1993), have been recorded at more depolarized holding potentials than in this study and that the effective concentrations have to be compared with great caution given the different methods of ligand application.

3 Neuromodulation of I_{ACh}

A full understanding of synaptic neurotransmission will never be achieved if the multiple and interacting modulatory effects of diverse signaling molecules will not be properly considered. In regard to the modulation of cholinergic neurotransmission in insects, only few investigations (Butt and Pitman 2002, 2005; David and Pitman 1996; Westmark et al. 2009) have focussed on the modulation of cholinergic currents by actions of neuromodulatory substances, such as biogenic amines and neurotransmitters.

In this dissertation, the modulatory effects of biogenic amines (octopamine and

serotonin) and muscarine (a plant alkaloid known to activate the mAChRs) upon cholinergic currents elicited in identified leg motoneurons by exogenous application of ACh were investigated.

Modulation of I_{ACh} by actions of biogenic amines

Bath applications of octopamine and serotonin did not induce any membrane currents but reduced the amplitude of ACh-induced currents (I_{ACh}) in a concentration-dependent manner, indicating that these biogenic amines act rather as modulators of excitatory cholinergic transmission in stick insects. The octopamine concentration that blocked 50 % (IC_{50}) of the fast-desensitizing ACh-induced current (I_{ACh1}) was lower than the octopamine IC_{50} observed to block cholinergic currents in cockroach neurons (Butt and Pitman 2002) but higher than the serotonin IC_{50} in this dissertation for both components of ACh-induced currents ($I_{ACh1}$and I_{ACh2}) and in other studies (Barajas-López et al. 2001; Butt and Pitman 2002; Fucile et al. 2002). The fact that octopamine and serotonin did not induce any membrane currents but abolished I_{ACh} with different affinities could indicate that these biogenic amines modulate the cholinergic currents by triggering different intracellular pathways. However, previous studies in cockroach motoneurons have reported that octopamine and serotonin most probably regulate cholinergic currents by activating the same intracellular signaling pathways (Butt and Pittman 2002).

Both, octopamine and serotonin, consistently suppressed the I_{ACh} in all motoneurons recorded. These findings are quite similar to those reported for a cockroach (Butt and Pitman 2002) and a locust (Leitch et al. 2003) motoneuron types. Other investigations on cholinegic synaptic transmission in insects, however, indicated that octopamine increased the ACh responsiveness of motoneurons while serotonin was responsible for down-regulating their sensitivity to ACh (Casagrand and Ritzmann

1992 b; Hill and Blagburn 2001). This additional evidence lead to the assumption that the modulation of cholinergic responses by action of biogenic amines might vary according to the type of neuron and synapse (i.e., sensory – interneuron, interneuron–interneuron, sensory–motoneuron and interneuron–motoneuron).

While serotonin and octopamine acted similarly on the cholinergic responsiveness of isolated stick insect motoneurons, the possibility of differential effects of these molecules on premotor neurons can not be discarded. Evidences for individual effects of octopamine among different leg motoneurons have been already described (Parker 1996). In locust, excitatory post-synaptic potentials (EPSPs) evoked by the fast extensor motoneuron in a fast flexor motoneuron were normally increased by octopamine. In contrast, EPSPs in slow flexor motoneurons were reduced by octopamine (Parker 1996). In a previous investigation, tonic depolarizations recorded in middle leg motoneurons and elicited by activation of foreleg stepping sequences were up-regulated by octopamine (Westmark et al. 2009), which might not be due to an up-regulation of the motoneurons' response to ACh. Such octopaminergic effect could be due to an octopamine-mediated increase in activity in premotor interneurons that in turn excites the recorded motoneurons (Westmark et al. 2009). Since the excitatory effect of octopamine on stick insect motoneurons usually prevails in semi-intact preparation conditions, octopaminergic actions on other neurons (e.g. sensory and dorsal unpaired neurons) has to be better elucidated.

Dorsal unpaired (DUM) and ventral unpaired (VUM) neurons are known to contain and release octopamine in insects (Roeder 1999). DUM neurons in crickets have been found to be active during walking (Gras et al. 1990), and an increased activity in mesothoracic DUM neurons in the stick insect has been shown during single middle leg walking (Mentel et al. 2008). While these DUM neurons are known to innervate leg muscles, it is unclear whether they release octopamine into the central nervous system. Terminals of middle leg sensory neurons may also release ACh and such re-

lease could be subject to modulation (Torkkeli and Panek 2002). Although most of the middle leg sense organs were removed with leg amputation in the *in situ* experiments described in Westmark et al. (2009), one can argue that the terminals of sensory neurons may still be functional for a while even though the sensory axons were cut. Therefore, an increase in tonic depolarization in motoneurons could be expected if octopamine increased the release of ACh from functional sensory terminals. However, the enhancing effects of octopamine on sensory neurons rather act peripherally than centrally (e.g., Bräunig and Eder 1998; Matheson 1997; Widmer et al. 2005).

Regulations of tonic depolarizations in stick insect motoneurons by actions of serotonin showed a more complex scheme. In contrast to the results obtained for octopamine under conditions where the central network stays intact, serotonin either increased or decreased the tonic depolarizations observed in these stick insect motoneurons (Westmark 2007). According with the results obtained in this dissertation, such opposing serotonergic effects might not be based on the motoneurons' response to ACh, since serotonin consistently suppressed the I_{ACh} in all motoneurons studied. Opposing effects of serotonin in invertebrates have been attributed to the activation of parallel intracellular signaling pathways through either different serotonin receptors (Bermudez et al. 1992; Tierney 2001) or different levels of a common initial second messenger (Teshiba et al. 2001). Serotonin may act presynaptically to decrease the amount of ACh released by sensory neurons in cockroaches (Hill and Blagburn 2001) but it has been found to increase EPSP amplitude at crayfish neuromuscular junctions by releasing more vesicles at a faster rate (Southard et al. 2000). In cockroach motoneurons, serotonin down-regulates postsynaptic nicotinic ACh responses by activating G-protein-dependent intracellular signaling pathways that are indepedent of intracellular Ca^{2+} concentrations (Butt and Pitman 2002, 2005).

Across insect species, responses regulated by serotonin (Baines et al. 1990; Blenau and Baumann 2001; Parker 1995; Tierney 2001) or octopamine (Achenbach et al. 1997;

Balfanz et al. 2005; Bischof and Enan 2004; Han et al. 1998; Parker 1996; Walther and Zittlau 1998) were mediated by cAMP-pathways. In stick insects, the involvement of cAMP pathways in an up-regulation of tonic depolarizations in leg motoneurons was achieved by bath-applying 8-Br-cAMP (Westmark et al. 2009). The results obtained in this dissertation, however, indicated that it was not based on direct up-regulation of motoneuron ACh responsiveness by 8-bromo-cAMP, since bath applications (2.0 x 10^{-6} M) of this membrane permeable cAMP did not alter the amplitude of the slow-desensitizing ACh-induced current (I_{ACh2}) but significantly suppressed I_{ACh1}. Under conditions where the central network stays intact, cAMP is more likely to be involved in up-regulating activity in interneurons that will depolarize motoneurons during stepping. Whether this upregulation is at some point associated with ACh is unclear. Such association is possible and has been shown in insects for the AC/PKA pathway and mAChRs and nAChRs (e.g., Thany et al. 2007; Trimmer 1995; Wenzel et al. 2002). In addition, Wenzel et al. (2002) have shown a positive coupling of mAChRs to AC in the grasshopper brain that led to an increased cAMP level. Therefore, specific octopamine- and serotonin-activated intracellular signaling cascades in stick insect motoneurons need to be explored.

Modulation of I_{ACh} by cholinergic ligands

Nicotine showed characteristics of a full ACh agonist for the receptors mediating I_{ACh1}, but acted only as an ACh partial agonist for the receptors mediating I_{ACh2} (see RESULTS, section 3.1). The amplitude of currents elicited by paired co-applications (*via* an U-tube application system) of ACh with nicotine corroborated to this assumption. Although not significantly different from the amplitude of I_{ACh}, co-application of ACh and nicotine differentially modified the amplitude of the fast-desensitizing co-application resulting currents ($I_{(ACh+NIC)1}$) and slow-desensitizing co-application re-

sulting currents ($I_{(ACh+NIC)2}$). $I_{(ACh+NIC)1}$ showed higher amplitudes than I_{ACh1}, while the amplitude of $I_{(ACh+NIC)2}$ was smaler than those observed to I_{ACh2}. Co-applications of muscarine with ACh or nicotine did not cause a current amplitude significantly different than that induced by ACh (or nicotine) alone. This indicates that muscarine did not compete for the binding site of ACh in the cholinergic receptors expressed in the motoneurons. However, because the acetylcholine fast- and slow-desensitizing currents ($I_{ACh1}$and I_{ACh2}, respectively) were significantly smaller when muscarine was bath applied (for 4 min) priorly the ACh application, the possibility of modulatory muscarine-triggered intracellular signaling cascades can not be excluded. Such regulation of nAChR-mediated responses by activation of mAChRs has been reported in vertebrates (Brown and Galligan 2003; Shen et al. 2009; Verbitsky et al. 2000; Zwart and Vijverberg 1997) as well as in invertebrates (Courjaret et al. 2003; David and Pitman 1996; Liu et al. 2007).

In insects, stimulation of muscarine-mediated intracellular pathways either increases or decreases currents elicited through activation of nAChRs. For example, in stick insect motoneurons (Westmark et al. 2009) and cockroach DUM neurons (Courjaret et al. 2003), up-regulations of cholinergic responses were achieved by triggering Ca^{2+}-dependent intracellular pathways. Attenuation of nAChR-mediated currents, however, was based on mAChR-mediated activation of Ca^{2+}-dependent intracellular pathways in cockroach motoneurons (David and Pitman 1996).

4 Actions of neonicotinoids in the I_{ACh}

Like imidacloprid, the recently discovered neonicotinoids used in this dissertation abolished the acetylcholine-induced currents (I_{ACh}) quite nicely, with similar efficacy than that showed by the nicotinic antagonist α-bungarotoxin (α-BGTX). neonicotinoids have been used as effective probes in structural (Matsuda et al. 2001; Nishiwaki

et al. 2003; Tomizawa and Casida 2003; Zhang et al. 2000) and funcitonal investigations of insect nAChRs (Bai et al. 1991; Brown et al. 2006; Ihara et al. 2006; Salgado and Saar 2004). Comparing the efficacies of neonicotinoids to activate nAChRs, not all compounds of this insecticide class elicited the same response pattern (Brown et al. 2006; Ihara et al. 2007b; Tan et al. 2007; Toshima et al. 2008). The diverse actions of neonicotinoids depend on the structure of the neonicotinoids as well on the subunit composition of the nAChRs tested. In insects, more than 10 nAChR genes have been cloned, and post-translational modification of subunit mRNAs has been demonstrated (Grauso et al. 2002; Lansdell and Millar 2002; Jones et al. 2005; Rinkevich and Scott 2009; Sattelle et al. 2005), suggesting the existence of many nAChR subtypes. In general, neonicotinoids are nAChR agonists and the compounds containing the nitroguanidine and chemically-related moieties (e.g. clothiadin) exhibit higher agonist efficacy than the neonicotinoids with the imidazolidine ring structure (e.g. imidacloprid) (Brown et al. 2006; Ihara et al. 2004; 2007b). Antagonistic actions, however, of some neonicotinoids have been described (Ihara et al. 2007a; Zhang et al. 2008).

All the neonicotinoid compounds used here acted slightly more efficiently upon the slow-desensitizing acetylcholine-induced current (I_{ACh2}), which corroborates the hypothesis that the stick insect motoneurons express more than one nAChR receptor type. Similar block action of neonicotinoids (imidacloprid and clothianidin) on desensitizing ACh-induced currents were found in cockroaches neurons (Salgado and Saar 2004). While the results obtained in this dissertation clearly showed that the I_{ACh} were conducted by at least two desensitizing nAChR subtypes, it did not allow an idetification of a non-desensitizing ACh-activated receptor type, as observed in cockroaches neurons (Salgado and Saar 2004; Tan et al. 2007).

5 Concluding remarks

The approach used in this study allowed the investigation of acetylcholine induced currents of unequivocally identified adult leg motoneurons under biophysically controlled conditions. Whole-cell patch-clamp recordings showed that somata of leg motoneurons posses functional AChRs. This study provides solid evidence that the motoneurons possess at least 2 types of nicotinic receptors that mediate current components with differing rates of desensitization. The functional roles of these receptors have yet to be verified in preparations with functionally intact synapses. Using quantitative Ca^{2+} imaging, it was found that ~ 18 % of I_{ACh} is carried by Ca^{2+}. Thus I_{ACh} contributes not only to the membrane potential, but might also induce Ca^{2+} triggered processes.

The biogenic amine octopamine abolished the I_{ACh}, indicating that the enhancing effects of octopamine observed in stick insect motoneurons in previous investigations, where the central network stays intact (Westmark et al. 2009), are not based on a direct upregulation of motoneuron responses to ACh. Serotonin consistently reduced the I_{ACh} in all neurons recorded. These results suggest that serotonin might activate counteracting effects among the neurons involved in the regulation of tonic depolarizations observed in stick insect motoneurons during walking (Westmark 2007). Imidacloprid and the recently discovered neonicotinoids used in this dissertation reduced I_{ACh} with high efficacy, but did not allow a pharmacological separation of I_{ACh} types as suggested in Salgado and Saar (2004). The expression of non-desensitizing ACh-activated currents was not detected in this dissertation, which might explain the similar efficacies of these neonicotinoids in the stick insect motoneurons.

The results obtained in this dissertation represent an important prerequisite to better understand cholinergic synaptic transmission in the sensory-motor system of the stick insect, which has served very successfully as a model for walking and locomotion. Future studies will have to focus on the analysis of transmitter receptors on

the dendrites of motoneurons, as well as on premotor interneurons, in order to unravel network interactions underlying walking pattern generation. In addition, it will be important to unravel the functional consequences of the increase of intracellular Ca^{2+}- elicited *via* cholinergic inputs.

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Appendix

1 Supplemental Methods

Calibration of the Ca^{2+} selective electrode

To calibrate the Ca^{2+} selective electrode we followed closely the approach described by McGuigan et al. (1991). To calibrate the Ca^{2+} selective macroelectrode five calibration solutions were prepared containing (in mM): 140 KCl, 2.5 KOH, 15 NaCl, 1 $MgCl_2$, 5 HEPES and 10, 4, 1, 0.4, 0.2 $CaCl_2$, respectively. Additionally an "EGTA solution" containing (in mM): 129.5 KCl, 13 KOH, 15 NaCl, 1 $MgCl_2$, 5 HEPES and 4 EGTA and a "Ca-EGTA solution" containing (in mM): 129.5 KCl, 13 KOH, 8 NaCl, 7 NaOH, 1 $MgCl_2$, 5 HEPES, 4 EGTA and 4 $CaCl_2$ were prepared. All solutions were adjusted to pH 7.2 with HCl. Twelve EGTA : Ca-EGTA solutions were mixed in ratios of: 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 3:1, 5:1, 9:1. Using a Ca^{2+} selective macroelectrode (Ca 800, Wissenschaftlich- Technische Werkstätten GmbH, Weilheim, Germany), the potentials of the 5 Ca^{2+} macroelectrode calibrating solutions, as well as the 12 EGTA : Ca-EGTA mixtures were measured, in order from high to low free Ca^{2+} concentration. After measurement of the last solution, the potential of the initial solution was measured again to check for a drift of the macroelectrode. The potentials were then drift corrected. The measured potentials in the Ca^{2+} calibration solutions can be described by the Nernst equation and can thus be used to determine the slope of the electrode (change in potential per change in 10 fold Ca^{2+} concentration in mV • pCa^{-1}). However, the potentials of the buffer solutions can not be described by the Nernst equation, because their free Ca^{2+} concentrations are in the nanomolar range, where the slope of the electrode becomes nonlinear. In this case the Nikolsky-Eisenmann equation relates the measured potentials in the buffer solutions to the free

Ca^{2+} concentrations (Kay et al. 2008):

$$E = E^0 + s\,log\left(\left[X^{2+}\right] + \sum\right)$$

E is the measured potential of the electrode in mV, E^0 is the standard electrode potential in mV, s is the slope of the electrode in mV • pCa^{-1}, $\left[X^{2+}\right]$is the free Ca^{2+} concentration in mol • l^{-1} and $\sum$ is an interference constant accounting for nonlinearity of the slope in the nanomolar range in mol • l^{-1}. The $\left[X^{2+}\right]$can be calculated from the total added Ca^{2+} concentration $[X]_T$, the total EGTA concentration $[ligand]_T$ and the EGTA dissociation constant K_{app}:

$$\left[X^{2+}\right] = \frac{b + \sqrt{b^2 + 4K_{app}\,[X]_T}}{2}$$

where,

$$b = -\left(K_{app} + [Ligand]_T - [X]_T\right)$$

There are three unknown parameters, which must be calculated, namely $[Ligand]_T$, K_{app}, and $\sum$ in the Nicolsky-Eisenman equation. In Luthi et al. (1997), a cyclic iterative scheme was used to calculate the $[Ligand]_T$ and K_{app} parameters, but a fixed value for the parameter $\sum$ (see McGuigan et al. [2006] for details) was used. Once these parameters are known, the free Ca^{2+} concentration of the buffer solutions can be calculated with the Nikolsky-Eisenman equation. All calculations were performed in R using the 'R function' ALE (Automatic determination of Ligand purity and Equilibrium dissociation constant, Kay et al.[2008]).

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Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium ion concentration
4-AP	4-aminopyridine
ACh	Acetylcholine
BAPTA	1,2-bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid
cAMP	Cyclic adenosine monophosphate
EC_{50}	Concentration that blocks half of the maximal currrent
EGTA	Ethylene diamine tetraacetic acid
IC_{50}	Concentration that blocks half of the maximal current
I_{ACh}	Acetylcholine-induced currents
$I_{(\mathrm{ACh+MUSC})}$	Currents elicited by co-application of ACh and muscarine (MUSC)
$I_{(\mathrm{ACh+NIC})}$	Currents elicited by co-application of ACh and nicotine (NIC)

I_{Ca}	voltage activated calcium (Ca) currents
I_{IMI}	Imidacloprid-induced currents
I_{NIC}	Nicotine-induced currents
$I_{(NIC+MUSC)}$	Currents elicited by co-application of NIC and MUSC
IMI	Imidacloprid
mAChR	Muscarinic acetylcholine receptor
nAChR	Nicotinic acetylcholine receptor
ncr	Nervus cruris
TEA	Tetraethylammonium chlorid
TTX	Tetrodotoxin

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut und Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von oben angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Ansgar Büschges betreut worden.

Köln, den 08.02.2010

List of Publications

Articles

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- April 2006- Februar 2010: Doktorarbeit an der Universität zu Köln, Abteilung für Tierphysiologie; Betreuer: Prof. Dr. Ansgar Büschges. Titel der Doktorarbeit:*"Properties of cholinergic currents in identified leg motoneurons of the stick insect Carausius morosus"*

- Juni 2007 - Juli 2007: Teilnahme am Sommerkurs „Neuroethology: The Neural Basis of Natural Behavior Using an Invertebrate System " an den Friday Harbor Laboratories in San Juan Island-WA, USA.

- März 2003 - Februar 2005: "Master Science Degree (MSc.)" in Entomologie an der Universidade Federal de Viçosa-MG (Brasilien); Betreuer: Prof. Dr. Raul Narciso C. Guedes. Titel der Masterarbeit: *"Competition among pyretroide resistants and susceptible strains of Sitophilus zeamais Motschulsky (Coleoptra: Curculionidae)". (in Portuguese).*

- März 1998- Februar 2003: "Bachelor Science Degree (BSc.)" in Agrarwissenschaften an der Universidade Federal de Viçosa-MG (Brasilien).

Auszeichnungen

- 2009 - Monsanto Company: Ausgezeichnet mit dem zweiten Platz des brasilianischen Agrar-Umweltschutz Monsanto Preises
- 2003 - Agrarwissenschaftskollegium der Universidade Federal de Viçosa-MG (Brasilien): Anerkennung für ausgezeichnete Studienleistungen während des "Bachelor Science Programms" in Agrarwissenschaften
- 2002 - Brasilianischer Ausschuss für Aktionen im Bereich Insektizidenresitenzen (IRAC-BR): Beste Grundstudiumsarbeit im Bereich Insektizidenresitenzen präsentiert auf der XIX. Bienalle des brasilianischen entomologischen Geselschaft (XIX CBE)

Forschungsstipendien

- 2006 - 2010: Brasilianisches Erziehungministerium (CAPES)/ Deutscher Akademischer Austauch Dienst (DAAD) – Ph.D Sipendium
- 2003-2005: Brasilianischer Forschungsrat (CNPq) – MSc. Stipendium
- 2001-2002: Stiftung zur Förderung der Forschung im brasilianischen Bundesstaat Minas Gerais (FAPEMIG) – Forschungsprojektstipendium
- 1999-2001: CNPq – Forschungsbeginn Stipendium

Berufserfahrung

- 2007 - 2009: Praktikumsbetreuung (eingeladen) in Grund- und Hauptstudiumskursen (Bachelor- und Masterkursen) in Tierphysiologie / Neurobiologie an der Universität zu Köln
- Sommer 2004: Grundstudiumvorlesungen (eingeladen) über Prinzipien ökotoxikologischer Verschmutzung an der Universidade Federal de Viçosa-MG (Brasilien).

Curriculum vitae

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Education

- April 2006 - February 2010: **Ph.D** in the department of Animal Physiology (Neurophysiology) – University of Cologne – Cologne - Germany; Advisor: Prof. Dr. Ansgar Büschges. Ph.D Thesis Title: *"Properties of cholinergic currents in identified leg motoneurons of the stick insect, Carausius morosus"*

- June 2007- July 2007: Participation at the summer course „Neuroethology: The Neural Basis of Natural Behavior Using an Invertebrate System " at Friday Harbor Laboratories in San Juan Island-WA, USA.

- March 2003 - February 2005: "Master Science Degree (**MSc.**)" in Entomology– Federal University of Viçosa – Viçosa-MG - Brazil; Advisor: Prof. Dr. Raul Narciso C. Guedes. Master Thesis: *"Competition among pyretroide resistants and susceptible strains of Sitophilus zeamais Motschulsky (Coleoptra: Curculionidae)". (in Portuguese)*.

- March 1998 - February 2003: "Bachelor Science Degree (**BSc.**)" in Agriculture at the Universidade Federal de Viçosa-MG (Brazil).

Awards

- 2009 - Monsanto Company: awarded Brazilian second-best agro-environmental Monsanto Research Award
- 2003 - College of Agriculture of Federal University of Viçosa (Brazil): honor by excellent student performance in agriculture undergraduate program
- 2002 - Brazilian Insecticide Resistance Action Committee (IRAC-BR): Best undergraduate paper on insecticide resistance management, XIX biennial Brazilian Congress of Entomology (XIX CBE)

Scholarship and Fellowships

- 2006 - 2010: Brazilian Ministry of Education Agency (CAPES)/ Germany Academic Exchange Service (DAAD) Fellowship – Ph. D Scholarship
- 2003-2005: Brazilian Council of Scientific and Technological Development (CNPq) Fellowship – MSc. Scholarship
- 2001-2002: Research Foundation of Minas Gerais State (FAPEMIG) Fellowship – Research project
- 1999-2001: CNPq fellowship - Scientific initiation

Professional Experiences

- 2007 - 2009: assistant teacher (invited), undergraduate and graduated courses in animal physiology/neurophysiology – University of Cologne - Germany
- Sommer 2004: Undergraduate classes (invited) in pollutant ecotoxicological principles - Federal University of Viçosa – Brazil