# Anatomical and chemical approaches to the development of model insect nervous systems

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**The more I see the less I know for sure.**

John Lennon

for family and friends

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## Zusammenfassung

Heuschreckenembryonen sind dazu geeignet, die Entwicklung des Nervensystems an einzelnen, identifizierten Nervenzellen zu untersuchen. In meiner Arbeit habe ich mögliche Auswirkungen des Neurotransmitters Histamin auf die Morphologie histaminerger Neurone und den Einfluss des Botenstoffes Stickstoffmonoxid (NO) auf die Navigation auswachsender Nervenzellen untersucht. Histamin ist der Neurotransmitter der Lichtsinnesrezeptoren bei Insekten, über das Verteilungsmuster histaminerger Zellen in der ventralen Ganglienkette ist aber nur wenig bekannt. Immunzytochemische Markierungen ermöglichen die Beobachtung der Entwicklung histaminerger Nervenzellen in den beiden Heuschreckenarten S. gregaria und L. migratoria. Erste Zellen erscheinen im Gehirn nach etwa 50% der Embryonalentwicklung und entlang der ventralen Ganglienkette ab 55%. Im Unterschlundganglion liegt ein Paar histaminerger Nervenzellen an ventro-lateraler Position. Außer dem prothorakalen Ganglion enthält jedes weitere thorakale und abdominale Ganglion ein Paar histaminerger Nervenzellen an ventro-medialer Position. Der Fortschritt des Neuritenwachstums und die Zunahme des Verzweigungsmusters wurde während der Embryonalentwicklung verfolgt und ähnelt kurz vor dem Schlupf dem adulten Verteilungsmuster. Da keine Evidenz für eine Histaminaufnahme im ZNS gefunden wurde, ist eine morphogene Wirkung von Histamin während der Embryonalentwicklung unwahrscheinlich.
Zyklische Nukleotide sind wichtige intrazelluläre Modulatoren von richtungsweisenden Signalen auf auswachsende Nervenzellen. Durch die NO-vermittelte Signalkaskade wird intrazellulär zyklisches Guanosinmonophosphat (cGMP) erhöht. Mit sensiblen Nachweismethoden kann cGMP in einigen früh auftretenden Pionierneuronen im zentralen und peripheren Nervensystem erkannt werden. Pionierneurone legen die ersten Verbindungen im Nervensystem. Nervenzellen aus dem Tibiasegment des Beines wachsen entlang eines Pfades mit zwei Richtungswechseln ins ZNS. Die Gegenwart chemischer Liganden, die auf die NO-Signalkaskade wirken, führt zu Fehlern beim Richtungswechsel. Durch Stimulation der löslichen Guanylatzyklase oder die Zugabe von membranpermeablem cGMP wurde das normale Aussehen wieder hergestellt. Änderungen von zyklischem Adenosinmonophosphat führen zu ähnlichen Fehlern im gerichteten Auswachsen. Pionierneurone im Bein werden durch räumliches und zeitliches begrenztes Auftreten von Semaphorinmolekülen geleitet. Störungen des Gehalts an zyklischen Nukleotiden bewirken fehlerhaftes Auswachsen. Meine Ergebnisse lassen daher auf eine wichtige Rolle zyklischer Nukleotide auf die Modulation von Richtungssignalen schließen.

**Schlagwörter**: axonale Navigation, Signalmoleküle, zyklische Nukleotide

## Abstract

Locust embryos are attractive model organisms to study the embryonic development of the nervous system at the level of identified neurons. In my thesis, I investigated putative morphogenetic actions of the transmitter histamine on histaminergic neurons and the atypical messenger nitric oxide on navigating growth cones. Histamine is the neurotransmitter of insect photoreceptor cells, but little information is available about the distribution pattern of histaminergic neurons in the locust ventral nerve cord. The onset and progress of histamine immunoreactivity (HA-IR) in the locust species *S. gregaria* and *L. migratoria* was observed. First HA-IR neurons appeared in the brain around 50% of embryonic development. Along the ventral ganglia chain HA-IR neurons could be reliably traced from 55% on. In the suboesophageal ganglion a pair of HA-IR cell bodies was located in a posterior-lateral position. Except for the prothoracic ganglion, a pair of HA-IR cell somata was ventro-medially located in each thoracic and abdominal ganglion. The progress of neurite extension and neuromere innervation could be followed through the embryonic stages till the mature pattern was expressed. Because I was unable to find evidence for direct histamine uptake into histaminergic neurons of the ventral ganglia, putative morphogenetic actions of histamine seem to be unlikely during early embryonic development.
Cyclic nucleotides are important modulators of guidance signals in navigating growth cones. The nitric oxide (NO)-signaling cascade is a potent mechanism to enhance cyclic GMP. Sensitive immunological staining enabled to detect cyclic guanosine monophosphate (cGMP) in response to nitric oxide in a subset of early arising central and peripheral pioneer neurons. Pioneer neurons are the first to establish connections within the developing nervous system. A pair of peripheral neurons emerges in the tibial segment of the limb. They grow along a highly stereotyped pathway which comprises two sharp turns. Exposure of cultured embryos to chemical ligands that affect NO-signaling caused severe pathway disruptions. Stimulation of soluble guanyly cyclase or application of a membrane permeant cGMP analog could rescue the normal morphology. Similar pathway disruptions were found when the formation of cyclic adenosine monophosphate (cAMP) was affected. Pioneer neurons in the limb are guided by spatio-temporal expression patterns of semaphorin proteins. Perturbation of cyclic nucleotide levels affected this directed outgrowth. These findings suggest an essential role for cyclic nucleotides in the modulation of growth cone responses to these extracellular signals.

**Keywords**: cyclic nucleotides, guidance cues, growth cone navigation

## List of abbreviations

| | |
|---|---|
| %E | percentage of embryonic development |
| A4 | abdominal gangion number 4 |
| AC | adenylate cyclase |
| ACh | acetylcholin |
| AChE | acetylcholin esterase |
| cAMP | cyclic adenosine 5`-monophosphate |
| cGMP | cyclic guanosine 5`-monophosphate |
| CI | common inhibitor |
| ChAT | cholin acetyl transferase |
| CNS | central nervous system |
| DMSO | dimethyl sulfoxide |
| DUM | dorsal unpaired median neuron |
| EDAC | 1-ethyl-3-(3`-dimethylaminopropyl) carbodiimide |
| Fas | fasciclin |
| FETi | fast extensor tibiae |
| Fig. | figure |
| GMC | ganglion mother cell |
| GTP | guanosine 5`-triphosphate |
| HA | histamine |
| HA-IR | histamine immunoreactivity |
| HRP | horseraddish peroxidase |
| IBMX | 3-isobutyl-methylxanthine |
| IR | immunoreactivity |
| L-15 | Leibowitz 15 cell culture medium |
| Meso | mesothoracic ganglion |
| Meta | metathoracic ganglion |
| min | minute |
| MP | midline precursor |
| NADPH | reduced nicotinamid adenine dinucleotide phosphate |
| NAME | nitro-arginin-methylester |
| NB | neuroblast |
| NGS | normal goat serum |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| NRS | normal rabbit serum |
| ODQ | 1H-(1,2,4)-oxadiazolo[4,3-a]quinoxalin-1-one |
| PBS | phosphate buffered saline |
| PDE | phosphodiesterase |
| PLP | posteriolateral cell cluster in the protocerebrum |

| | |
|---|---|
| PN | pioneer neuron |
| PNS | peripheral nervous system |
| PP IX free acid | protoporphyrin IX free acis |
| Pro | prothoracic ganglion |
| PTIO | 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide |
| PTX | PBS supplemented with Triton X-100 |
| SERT | serotonin uptake transporter |
| SETi | slow extensor tibiae |
| Sema | semaphoring |
| SNP | sodium nitroprusside |
| sGC/s-GC | soluble guanylate cayclase |
| SOG | suboesophageal ganglion |
| STO | stomodaeum |
| Ti1 | pioneer neuron of the tibial segment of the limb |
| TG | terminal ganglion |
| VNC | ventral nerve cord |

# 1. Introduction

Development is a continuing process of changes. Starting with an undifferentiated array of cells, ongoing proliferation and differentiation affect the internal embryonic environment. Because newly arising cells have to integrate into this changing environment, there is a need for transiently expressed compounds which regulate certain processes for individual development. Such signals are provided by the surrounding environment acting on the individual development. Such ectopic signals can serve as checkpoint control system for particular ongoing developmental phases (Ben-Ari and Spitzer, 2010). The absence of the signals from adjacent cell populations would then lead to an arrest of a particular developmental phase. Observations on the development of the relatively simple organized insect nervous system provide large advantages for studying distinct developmental processes. The insect central nervous system (CNS) consists of a frontal brain and a ventral nerve cord (VNC) with single ganglionic masses corresponding to each body segment. All ganglia are linked longitudinally by paired connectives. Afferent and efferent fibers connect to the ganglia via peripheral nerves (Fig. 1).

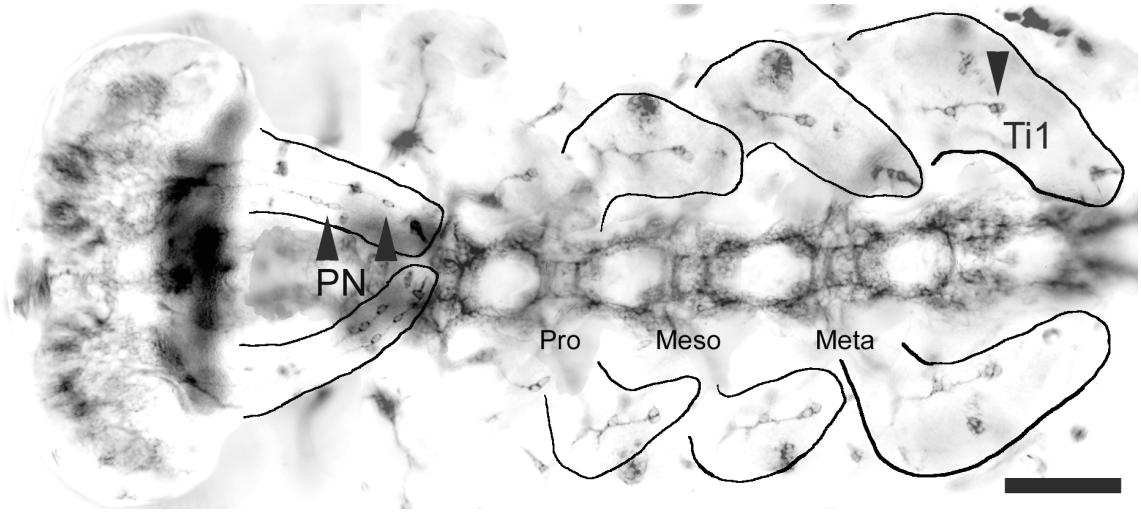


**Figure 1. Locust embryo around 40%E**
The brain and the ventral ganglia chain can be distinguished from the surrounding tissue. The thoracic ganglia (Pro-, Meso, Meta-) receive afferent connections by sensory neurons (Ti1 in the limb, PN in the antenna). Horseradish peroxidase (HRP)-labeling that selectively detects neuronal tissue (Jan and Jan, 1982; Haase et al. 2001). Anterior to the left, scale bar: 100 µm

## 1.1 Formation of the nervous system

The CNS is formed by progenitor cells that emerge from the ventral neurogenic zone. Starting around 30% of development (%E) lateral neuroblasts (NB) and midline precursors (MP) differentiate (Bate, 1976a; Boyan et al., 1995; Shepherd and Bate, 1990). Thoracic NBs arise in a highly ordered fashion along distinct rows and columns. This enables to identify single NBs and to follow their neuronal progeny (Fig. 2A and B). Lateral NBs give rise to a line of ganglion mother cells (GMC) which undergo a final cell division to give rise to sibling neurons (Doe and Goodman, 1985; Goodman and Bate, 1981; Thomas et al., 1984). Each neuron develops further individually (Fig. 2C), indicated by their distinct neurotransmitter and the kind of axonal projections (Fig. 2D) (Bastiani et al., 1984). A different mode of neurogenesis is performed by midline precursors. Their lineage is direct with a single cell division that gives rise to two prospective neurons (Doe and Goodman, 1985). Prominent members of MP progeny are the MP1 and MP2 neurons that pioneer the first longitudinal pathways (Fig. 2D) along the developing CNS (Bate and Grunewald, 1981). Peripheral neurons emerge from epithelial progenitors, e.g. in the antenna and in the developing limbs (Bate, 1976b; Keshishian, 1980; Fig. 1).

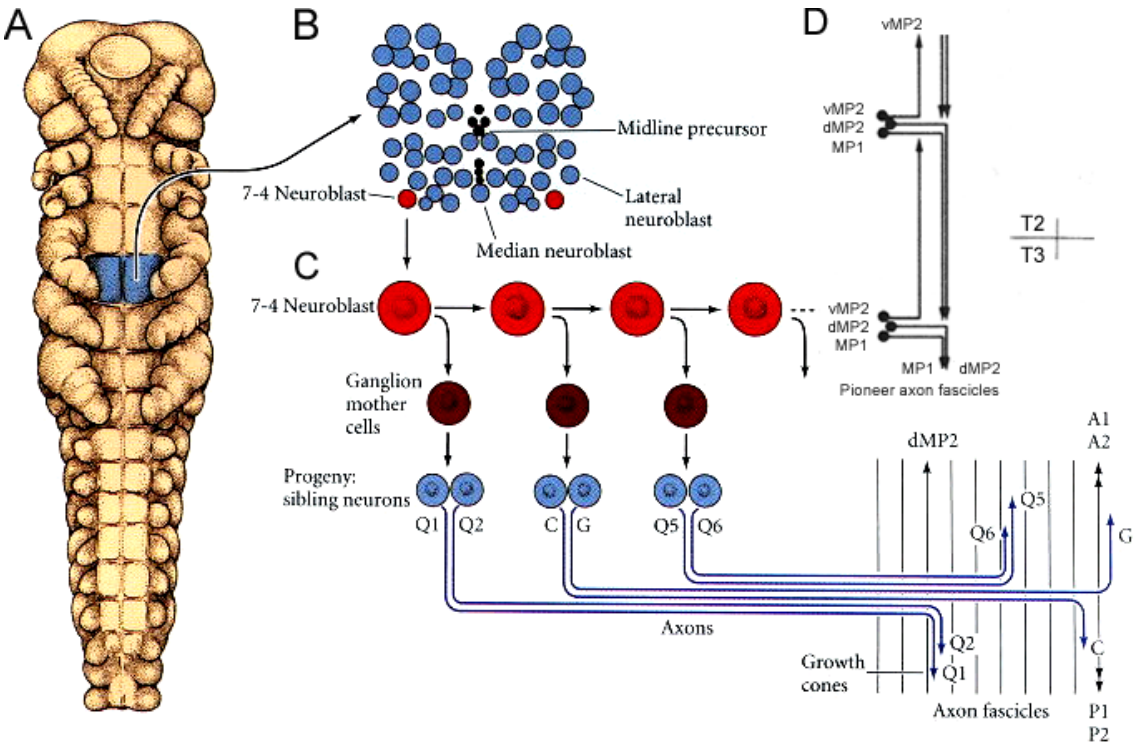


**Fig. 2. Neurogenesis in the locust embryo.**
**A** Ventral view of an embryo at around 35% of development. **B** Neuroblast map of a thoracic segment. Laterally reversed neuroblasts of both hemispheres (blue) are arranged in distinct rows and columns. Unpaired midline precursors arise along the ventral midline (black). They give rise to two midline precursor neurons by a single cell division. **C** Contrarily, each lateral precursor produces a line of GMCs (brown). Neuronal progeny develops individually. As an example the lineage of NB 7-4 is

depicted with such different neurons as, e.g. Q1, Q2, C, G, etc., choosing different axon fascicles. **D** Pioneer axon fascicles of the MP1 and MP2 neurons. T2 and T3 indicating for thoracic segment boundaries.
A-C Modified from.Goodman and Bastiani 1984. D Modified from Bastiani et al. 1987.

During the formation of the nervous system, neurons send out an axon to establish the connectivity of a functional nervous system. The formation of the major tracts and the wiring of the nervous system occur in a stereotyped way with remarkable accuracy (Shepherd and Bate, 1990). During development the growing axons are guided by extracellular cues provided by the surrounding tissue. Commonly, members of the netrin, slit, semaphorin, and ephrin families of guidance molecules are involved in particular growth cone guidance and target recognition (Charron and Tessier-Lavigne, 2005; Chisholm and Tessier-Lavigne, 1999; O`Connor, 1999, Raper, 2000, Tamagnone and Comoglio, 2000; Yu and Bargmann, 2001). Further effects were assumed for certain morphogens and neurotrophic factors (Charron et al., 2003). The fine tuning of growth cone responses to distinct cues is orchestrated by spatially and temporally restricted synthesis of guidance molecules and by their specific receptors on growth cone filopodia. For instance, axons with contralateral projections crossing the midline should not return back into the ipsilateral side. Coordinated expression of receptors to certain cues thus directs axonal outgrowth and navigation (Rajagopalan et al., 2000).

In the first part, this thesis addresses trophic and morphogenetic actions on identified neurons by a distinct neurotransmitter.

### 1.2 The role of neurotransmitters in development

Apart from their role as mediators of neuronal communication, several neurotransmitters are thought to control developmental processes prior to the formation of synaptic connections. For example, biogenic amines are assumed to promote axonal survival, axonal outgrowth, and axonal navigation in both vertebrate and invertebrate embryogenesis (Buznikov et al., 1995, Buznikov et al., 1996; Daubert and Condron, 2010; Haydon et al., 1987; Koert et al., 2001; Nguyen et al., 2001).

The biogenic amine serotonin, for instance, can serve as a chemical signal for the development and regeneration of distinct neurons. The presence of serotonin in the medium leads to the inhibition of axonal elongation of cultured *Helisoma* neurons (Haydon et al., 1987). Moreover, serotonin is important during early embryogenesis. In the sea urchin, serotonin is essential for gastrulation and neurogenesis (Buznikov et al., 2001). Further actions are evident in cell proliferation and migration. Regenerating growth cones of serotonergic fibers in the pond snail *Lymnea* are retracted in the presence of serotonin, which would indicate for an

autocrine action of serotonin on serotonergic axons (Koert et al., 2001).

### 1.3 Histamine in the insect nervous system

The biogenic amine histamine was surmised to play a likely role in insect nervous system formation. Unlike in vertebrates, this amine acts as a transmitter of certain sensory cells in arthropods (Nässel et al., 1990). Mainly, the fast synaptic transmission of photoreception is mediated via histamine gated chloride conductances (Hardie, 1987; 1988; Sarthy et al., 1991). Although histamine is present in vertebrate embryonic tissue and later in stem and cancer cells, its role in nervous system development remains unclear (Haas et al., 2008). Shifts in temporal and spatial expression of histamine could indicate for prospective actions of histamine during nervous system development (Häppöllä et al., 1991).

In insects, histamine is present in the CNS including the brain and all ventral ganglia (Elias and Evans, 1983). Despite the existence of detailed maps of histaminergic neurons in the locust brain (Gebhardt and Homberg, 2004), there is less information available about the ground pattern of histaminergic neurons and the developmental course of histamine immunoreactivity in the ventral nerve cord. To obtain precise information about a possible role in nervous system development, I decided to compare the spatio-temporal pattern of histamine immunoreactivity in the embryonic development of the two locust species *Schistocerca gregaria* and *Locusta migratoria*.

Apart from the investigation of potential actions mediated by histamine on the development of histaminergic neurons, I started with parallel investigations regarding the influence of cyclic guanosine 5`-monophosphate (cGMP) on growth cone navigation. Evidence emerges that growth cone responses to particular guidance cues are modulated by cyclic nucleotide contents in the growth cone (He et al., 2003; Yu and Bargmann, 2003). My approach is based on former findings about pioneer outgrowth in the antenna (Seidel and Bicker, 2000) which is affected by perturbation of the nitric oxide-cGMP signal transduction cascade.

### 1.4 Nitric oxide signaling

The nitric oxide (NO) signaling cascade is a potent signaling pathway to stimulate the formation of cyclic guanosin 5`-monophosphate in target cells. NO is a gaseous transmitter which regulates several developmental processes. The actions of NO are various including cell cycle regulation (Cheng et al., 2003; Kuzin et al., 1996), neuronal migration (Haase and Bicker, 2003), target recognition and synaptic patterning of photoreceptors (Gibbs et al., 2001). Additional effects can be observed on axonal regeneration (Stern and Bicker, 2008), and on outgrowth and elongation of antennal pioneer axons (Seidel and Bicker, 2000).

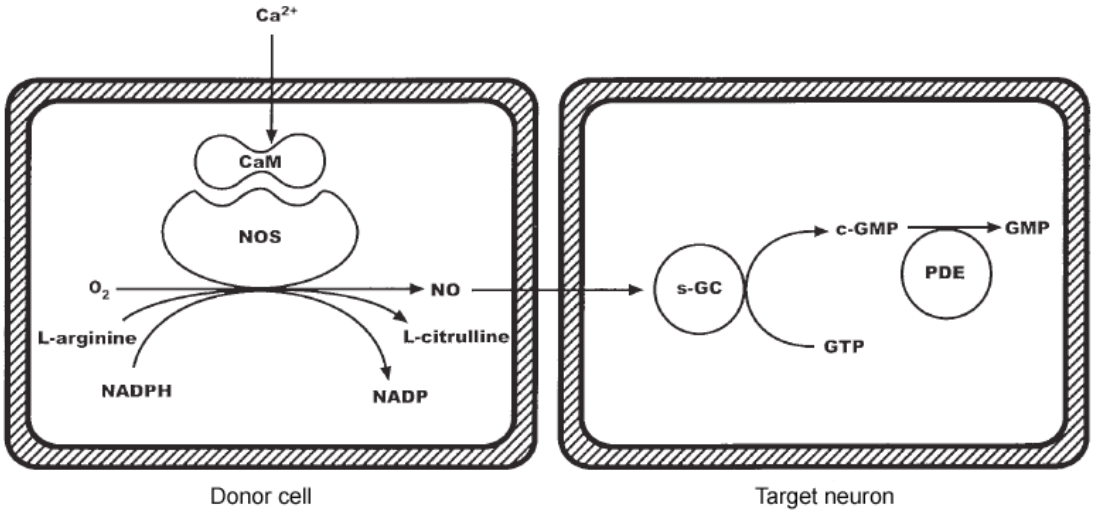


**Figure 3. Scheme of nitric oxide signaling.**
In the donor cell the formation of nitric oxide by $Ca^{2+}$/Calmodulin dependent nitric oxide synthase is shown. After activation, NOS protein converts L-arginin into NO and L-citrulline in the presence of $O_2$ and NADPH. NO is membrane permeable and can diffuse into the surrounding tissue. The main receptor for NO is soluble guanylyl cyclase (s-GC) that converts GTP into cGMP after activation by NO. Elevated levels of cGMP then lead to cellular responses in the target neuron. Finally, cyclic GMP was degraded by phosphodiesterases (PDE) into GMP. (Modified from Bicker, 2001)

In the nervous system nitric oxide is formed by $Ca^{2+}$ -regulated nitric oxide synthase (NOS). After activation by $Ca^{2+}$/Calmodulin, NOS converts L-arginine into L-citrulline in the presence of $O_2$ and of NADPH as a cofactor (Fig. 3). Nitric oxide is a membrane permeant molecule that diffuses from its site of production into the surrounding tissue. In the target cells, the main receptor molecule for NO is soluble guanylyl cyclase (sGC). After binding to NO, this enzyme is activated and converts guanosine 5`-triphophate (GTP) into cyclic guanosine 5´-monophosphate (cGMP) (Bicker, 2001; Denninger and Marletta, 1999; Garthwaite, 1991; Hall and Garthwaite, 2009; Müller, 1994; 1997; Roy and Garthwaite, 2006).

### 1.5 Pioneer neurons in the limb

Seidel and Bicker (2000) show perturbation of pioneer axon outgrowth in the antenna after inhibition of NO-cGMP signaling. Based on their findings, I used a more complex pathway of axonal outgrowth and navigation. Around 30%E a pair of sibling neurons emerges in the tibial segment (Ti1) of the metathoracic limb and subsequently started axonogenesis (Bate, 1976b; Bentley and Keshishian, 1982; Keshishian, 1980). Within the next 5%E the path into the CNS is laid. Along their pathway, the Ti1 growth cones get in contact with guidepost neurons of adjacent segments (Fe1, Tr1, and Cx1 in Fig. 4), promoting axonal outgrowth (Bentley et al., 1991; Bentley and O`Connor, 1992; Klose and Bentley, 1989). The pioneer neurons of the metathoracic limb follow a characteristic path including two sharp turns (Caudy and Bentley, 1987; O'Connor et al., 1990; Taghert et al., 1982).

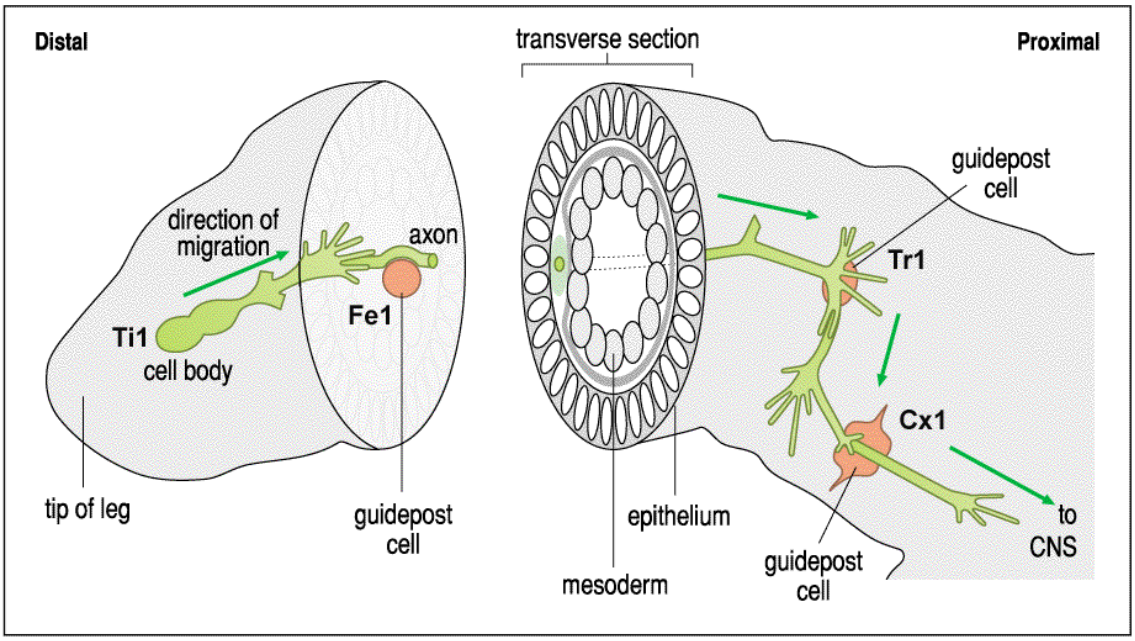


**Fig. 4. Ti1 pioneer pathway in the locust metathoracic limb.**
Path of the migrating Ti1 growth cones including a first ventral turn at the Tr1 guidepost cell. After filopodial contact to the Cx1 guidepost cells a second turn follows proximally. After axon initiation and elongation, the first guidepost cell of the femur segment (Fe1) helps Ti1 growth cones to grow proximally. Abbreviations: Ti tibia; Fe femur; Tr trochanter; Cx coxa. (Modified from Wolpert et al., 2002 ).

### 1.6 Guidance of pioneer neurons in the limb

The guidance of Ti1 growth cones along their characteristic path is likely to be mediated by alternating repulsion and attraction by guidance cues in the limb epithelium. A pronounced effect on Ti1 outgrowth is mediated by members of the semaphorin (Sema) family of guidance molecules. Sema 1A, a membrane bound glycoprotein, is ectopically present on epithelial cells along the segment borders (Kolodkin et al., 1992; Singer et al., 1995). Sema 1A provides an attractive cue for Ti1 growth cones. Sema 1A is associated with Ti1 growth promotion and axon fasciculation, indicating for potent actions on Ti1 growth cone guidance (Wong et al., 1997; 1999). A repulsive effect on Ti1 growth cones is shown for the secreted protein Sema 2A. Expression of Sema 2A begins at the tip of the limb bud before Ti1 neurons are differentiated (Isbister et al., 1999). During further development two gradients emerge over the limb epithelium (Fig. 5). Highest concentrations are found at the tip and along the dorsal edge. The concentrations of the overlapping Sema 2A gradients decrease proximally and ventrally. Antibody masking perturbs normal Ti1 pathfinding such as the growth cones fail to turn correctly and enter dorsal parts of the limb. Further, totally misrouted fibers appear growing distally (Isbister et al., 1999; Isbister et al., 2003; Legg and O`Connor, 2003; O'Connor, 1999).

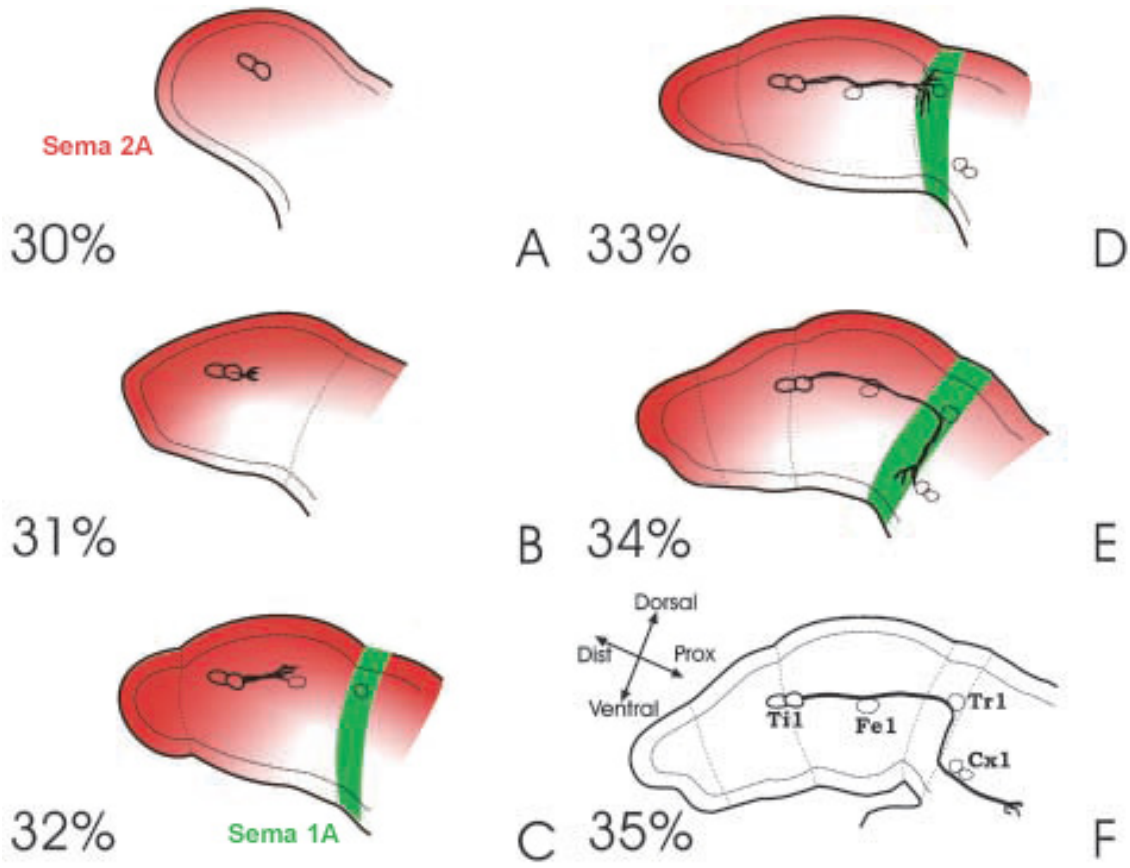


**Fig. 5. Developmental expression of two semaphorins in the locust metathoracic limb.** The development of the metathoracic limb and the progress of Ti1 outgrowth are depicted schematically. Ti1 growth cones are driven by members of the semaphorin family of guidance cues. Sema 2A (red) is a diffusible protein that is secreted into the limb epithelium forming a pronounced gradient. The highest concentrations are present at the tip and along the dorsal edge. **A** The Ti1 pioneer neurons emerge at 30%E and subsequently start axonogenesis. **B** At 32%E the first guidepost neuron in the femur segment is in filopodial contact. **C.** At 33%E the growth cone contacts guidepost neurons of the trochanter segment. **D** The growth cones orient along a circumferential path ventrally. Along the coxa-trochanter segment boundary the permissive cue Sema 1A is ectopically expressed by epithelial cells (green).**E** Ti1 growth cones orient along this path until they get in contact with Cx cells. **F** Semischematic standard Ti1 pathway (modified from Legg and O`Connor, 2003)

Turning assays in cultured *Xenopus* neurons show that the modulation of growth cone responses to an applied Sema 3A gradient is dependent on the intracellular ratio of cyclic nucleotides (Song et al., 1998). In *Drosophila*, responses to semaphorin-signaling are associated with intracellular cGMP mediated by particulate guanyly cyclase (Ayoob et al., 2004).

## 1.7 Thesis outline

A main goal of my doctoral thesis is to identify chemical signals which affect the formation of the complex wiring of the nervous system. My thesis is divided into two distinct approaches. In the first part, I describe the development of a distinct neurotransmitter system. In this neuroanatomical investigation the time course of development and the distribution pattern of histaminergic neurons are compared in the two locust species, *Schistocerca gregaria* and *Locusta migratoria*. For instance, locust serotonergic neurons express the serotonin transporter (SERT) before neurotransmitter synthesis started (Condron, 1999). Hence, extracellular serotonin can be taken up directly into presumptive serotonergic neurons. Immunolabeling allows to identify particular neurons and to follow their early development. One aspect of my anatomical description of histamine immunoreactivity (HA-IR) was to test for a putative uptake mechanism of extracellular histamine into histaminergic neurons of the ventral nerve cord. First, I started to map the cellular distribution of histamine immunoreactive neurons in the ventral nerve cord of third instar larvae. Second, the embryonic development of HA-IR was followed from the first onset of immunolabeling until hatching. These findings should be compared with the distribution pattern of larval stages. Then, a prospective action of bath applied histamine could be tested for putative actions on axonal outgrowth.

In the second part, I investigate the influence of the gaseous messenger nitric oxide on axonal outgrowth and growth cone navigation. Due to its relatively simple organized nervous system, I chose the locusts as a model for the study of growth cone guidance. Moreover, the embryos are relatively large and easy to handle. Especially locust embryos can be kept in culture and are accessible for bath applied chemicals (Bonner et al., 2003; Haase and Bicker, 2003; Lefcort and Bentley, 1987; Seidel and Bicker, 2000). Immunocytochemical staining techniques allow for labeling and identification of single neurons. Therefore, the development of identified neurons can be followed and presumptive effects on nervous system formation by applied chemical ligands regarding specific signaling cascades can be investigated.

# 2. Publications

## 2.1 Contributions to publications

**Development of histamine-immunoreactivity in the central nervous system of the two locust species *Schistocerca gregaria* and *Locusta migratoria.***
Arne Pätschke and Gerd Bicker

I carried out the preparations, the immunocytochemical labeling, the documentation, and the preparation of the photomicrographs and schemes. I also wrote the manuscript under supervision of Prof. Dr. G. Bicker. Prof. Dr. G. Bicker helped with the interpretation and discussion of the results.

**A role for nitric oxide-cGMP signaling in directed axonal outgrowth of insect pioneer neurons**

Arne Pätschke and Gerd Bicker

I carried out all preparations and experiments. Further, I carried out immunocytochemical labeling, documentation, and statistical analysis. I prepared the figures and schemes. Finally,I wrote the manuscript with input from Prof. Dr. G. Bicker to the interpretation and the discussion of the results.

## 2.2 Development of histamine-immunoreactivity in the central nervous system of the two locust species *Schistocerca gregaria* and *Locusta migratoria*

Arne Pätschke and Gerd Bicker*

University of Veterinary Medicine Hannover
Division of Cell Biology - Institute of Physiology
Bischofsholer Damm 15/102, D-30173 Hannover, Germany

* Correspondence to: Gerd Bicker, University of Veterinary Medicine Hannover, Division of Cell Biology – Physiological Institute, Bischofsholer Damm 15/102, D-30173 Hannover
Email: Gerd.Bicker@tiho-hannover.de
Phone +49 (0) 511-8567765

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**Abstract**

Locusts are attractive model preparations for cellular investigations of neurodevelopment. In this study, we investigate the immunocytochemical localization of histamine in the developing ventral nerve cord of two locust species, *Schistocerca gregaria* and *Locusta migratoria*. Histamine is the fast neurotransmitter of photoreceptor neurons in the compound eye of insects, but it is also synthesized in interneurons of the central nervous system. In the locust ventral nerve cord, the pattern of histamine-immunoreactive neurons follows a relatively simple bauplan. The histaminergic system comprises a set of single, ascending projection neurons that are segmentally arranged in almost every neuromere. The neurons send out their axons anteriorly, forming branches and varicosities throughout the adjacent ganglia. In the suboesophageal ganglion the cell bodies lie in a posterio-lateral position. The prothoracic ganglion lacks histaminergic neurons. In the posterior ganglia of the ventral nerve cord the somata of the histaminergic neurons are ventro-medially positioned. Histamine-immunoreactivity starts around 50% of embryonic development in interneurons of the brain. Subsequently, the neurons of the more posterior ganglia of the ventral nerve cord become immunoreactive. From 60% embryonic development, the pattern of soma staining in the nerve cord appears mature. Around 65% of embryonic development, the photoreceptor cells show histamine-immunoreactivity. The histaminergic innervation of the neuropile develops from the central branches toward the periphery of the ganglia and is completed right before hatching.

Keywords: biogenic amine; homology; insect; invertebrate; ventral nerve cord

## Introduction

Since its discovery as a fast neurotransmitter of insect photoreceptor cells, the biogenic amine histamine has been in the focus of considerable research. Histamine hyperpolarizes the postsynaptic monopolar neurons by directly gating a chloride conductance (Hardie, 1987, 1988). The cloning of the histamine receptors in *Drosophila* revealed indeed their identity as members of the ligand-gated ion channel family (Gisselmann et al., 2002; Witte et al., 2002). The visual system of *Drosophila* has also been used for the study of histamine metabolism, synaptic release, and complex re-uptake mechanisms involving glial cells and photoreceptors (Borycz et al., 2002; Romero-Calderon et al., 2008; Sarthy, 1991). Although the large amount of histamine found in the insect optic lobes is mainly due to the high number of photoreceptor cells, some histamine is also present in other brain regions and in all ganglia of the ventral nerve cord (Elias and Evans, 1983).

A rather straightforward method to visualize histaminergic neurons is immunocytochemical staining with antisera raised against histamine conjugated to protein carriers. Several studies have resolved the distribution of histamine-immunoreactive (HA-IR) neurons in the insect brain, such as cockroach (Loesel and Homberg, 1999; Pirvola et al., 1988), cricket and honeybee (Bornhauser and Meyer, 1997), locust (Gebhardt and Homberg, 2004), several hymenopteran species (Dacks et al., 2010), the sphinx moth (Homberg and Hildebrand, 1991), and *Drosophila melanogaster* (Monastrioti, 1999; Pollack and Hofbauer, 1991). Some chemoneuroanatomical studies including HA-IR have also been dedicated to the developing brain, like the analysis of the *Drosophila* larval chemosensory system (Python and Stocker, 2002). Although in some crustacean species, the development of histaminergic neurons has been traced in brain and ventral nerve cord (Harzsch and Glötzner, 2002; Rieger and Harzsch, 2008), there is no information available about the development of HA-IR in the ventral nerve cord of insects.

Embryonic locusts are especially useful models of developmental biology, because cellular mechanisms of axon guidance can be studied at the level of single identified neurons. The unique identity of the invidual neuroblasts together with detailed lineage studies of their progeny facilitated the developmental analysis of neurite formation in the segmentally arranged neuromeres (Bastiani et al, 1984; Bate 1976a; Goodman and Bate, 1981). In particular, the development of a single group of segmentally repeated serotonergic neurons, which are the progeny of neuroblast 7-3, has been studied in the locust embryo (Condron 1999; Taghert and Goodman, 1984). Because of the stereotyped arrangement of neuroblasts among rather distantly related insects (Thomas et al., 1984) and conservation of early embryonic cell lineages, the development of these serotonergic interneurons could be compared to homologous neurons in *Drosophila* (Lundell and Hirsh, 1998; Sykes and Condron, 2005; Valles and White, 1988).

Similar to the set of segmentally repeated serotonin-IR neurons, insects seem to develop only a rather limited number of serially homologous HA-IR neurons in the neuromeres of the ventral nerve cord. So far, these neurons have only been delineated in the adult nervous system of flies (Nässel et al., 1990) and crickets (Hörner et al., 1996; Hörner, 1999). To bridge this gap, we here present the distribution pattern of histaminergic neurons in the ventral nerve cord of locusts.

In this work, we address four issues. First, we provide a neuroanatomical survey of the segmental organization of HA-IR in the ventral nerve cord. Second, we describe the emerging HA-IR in the visual system of the embryo as a reference. Then, we follow the appearance and outgrowth pattern of HA-IR during embryonic development of the ventral nerve cord, including the ascending projections into the brain. For phylogenetic considerations, we finally compare how the pattern of HA-IR matches in the two locust species *Schistocerca gregaria* and *Locusta migratoria*.

## Materials and Methods

*S. gregaria* and *L. migratoria* eggs were collected from crowded colonies at our institute. The egg pods, laid in moist vermiculite or a mixture of sand and vermiculite, were collected daily and kept at 30°C until use. Embryos were staged percentage of embryogenesis (%E) based on the system of Bentley et al. (1979). Especially for the staging of *L. migratoria*, we applied additional criteria (Ball and Truman, 1998). These criteria were supplemented with our own observations, such as the morphology of the metathoracic leg. According to morphological observation from our locust colonies, embryonic staging is quite reliable, because embryos obtained from one clutch showed a developmental variability within 5%. In addition to the embryos, we used third instar nymphs to investigate the pattern of histamine immunoreactivity in the ventral nerve cord of *S. gregaria*.

### Dissection of young embryos between 40%E and 55%E

Embryos up to 55%E were prepared as whole mount preparations. Embryos were pulled out of their egg shell in ice-cold cell-culture medium (Gibco, Leibowitz, L-15). Remaining amnion sheath around the embryo was removed with fine forceps. Embryos were collected in cooled fresh L-15 medium in 48 well plates on a shaker with one embryo per chamber until fixation.

### Dissection of embryos older than 60%E

From 60%E on the embryos produce a water repellent cuticle and therefore can be placed on liquid unpolymerized sylgard (Dow Corning). The embryos were pulled out of their egg shell in a dry petri dish. All appendages were removed. The embryo was dried on a tissue and subsequently placed on a drop of liquid unpolymerized sylgard in a wall rounded chamber on a slide (Stern and Bicker, 2008). The embryo was opened from the dorsal side and a drop of added medium caused the embryo to expand on the sylgard. After removal of the gut, the membrane covering the CNS was opened to provide access of the antibodies. Because the embryo was closely attached to the sylgard, immunohistochemistry could be performed in the chamber.

### Ventral nerve cord preparation in nymphs

Nymphs were anesthetized on ice for 45 minutes. The hind end of the larva as well as the mouthparts was removed. The foregut was cleared from the surrounding mouthparts, and then the whole gut was pulled out through the opened abdomen. The ventral nerve cord including all ganglia from the suboesophageal ganglion (SOG) to the terminal ganglion (TG) was dissected and collected in ice cold medium until fixation.

### Histamine immunocytochemistry

Embryos or nervous tissue were fixed in freshly prepared 4% 1-ethyl-3-(3′-dimethyl-aminopropyl) carbodiimide (EDAC, Calbiochem or Sigma either dissolved in L-15 medium or PBS) (Panula et al., 1988). In early experiments, the tissue was fixed over night at 4°C, but even in younger embryos, this resulted in an unsatisfactory staining with high background. Therefore, we reduced the fixation time to 45-60 minutes at room temperature. After fixation, the tissue was permeabilized with 0.3% saponine in PBS containing 0.5% Triton X-100 (PTX 0.5%). Embryonic stages were permeabilized for 45-60 minutes, the ventral nerve cords of third instar larvae for up to one and a half hours. Afterward, tissue was blocked in 5% normal goat serum (Vector) for at least one hour at room temperature. Then, an antiserum raised against histamine (ImmunoStar, 1:1000 in PTX 0.5%) was added overnight at 4°C. After several washes in PTX 0.5%, a biotinylated secondary antibody (Vector, 1:250) was added for two and a half hours at room temperature. HA-IR was visualized by CY3 conjugated strepavidin (Sigma, 1:300).

## Antibody specificity

The polyclonal antibody was raised against synthetic histamine conjugated to succinylated limpet hemocyanine with carbodiimide. No cross reaction with L-histidine or L-histidine containing peptides are documented (ImmunoStar Inc., data sheet cat. #22939). The observed pattern of immunolabeled cells in the ventral nerve cord was very similar to that found in crickets (Hörner et al., 1996). Dacks et al. (2010) used the same antibody for a comparative study within hymenopteran species. The total absence of immunolabeling in Drosophila null lines for histidine decarboxylase (Melzig et al., 1996) indicates for a high specificity of the antibody. In control experiments for possible unspecific binding of the detection system, we omitted the primary antibody, replaced it with blocking solution, and followed the labeling protocol as described earlier. In theses cases, staining was absent.

## Preincubation with bath applied histamine

To test for a putative uptake mechanism, histamine was added into the medium. Histamine (Fluka) was dissolved in PBS to a final concentration of 100 mM. Aliquots were kept in a freezer. For application, the stock solution was dissolved in L-15 medium to a final concentration of either 10 or 20 µM. Embryos at different stages were preincubated for up to 1h at room temperature. After washing with fresh L-15 medium, the embryos were fixed in freshly prepared EDAC, and immunocytochemistry was performed.

## Tissue embedding and microscopy

Whole embryos and ventral nerve cord preparations were cleared in 50% glycerol and mounted in 90% glycerol on glass slides. Preparations were analyzed under a Zeiss Axioskop and pictures were taken with a Zeiss Axiocam HRc. Photomicrographs were arranged using Adobe Photoshop 7.0. Fluorescence values were inverted and slightly enhanced for contrast.

## Results

### General pattern of HA-IR interneurons in the ventral nerve cord

The locust ventral nerve cord consists of the SOG, three thoracic ganglia (Pro-, Meso-, and Metathoracic with the fused abdominal ganglia A1-3), four unfused abdominal ganglia (A 4-7) and the TG. To facilitate antibody penetration for detection of HA-IR in whole-mounts, immunocytochemical stainings were performed on third instar nymphs instead of the larger adult specimen. These immunocytochemical stainings revealed a specific pattern of intersegmentally projecting HA-IR interneurons. Figure 1 provides a schematic drawing of the pattern in the ventral nerve cord.

In the SOG, we found one brightly stained cell body on each side of the posterio-lateral soma rind (Figs. 1, 2a). The medially oriented primary neurite branched before reaching the midline into an anterior and a posterior process. With the exception of the prothoracic ganglion, we could identify one pair of HA-IR interneurons in each neuromere of the thoracic and abdominal ganglia. The axons of these bilaterally symmetrical interneurons ascended bundled together along the ganglionic chain of the ventral nerve cord. In each ganglion, the immunoreactive somata occupied a rather characteristic position in the cell body layer. This position depended on neuromere identity (Fig. 1). The medio-ventrally located somata sent out a primary neurite that was initially looping toward the midline before turning laterally and connecting into the ipsilaterally ascending projection. Neurite branches of the interneurons arborized into a dense network of HA-IR processes in the neuropile of each hemiganglion. Although HA-IR cell bodies were absent in the prothoracic ganglion, the neuropile was densely filled with immunoreactive processes (Fig. 2b). The neuropilar processes formed varicosity like structures that may correspond to putative releasing sites of histamine (Fig. 2). Because of the medio-ventral cell body position, the medially looping primary neurite, and the ascending projection, we surmise that the HA-IR interneurons of the thoracic (Fig. 2b-d), abdominal (Fig. 2e) and terminal neuromeres (Fig. 2f) comprise a set of serial homologues. The HA-IR neurites formed an extensive network in the ganglia of the ventral nerve cord, whereas all peripheral nerves remained unstained.

In Figure 1 the path of the ascending HA-IR fibers running through the connectives was indicated by an uninterrupted line, although mainly four HA-IR axons were forming this fiber bundle. The number of closely attached processes did not increase from posterior to anterior. Most likely, the intersegmental projections terminated in an adjacent ganglion. The ascending axons emanating in a distinct ganglion always chose the most lateral trajectory in their fiber bundle.

Similar to the thoracic ganglia, the unfused abdominal ganglia contained also one pair of HA-IR interneurons with medio-ventral cell body position and medially curving primary neurite (Fig 2e). In the fused TG, we consistently found six cell bodies. Based on their characteristic morphology, the three pairs could be attributed to the system of ascending serial homologues. One or two pairs of more faintly stained somata could be detected in a more lateral position (Figs. 1, 2f). In all abdominal ganglia, a dense network of immunoreactive fibers in both ganglionic hemispheres became apparent.

**Onset of HA-Immunoreactivity in the Central Nervous System**

The first expression of HA-IR appeared after completing half of the embryonic development. The exact onset of histamine expression seemed to be slightly variable, since we observed differences in the exact timing of HA-IR expression in embryos of the same clutch. Around 50% of embryonic development the first HA-IR neurons became visible in the brain (Fig. 3a). They belonged to a cell cluster of HA-IR neurons in the posterior lateral protocerebrum that consisted of six large cell bodies in later stages. At 55%E, the tip of an axon entered the brain and could be found at the level of the stomodaeum (Fig. 3a). To increase levels of HA-IR via potential uptake mechanisms, we supplied exogenous histamine to the nervous tissue before fixation. However, bath application of histamine at concentrations of 10 µM did not enhance staining intensities. Moreover, adding histamine before 50%E did not result in cellular detection of HA-IR in the nerve cord.

The photoreceptor neurons of the compound eyes started with their expression of HA-IR between 60% and 65%E (Fig. 3b). Staining was visible in the cell bodies and axons. The axons traversed the fenestrated layer in bundles and reached the developing lamina or medulla. Figure 4 provides a summary of the developmental expression of HA-IR in the brain and ventral nerve cord.

**Development of HA-IR in the Ventral Nerve Cord**

In the ventral nerve cord, HA-IR neurons could be detected as early as 50%E (Fig. 5a). Between 50 and 55%E a first longitudinal axon extended without any branching through the thoracic connectives (Figs. 5a, b). From 55%E on, the HA-IR neurons of the thoracic ganglia were consistently stained in the majority of preparations (Fig. 5).

Figure 5 displays distinctive events in the formation of HA-IR neurons in the ventral nerve cord. From 60%E on, the number of HA-IR cells was constant, and the connections between their cell bodies and their neuropilar branches could be reliably traced. The developmental pattern of HA-IR in the neuromeres of the ventral nerve cord of younger embryos displayed

a gradient, with the highest expression in the anterior thoracic and the lowest in the most posterior abdominal ganglia (not shown). During embryonic development, the metathoracic ganglions are assembled by the sequential fusion of the metathoracic with the first three abdominal neuromeres (Fig. 5). The condensation of the ganglia proceeds from anterior to posterior, beginning with the fusion of the metathoracic with the A1 neuromere.

In every ganglion initially, HA-IR fibers started to ramify toward more medial parts of the neuropile (Figs. 5c, d). These branches became more elaborated during the next developmental stages. In the thoracic ganglia, three main branches gave rise to a dense innervation of central neuropilar regions from 70%E on (Figs. 5e). The first fibers that were growing into the lateral hemispheres could be observed at the same stage (Figs. 5e, f).

The innervation of the lateral hemispheres in the thoracic ganglia started around 70%E with fine fibers growing into the hemispheres at the anterior and posterior edge of the neuropile (Figs. 5e, f). Through the next percentages of development, these fibers continued to grow further along the edge of the neuropile. Around 75-80%E, they nearly met each other close to the lateral edge of the neuropile and started to turn and grow inwards. By further extension, they innervated both hemispheres and formed a dense network of arborizations in the neuropile. From 85%E on, varicosities were apparent as putative releasing sites of histamine, first in the central regions of the neuropile and later in the lateral hemispheres, too (Fig. 7). At 95%E, the pattern of immunoreactive fibers in the ventral nerve cord resembled the one of larval stages, as indicated by the HA-IR distribution in third instar nymphs (Fig 1; 5 g, h). This ground pattern was elaborated during development of the larval stages. Our immunocytochemical stainings of the ventral cord provided neither evidence for peripherally projecting HA-IR motoneurons nor for afferent sensory neurons projecting into the CNS.

In the first unfused abdominal ganglion A4, one axon arose from the central neuropile and left the ganglion posteriorly through a more median pathway into the connective (Fig. 6). The detailed projection of this descending neurite could not be traced. In contrast to this medially located pathway, the ascending fibers chose a more lateral path through the connectives.

**Comparison of HA-IR in the ventral nerve cord of two locust species**

A comparison of the developmental onset and distribution of HA-IR in the ventral nerve cord of *S. gregaria* and *L. migratoria* showed that the HA-IR neuroarchitecture corresponded well between the two species. For example, the prothoracic (Figs. 7a, b) and the metathoracic ganglion (Figs. 7 c, d) at a stage between 80-85%E displayed a similar pattern of developmental progress. At this stage, the innervation of both hemispheres was not yet completed, but, in both species, the developmental progression appeared to be at the same level. However, we found one exception regarding the timing of a specific developmental

event. In the first unfused abdominal ganglion, a neurite emerged from the central neuropile leaving posteriorly through a median path. In *S. gregaria* this neurite could be found as early as 70%E (Fig. 6a), whereas the immunoreactivity in *L. migratoria* could not be detected before 85%E (Fig. 6b).

## Discussion

A fundamental aspect of nervous system development is the differentiation of neurotransmitter phenotype. Here, we present a survey on the embryonic development and the larval distribution pattern of HA-IR neurons in the locust ventral nerve cord. Only a small subset of neurons labeled with an antiserum raised against histamine. This set of neurons was arranged in a rather simple bauplan.

Although mainly in younger embryos, additional cell-like structures appeared immunoreactive in single ganglia (Figs. 5 a, c, d). This staining most likely results from unspecific binding of the detection system after long-time fixation. In contrast to the intense staining of interneurons and axons at older stages, these cell-like structures were variable in appearance and only weakly stained. A reduction of fixation time diminished this unspecific labeling. The pattern of true immunoreactivity in our neuroanatomical description is based on the detection of labeling in identified cell bodies and neurites of at least five specimens.

We found a consistent staining pattern starting in young embryos around 55%E up to third instar larvae. The onset of histamine expression in the VNC of both locust species occurred rapidly within a few percentages of development. After the beginning of neurotransmitter expression, the progress of neurite outgrowth could be followed in detail, like the axonal tips growing into the brain (Fig. 3a) or the expansion of HA-IR branches into the neuropile of ventral ganglia.

In embryonic locusts, histamine expression started within a similar developmental time frame, when markers for other neurotransmitter systems became immunopositive. In the ventral nerve cord, GABA-immunoreactivity appears at 55%E (O´Dell and Watkins, 1988), serotonin-immunoreactivity between 54%E and 56%E (Taghert and Goodman, 1984), proctolin-IR between 50%E and 60%E (Keshishian and O´Shea, 1985), allatostatin-IR at 45%E to 50%E (Kreissl et al., 1999), and a histochemical marker for nitric oxide synthase is expressed at 47%E (Stern et al., 2010).

Monoaminergic neurotransmission is commonly terminated by reuptake of the amine via specific membrane transporters. The appearance of transporter molecules does not necessarily coincide with transmitter synthesis. The locust embryo provides an example, where serotonergic neurons express the serotonin transporter long before the neurotransmitter is synthesized (Condron, 1999; Stern et al., 2007). Bath applied serotonin is taken up by discrete neurons and detected by immunocytochemistry. The immunocytochemical staining could be conveniently used to follow the morphological differentiation of presumptive serotonergic neurons at stages before the appearance of intracellular transmitter synthesis.

Within the detection threshold of our immunofluorescence method, we found no evidence for specific uptake mechanisms of histamine into the locust neurons. Preincubation of the locust embryo with histamine at earlier developmental stages did not reveal any further HA-IR neurons. Moreover, bath application of histamine caused no increase in staining intensities of the identified HA-IR neurons. Rather, the staining levels were comparable to untreated preparations. Differences in the neuronal expression of transport mechanisms for histamine and serotonin are not unexpected.

Histamine metabolism in *Drosophila* eyes is thought to involve rather complex shuttling mechanisms between photoreceptor and glial cells. Histamine released by photoreceptor cells is taken up into glial cells and modified into a metabolite, carcinine. In the photoreceptors, carcinine is hydrolyzed, and histamine is packed into vesicles ready for re-release (Edwards and Meinertzhagen, 2010; Stuart et al., 2007; True et al., 2005). Apart from its firmly established role as neurotransmitter of photoreceptor neurons (Hardie, 1987; Pirvola et al., 1988; Pollack and Hofbauer, 1991; Simmons and Hardie, 1988), other physiological functions of histamine in the insect nervous system are gradually emerging. One early electrophysiological study presented histamine as a modulator of interneurons in sound reception (Skiebe et al., 1990).

Genomic sequence comparisons among metabotropic amine receptors have shown that invertebrates lack metabotropic histamine receptors, such that histaminergic neurotransmission appeared to be exclusively mediated via ionotropic receptors (Roeder, 2003). In some, but not all insect species, the antennal lobe was innervated by HA-IR interneurons (Nässel, 1999). Optical recordings of odor-induced calcium signals in the antennal lobe of the honeybee showed physiological evidence (Sachse et al., 2006) that histamine may not only act as inhibitory transmitter in the eye but also in other parts of the insect CNS. Electrophysiological results indicate for histamine-gated chloride channels on a subset of dorsal unpaired median (DUM) neurons of *L. migratoria* (Janssen et al., 2010). Thus, it is possible that the HA-IR interneurons in the VNC (Fig. 1) may provide intersegmentally projecting fast inhibition onto postsynaptic circuits.

In flies, the innervation of putative neurohaemal releasing sites has been described (Nässel et al., 1990), suggesting a neurohormonal action of histamine, although functional studies are lacking. Interestingly, immunocytochemical studies combined with the genetic depletion of histamine levels have shown that most cuticular mechanoreceptors in *Drosophila* use this amine as a transmitter (Buchner et al., 1993; Melzig et al., 1996). However, the histaminergic mechanoreceptors may be a specialty of fruit flies, because arthropod mechanosensory neurons are predominantly cholinergic (Sattelle and Breer, 1990). Many sensory neurons of the adult locust stained with an antibody against the acetylcholine synthesizing enzyme ChAT (Lutz and Tyrer, 1987). A cholinergic phenotype of locust mechanosensory neurons was also supported by the expression of AChE in sensory cells of the body wall and appendages

(Bicker et al., 2004). In this study, we found no evidence for HA-IR mechanosensory cells and motoneurons innervating skeletal muscles.

Currently, the phylogenetic relationship of crustaceans and insects is intensely discussed. The Tetraconata (Pancrustacea) concept holds that Insecta are a sister clade of Crustacea (Jenner, 2010; Koenemann et al., 2010). Insects and crustaceans share common features in neurogenesis with neuroblasts arising in the ventral neuroectoderm giving rise to ganglion mother cells through asymmetrical cell divisions (Dohle et al., 2003; Stollewerk and Simpson, 2005; Stollewerk and Chipman, 2006; Whitington, 2003). The expression of molecular markers provided evidence for homologous structures during early nervous system development (Duman-Scheel and Patel, 1999). Comparative studies revealed a small subset of neurons to be homologous regarding to their relative position in the neuroblast map and later in the ganglion, to their cell lineage, and to their axonal growth properties (Ungerer and Scholtz, 2008; Whitington, 2003).

In the CNS of several crustaceans such as in *Calanus finmarchicus*, Copepoda, (Hartline and Christie, 2010) and in *Artemia salina*, Anostraca, (Harzsch and Glötzner, 2002) HA-IR has been mapped. In Malacostraca, such as crayfish, lobster (Mulloney and Hall, 1991), and the marbled crayfish (Rieger and Harzsch, 2008) the number of HA-IR neurons was higher than in the insect ventral nerve cord. A developmental similarity between locust and marbled crayfish is the rapid onset of histamine-IR after the first half of embryogenesis. Usually, one pair of HA-IR neurons appeared in each ganglion in a ventro-median position (Mp cells in Rieger and Harzsch (2008)). Similar neurons were found in crayfish and lobster (Mulloney and Hall, 1991). Because of their relative soma position, neuronal morphology with ascending fiber arborizations in the neuropile of adjacent ganglia and common neurotransmitter phenotype, these interneurons may be candidates for homologous cells in the crustacean and insect ventral nerve cord. Apart of these segmentally repeated neurons, a small number of additional HA-IR neurons appeared in the crustacean ventral nerve cord in more lateral positions or as unpaired midline neurons (Rieger and Harzsch, 2008).

In contrast, the pattern of HA-IR in insect ventral nerve cord was highly consistent and followed a relatively simple bauplan as revealed in flies (Nässel at al., 1990; Nässel and Elekes, 1992) and in crickets (Hörner et al., 1996). We observed only little variations in the time course of neuropil innervation in the two locust species (Fig 6 and 7). A comparison with cricket ventral nerve cord preparations revealed a similar pattern of innervation in the thoracic and abdominal ganglia (Figs. 1, 2) with segmentally repeated interneurons, which are presumably homologous (Figs. 3, 5 in Hörner et al. (1996)). Intriguingly, a characteristic insect feature seems to be the absence of the histaminergic homologues in the prothoracic ganglia. The additionally stained lateral neurons of the cricket SOG (Hörner et al, 1996) were not observed in the locust. Among the investigated insects, the two locust species appear to have the simplest ground pattern of HA-IR interneurons in the ventral nerve cord. Since the

locust nervous system can be manipulated in embryo culture (Seidel and Bicker, 2000), it will be possible to test whether pharmacological manipulation of histaminergic transmission affects neural development.

**Acknowledgement**

We would like to thank Torben Stemme for help with some preparations and the care of our locust colony. This study was supported in parts by grants of the Deutsche Forschungsgemeinschaft (DFG).

## Figures

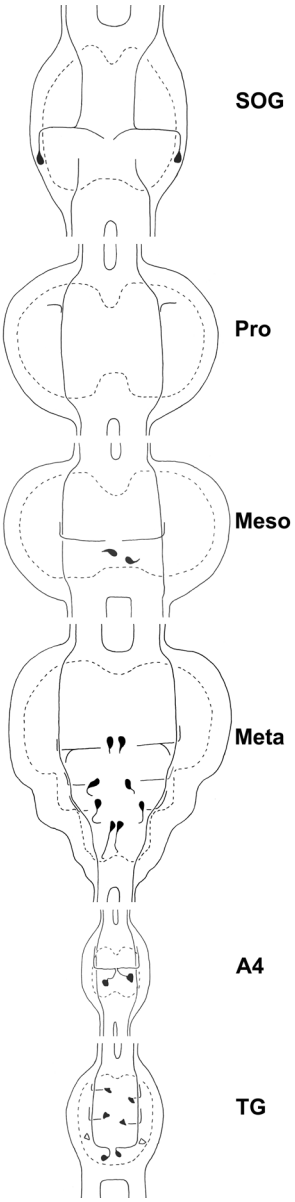


**Fig. 1. Schematic drawing of HA-IR in the locust ventral nerve cord.**
The drawing illustrates the relative cell body position and course of the primary neurites through the ganglionic hemispheres and connectives. The anteriorly directed longitudinal path, composed of at least four independent axons, is presented as a single continuous line along the ventral nerve cord. Details of the neuropile innervation are left out, but the borders of neuropile innervation are indicated as a dashed line. Some axons bifurcate within a distinct ganglion, which is indicated by lines that end up in a hemisphere. Consistently and heavily stained cell bodies are compelety filled out, faintly stained somata are left open.
Abbreviations: SOG: suboesophageal ganglion; Pro: prothoracic-; Meso: mesothoracic-; Meta: metathoracic ganglion; A4: first free abdominal ganglion; TG: terminal ganglion

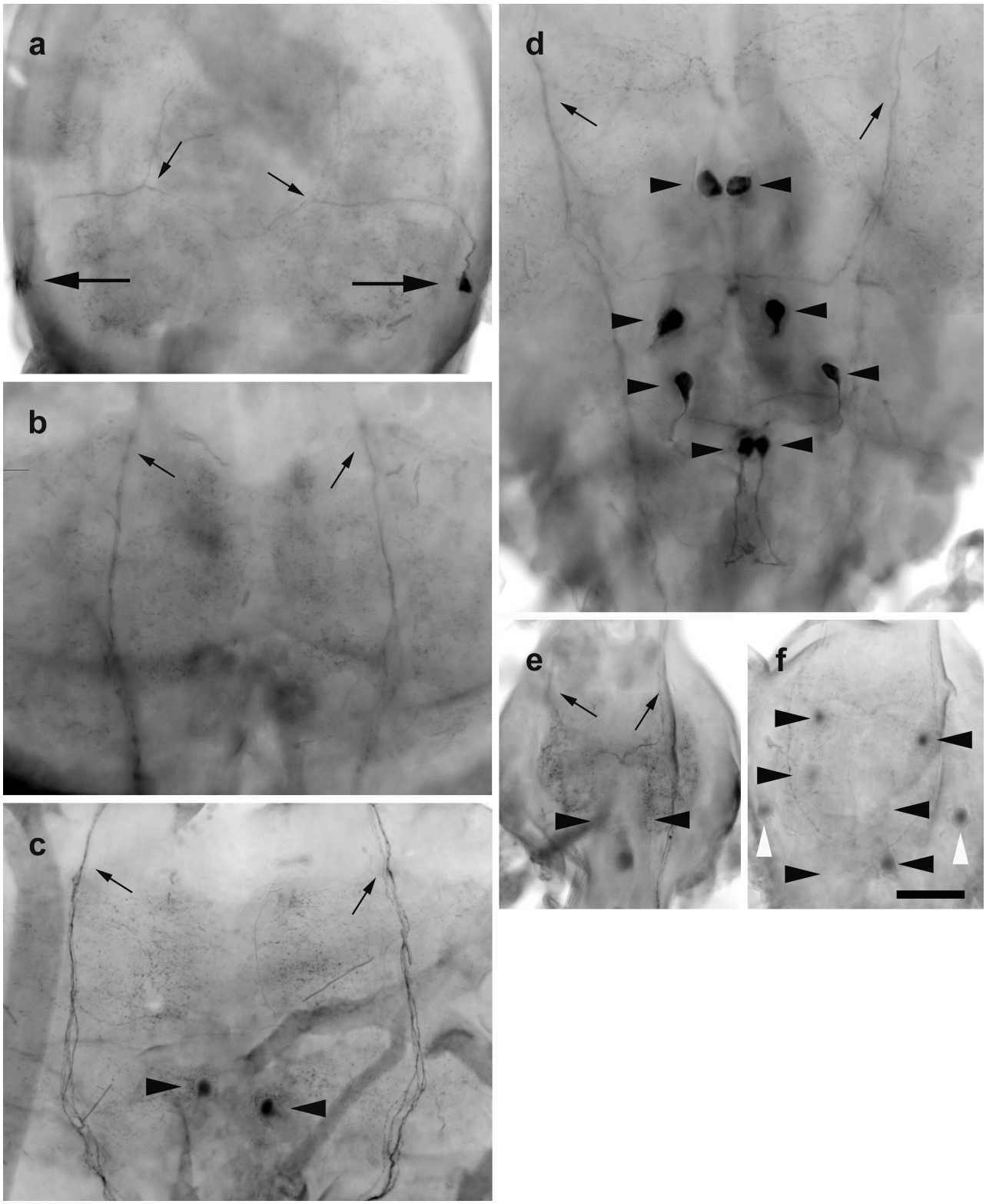
a
b
c
d
e
f

**Fig. 2. Distribution pattern of HA-IR cell bodies and fibers in the ventral nerve cord of third instar nymphs.**

Photomicrographs of whole mount preparations. In the suboesophageal ganglion (**a**) a pair of HA-IR neurons lies in a ventro-posterior position (arrows). These neurons send out an axon that bifurcates (small arrows) into one neurite running anteriorly, whereas the other runs close to the midline. The further course is covered by the highly arborized fibers in the central neuropile. Although the neuropile of the prothoracic ganglion (**b**) is traversed by histaminergic fibers (arrows), no HA-IR cell bodies are apparent. Segmentally repeated interneurons project anteriorly through the thoracic and abdominal ganglia (arrowheads in c-f). The longitudinal ascending fiber tract traversed all ventral ganglia (arrows in b-e). In the neuropile, the fibers arborize and form a dense network with a sharp border to the adjacent soma rind. In the meso- (**c**) and metathoracic ganglion (**d**) the cell bodies lie in ventro-median positions (arrowsheads). The primary neurites grow laterally turning toward the midline. Near the midline the primary neurites of both opponent neurons are closely attached but then grow straight into the hemispheres where they turn anteriorly to leave the ganglion. The first free abdominal ganglion (**e**) serves as an example for the abdominal ganglia. Cell bodies are located in a ventro-median position, covered by the network of histaminergic fibers in both hemispheres (the relative cell body positions are indicated by arrowheads). In the terminal ganglion (**f**), three pairs of cell bodies are in a ventro-median position (arrowheads), whereas an additional pair is located more laterally (white arrowheads). Dorsal view, anterior to the top. Scale: 100 µm in a-e, 118 µm in f.

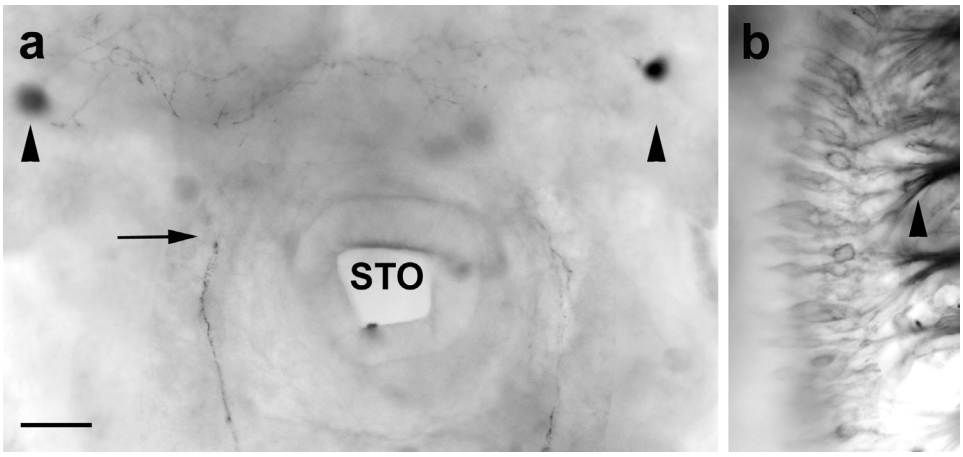


**Fig. 3. Developmental progress of histamine immunoreactivity in the embryonic brain and ventral nerve cord.**
(**a**) Initial HA-IR in single neurons in the brain at around 50%E (arrowheads). Cells belong to a cluster of neurons located in the posterior lateral protocerebrum. At this stage, one axon traversing the thoracic ganglia enters the brain. Here, the tip of the axon (arrow) is at the level of the stomodaeum (STO). (**b**) Photoreceptor cells express HA-IR from around 60%E on. Immunoreactive photoreceptor axons wind in bundles through the fenestrated layer (arrowhead). Photomicrograph as a combination of several focal planes of a whole-mount preparation. Photoreceptor layer is to the left. Anterior to the top. Scale: 50 µm.

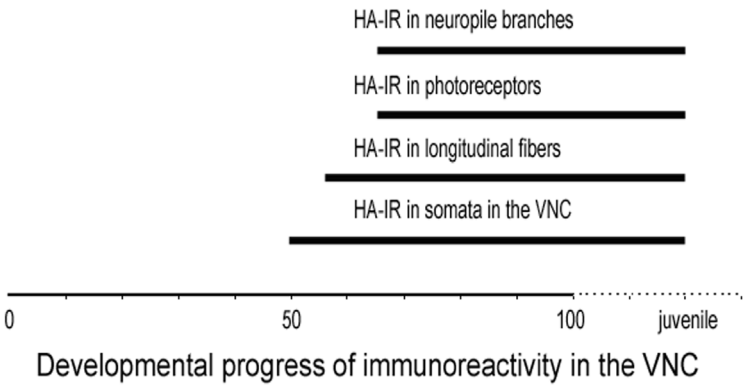


**Fig. 4. Time scale of the onset and the progress of histamine immunoreactivity during embryonic and larval development**.
From around 50%E on, the labeling of neurons in the brain and ventral nerve cord becomes consistent. From 55%E, a longitudinal fiber tract can be traced along the ventral nerve cord. Photoreceptors are detectable from 60%E on. Between 65 and 70%E, HA-IR branches start to grow into peripheral parts of the ventral ganglia. After embryogenesis, the general pattern of immunoreactivity appears to be stable.

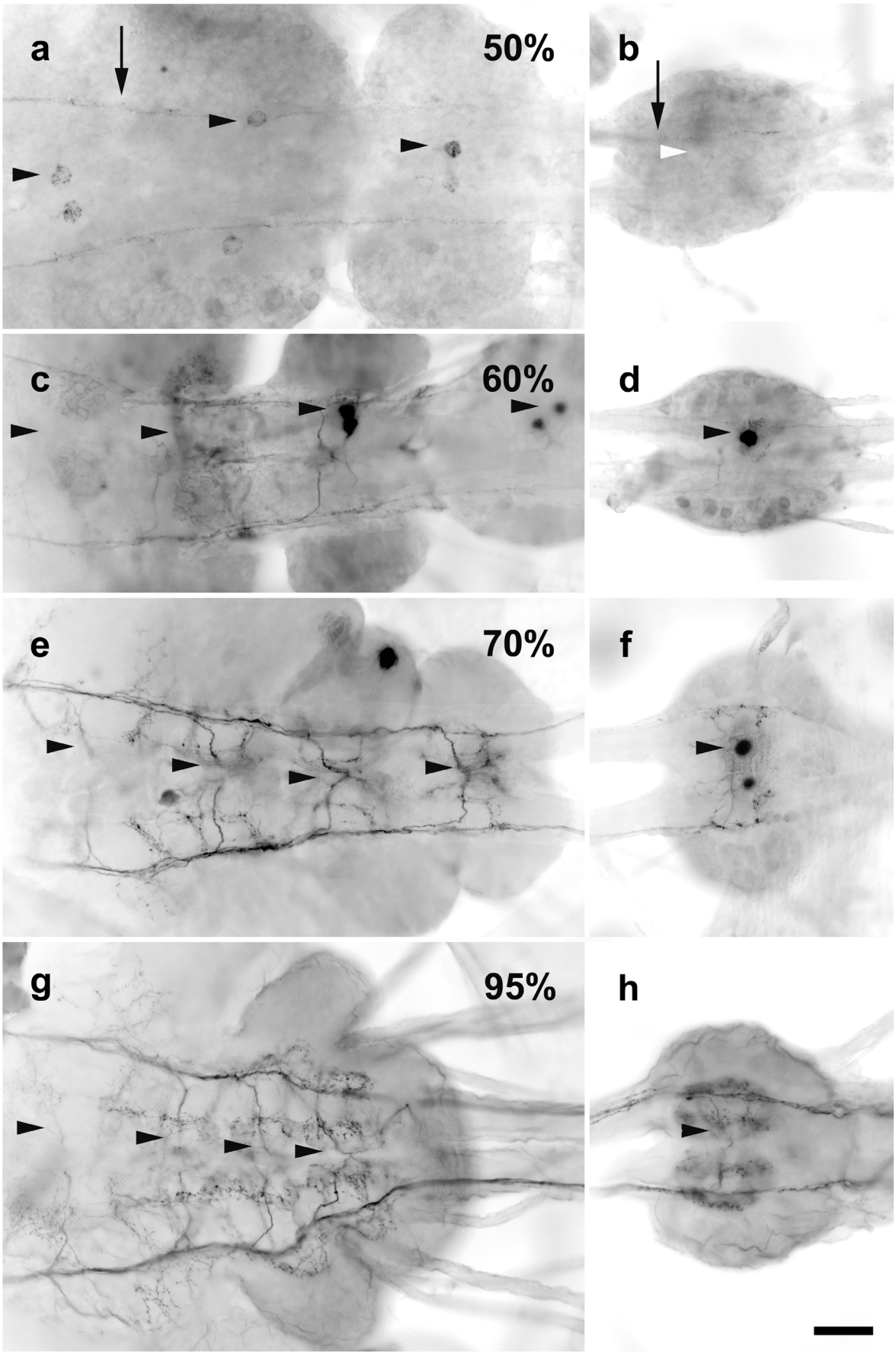
a
50%
b
c
60%
d
e
70%
f
g
95%
h

**Fig. 5. Development of HA-IR in *S. gregaria* embryos.**

The time course of HA-IR onset and axonal branching in the neuropile in the fused metathoracic (left panel) and the first free abdominal ganglion (right panel) is presented. Arrowheads point toward the positions of the cell bodies that are out of focal plane. Although two bilaterally symmetric neurons were found in each neuromere, the arrowheads indicate the positions of HA-IR interneurons of only one ganglionic hemisphere. (**a**) First appearance of HA-IR at 50%E. Despite a high background staining (a and d) the HA-IR labeling could be clearly detected in the segmental interneurons (arrowheads). The longitudinal tract is established by ascending immunoreactive fibers (arrow). (**c**) From 60%E on, the immunoreactivity is consistently found in the interneurons. (**e**) At 70%E, branches started to arborize at distinct sites into the central and lateral neuropile (see also Figure 6). The longitudinal fiber tract is fully established at this stage. (**g**) At 95%E, the neuropile is filled with a dense network of HA-IR fibers forming varicosity-like structures.

As an example for the abdominal ganglia, the second row depicts the onset of HA-IR in the first abdominal ganglion (A4). (**b**) At 50%E, a developmental delay becomes obvious. Although the longitudinal tract is filled with immunoreactive fibers (arrow) the abdominal neurons are not yet immunoreactive (white arrowhead points to the corresponding cell body position). (**d**) From 60%E on, the immunoreactivity of the interneurons was consistently established. (**f**) At 70%E, branches start arborization into the central neuropile giving rise to the dense network of HA-IR fibers. (**h**) At 95%E, the neuropile is densely innervated and sharply demarcated from the border of the unlabeled soma rind.

Dorsal view, anterior to the left. Scale: 100 µm.

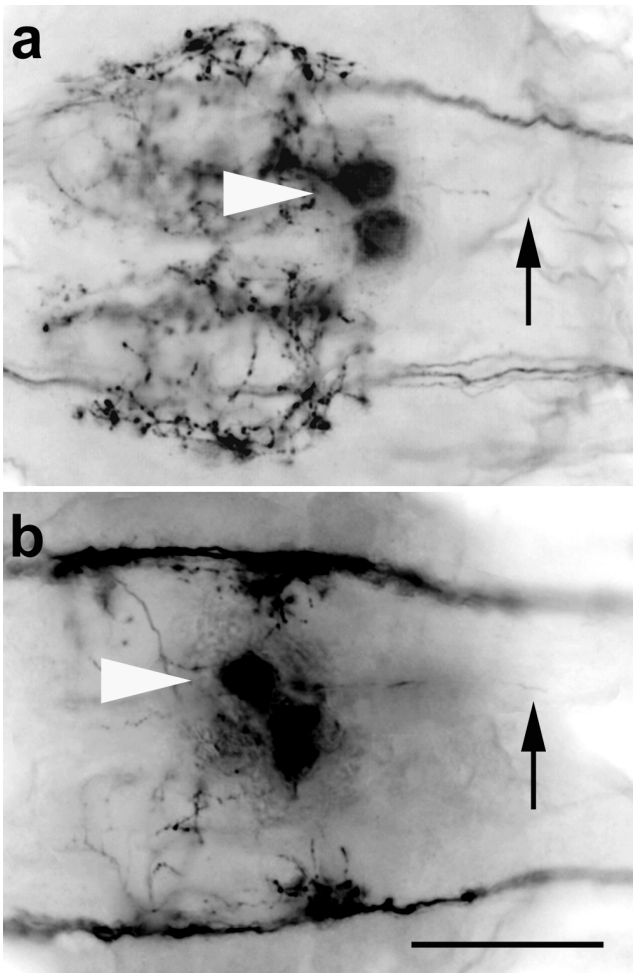


**Fig. 6. Temporal differences in the development of HA-IR in the first free abdominal ganglion (A4).**

(**a**) Demonstration of A4 at 75%E in *S. gregaria* and between 80% and 85%E in *L. migratoria* (**b**). Temporal differences in the innervation pattern become evident. In *S. gregaria*, the central neuropile is already filled with fine arborizations, whereas in *L. migratoria* they just start to ramify. In both specimens, a single neurite grows posteriorly from the central neuropile (arrows). In contrast to the ascending axons of the interneurons, this neurite follows a median path through the connectives. The origin of the descending processes remained unclear. The arrowheads point to cell bodies of one ganglionic hemisphere.

Dorsal view, anterior to the left. Scale: 50 µm.

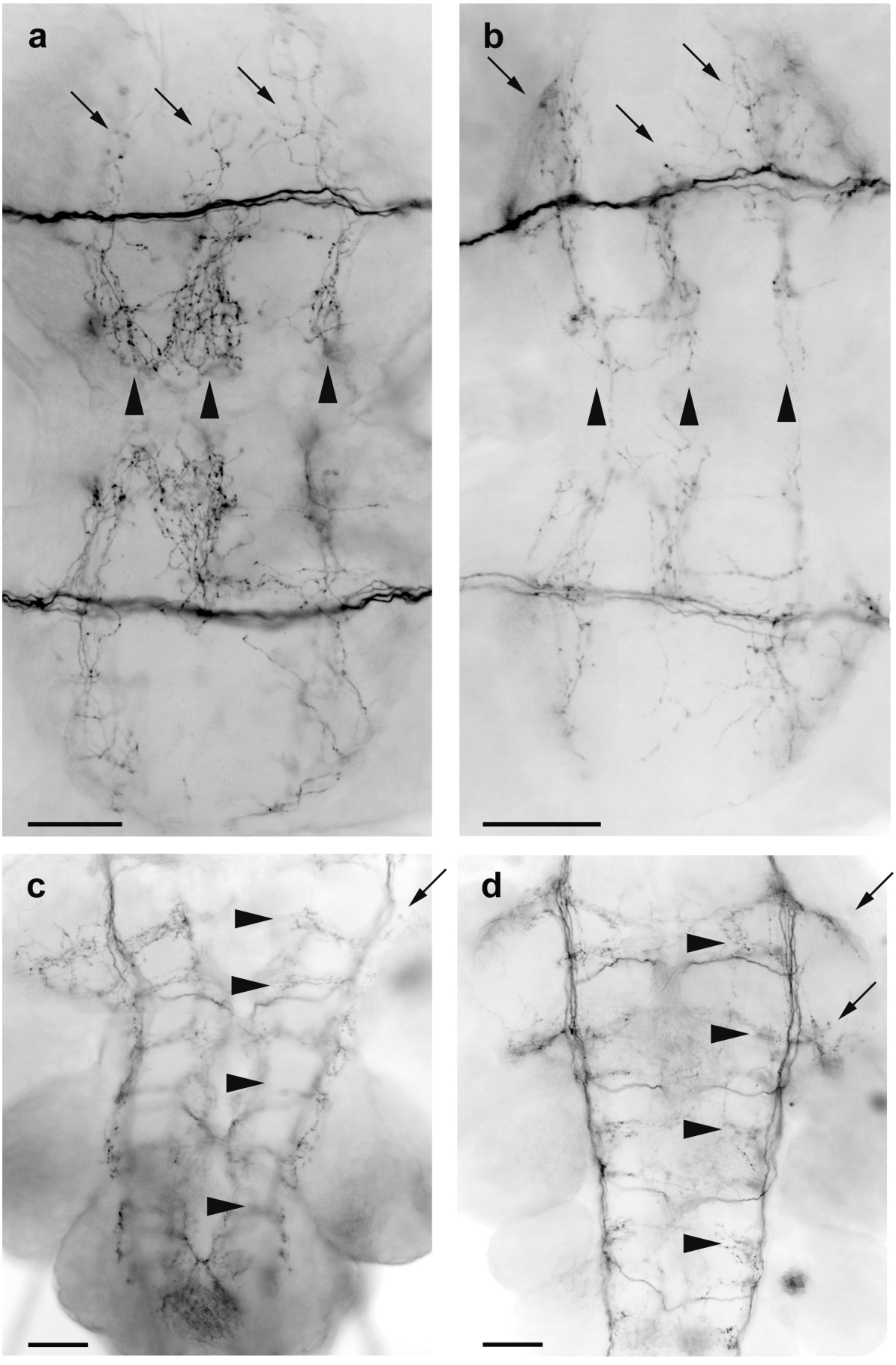
a
b
c
d

**Fig. 7. Comparison of HA-IR distribution in thoracic ganglia of two locust species at 85%E.**

On the left, the prothoracic (**a**) and metathoracic (**c**) ganglia of *S. gregaria* and on the right the same ganglia (**b** and **d**) of *L. migratoria*. The preparations are derived from specimens of the same developmental stage, identified by morphological characters (Bentley et al., 1979). At this developmental stage, there are no differences evident in the neuroarchitecture of the HA-IR pattern. In the prothoracic ganglia of both species, HA-IR interneurons are absent. Varicosity-like structures as putative releasing sites of histamine are distributed all over the neuropile (a and b). (a-d) The ganglionic neuropile is innervated at three distinct locations starting with ramifications into central (arrowheads) and lateral (arrows) regions. A similar shape is presented in the metathoracic ganglion, although the general shape in this fused ganglion is somewhat different from the other thoracic ganglia.

a and c: anterior to the left; b and d: anterior to the top; dorsal view. Scale: 50 µm.

### 2.2.1 Appendix

#### Histamine uptake

The early developmental expression of neurotransmitter uptake mechanisms can be used as a convenient method for labeling identifiable neurons by immunocytochemistry prior to neurotransmitter synthesis. For example, serotonin is taken up into prospective serotonergic neurons prior to serotonin synthesis via specific transporters (Condron, 1999). To test for putative uptake mechanisms of histamine into neurons of the CNS, histamine was added to the medium.

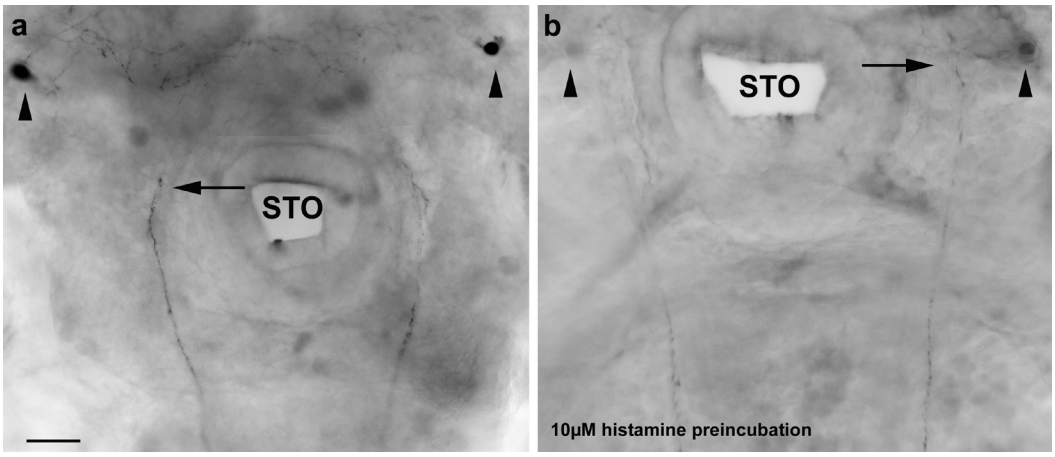


**Fig.8. Developmental progress of histamine immunoreactivity in the embryonic brain.**
(**a**) Initial HA-IR in single neurons in the brain at around 50%E (arrowheads). Cells belong to a cluster of neurons located in the posterior lateral protocerebrum. At this stage, one axon traversing the thoracic ganglia enters the brain. Here, the tip of the axon (arrow) is at the level of the stomodaeum (STO). (**b**) Preincubation with bath applied histamine neither enhances the staining intensities nor the number of immunoreactive neurons in the brain. Anterior to the top, scale 50 µm.

Preincubation with histamine prior to histamine synthesis did not label presumptive histaminergic neurons in the CNS. Identified neurons in the brain (Fig. 8) and in the VNC (not shown) show comparable intensities of labelling. Within the detection threshold of the immunofluorescence method, I was unable to find evidence for a specific uptake mechanism of histamine.

## 2.3. A role for nitric oxide-cGMP signalling in directed axonal outgrowth of insect pioneer neurons.

Arne Pätschke and Gerd Bicker

University of Veterinary Medicine Hannover
Division of Cell Biology
Bischofsholer Damm 15, D-30173 Hannover, Germany

Email: Arne.Paetschke@tiho-hannover.de
Email: Gerd.Bicker@tiho-hannover.de

Phone: +49 511 856 7766
Fax: +49 511 856 7687

## Abstract

The dynamic regulation of nitric oxide synthase (NOS) activity and cyclic GMP levels suggest functional roles of NO-cGMP signalling in the development of nervous systems. NO-cGMP signaling is required for axonal elongation of pioneer neurons in the antenna of the grasshopper embryo. Here, we report evidence for an essential role of this signaling cascade in directed axonal outgrowth of limb bud pioneer neurons. A sensitive immunocytochemical staining method revealed NO-induced cGMP synthesis in identified pioneers of the metathoracic limbs. NO-induced cGMP synthesis was not only found in peripheral, but also in certain central nerve cells, such as the MP1 and the dorsal MP2 midline precursor neurons which pioneer the first longitudinal pathway of the ventral nerve cord. Moreover, we resolved transient NO sensitivity in the outgrowing identified motor axons of the slow and fast extensor tibiae (FETi, SETi) prior to the formation of neuromuscular junctions. These findings suggest a role of NO-cGMP signaling during axonal outgrowth rather than in synaptogenesis. To investigate a role of NO-cGMP signaling in directed axon outgrowth, we focussed on the pair of Ti1 pioneers originating in the tibiae of the metathoracic limbs. We used chemical manipulations in whole embryo culture together with immunocytochemical staining for a neuronal epitope to analyze the resulting axon guidance phenotypes. The highly stereotyped pathway of the afferent Ti1 pioneer neurons includes two characteristic turns along the limb epithelium. Chemical inhibition of NO and cyclic GMP formation caused defects in pathway formation mainly at the turning points. These defect phenotypes could be rescued by application of membrane-permeant cGMP or a direct activator of the enzyme soluble guanylyl cyclase. Our results show that NO-cGMP signaling is required for oriented growth cone steering. Since western blot analysis revealed the presence of NOS in metathoracic limb bud homogenates, we propose that tissue endogenous NO signaling stimulates the formation of cGMP in pioneer neurons. Upregulation of cGMP levels may tune the growth cone to respond correctly to secreted and membrane-bound guidance cues of the limb bud.

Keywords:
axonal navigation; development; growth cone steering; cyclic nucleotide; guidance cue; immunocytochemistry; *Schistocerca gregaria*

## Introduction

Nitric oxide (NO) is an atypical cellular messenger that plays multiple functions in the vascular, immune, and nervous system. A major receptor molecule for NO is the cyclic guanosine-monophosphate (cGMP)-synthesizing enzyme soluble guanylyl cyclase (sGC). In the vertebrate nervous system, NO-cGMP signalling has been implicated in cell proliferation, cell migration, synaptogenesis, and synaptic plasticity (Boehning and Snyder, 2003; Garthwaite, 2008; Godfrey et al., 2007; Nikonenko et al., 2008; Packer et al. 2003; Tanaka et al. 1994; Taqatqeh et al. 2009). NO has also emerged as a signalling molecule regulating similar aspects of neural plasticity and neurodevelopment in insects (Champlin and Truman 2000; Gibbs and Truman, 1998; Haase and Bicker 2003; Kuzin et al., 1996; Shakiryanova and Levitan 2008; Wildemann and Bicker 1999).

Immunocytochemical studies of NO-induced cGMP synthesis in embryonic grasshoppers suggested a role of NO-cGMP signaling during synapse formation. Many identifiable nerve cell types respond to exogenous nitric oxide application by producing cGMP (Truman et al., 1996). Some of the NO-responsive cells are identified motoneurons showing cGMP-IR axonal growth cones. The sensitivity to NO appears after the growth cone has arrived at its target but before branches have started to explore the muscle, reflecting the transition from longitudinal elongation to the formation of lateral branch growth. Moreover, certain sensory and interneurons also become NO receptive as they change from axonal outgrowth to synaptogenesis. The discovery of a transient NO sensitivity led to the hypothesis (Ball and Truman, 1998) that cGMP plays a role in the early stages of communication between a postsynaptic target and specific innervating neurons.

An investigation of pioneer neuron outgrowth shows that NO signaling affects growth cone extension, indicating that one mechanism how NO may influence the establishment of synaptic connectivity could simply be via regulation of cell motility (Seidel and Bicker 2000). Pioneer neurons of the central and peripheral nervous system establish the first axonal pathways that are followed by later-growing axons. In the antenna and limb buds identified pairs of peripheral pioneer neurons extend their axons along a basal lamina from their origin near the tip of each appendage to the CNS (Bate, 1976b; Bentley and O'Connor, 1992). Pathfinding involves selective adhesion of the growth cones to secreted/substrate bound guidance cues and recognition of guide post cells (Bentley and O'Connor, 1992). In the thoracic limb bud, two secreted gradients of the semaphorin cell recognition molecule Sema-2A have been identified, that repel the pioneer growth cones away from the periphery while extending towards the CNS (Isbister et al., 1999; Legg and O'Connor, 2003). A circumferential band of the transmembrane semaphorin (Sema-1A) molecule in the epithelium of the trochanter segment seems to contribute as attractive guidance cue to the characteristic ventral turn of the pioneer axons (Legg and O'Connor, 2003; Wong et al., 1999).

Outgrowing pioneer neurons in the antennal appendage (Bate, 1976b; Ho and Goodman, 1982, Boyan and Williams, 2004) synthesize cGMP in response to exogenous NO treatment (Seidel and Bicker, 2000). Inhibition of endogenous NO synthase (NOS) and sGC activity by small-molecule compounds results in a retardation of axon outgrowth from the pioneer neurons. The pharmacological disruption of pioneering pathways can be rescued by supplementing the cultured embryos with membrane permeant cGMP and with a NO-independent activator of sGC. These results indicate that an endogenous NO signal is necessary for pioneer axonogenesis in the antenna (Seidel and Bicker, 2000).

To detect NO-induced cGMP synthesis in the grasshopper neurons, Truman et al. (1996) and Seidel and Bicker (2000) used a routine immunocytochemical technique developed by De Vente et al. (1987). This method involves incubation of the intact tissue in a NO-donor and blocking of cGMP degradation by a phosphodiesterase inhibitor prior to fixation and immunostaining. In the present investigation, we have revisited the analysis of cGMP formation during early embryonic development. We preincubate the embryos with the NO donor sodium nitroprusside (SNP), the phosphodiesterase inhibitor IBMX and the compound A-350619, an agent which synergizes with NO to stimulate sGC enzymatic activity (Miller et al., 2003). This leads to enhanced formation of cGMP and improves immunocytochemical labeling. Using the more sensitive immunocytochemical staining, we find robust NO sensitivity in the antennal and also in the pioneer neurons of the limb buds. NO-induced cGMP synthesis is not only confined to peripheral but also expressed in pioneer neurons of defined central pathways. Moreover, we resolve NO sensitivity in outgrowing identified motor axons prior to the formation of neuromuscular junctions. Finally, we use chemical manipulations in whole embryo culture to analyze the role of NO-sGC-cGMP signaling for the directed outgrowth of pioneer neurons in the metathoracic limb buds.

## Materials and Methods

*Schistocerca gregaria* eggs were collected from a crowded colony at our institute. The egg pods, laid in moist vermiculite, were collected daily and kept at 30°C until use. Embryos were staged according to the system of Bentley et al. (1979) supplemented with our own observations, such as the morphology of the metathoracic limb. For *in vivo* culturing experiments the embryos were staged very carefully to 32%E (percentage of embryonic development).

### *In vivo* culturing experiments

For preparation, the eggs were cleaned and sterilized in 70% ethanol. Egg shells were opened in cold L-15 cell culture medium (L-15, Gibco, Life Technologies) containing 1% penicillin-streptomycin (10,000 units per ml). The amnion surrounding the embryo was removed carefully and the dorsal membrane was opened to allow access of the chemicals. After dissection, the embryos were collected in fresh L-15 supplemented with 1% Penicillin-Streptomycin into 48-well plates on a shaker. The embryos were allowed to develop for 18 hours at 30°C in the presence of chemicals affecting compounds of the NO-signaling pathway. After incubation the embryos were fixed immediately in 4% PFA (0.1M phosphate buffered saline (PBS) supplemented with 4% paraformaldehyde) and horseradishperoxidase immunoreactivity (HRP-IR) was performed as described later.

### Pharmacological treatment

All chemicals in this study were purchased from Sigma, exceptions were especially mentioned. The NOS inhibitors 7-nitroindazol (7-NI), a sodium salt of 7-nitroindazol (7-Nina), the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), the sGC activator protoporphyrin IX free acid (PP IX free acid, Alexis), and the phosphodiesterase inhibitor 3-isobutyl-methylxanthine (IBMX) were dissolved in dimethyl sulfoxide (DMSO, Roth). The cGMP analogs 8-Bromo-cGMP (8-Br-cGMP), nitro-L-arginine methyl ester (L-NAME) and the D antiomer (D-NAME), 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), sodium nitroprusside (SNP), and the sGC stimulator A- 350619 (Miller et al., 2003) were dissolved in L-15. Stock solutions of the reagents were kept in a freezer until use. For culturing experiments solutions were diluted to provide a final concentration of 0.5% DMSO in the medium. The same amount of solvent was added to the control medium.

### Cyclic GMP immunoreactivity

To enhance the formation of cGMP, the embryos were incubated in the presence of the nitric oxide donor SNP (100µM), the NO independent sGC stimulator A- 350619 (50µM), and the phosphodiesterase inhibitor IBMX (0.1mM). Early stages were incubated for up to 19 hours at 30°C. From 36%E on the incubation time was reduced to 70min at 30°C. Subsequently, embryos were fixed in 4% PFA for 45-60min at room temperature. Embryonic tissue was permeabilized in 0.3% saponine in PTX 0.5% for 45 min. After several washes in PTX 0.5% an antibody against cyclic GMP (courtesy of Dr. J. de Vente; for specificity see de Vente et al., 1987), raised in sheep, was applied (1:7500 or 1:10000 in PTX 0.5%) over night at 4°C. A secondary antibody (anti-sheep IgG, Vector; 1:250) conjugated to biotin was added for up to 4 hours at room temperature. For visualization either CY3 or AlexaFluor 350, both conjugated to streptavidin, was added (1:300 in PTX 0.5%) for at least one hour at room temperature.

### Cyclic GMP and Fasciclin II double labeling

Anti cGMP-IR was performed as described above. Instead of mounting, the embryos were washed in PTX 0.5% several times and blocked in 5% normal horse serum (NHS). An antiserum against Fas II raised in mouse (cell culture supernatant, Developmental Hybridoma Studies Bank) was added (1:50 in PTX 0.5%) over night at 4°C. After several washes in PTX 0.5% a HiLyte Fluor coupled secondary antibody was added (1:200 in PTX 0.5%) for 4 hours at room temperature.

### NOS detection by Western Blot analysis

For the detection of NOS we used both whole embryos at 30%E (N=10) and metathoracic limbs of 33%E embryos (N=15). Whole embryos and the metathoracic limbs were collected in 0.3% Saponine dissolved in PTX 0.5% containing 1% protease inhibitor cocktail (HALT, Pierce, Rockford, IL, USA) buffer to prevent decay of proteins. Samples of 10 (whole embryos and 30 (metathoracic limbs) were collected in 200 µl lysis buffer. Homogenisation was performed using a Kontes Duall tissue grinder with PTFE pestle (Landgraf Laborsysteme, Langenhagen, Germany). Homogenates were centrifuged for 10 min at 6000x g. Protein pellets were subsequently redissolved in 2x Laemmli puffer (100 mM Tris-HCl, pH 6.8 with 4% SDS, 20% glycerol, 0.02% bromphenol blue). Samples were denaturated at 95°C for at least 3 min in loading buffer (2x Laemmli buffer with 2% SDS, 10% 1 M DTT). The samples were separated on 8% PAGE and transferres to a PVDF membrane (Roth). After equilibration of the membrane in PBS immunocytochemistry was performed. The membrane was blocked in 5% low fat milk powder (Humana, Germany) in PBS containing 1% BSA for one hour at

room temperature and subsequently an antiserum against a universal NOS (uNOS, Affinity BioReagents, Golden, CO, USA) raised in rabbit was added at a concentration of 1:100 in PTX 0.05% over night at 4°C. After several washes in PTX 0.05% a biotinylated secondary antibody against rabbit (1:250) was added for 2 hours at room temperature. Subsequently uNOS-IR was visualized by peroxidase staining technique using the Vector ABC kit. The blots were scanned and prepared for presentation.

**Visualization of the developing Peripheral System Nervous**

Invertebrate nerve cells express an epitope that could be detected by immunocytochemistry with an antiserum raised against the horseradish peroxidase (Jan and Jan, 1982, Haase et al., 2001). We used this technique for the observation of developing Ti1 pioneer neurons. Embryos were fixed in 4% PFA for 45min. After permeabilization in 0.3% saponine in PTX 0.5% up to one hour at room temperature, unspecific binding sites were blocked in 5% normal rabbit serum (NRS) for 1h. Subsequently an antiserum against the HRP epiotope was added (1:5000 in PTX 0.5%) over night at 4°C. After several washes in PTX 0.5%, a biotinylated secondary antibody (Vector, 1:250) was added for two and a half hour at room temperature. HRP-IR was visualized by CY3 conjugated streptavidin (1:300).

**Analysis of pathfinding defects**

After 18 hours in culture embryos were prepared for HRP immunocytochemistry. In each trial, five embryos were fixed right after dissection as an internal control of the original stage at the beginning of the experiment. An untreated group was used as a control for Ti1 outgrowth and elongation. Abnormalities in Ti1 morphology were analyzed compared to these control groups. For analysis, several criteria of abnormal growth were specified: Presence of defasciculated fibers (for more than one soma diameter in length), abnormal branching pattern, and misrouted fibers. As the Ti1 growth cones have to make two distinct choices for turning, the errors at the first and the second turn were observed separately. Often, such mistakes at the turning points led to aberrant growth into dorsal parts of the limb (failure of first turn) and growth into distal direction (failure of second turn). The limbs, where at least one of these criteria was found, were summarized and tested by chi-square-test for significance. The degree of significance is mentioned as $p<0.05$ *, $p<0.01$ ** and $p<0.001$ ***.

### Tissue embedding and microscopy

Whole embryos were cleared in 50% glycerol and mounted in 90% glycerol on glass slides. Preparations were analyzed under a Zeiss Axioskop. To document certain stages pictures were taken by a Zeiss Axiocam HRc. Then, photomicrographs were arranged using Photoshop 7.0. Values of fluorescence were inverted and slightly enhanced for contrast.

## Results

### Nitric oxide induced cGMP immunoreactivity

### Central pioneer neurons

Using the compound A-350619 as enhancer of NO-stimulated sGC activity we re-examined an immunocytochemical study on responses of neurons to agents that generate nitric oxide. Truman et al. (1996) reported that that the onset of NO sensitivity in embryonic neurons is confined to later stages of embryonic development, but a few neurons became responsive. During early development, a small subset of central neurons expressed NO-induced cGMP-IR in the thoracic and abdominal ganglia of the ventral nerve cord.

A first longitudinal path in the ventral nerve cord is pioneered by midline precursor neurons (MP). Axons of the MP1 and the dorsal MP2 (dMP2) grow posteriorly whereas the ventral MP2 (vMP2) choose an anteriorly directed pathway (Bate and Grunewald, 1981; Bastiani et al., 1984; 1987). Around 34%E, the MP 1 and dMP2 neurons produced cyclic GMP in response to exogenous NO stimulation. At this stage the neurons have initiated axon outgrowth (Fig. 1A). At 36%E the axons have entered the adjacent ganglia (Fig. 1B). At this stage the ventral MP2 neurons have started axonogenesis. The tip of the growing axon could be identified leaving the ganglion anteriorly (arrow in Fig. 1B). The vMP2 was only transiently responsive to NO, whereas MP1 and dMP2 remained responsive over the next stages (Fig. 1C). MP1/dMP2 neurons could be identified until approximately 40%E (Fig. 1D) but from 45 % on the immunoreactivity decreased (not shown). The MP1/dMP2 neurons can be unambiguously identified by their expression of the neuronal adhesion molecule fasciclin II (Fas II) (Bastiani et al., 1987). A double labeling approach with antibodies against cGMP-IR and Fas II confirmed their identity (Fig. 2).

### Motor neurons

The number of cGMP-IR neurons increased rapidly from the first appearance at 34%E over the next percentages of development. In the metathoracic ganglion, small extensor tibiae motor neuron (SETi) cell bodies were located at the anterior edge of the ganglion, laterally to the connective. At 34%E the SETi cell bodies became cGMP-IR. At this time they eventually started axonogenesis and their initial neurite grew apart of the ganglion (Fig. 1A). In the metathoracic ganglion the axon of SETi left the ganglion through nerve root 3 (Myers et al., 1990; Whitington and Seifert, 1981). Cyclic GMP-IR remained over the next percentages of development and axonal outgrowth could be followed (Fig. 1). At 37%E the fast extensor tibiae (FETi) became cGMP-IR. The initial neurite grew into a loop through the lateral hemisphere and left the ganglion via nerve root 5 (Fig. 1B). However, we could observe cGMP-IR in FETi only at this distinct developmental stage.

## Peripheral neurons

Antennal pioneer neurons respond to an exogenous NO stimulus with the formation of cGMP (Seidel and Bicker, 2000). Here we show that cGMP-IR is also expressed in the Ti1 pioneer neurons of the metathoracic limb. Detection of cGMP-IR in the Ti1 neurons was observed at around 35%E. At this stage the growth cones have already entered the CNS (Fig. 3A).

The observations of cGMP-IR in central neurons and in peripheral pioneer neurons after exposure to an external NO donor raise the following questions. Is there a natural source of nitric oxide in the locust embryo and what might be the role of NO/cGMP in nervous system formation? To test for a potential role of this signaling cascade (Fig. 3C) in axonal outgrowth and navigation, we chose to examine the well known Ti1 pathway (Fig. 3B) instead of the identified central neurons.

## Western Blot analysis of NOS

A sensitive method for the detection of proteins is the Western blot analysis. For detection of NOS, lysates of both, whole embryos at 30%E (N=10), and of metathoracic limbs at 33%E (N=15) were separated by SDS-PAGE. Figure 4 presents clearly restricted. A strong immunoreactive band could be detected at approximately 130 kDa in homogenates of whole embryos (30%E) and of metathoracic limbs (33%E), indicating for NOS-like protein (Fig.4). Less intense bands appeared at approximately 70 kDa.

## NO-cGMP-signaling is essential for correct axonal navigation

In whole embryo culture, starting at 32%E, the Ti1 axons grew within 18 hours into the CNS (Figs. 5 A and B). In each experiment we prepared five embryos separately to confirm the correct stage at the start of the experiment. Each trial was performed following a standardized experimental design. Embryos were randomly divided into single groups. One group served as an untreated control. The same amount of vehicle, used to solve inhibitors or activators of the NO-signaling cascade (Fig. 3C), was added into the medium of the control group. After treatment the Ti1 pioneer outgrowth was carefully analyzed for malformations in axonal pathfinding and navigation using HRP-immunofluorescence.

Except for the group where D-NAME was used, each control group revealed a comparable level of correct axonal outgrowth. In summary, the levels of normal outgrowth ranged from 70% to around 85% in the control groups (Fig. 7). This level was actually reached in the rescue trials.

To test the influence of NO-cGMP signaling on directed Ti1 pioneer axon outgrowth, single components of the transduction cascade were blocked (Fig. 3C). The nitric oxide synthase inhibitor 7-NI (500µM) reduced the correct outgrowth to 54.2% (N=59). This effect could either be rescued by NO independent stimulation of sGC with 1mM PP IX free acid or by addition of the membrane permeant cGMP analog 8-Br-cGMP (500µM) into the medium. Protoporphyrin IX free acid rescued the defect to a correct outgrowth of 77.5% (N=40). Adding 8-Br-cGMP resulted in 88.9% (N=27) correct outgrowth. This rescue effect was significant compared to the phenotype resulting from NOS inhibition by 7-NI (47.5%, N=61, Figs. 7A and B).

Normally, Ti1 growth cones turn ventrally and grow circumferentially along the femur-trochanter (Fe-Tr) segment border (Fig. 3B). After inhibition of NOS by 7-NI approximately 50% of Ti1 growth cones failed to perform the correct ventral turn. The Ti1 growth cones were misrouted and could be observed in dorsal parts of the limb proximally to the Fe-Tr segment boundary (not shown).

The sodium salt 7-Nina is a more water soluble NOS inhibitor than 7-NI, allowing for a lower concentration of DMSO in the medium. After inhibition with 7-Nina a total of 33.9% (N=39) of the Ti1 growth cones grew along the correct path (Fig. 7C). In 10 of 39 limbs at least one axon failed to turn ventrally at the first steering point, instead growing further into dorsal parts of the limb (Fig. 5C). In 8 of 39 limbs the axons turned ventrally in a correct way but failed to turn proximal at the second steering point correctly (Fig. 5D). The pathfinding errors could be rescued by adding 500µM 8-Br-cGMP into the medium to 77.8% (N=36) of correct outgrowth (Figs. 5E and 7C).

The competitive inhibitor L-NAME caused a significant decrease in correct Ti1 outgrowth (Fig. 7D). Compared to controls with the inactive enantiomer D-NAME showing correct outgrowth at a level of 55.0% (N=20), L-NAME caused a significant reduction of the pathfinding precision to only 21.7% (N=23). Most of the navigational errors were made at the first turning point (12 in 23 limbs). In rescue trials 500µM 8-Br-cGMP was added to the medium. Here, 60.0% (N=10) of Ti1 axons performed a correct path resembling the levels of the control group (Fig. 7D).

A further method to test for functional NO-signaling was to capture tissue generated NO (Fig. 7E). By scavenging NO with 500µM PTIO the correct pattern of outgrowth was reduced to 33.3% (N=30). This effect could again be significantly rescued to 65.9% (N=29) by adding 8-Br-cGMP.

To test for further downstream effects in the NO-sGC-cGMP signaling cascade sGC was inhibited by the potent inhibitor ODQ (Fig. 3C). In the presence of 200µM ODQ, axonal outgrowth was nearly completely blocked (data not shown). At concentrations of 100µM which enabled axonal outgrowth, significant pathfinding defects could be observed. The percentage of limbs with the correct path was reduced to 32.3% (N=37). The majority of errors (25 of 37 limbs) could be observed at the first steering point (Fig. 6A). Adding 8-Br-cGMP into the medium rescued the pathfinding significantly to 58.3% (N=36). Although the overall morphology of the Ti1 pathway could be rescued by exogenous 8-Br-cGMP, the shape of the axons was still affected such that many filopodia-like extensions protruded along the course of the entire axons (Figs. 6B and 7F). In summary, the results of blocking of NOS and sGC enzymes in combination with the rescue experiments argue for an essential role of NO-cGMP signaling in the correct navigation of the Ti pioneer pathway.

## Discussion

In the current study, we have examined the spatiotemporal expression of NO-induced cGMP-IR during onset of axonal outgrowth in the ventral nerve cord and leg appendages of the locust. Using a sensitive cytochemical method, we detected the synthesis of cGMP in identified pioneer neurons of central and peripheral pathways. To analyse a potential function of NO-cGMP signalling in neurite development, we focussed on the powerful model system of Ti1 pioneer axon outgrowth in the limb buds (Fig. 2-3A). Western blotting revealed the presence of NOS in the embryonic limbs. Chemical manipulation of endogenous NOS and sGC activity in an embryo culture system influenced directed axon outgrowth. The combined loss and gain of function experiments suggest that NO-cGMP signaling is an essential component of growth cone navigation along the complex Ti1 pioneering pathway. These results contrast somewhat with the results of Seidel and Bicker (2000), which considered the effects of NO-cGMP signaling only on the initial outgrowth of antennal pioneers. Blocking of NOS or sGC retarded axon extension of the antennal pioneers, while no pathfinding defects were observed. Similarly, there is clear evidence for a permissive role of this signaling cascade in neuronal migration on the embryonic locust gut, with no pathfinding defects after blocking of transcellular NO-cGMP signaling (Haase and Bicker, 2003, Knipp and Bicker, 2009).

### NO-induced cGMP formation in pioneering pathways

In insect central nervous systems, the first longitudinal pathway is pioneered by the MP1/dMP2 neurons (Bastiani et al., 1984; Bastiani et al., 1985; Lin et al., 1995; Taghert et al., 1982). These prominent neurons showed cGMP-IR during outgrowth in the locust embryo (Fig. 1). For an unambiguous identification of these central pioneers, we performed double labeling against a characteristic cell surface molecule (Fig.2). The neuronal cell adhesion molecule Fasciclin II (Fas II), expressed by only a small subset of neurons, served as a marker for MP1/2 neurons (Seaver et al., 1991). The MP1/dMP2 cells started to express Fas II when the axons covered half of the way into the next posterior ganglion. Afterwards, they fasciculated with the axons of the adjacent MP1/dMP2 processes, thus, forming the MP1/dMP2 longitudinal fascicle (Fig. 2). The later arising vMP2 grew along a different fascicle anteriorly and did not express Fas II (Bastiani et al., 1987; Harrelson and Goodman, 1988). The sensitivity to NO of the MP1/2 neurons was maintained until approximately 50%E when the longitudinal fascicle has already been formed. Since other cells that pioneer the first three longitudinal axon fascicles (Bastiani et al., 1986) did not express cGMP-IR, NO-induced cGMP formation seems to be restricted to a set of individual cell types and cannot be considered as a general cellular property of pioneering neurons. However, the restricted cellular responsiveness illustrates how a NO signal spreading in a tissue volume might address selected target neurons of the developing embryo. Because the NO-cGMP signaling cascade is a positive regulator for axonal extension of antennal pioneers (Seidel

and Bicker, 2000) and regeneration in the developing locust CNS (Stern and Bicker, 2008), it is possible that tissue endogenous NO release provides a signal for axon outgrowth of the MP1/2 neurons.

The extensor muscle of the metathoracic limb is innervated by two motor neurons SETi and FETi which sent out their axons via a different nerve roots (Myers et al., 1990; Whitington, 1989; Whitington and Seifert, 1981). Both motor neurons expressed cGMP-IR during axonal elongation. In previous investigations, Truman et al. (1996) and Ball and Truman (1998) detected NO-induced cGMP-IR in these motor neurons after the growth cone has arrived at its target, but before branches have started to explore the muscle, reflecting the transition from longitudinal extension to the formation of lateral branches. Ball and Truman (1998) interpreted their results as indication that cGMP plays a role in the early stages of communication between a postsynaptic target and specific innervating neurons. Our more sensitive immunocytochemical technique revealed cGMP expression already at stages of motor axon elongation (Fig.1) which challenges this view. Even though we have currently no technique at hand that would resolve actual cGMP concentrations during extension of the motor axon, we favor an interpretation that links the response to NO by increased cGMP synthesis to a signal for axonal motility. The interpretation of the cGMP increase as a timing signal for axonal motility receives also support from our demonstration of transient NO-induced cGMP synthesis during the migration of neurons on the embryonic midgut (Haase and Bicker 2003). Nevertheless, our interpretation does not exclude a role for the continuing cGMP response during later phases of synaptogenesis or synaptic maturation.

In the periphery, Ti1 pioneer neurons responded to NO stimulation with the formation of cGMP around 35%E (Fig. 3A). At this stage the Ti1 growth cones have nearly reached the CNS. Similar to the outgrowing antennal pioneers (Seidel and Bicker 2000) the Ti1 pioneers did not show NADPHd staining. However, the NO-induced cGMP-IR implicates the presence of sGC in the Ti1 pioneers. These results suggest that the Ti1 pioneers may receive an endogenous NO signal from the surrounding limb bud tissue.

To detect the presence of low concentrations of NOS enzyme in early embryonic tissue, we performed western blotting of whole embryos and isolated metathoracic limb buds at stages when major axonal outgrowth occurred. In the absence of molecular sequence data for the NOS of locusts, we used a universal NOS antibody that recognises a highly conserved sequence of the three mammalian NOS isoforms and also detects arthropod NOS (Bicker, 2001; Bullerjahn and Pflüger, 2003; Christie et al. 2003; Settembrini et al., 2007). This uNOS antibody stains interneurons in the adult locust antennal lobe (Bicker, 2001). In a cell culture assay these interneurons have been shown to release NO in a $Ca^{2+}$/calmodulin dependent way (Müller and Bicker, 1994). Western blot analysis of homogenates of whole embryos at 30%E revealed the presence of a band of NOS-like protein at approximately 132 kDa and of a lower molecular weight band, which is presumably a protein fragment (Fig. 4). Western

blotting of isolated metathoracic limb buds resulted in one stained band around 132 kDa and a more faintly stained band around 127 kDa. Similar results have been obtained using a different batch of the uNOS antiserum (Stern et al., 2010). Thus there is evidence for NOS in the developing limb at stages when the Ti1 growth cones navigate the limb bud.

The approximate size of NOS has been independently determined as 135 kDa in the locust brain (Elphick et al., 1995), between 116 to 180 kDa in the locust abdominal nervous system (Bullerjahn and Pflüger, 2003), and between 130-150 kDa in other insects (Gibson and Nighorn, 2000; Settembrini et al., 2007; Watanabe et al., 2007). The genome of *Drosophila* contains only a single NOS gene (Enikolopov et al., 1999, Regulski and Tully, 1995) and immunoblotting showed a band of the corresponding protein at about 150 kDa (Regulski et al., 2004). We assume that NOS is present in the epithelium of the limb buds similar to the antennal epithelium (Seidel and Bicker, 2000), and that it provides a transcellular NO signal to the cGMP-IR Ti1 pioneers.

NOS catalyzes the synthesis of NO and L-citrulline from L-arginine and $O_2$. Since this reaction requires nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, NADPH-diaphorase histochemistry (NADPHd) following formaldehyde fixation of neural tissue is a common method for staining NOS-expressing cells (Matsumoto et al., 1993). In various regions of the adult locust nervous system, measurements of NOS activity in cell homogenates correlate rather nicely with the biochemical determination of NADPHd activity and the histochemical staining pattern of NADPHd positive cells (Elphick et al., 1995; Müller and Bicker; 1994). Nevertheless, the results of the diaphorase staining are rather sensitive to variations in fixation conditions (Ott and Burrows, 1999). Unlike in the antennal epithelium (Seidel and Bicker 2000), we found no discrete labeling with the NADPH diaphorase technique in the embryonic limb bud. The absence of NADPH diaphorase staining with discrete cellular resolution may have been due to the lower sensitivity of the histochemical technique in comparison to the immunoblotting. This observation is also in accordance with data from the ventral nerve cord of *L. migratoria,* where NADPHd labeling has not been visualized prior to 47%E, but western blotting of whole embryos resulted in a NOS signal already at 30% E (Stern et al., 2010).

**Nitric oxide-cGMP signaling and directed axon growth**

Because the Ti1 neurons synthesize cGMP in response to an exogenous nitric oxide stimulus (Fig. 3A) and because we found evidence for NOS protein in the metathoracic limb, we surmised an effect of the NO-cGMP signaling cascade on axonal outgrowth. The pathway of the afferent Ti1 pioneer neurons includes two characteristic turns along the limb epithelium (Fig. 3B) (Bate, 1976b; O`Connor et al., 1990). We observed defects in pathway formation after inhibition of single components of NO signaling. Both, inhibition of NO formation and inhibition of cyclic GMP formation caused similar defects in pathway formation (Fig 5, 6) Finally, Ti1 growth cones did not grow along the highly stereotyped path along the limb epithelium after perturbation of the NO signaling pathway but entered abnormal regions. These phenotypes resemble the defects which were described by antibody blocking experiments of semaphorin signaling (Isbister et al., 1999; Wong et al., 1997; 1999).

Indeed, cGMP signaling has been found to be essentially linked to a certain semaphorin-mediated growth cone behavior in Drosophila (Ayoob, 2004), although in this case the biosynthetic enzyme was of a receptor type guanylyl cyclase. Cell culture experiments using *Xenopus* neurons have shown that elevated cGMP concentrations change the response to semphorin 3A from repulsion to attraction (Song et al., 1998). Thus the relative amounts of cyclic nucleotides influence growth cone behavior and the direction of growth cone turning (Song and Poo 2001). Moreover, growth cones can change their turning responses also to other substrate guidance cues by modulating cytosolic calcium signals via NO-cGMP signaling (Tojima et al., 2009)

We interpret the undirected extension of Ti1 axons as reduced capability of the growth cones to process guidance information. Intriguingly, the disruptive effects after perturbation of NO signaling could be rescued by activation of sGC by protoporphyrin free acid (Fig. 7). Similarly, the inhibition of NO signaling in the presence of a membrane soluble cGMP analog prevented Ti1 pathway disruptions, indicating that NO-cGMP signaling is essential for growth cone navigation. Since the rescue trials eventually reached the rate of correct outgrowth of the corresponding control groups (Fig. 7) we can also exclude unspecific side effects of the bath applied chemical agents.

Our *in vivo* data indicate that the pioneer neurons may receive extracellular NO signals from the limb tissue and that elevated cGMP levels are essential for growth cone guidance. Could site specific release at the turning points or a gradient of tissue-intrinsic NO signal play a role as a guidance factor for the cell migration? Since homogeneous bath application of NO donors and cGMP analog is sufficient to rescue the pharmacologically induced pathfinding defects, it is rather unlikely that NO release can prefigure the migratory route of the pioneers. Rather, the extracellular distribution of the two Sema molecules will provide critical guidance signals for pioneer growth cone navigation (Kolodkin et al., 1992; Isbister et al., 1999).

However, the presence of NO-inducible sGC activity in the pioneer neurons (Fig. 3) suggests that enhanced levels of cGMP are required for correct interpretation of the Sema signals.

Since production of NO is a tightly regulated process (Bredt and Snyder 1992; Garthwaite and Boulton 1995), increases in cytosolic $Ca^{2+}$ levels could also provide a developmental timing signal for the production of NO in the insect embryo (Müller and Bicker, 1994). The timed generation of NO signals might then affect a rearrangement of actin filaments in the neuronal cytoskeleton as shown for the migrating enteric neurons (Haase and Bicker 2003). In summary, transcellular NO-cGMP signaling from the expanding limb tissue to the Ti1 neurons may orchestrate the development of the rather complex pioneer pathway by providing timing information for axonal extension and by fine tuning the response of the growth cone to the Sema guidance cues.

## Figures

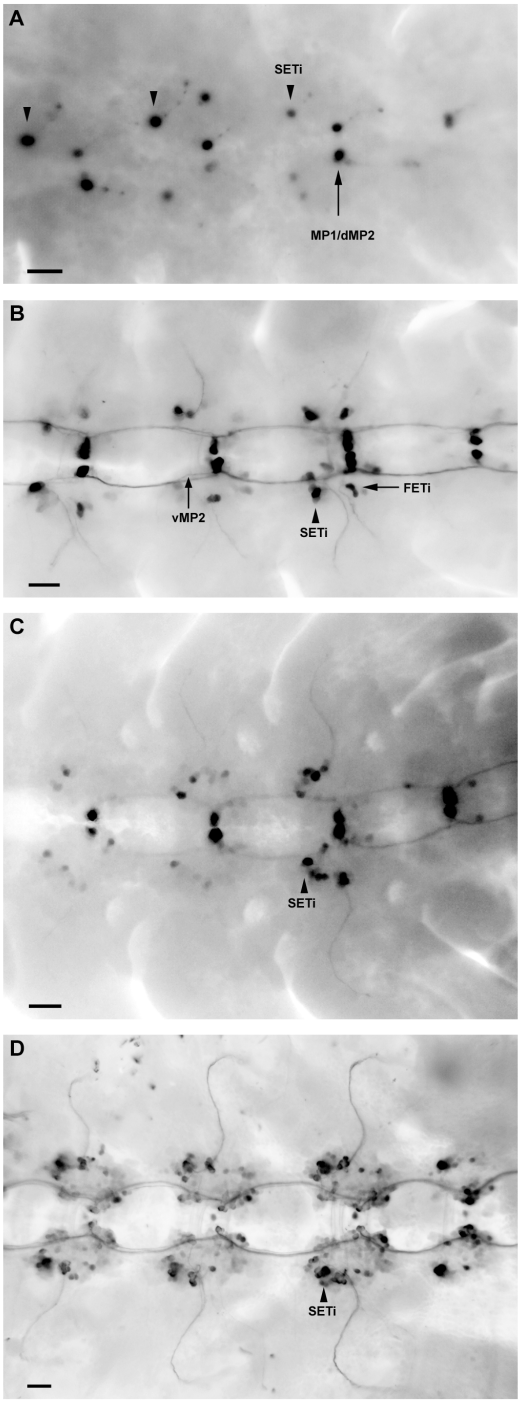

**Fig. 1. Time course of NO induced cGMP-IR in the thoracic ganglia during early embryonic development**

Photomicrographs of the thoracic (Pro-, Meso-, and Metathoracic) and the first abdominal ganglion. (**A**) At 34%E NO-induced cGMP-IR appeared in the MP1/dMP2 neurons (arrow in the metathoracic ganglion) of the thoracic ganglia. The somata were labeled and descending axons could be followed. In the thoracic ganglia cGMP-IR (arrowheads) appeared also in SETi. (**B**) At 36%E the MP1/dMP2 fascicle and SETi remain cGMP-IR. In addition, an outgrowing vMP2 (arrow) and the immunoreactive FETi motorneuron could be identified by cell body position and the course of the primary neurite (arrow). (**C**) From 37% on, the number of cGMP labeled neurons increases. FETi can not be detected anymore, whereas SETi and MP1/dMP2 fascicle remain IR. (**D**) From 40% on, the number of cGMP labeled neurons is further increased. Dorsal view, anterior is to the left. Scale: 100 µm.

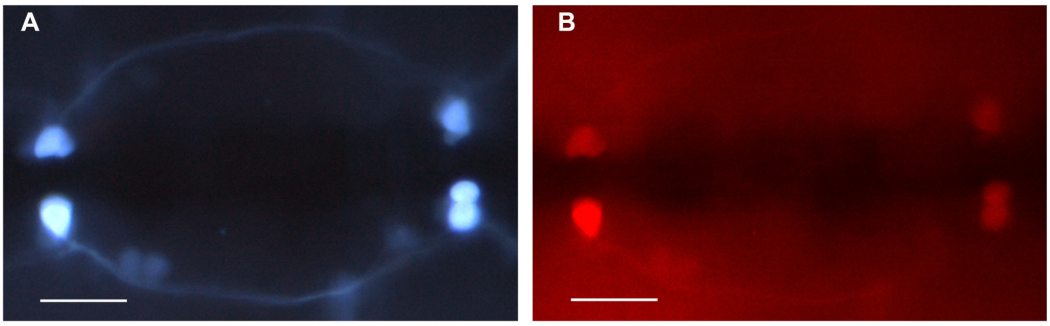


**Fig. 2. MP1/dMP2 pathway in a thoracic ganglion**
Labeling of thoracic MP1/dMP2 pioneers for cGMP and Fas II immunofluorescence at 35%E. (**A**) Nitric oxide induced cGMP-IR. (**B**) Double labeling of somata with Fas II antiserum in the same preparation revealed the identity of the MP1/dMP2 pioneers. Dorsal view, anterior to the left. Scale: 50 µm.

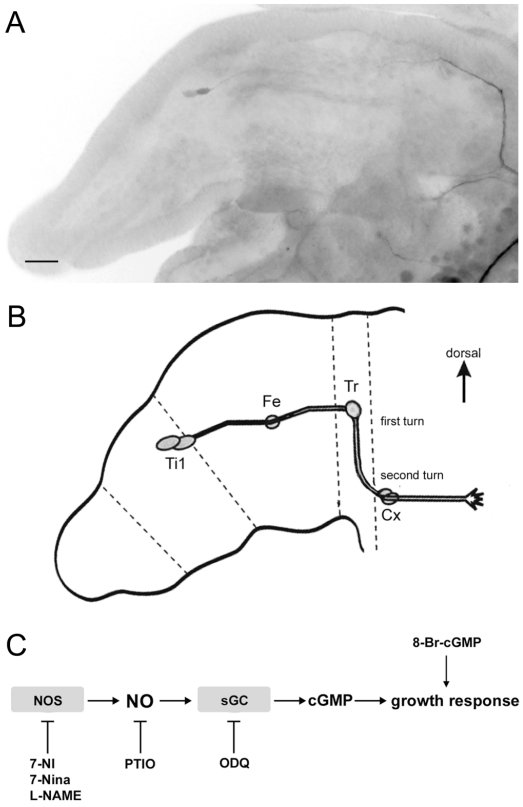


**Fig. 3. Pioneer pathway in the limb**

(**A**) cGMP formation in response to NO stimulation. Ti1 neurons show cGMP-IR after combined stimulation by NO and A350619 with parallel inhibition of phosphodiesterase by IBMX. (**B**) Scheme of the Ti1 pathway through the metathoracic limb (modified from Isbister and O`Connor, 2000) including guidepost neurons of the femur (Fe), trochanter (Tr), and coxa (Cx) segments. Along the Tr-Cx boundary the growth cone follow a ventral path befor turning proximally into the CNS. (**C**) Chemical activators (above) and inhibitors (below) affecting the NO-sGC-cGMP signaling cascade. Scale in A: 50 µm

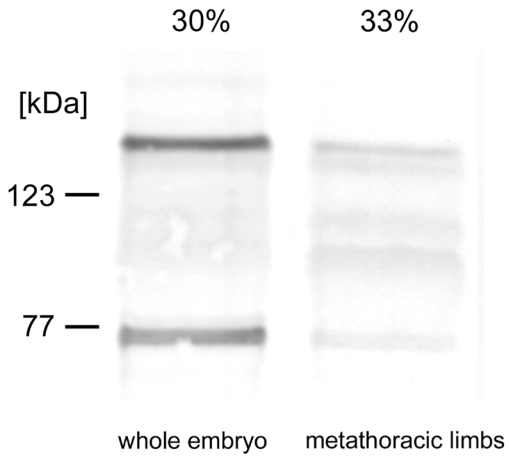


**Fig. 4. Immunoblotting of NOS in the embryo**
Strongly immunoreactive bands at approximately 130 kDa indicating the presence of NOS in lysates of whole embryos at 30%E (left) and in the metathoracic limbs at 33%E (right). The used batch of the polyclonal uNOS antiserum shows also unspecific labeling of lower molecular weight protein bands in both lanes.

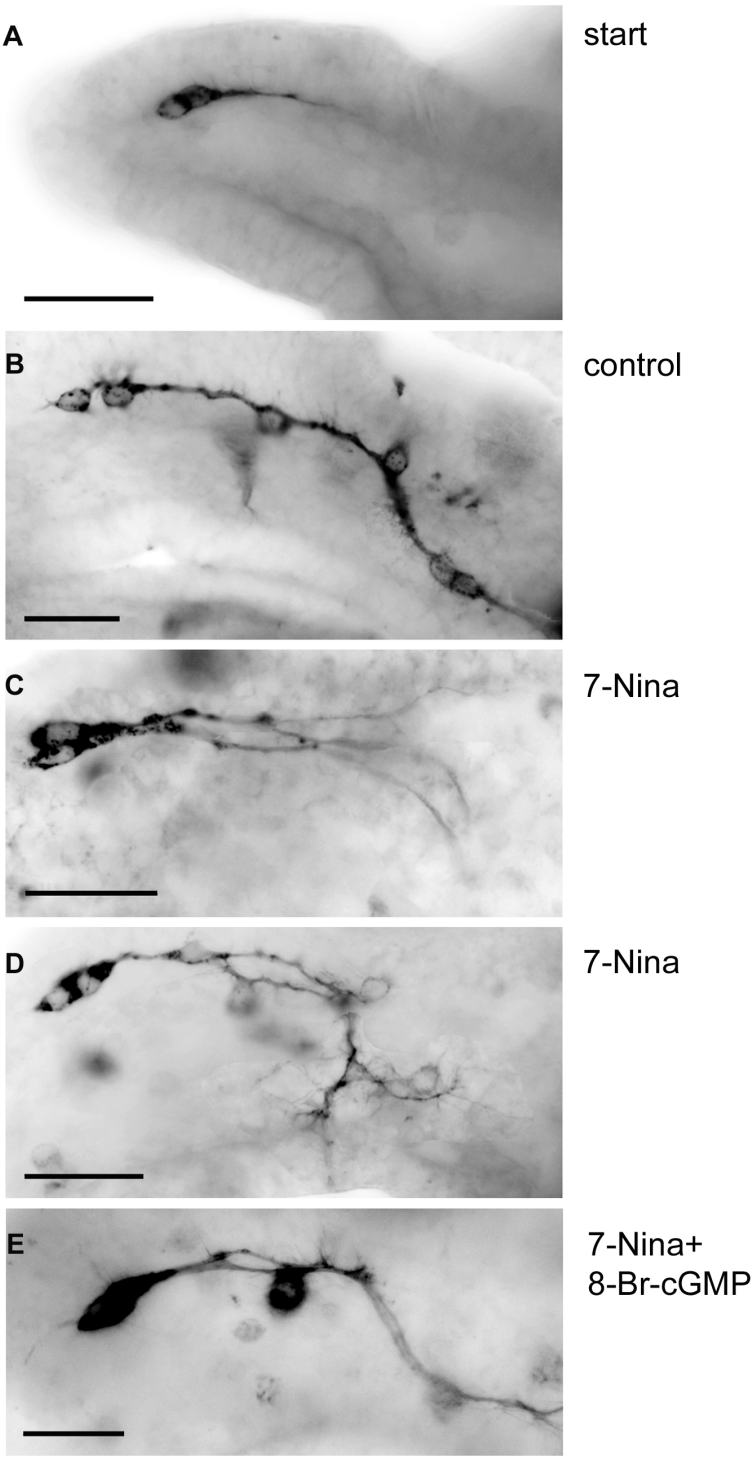
A
start
B
control
C
7-Nina
D
7-Nina
E
7-Nina+
8-Br-cGMP

**Fig. 5. Effects of NOS inhibition on directed Ti1 outgrowth**
(**A**). Initial axonogenesis of Ti1 neurons at the beginning of cell culture experiment. (**B**) Development of Ti1 neurons in untreated controls. Normal outgrowth pattern of Ti1 neurons is shown after development under control conditions. The cell culture medium contains the vehicle DMSO. Under these conditions no morphological abnormalities were found. Pioneer neurons showed pathway disruptions in an embryo that was exposed to 500 µM 7-Nina right after Ti1 axon initiation. Two morphological pathway disruptions were found with errors occurring at the first turn (**C**) or at the second turn (**D**). (**E**). Limb pioneer neurons after treatment with 7-Nina in the presence of membrane permeable cGMP analog (8-Br-cGMP) showed rescue to normal outgrowth pattern. Anterior to the top, CNS to the right. Scale: 50 µm.

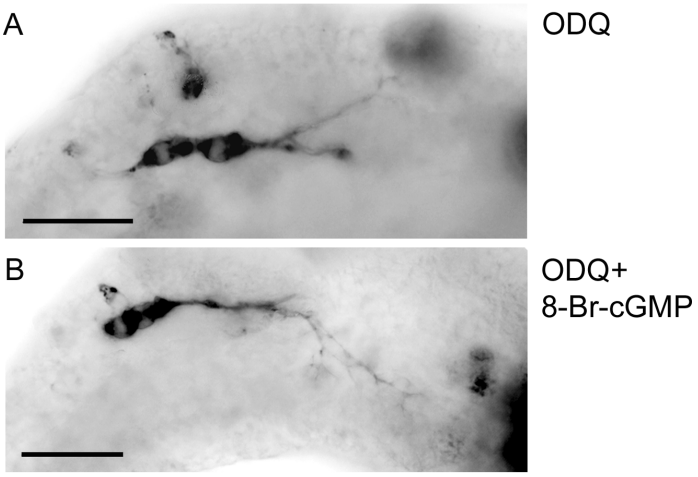


**Fig. 6. Effects of sGC inhibition of pioneer neuron morphology**
(**A**) Limb pioneer neurons in an embryo that was treated with 100 µM ODQ. This treatment caused severe pathway disruption in directed Ti1 outgrowth. (B). Embryo culture in 100 µM ODQ plus 8-Br-cGMP rescued Ti1 morphology to almost normal morphology. Although correct pathfinding increased the overall morphology of Ti1 axons differed from the normal morphology. Anterior to the top, CNS to the right. Scale: 50 µm.

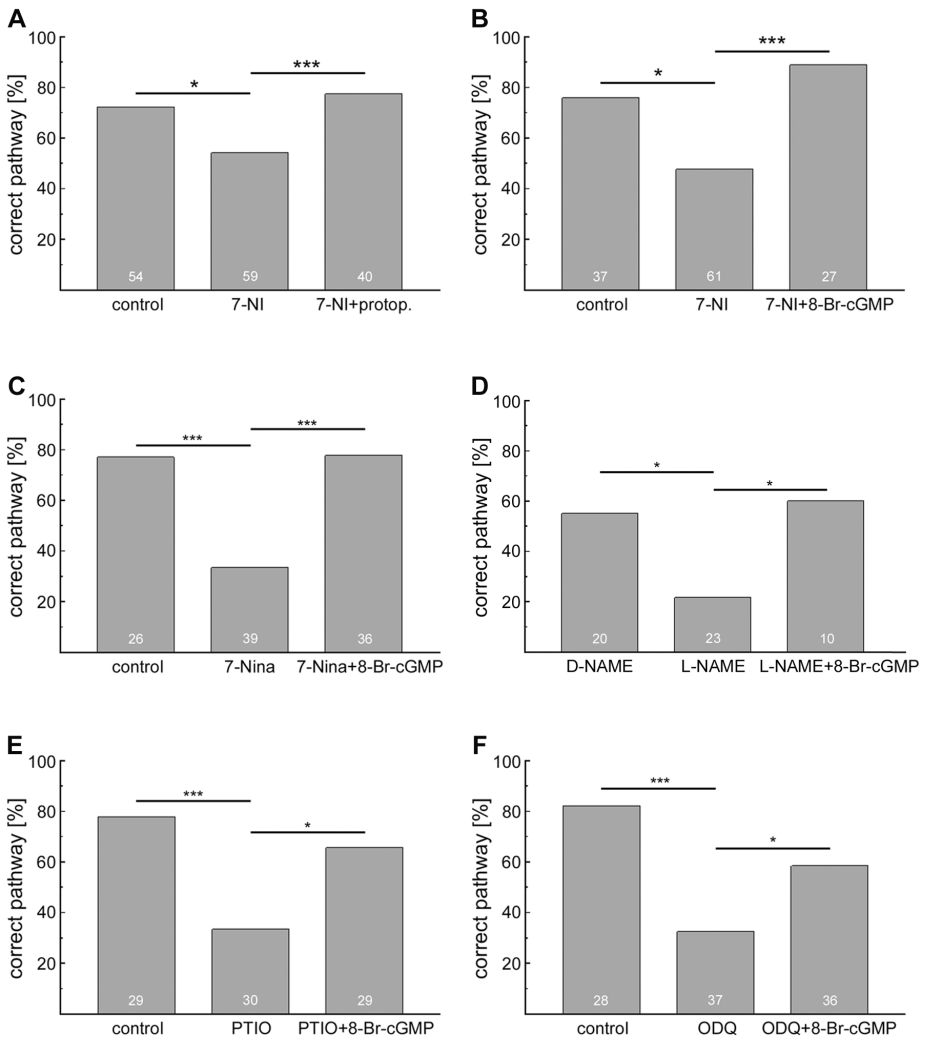
A
B
C
D
E
F
correct pathway [%]
100
80
60
40
20
*
***
54
59
40
control
7-NI
7-NI+protop.
37
61
27
7-NI+8-Br-cGMP
26
39
36
7-Nina
7-Nina+8-Br-cGMP
20
23
10
D-NAME
L-NAME
L-NAME+8-Br-cGMP
29
30
29
PTIO
PTIO+8-Br-cGMP
28
37
36
ODQ
ODQ+8-Br-cGMP

**Fig. 7. Effects of compounds that affect NO-cGMP signaling**

Quantification of pathway disruptions after treatment. (**A-C**) Inhibition of NOS caused errors in directed Ti1 outgrowth. Treatment in the presence of protoporphyrin IX free acid or of 8-Br-cGMP increased correct Ti1 outgrowth. (**D**) Treatment with the competitive inhibitor L-NAME caused reduction in correct Ti1 outgrowth. A mild effect on Ti1 outgrowth was obtained in the presence of the inactive form D-NAME. (**E**) Elimination of tissue endogenous NO by 500 µM PTIO showed a reduction in directed Ti1 outgrowth. (**F**) Quantification of Ti1 outgrowth in the presence of the sGC inhibitor ODQ. Treatment with 100 µM ODQ showed reduced ability of directed Ti1 outgrowth which nearly reached normal levels in the presence of 8-Br-cGMP. $X^2$-test * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

## 2.3.1 Appendix

Since reduced endogenous cGMP levels led to significant pathway disruptions, I was also interested in effects of increased intracellular cGMP concentration on Ti1 outgrowth. The classical phosphodiesterase type 5 blocker sildenafil has also been shown to elevate cGMP levels in an insect preparation (Zayas et al., 2002). In my whole animal culture approach, embryos of 32 %E were allowed to develop for 18 hours in the presence of sildenafil citrate (Biotrend AG, Zürich, CH) for 18 hours. Enhanced intracellular cGMP resulted in pathway disruptions similar to that found after reducing intracellular cGMP. The abnormal Ti1 morphology was analyzed and and compiled in (Fig. 8). At a concentration of 100 µM sildenafil, correct pathfinding was significantly reduced to 36.9% (N=19). In the presence of 50 µM sildenafil, correct Ti1 outgrowth was reduced to similar levels of 37.5% (N=8).

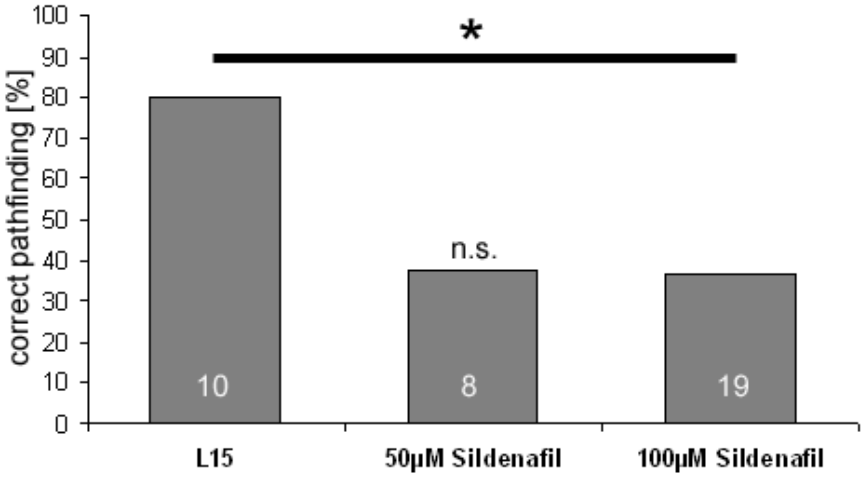


**Fig. 8. Influence of elevated cytosolic cGMP on Ti1 navigation.**
Correct Ti1 outgrowth was reduced by sildenafil citrate. Concentrations of 50 µM reduced correct outgrowth to 37.5% and of 100 µM to 36.9%. $X^2$-test for significance, $p<0.05$ *

Sema 1A is present in the metathoracic limb and antibody masking results in perturbed Ti1 outgrowth (Wong et al., 1997). In *Drosophila*, growth cone repulsion of Sema 1A is attributed to cAMP signalling (Bashaw, 2004, Terman and Kolodkin, 2004). Effects of altered cAMP levels were tested by stimulation and by inhibition of cAMP formation. A potent stimulator of cAMP formation is forskolin (Seamon et al., 1981). In the presence of 100µM forskolin Ti1 morphology was disturbed. Main navigational errors appeared at the steering points (Fig. 9A). Inhibition of adenylate cyclase (AC) by Sq 22,356 (Sigma) reduced correct Ti1 outgrowth. 50 µM Sq 22,536 led to defasciculated Ti1 fibers (Fig. 9B). Additional errors occurred at the turning points (not shown). In only 20.0% (N=35) of the treated limbs, Ti1 neurons show correct pathway formation, whereas 78.3% (N=23) reached the CNS along the correct pathway in the untreated controls (Fig. 9C). To test whether the disruptive effect of reduced endogenous cAMP could be reverted, I added the membrane permeant cAMP

analog (8-Br-cAMP). This treatment completely rescued the defect phenotype to control levels. In 92.9% (N=14) of the limbs the Ti1 pathway was not affected (Fig. 9C). These results suggest that in addition to cGMP, a certain level of cAMP may also be required for correct Ti1 growth cone guidance.

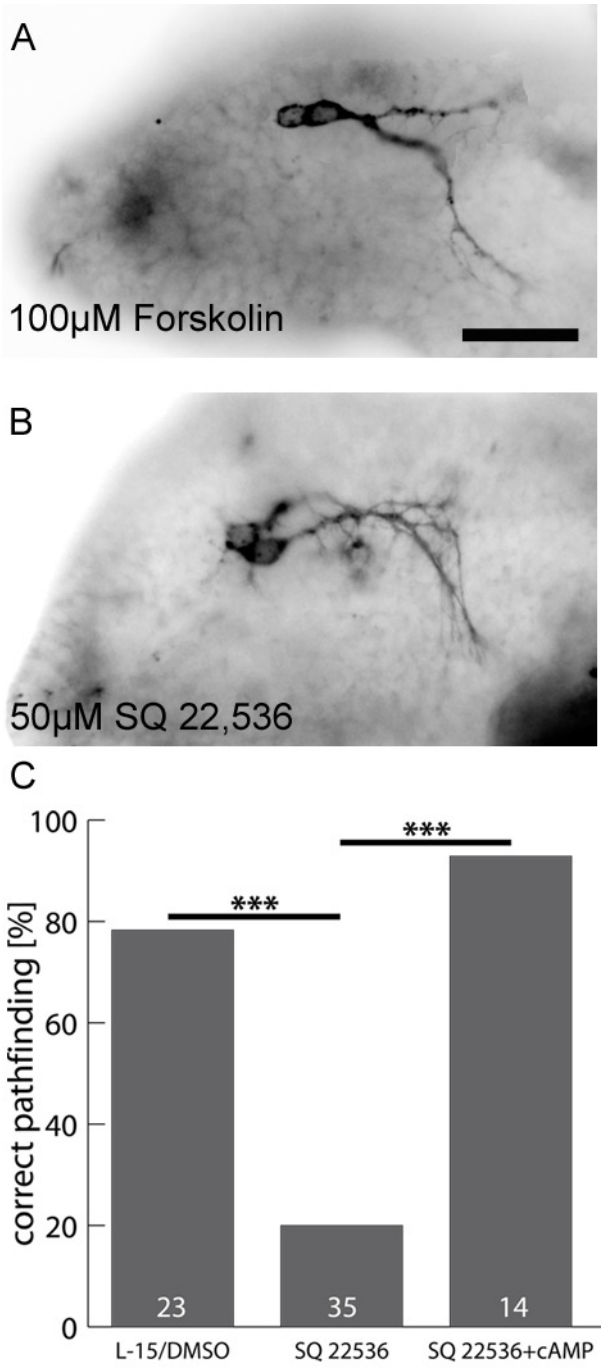


**Fig. 9. Altered levels of cAMP perturbed Ti1 pioneer outgrowth.**
(A) Navigational errors in Ti1 outgrowth by enhanced cytosolic cAMP after stimulation with 100 µM forskolin. One growth cone failed to turn ventrally and grew into the dorsal regions of the limb while the other grew along the normal path. (B) Decreased cytosolic cAMP resulted in defasciculated fibers. (C). Quantification of correct pathfinding. Inhibition of adenylate cyclise prevents Ti1 neurons from correct pathway formation. The number of limbs observed is indicated in the bar. Scale in A: 50 µm. $X^2$-test $p<0.001$ ***

## 3. Discussion

Neuronal development is not a serial progress but integrates phenotypic milestones provided by ectopic signals (Ben-Ari and Spitzer, 2010). Such milestones include, for instance, intermediate targets, guidepost and target cells. To reach their proper targets, navigating growth cones are equipped with appropriate receptors to recognize such signals on their particular pathway. Further actions on growth cone navigation are mediated by trophic support and morphogenetic control. Neurotransmitter compounds are candidates for mediating developmental actions on particular neurons before synaptic contacts are established.

I tested for morphogenetic actions of histamine on histaminergic neurons. Temporally and spatially restricted expression of certain molecules is an indicator for actions during nervous system development. In the developing rat peripheral nervous system, histamine is present in different nerves and surrounding tissue, suggesting a trophic role (Häppöllä et al., 1991). In rat neural stem cells histamine stimulates proliferation and survival (Molina-Hernández and Velasco, 2008). However, in the locust embryo there was no evidence for additional structures which were temporarily HA-IR. Rather, the onset of HA-IR was rapid over only a few percentages of embryonic development, but started before synaptogenesis. In contrast, the biogenic amine serotonin mediates both, trophic and morphogenetic functions, not only during early embryonic events (Buznikov et al., 1996) but also during the formation and maintenance of synapses. In *Drosophila* embryos, serotonin influences axonal branching by the total amount of serotonin in the tissue (Sykes and Condron, 2005). In *Helisoma* B19 neurons the axonal elongation is inhibited by serotonin (Haydon et al., 1984). This inhibitory action of serotonin onto a subset of growth cones is transduced by the activation of voltage gated $Ca^{2+}$ conductances (Goldberg et al., 1991). Interestingly, serotonin can be taken up via SERT into prospective serotonergic neurons prior to serotonin synthesis (Condron, 1999; Stern et al., 2007). To test for a similar uptake mechanism, I treated embryos at different stages with 10 or 20 µM histamine. Within the sensitivity limitations of the immunofluorescence method, I was unable to detect any alterations in immunoreactivity after preincubation with histamine.

In several vertebrates a vesicular uptake of histamine through vesicular monoamine transporter (VMAT) has been shown (Amara, 1995; Hoffman et al., 1998). Evidence for an uptake mechanism in insects is revealed by *Drosophila* knock-outs for histidine decarboxylase that recovers photoreception by nutrient histamine (Melzig et al., 1996; Melzig et al., 1998). In adult locusts, an uptake mechanism is shown for glial but not for photoreceptor cells in the optic neuropiles (Elias et al., 1984). Recent findings show that in *Drosophila* small subsets of glial cells in the lamina express VMAT. Here, histamine is taken up from the synaptic cleft into glial cells (Romero-Calderon et al., 2008). The transport back into photoreceptors involves molecular modifications. Under the influence of the *Ebony/Tan* protein pathway the

amino acid alanine is bound to histamine, forming carcinine. Carcinine is able to cross glial and photoreceptor membranes. In the photoreceptor cell, the formerly bound amino acid is released under the action of *Tan* protein and histamine is ready for re-use (Edwards and Meinertzhagen, 2010; Stuart et al., 2007; True at al., 2005). This modification of histamine into carcinine provides for a major pathway in neurotransmitter recycling. Unfortunately, it remains unclear, whether this is a specialty of photoreceptors in the fruit fly or a common way to recycle histamine in the insect CNS. Because my neuroanatomical study provided no evidence for an influence of histamine on the development of histaminergic neurons, I began to investigate modulatory actions of cyclic nucleotides on growth cone behavior.

Cellular motility, as in filopodial extension and retraction, depends on the polymerization of actin in cellular protrusions (Lauffenburger and Horwitz, 1996). In locust midgut neurons NO-cGMP signaling stimulates migration by activation of protein kinase G and is associated with rearrangement of actin (Haase and Bicker, 2003). Navigating growth cones are guided by several classes of molecules that are either presented on the cellular surface of adjacent cells or are secreted, able to diffuse through the tissue (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Extracellular guidance signals are transduced into growth cone behavior by intracellular signaling cascades. The Rho family of small GTPases regulates actin-associated proteins. Cyclic nucleotides modulate the actions of actin-associated proteins (Castellani and Rougon, 2002; Dickson, 2001; Song and Poo, 2001).

My *in vivo* approach affects the levels of cyclic nucleotide during axonal navigation. Perturbation of cyclic nucleotide levels showed dramatic effects on correct Ti1 pathway formation. I interpret this misrouting with a reduced capability to respond to exogenous Sema signals which appear along the metathoracic limb in a spatio-temporal orientation that could be associated with Ti1 outgrowth (Fig. 4). Both, enhanced and reduced levels of cGMP showed similar pathway disruptions (2.3 Fig. 5, 6, 8). This suggests a capability of intracellular cGMP to modulate growth cone responses to exogenous signals. Ti1 pathway disruption suggested these effects as reduced capability of the growth cones to respond to the repellent Sema 2A signal (Isbister et al., 1999). This interpretation resembles former findings in Drosophila, where Sema-signaling is associated with receptor coupled particular GC (Ayoob et al., 2004). Along the Ti1 pathway two sharp turns are essential to establish the normal path. At these critical choice points the growth cone has to combine exogenous signals into a turning behavior. Behind the trochanter guidepost neuron the growth cone enters a region covered by Sema 1A, which is a permissive cue for Ti1 axons (Kolodkin, 1992; Wong et al., 1997). At this point, repulsive Sema 2A forms a molecular gradient ranging from high concentrations at the dorsal edge to ventrally low concentrations (Isbister et al., 1999; Legg and O`Connor, 2003). Local increase of cGMP in cultured neurons is associated with growth cone collapse (Polleux et al., 2000; Shelly, 2011). In normal Ti1 outgrowth, the growth cone turns ventrally and grows further along a circumferential path. But, after inhibition of NO mediated cGMP formation, the growth cones failed to turn and instead oriented proximally,

thus entering dorsal regions behind the trochanter-coxa boundary (see 2.3. Fig. 3-5). This effect could be rescued by independent stimulation of sGC by protoporphyrin IX free acid or by application of membrane permeable cGMP analog.

In further experiments, I investigated the influence of altered levels of cAMP on Ti1 outgrowth (see 2.3.1 Appendix, Fig. 9). In this approach, intracellular cAMP formation was stimulated by forskolin (Seamon et al., 1981) or inhibited by Sq 22,536 (Yamada et al., 2005). Both approaches showed an increase in abnormal Ti1 pathway formation. Similar to the findings after perturbing cGMP levels, Ti1 fibers showed aberrant growth with an increase in defasciculated fibers and errors in correct turning behavior. Cultured *Drosophila* neurons show reduced capability in growth cone motility when physiological levels of cAMP are altered suggesting a role in growth cone navigation and synaptic plasticity (Kim and Wu, 1996). In *Drosophila*, repulsive Sema 1A signaling is linked by *nervy* to cAMP dependent protein kinase A signaling (Bashaw, 2004; Terman and Kolodkin, 2004). Contrarily, in the locust limb Sema 1A is a permissive cue for Ti1 axons. Blocking of Sema 1A does not completely prevent Ti1 axons from correct turning. Abnormal growth patterns are reflected in an increase of defasciculated fibers, but no mistakes at the turning points (Wong et al., 1997; 1999). The pathfinding errors found after perturbation of cAMP signaling can not be contributed to the modulation of Sema 1A-signaling alone. Moreover, these pathway disruptions suggest for a balanced action of cyclic nucleotides on correct growth cone navigation. Cultured spinal cord neurons, exposed to exogenous guidance molecules, show a capability to switch their response from repulsion to attraction by modification of intracellular cyclic nucleotides (Song et al., 1998). Higher levels of cAMP are attributed to growth cone attraction while increased levels of cGMP are attributed to growth cone retraction (He et al., 2003; Song and Poo, 1999; Yamada et al., 2005).

A correct balanced ratio of cyclic nucleotides is likely to be essential for normal growth cone behavior. Exogenous signals affecting this balance can serve as a modulator for distinct growth cone responses to certain exogenous guidance signals. Spatiotemporally restricted regulation of cyclic nucleotides mediates regulatory mechanisms for growth cone behavior (Han et al., 2007) or axon initiation (Polleux et al., 2000; Shelly et al., 2011).

Finally, correct pathfinding *in vivo* is attributed to a spatiotemporally controlled expression of guidance signals. Growth cone behavior in response to extracellular signals has been shown to be modulated by intracellular levels of cyclic nucleotides. However, it remains unclear which intracellular signaling cascade or cascades are activated by a single guidance signal. I have shown that extracellular guidance signals which affect growth cone motility and turning behavior can be modulated by the level of cyclic nucleotides. Growth cone behavior in response to guidance signals is preferentially observed in cell culture. So, here I provide an *in vivo* approach with which both, intracellular signaling cascades in response to extracellular guidance signals, and the modulation by cyclic nucleotides could be studied.

## 4. Conclusion and outlook

In this thesis, I have analyzed a rather simple *in vivo* model for axonal outgrowth. Histamine is rather unlikely to mediate morphogenetic actions during early axonal outgrowth in the locust embryo. Unlike the biogenic amine serotonin, histamine is not taken up directly via a transporter into histaminergic neurons but involve passages through adjacent glial cells (Edwards and Romero-Calderon, 2010).

I could show an essential effect of cyclic nucleotide levels on Ti1 growth cone guidance. In my approaches, alterations of cyclic nucleotide levels caused severe Ti1 pathway disruptions. The experimental changes in cAMP or cGMP levels caused a reduced capability of Ti1 growth cones to react to semaphorins in the limb epithelium. Since these findings indicate for a combined action of cyclic nucleotides in this *in vivo* model, further examinations could address the intracellular signal transduction regarding the dynamics of cytoskeletal rearrangement, especially at the turning points, where the influence of cyclic nucleotide modulation on turning behavior was apparent. At these points, actin acculmulation is associated with attractive guidance signals (Isbister and O`Connor, 2000). It would be of interest, if the accumulations could be disturbed by alterations of cyclic nucleotides levels. The locust embryonic limb provides the advantage of an open fillet preparation that could serve as a model for studying growth cone responses at the level of single filopodia (Lefcort and Bentley, 1987).

Navigating growth cones have to integrate a variety of exogenous guidance signals. In the peripheral nervous system NO-cGMP signaling regulates axonal navigation in the limb and the initial stages of outgrowth in the antenna (Seidel and Bicker, 2000). In my thesis, I interpret the transient sensitivity to NO-cGMP signaling as a milestone during the progress of development. Ti1 neurons are sensitive at stages, when their growth cones have to make critical pathway decisions. In the CNS only a subset of pioneer neurons shows this transient sensitivity to NO. Further studies therefore might address whether these central pioneers do also require NO-cGMP signaling for correct growth cone guidance.

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At this point, I have the opportunity to say thanks to my family and to all of my friends encouraging me from time to time.

## 7. Curriculum vitae

Dipl.-Biol. Arne Pätschke
geb. am 26.01.1973 in Stadthagen

**Schulischer Werdegang**

| | |
|---|---|
| 1979-92 | mit Abschluß Abitur am Gymnasium Bad Nenndorf |
| 1992-93 | Wehrdienst in Wolfenbüttel und Nienburg |
| 1993-02 | Studium der Biologie an der Universität Hannover<br>Abschluß Dipl.-Biol. |
| 2004-11 | Promotion an der Leibniz Universität Hannover<br>erstellt an der Tierärztlichen Hochschule Hannover -<br>Physiologisches Institut-Zellbiologie |

**beruflicher Werdegang**

| | |
|---|---|
| 1998-2004 | Deutsche Post AG |
| 2003-2004 | wissenschaftliche Hilfskraft:<br>Stiftung Tierärztliche Hochschule Hannover<br>Physiologisches Institut-Zellbiologie |
| 2004-08 | wissenschaftlicher Mitarbeiter:<br>Stiftung Tierärztliche Hochschule Hannover<br>Physiologisches Institut-Zellbiologie |
| seit 2009 | freiberuflicher Nachhilfelehrer |

## 8. Veröffentlichungen

**Pätschke A, Bicker G.** 2011. Development of Histamine-Immunoreactivity in the Central Nervous System of the Two Locust Species *Schistocerca gregaria* and *Locusta migratoria*. Microsc Res Tech 74: 946-56

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**Pätsche A.** 2002 Diplomarbeit. Regeneration im Zentralen Nervensystem der Heuschrecke *Locusta migratoria*. Universität Hannover

### Präsentationen

| | |
|---|---|
| 2006 | Development and Plasticity of the Insect Brain<br>Universität Marburg |
| 2004 | Regeneration im Zentralen Nervensystem der Heuschrecke<br>Jahrestagung der DZG in Rostock |

### Posterpräsentationen

| | |
|---|---|
| 2009 | Developmental Biology Conference, Hannover |
| 2007 | Neurobiology Conference, Göttingen |
| 2006 | Neurobiologischer Doktorandenworkshop in Berlin |
| 2003 | Neurobiologischer Doktorandenworkshop in Göttingen |
| 2003 | Neurobiology Conference, Göttingen |
| 2002 | Neurobiologischer Doktorandenworkshop, München |

# 9. Erklärung

## Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

Anatomical and chemical approaches to the development of model insect nervous systems

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

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(Unterschrift)

Name: Dipl.-Biol. Arne Pätschke