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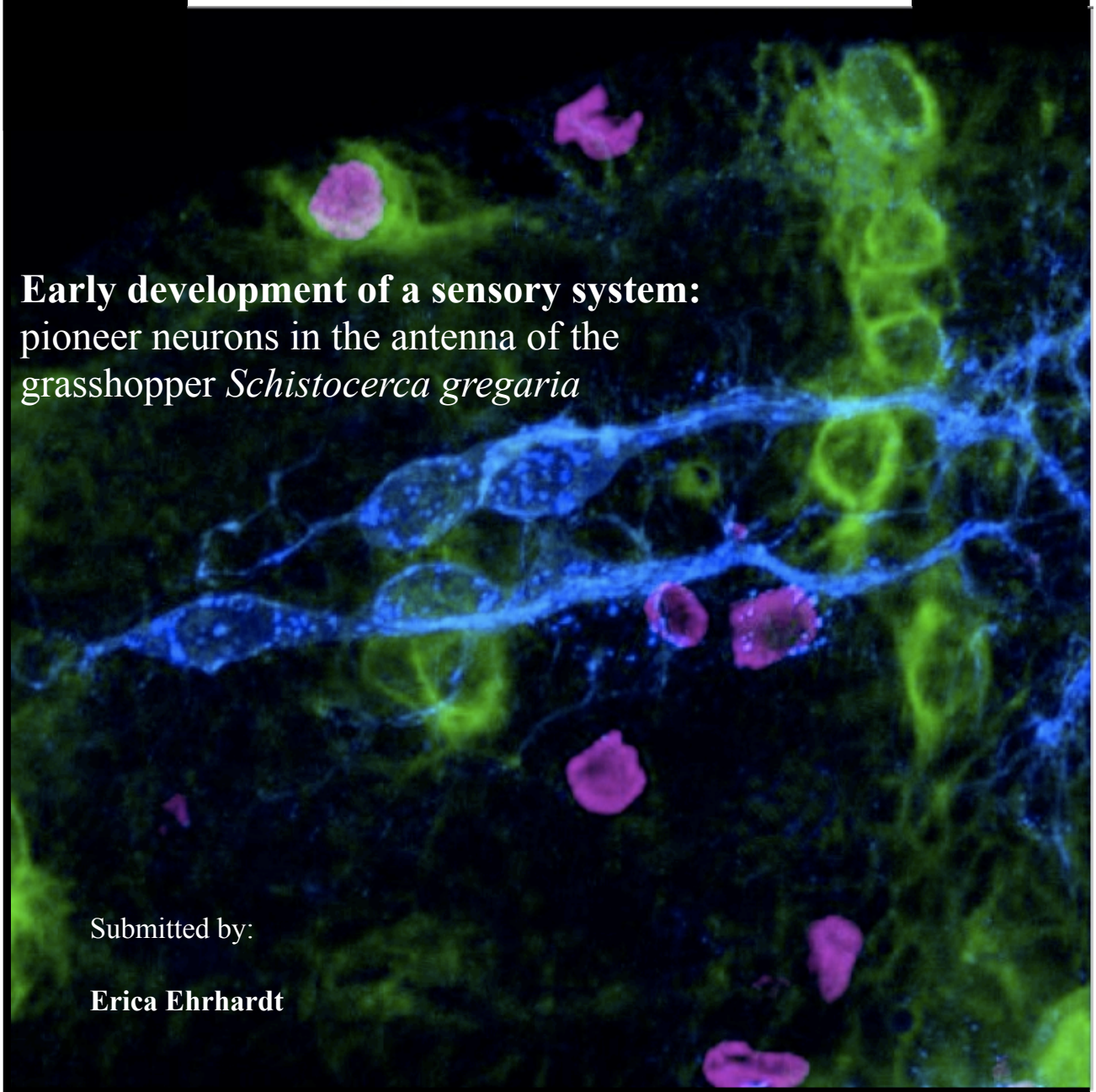

# **Early development of a sensory system:** pioneer neurons in the antenna of the grasshopper *Schistocerca gregaria*


Submitted by:

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*Dedicated to Dr Hugo Hildebrand.*
*Thank you for making me feel welcome in Germany.*

Cover image by Tatjana Kleele, George Boyan, and Erica Ehrhardt.
Taken from the cover of Arthropod Structure and Development, 2016.

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# 1. Abstract

The insect antenna is a key sensory organ involved in olfaction, audition, flight, and reproductive behavior. In the hemimetabolous grasshopper, *Schistocerca gregaria*, the nervous system of the antenna is a well-established model system for the study of axogenesis. The nervous system of the antenna consists of two nerve tracts, one dorsal and one ventral, which are each first navigated by a set of pioneer neurons: a pair of sibling cells at the tip of the antenna, and a single cell at the base. As the axons of the tip pioneers grow towards the base of the antenna, their axons make contact with the base pioneer. The pioneer growth cones then progress into the deutocerebrum. The axons of sensory neurons fasciculate with the pioneer axons and grow along them to their targets in the brain.

While the tip pioneers are known to delaminate from the epithelium into the lumen, the origins of the base pioneers are unknown. The role of the base pioneers in axon guidance remain unclear, as do the molecular guidance cues regulating axogenesis in the antenna. Although many studies have investigated the targets innervated by sensory neurons of the antenna, the targets of the pioneer growth cones themselves, and the extent to which the pioneer growth cones progress into the brain neuropil proper, have remained unclear. Furthermore, the fates of the pioneer neurons later in development are poorly understood.

The results presented in this thesis show that:

(1) the base pioneers derive from the mesoderm while tip pioneers originate in the ectodermal epithelium;
(2) although tip pioneers express cell surface lipocalin Lazarillo continuously, the base pioneers express Lazarillo only during the time period when the tip pioneer filopodia first contact them;
(3) Lazarillo is necessary for the proper navigation of tip pioneer growth cones;
(4) the apical antennal pioneer axons project into the deutocerebral, then protocerebral neuropils, where they target a set of Lazarillo-positive cells which themselves pioneer the primary axon scaffold of the brain;
(5) the apical pioneers are removed by apoptosis shortly after mid-embryogenesis, a developmental stage similar to that reported for the death of pioneers in the leg, a homologous appendage, while base pioneers progressively change their molecular profile and can no longer be identified with certainty by mid-embryogenesis, yet do not exhibit death labels.

Pioneer neurons have also been observed in many other systems, both vertebrate and invertebrate, but little is known about the pioneers of other insect antennae. My–work opens the possibility of a comparative study to determine whether insects, such as fruit flies and beetles, establish their antennal nerve tracts via mechanisms similar to those described here.

# 2. Introduction

## 2.1. Function of the insect antenna

An insect's antennae are a pair of articulated appendages associated with the head's deutocerebral segment (Snodgrass, 1935; Butt, 1960; Bullock and Horridge, 1965). Arthropod appendages can be either uniramous, a linear series of segments, or biramous, a branched limb. Insects have uniramous antennae (Emerson and Schram, 2012).

The antennae are key sensory organs for insects. Antennae are the main insect olfactory sense organs (Hansson *et al.*, 1996; Hildebrand, 1996; Kaissling and Leal, 2004; Jin *et al.*, 2005) and therefore serve as a model system for studying olfaction. The olfactory system is particularly scientifically interesting because the molecular and cellular mechanisms underlying sensory signaling are remarkably conserved between different animals from fruit flies to mammals (Buck, 2000; Axel, 2005; Galizia and Rössler, 2010). Olfactory cues affect locusts' aggregation, feeding, mating and oviposition (Torto *et al.,* 1994*;* Chen *et al.,* 2004; Hassanali *et al.,* 2005).

Antennae are involved not only in olfaction, but also in the detection of mechanical, chemical and thermal stimuli (Gewecke, 1972; Altner *et al.,* 1981). The appendages also play a role in audition (Göpfert and Robert, 2001; Göpfert *et al.*, 2002; Göpfert, 2007); gravity sensing (Kamikouchi *et al*., 2009); swimming (Gewecke, 1996); flight (Gewecke, 1972; Gewecke and Heinzel, 1980); optomotor behavior (Honegger, 1981) and reproductive behavior (Loher and Dambach, 1989; Stevenson *et al.*, 2000). In orthopteran insects, escape behavior can be initiated by stimulation of the chordotonal organs near the base of the antenna, which excite descending mechanosensory interneurons. Input from sensilla on other parts of the antenna also plays a role in determining the insect's response to threatening stimuli (Comer and Baba, 2011).

## 2.2. The model organism: The desert locust *Schistocerca gregaria*

Grasshoppers are insects of the Orthoptera order and the Caelifera suborder. Locusts are short-horned grasshoppers of the family Acrididae that, at high population densities, gather and fly in swarms. The desert locust (Fig. 2.1a) is a major agricultural pest which has caused massive economical damage by consuming crops in parts of Africa and Asia. The hardiness and prolific reproduction which enable the animal to act as such a destructive plague in the wild also make it a relatively easy organism to breed and maintain in a lab. The grasshopper is a hemimetabolous insect, which allows its individually-identifiable neurons to be followed from their birth during embryogenesis through the rest of the insect's development, in contrast to holometabolous insects which rebuild their nervous systems during metamorphosis (see Boyan and Ball, 1993).

## 2.3. The morphology of the antenna

A locust antenna consists of two basal segments– the scape and the pedicel– and a flagellum which comprises a series of similarly-shaped, smaller segments (Fig. 2.1b). The antenna is moved by two pairs of muscles (Fig. 2.1c). The tentorio-scapal muscles in the head move the scape up and down. A pair of muscles which insert at the base of the scape move the pedicel from side to side. The flagellum is not moved actively by muscles, but moves passively when the head, scape and pedicel move (Gewecke, 1972).

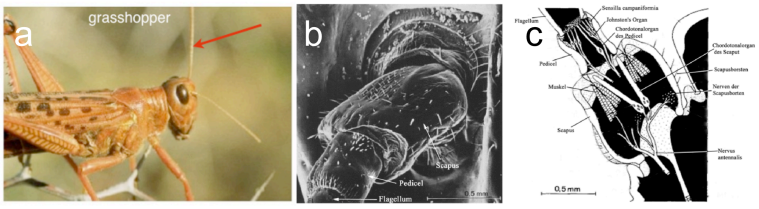



**Figure 2.1.** *Schistocerca gregaria* and the insect antenna. (**a**) The grasshopper antenna. (**b**) Proximal part of the left antenna. The grasshopper antenna comprises flagellum, pedicel and scape, as seen in this image from a scanning electron microscope. (**c**) The grasshopper antenna is moved by muscles in the scape and pedicel. **a** modified from lecture slides of G.S. Boyan. **b** and **c** modified from Gewecke (1972).

### 2.3.1. Types of sensilla in the grasshopper embryo

The functions of the insect antenna are made possible by sensilla, innervated by one or several neurons and support cells, located in or below the antennal cuticle (Keil, 1997). The morphology and activity of these neurons vary between insect species (Keil, 1984), as does the number and distribution of sensilla (Chen *et al*., 2003). The locust antenna has three kinds of olfactory sensilla– sensilla basiconica, sensilla trichodea and sensilla coeloconica (Ochieng *et al*., 1998)– as well as the mechanosensory and gustatory sensilla chaetica (Fig. 2.2; Jin *et al*., 2005). Different kinds of sensilla can be identified by their morphology and are distributed in different patterns along the length of the antenna. All sensilla must innervate the brain to fulfill their function as sensory organs.

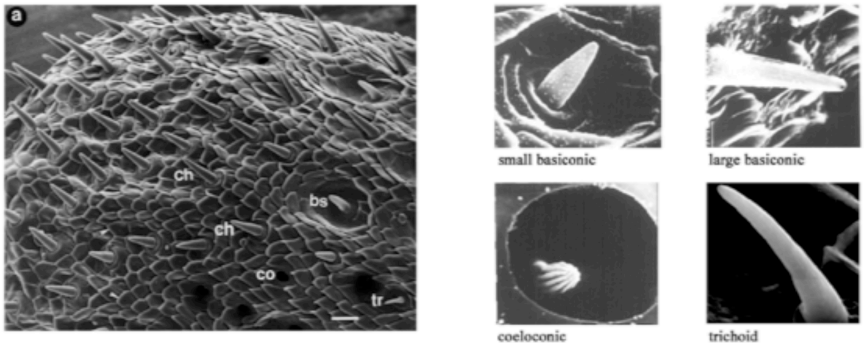



**Figure 2.2.** The different types of chemosensitive sensilla on an antenna of adult *L. migratoria* as seen in the scanning electron microscope. Sensilla chaetica (Ch) are mainly localized close to the segmental borders. S. basiconica (Ba) and s. coeloconica (Co) are the most common types, whereas s. trichodea (Tr) are fairly rare. The surface of the antenna is sculptured by scale plates. Bar, 20 µm. Modified from Chen *et al.* (2003) and Jin *et al*. (2005).

### 2.3.2. Innervation of the brain by sensory neurons in the antenna

The sensory neurons of the antenna innervate the deutocerebrum, which comprises two neuropils. The posterior region of the deutocerebrum, the antennal mechanosensory and motor center (AMMC) or the dorsal lobe, is the site where the brain performs the initial processing of mechanosensory information. Most olfactory sensory neurons (OSNs) innervate the other neuropil of the deutocerebrum, the antennal lobe, which is the initial processing center for olfactory information in the insect brain (Homberg *et al*., 1989; Hansson and Anton, 2000). The insect antennal lobe is organized into invariant spheroid structural units, the glomeruli, which are arranged stereotypically (Flanagan and Mercer, 1989; Galizia *et al*., 1999; Laissue *et al*., 1999). The number of glomeruli varies dramatically between species. Mosquitos have only 35 glomeruli (Bausenwein and Nick, 1998); *Drosophila* has 43 (Laissue *et al*., 1999); most butterflies and moths have about 60 (Rospars, 1983; Masante-Roca *et al*., 2005; Varela *et al*., 2009; Heinze and Reppert, 2012); honeybees have fewer than 200 (Arnold *et al*., 1985); hornets have 265 (Couto *et al*., 2016); while locusts have approximately 1,000 glomeruli (Ernst *et al*., 1977). From the antennal lobe, projection neurons carry information to higher brain centers via the inner antennocerebral tract to the calyx of the ipsilateral mushroom body and protocerebrum, or the medial and outer antennocerebral tracts which innervate the protocerebrum but bypass the mushroom body (Wong *et al*., 2010).

Although the OSNs are known to target the glomeruli of the antennal lobe, it is unclear where the antennal pioneers grew after extending beyond the antenna. A goal of this project is to clarify which cells or brain regions serve as targets for the pioneers in the brain.

## 2.4. Development of the antenna

Insect embryogenesis begins with the formation of the blastoderm, a layer of single cells surrounding the yolk. Gastrulation produces two germ layers from the blastoderm. The inner layer, which is composed of cells that have migrated through the gastral groove, becomes the mesoderm, which will give rise to muscles and hemolymph among other body parts. The outer layer, the ectoderm, produces the epidermis and most of the nervous system (Ho *et al*., 1983). The third germ layer of insects, the endoderm, forms the midgut. Early in the embryonic development of hemimetabolous insects, the ectoderm of the head bulges forward and out, creating a pair of extrusions of the epithelium– the antennal rudiments (Butt, 1960; Manton, 1960). Bentley

*et al.* (1979) describe these early antennae as appearing at 20% of embryogenesis in the grasshopper (Fig. 2.3).

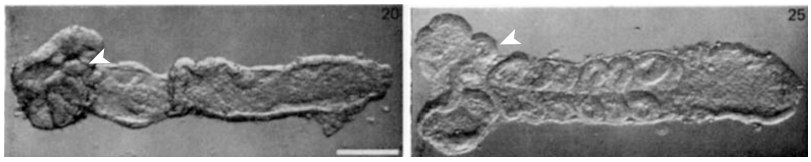



**Figure 2.3.** The locust antenna (white arrowheads) becomes identifiable at 20%. Modified from Bentley *et al*. (1979). Adapted with permission from Development (dev.biologists.org/content/54/1/47.short).

### 2.4.1. Gross morphology, the mesoderm, and the cuticle

Ullmann (1964) described the morphology of the embryonic antennal rudiments of the beetle *Tenebrio molitor:* the center of the antenna, the lumen, contains mesodermal tissue– a coelomic sac. The lumen includes haemocyte precursors and what will become the median strand. The insect basement membrane, which separates the mesoderm and epithelium, comprises collagen fibers (Blumberg *et al*., 1987) and a basal lamina composed of muccopolysaccarides (Fig.. 2.4a; Wigglesworth, 1956). Wigglesworth (1956, 1973) presented evidence that haemocytes secrete basement membrane components in *Rhodnius* assassin bugs. Ball *et al*. (1987) confirmed that haemocytes contribute to the basement membrane in grasshopper embryos as well (Fig. 2.4b). It has not been shown conclusively which germ layers give rise to the pioneer neurons of the antenna.

The outer surface of the epithelium is covered by a cuticle. The primary cuticle of the locust embryo is identifiable as early as 35%. At 50-55%, the primary cuticle undergoes apolysis, the separation of the cuticle from the epidermis, as a secondary cuticle forms below it. The secondary cuticle in turn goes through apolysis at 80%, as a third cuticle grows underneath. The secondary cuticle is not shed until the animal hatches; thus embryos at this age are covered by multiple cuticular layers (Bentley *et al*., 1979). The formation of the secondary cuticle at 55% interferes with immunolabeling, by preventing antibodies from diffusing into the tissue.

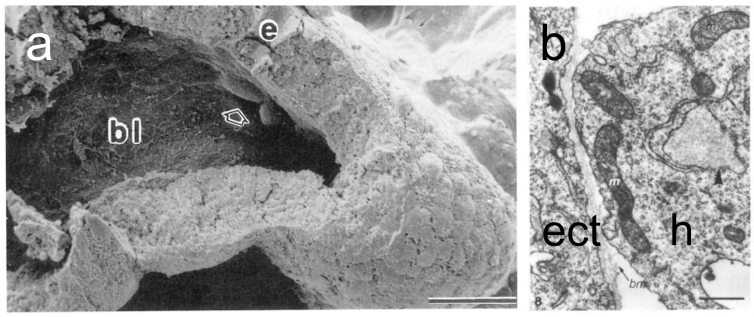



**Figure 2.4.** The basement membrane of the grasshopper embryo. (**a**) Scanning electron micrograph of a grasshopper limb after 32% of embryogenesis with the mesoderm removed. The basal lamina (bl) covers the basal surface of the epithelium (e). The soma of a pioneer neuron is detectable below the basal lamina (arrow). (**b**) During embryogenesis, the basement membrane (bm) is laid down between a haemocyte (h) and the ectoderm (ect). Immunoreactive material is contained in a cisterna of rough endoplasmic reticulum (large arrowhead). **a**. Taken from Condic and Bentley (1989a). Adapted with permission from Elsevier (www.sciencedirect.com/science/article/pii/089662738990202X). **b.** Modified from Ball *et al*. (1987). Adapted with permission from Development (dev.biologists.org/content/99/2/255.short).

### 2.4.2. Meristal annuli and segmentation

Segmentation initially develops during embryogenesis. The surface of the antenna does not remain smooth over the course of embryogenesis, but develops bulges, which may represent an early form of segmentation. Butt (1960) described three lobes in the early embryonic antenna. The number of antennal segments increases during both embryonic and post-embryonic development. Bentley *et al*. (1979) interpreted the antenna's meristal annuli as pronounced furrows which divide the antennal epithelium into serrations starting at 55% of embryogenesis. Each serration gains clusters of sensory neurons. After the animal hatches, the antenna continues to grow, and the number of sensilla continues to increase whenever the insect sheds its current exoskeleton and grows a new cuticle, until the grasshopper reaches adulthood. At the time of

hatching, the flagellum is divided into eleven meristal annuli. After hatching, the five most distal annuli are preserved, while annuli 6 to 11 divide to produce more annuli (Chapman, 2002; Fig. 2.5).

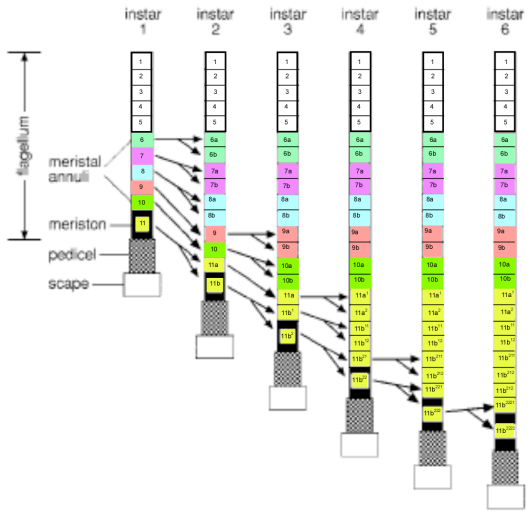
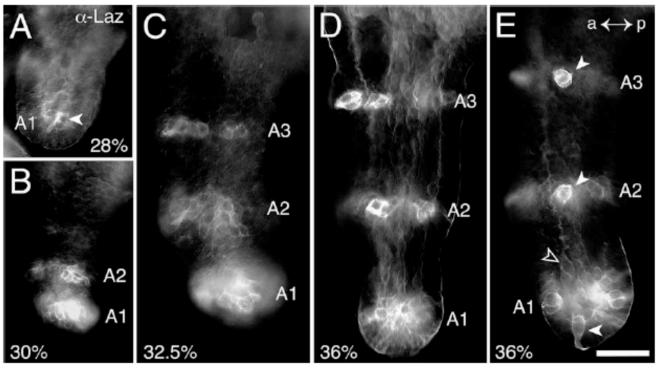



**Figure 2.5.** Diagram of the post-embryonic antennal development of the grasshopper. Arrows and colors represent the fate of proximal annuli between each instar. Annuli which are not indicated by arrows do not divide. Modified from Chapman (2002), with ideas contributed by J.L.D. Williams.

The segmentation of the embryonic grasshopper antenna is observable not only in the morphology of the epithelium, but also in the expression patterns of several molecules, which in early embryogenesis are restricted to a few expression zones. The tip expression zone is called A1, the midline area is A2, and the base is A3; a fourth region, A4, appears in the area where the scape will develop (Fig. 2.6; 2.7; Chapman, 2002; Boyan and Williams, 2004). The molecular markers whose expression during early development corresponds to these zones include the membrane-associated protein Annulin (Bastiani *et al*., 1992), the neuronal marker horseradish peroxidase (HRP; Jan and Jan, 1982), the glial marker Repo (Halter *et al*., 1995; Boyan and Williams, 2004), and Lazarillo, a cell surface glycoprotein (Fig. 2.6; Ganfornina *et al*., 1995; Sanchez *et al*., 1995; Boyan and Williams, 2004). The nerve tracts of the antenna are pioneered in a stepwise manner by sets of pioneer neurons which appear in each expression band (Fig. 2.7; Boyan and Williams, 2004).

It is uncertain exactly how the embryonic regions of expression correspond to segmentation in the adult antenna. Johnston's organ, the chordotonal organ found in the pedicel of the adult grasshopper (Gewecke, 1972), develops in the A3 zone. Therefore I propose that the A1 and A2 expression zones represent the flagellum, while the A3 zone represents the pedicel and A4 forms the scape. Chapman found that after the grasshopper hatches, meristal annuli 1-5 remain the same, while the more proximal annuli divide to produce new annuli. Because annuli 1-5 share the same segmental identity as the tip of the flagellum and the same fate of not dividing, I speculate that annuli 1-5 may also share a common origin; they may be derived from the A1 region of the embryonic antenna. If this is the case, then annuli 6-11 of the first instar nymph may represent the embryonic A2 region. However, this proposed correspondence between Chapman's meristal annuli and the embryonic molecular expression regions is highly speculative.

**Figure 2.6**. Immunolabeling against Lazarillo in grasshopper antenna illustrates the embryonic development of the meristal annuli. (**a**) At 28%, only a few cells (white arrowhead) at the tip (A1) express Lazarillo. (**b**) At 30%, a second band of Lazarillo immunoreactivity (A2) appears proximal to the A1 band. (**c**) After 32%, a third band (A3) proximal to the A1 and A2 bands appears. (**d**) The dorsal epithelium contains three bands of Lazarillo expression. (**e**) The ventral epithelium of the same antenna as d shows the same expression pattern. Taken from Boyan and Williams (2004).



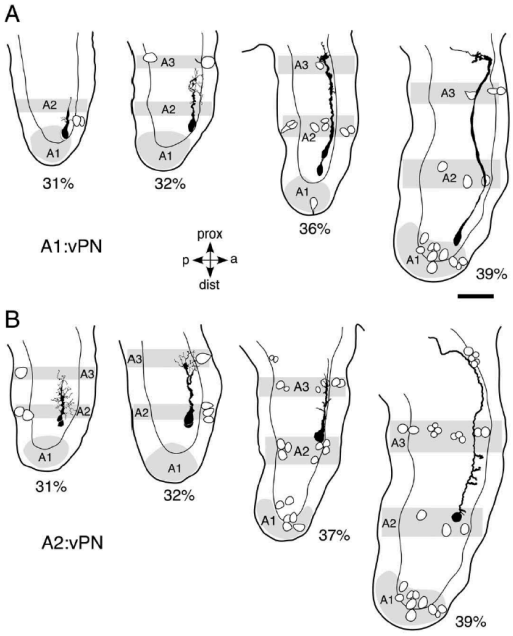


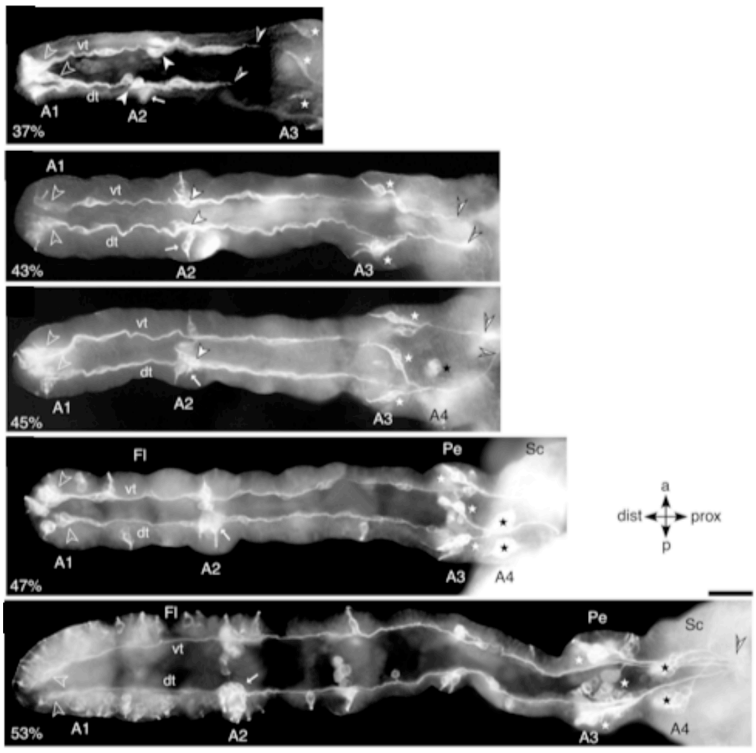

**Figure 2.7.** Drawings of ventral pioneer neurons (black cells) from the A1 (**A**) and A2 (**B**) annuli during early embryogenesis in wholemount antennae of *S. gregaria*. White cell outlines depict HRP-expressing cells in the epithelium which develop into sensory cells. Gray indicates A1, A2, A3 regions. The posterior side of each antenna is on the left. Scale bar represents 45 µm. Taken from Boyan and Williams (2004).

### 2.4.3. Sensory neurons

Sensory neurons, identifiable by their apical dendrites and their somata's location in the epithelium, appear by 37% of embryogenesis (Fig. 2.8), initially usually at the tip of the grasshopper antenna, and soon also at the midline and base. Until mid-embryogenesis, the sensory neurons are found in circumferential bands in the A1, A2 and A3 regions; later, sensory neurons also differentiate between the bands (Seidel and Bicker, 2000).

During the postembryonic development of the locust antenna, sensilla differentiate first in more distal annuli, before differentiating in more promixal annuli. The most distal annuli of the antennae of a locust nymph are already fully differentiated when the animal hatches and will not develop any new olfactory sensilla when the insect molts. In proximal annuli of the antenna, trichoid contact chemoreceptors differentiate before olfactory sensilla develop (Chapman and Greenwood, 1986).

**Figure 2.8.** Embryonic development of the sensory nerves in the antenna of *S.gregaria*. HRP immunolabeling reveals the development of the antennal nerve tracts (ventral, vt and dorsal, dt). Note the development of a cluster of sensory neurons in the dorsal epithelium of the A2 annulus (white arrow). Pioneer neuron somata of the A1 (open arrowheads) and A2 (white arrowheads) annuli are found at the lumen/epithelium border. At 37%, the growth cones (white/open arrowheads) of A2 pioneers have not yet reached the neuronal clusters of Johnston's organ (white stars) in the A3 region at the base. By 47%, regions of the embryonic antenna can be identified as corresponding to the flagellum (Fl), pedicel (Pe), and scape (Sc). Scale bar represents 70 µm. Appendage axes are shown. Taken from Boyan and Williams (2004).



### 2.4.4. Nerve tract associated cells

Sensory neuron axons grow along the nerve tracts to the deutocerebrum, after these nerve tracts are first established by pioneer neurons, but not all of the cells associated with the nerve tracts are neurons. Nerve tract associated (NTA) cells are found along the nerve tracts during development, but are neither neurons nor glia, as demonstrated by their lack of HRP and Repo expression. Unlike the glia and neurons in the antenna during early embryogenesis, the NTA cells are not organized in segments but are found all along the nerve tracts (Fig. 2.9). NTA cells originate in the mesodermal tissue of the antennal coelom, although they soon migrate to the nerve tracts at the epithelium/lumen border (Boyan and Williams, 2007). NTA cells are dye coupled to one anther and to pioneers (Boyan and Williams, 2007). NTA cells form a scaffold along the nerve tracts, but their function is unknown.

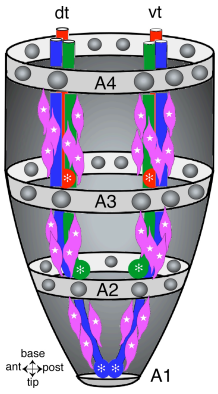



**Figure 2.9.** Schematic of the locations of NTA cells and pioneers in the antenna. A1 pioneers (blue) have somata located neuron the tip, while other pioneers are found in the A2 (green) and A3 (red) annuli. NTA cells (magenta) are associated with both dorsal (dt) and ventral (vt) nerve tracts. Taken from lecture slides of G.S. Boyan.

### 2.4.5. Serial homology

The antennae are serially homologous to other insect appendages, including the legs (Gibson and Gehring, 1988). The nervous systems of grasshopper appendages exhibit homologous development (Meier and Reichert, 1991; Boyan and Williams, 2004). The embryonic legs and antennae show similar expression patterns of molecules such as Annulin (Fig. 2.10a-d). Glial cells appear at similar locations in the embryonic antennae and legs (Fig. 2.10e-f; Boyan and Williams, 2004). The nerve tracts in the antenna are established by pioneer neurons associated with each expression region (Fig. 2.7). The nerve tracts of the leg are likewise navigated by a series of pioneer neurons. From tip to base, the segments of the leg are the tarsus, tibia, femur, trochanter and coxa (Anderson and Tucker, 1989). The first pioneers of the leg are a pair of tibial 1 (Ti1) sibling cells which establish nerve 5B1 in the tibia, femur, and coxa-trochanter (Keshishian and Bentley, 1983a), and a pair of Cx1 afferent neurons which arise near the base of the leg (Bentley and Toroian-Raymond, 1989). The Ti1 somata are found in the tibia, while their growth cones extend along a stereotyped path to the base of the leg, growing from cell to cell in a chain of preaxogenesis neurons (Anderson and Tucker, 1989; Keshishian and Bentley, 1983a).

The identities of appendages such as the antennae and legs are determined by homeotic selector genes which code for transcription factors. If all selector gene activity is silenced, the insect develops leg-like ventral appendages divided into a proximal and a distal segment (Casares and Mann, 2001). Insects may be descended from an ancestor which possessed such appendages before a system of selector genes evolved and diversified body structures which had previously been identical. The identity of the antenna is selected by the genes *extradenticle (exd)* and *homothorax (hth)*, which is necessary for the Exd protein's nuclear localization. When the activity of these genes is suppressed, the antenna is transformed into a leg. When *hth* is expressed ectopically, other body parts take on the identity of the antenna (Casares and Mann, 1998).

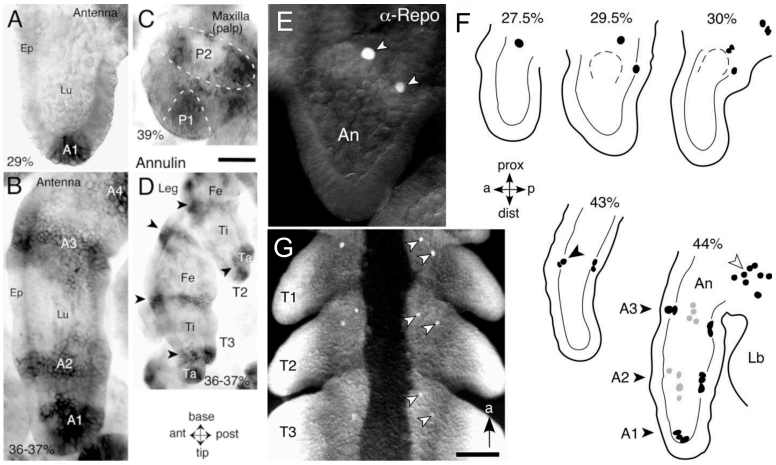



**Figure 2.10.** Annulin and Repo immunolabeling in the locust embryo illustrates the similarities of the leg, maxilla, and antenna. (**a**) At 29%, Annulin appears at the tip and base of the antenna. (**b**) After 35%, Annulin is expressed in bands A1, A2, A3, A4. (**c**) The maxilla shows similar Annulin expression. (**d**) Annulin is found in bands (black arrowheads) in the leg. (**e-f**) Repo-expressing glia appear first at the antennal base during early embryogenesis and later in the A1, A2, A3 bands. (**g**) Legs show similar expression. **a-d** from G.S. Boyan (personal communication). **e-g** taken from Boyan and Williams (2004).

## 2.5. Pioneer neurons

Pioneer neurons are defined as the cells whose axons first navigate a particular pathway in the developing nervous system. Ramón y Cajal (1890) first observed pioneer axons in the central nervous system of the chicken embryo (Fig. 2.11a). Since then, pioneer neurons have been studied in a wide variety of animals. Pioneer neurons have been investigated in the central nervous system of mammals (Fig. 2.11b; McConnell *et al*., 1989; Soria and Fairén, 2000; Supèr *et al*., 1998), insects (Fig. 2.11c; Hidalgo and Brand, 1997; Garbe and Bashaw, 2007; Biffar and Stollewerk, 2015), crustaceans (Ungerer and Scholtz, 2008; Fischer and Scholtz, 2010; Ungerer *et al*., 2011), and flatworms (Younossi-Hartenstein *et al*., 2000). Pioneer neurons from the periphery extend axons into the central nervous system and guide the axons of sensory neurons in mammals (Gong and Shipley, 1995; Stainier and Gilbert, 1990), amphibians (Davies *et al*., 1982), zebrafish (Whitlock and Westerfield, 1998; Pittman *et al*., 2008), snails (Voronezhskaya and Ivashkin, 2010), crustaceans (Ungerer *et al*., 2011) and both holometabolous (Nardi, 1983; Williams and Shepherd, 2002) and hemimetabolous insects (Denburg and Norbeck, 1989; Maynard *et al*., 2007).

Bate (1976) first described the pioneers of the grasshopper appendages (Fig. 2.12a,b). The axons of these neurons first establish the pathways of the ventral and dorsal nerve tracts of the antenna (Fig. 2.12c-e; Ho and Goodman, 1982).

It is unclear whether and how pioneer neurons differ from other neurons at similar locations and stages of development. Kulkarni *et al*. (2007) described differences in protein mobility between the pioneer growth cones– the dynamic and expanded ends of growing axons– and the growth cones of the axons which followed them in the developing zebrafish nervous system. They determined the molecular diffusion rates within growth cones of GFP-expressing neurons in transgenic zebrafish by photobleaching the GFP within growth cones, using a laser which was too weak to interfere with the growth of the axon. Then they recorded the time required for the area to fill with unbleached GFP. Pioneer growth cones had longer recovery times than growth cones which were fasciculated and following preexisting axons rather than navigating independently. Kulkarni *et al*. (2007) suggest that the slower diffusion of GFP in pioneer growth cones is related to actin. Actin networks are more elaborate in actively navigating growth cones, and depolymerizing actin erases the differences in GFP diffusion between different growth cones (Kulkarni *et al*., 2007).

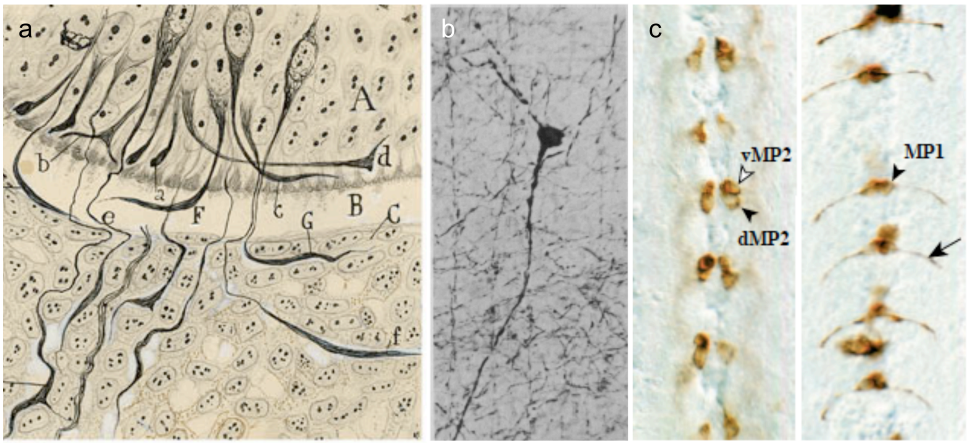



**Figure 2.11.** Pioneer neurons in the vertebrate and insect central nervous systems. (**a**) Ramón y Cajal's drawing of pioneer neurons of the embryonic chicken spinal cord. (**b**) Backfill of a pioneer neuron in the mammalian brain. (**c**) GAL4-expression in the vMP2, dMP2, and MP1 neurons which establish the longitudinal pathways in *Drosophila*. **a** taken from Garcia-Lopez *et al*. (2010). **b** taken from McConnell *et al.* (1989). Reprinted with permission from AAAS. (science.sciencemag.org/content/245/4921/978.short). **c** taken from Hidalgo and Brand (1997). Adapted with permission from Company of Biologists (dev.biologists.org/content/124/17/3253.short).

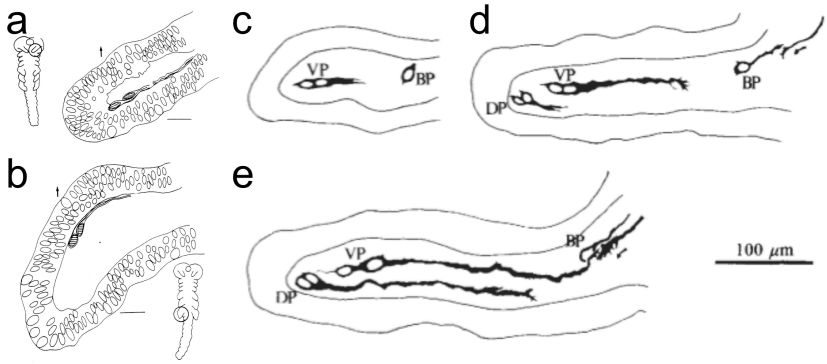



**Figure 2.12.** Early work on the pioneers of the embryonic grasshopper appendages. (**a**) The nerve tracts of the embryonic grasshopper antennae are pioneered by pairs of sibling cells. (**b**) The nerve tracts of the embryonic grasshopper legs are pioneered by similar cells. (**c**) The first neurons observable in the embryonic antenna are a pair of ventral pioneer neurons near the tip, and a single ventral base pioneer. (**d**) The ventral tip pioneers are soon joined by a pair of dorsal tip pioneers. Tip pioneer axons grow towards the base. Ho and Goodman (1982) report that the axon of a single base pioneer grows into the brain as a motoneuron axon grows from the deutocerebrum into the antenna. (**e**) Ventral pioneer axons make contact with a base pioneer on the same side of the antenna. **a,b** taken from Bate (1976). **c-e** taken from Ho and Goodman (1982).

The growth kinetics of pioneer axons may also be different from those of follower axons. When zebrafish axons grow across the midline, the growth cones of pioneer neurons slow down when they are close to the midline. Follower growth cones on the other hand, grow at a relatively constant rate regardless of their location (Bak and Fraser, 2003).

Pioneers may influence the development of tissues in their target regions. In rats, after olfactory pioneers grow into the ventricular zone of the developing telencephalon, the cell cycle kinetics in the olfactory bulb primordium change significantly. The duration of the cell cycle in the olfactory bulb primordium lengthens when the pioneer axons arrive, and twice as many olfactory bulb primordium cells exit the mitotic cycle compared to cells in the adjacent cortex which are not near olfactory pioneer axons. About a day after the pioneer axons grow into the telencephalon, the telencephalon evaginates to form the olfactory bulb. The olfactory pioneer axons may contribute to the induction of the olfactory bulb by modulating cell cycle kinetics of nearby cells (Gong and Shipley, 1995).

### 2.5.1. Pioneer neurons in arthropods

Some pioneer neurons in insects appear to be conserved. The developing central nervous systems of *Drosophila* and the beetle *Tribolium castaneum* rely some of the same pioneer neurons, including aCC and pCC, to establish the intersegmental nerves and longitudinal tracts. The pioneers show little variation in their organization between these two organisms (Biffar and Stollewerk, 2015). Some crustacean pioneers are

similar with regard to position, axon morphology, and timing of axogenesis to insect pioneers, supporting the homology of the crustacean and hexapod central nervous systems (Ungerer and Scholtz, 2008).

Pioneer neurons in the grasshopper embryo can project their axons deep into central neuropils. The pioneer axons of the cerci, which first navigate the nerve tract used by the sensory neurons that innervate wind-sensitive filiform hairs, grow into the terminal ganglion and then travel anteriorly through the primary longitudinal tract of the ventral nerve cord (Shankland, 1981). The pioneer axons of the grasshopper legs grow into the thoracic ganglia and travel anteriorly through the longitudinal tract towards the brain (Lakes-Harlan and Pollack, 1993). It has not previously been shown which brain areas are targeted by the pioneer axons of the antenna.

The pioneers of the grasshopper embryo express HRP, suggesting that they possess a neuronal identity (Jan and Jan, 1982). Some grasshopper pioneer neurons clearly take on a neuronal role at some point during development; the Term-1 expressing pioneers of the primary brain commissure eventually develop into Leucokinin-1-positive neurosecretory interneurons (Ludwig *et al.*, 2002). However, it is not clear whether the pioneers of the antenna function as neurons while guiding follower axons during embryogenesis. Grasshopper antennal pioneer neurons have a resting potential of about 50 mV and do not fire action potentials during early embryogenesis (Boyan and Williams, 2007). Similar results have been found in the leg; up to 45% of embryogenesis, pioneer neurons in the grasshopper leg did not generate any spiking activity (Keshishian, 1980). However, Selzer and Schaller-Selzer (1987) report that they recorded action potentials in cockroach luminal cells, which may be equivalent to pioneer neurons, at only 23% of embryogenesis, soon after their delamination from the epithelium. These data await independent confirmation.

The nerve tracts of the antenna have similar organization to the nerves of other appendages such as the maxilla and the leg, confirming that the antenna is homologous with these other appendages of the grasshopper (Meier & Reichert, 1991). The nerve tracts of the antenna, maxilla and leg are each established by comparable sets of pioneer neurons and guidepost cells; each segment of each appendage is associated with a set of pioneers (Boyan and Williams, 2004). Given the similarities between the developing nervous systems of the different appendages (Fig. 2.13), work done on the pioneer neurons of other parts of the grasshopper peripheral nervous system, such as the legs, may suggest answers to questions about the antennal pioneers as well. This aspect will be explored in this thesis with respect to delamination and fate of the pioneers.

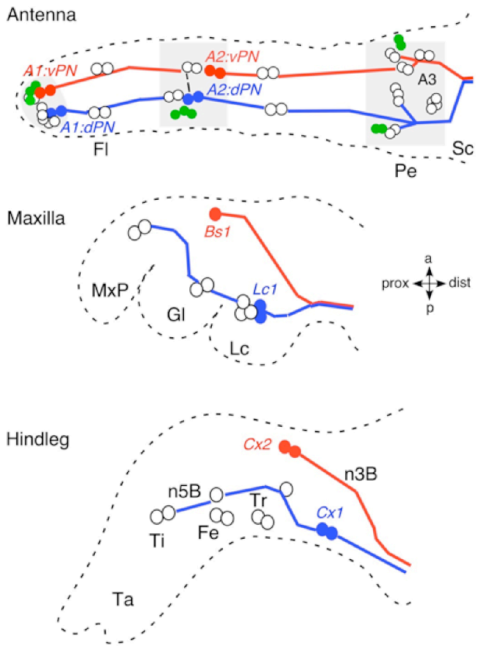



**Figure 2.13.** Schematic diagrams of the metamerically conserved system of the nerve tracts in the grasshopper antenna, maxilla, and metathoracic leg. The ventral nerve tracts are red and the dorsal nerve tracts blue. Homologous neuronal somata are colored red or blue, while other neuronal somata are represented as white circles. Green circles represent glia in the antenna. Not to scale. Appendage axes are shown. Modified from Boyan and Williams (2004).

## 2.6. Pioneer neurons of the grasshopper antenna

The grasshopper is a useful model organism for studying pioneer neurons. In contrast to mammals, the developing nervous system of the grasshopper is easily accessible for biochemical and physiological investigations, and the grasshopper has large, individually identifiable neurons (Boyan and Ball, 1993). Soon after 30%, two pairs of pioneer neurons, first the ventral pair termed vA1 pioneers here and then the dorsal pair termed dA1 pioneers here, appear in the ectodermal epithelium at the tip of the antenna and, after delaminating into the lumen, extend their growth cones along the lumen/epithelium border towards the deutocerebrum, establishing the pathways of the ventral and dorsal nerve tracts (Ho and Goodman, 1982). The ventral pioneers initiate axogenesis almost immediately after delaminating into the lumen, at about 31%; the dorsal pioneers initiate axonal outgrowth slightly later (Seidel and Bicker, 2000).

A base pioneer (BP) is reportedly born nearly simultaneously with the vA1 pioneers, but at the proximal end of the antenna (Ho and Goodman, 1982). At the same time, a motoneuron in the deutocerebrum extends an axon into the antennal base. Ho and Goodman (1982) wrote that the axons of the motoneuron and the BP run parallel to each other, but do not fasciculate with one another (Fig. 2.12d), while Berlot and Goodman (1984) wrote that the motoneuron and BP axons do fasciculate. The motoneuron will innervate a muscle at the lateral edge of the base of the antenna (Ho and Goodman, 1982).

Previous work (Ho and Goodman, 1982; Berlot and Goodman, 1984; Seidel and Bicker, 2000) reported only a single base pioneer, on the ventral side. This finding was questioned, because the antenna has two nerve tracts, each with its own A1 pioneers. The ventral base pioneer reportedly serves as a target for the vA1 pioneers (Seidel and Bicker, 2000), suggesting that it could act as a guidepost cell in this system, yet the ventral base pioneer seems to disappear before the dA1 axons reach the base (Berlot and Goodman, 1984). It seems unlikely that the ventral pathway would need a base pioneer, while the dorsal pathway forms without any cues from such a cell.

If base pioneers act as guidepost cells for apical pioneers, the molecular mechanism through which they guide the A1 pioneers was unknown. Lazarillo, a highly glycosylated cell surface glycoprotein, is involved in the guidance of pioneer axons in the locust central nervous system (Sánchez *et al.*, 1995) and could be a candidate molecule for peripheral development as well as will be tested in this thesis. In addition to signals from guidepost cells, pioneers in grasshopper appendages may also navigate using cues from the substrate on which they grow (Caudy and Bentley, 1986; Lefcort and Bentley, 1987), but it is unclear what signals might be used in the antenna.

## 3. Aims of this thesis

The focus of this project is to investigate the role of the pioneer neurons in the development of the nervous system of the antenna of the grasshopper *Schistocerca gregaria*, in particular to explore the following unanswered questions:

(1) The origin of the pioneer neurons is unclear, in particular the ontogeny of the base pioneers is entirely unknown;
(2) Although the lipocalin Lazarillo has been shown to play an essential role in axogenesis in the central nervous system and to be expressed by pioneers in the antenna, the role of Lazarillo in axogenesis in the antenna has not been investigated;
(3) Although the pioneer axons of the legs and cerci in orthopterans have been shown to penetrate well into the central nervous system, the extent to which antennal pioneers grow into the brain and the targets of the pioneer axons after they extend beyond the antenna have not been elucidated;
(4) Although the leg pioneers have been observed to undergo programmed cell death after mid-embryogenesis, the fates of the antennal pioneer neurons are unclear. Base pioneers have been described either as dying or as losing their HRP immunoreactivity at approximately 40% of embryogenesis. A goal of this project is to determine what happens to base pioneers at this age.
(5) Previous literature has described a base pioneer that may act as a guidepost cell for the apical pioneers of the ventral pathway, yet no such cell had been observed for the dorsal pathway. It is unclear why a base pioneer would be necessary for one of the antennal pathways but not the other.

# 4. Materials and Methods

## 4.1. Animals and preparation

Eggs were produced by a crowded colony of *Schistocerca gregaria* with a 12/12 h light/dark regime, 35% air humidity, a day temperature of 30°C and a night temperature of 20°C. Eggs were incubated in moist, aerated containers under this same regime. Embryos were staged according to Bentley *et al*. (1979). The staging protocol becomes less accurate with age, so that at mid-embryogenesis an uncertainty of +/- 1% is not uncommon over 20 days of embryogenesis. Staged embryos were removed from the egg and embryonic membranes in 0.1 M phosphate buffered saline (PBS: 2 mM $NaH_2PO_4$ monohydrate; 16 mM $Na_2HPO_4$ anhydrous; 175 mM NaCl, adjusted to pH 7.4 with NaOH) (Ehrhardt *et al*., 2015c).

For first instar stages, grasshoppers from a crowded culture were cold anesthetized and their antennae dissected from the head, after which the insects were returned to their cages (Ehrhardt *et al*., 2015b).

## 4.2. 5-ethynyl-2'-deoxyuridine (EdU) incorporation

EdU is a thymidine analogue which is incorporated into the DNA of proliferating cells during S phase (Sousa-Nunes *et al*., 2011; Takagi *et al*., 2012). A Click-iT® EdU imaging kit (Invitrogen, C10337) was used for EdU incorporation experiments. Unfixed embryos were incubated in a 50 µM solution of EdU in PBS for 2-4 h at room temperature on a shaker in the dark. The embryos were then fixed in 3.7% PFA (paraformaldehyde in PBS), washed and incubated in the Click-iT® reaction solution according to the kit instructions.

## 4.3. Apoptosis labels

### 4.3.1. Acridine orange (AO)

This green fluorescent dye (3,6-bis(dimethylamino) acridine; Sigma-Aldrich, 235474) is a vital stain which binds to nuclei acids (Söderström *et al.,* 1977). Acridine orange has previously been used to detect programmed cell death during insect development (Spreij, 1971; Abrams *et al*., 1993; Jiang *et al*., 1997; Liu and Boyan, 2013; Boyan and Liu, 2014). Apoptotic cells are selectively labeled by AO and the dye does not mark necrotically dying cells (Abrams *et al*., 1993). After removal from the egg, wholemount preparations were incubated in 5 µg/ml AO in PBS for 30 min at room temperature on a shaker (Ehrhardt *et al*., 2015c).

### 4.3.2. TUNEL

This method (terminal deoxynucleotidyl transferase deoxyuridine triphosphate nucleotides (dUTP) nick end labeling) detects the fragmentation of DNA which occurs during apoptosis (Gavrieli *et al*., 1992). TUNEL has been used to investigate the DNA fragmentation accompanying programmed cell death during development in insects (Jiang *et al*., 1997; Bello *et al*., 2003; Lobbia *et al.,* 2003; Page and Olofsson, 2008; Boyan and Liu, 2014). After being fixed in 3.7% PFA overnight, embryos were permeabilized to enable TUNEL labeling. Embryos were sonicated as described below. Embryos were then placed in 0.1 M citrate buffer (19 mM citric acid; 81 mM sodium citrate, adjusted to pH 6.0 with NaOH), and placed in a microwave oven for 5 min at 360 W in order to permeabilize the tissue. The embryos were then washed overnight in PBS at 4°C. A preincubation medium of 20% normal goat serum (NGS, Sigma) and 3% bovine serum albumin (BSA, Sigma) in TrisHCl (0.1 M Tris in distilled water, adjusted to pH 7.5 with HCl) was applied for 30 min at room temperature to denature the DNA. Embryos were washed three times (10 min each) in 0.1 M PBS at room temperature. The preparations were then labeled using the *In Situ* Cell Death Detection Kit, TMR red (Roche) for 2 h at 37°C. They were washed in 0.1 M PBS overnight before HRP immunolabeling (Ehrhardt *et al*., 2015c).

## 4.4. Embedding and sectioning

Embedding and sectioning of antennae was done as previously described in Boyan and Williams (2004) for agarose embedding and vibratome sectoning; and in Boyan and Williams (2007) for Epon 812 embedding.

## 4.5. Sonication

As the cuticle of acridids differs in thickness and composition from that of larval *Drosophila* (see Chapman, 1982), the sonication protocol had to be modified from that of Patel (1994). Embryos and antennae of first instar stages were first fixed overnight at 4°C in the dark in 3.7% PFA. Embryos and antennae were washed four times (10 min each) in 0.1% PBT (0.1 M PBS with 0.1% Triton-X, Sigma) and placed into 500 µl of fresh 0.1% PBT in an Eppendorf tube which was then positioned in a Bandelin SONOREX™ rk100 ultrasound (35 kHz) bath sonicator. Embryos under 55% were sonicated at half power. The sonicator produces high intensity (320 W) ultrasound which renders the cuticle porous, but also generates unwanted tissue heating. To counteract this, embryos were sonicated in cycles of 3 s duration with the Eppendorf being removed and placed in an ice bath for 15 s between each cycle. The protocol applicable to each age is shown in Table 4.1 (Ehrhardt *et al.,* 2015b).

| Age | No. cycles |
|---|---|
| 65-70% embryogenesis | 20 |
| 80% embryogenesis | 25 |
| 90% embryogenesis | 30 |
| 1st instar | 35 |

**Table 4.1.** Sonication times (number of cycles, each of 3 s duration) required to render the cuticle porous to antibodies at each age tested. Note that after each cycle, the container with the preparation was place in an ice bath for 5 s. Modified from Ehrhardt *et al.* (2015b).

Following sonication, PBT was removed. In order to digest the cuticle, further breaking it down and rendering it porous, the sonicated preparations of all ages were incubated in 0.2% collagenase (*Clostridium histolyticum*: Sigma-Aldrich C9572) in TES buffer (5 mM TES (N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid); 150 mM NaCl; 3 mM KCl; 2 mM $CaCl_2$; 1 mM $MgSO_4$; adjusted to pH 7.0 with NaOH) for 8-20 min (according to age), before washing three times in 0.1 M PBS (total 1 h washing time) at room temperature and then proceeding to the immunolabeling steps (Ehrhardt *et al.,* 2015b).

## 4.6. Whole embryo culture and immunoblocking

Eggs were disinfected in 70% ethanol for 1 minutes before dissection. Unfixed embryos were removed from the egg and placed in a sterile culture medium which consisted of Grace's Insect TC Medium (Bio & SELL 2.12G07J), 600 mg/l L-glutamine (Roth), 20 µl/ml 20-hydroecdysone (Sigma), 25 µg/ml juvenile hormone III (Sigma-Aldrich), 120 units/ml penicillin, 120 units/ml streptomycin, and 2.5 µg/ml Fungizone (Gibco). Control embryos were allowed to grow in this medium under sterile conditions at 30°C (whole embryo culture). Three experimental batches of five embryos of the same age (32%) were grown in parallel in the same culture medium, but to each of which, a different dosage of mAb 10E6 (anti-Lazarillo), between 0.176 and 17.6 µM, had been added, as described in Sánchez *et al*. (1995). The immunoblocking data presented here refer to an antibody concentration of 2 µM in the culture medium above. This was within the concentration spectrum previously shown to be effective in disrupting growth cone guidance in the ventral nerve cord of the grasshopper and over the same embryonic ages (Sánchez *et al*., 1995). Only the most successful data are shown. Embryos in control and experimental groups grew an average of 4% (from 32 to 36%) over the course of the experiment (2 days). Control and experimental embryos were subsequently fixed

in 3.7% PFA and processed for HRP-labeling (Ehrhardt *et al*., 2015a). The protocol followed that of B. Niederleitner and M. Güntner (personal communication).

## 4.7. Fixation

For Lazarillo or Mes3 experiments, preparations were fixed in PIPES-FA (3.7% paraformaldehyde in 100 mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$) overnight at 4°C. For I-5 immunolabeling, preparations were fixed in 2% paraformaldehyde in Millonig's buffer (Millonig, 1963) for 30-60 min at room temperature. For all other experiments, preparations were fixed in 3.7% PFA overnight at 4°C. After fixation, preparations were washed in PBS for 6 h.

## 4.8. Immunolabeling

Preparations were incubated for 1 h at room temperature in a preincubating medium which used normal goat serum (NGS, Sigma), normal horse serum (NHS, Sigma), bovine serum albumin (BSA; Sigma), or fetal calf serum (FCS) to block non-specific labeling. Primary antibodies were then diluted in this same solution. After incubation in the primary antibodies, preparations were washed six times in 0.1 M PBS (3 h total washing time) before being transferred to the relevant incubation medium containing the secondary antibody. Wholemount embryos were incubated in the secondary antibodies for 2-3 days at 4°C in the dark, while sectioned preparations were incubated in the secondary antibodies for 24 h at 4°C. After the application of secondary antibodies, preparations were washed overnight (six changes) in 0.1 M PBS. Preparations which were immunolabeled using secondary antibodies conjugated with peroxidase (PO) were treated with the substrate diaminobenzidine using Sigma Fast DAB tablets and the staining intensified with 0.075% ammonium nickel sulphate. Preparations were cleared in 90% glycerol in 0.1 M PBS for at least 3 h, positioned onto microscope slides (Menzel), covered in Vectashield® (Vector laboratories), and coverslipped for confocal microscopy.

The protocols for the application of primary antibodies are shown in Table 4.2 and the secondary antibodies in Table 4.3.

## 4.9. Specificity controls

Controls for the specificity of all antibodies employed involved (a) application of the relevant secondary in the absence of the primary antibody (in no case was a staining pattern observed) and (b) comparison of our experimental results with the originally published data (see above). In all cases, an identical staining pattern to that first description was observed (Ehrhardt *et al*., 2015b).

As a negative control in TUNEL experiments, embryo appendages were exposed to the label solution alone, without terminal transferase, instead of the TUNEL reaction mixture. In this control experiment, no labeling was observed.

| antigen | embryos | primary antibody | incubation medium | dilution | incubation time | experiments | Primary description |
|---|---|---|---|---|---|---|---|
| Horseradish peroxidase (HRP) | wholemount | polyclonal in rabbit (Dianova, 323-005-021) | 0.1 M PBS, 0.5% Triton-X, 1% NGS, 3% BSA | 1:200 | 3 days at 4°C | single-labeling; double-labeling with 1C10, Mes3, 10E6, or α-Tubulin | Jan and Jan (1982) |
| Horseradish peroxidase (HRP) | sectioned | in goat (Dianova, 123-005-021) | 0.1 M PBS, 0.5% Triton-X, 1% NHS, 3% BSA | 1:200 | 1 day at 4°C | single-labeling | Jan and Jan (1982) |
| Lachesin (1C10) | wholemount | in mouse (gift of M. Bastiani) | 0.1 M PBS, 0.5% Triton-X, 1% NHS, 3% BSA | 1:5000 | 3 days at 4°C | single-labeling; double-labeling with HRP or PH3 | Karlstrom *et al*. (1993) |
| Lazarillo (10E6) | wholemount | in mouse (gift of D. Sánchez) | 0.1 M PBS, 0.1% Tween20, 5% FCS | 1:3 | 5 days at 4°C | single-labeling; double-labeling with HRP | Sánchez *et al*. (1995); Ganfornina *et al*. (1995) |
| Mes3 | wholemount | in mouse (gift of C. Goodman) | 0.1 M PBS, 0.1% Triton-X, 5% FCS | 1:4 | 3 days at 4°C | double-labeling with HRP, or in combination with AO | Kotrla and Goodman (1984); Boyan and Williams (2007) |
| Phosphohistone -3 (PH3) | wholemount | polyclonal in rabbit (Millipore, 06-570) | 0.1 M PBS, 0.5% Triton-X, 1% NHS, 3% BSA | 1:250 | 3 days at 4°C | double-labeling with 1C10 | Takizawa and Meshorer (2008) |
| α-Tubulin | wholemount | monoclonal in rat (abcam, ab6161) | 0.1 M PBS, 0.5% Triton-X, 1% NGS, 3% BSA | 1:600 | 3 days at 4°C | single-labeling; double-labeling with HRP | Konenko and Pflüger (2007) |
| I-5 | sectioned | monoclonal in mouse (gift of E. Ball) | 0.1 M PBS, 0.25% Triton-X, 2% BSA | | 1-2 days at 4°C | single-labeling | Ball *et al*. (1985) |

**Table 4.2.** Primary antibodies.

## 4.10. DAPI

This fluorescent probe (4,6-diamidino-2-phenylindole) binds to the minor groove of double-stranded DNA, and thus labels cell nuclei (Naimski *et al*., 1980). DAPI (Sigma) was diluted 1:100 in 0.1 M PBS and applied to preparations for 30 min at room temperature, followed by six washing cycles (20 min each) in 0.1 M PBS (Ehrhardt *et al*., 2015c).

| primary antibody | embryos | secondary antibody | incubation medium | dilution | experiments |
|---|---|---|---|---|---|
| Horseradish peroxidase (HRP) in rabbit | wholemount | goat anti-rabbit Alexa® 488 (Invitrogen, A11034) | 0.1 M PBS, 0.05% Triton-X, 1% NGS, 3% BSA | 1:450 | single-labeling; double-labeling with 1C10, 10E6, or α-Tubulin |
| Horseradish peroxidase (HRP) in rabbit | wholemount | goat anti-rabbit Cy3 (Dianova, 111-165-003) | 0.1 M PBS, 0.05% Triton-X, 1% NGS, 3% BSA | 1:200 | labeling in combination with AO, double-labeling with Mes3 |
| Horseradish peroxidase (HRP) in rabbit | sonicated 90% | goat anti-rabbit FITC (Dianova, 111-095-003) | 0.1 M PBS, 0.05% Triton-X, 1% NGS, 3% BSA | 1:200 | double-labeling with α-Tubulin |
| Horseradish peroxidase (HRP) in goat | sectioned | donkey anti-goat Cy3 | 0.1 M PBS, 0.05% Triton-X, 1% NHS, 3% BSA | 1:200 | single-labeling |
| Lachesin (1C10) in mouse | wholemount | goat anti-mouse Cy3 (Dianova 115-165-044) | 0.1 M PBS, 0.05% Triton-X, 1% NGS, 3% BSA | 1:150 | single-labeling; double-labeling with HRP or PH3 |
| Lazarillo (10E6) in mouse | wholemount | goat anti-mouse Cy3 (Dianova 115-165-044) | 0.1 M PBS, 0.5% Tween20, 1% NGS, 3% BSA | 1:150 | single-labeling; double-labeling with HRP |
| Lazarillo (10E6) in mouse | wholemount | goat anti-mouse PO (Jackson ImmunoResearch) | 0.1 M PBS, 0.5% Tween20, 1% NGS, 3% BSA | 1:4 | single-labeling |
| Mes3 in mouse | wholemount | donkey anti-mouse Alexa 488 (Invitrogen, A21202) | 0.1 M PBS, 0.05% Triton-X, 1% NGS, 3% BSA | 1:450 | double-labeling with HRP |
| Mes3 in mouse | wholemount | goat anti-mouse Cy3 (Dianova 115-165-044) | 0.1 M PBS, 0.05% Triton-X, 1% NGS, 3% BSA | 1:200 | labeling in combination with AO |
| Phosphohistone-3 (PH3) in rabbit | wholemount | goat anti-rabbit Alexa® 488 (Invitrogen, A11034) | 0.1 M PBS, 0.05% Triton-X, 1% NGS, 3% BSA | 1:250 | double-labeling with 1C10 |
| α-Tubulin in rat | wholemount | goat anti-rat Cy3 (abcam, ab6953) | 0.1 M PBS, 0.05% Triton-X, 1% NGS, 3% BSA | 1:150 | single-labeling; double-labeling with HRP |
| I-5 in mouse | sectioned | goat anti-mouse PO (Jackson ImmunoResearch) | 0.1 M PBS, 0.25% Triton-X, 2% BSA | 1:4 | single-labeling |

**Table 4.3.** Secondary antibodies.

## 4.11. Intracellular dye injections

For combined intracellular staining and immunolabeling following AO labeling antennae were embedded in 5% agarose and sectioned as previously described in Boyan and Williams (2007). Agarose sections were mounted on HistoBond© slides (Marienfeld) and covered in PBS. Apoptotic cells were identified and

photographed using a Zeiss Axioskop 2 microscope. Intracellular dye injection was performed as previously described in Boyan and Williams (2007) using a thin-walled glass capillary microelectrode (Clarke Instruments) prepared on a Sutter electrode puller with a resistance of 30-40 MΩ filled with a solution of Alexa Fluor® 568 hydrazide (10 mM in 200 mM KCl).

## 4.12. Imaging

Preparations were imaged using a Leica TCS SP5 confocal laser scanning microscope with x20 and x63 oil immersion objectives. Fluorophores were visualized using excitation wavelengths of 405 nm for DAPI; 488 for Alexa® 488, AO, FITC, and EdU; 561 for Cy3, TUNEL, and Alexa® 568. Intracellularly injected Alexa® Fluor 568 was also excited at 430-440 nm and captured at 535 nm on the Zeiss Axioskop 2 fluorescent microscope with a 1.3 MP Color CCD camera (Scion Corporation) and Scion Visicapture™ software. Preparations labeled with PO-conjugated secondary antibodies were also viewed under the Zeiss microscope. Confocal images were processed using ImageJ software (open source). Figures were formatted using Canvas™ X software (ACD Systems) (Ehrhardt *et al*., 2015c).

# 5. Results

## 5.1. Origin of pioneer cells

In arthropod appendages, two tissues have been identified as generating proliferative cells: the ectodermal epithelium, and the mesoderm, which in the antenna is represented by the lumen. The apical pioneers have been reported to delaminate from the ectodermal epithelium (Ho and Goodman, 1982). We provided further support for these previously published results using different immunolabeling methods. The origin of the base pioneers has been unknown. We investigated the ontogeny of the pioneers using immunolabeling against Mes3, an antigen expressed by cells of mesodermal origin, to distinguish cells of mesodermal from those of epithelial origin and so establish whether these various pioneers derive from a common or different embryonic Anlagen (Ehrhardt *et al*., 2015a).

### 5.1.1. The origin of apical pioneers

The origin of the apical pioneers in the ectodermal epithelium at approximately 30% has been reported by Ho and Goodman (1982). However, Ho and Goodman (1982) did not publish any images of immunolabeled pioneers in the epithelium or during delamination to support this observation. Therefore, we performed immunolabeling experiments using several markers expressed by pioneers to confirm this finding (Fig. 5.1). We used antibodies against HRP, a neuron-specific marker (Jan and Jan, 1982); against Lazarillo, a cell-surface glycoprotein which is expressed by a subset of neurons including pioneers and sensory neurons (Ganfornina *et al.,* 1995; Sánchez *et al*., 1995; Ehrhardt *et al*., 2015a); and against Lachesin, which is expressed by neurons and their progenitors (Karlstrom *et al*., 1993). All three labels revealed putative apical pioneers in the ectodermal epithelium of the antenna during early embryogenesis prior to delamination. While in the epithelium, pioneers are connected to the outer epithelium via thin processes (Fig. 5.1a-d). We also observed pairs of apical pioneers in the process of delaminating (Fig. 5.1e).

We combined Lachesin with EdU in order to search for possible progenitors or mother cells which could give rise to the pioneer neurons (Fig. 5.2). At 28%, we observed EdU labeling within a Lachesin-positive putative pioneer cell. EdU is incorporated into DNA during S phase, meaning that either this Lachesin-expressing cell synthesized DNA during the EdU pulse, or that it was generated by the mitotic division of another cell which had copied its DNA during the EdU pulse. A second EdU-labeled cell was directly next to the Lachesin-positive cell. We speculate that this second cell could be a mother cell.

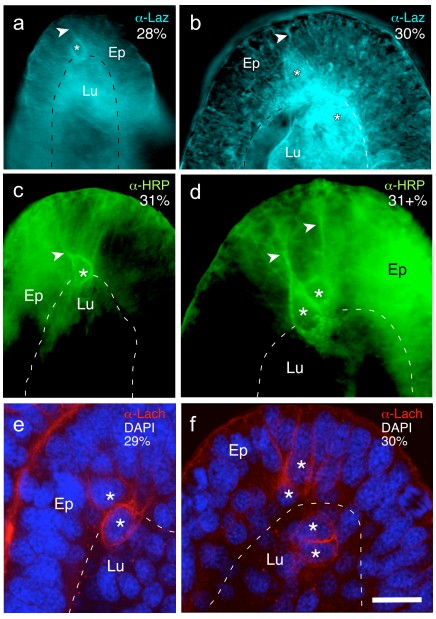



**Figure 5.1.** Origins of apical pioneers in wholemount antennae. (a-b) DIC images of Laz-expressing putative vA1 pioneers at 28% and 30% of embryogenesis. (**a**) At 28% of embryogenesis, a single Laz-expressing cell (white star) is visible in the epithelium extending a projecting towards the outer epithelium (white arrowhead). (**b**) At 30% of embryogenesis, one Laz-expressing cell is visible in the epithelium while another Laz-positive cell is stained in the lumen. (**c-d**) DIC images of HRP-expressing putative dA1 pioneers at approximately 31%. (**c**) At 31%, a single HRP-expressing cell (white star) is visible in the epithelium. (**d**) In a slightly older antenna from the same experiment, a pair of cells (white stars) express HRP in the epithelium. (**e-f**) Confocal images of Lachesin-expressing putative pioneers at approximately 30% of embryogenesis. (**e**) At 29% of embryogenesis, a pair of Lachesin-expressing vA1 pioneers (white starts) delaminate. (**f**) At 30% of embryogenesis, the Lachesin-expressing vA1 pioneers are in the lumen but have not yet initiated axogenesis. A second pair of Lachesin-expressing cells are visible in the epithelium. White stars indicate putative pioneers. White arrowheads indicate projections connecting the putative pioneers to the outer epithelium. Dashed lines indicate the border between the epithelium and lumen in each antenna. Scale bar in f represents 25 µm in **a**; 15 µm in **b**; 20 µm in **c**; 15 µm in **d**;10 µm in **e, f**.

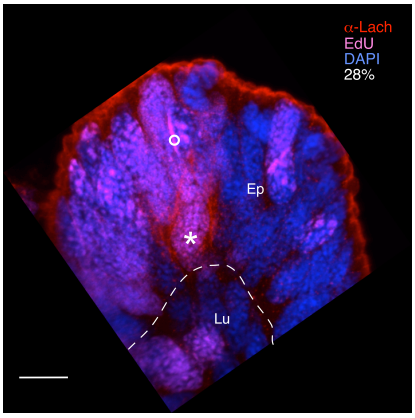



**Figure 5.2.** Confocal image of Lachesin immunolabeling combined with EdU and DAPI staining in a wholemount antenna at 28%. Lachesin (red) is expressed by a putative vA1 pioneer (white star) in the epithelium (Ep). The border of between the lumen and the epithelium, as determined by DAPI staining, is indicated with a dashed white line. EdU (magenta) labels the putative pioneer, indicating that this cell or its progenitor was in S phase during the EdU pulse. Directly next to the Lachesin-expressing, EdU-positive cell, another cell (white circle) is also labeled with EdU. Scale bar represents 10 µm.

### 5.2.2. The origin of base pioneers

As the axons of the apical pioneers grow towards the base, they encounter base pioneers. We have identified a single HRP-positive base pioneer associated with each nerve tract (Fig. 5.3). The two base pioneers are not at the same position in the antenna. The ventral base pioneer is close to the midline, while the dorsal base pioneer is right next to the border of the antenna and the deutocerebrum. This difference in location may explain why previous reports of the embryonic antennal nervous system failed to identify the dorsal base pioneer.

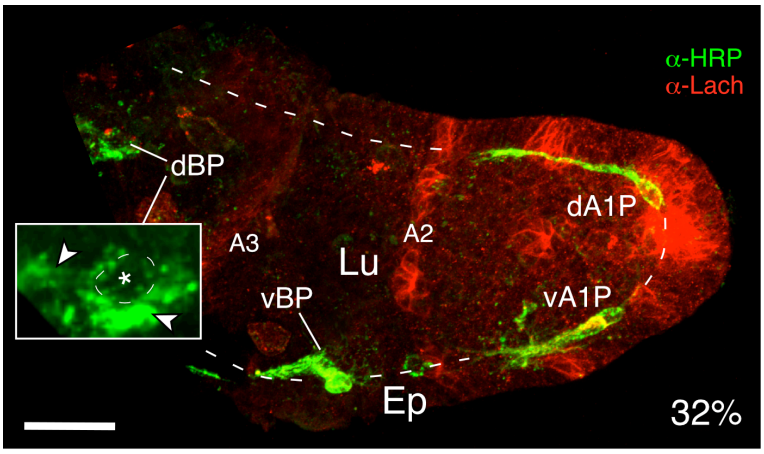



**Figure 5.3.** Confocal image of the early embryonic antenna (32%) following double immunolabeling (α-HRP, α-Lach) shows ventral and dorsal A1 pioneer neurons (vA1P and dA1P) establishing the initial axon scaffold of the antennal nervous system. Each apical pioneer targets its own so-called base pioneer (vBP, dBP) before projecting to the brain (not shown). Inset (white rectangle) shows the dBP (white star, soma outlined dashed white) at higher magnification and extending HRP-positive filopodial processes (white arrowheads). A1 apical pioneers occur as sibling cells but may, on occasion, lie directly superimposed preventing individual imaging. The approximate border between the outer ectodermal epithelium (Ep) and inner mesodermal lumen (Lu) is indicated (dashed white line). Scale bar in a represents 50 µm, 20 µm in inset. Modified from Ehrhardt *et al*. (2015a).

Double-labeling with antibodies against HRP and Mes3 (Fig. 5.4a) reveals the dA1/vA1 pioneers of the antennal tip to be HRP-positive/Mes3-negative, confirming they are neurons and derive from the epithelium (ectoderm). This same double-labeling also confirms the presence of two BPs, one for each antennal pathway (Fig. 5.4b). Rotating the confocal Z-stack to a side view (Fig. 5.4b) clearly shows the ventral tip pioneer extending a process to a ventral BP and the dorsal tip pioneer likewise extending a process to a dorsal BP. Further, both the BPs appear to be HRP-positive/Mes3-positive, consistent with a derivation from the mesoderm and not the ectoderm. Confocal images at higher resolution (Fig. 5.4c) confirm that both BPs, but not the A1 tip pioneers, are Mes3-positive and, therefore, derive from the mesoderm which is represented by the lumen of the antenna (Ehrhardt *et al*., 2015a).

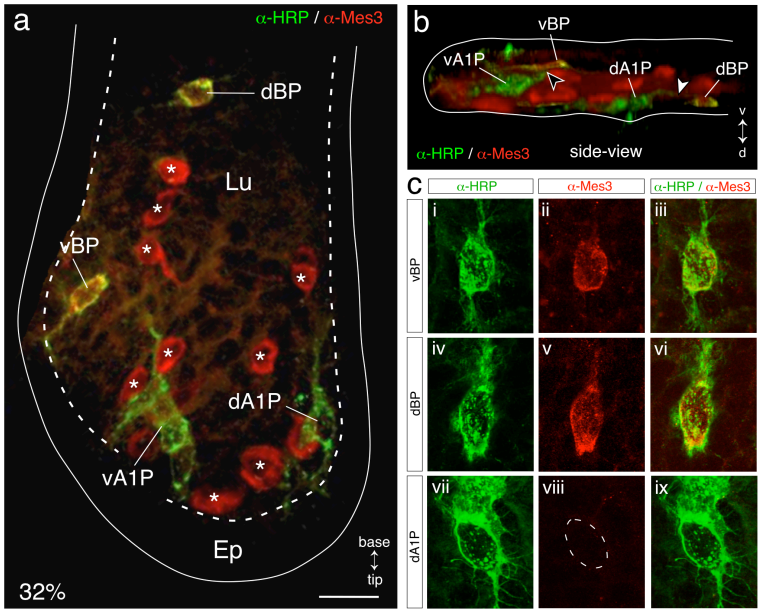



**Figure 5.4.** Molecular markers confirm different ontogenies for tip and base pioneers. (**a**) Confocal image of the early embryonic antenna (32%) following double immunolabeling with the neuron-specific marker HRP and the mesodermal marker Mes3. Tip pioneers (vA1P, dA1P) appear HRP-positive/Mes3-negative, while base pioneers (vBP, dBP) appear HRP-positive/Mes3-positive. Mes3-positive putative hemocytes (white stars) likely secrete basement membrane components for the appendages (Ball *et al*., 1987). (**b**) Confocal image of the antenna as in a but in side view confirms that there is a tip and base pioneer associated with each of the ventral (vA1P, vBP) and dorsal (dA1P, dBP) pathways. Growth cones of the vA1P (black arrowhead) and dA1P (white arrowhead) are in the process of establishing contact with their respective base pioneers. (**c**) Confocal images following double labeling with the neuron-specific marker HRP and the mesodermal marker Mes3 reveal both the vBP (**i-iii**) and dBP (**iv-vi**) that are HRP-positive/Mes3-negative (**vii-ix**), consistent with a derivation from the ectoderm. Scale bar in **a** represents 25 µm in **a, b**; 15 µm in **c**. Taken from Ehrhardt *et al*. (2015a).

At approximately 30%, at the stage of development at which base pioneers first become identifiable, we observe that in some cases base pioneers make contact with cells in the epithelium (Fig. 5.5). Immunolabeling against mitosis marker phospho-Histone H3 (PH3) reveals that these cells are proliferative (Fig. 5.5a,b). However, it is not clear what the relationship of this base pioneer is to these cells. If these proliferative cells are mother cells which give rise to the base pioneers, that would mean that Mes-3 is not the mesodermal marker which it is believed to be, as these cells are part of the ectodermal epithelium. Perhaps the contact of base pioneers with these cells does not reflect the origin of the base pioneers, but instead indicates some sort of information transfer between base pioneers and the proliferative cells.

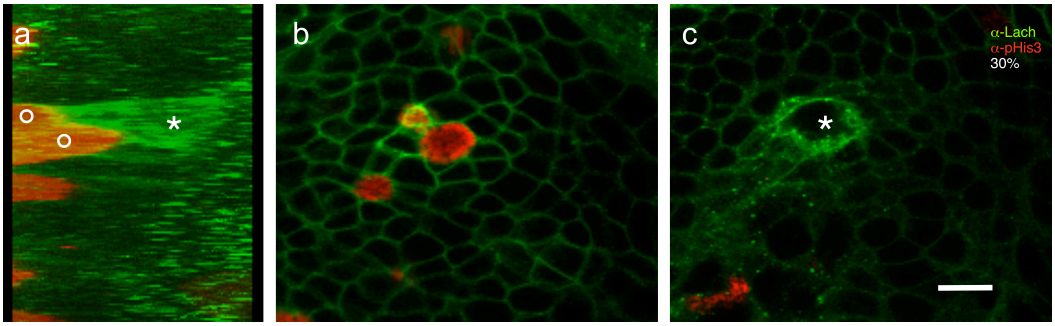



**Figure 5.5.** Confocal images from a wholemount 30% antenna immunolabeled against Lachesin and PH3. (**a**) The side view reveals that the Lachesin-expressing ventral base pioneer (white star) in the lumen makes contact with a pair of PH3-positive cells in the epithelium (white circles). (**b**) Top view of the PH3-expressing cells in the epithelium. (**c**) Optical cross-section of the base pioneer in the lumen. Scale bar represents 8 µm in each panel.

## 5.2. Axogenesis

The BPs on each side are not in equivalent locations along the antennal axis; the dBP is consistently closer to the base than is the vBP. This means that the growth cones of the tip pioneers must navigate different distances in order to contact their respective BPs. Comparisons (Fig. 5.6) confirm putative filopodial contacts between both the ventral (vA1) and dorsal (dA1) tip pioneers and their respective BP targets. Further, filopodial intermingling occurs slightly earlier in the ventral pathway (34%) than the dorsal pathway (36%). Differential growth patterns consistent with the data we present here have previously been reported for the ventral and dorsal tip pioneers (Ho and Goodman, 1982; Berlot and Goodman, 1984; Seidel and Bicker, 2000). We have shown that there is a BP possessing filopodia associated with each antennal tract and

that these filopodia intermingle with those of the respective dA1/vA1 pioneers from the antennal tip. Such putative reciprocal filopodial contacts could provide a morphological substrate for a molecular recognition process which we investigate below (Ehrhardt *et al*., 2015a).

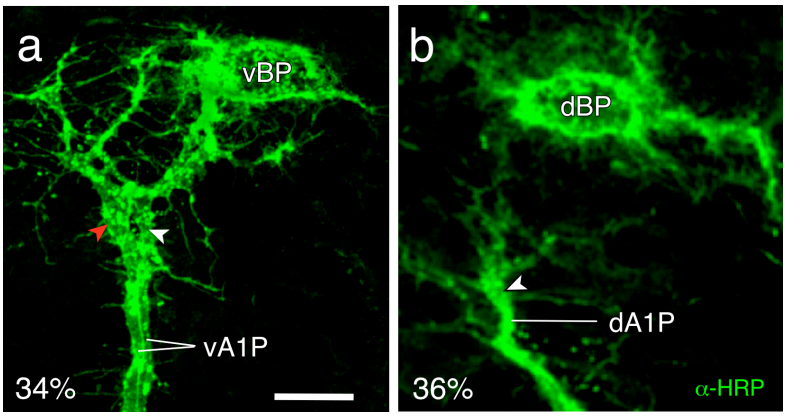



**Figure 5.6.** A1 axons contact base pioneers. (**a**) Confocal image following immunolabeling (α-HRP) at 34% of embryogenesis reveals putative filopodial contacts between an HRP-positive ventral tip pioneer (vA1P, white arrowheads) and its target HRP-positive base pioneer (vBP, red arrowheads). (**b**) Confocal image following immunolabeling (α-HRP) at 36% of embryogenesis shows the growth cone and filopodia of an HRP-positive dorsal tip pioneer (dA1P, white arrowheads) putatively contacting the filopodia (red arrowheads) and soma of its target base pioneer (dBP). Scale bar in a represents 20 µm in **a**, 15 µm in **b**. Taken from Ehrhardt *et al*. (2015a).

In the early embryonic antenna, double immunolabeling reveals that the tip pioneers (Fig. 5.7a (i-vi) and base pioneers (Fig. 5.7b (i.vi)) are HRP-positive/Lazarillo-positive, consistent with their being pioneer neurons. However, the temporal expression pattern of the Lazarillo antigen differs among the various pioneers Whereas Lazarillo expression in the tip pioneers is continuous during embryogenesis (Fig. 5.7a (iii, vi) and see Boyan and Williams (2004) for later ages), the base pioneers initiate Lazarillo expression at the time their filopodia and those of the tip pioneers first recognize one another (Fig. 5.7b (iii)). They subsequently downregulate this antigen (Fig. 5.7b (ii, v)) although they still express the HRP antigen (Fig. 5.7b (i,iv)) allowing them to be identified (Ehrhardt *et al*., 2015a).

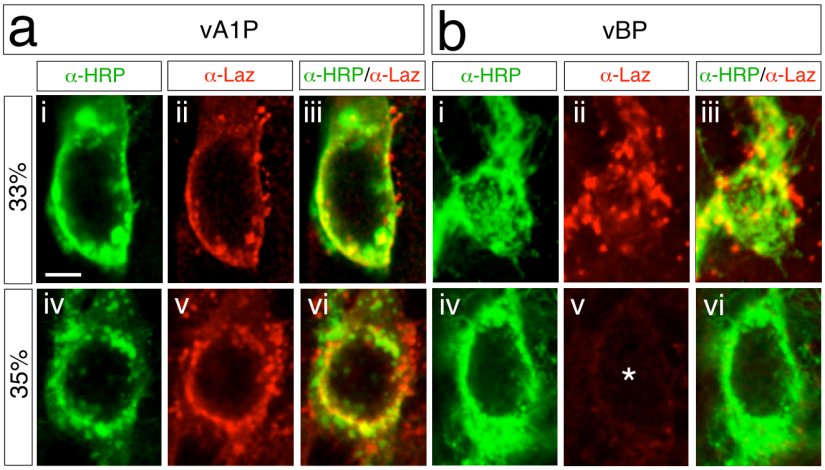



**Figure 5.7.** Dynamic expression of a cell surface antigen involved in axon guidance by pioneer neurons. (**a**) Confocal images of representative tip pioneers (vA1P) following double labeling with the neuron-specific marker HRP and the cell surface guidance molecule Lazarillo (Laz) at two different ages (33 and 35%) during embryogenesis. At 33% (**i-iii**) and 35% (**iv-vi**), the vA1P co-expresses HRP and Lazarillo. (**b**) Confocal images of representative base pioneers (vBP) from the same antennal pathway as in a following double labeling with the neuron-specific marker HRP and Lazarillo (Laz) at two different ages (33% and 35%) during embryogenesis. The vBP co-expresses HRP and Lazarillo at 33% (**i-iii**) but downregulates Laz expression at 35% (**iv-vi**, white star in **v** indicates soma location). Scale bar represents 5 µm in each panel. Taken from Ehrhardt *et al*. (2015a).

In order to use immunoblocking to investigate the mechanisms of axon guidance in the embryonic antenna, it was necessary to develop a method for keeping embryos alive in culture. This method allows us to remove an embryo from the egg, manipulate it experimentally, for example by adding an antibody to the culture medium, and keep it alive for several days during which time it can undergo fairly normal growth (Fig. 5.8), although at a slower rate than it would in the egg.

We examined whether Lazarillo also contributes to axon guidance during early embryogenesis in the antennal nervous system, consistent with its role in the ventral nerve cord (Sánchez *et al*., 1995). Immunoblocking of the Lazarillo epitope under whole embryo culture conditions was indeed found to disrupt axogenesis in the early embryonic antenna (Fig. 5.9). Blocking Lazarillo expression caused the

growth cones of both dorsal (Fig. 5.9b) and ventral (Fig. 5.9c) apical pioneers to miss their correct basal pioneers despite their maintained physical presence (c.f. Fig. 5.9a). We interpret this as a failure to molecularly identify the respective targets. In our experiments, the growth cones of the tip pioneers from both pathways stalled and extended uncharacteristic wide-ranging filopodia in an apparent search process (Ehrhardt *et al*., 2015a).

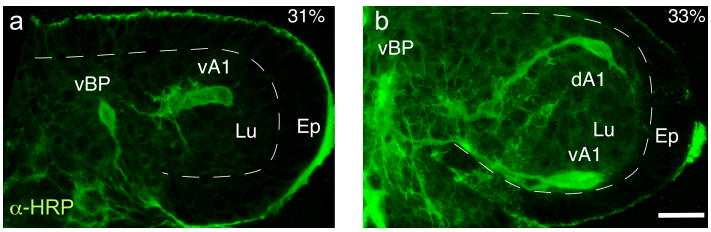



**Figure 5.8.** Confocal images of HRP-immunolabeled antennae that have have been cultured for 24 h starting from an age slightly under 30%. (**a**) This embryo has grown to a stage of development equivalent to 31% of embryogenesis while in culture, as demonstrated by the presence of HRP-expressing vA1 and vBP neurons in the lumen. (**b**) This embryo has grown to a stage of development equivalent to 33% while in culture, as revealed by the presence of vA1 and dA1 pioneers whose axons have extended to an HRP-expressing vBP at the base. Scale bar represents 20 µm in each panel.

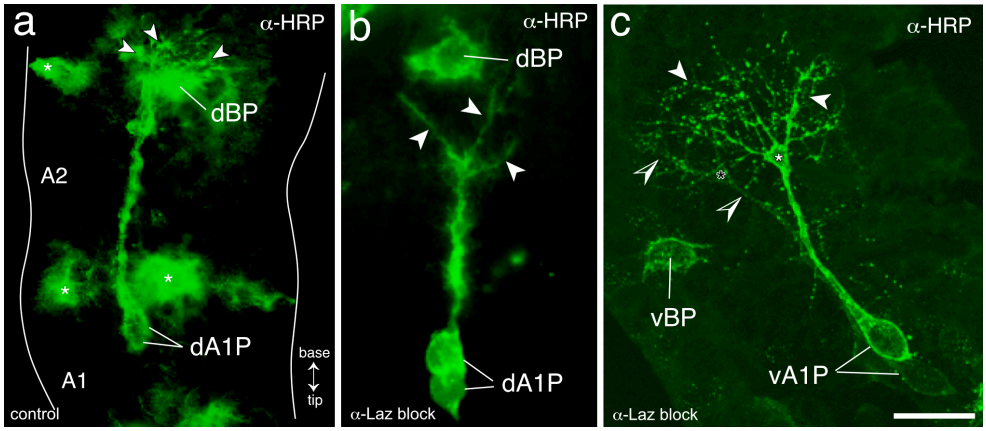



**Figure 5.9.** Disrupted target recognition following antibody blocking. (**a**) Normal growth. Fluorescence microscope image of the antenna (edge is outlined white) from an embryo grown in culture for 2 days (from 32% up to 36% of embryogenesis) and subsequently immunolabeled with neuron-specific HRP. The growth cone filopodia (white arrowheads) of the sibling tip pioneers (vA1P) have contacted a target HRP-positive vBP en route to the brain (not shown). Other putative guidepost cells (white stars) are also labeled. (b,c) Disrupted target recognition in the antennae of embryos of the same age and grown under the same conditions, as in a except that Lazarillo antibody was added to the initial culture medium (see Materials and methods). (**b**) Fluorescence microscope image following neuron-specific HRP immunolabeling shows a sibling pair of dA1P neurons whose growth cone appears to have stalled and whose extensive filopodia (white arrowheads) have apparently failed to recognize the target dBP. (**c**) Confocal image following neuron-specific HRP immunolabeling shows a sibling pair of vA1P neurons whose respective growth cones (open, solid white stars) have by-passed their target vBP, stalled, and now extend wide-ranging filopodia (open, solid white arrowheads) in search of the target. Antenna orientation in **a** applies to all panels. Scale bar in **c** represents 18 µm in **a**, 17 µm in **b**, and 15 µm in **c**. B. Niederleitner, M. Güntner, and G.S. Boyan contributed data to this figure. Taken from Ehrhardt *et al*. (2015a).

## 5.3. Targets of the pioneer neurons

Although the axon growth of the apical pioneers to the base pioneer and the border of the deutocerebrum have been well studied, the targets of the antennal pioneers in the brain were unknown. We used immunolabeling against HRP and Lazarillo to identify the projections of the antennal pioneers as they grow in a stereotypic manner out of the antenna and into the first deutocerebral, then protocerebral, brain neuropils. We identified that their targets in the protocerebrum as the Lazarillo-expressing LC cells (Boyan and Ehrhardt, 2015).

### 5.3.1. A motoneuron in the deutocerebrum

Ho and Goodman (1982) reported that base pioneer growth cones are the first peripheral processes to reach the CNS. Berlot and Goodman (1984) observed that base pioneer axons fasciculate with the axons of motoneurons. We also observed base pioneers extending growth cones into the deutocerebrum (Fig. 5.10). In this preparation, proliferative cells were labeled using 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analog of thymidine which is incorporated into DNA during DNA synthesis in living tissue (Sousa-Nunes *et al.,* 2011; Takagi *et al*., 2012). A large round cell is labeled with EdU, indicating that it went through S phase while the EdU pulse was applied. The EdU labeling and morphology of the cell suggest that it is a

neuroblast. The presence of this putative neuroblast next to the dorsal base pioneer provides further evidence that the dorsal base pioneer's growth cone is in the deutocerebrum rather than the antenna, as Zacharias *et al*. (1993) documented the presence of similar large, mitotically active neuroblasts in the grasshopper deutocerebrum at a similar stage of embryogenesis using BrdU incorporation. The motoneuron which will serve as a target for this pioneer is also revealed by HRP labeling (Fig. 5.10).

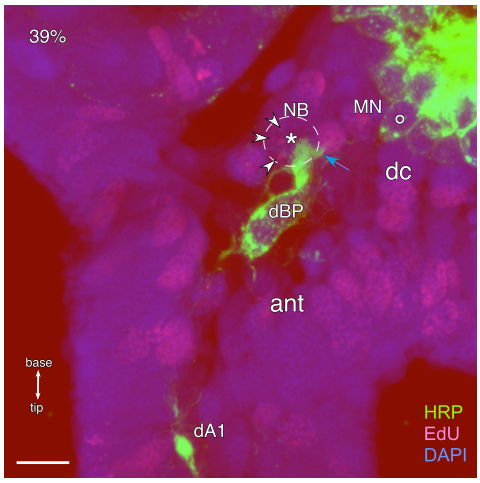



**Figure 5.10.** HRP immunolabeling combined with EdU and DAPI staining reveal that a BP in a sectioned 39% antenna is extending its growth cone (blue arrow) into the deutocerebrum (dc) toward a motoneuron (MN, white circle). HRP (green) is expressed by the axon of the dorsal apical pioneers (dA1), the dorsal base pioneer (dBP) and other neurons in the brain. EdU (magenta, white arrowheads) labels a large, round cell (white dashed circle), which based on its morphology and proliferative nature appears to be a neuroblast (NB). Scale bar represents 10 µm.

### 5.3.2. The primary axon scaffold of the brain

Immunolabeling with neuron-specific markers reveals that the initial set of axon projections in the embryonic insect brain comprises a simple scaffold of commissural and descending pathways centered around the stomodeum (Fig. 5.11a; Reichert and Boyan, 1997). The scaffold is generated by sets of identified central pioneer axons located at stereotypic sites and is used for fasciculation by later differentiating axons. These sites include a complex of seven cell clusters spread across the protocerebrum and deutocerebrum (Fig. 5.11b) and recognizable by the fact that they are the only cells in the early brain to co-express Lazarillo and HRP (Graf *et al.,* 2000). The bilateral, unpaired LC cells of cluster 2 from this complex (Fig. 5.11c) have been shown to pioneer a fascicle of the primary protocerebral commissure (Graf *et al*., 2000), and these cells also prove to be targets for the projections from the pioneers of the developing antennal system (Boyan and Ehrhardt, 2015).

### 5.3.3. Projections of pioneer neurons into the brain

A maintained co-expression of the neuronal label HRP and the lipocalin Lazarillo by the apical pioneer neurons allows their unequivocal identification in the developing antennal nervous system (Fig. 5.11d). We used this feature to conduct a staged analysis of apical pioneer cell projections beyond the antenna and into the brain (Fig. 5.12; Boyan and Ehrhardt, 2015).

At 37% of embryogenesis (Fig. 5.12a-c), Lazarillo-positive axons of the ventral and dorsal apical pioneers have reached the antennal base and can be seen to turn medially and anteriorly toward the deutocerebrum where their growth cones approach the Lazarillo-positive C3 cell cluster. At this stage, the pioneers often exhibit slightly different growth rates so that their projections extend different distances into the deutocerebrum. This asymmetry is resolved as the pioneer fascicles subsequently merge within the deutocerebrum. Further anterior in the protocerebrum of the brain, an identified Lazarillo-positive cell (the

LC, see Graf *et al*., 2000) of the C2 cluster at the mid-line projects an axon laterally toward the C1 and C6 clusters. Other Lazarillo-positive cell clusters are present in the protocerebrum but have not yet commenced axogenesis so that the axon scaffold is still incomplete. At 39% of embryogenesis (Fig. 5.12d-f), the growth cones of the antennal pioneers now extend equidistantly into the deutocerebrum of the brain, their fascicles have merged, and their filopodia intermingle with those from cells of the C3 cluster. At the same time, the growth cone of the LC cell from the C2 cluster in the protocerebrum has turned posteriorly and also projects toward the C3 cluster. At 41% of embryogenesis (Fig. 5.12g-i), the growth cones of the apical pioneers are indistinguishable so that we can only refer to a combined projection. Filopodia from the antennal pioneers extend anteriorly beyond the C3 cluster and project onto an axonal pathway which now links the C1 and C2 clusters of the protocerebrum with the C3 cluster of the deutocerebrum. This sequential extension of the projections from the antennal pioneers onto the various Lazarillo positive cell clusters of the brain is summarized schematically in Fig. 5.13a-c (Boyan and Ehrhardt, 2015).

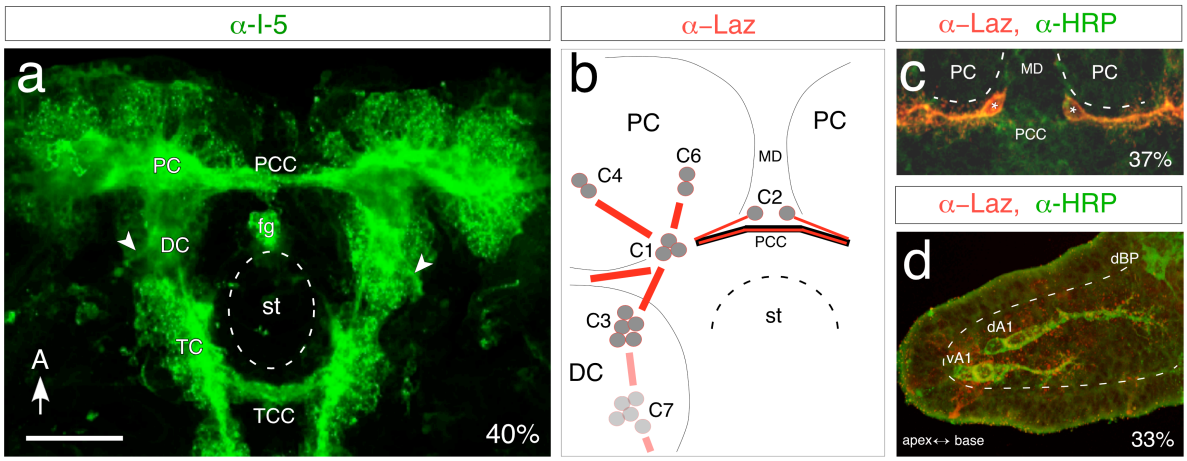



**Figure 5.11.** The initial axon tracts of the brain and antenna of the embryonic grasshopper. (**a**) Confocal image following immunolabeling with the neuronal marker I-5 in wholemount preparations reveals the primary axon scaffold of the brain as at 40% of embryogenesis. Commissural fibers cross the mid-line anterior to the stomodeum (st, approximate outline dashed white) via the protocerebral commissure (PCC), and posterior to the stomodeum via the tritocerebral commissure (TCC). Axons (white arrowheads) are seen in the deutocerebral (DC) neuropil at a location where the antennal nerve will form and may be those of motoneurons. Anterior A is at the top. (**b**) Schematic (not to scale) shows identified clusters of Lazarillo-positive cells (C, red outline) which contribute axons (red) to the primary axon scaffold in the protocerebrum (PC) and deutocerebrum (DC) of the brain (as seen at 41% if embryogenesis from a frontal prospective). Not all clusters of the scaffold are depicted here (See Graf *et al*., 2000) and only the left brain hemisphere is shown. Cluster 7 with its projections to the tritocerebrum (not shown) is located deeper (more dorsally) in the brain and so is rendered less opaque. A subset of axons (red) of the primary brain commissure (PCC, black) originating from the C1 cluster is Lazarillo-positive. (**c**) Confocal image of the brain midline at 37% of embryogenesis following double-labeling against the neuronal markers Lazarillo (red, Laz) and horseradish peroxidase (green, HRP) reveals co-expression (yellow) by the bilateral unpaired LC cells (white stars) comprising the C2 cluster of each protocerebral hemisphere (PC). These cells pioneer a fascicle of the PCC (green) (see Graf *et al*., 2000). (**d**) Confocal image of an antenna at 33% of embryogenesis following HRP and Lazarillo immunolabeling reveals that Lazarillo is expressed by the two sets of sibling apical pioneers (vA1, dA1). Other abbreviations: TC, tritocerebrum. Scale bar represents 70 µm in **a**; 60 µm in **c**; 25 µm in **d**. Modified from Boyan and Ehrhardt (2015).

A reconstruction of the arborizations from the antennal pioneers and those of the LC cell (Fig. 5.13d) at higher resolution confirms that their terminals closely appose one another so that their filopodia intermingle in the protocerebrum. The data allow us to speculate that their may be filopodial contacts between these peripheral and central pioneers. We suggest that in projecting onto this central scaffold, that apical pioneers of the antenna represent a peripheral component of a molecularly continuous brain circuit (Boyan and Ehrhardt, 2015).

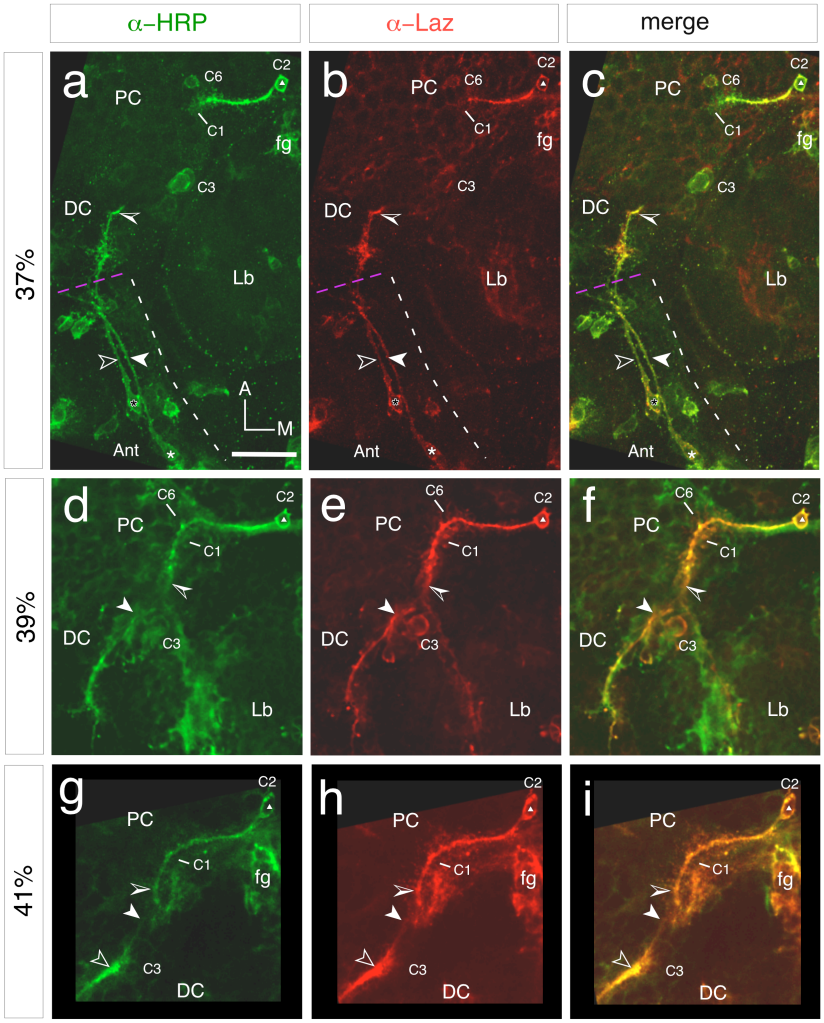



**Figure 5.12.** The projections of A1 pioneer axons into the brain at successive stages of embryonic development as revealed by double-labeling against HRP (green), Lazarillo (red), co-expression (yellow, merge). Only the left brain hemisphere is viewed from ventral (frontally) is depicted throughout. Approximate outline of the antenna is shown dashed white. (**a-c**) 37% of embryogenesis. Lazarillo/HRP-positive antennal pioneers (black star dA1P, white star vA1P) generate axons (open white arrowhead dA1P, white arrowhead vA1P) whose growth cones have projected beyond the antennal base (approximate location given by magenta dashed line) and into the deutocerebrum (DC). The growth cones are still divergent at the early stage and that of the dA1P is out of the picture here. The growth cone of the vA1P (open/white arrowhead) has begun to turn medially toward the C3 cluster in the DC. In the protocerebrum (PC), the growth cone from the LC cell (white triangle) of the C2 cluster projects laterally toward the C6 cluster. (**d-f**) 39% of embryogenesis. The vA1P and dA1P fascicles have merged at this stage. The growth cones (white arrowhead) from the Lazarillo/HRP-positive apical pioneers project anteriorly beyond the C3 cluster of the DC and toward the C1 cluster in the PC. The growth cone (open/white arrowhead) of the LC cell (white triangle) has turned posteriorly around the C1 cluster and now projects toward the C3 cluster. (**g-i**) 41% of embryogenesis. Lazarillo/HRP-positive filopodia (white arrowhead) from the growth cones (open white arrowhead) of the antennal pioneers have extended into the PC and intermingle with those from the Lazarillo/HRP-positive growth cone (open/white arrowhead) of the antennal pioneers have extended into the PC and intermingle with those from the Lazarillo/HRP-positive growth cone (open/white arrowhead) of the LC neuron (white triangle) from the C2 cluster of the PC Other abbreviations: fg, frontal ganglion; Lb, labrum. Axes: anterior (A), medial (M). Scale bar represents 40 µm throughout. Taken from Boyan and Ehrhardt (2015).

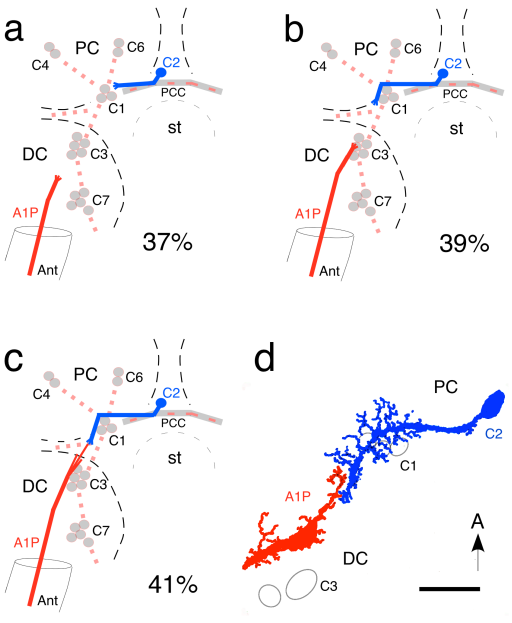



**Figure 5.13.** Schematics (**a-c**) summarizing the progressive ingrowth of axons from A1 pioneers of the antenna (Ant) into the brain during early embryogenesis (37-41%) as revealed by double-labeling against Lazarillo and HRP (see Fig. 5.12) As the pioneer fascicles from the antenna merge in the brain, their projections are grouped for the purposes of this representation. The growth cones of the antennal pioneers (red) project from the C3 cluster of the DC toward the C1 cell cluster in the protocerebrum and encounter the growth cone (blue) of the protocerebral LC cell from the C2 cluster. Other axon pathways linking the cell clusters are shown dashed. (**d**) Reconstruction based on a Z stack of confocal images following Lazarillo labeling at 41% of embryogenesis shows that the projections from the antennal pioneers (red) lie in close proximity to those of the LC cell (blue) at the border of the protocerebrum and deutocerebrum. Cells of the C3 and C1 clusters are shown schematically but in their correct locations. Scale bar represents 30 µm in d. Taken from Boyan and Ehrhardt (2015).

## 5.4. Fates of the pioneers

Previously the fates of the antennal pioneers were unknown. We used two methods for labeling apoptotic cells to investigate when and whether antennal pioneers undergo programmed cell death, as the pioneers of the leg do. Sectioning and sonication allowed us to label antennae after the thickening of the cuticle at mid-embryogenesis.

### 5.4.1. Fates of apical pioneers

The apical pioneers can be reliably identified from the time of their appearance (31% of embryogenesis) up to mid-embryogenesis (53%) via several molecular labels including HRP (Fig. 5.14) and the cell surface lipocalin Lazarillo (not shown, but see Boyan and Williams, 2004; Ehrhardt *et al.*, 2015a). These data reveal their morphology to be consistent over this period, although their slightly oblong somata sometimes remain in contact (Fig 5.14a,b,f; Fig. 5.15) and other times separate slightly (Fig 5.14c-e) during subsequent development, while always remaining in axonal contact (Ehrhardt *et al.,* 2015c).

In order to investigate the pioneer fates, we employed two established markers of programmed cell death: acridine orange (AO) and TUNEL (see Methods 4.3.2) in combination with the neuron-specific label HRP and, in the case of the base pioneers, also the mesodermal label Mes3. Control experiments confirm that while TUNEL may be detected a little earlier than AO (DNA fragmentation occurring prior to pycnotic body formation), all apoptotic cells examined in the antenna are eventually co-labeled with these death markers (Fig. 5.16; Ehrhardt *et al*., 2015c).

Application of AO revealed cell death to be prominent in both the epithelium and lumen of the embryonic antenna (Fig. 5.17), even as early as 35%. The cell types involved remain unidentified, but the band-like pattern of apoptotic cells in the epithelium correlates with the meristal segmentation of the antenna (Fig. 5.17; see Chapman, 1982, 2002), while AO-positive cells in the lumen appear to be associated more with the median strand (Fig 5.17). AO labeling does not appear in identified apical pioneers prior to mid-embryogenesis. At 53% (Fig. 5.18a), HRP immunolabeling identifies the apical pioneers of both nerve tracts at the lumen epithelium border, but AO is not found within the apical pioneers themselves although it labels nearby cells in the A1 region. At 55% (Fig. 5.18b), AO labeling of a putative pycnotic body is now found within the soma of an apical pioneer associated with the ventral nerve tract, providing the first evidence that an apical pioneer is undergoing apoptosis. At 57% (Fig. 5.18c), AO labeling is present within one apical pioneer but not its sibling, suggesting that siblings do not necessarily die concurrently. By 60% (Fig. 5.18d), AO labeling is visible within the soma of an apical pioneer, and its HRP labeling has become faint and spotty, consistent with a degeneration of the somal membrane (Ehrhardt *et al*., 2015c).

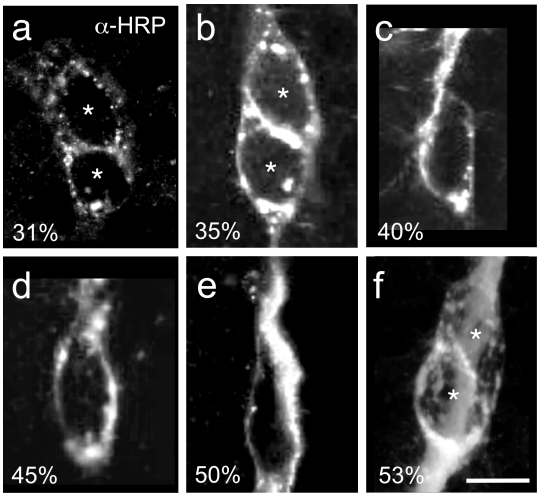



**Figure 5.14.** Confocal images of A1 pioneers at successive developmental stages (**a-f**) following labeling against HRP. Apical pioneers are molecularly identifiable from their delamination at 31% (**a**) up to mid-embryogenesis (**f**). Apical pioneers develop as paired siblings (white asterisks) but in some preparations these siblings separate so that high resolution images then show one cell (**c, d, e**). Scale bar in **f** represents 10 μm in **a**, **b**; 15 μm in **c**; 10 μm in **d**; 15 μm in **e**; 10 μm in **f**. Taken from Ehrhardt *et al*. (2015c).

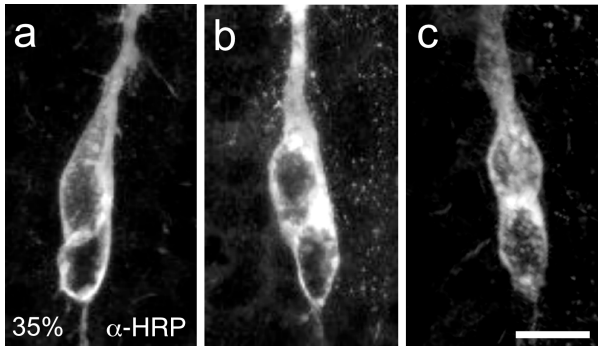



**Figure 5.15.** Confocal images of sibling A1 pioneers from three different antennae (**a-c**) at the same age (35%) following HRP immunolabeling demonstrate the consistency of A1 pioneer morphology. Base of the antenna is to the top. Scale bar represents 15 µm in all panels. Taken from Ehrhardt *et al*. (2015c).

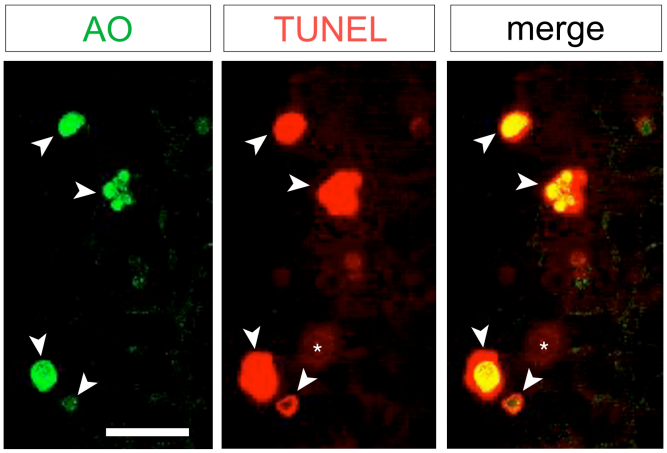



**Figure 5.16.** Cell death markers AO (green) and TUNEL (red) co-label (merged, yellow) the nuclei of apoptotic cells at mid-embryogenesis. Some cells (white star) show TUNEL earlier than AO so that co-labeling is not evident yet. Scale bar represents 15 µm in all panels**.** Taken from Ehrhardt *et al*. (2015c).

Apoptosis of apical pioneers revealed by AO labeling was confirmed via a TUNEL assay which detects DNA fragmentation (see Methods). TUNEL did not label any apical pioneer neurons at 50-53% (data not shown), but at 56%, HRP-positive pioneers associated with the ventral (Fig. 5.19a) and dorsal (Fig. 5.19b) pathways both show clear TUNEL labeling in the soma. Further, the sibling pioneers of a given pathway may undergo apoptosis concurrently (Fig. 5.19a), or not (Fig. 5.19b). Our data for AO and TUNEL are consistent in revealing apoptosis of the apical pioneers at around 56% of embryogenesis (Ehrhardt *et al*., 2015c).

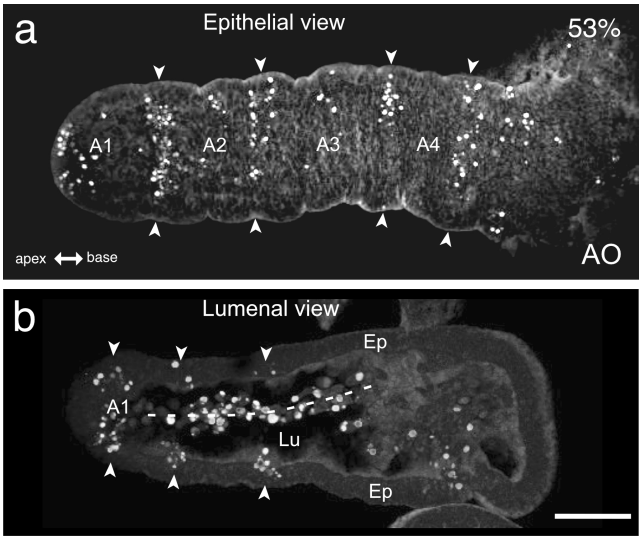



**Figure 5.17.** Fluorescence microscope images of antennae at mid-embryogenesis show the distribution of apoptotic cells labeled by AO. (**a**) Epithelial view shows apoptotic cells occurring as bands (white arrowheads) at the borders of the meristal annuli of the antenna (A1-A4). (**b**) AO also labels cells in the lumen (Lu) of another antenna of approximately the same age. Most dying cells in the lumen appear closely associated with the median strand (dashed white line). Scale bar in **b** represents 100 µm in all panels. Taken from Ehrhardt *et al*. (2015c).

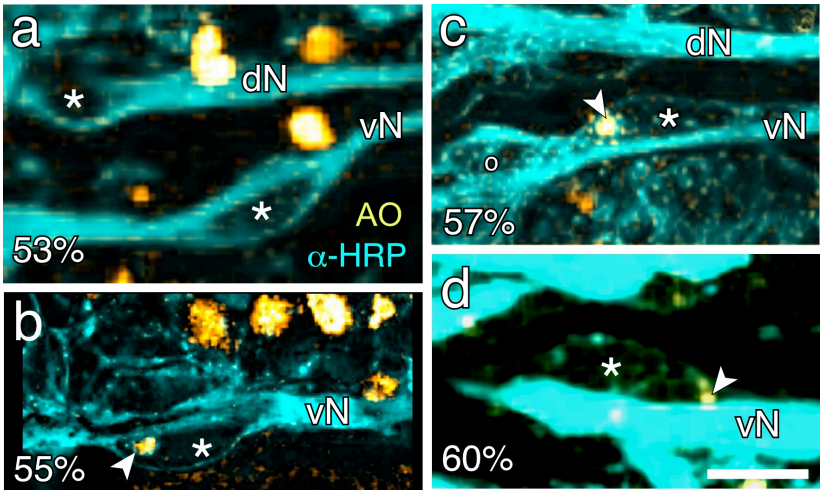



**Figure 5.18.** Confocal images of A1 pioneers in sectioned antennae following application of AO (yellow) and subsequently co-labeled against HRP (cyan). (**a**) At 53%, the apical pioneers (white stars) of the dorsal (dN) and ventral (vN) nerve tracts show no signs of cell death, although other cells in the vicinity are labeled by AO. (**b**) At 55%, acridine orange (AO) stains a pycnotic body (white arrowhead) in the soma (white star) of the ventral nerve tract (vN). (**c**) At 57%, one apical pioneer (white star) of the ventral nerve tract (vN) contains an AO-labeled pycnotic body (white arrowhead), while its sibling (open white circle) shows no signs of apoptosis. Sibling pioneers of the same antenna do not necessarily undergo apoptosis concurrently. (**d**) At 60%, an apical pioneer (white star) is labeled by AO (white arrowhead), consistent with apoptosis. The HRP labeling of the membrane has become fainter and patchy (open white arrowheads). Scale bar in **d** represents 12 µm in all panels. Taken from Ehrhardt *et al*. (2015c).

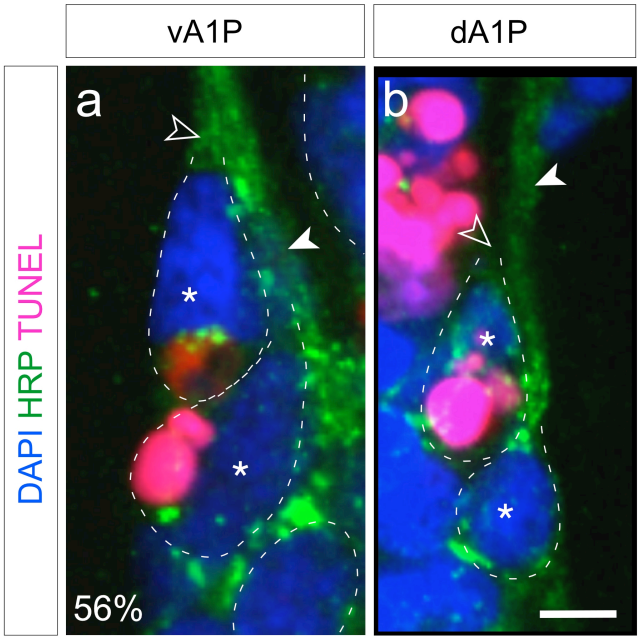



**Figure 5.19.** Confocal images following TUNEL labeling of A1 pioneers in the antenna at 56% of embryogenesis. DAPI (blue) labels nuclear DNA. (**a**) TUNEL (red) labels pycnotic bodies (white stars) in the somata (outline dashed white) of a sibling pair of apical pioneers (vA1P) of the ventral nerve tract. Neuron-specific HRP (green) labels their axons (open and solid white arrowheads) projecting towards the antennal base (top of figure). (**b**) Labeling as for a. Only one of the sibling pair of dorsal A1 pioneers shows TUNEL. Scale bar in **b** represents 7 µm in all panels. Taken from Ehrhardt *et al*. (2015c).

Finally, programmed cell death in apical pioneers was also confirmed in several preparations at 55% of embryogenesis via intracellular dye injection combined with immunolabeling (Fig. 5.20). After first locating a dying putative pioneer in the A1 region using the AO marker (Fig. 5.20a), the cell soma in the preparation shown was intracellularly filled with Alexa® 568 dye (Fig. 5.20b) and a co-localization of both labels confirmed. Subsequent HRP immunolabeling confirmed that the dying cell is a neuron with an axon associated with one of the antennal nerve tracts (Fig. 5.20c). At higher magnification, a confocal view of the dye-filled, HRP-labeled dying cell from a further preparation (Fig. 5.20d) reveals the axonal and somal morphologies to be characteristic of an apical pioneer (Ehrhardt *et al*., 2015c).

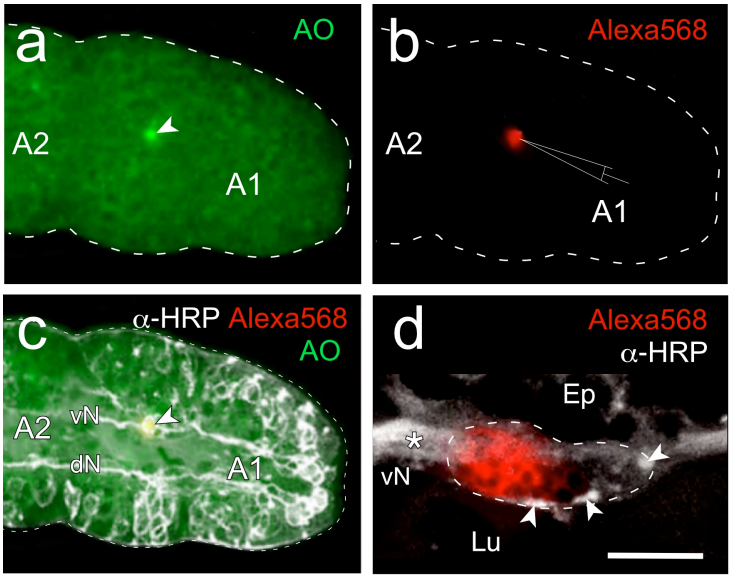



**Figure 5.20.** The pioneer identity of an AO-labeled cell in a section of the antenna at 55% of embryogenesis is confirmed with dye-filling and immunolabeling against HRP. The antenna is outlined with a dashed white line. (**a**) Photomicrograph shows that the apoptosis marker AO labels a cell (white arrowhead) in the lumen of the A1 region near the tip of the antenna. (**b**) Same preparation as in a. The cell in a is subsequently filled with Alexa® 568 dye using a sharp glass electrode (drawn schematically). (**c**) HRP immunolabeling of the preparation in a,b confirms the co-localization of AO and Alexa® 568 dye (yellow), and that the dying cell of this preparation (white arrowhead) generates an axon associated with the ventral nerve tract (vN). (**d**) A higher magnification view of the dye-filled nucleus (red) of an HRP-labeled (white arrowheads) dying cell from another preparation to **a-c**. The axon (white star) in the vN and the somal morphology (dashed white line) are characteristic of the apical pioneers. Scale bar in **d** represents 80 µm in **a, b, c**; 10 µm in **d**. Taken from Ehrhardt *et al*. (2015c).

### 5.4.2. Fates of base pioneers

Double-labeling with Mes3 and HRP allows the base pioneers to be identified over a period of 31-45% of embryogenesis (Fig. 5.21). These labels reveal a consistent cellular morphology that is considerably different from that of the apical pioneers. The BPs possess characteristically more spherical somata bearing, in addition to the axon, many small dendritic projections not present in their apical counterparts (c.f. Fig. 5.14). The Mes3 expression indicates a derivation from the mesoderm (lumen) in contrast to the ectodermal (epithelial) origin of the apical pioneers which are Mes3-negative (see Ehrhardt *et al*., 2015a). Significantly, the BPs are single cells, not siblings like the apical pioneers. The BPs are present at the time the apical pioneers delaminate and commence axogenesis (c.f. Fig. 5.3b), and their dual expression of HRP and Mes3 continues up to 40% of embryogenesis (Fig. 5.21a-f). After this stage, HRP expression becomes spotty (Fig. 5.21g) and is finally downregulated (Fig. 5.21g and j). Mes3 expression, on the other hand, is retained until at least 45% (Fig. 5.21h-l) after which the BPs can no longer be reliably identified against the background of other Mes3-expressing cells in the vicinity (Ehrhardt *et al*., 2015c).

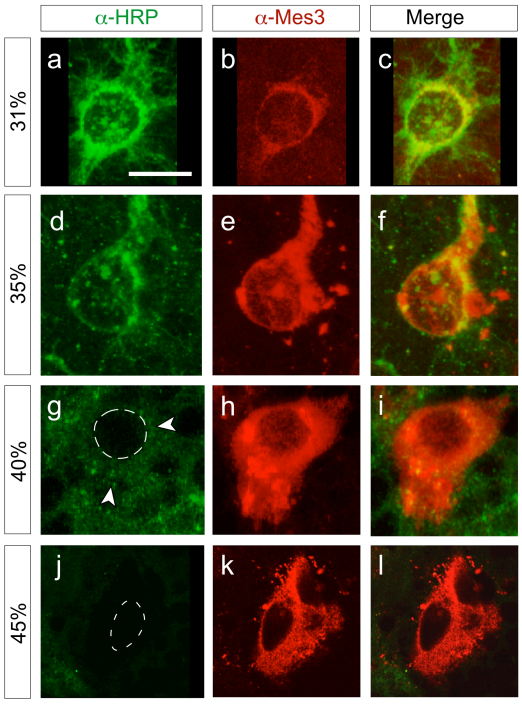



**Figure 5.21.** Confocal images of BPs from antennae of different embryonic ages following double labeling with HRP (green) and Mes3 (red). Merged images show co-expression (yellow). Panels **a-f** show dorsal base pioneers, panels **f-l** show ventral base pioneers. All base pioneers co-expression HRP and Mes3 from 31% up to 40% of embryogenesis (**a-i**). At 40% (**g-i**), the HRP immunolabeling has been downregulated while the ontogenetic label Mes3 is maintained. Scale bar in a represents 10 µm in **a-c**; 8 µm in **d-i**; 15 µm in **h-l**. Taken from Ehrhardt *et al*. (2015c).

The period over which we can currently search for evidence of cell death in base pioneers is therefore narrower than in the apical pioneers. Nevertheless, neither AO- (Fig. 5.22) nor TUNEL-labeling (data not shown) revealed evidence of cell death in the BPs up to mid-embryogenesis, although apoptotic cells are clearly present in their vicinity (Fig. 5.22c). DAPI staining also shows no evidence of pycnotic bodies forming in their nuclei, and there is no obvious degeneration of cell membranes at a time when HRP has already been downregulated (Fig. 5.21g-l; Fig. 5.22a-c). The BPs therefore begin to alter their molecular profiles with respect to HRP-, Mes3-, and Lazarillo-

expression (see Ehrhardt *et al*., 2015a) in a stepwise manner without any evidence of death signals. At present we must allow for the possibility that the base pioneers may persist in the antenna in a new, and as yet unclear role (Ehrhardt *et al*., 2015c).

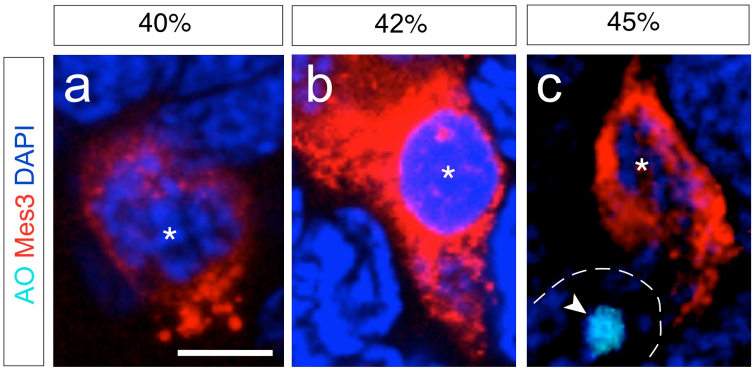



**Figure 5.22.** Confocal images of BPs at successive ages (**a**, 40%; **b**, 42%; **c**, 45%) following labeling with AO (cyan), Mes3 (red), and DAPI (blue). Base pioneers are not labeled by AO over the ages shown. DAPI staining shows their nuclei (white stars) to be free of pycnotic bodies, and the cell membranes show no signs of degeneration. AO (white arrowhead) does, however, label the nucleus of a nearby dying Mes3-negative cell (soma outline dashed white). Scale bar in **a** represents 8 µm in **a-c**. Taken from Ehrhardt *et al*. (2015c).

## 5.5. Sensory cell clusters

The proper navigation of the pioneer axons is likely critical for the development of the antennal nerve tracts, because the axons of sensory neurons fasciculate with pioneer axons and follow them to their targets (Fig. 5.23). Each cluster of sensory neurons projects axons onto the pioneers associated with one nerve tract (Fig. 5.23c,f), but not both nerve tracts. It is unclear how the sensory neurons determine which nerve tract to fasciculate with. Apical pioneers usually have centrifugal projections (Fig. 5.23a-c) directed towards the sensory neurons at the tip, which could help guide the sensory axons.

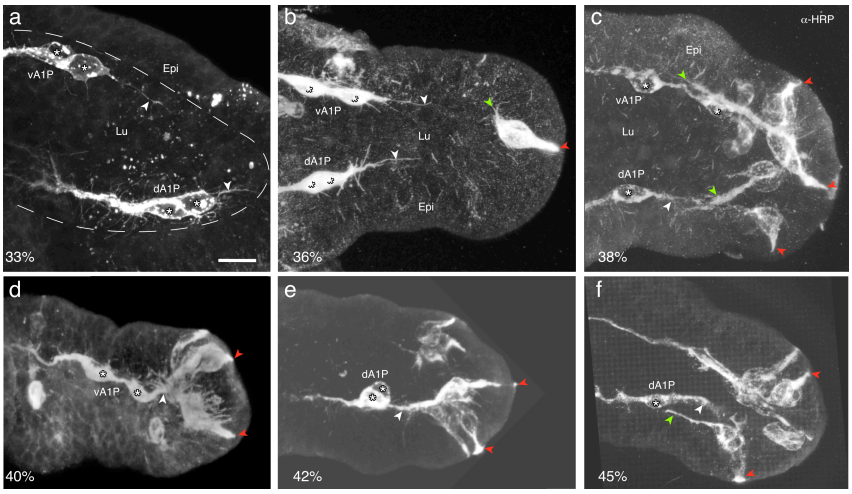



**Figure 5.23.** HRP immunolabeling reveals centrifugal projections of A1 pioneers in wholemount antennae at 33-45% of embryogenesis. (**a**) At 33%, before the first sensory neurons differentiate, the vA1 and dA1 pioneers possess thin centrifugal projections (white arrowheads). (**b**) After 35%, sensory neurons appear in the antenna, including a pair at the tip of the antenna. The vA1 and dA1 pioneers still possess centrifugal projections (white arrowheads). (**c**) At 38%, more and more sensory neurons (red arrowheads) are appearing near the tip. On the dorsal side in this antenna, a pioneer's centrifugal projection is directed towards the projections of the sensory neurons. (**d**) Several sensory neurons in the epithelium are connected (white arrowhead) to the A1 pioneers. (**e**) Several sensory neurons are connected (white arrowhead) to the A1 pioneers. (**f**) Sensory neurons (red arrowheads) extend projections onto A1 pioneers (white star). White arrowheads indicate centrifugal projections of pioneers. Red arrowheads indicate apical dendrites of sensory neurons. Green arrowheads indicate axons of sensory neurons. White stars indicate pioneer somata. Scale bar in a represents 8 µm in **a**, 12 µm in **b**, 12 µm in **c**, 15 µm in **d-f**.

Until recently, the later development of the antenna was difficult to study due to the formation of a secondary cuticle at 55% (Bentley *et al*., 1979), which is impermeable to antibodies. One method to immunolabel older embryos is to slice the antenna into thin sections, allowing antibodies to be applied to the nervous system without the cuticle interfering (Fig. 5.24). However, this process often results in unwanted

distortion of the tissues involved or in the disturbance of molecular binding sites. Further, depending on the thickness of the tissue, elements of the nervous system may be located across several sections which must then be aligned either manually or via an image-processing algorithm to allow reconstruction (Ehrhardt *et al*., 2015b).

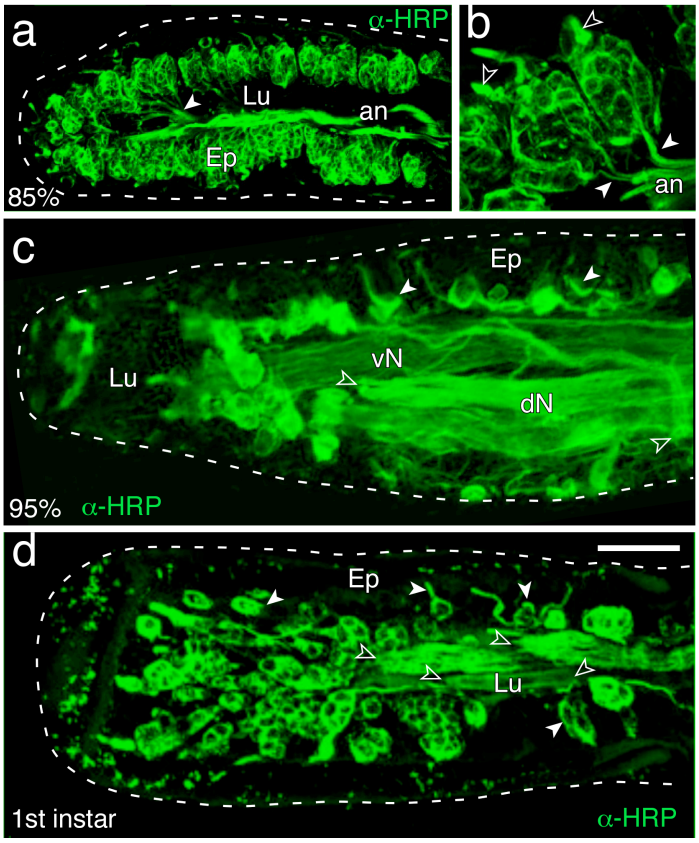



**Figure 5.24.** The nervous system of sectioned antennae. (**a**) 85% antenna in longitudinal section. Immunolabeling (HRP, green) was performed after sectioning. Clusters of HRP positive sensory cells in the epithelium (Ep) direct axonal projections (e.g. white arrowhead) onto the antennal nerves (an) in the lumen (Lu). Approximate outline of the cuticularized antenna is dashed white. (**b**) Higher-power confocal view reveals single clusters of HRP-positive sensory cells with dendritic projections (open white arrowheads) that will innervate sensory hairs and axons (white arrowheads) projecting into the antennal nerve (an). (**c**) 95% antenna in longitudinal section. Immunolabeling against HRP (green) was performed after sectioning which has disrupted both sensory cell clusters (white arrowheads) in the epithelium (Ep), and nerve pathways (open white arrowheads) in the lumen. (**d**) First instar, antenna in longitudinal section and subsequent immunolabeling against HRP (green). Sectioning has disrupted cell cluster integrity (white arrowheads) and bisected the antennal nerves (open white arrowheads). Scale bar in d represents 80 µm in **a**, 15 µm in **b**, 40 µm in **c**, 50 µm in **d**. Modified from (Ehrhardt *et al*., 2015b).

We developed a method of using sonication to disrupt the cuticle, which for the first time allows immunolabeling of the intact sensory nervous system of wholemount antennae during late embryogenesis and the first instar after hatching. This sonication technique enables us to observe how more and more sensory neurons differentiate in the epithelium along the length of the antenna during late embryogenesis (Fig. 5.25). The axons of these sensory neurons project into the antennal nerve tracts (Fig. 5.25d,g). The dendritic terminals of sensory neurons are rich in α-tubulin, as are their axons, but the somata of the neurons show little α-tubulin expression at 70% of embryogenesis (Fig. 5.25e,h). In first instar nymphs, immunolabeling against α-tubulin (Fig. 5.26) and HRP (Fig. 5.27) reveal the two antennal nerve tracts and the sensory cell clusters which are segmentally organized into meristal annuli. Although sensory somata at 70% of embryogenesis lack α-tubulin, α-tubulin expression extends into the somata by 90% of embryogenesis (Fig. 5.25k) and remains in the sensory cell bodies in first instar nymphs (Fig. 5.26b-d).

When we compared the immunolabeling of the antennal nervous system after sonication (Fig. 5.25, 5.27) with that seen in antennae of equal age but after sectioning (Fig. 5.24), we found that the general organization of sensory cell clusters and axon pathways is preserved. Sonication does not therefore disrupt cellular integrity. Further, the pattern of neuronal labeling is consistent with previously published data from the grasshopper antenna (Seidel and Bicker, 2000; Boyan and Williams, 2004; Boyan and Williams, 2007; Ehrhardt *et al*., 2015a), confirming that sonication also does not disrupt antigen distribution. This cellular and molecular integrity ensures that confocal microscopy can be employed to generate three-dimensional reconstructions of the peripheral nervous system directly in intact antennae obviating the need for serial reconstructions from tissue sections (Ehrhardt *et al*., 2015b).

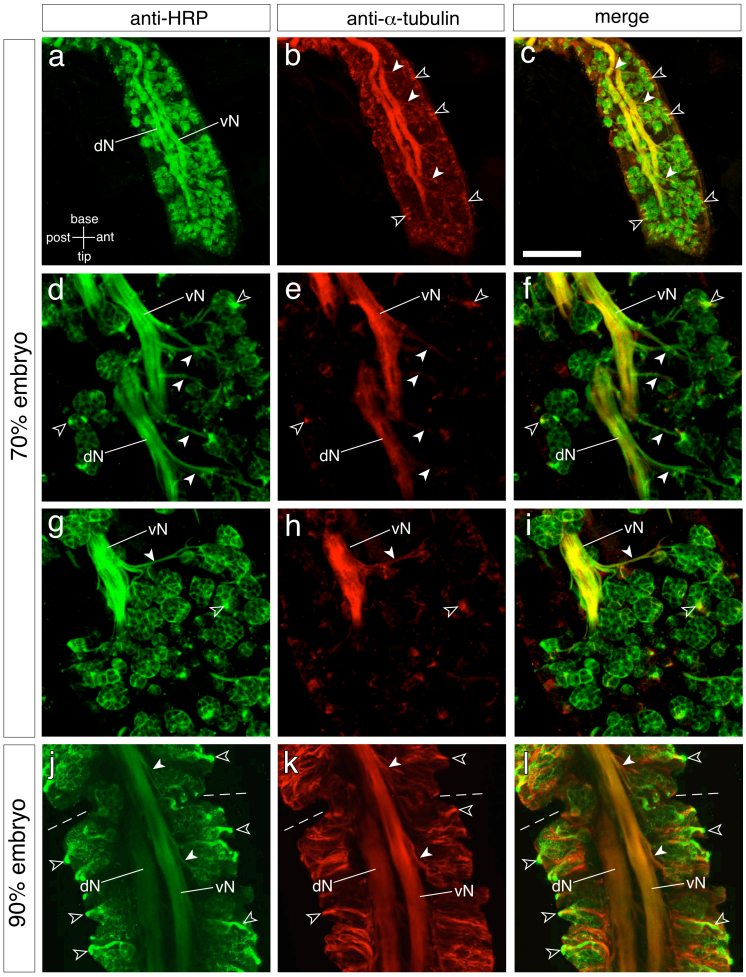



**Figure 5.25.** Confocal images of intact antennae at two embryonic ages (**a-i**, 70%; **j-l**, 90%) viewed in wholemount following sonication and double labeling with anti-HRP (green) and microtubule specific anti-α-tubulin (red). Merge images show co-expression (yellow). (**a**) HRP labeling of sensory cell clusters along with their axonal projections into the dorsal (dN) and ventral (vN) antennal nerves. (**b**) α-Tubulin labeling is predominantly in the dendritic terminals of sensory cells (open white arrowheads) but not their somata. (**c**) Axon projections (white arrowheads), dendritic terminals of sensory cells (open white arrowheads), and the antennal nerves co-express these labels. At higher power (**d-i**), HRP labeling reveals the axonal processes (**d**, white arrowheads) and somata (**g**) of sensory cells, as well was axons in the ventral antennal nerve (vN). α-Tubulin (**e, h**) labels sensory cell axons (white arrowheads) and their dendritic terminals (open white arrowheads) as well as the antennal nerves (vN, dN). Merged images (**f, i**) confirm co-expression (yellow) in sensory cell axons (white arrowheads) and dendritic terminals (open white arrowheads). At 90% of embryogenesis, HRP labeling (**j**, green) is seen in the antennal nerves (dN, vN), in axons of sensory cells (white arrowheads), and in dendrites (open white arrowheads) of sensory cell somata. Dashed line indicates meristal border. (**k**) α-Tubulin labeling (red) is seen in the antennal nerves, axons of sensory cells (white arrowheads), and dendritic terminals (open white arrowheads). (**l**) Double labeling is seen in the antennal nerves (dN, vN), axons of sensory cells (white arrowheads) and dendrites (open white arrowheads) of sensory cells. Scale bar in **c** represents 150 µm in **a-c**, 45 µm in **d-i**, 55 µm in **j-l**. Taken from Ehrhardt *et al* (2015b).

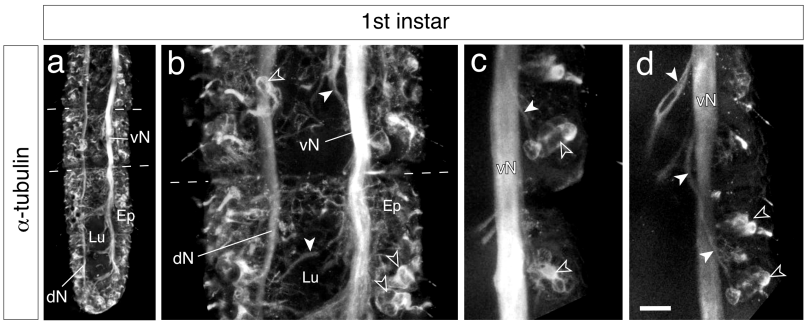



**Figure 5.26.** Confocal images of intact antennae from first instar nymphs viewed in wholemount following sonication and immunolabeling against anti-α-tubulin. At low magnification (**a**), labeling against α-tubulin reveals the two antennal pathways (vN, dN) in the lumen (Lu), as well as the epithelial (Ep) sensory cell clusters clearly organized according to segmental pattern. Dashed white lines indicate borders of meristal annuli. At higher magnification (**b-d**), α-uubulin labeling is present in differentiated sensory cell clusters (open white arrowheads) into an antennal nerve (dN, vN). Scale bar in **d** represents 90 µm in **a**, 30 µm in **b**, 20 µm in **c,d**. Taken from Ehrhardt *et al* (2015b).

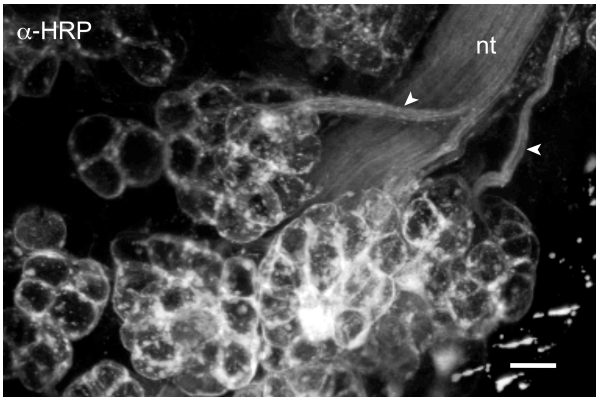



**Figure 5.27.** Confocal image of intact antenna from a first instar nymph viewed in wholemount following sonication and immunolabeling against anti-HRP. The axons (white arrowheads) of sensory cell clusters project into one of the antennal nerve tracts (nt). Scale bar represents 10 µm.

# 6. Discussion

## 6.1. Ablation of the pioneer neurons

In order to investigate the role of pioneer neurons in the establishment of neural pathways, experiments have been performed in which pioneer neurons are ablated, and the development of the system observed and compared to a control. However, these experiments have produced mixed results with regard to the importance of pioneer neurons.

### 6.1.1. Ablation in vertebrates

Pioneer ablation has been performed in vertebrates. When the pioneers which establish the nerve from the olfactory placode to the olfactory bulb in the zebrafish are ablated by being cut out using glass microneedles, the sensory neuron axons are severely misrouted (Whitlock and Westerfield, 1998). On the other hand, Eisen *et al*. (1989) reported that when the motoneurons which pioneer the zebrafish peripheral motor pathways are ablated using a laser, motoneurons which develop later are still capable of navigating the correct pathways. When the pioneer neurons which form the axonal scaffold of the forebrain commissure in the zebrafish are ablated, later axons take on the role of the pioneers and alter the kinetics of their growth cones to match those of pioneers (Bak and Fraser, 2003).

Some pioneer neurons may be necessary for nerve tract formation while others are not, even within the same animal. Pike *et al*. (1992) ablated pioneer neurons in zebrafish with a laser and observed the effect, or lack thereof, on the development of the peripheral motor nerves. When the pioneers of the ventral nerves were ablated, the nerves formed more slowly than usual, but normal nerves were still eventually established. Thus, the presence of the pioneer sped up the formation of the ventral nerve, but was not required for it. However, a pioneer was absolutely necessary for the navigation of the dorsal nerve in the zebrafish. When the dorsal nerve pioneer was ablated, the follower axons consistently grew in an incorrect direction. The atypical path of the nerve in the absence of the pioneer was always similar, revealing that the follower neurons were led astray by some other consistent guidance cue if the pioneer neuron was not available to keep them on track (Pike *et al*., 1992). The development of the zebrafish dorsal nerves appears to more sensitive to perturbation in general than that of ventral nerves. Melançon *et al*. (1997) found that the ventral nerves form completely normally in the absence of the muscle pioneers which act as targets of the pioneer motoneurons, but the dorsal nerve primary motoneuron axons display abnormal branching and grow away from their normal pathway when their muscle pioneer targets are ablated. The major differences between the effects of ablation of the pioneers or of their targets in two different nerve tracts in the same organism using the same method warn that we should not generalize about the role of pioneer neurons as a class. Each pioneer neuron or network of pioneer neurons must be tested and evaluated separately.

### 6.1.2. Ablation in invertebrates

Pioneer neurons in insects have been ablated in attempts to determine the roles which these cells play in development and in the guidance of follower axons.

#### 6.1.2.1 Ablation *via* mercury arc lamp radiation

Keshishian and Bentley (1983b) ablated the Ti1 pioneer neurons in grasshopper legs by filling the neurons with dye and then irradiating them with a mercury-arc lamp. Following this treatment, the nerve pathways in the legs still developed normally. Keshishian and Bentley (1983b) concluded that the nerve tracts of the grasshopper peripheral nervous system can be established by follower neurons alone, that the pioneer neurons do not possess any unique ability to navigate and that their function can be taken over by other cells. However, a possible flaw in this method of ablation is that, although the cells are killed, the remnants of the pioneer axons may still guide follower axons via molecular cues or an adhesive surface which remains intact on the axonal surface of the pioneers despite the cells themselves being dead and non-functional. Therefore in this thesis we instead used molecular methods to interfere with the growth of pioneer axons.

#### 6.1.2.2 Laser ablation

The timing of pioneer ablation may determine its effects. When pioneer neurons of the cricket cerci are ablated before they can initiate axogenesis, the cercal nerve tracts fail to develop and multiple nerve bundles form in their place. If the pioneers are killed later in development, after their axons have navigated the nerve tracts and become associated with glial cells, the cercal nerves continue to develop normally (Edwards *et al*., 1981). This suggests that the presence of pioneer axons is necessary for the development of these nerve tracts due to the essential structural role of the pioneers as a guide for other neurons and glia, but that the pioneers need not remain alive to play any functional role in later development. This further supports our conclusion that killing post-axogenesis pioneer neurons is not sufficient to determine their role in development, due to the structural guidance which even dead pioneer axons may provide, but that other methods are necessary for investigating this system.

Experiments suggest that pioneer neurons are essential for the normal development of pathways in the grasshopper central nervous system. The intersegmental nerve in the locust central nervous system is established by U neurons and aCC neurons. Du Lac *et al*. (1986) ablated the U neurons which help pioneer the intersegmental nerve in the central nervous system with a laser microbeam in cultured grasshopper embryos. When the grasshopper U neurons were ablated, the aCC axon stalled and no fasciculation was observed (du Lac *et al*., 1986).

#### 6.1.2.3 Heat shock ablation

When heat shock is used to prevent the differentiation of pioneer neurons in the grasshopper leg, the sensory neurons of the leg fail to grow to their targets in the central nervous system (Klose and Bentley, 1989). Thus in the appendages of both the grasshopper and the cricket, it appears that living pioneers are essential early in development when their axons have not yet completed their pathway to the brain, but that the cells can be killed later in development without disrupting the nerve tracts. This suggests that after navigating to their targets, pioneer neurons in grasshopper appendages play a structural but not functional role in the establishment of the nerve tracts.

#### 6.1.2.4 Sharp electrode ablation

Raper *et al*. (1984) ablated pioneer neurons in the grasshopper central nervous system by using a sharp electrode to puncture the somata and sever the axons. In normal development, the G growth cone fasciculates with the A/P fascicle. When the pioneer neurons are ablated, the G growth cone could no longer navigate properly (Raper *et al*., 1984). Pioneers are required to establish the MP1/dMP1 pathway, the earliest longitudinal nerve tract to develop, in the grasshopper. When the pioneers are ablated, the pCC follower axon either stalls, or else fasciculates with another incorrect axon, which leads it astray (Bastiani *et al*., 1986). Since these experiments suggest that in the grasshopper central nervous system, specific pioneer neurons are required to guide follower axons in order for nerves to develop normally, we would expect that the pioneers in the peripheral nervous system of this animal play a similarly important role in the development of the nerve tracts in the appendages.

#### 6.1.2.5 Genetic ablation

The GAL4/UAS system allows a protein to be expressed in a subset of cells (Brand and Perrimon, 1993). The driver GAL4 gene encodes a yeast transcription activator protein, while the response element UAS (Upsteam Activation Sequence) is an enhancer which activates gene transcription when bound by GAL4. When the protein expressed in a restricted subset of cells using this system is a toxin which disrupts protein synthesis such as ricin from castor beans or diptheria toxin A from bacteria, the GAL4/UAS system can be used to ablate specific cell types, including early in development. Flp recombinase can be used to prevent the expression of the protein throughout the entire set of cells, but to allow it to be express stochastically (see Simpson, 2009; Venken *et al*., 2011). These genetic tools are a promising method for future work investigating the details of the roles of cells such as pioneers, guidepost cells, and glia in the developing insect nervous system.

Lin *et al.* (1995) ablated random neurons in the embryonic fly central nervous system by injecting flp sense RNA to trigger the expression of diptheria toxin via the GAL4/UAS in some neurons. After the aCC pioneers were ablated by this method, the follower U neurons were still able to establish the pathway of the intersegmental nerve, although the development of the nerve tract was delayed in the absence of pioneers. Lin *et al.* (1995) concluded that pioneer neurons are not necessary for nerve tract formation in the fly central nervous system, in contrast to the grasshopper results. However this finding may have been questionable, due to their use of the random expression of a toxin to ablate neurons. If any nearby pioneer neurons were still alive, the pioneer neurons of a neighboring segment could replace the ablated pioneer neuron (Bastiani *et al*., 1986).

Sánchez-Soriano and Prokop (2005) found similar results to Lin *et al.* (1995) by using *RN2-Gal4*-induced expression of ricin to ablate aCC neurons. The ablation of aCC neurons had a more dramatic effect on the development of the intersegmental nerve than U neuron ablation. Ablating the aCC neurons affected the growth of the entire intersegmental nerve in 26% of the embryos. Ablation-caused defects mainly took the form of early stalling of the nerve. The U neurons were usually able to reach their targets even following the ablation of the aCC neurons. Ablating the U neurons instead of the aCC neurons had even less of an effect; the growth of the intersegmental nerve was affected by the ablation of U neurons in only 2.9% of embryos. Sánchez-Soriano and Prokop (2005) found, however, that the results of ablation do not tell the whole story of the importance of pioneer neurons. When pioneer neurons are ablated, other, normally less important pathfinding cues may become critical for the guidance of follower neurons and compensate for the ablation of the pioneer neurons. When the ionotropic GABA receptor Rdl is expressed in neurons, it causes their growth cones to stall. Sánchez-Soriano and Prokop (2005) expressed this receptor in aCC neurons, causing their axons to stall before reaching their targets. When these axons stalled, the growth of the intersegmental nerve was also arrested in 70% of embryos. Thus, follower neurons may navigate using other cues in the absence of pioneers, but when pioneer axons are present, follower axons rely on them to travel to their targets (Sánchez-Soriano and Prokop, 2005).

Pioneers of the fly ventral nerve cord were also ablated by Hidalgo and Brand (1997) using the GAL4/UAS system to express ricin in longitudinal tract pioneer neurons. When all pioneers were ablated, the follower neurons could not establish the longitudinal pathways, but stalled or went wildly astray, even crossing to the wrong side of the midline, and the nerve tracts were never able to correct the defects that resulted from the loss of the pioneers (Fig. 6.1). In the absence of pioneers, nerve tract development was not merely delayed, but halted or greatly perturbed. However, when only a single pioneer neuron was ablated, the axon tract formed fairly normally. The ablation of a pair of pioneer neurons had a noticeably more dramatic impact on the development of the nerve tract, and ablating three pioneer neurons further increased the frequency of growth cones stalling or growing abnormally. These results suggest that pathways are established by networks of pioneers working together. Each fascicle in the fly can only be established through the interaction of two or more pioneer neurons functioning interdependently. No individual pioneer seems to be necessary for the establishment of the fly nerve tract, but systems of pioneers work together to build the nerves and guide follower neurons (Hidalgo and Brand, 1997).

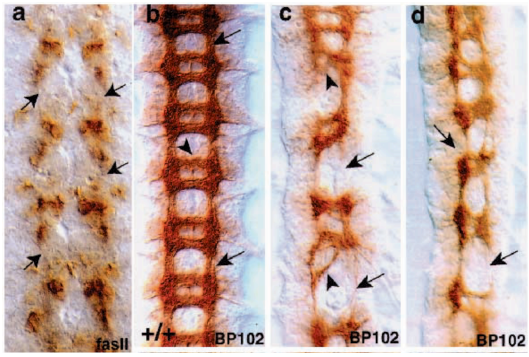



**Figure 6.1.** Effects of ablation of all pioneers in the *Drosophila* embryo. (**a**) At stage 14, the fly neurons have not yet formed the nerve pathways. (**b**) In a control animal, no neurons are ablated and the logitudinal bundles (arrows) and commissures (arrowhead) form normally. (**c,d**) Following ablation of pCC, MP1, dMP2 and vMP2, the longitudinal bundles are missing or unusually thin (arrows), while the commissures are broken or disorganized (arrowheads). Taken from Hidalgo and Brand (1997).

Nevertheless, I wish to point out that, despite the genetic advantages of *Drosophila*, the grasshopper antennal nervous system offers several benefits as a model system: large, identifiable pioneer cells and access at all developmental stages via immunohistochemistry.

## 6.2. Axon guidance in the insect nervous system

Bate (1976) noted that the grasshopper leg nerves do not develop in a straight line directly to the central nervous system, but turn at consistent locations. Therefore, the pioneer neurons must respond to some signal which tells them when or where to turn. A number of different mechanisms have been proposed for pioneer navigation in the insect peripheral nervous system. Pioneer axons in insects may be guided by signals from other cells, by molecular cues, or by contact with the basement membrane along which they grow. Through our immunoblocking experiments, we have found evidence that the base pioneers provide a signal for the apical pioneers, via the cell surface glycoprotein Lazarillo.

Berlot and Goodman (1984) suggested that the pioneer axons of the antenna are guided by an adhesive hierarchy of epithelial and neuronal surfaces. Their model was that the initial direction of pioneer growth cones is determined by the polarized epithelium, that the axons are subsequently guided towards the base by an adhesive gradient along the epithelium, that landmark neurons then lead the growth cones away from this axial polarity, and finally that once in the central nervous system, pioneer growth cones follow labeled axonal pathways to establish their stereotypical routes (Berlot and Goodman, 1984).

Pioneer growth cones in grasshopper appendages extend filopodia further in the direction they will steer towards than in incorrect directions. Isbister and O'Connor (1999) tested whether these observed differences in filopodial extension were due to differential substrate adherence; however they could not find evidence that a gradient of adhesiveness guided grasshopper limb pioneer axons as their growth cones steered towards the central nervous system. Isbister and O'Connor (1999) used cytochalasin D to disrupt F-actin, which resulted in pioneer filopodia being pulled by inner tension into the growth cone from which they were extended. Any adhesive contacts which the filopodia had made countered this tension, so by comparing the rates at which different filopodia withdrew, Isbister and O'Connor (1999) could compare the substrate adhesion experienced by different filopodia. However, filopodial-substrate adhesion appeared to be uniform across the entire pioneer growth cone, regardless of whether the filopodia were extending towards the pioneer's target or in an incorrect direction (Isbister and O'Connor, 1999).

### 6.2.1. Cellular navigation mechanisms

Bate (1976) proposed that sheathing cells found at intervals along developing grasshopper appendages could act as stepping stones that allow pioneer growth cones to determine their location. Bentley and Keshishian (1982) suggested that the pioneer neurons in the grasshopper leg might navigate using guidepost cells, i.e. neurons which are found along the route of the axon, which act as cues for the pioneer growth cone. In the grasshopper leg, these cells are found at every turning point of the leg nerves. When pioneer growth cones encounter these guidepost cells, the pioneers and the guideposts become coupled, probably via gap junctions, such that dye injected into one cell can fill the other neuron as well (Bentley and Keshishian, 1982). Pioneers in the antenna are dye coupled (Boyan and Williams, 2007)

Part of the mechanism through which guidepost cells influence the growth of pioneer axons may be an effect on calcium concentration. Bentley *et al*. (1991) found that the calcium concentration in pioneer axons of the grasshopper leg is altered when the axons make contact with guidepost cells. Grasshopper leg pioneer neurons have a high calcium concentration, over 100 nM, while undergoing axogenesis, with the highest calcium concentration found in the growth cone of the cell. Guidepost cells tend to have low calcium concentrations of 65 nM when contacted by pioneer axons. When the pioneers form a connection with a guidepost cell, calcium flows out of the pioneer axon into the guidepost cell (Bentley *et al*., 1991).

It is not known how similar the base pioneers are to the guidepost cells of the leg. Further experiments could compare these cells. Dye injection experiments could test when and whether the base

pioneers are dye-coupled to the A1 pioneers. Calcium indicators could reveal whether the calcium concentration in A1 pioneer axons change when they make contact with base pioneers.

Pioneer axons can be guided not only by neurons or neuronal precursors, but also by glia. For example, as the growth cones that pioneer the grasshopper intersegmental nerve exit the central nervous system, they follow a pre-existing path of glia. The pioneer growth cones are drawn to and make contact with the segment boundary cell, which signals to the pioneers where the intersegmental nerve root should be located (Bastiani and Goodman, 1986). Glia play similar roles in other insects (Oland and Tolbert, 2003). In the fly central nervous system, glia are responsible for axonal guidance and regulate fasciculation or defasciculation of pioneer axons (Hidalgo and Booth, 2000). In vertebrate embryos as well, neurites can be guided to their targets by pre-laid glial pathways (Singer *et al*., 1979). During early embryogenesis, when the A1 pioneer axons are navigating to the base of the antenna, only a few glia are present in the antenna, at the base (Boyan and Williams, 2004). It is unclear whether these glia play any role in guiding axogenesis. Muscle pioneers seem to play a role in the guidance of cockroach leg pioneer axons (Rajan and Denburg, 1997), but have not been studied in axonal navigation of the antennal pioneers.

Environmental guidance cues may affect the behavior of pioneer growth cones by altering the organization of the growth cone's cytoskeleton. The growth cones of grasshopper leg pioneers contain a rich microtubule network which often forms transient, complex microtubule loops. When the pioneer growth cone is in the process of turning towards a guidepost cell, microtubules selectively invade branches that derive from filopodia which have contacted the guidepost cell. When the pioneer growth cone extends across a segmental border, microtubules selectively invade branches orientated in the correct direction. Selective invasion generates an asymmetrical microtubule organization pattern within the growth cone, which may influence the steering of the growth cone (Sabry *et al*., 1991). In grasshopper limbs, actin accumulation is associated with continued growth of filopodia, while withdrawal of filopodia is accompanied by reduction in actin. When pioneer growth cones turn towards a target, actin selectively accumulates in the proximal areas of filopodia which have contacted target cells (O'Connor and Bentley, 1993).

### 6.2.2. Molecular navigation mechanisms

Lazarillo is a lipocalin, a member of a large family of proteins which transport small hydrophobic molecules. Lazarillo's glycosyl-phosphatidylinositol (GPI) tail keeps the molecule attached to the plasma membranes of the cells that express the glycoprotein. In the grasshopper embryo, sensory neurons of the peripheral nervous system express Lazarillo. The glycoprotein is also found in subsets of neuroblasts, ganglion mother cells, and neurons of the central nervous system and enteric nervous system. Additionally, a few non-neuronal cells, mainly associated with the excretory system, express Lazarillo. When an antibody against Lazarillo is applied to embryos growing in culture, the growth of commissural pioneer neurons is disrupted; growth cones stall or extend in abnormal directions (Sánchez *et al*., 1995). Sánchez *et al*. (1995) proposed that Lazarillo enables axonal guidance by acting as a receptor which detects a signaling molecule that is involved in the outgrowth and navigation of the axons. The A1 pioneers of the grasshopper antenna also express Lazarillo (Boyan and Williams, 2004), suggesting that this glycoprotein may act as an axon guidance molecule not only in the central nervous system but also in the peripheral nervous system. Our immunoblocking experiments confirmed that Lazarillo is necessary for the navigation of the A1 pioneers in the antenna, showing that Lazarillo functions as a common axon guidance mechanism in both the central and peripheral nervous systems of the grasshopper.

Pioneer axogenesis is influenced by nitric oxide (NO), a gas which acts as a signaling molecule in the nervous system. NO activates soluble guanylyl cyclase (GC) in neurons. When exposed to NO, pioneer neurons in the grasshopper antenna produce cyclic GMP. cGMP expression causes the dorsal and ventral A1 pioneer axons in the antenna to grow during early embryogenesis. If nitric oxide synthase or GC are blocked, the navigation of the pioneer growth cones is perturbed (Fig. 6.2). Nitric oxide synthase activity is found in the basal lamina. The NO/cGMP system may be involved in stimulating the growth of pioneer axons along the basal lamina of the antenna; the epithelial cells may be producing NO as a growth signal which affects pioneer axons (Seidel and Bicker, 2000).

Other molecules have been shown to be important for axon navigation in insect nervous systems, but have not been tested in the developing antennae.

Semaphorin I is a transmembrane glycoprotein expressed by bands of epithelial cells in the embryonic grasshopper leg. Semaphorin I acts as an attractive cue which guides growth cones and is required for axon outgrowth of the neurons of the subgenual organ, a hearing organ located in the tibia (Wong *et al*., 1997).

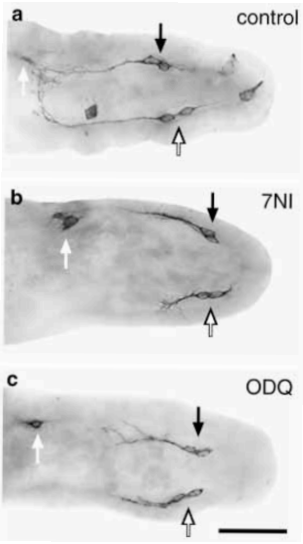



**Figure 6.2.** Effects of pharmacological agents on the growth of the HRP-labeled pioneer axons of the antenna. (**a**) Normal axon outgrowth in ventral (black arrow) and dorsal (open arrow) nerve tracts of an embryo cultured under control conditions. (**b**) Ventral (black arrow) and dorsal (open arrow) pioneer neurons of an embryo exposed to 7-nitroindazole (7NI) just after initiating axogenesis. 7NI selectively inhibits nitric oxide synthase. The axon elongation of these neurons was abnormally slow and the axons failed to reach base pioneers. (**c**) Pioneer neurons (black and open arrow) of an embryo exposed to oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). ODQ blocks the activity of soluble guanylyl cyclase. The axon elongation of these neurons was abnormally slow and the axons failed to reach base pioneers. Scale bar represents 50 μm in all panels. Taken from Seidel and Bicker (2000). Adapted with permission of Company of Biologists (dev.biologists.org/content/127/21/4541.short).

Fasciclins are membrane-associated glycoproteins expressed in a subset of neurons in the developing insect nervous system. Neural cell adhesion molecule Fasciclin I is composed of four homologous domains of approximately 150 amino acids each and contains a signal sequence but lacks a transmembrane domain (Zinn *et al*., 1988). Fasciclin I is expressed in some commissural axons in the embryonic central nervous system, in all sensory axons of the peripheral nervous system, and in the Ti1 pioneers of the embryonic grasshopper leg (Bastiani *et al*., 1987; Zinn *et al*., 1988). Fasciclin I is required for the fasciculation of the two Ti1 pioneer axons in the grasshopper leg. When Fasciclin I is denatured with a laser-excited dye-labeled antibody, grasshopper leg pioneer axons fail to adhere to each other, although the cells do not appear to be damaged by the laser. The same method for inactivating Fasciclin I does not cause any changes in the growth or guidance of the pioneers (Jay and Keshishian, 1990; Diamond *et al*., 1993).

In the grasshopper leg, Fasciclin II is required for the initiation of Ti1 axogenesis. When fasciclin II is inactivated with a laser early in embryogenesis, the Ti1 neurons fail to exhibit any axonal outgrowth (Diamond *et al*., 1993). In both grasshoppers and flies, Fasciclin II is expressed on the cell membrane of the neurons of the intersegmental nerve. In Fasciclin II mutant flies, the MP1 fascicle fails to develop because the MP1, dMP2, and vMP2 growth cones do not recognize each other; instead, their growth cones stall (Grenningloh *et al*., 1991). Altering the expression of Fasciclin II in *Drosophila* changes the likelihood of the intersegmental nerve being arrested when the pioneer growth cones are stalled via the targeted expression of an ionotropic GABA receptor. When Fasciclin II is over-expressed, the intersegmental nerve is arrested in 97% of fly embryos when the aCC growth cones stall. In mutant embryos that lack Fasciclin II, the intersegmental nerve is only arrested in 36% of embryos when the aCC growth cones stall. Since the expression of Fasciclin II determines whether the intersegmental nerve can still form when the pioneer axons are stalled, it appears that fly pioneer neurons influence follower axons via Fasciclin II (Sánchez-Soriano and Prokop, 2005). Fasciclin III is expressed in a subset of axons in *Drosophila* during development (Patel *et al*., 1987; Snow *et al*., 1989). In flies, Fasciclin III mediates cell adhesion in some neurons and plays a role in growth cone guidance (Snow *et al*., 1989).

Fasciclin IV is found in the grasshopper central nervous system and shows a banded expression pattern in the epithelium of appendages. In the metathoracic leg, a band of Fasciclin IV expression corresponds to the location where the axons of the pioneer neurons must change direction. When an antibody against Fasciclin IV is applied to embryos growing in culture, leg pioneer axons grow in aberrant directions (Kolodkin *et al*., 1992).

Some molecular gradients may guide pioneer axon navigation in insect appendages. In the cockroach leg, antibodies have been produced that bind to molecules within the basal lamina or extracellular matrix during the stage of development at which the pioneer axons grow towards their targets. Two of these antibodies, PROD-1 and PROD-2, strongly label the base of the leg, while another antibody, DIP-1, strongly labels the tip. The proteins bound by these antibodies could act as environmental guidance cues for the growth of pioneer axons (Norbeck *et al*., 1992). Cockroach leg pioneer axons also appear to be guided by heparan sulfate proteoglycans and GPI-linked proteins (Rajan and Denburg, 1997).

### 6.2.3. The basement membrane

As the leg pioneers grow, they remain closely apposed to the basal lamina (Fig. 6.3). The filopodia of the pioneers make some contacts with ectodermal cells and few contacts with mesodermal cells (Anderson and Tucker, 1988). In the embryonic leg, the continuity and thickness of the basal lamina varies greatly from one region to another. The tip and nearby regions may lack basal lamina altogether or only contain small laminar segments. More proximal regions have a continuous basal lamina. The embryonic basal lamina is thickest closest to the base of the leg, where it may be as thick as 150 nm, and gradually thins out to under 40 nm as it approaches the tip. Unlike larval appendages, embryonic legs may have multiple layers of basal lamina. The dorsal ectoderm and the base of the leg are most likely to have multilayered basal lamina, while the ventral ectoderm is usually associated with a single layer of basal lamina (Anderson and Tucker, 1989).

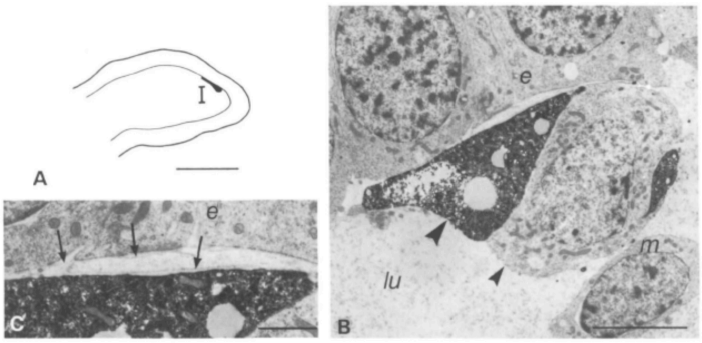



**Figure 6.3.** Pioneer neurons and basal lamina in the leg. (**a**) Drawing of a pioneer neuron in the leg. (**b**) The cell bodies (black arrowheads) of a pair of pioneer neurons. The pioneer on the left has been injected with HRP. A mesodermal cell (m) is also nearby in the lumen (lu). (**c**) Higher magnification of b shows the basal lamina (black arrows) between the pioneer somata and the ectoderm (e). Scale bars represent 0.1 mm in **a**, 5 µm in **b**, 1 µm in **c**. Taken from Anderson and Tucker (1988). Adapted with permission of Company of Biologists (dev.biologists.org/content/104/4/601.short).

The morphology of pioneer growth cones in the grasshopper leg changes as the axon grows from the tibia region towards the base of the appendage. Growth cones extend branches and lamellae circumferentially along segment boundaries and keep their filopodia and lamellae extended longer at these locations. Where the projections encounter a well-differentiated segment boundary, the growth cone immediately realigns itself circumferentially. In the proximal region of each limb segment, pioneer growth cones show a greater amount of branching and lamellae. This suggests that the affinity between pioneer growth cones and the substrate on which they grow increases in the proximal region of each segment, before peaking at the segment boundary. This increased epithelial affinity could act as a guidance cue for pioneer growth cones (Caudy and Bentley, 1986).

Surgical manipulation of cultured grasshopper legs suggests that pioneer growth cones can navigate correctly to the base of the leg in the absence of all tissue outside the leg, contact with the limb contour, an axial electrical field, a diffusion gradient from a localized source, mesodermal tissue or guidepost cells. The leg pioneers appear to be able to navigate to the base solely based on the epithelium including the basal lamina, and the pioneer neurons' own internal polarity (Lefcort and Bentley, 1987).

The role of the basal lamina in axogenesis of grasshopper leg pioneers may depend on the stage of development. Up to 31%, Ti1 pioneer axons can grow normally, recognize limb segmental borders, and selectively interact with other neurons, even when the basal lamina has been removed. However, at 32% of embryogenesis, removal of the basal lamina often results in displacement of the Ti1 cell bodies to ectopic locations and in aberrant pathfinding (Condic and Bentley, 1989a). Removing the basal lamina after the

initiation of axogenesis but before growth cones have contacted other neurons or segment boundaries results in the pioneer axons retracting to the somata. When microfilaments are depolymerized by cytochalasin D, the axons do not retract even when the basal lamina is removed, suggesting that the retraction of axons in the absence of the basal lamina is caused by microfilament-based cytoskeletal components. These findings are evidence that pioneer axons in the leg are under tension, which they resist due to adhesive interactions between the growth cones and the basal lamina (Condic and Bentley, 1989b). Removing the basal lamina after pioneer growth cones have contacted other neurons does not cause growth cone retraction. Instead, the pioneer axons remain in place and their somata are pulled proximally when the basal lamina is destroyed. A similar phenomenon occurs when the pioneer axons contact segment boundaries. Axons which are closely apposed to two segment borders remain in place even the basal lamina is digested by enzymes. Instead their somata reposition proximally. Thus when pioneer axons contact other neurons or segment boundaries, they appear to establish adhesive interactions with other cells which are independent of the basal lamina (Condic and Bentley, 1989c).

## 6.3. Origin of neurons in insect appendages

The origin of the apical pioneers in the epidermal epithelium was observed by Ho and Goodman (1982). However, they did not present any images of the birth of these cells. We used immunolabeling against HRP, Lazarillo and Lachesin to investigate the origin of the apical pioneers (Fig. 5.1). Our results confirm Ho and Goodman's (1982) report that these cells originate in the epithelium. We have also shown that the apical pioneers lack Mes3 expression, which provides further evidence that they are derived from the ectoderm and not the mesoderm (Fig. 5.4). The ontogeny of the base pioneers had not been previously reported. We showed that these cells express Mes3, suggesting that they originate from the mesodermal tissue in the lumen (Fig. 5.4).

Many aspects of the origin of the antennal pioneers remain unclear. It is not known what progenitor or mother cell gives rise to the antennal pioneers, nor whether they are produced by symmetrical or asymmetrical cell division. Keshishian (1980) reported that the Ti1 pioneers of the leg are produced in the epithelium by a large mother cell in the ectodermal epithelium which divides once, resulting in a pair of sibling cells. Given the serially homologous nature of the appendages, the apical pioneers of the antenna could have a similar origin. Although Keshishian (1980) did not use immunolabeling to unambiguously identify the mother cell or the pioneers, this paper represents some of the best published evidence for the origins of pioneers of a grasshopper appendage.

Unlike the sensory cells, whose somata remain within the epithelium as their axons grow into nerves, pioneers delaminate into the lumen. Keshishian (1980) observed the delamination of the leg pioneers. In this thesis, I have shown that the apical pioneers of the antenna delaminate in a similar manner (Fig. 5.1e).

Although little is known about the origin of the pioneer neurons in the insect antenna, the origin of the sensory neurons of the antenna has been investigated in several species. In holometabolous insects such as the moth, the antenna develops from an imaginal disc of epithelium during the pupal phase. After apolysis, the disc differentiates into sensillogenic and nonsensillogenic regions (Keil and Steiner, 1990). Within the sensillogenic regions, the proneural clusters are specified by the activity of proneural genes such as those of the *achaete-scute* complex. In each proneural cluster, one sense organ precursor (SOP) gains its identity from the interaction of neurogenic genes such as *notch* and *delta*. The SOP inhibits the ability of surrounding cells to become SOPs so that they instead develop as epidermal cells. Neural type selector genes such as *cut* or *rhomboid* determine the identity of the sensilla which will develop from the SOP (Keil, 1997).

Within two days after apolysis, sensillar precursors divide, producing the Anlagen of the sensilla (Fig. 6.4; Keil, 1992). In each sensilla trichodea Anlage of the moth, six or seven cells are arranged side by side. This cell cluster includes two or three prospective sensory neurons and a thecogen cell located basally, whose apical processes form one fascicle. A trichogen and two tormogen cells are found apically in each cluster. One of the glia-like tormogen cells in each cluster degenerates during development. Extensive cell death also occurs in the basal region of the moth antennal epithelium (Keil and Steiner, 1990). At

approximately the same time that the sensillar Anlagen are forming, the axons of the sensilla begin to grow towards their targets (Keil, 1992).

In *Drosophila*, sensory neurons of the antenna, whether olfactory or mechanosensory, are derived from SOPs which appear starting in the late larval stage (Jarman, 2014). The *Drosophila* proneural gene *atonal* specifies sensilla coeloconica (Gupta and Rodrigues, 1997). All *Drosophila* chordotonal organs also require *atonal* to specify the scolopidial SOPs (Jarman *et al*., 1995). There is evidence that the proneural genes for all olfactory sensilla in the *Drosophila* antenna are basic helix-loop-helix transcription factors (Gupta and Rodrigues, 1997).

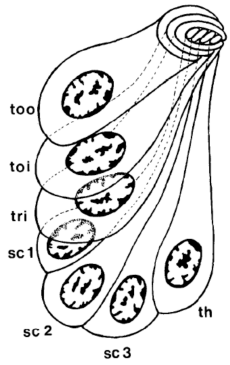



**Figure 6.4.** Anlage of a sensillum in the moth antenna. Taken from Keil and Steiner (1990).

In the grasshopper, the origins of sensory neurons of the antenna have not yet been investigated in detail. It is also not know whether the pioneer neurons of the grasshopper share a similar origin with the sensory neurons, as the precursor of the pioneers has not been identified. It has not been shown which genes determine the identity of the pioneers of the grasshopper antenna.

## 6.4. Sensilla of the antenna

The variety of sensilla on the antenna enable the grasshopper to detect and respond to different stimuli. Sensilla trichodea respond to a sex pheromone and to odorants from locust feces (Ochieng and Hansson, 1999; Cui *et al.*, 2011). Neurons of sensilla basiconica are excited by aggregation pheromones from both locust nymphs and adults, and by a plant odorant (Hansson *et al*., 1996). Sensilla coeloconica respond to organic acids, plant odorants and the odors of locust nymphs, but these sensilla are inhibited by aggregation pheromones (Ochieng and Hansson, 1999; Hansson *et al*., 1996).

### 6.4.1. Types of sensilla

The types of sensilla of the grasshopper antenna, sensilla basiconica, sensilla trichodea, sensilla coeloconica and sensilla chaetica, have distribution patterns that vary over the length of the antenna. The highest density of sensilla chaetica is at the terminal segment of the flagellum (Ochieng *et al*., 1998). Sensilla basiconica and coeloconica are spread out over the flagellum, but have the highest densities in the middle segments. Sensilla trichodea are concentrated in the fifth, tenth and fourteenth segments of the flagellum (Ochieng *et al*., 1998). The time course over which this pattern of sensillar distribution develops in the grasshopper is not known. It is unclear which genes regulate the development of the sensilla in the embryonic grasshopper antenna. During early embryogenesis, the sensory neurons first appear in bands within the meristal annuli. However, the development of the sensory nervous system during late embryogenesis has not been investigated in detail and the sensilla types of the sensory clusters in early embryogenesis have not been identified, so it is unknown how the distribution patterns of the sensilla after hatching correspond to the segmental organization of sensory clusters observed during early embryogenesis.

Different kinds of sensilla can be identified by their morphology (Fig. 6.5). Sensilla chaetica contain five sensory neurons. The dendrite of one neuron terminates at the base of the hair. The dendrites of the other

four sensory neurons pass the sensillar wall through the terminal pore (Ochieng *et al*., 1998). Sensilla basiconica can have up to 50 OSNs, while sensilla trichodea only house up to three OSNs and sensilla coeloconica up to four (Ochieng *et al*., 1998). Sensilla trichodea and basiconica have a single wall, while sensilla coeloconica are double-walled (Jin *et al*., 2005). In sensilla basiconica, the dendrites of the sensory neurons form large numbers of branches and the thin sensillar wall is full of large pores (Ochieng *et al*., 1998; Jin *et al*., 2005). The thick cuticular walls of sensilla trichodea have few pores; each sensory neuron has few if any branches, and the dendrites mainly remain within the sensillar lumen rather than crossing the sensillar wall through pores (Ochieng *et al*., 1998; Jin *et al*., 2005). Sensilla coeloconica are found within round cuticular pits in the surface of the antenna (Ochieng *et al*., 1998).

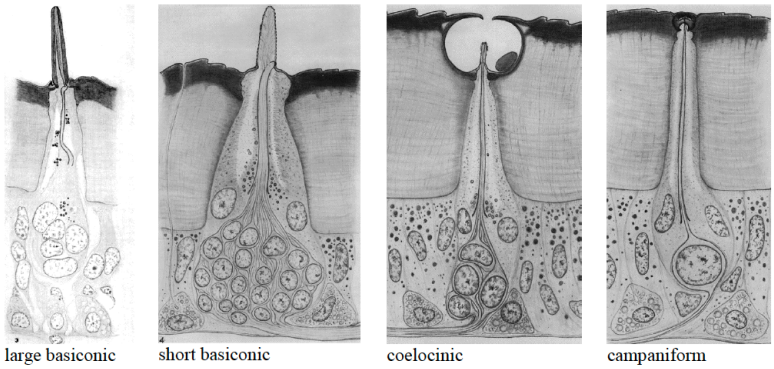



**Figure 6.5.** Schematic representation of cross sections of sensilla in the grasshopper antenna. Taken from Slifer *et al*. (1957) and Slifer *et al*. (1959).

Given the differences in appearance of different types of sensilla, it should be possible to identify sensory clusters during development. Mapping sensory neurons during development could help investigate many unanswered questions about the embryonic development of the sensory nervous system of the antenna, such as what mechanisms generate the distribution patterns of types of sensilla during development, or whether some sensilla types appear earlier in development than others. Now that our sonication method enables us to immunolabel the intact nervous systems of wholemount antennae in late embryogenesis and first instar nymphs, we are now in a position to do this analysis. Confocal imaging of wholemount antennae following sonication and HRP-immunolabeling allows us to identify sensilla types such as sensilla coeloconica (Fig. 6.6a) and sensilla basiconica (Fig. 6.6b) during late embryogenesis based on their morphology. The same method also works on first instar nymphs (Fig. 5.27).

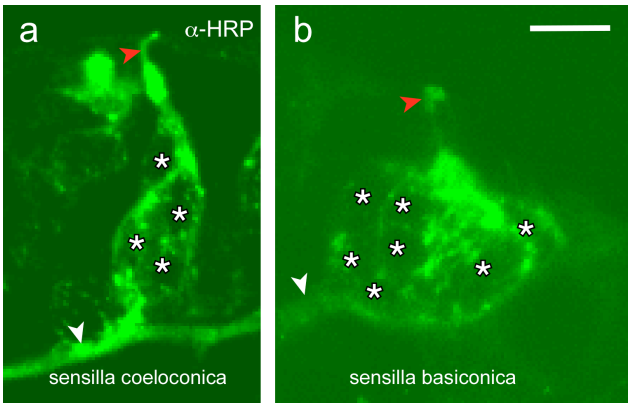



**Figure 6.6.** Confocal images of HRP-labeled sensilla in sonicated, wholemount embryonic antennae. Red arrowheads indicate apical dendrites of the sensory neurons. White arrowheads indicate the axons projecting from the sensory clusters onto the antennal nerve tracts. Stars indicate the somata of the sensory neurons. Scale bar indicates 20 µm in both panels.

### 6.4.2. Molecules expressed in insect sensilla

Insect antennae contain both olfactory and gustatory sensilla, which may express receptor proteins encoded by the same gene families (Scott *et al.*, 2001). In *Drosophila,* olfactory receptor (OR) proteins and gustatory

receptor (GR) proteins have seven transmembrane domains and often possess a similar 33 amino acid signature motif, suggesting that many gustatory and olfactory receptor gene families may have diverged from a common ancestral gene (Vosshall *et al.,* 1999; Scott *et al*., 2001). In *Drosophila*, each sensory neuron usually expresses only one of the genes which encode ORs (Vosshall *et al.,* 2000).

The number of ORs and GRs vary greatly between different insect species. Honeybees have over twice as many different ORs as fruit flies or mosquitos. In contrast to the diversification of ORs in bees, honeybees express only ten GRs, while fruit flies and mosquitos each have approximately 70 GRs (Robertson and Wanner, 2006).

ORs heteromerize with an OR-coreceptor (ORco) to produce a receptor complex. Odorants can activate this receptor complex by binding to it, which depolarizes the OSN via ionotropic or metabotropic mechanisms (Sato *et al*., 2008; Wicher *et al*., 2008). In the grasshopper, ORco is expressed in the OSNs of sensilla basiconica and sensilla trichodea, but not in the OSNs in sensilla coeloconica (Yang *et al*., 2012).

Insect antennae express ionotropic receptor (IR) proteins, another kind of olfactory receptor whose sequences and molecular structures are similar to ionotropic glutamate receptors (Benton *et al*., 2009; Rytz *et al.,* 2013). IRs enable insect OSNs to detect organic acids, amines and alcohols (Benton *et al.,* 2009; Ai *et al*., 2010). IR-subtypes IR8a and IR25a function as coreceptors, similar to the role of ORco proteins (Ai *et al*., 2013; Abuin *et al.,* 2011). In the grasshopper, IR8a and IR25a are expressed in the neurons of sensilla coeloconica but not in sensilla basiconica or sensilla trichodea (Guo *et al.,* 2014).

Odorants may be transported through the sensillum lymph to receptor proteins by small polypeptides classified as odorant-binding proteins (OBPs) or chemosensory proteins (CSPs; Vogt & Riddiford, 1981) based on conserved motifs including paired cysteine residues which are unique to OBPs or CSPs (Jin *et al*., 2005). The *S. gregaria* OBP is found in the sensillum lymph of sensilla tichodea and basiconica. Some CSPs have been identified in the outer sensillum lymph of sensilla chaetica as well as in the space between the epidermis and cuticle of the desert locust antenna. The *S. gregaria* OBP is expressed specifically in antennae, while CSPs show broader expression patterns which include sensilla chaetica on other parts of the insect (Jin *et al*., 2005).

Expression of CSPs in grasshoppers can vary with age, and expression can also differ between species. Jin *et al.* (2005) found that CSP III was expressed in adult *S. gregaria*, but not in nymphs of the same species, while related grasshopper species *Locusta migratoria* expressed a homologous polypeptide in nymphs but not in adults. In grasshoppers, the expression of OBPs and CSPs does not usually seem to vary depending on the sex or phase of the animal (Jin *et al*., 2005).

### 6.4.3. Chordotonal organs

Insect antennae contain chordotonal organs, i.e. mechanosensory organs (Yack, 2004). Chordotonal organs are composed of scolopidia which lie below the cuticle. Each scolopidium contains up to four bipolar sensory cells and several accessory or support cells, such as scolopale cells and scolopale cap cells (Yack, 2004). In a scolopale cell, rigid actin-rich rods surround and confine a liquid-filled lumen. The sensory cells each have an unbranched dendrite. At the tip of each dendrite is a specialized mechanosensory organelle. The dendrites are found in the lumen formed by the scolopale cell, immersed in a potassium-rich fluid. The scolopale cell most likely produces this liquid itself, and it also secretes an electron dense extracellular dendritic cap structure which attaches both to the tips of the dendrites and to the cuticle (Yack, 2004; Jarman, 2014).

Johnston's organ, a chordotonal organ in the pedicel which contains hundreds of scolopidia, monitors the stretching of the antenna cuticle. The sensory neurons in Johnston's organ are activated when stretched and deactivated when compressed. Thus Johnston's organ keeps track of how the antenna moves relative to the insect's head. Johnston's organ can play a role in insect audition, by detecting the movement of the antenna when it vibrates sympathetically in response to sound (Kamikouchi *et al*., 2009). The neurons of Johnston's organ innervate the AMMC. There, the axons make extensive contacts with other neurons. The axons from Johnston's organ connect to other cells in the antennomechanosensory center via both gap junctions and chemical synapses (Sivan-Loukianova and Eberl, 2005).

## 6.5. Fates of the pioneer neurons

Some pioneer neurons appear to be transient populations of cells; they are vital to the developing nervous system, but absent in the adult. Other pioneers persist and take on a new functional role at later developmental stages. We have shown that the apical pioneers of the antenna are removed by apoptosis long before the embryo is ready to hatch. The fates of the base pioneers are still unclear.

### 6.5.1. Fates of pioneer neurons in vertebrates

In both the cat and the mouse brains, the vast majority of pioneer neurons disappear during development, leading researchers to speculate that the pioneer neurons have died (McConnell *et al*., 1989; Supèr *et al*., 1998). A small minority of mammalian embryonic pioneer neurons have been observed to persist and maintain their connections in the neonatal brain (McConnell *et al*., 1989).

In zebrafish, the neural pathways from the olfactory placode in the peripheral nervous system to the olfactory bulb in the central nervous system are established by a transient population of pioneer neurons which are dissimilar both morphologically and histochemically to the OSNs, and do not express ORs. These vertebrate olfactory pathway pioneers arise from the lateral neural plate, grow axons to the olfactory bulb, and undergo apoptosis, as revealed by TUNEL (Whitlock and Westerfield, 1998).

### 6.5.2. Fates of pioneer neurons in other insect systems

In the fly central nervous system, pioneer neurons undergo programmed cell death in some segments, but not others. MP1 and dMP2 neurons play a role in pioneering longitudinal pathways. Apoptosis removes dMP2 and MP1 pioneer neurons unless the process is prevented by Hox gene *Abdominal B* repressing *reaper* and *grim.* Thus, this Hox gene's role in regulating apoptosis allows it to shape the neural architecture of different segments. In the posterior segments where *Abdominal B* is expressed, pioneer neurons persist, while in other segments they die (Miguel-Allaga and Thor, 2004).

Some pioneers in the grasshopper nervous system also undergo apoptosis. Kutsch and Bentley (1987) reported that pioneers of the leg die after mid-embryogenesis. The Ti1 pioneers disappear after mid-embryogenesis and Kutsch and Bentley (1987) showed that cell death marker trypan blue is taken up by a pair of dying cells in the region of the pioneers. However, Kutsch and Bentley (1987) were not able to use a neuron-specific label such as HRP or Lazarillo to provide an unequivocal identification of these dying cells.

Insect neurons produced during embryogenesis can also persist and guide the growth of axons during the development of the adult nervous system. In *Drosophila*, a cluster of multidendrite neurons in the mesothorax survives metamorphosis and acts as a population of pioneer neurons which are responsible for guiding the peripheral pathfinding of sensory bristle neurons in the adult (Usui-Ishihara *et al*., 2000). Drosophila larval sensory neurons pioneer the pathways which are followed by adult sensory neurons into and through the central nervous system (Williams and Shepherd, 2002).

Some pioneer neurons in the brain of *S. gregaria* persist in the adult. Meier *et al.* (1993) and Boyan *et al.* (1995) described the Term-1-expressing pioneer neurons of the primary brain commissure in the desert locust. These pioneers persist throughout development and, by the time the animal reaches adulthood, the Term-1 expressing pioneers have transformed into Leucokinin-1-positive neurosecretory interneurons (Ludwig *et al.*, 2002). These neurons express SIFamide neuropeptides in the adult grasshopper (Gellerer *et al*., 2015). The pioneers of the primary brain commissure, as well as lateral cells, delaminate from the ectoderm, displaying a peripheral-like ontogeny, which may hint that they could be similar to the pioneers of the peripheral nervous system in other ways as well (Ludwig *et al.,* 1999; Boyan and Williams, 2008). The adult morphology of these cells are conserved across insect orders, which is again a similarity to the highly conserved organization of the nervous system of the appendages (Ludwig *et al*., 2002).

### 6.5.3. Fates of pioneer neurons in the grasshopper antenna

After a certain stage of development, base pioneers are no longer identifiable. Berlot and Goodman (1984) claim that the BP dies before 40%. Seidel and Bicker (2000) offer a different explanation, that the BP loses its HRP immunoreactivity after being contacted by the axons of the vA1 and dA1 pioneers. The fates of the A1 pioneers had also been unclear, although Seidel and Bicker (2000) reported that the A1 pioneers can no longer be labeled with HRP in at least some 55% antennae. The serially homologous nature of the grasshopper appendages suggested that the A1 antennal pioneers might be removed by apoptosis similar to the fate of the leg Ti1 pioneers (Kutsch and Bentley, 1987). In this PhD project, we performed a time series of immunohistochemistry experiments in order to shed new light on the fates of the pioneer neurons.

The apical pioneers are reliably identifiable due to their consistent expression of HRP and Lazarillo until after mid-embryogenesis, when acridine orange and TUNEL reveal the death of these cells (Fig. 6.7). The somata of the dying apical pioneers contain dense structures resembling the osmophilic dense-core pycnotic bodies described in other dying insect neurons (Booker and Truman, 1987; Boyan *et al*., 2010). The dying apical pioneers exhibit shrinking cytoplasmic volume and degrading cell membranes, which are further signs of cell death (Baehrecke, 2002).

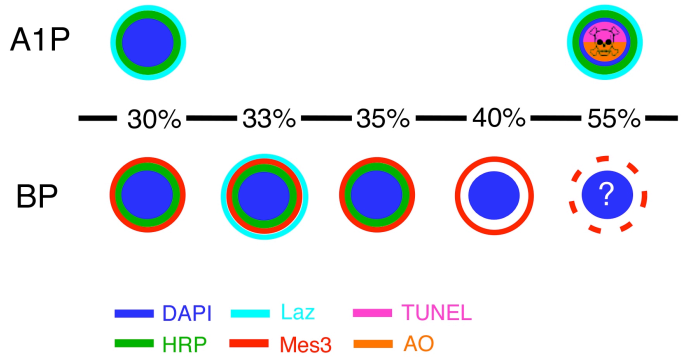



**Figure 6.7.** Schematic summarizing molecular expression patterns during thlife history of the apical (A1P) and base pioneer (BP) neurons of the grasshopper antenna. The nuclear label DAPI (blue) identifies both types of pioneers throughout. At approximately 30% of embryogenesis, the A1P cells co-express HRP (green) and Lazarillo (Laz, cyan), and this expression is maintained until around 55% of embryogenesis when they are additionally labeled by acridine orange (AO, orange) and TUNEL (magenta) indicating the onset of programmed cell death. The BP cells, on the otherhand, co-express HRP (green) and Mes3 (red) early in embryogenesis (30%), then additionally Lazarillo (33%, cyan). The Lazarillo expression ceases at 35% of embryogenesis while the HRP expression continues at least to 55% of embryogenesis after which time it too is downregulated. The Mes3 labeling continues at least to 45% of embryogenesis after which time the cells are no longer uniquely identifiable. Base pioneers do not show death labels up to mid-embryogenesis leaving their fate uncertain. Taken from Ehrhardt *et al*. (2015c).

The apical pioneer neurons of the antenna die at the same developmental stage (56-60%) when the Ti1 pioneers of the leg disappear (Kutsch and Bentley, 1987). Although Kutsch and Bentley (1987) observed that a pair of cells in the region of the Ti1 pioneers take up cell death marker trypan blue, the lack of a neuron-specific label in combination with the trypan blue means that the identification of the dying cells as pioneers cannot be certain. However, if we assume that Kutsch and Bentley (1987) were correct in identifying the pair of leg cells as Ti1 pioneers based on their location and morphology, then the fates of these leg pioneers are comparable to the fates of the apical pioneers of the antenna that we have reported here. We speculate that the similarity in antennal and leg pioneer cell fates are not a matter of coincidence, but constitute further evidence for the homologous nature of the mechanisms which generate the nervous system of these appendages (see Meier and Reichert, 1991).

In contrast to the constant HRP and Lazarillo expression of the apical pioneers until their deaths, the base pioneers alter their molecular profile over time. As summarized in Fig. 6.7, we have demonstrated that base pioneers express at least three labels: cell surface lipocalin Lazarillo, neuron-specific label HRP, and mesodermal label Mes3. Over the course of development, the base pioneers modify their expression of these molecules. Base pioneers express Lazarillo from 33% to around 35% of embryogenesis, HRP from 30 to 40% of embryogenesis, and maintain their Mes3 expression at least up to 45% of embryogenesis. Mes3 expression may persist longer than the other two labels because Mes3 represents an ontogenetic marker (Kotrla and Goodman, 1984), while HRP and Lazarillo are cell identity markers (Jan and Jan, 1982; Ganfornina *et al*., 1995; Sánchez *et al*., 1995).

Before mid-embryogenesis, the number of Mes3-expressing cells in the base of the antenna proliferates, such that the base pioneers are lost in this background of other cells showing similar expression in the same location. However, none of these Mes3-expressing cells are labeled by death markers. The base pioneers are located near the border of the antenna and the deutocerebrum, so their changing molecular profile may constitute a form of plasticity which occurs as part of boundary formation in other nervous systems such as the developing spinal cord (Cooke and Moens, 2002).

We speculate that the base pioneers may adopt a new role in the developing nervous system, consistent with their changing molecular profile and their lack of death signal or other signs of apoptosis such as pycnotic bodies or membrane degradation. The base pioneers of the grasshopper central nervous system could morph into a different cell type, similar to the way that the Term-1 pioneers of the primary brain commissure (Meier *et al*., 1993; Boyan *et al*., 1995) persist throughout development and transform into leucokinin-1-positive neurosecretory interneurons in the adult grasshopper brain (Ludwig *et al*., 2001).

## 6.6. Identity of the pioneer neurons

The pioneer neurons of the antenna are not classical sensory neurons, although they may both express many of the same molecular markers such as HRP, Lazarillo, and Lachesin (Ganfornina *et al*., 1995; Sanchez *et al*., 1995; Ehrhardt *et al*., 2015a). Both sensory neurons and apical pioneer neurons first appear in the epithelium, but the pioneer neurons do not remain there and instead delaminate into the lumen. Olfactory and gustatory sensory neurons target the glomeruli of the antennal lobe (Homberg *et al*., 1989), while the sensory neurons of chordotonal organs project to the AMMC (Sivan-Loukianova and Eberl, 2005). We have shown that although the pioneer axons guide sensory neurons into the deutocerebrum, the pioneers do not terminate there but rather go on to make contact with the Lazarillo-positive C3 and C2 neuronal clusters of the protocerebrum (Boyan and Ehrhardt, 2015). Sensory neurons may persist into adulthood in hemimetabolous insects, and in fact their numbers increase throughout embryonic and post-embryonic development (Chapman, 2002). Apical pioneers, in contrast, are removed by apoptosis after mid-embryogenesis, suggesting that apical pioneers play an important structural role in the developing nervous system but may be eliminated without playing a functional role (Ehrhardt *et al.*, 2015c).

It is unclear whether the pioneers of the antenna ever function as classical neurons. They have not been shown to release any neurotransmitter. The pioneers of the grasshopper antenna do not generate action potentials up to 45% (Boyan and Williams, 2007). Electrical excitability is reported to begin in the pioneers of the grasshopper leg at 45% (Keshishian, 1980; Keshishian and Bentley, 1983a). It may be possible that antennal apical pioneers initiate action potentials at some point before their deaths at 55%; further experiments at mid-embryogenesis may be necessary to find whether locust antennal pioneers are really as electrically inactive as they seem.

Selzer and Schaller-Selzer (1987) reported action potentials in the luminal cells of the cockroach at 23% of embryogenesis, within three percentage points of pioneer delamination in the cockroach. Selzer and Schaller-Selzer (1987) considered these luminal cells to be pioneer neurons; the lumenal cells derive from the epithelium and are dye-coupled to one another. If the lumenal cells are indeed pioneers, that would mean that the pioneers of the cockroach show activity at a much earlier stage of development than locust pioneers. However, the grasshopper antennal lumen also contains cells which are not pioneers, such as the NTA cells, which resemble the Selzer and Schaller-Selzer's (1987) putative pioneers morphologically (Boyan and Williams, 2007). Thus it is not clear whether the putative pioneers of Selzer and Schaller-Selzer (1987) were all really homologous to the grasshopper pioneers, or whether some actually corresponded to other cell types.

Selzer and Schaller-Selzer (1987) speculated that the early electrical activity of putative pioneers in the cockroach could be due to an embryonic cockroach hemolymph with very different concentrations of ions than the hemolymph in adult cockroaches or in other species. The composition of hemolymph in the embryonic cockroach antenna is not known. Selzer and Schaller-Selzer's (1987) electrophysiology method involved placing cockroach embryos in saline, which might also have affected the recorded activity.

Given the apparent lack of action potentials or neurotransmitters in the grasshopper antennal pioneers, and the death of the apical pioneers after mid-embryogenesis, we question whether their expression of HRP indicates an identity as classical neurons.

## 6.7. Targets of the pioneer neurons

Berlot and Goodman (1984) observed that the axon of the base pioneer fasciculates with a motoneuron, but did not identify which motoneuron. It was unknown which cells or brain regions were targeted by pioneer axons after they grew beyond the antenna. We used immunolabeling against Lazarillo to show that the axons of the apical pioneers project onto the Lazarillo-positive cells which contribute to the primary axon scaffold in the deutocerebrum and protocerebrum.

### 6.7.1. A motoneuron in the deutocerebrum

Each scape muscle is innervated by three or more excitatory motoneurons (Saager and Gewecke, 1989). The motoneurons which innervate the antenna muscles have their somata and dendritic fields in the AMMC. The exact location of a motoneuron soma varies between individual animals, so the identity of a motoneuron cannot be reliably determined based solely on the location of its soma (Bauer and Gewecke, 1991).

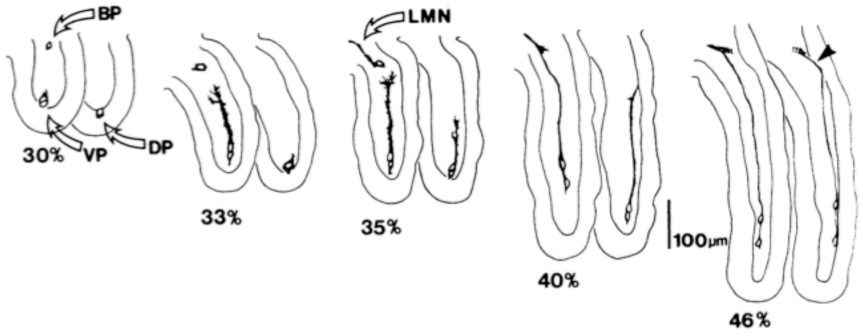



**Figure 6.8.** Growth of the pioneer axons within the antenna. At 30%, the only neurons observed in the antenna by Berlot and Goodman (1984) are the dorsal A1 pioneers (DP), the ventral A1 pioneers (VP) and a base pioneer on the ventral side (BP). At 35%, the axon of the BP fasciculates with a lateral motoneuron (LMN). Taken from Berlot and Goodman (1984). Reprinted with permission from AAAS (science.sciencemag.org/content/223/4635/493.short).

The axons of the motoneurons innervating the scape muscles reach the antenna via the lateral antennal nerve. This nerve gives off several branches, including the lateral scapal nerve and a motor nerve which innervates the median scape muscle. The lateral scapal nerve splits, giving rise to a sensory nerve and a motor nerve which innervates the lateral scape muscle (Gewecke, 1972). Each of the motor nerves innervating the scape muscles contain five axons (Bauer and Gewecke, 1991). The tentorio-scapal muscles in the head, which comprise the antennal levator muscle and the antennal depressor muscle, are innervated by nerves which branch from the lateral tegumentary nerve, a nerve that leaves the deutocerebrum lateral to the paired antennal nerve (Saager and Gewecke, 1989). The nerve innervating the levator muscle contains four axons, while the depressor nerve muscle includes six axons (Bauer and Gewecke, 1991).

Berlot and Goodman (1984) reported that one motoneuron fasciculates with an axon of a base pioneer (Fig. 6.8). Ho and Goodman (1982) wrote that this motoneuron eventually innervates a muscle at the base of the antenna. I have also imaged the connection between a base pioneer and a motoneuron (Fig. 6.9) at the age that Berlot and Goodman (1984) describe this connection existing, 35%. I speculate that this motoneuron may be one of the motoneurons innervating the tentorio-scapal muscles (Fig. 6.10).

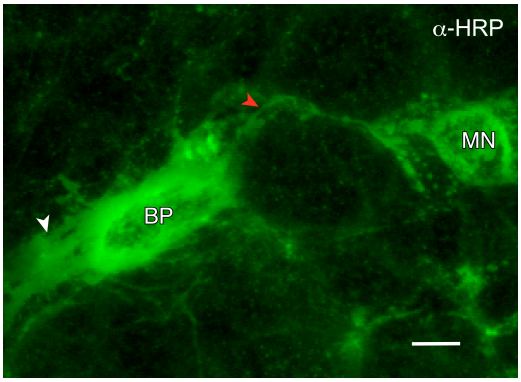



**Figure 6.9.** Confocal image of HRP-labeled neurons at the border of the antenna and deutocerebrum at approximately 35% of embryogenesis. Antenna is on the left, the deutocerebrum on the right. A growth cone of the apical pioneers (white arrowhead) is making contact with a base pioneer (BP). A thinner projection (red arrowhead) extends from the BP to a motoneuron (MN). The scale bar represents 5 µm.

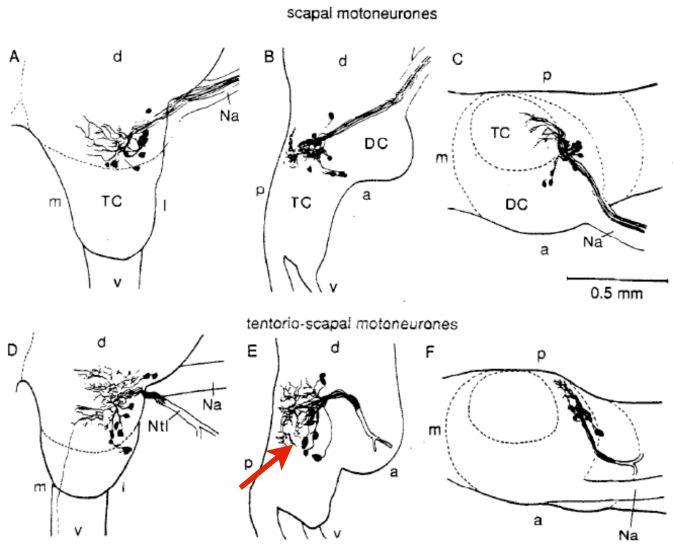



**Figure 6.10.** Camera lucida drawings of motoneurons stained with cobalt backfilling of the scape muscles (**a-c**) and the tentorio-scapal muscles (**d-f**). The red arrow emphasizes the tentorio-scapal motoneurons, which we propose include the motoneuron which fasciculates with antennal base pioneers. Modified from Bauer and Gewecke (1991).

### 6.7.2. Primary axon scaffold of the brain

In order to provide the locust with sensory information, the axons of the olfactory sensilla must transmit signals to the glomeruli of the antennal lobe. They find their way to the antennal lobe by growing along the pioneer axons which navigate this pathway earlier in development. However, we have shown that the glomeruli of antennal lobe are not the targets of the pioneer axons themselves; instead, these axons project onto the primary axon scaffold of the embryonic brain and contact Lazarillo-expressing cell clusters in the deutocerebrum and the protocerebrum, including the bilateral, unpaired LC cells of cluster 2, which have been demonstrated to pioneer a fascicle of the primary protocerebral commissure (Graf *et al*., 2000). Within the brain, the A1 pioneer axons grow from one Lazarillo-positive cell cluster to another in a stepping stone projection pattern reminiscent of the growth pattern described for pioneers in the grasshopper leg (see Bentley and O'Connor, 1992).

It is not possible for the glomeruli of the antennal lobe to serve as targets for the pioneer neurons, because the pioneer neurons grow into the deutocerebrum well before mid-embryogenesis, but the glomerular organization of the antennal lobe only forms after mid-embryogenesis, when the sensory axons of the antennal sensilla reach the area and integrate into the neuropil being formed by the progeny of the deutocerebral neuroblasts (see Boyan and Williams, 2000). We speculate that the pioneer neurons may pioneer a pathway such as the antenno-glomerular tract and guide the axons of the projection neurons from the antennal lobe to their targets in the protocerebral association centers of the brain (see Williams, 1975).

The primary axon scaffold of the embryonic brain includes components within the protocerebrum and deutocerebrum. Williams and Boyan (2008) showed that this scaffold serves as a target for pioneer neurons from the W, X, Y and Z lineages which generate the neurons which construct the neuroarchitecture of the central complex. We have now shown that the antennal pioneer axons also project onto the primary

axon scaffold, which suggests that this scaffold serves as a common target for multiple sets of pioneer neurons in the head. The Lazarillo-positive A1 pioneers contact the Lazarillo-expressing neurons of this scaffold to form a molecularly-contiguous pathway.

The A1 pioneers die by 60% of embryogenesis, well after they have established the initial pathway to the brain, but before the circuit becomes functional, and before any major sensory projections to the antennal lobe are complete. This sequence of events suggests that the A1 pioneers play a key structural role in the formation of the pathway, yet contributes only indirectly to the functional aspects of this circuitry.

## 6.8. Open questions and future work

I have investigated in detail the antennal pioneer neurons of the desert locust *Schistocerca gregaria*. I have provided answers for several previously unsolved questions related to the embryonic development of the antennal nervous system, such as the targets of the pioneers, the fates of the apical pioneers, and the origins of the base pioneers. However, much remains unknown with regards to the comparative biology of pioneer neurons. It would be illuminating to investigate the development of the antenna in other insect species such as the red flour beetle *Tribolium castaneum*, the fruit fly *Drosophila melanogaster*, and the cockroach *Periplaneta americana*. We could determine whether the antennal nerves of these animals are also established using a scaffold of pioneer neurons, and if so, how these pioneers compare to those in the grasshopper. Immunolabeling against Lazarillo could reveal whether the same glycoprotein is involved in axon navigation in the antennae of other species. Mes3 immunolabeling could show whether the pioneers in other species are derived from the ectoderm or mesoderm. Comparing different species of insects could provide information about when this system of pioneer neurons evolved, and whether it is conserved between orders.

The time limitations on this PhD project prevented us from carrying out a series of laser ablation experiments in cultured embryos. This method could be used to investigate questions such as whether both siblings of each pair of apical pioneers are necessary for the normal development of a nerve tract, whether nerve tracts can develop normally in the absence of NTA cells, and whether the pioneers require contact with sensory cells or motoneurons during their development.

Another open question is the role of gap junctions in the embryonic development of the antennal nervous system. Pioneer neurons in grasshopper appendages are dye-coupled to each other (Keshishian, 1980) and to other cells such as guidepost cells (Keshishian and Bentley, 1983a) and NTA cells (Boyan and Williams, 2007), but it is not known what function these connections have. Gap junctions could be blocked with *n*-heptanol in cultured embryos, revealing how the nervous system would develop in the absence of these connections.

It is unknown whether the pioneer neurons of the antenna ever release any neurotransmitter. It is unclear what neuroactive molecules are expressed by pioneer neurons (if any), sensory neurons and NTA cells (if any) in the grasshopper, and at what stages of development these substances are expressed.

The first sensory neurons of the antenna appear before 37% of embryogenesis (Boyan and Williams, 2004). However, few of the details of the development of these cells are known. The function and type of the sensory clusters which have been observed developing during embryogenesis have not been identified. If the sensory clusters were mapped, or if a system for identifying types of sensory clusters in the embryo were developed, we could look for patterns in the development of the sensory system, such as whether sensilla chaetica, sensilla basiconica, sensilla trichodea or sensilla coeloconica develop simultaneously, or whether the receptor types form during different stages of embryogenesis. It is unclear what developmental mechanisms produce the segment-specific distribution of sensory cells. It is unknown whether the centrifugal projections of pioneers help guide sensory axons from the tip, or whether these projections play some other role in development.

I have shown that the antennal pioneer axons project into the deutocerebrum by 40% of embryogenesis and that their targets are Lazarillo-positive cells which contribute to the primary axon scaffold of the embryonic brain. This occurs before the glomeruli of antennal lobe develop. What is still unclear are the next steps in development. It is not known how the pattern of sensory afferent projections into

the glomeruli are constructed in the grasshopper embryo. It is unclear whether the sensory projections are organized into a topography based on their age of position in the antenna in the grasshopper. It is not known whether the sensory afferents of the antenna in some way shape the development of the glomeruli. The details of how glia, neuroblasts, and their contacts with one another and with neurons contribute to the formation of the glomeruli in the grasshopper remain to be clarified. The ontogeny and cell types of the glia in the antennal lobe of the grasshopper are poorly understood.

I have investigated the role of the base pioneer in axogenesis, but other cells could also be involved in guiding the axons of antennal pioneers or sensory neurons, in the antenna itself or after these axons how grown into the brain. It is unknown whether glia or motoneurons play a role in guiding pioneers or sensory axons of the antenna.

# 7. References


Abrams, J.M., White, K., Fessler, L.I., Steller, H. (1993). Programmed cell death during *Drosophila* embryogenesis. Development 117:29-43.

Abuin, L., Bargeton, B., Ulrich, M.H., Isacoff, E.Y., Kellenberger, S., Benton, R. (2011). Functional architecture of olfactory ionotropic glutamate receptors. Neuron 69:44-60.

Ai, M., Blais, S., Park, J.Y., Min, S., Neubert, T.A., Suh, G.S. (2013). Ionotropic glutamate receptors IR64a and IR8a form a functional odorant receptor complex in vivo in *Drosophila*. J Neurosci 33:10741-10749.

Ai, M., Min, S., Grosjean, Y., Leblanc, C., Bell, R., Benton, R., Suh, G.S. (2010). Acid sensing by the *Drosophila* olfactory system. Nature 468:691-695.

Altner, H., Routil, C., Loftus, R. (1981). The structure of bimodal chemo-, thermo- and hygroreceptive sensilla on the antenna of *Locust migratoria*. Cell Tissue Res 215:289-308.

Anderson, H., Tucker, R.P. (1988). Pioneer neurones use basal lamina as a substratum for outgrowth in the embryonic grasshopper limb. Development 104:601-608.

Anderson, H., Tucker, R.P. (1989). Spatial and temporal variation in the structure of the basal lamina in embryonic grasshopper limbs during pioneer neurone outgrowth. Development 106:185-194.

Arnold, G., Masson, C., Budharugsa, S. (1985). Comparative study of the antennal lobes and their afferent pathway in the worker bee and the drone (*Apis mellifera*). Cell Tissue Res 242:593-605.

Axel, R. (2004). Scents and sensibility: a molecular logic of olfactory perception. Nobel Prize Lecture. Retrieved December 14, 2015.

Baehrecke, E.H. (2002). How death shapes life during development. Nat Rev Mol Cell Biol 3:779-787.

Bak, M., Fraser, S.E. (2003). Axon fasciculation and differences in midline kinetics between pioneer and follower axons within commissural fascicles. Development 130:4999-5008.

Ball, E.E., Gert de Couet, H., Horn, P.L., Quinn, J.M.A. (1987). Haemocytes secrete basement membrane components in embryonic locusts. Development 99:255-259.

Ball, E.E., Ho, R.K., Goodman, C.S. (1985). Muscle development in the grasshopper embryo. I. Muscles, nerves, and apodemes in the metathoracic leg. Dev Biol 111:383-398.

Bastiani, M.J., de Couet, H.G., Quinn, J.M.A., Karlstrom, R.O. (1992). Position-specific expression of the Annulin protein during grasshopper embryogenesis. Dev Biol 154:129-142.

Bastiani, M.J., du Lac, S., Goodman, C.S. (1986). Guidance of neuronal growth cones in the grasshopper embryo. I. Recognition of a specific axonal pathway by the pCC neuron. J Neurosci 6:3518-3531.

Bastiani, M.J., Goodman, C.S. (1986). Guidance of neuronal growth cones in the grasshopper embryo. III. Recognition of specific glial pathways. J Neurosci 6:3542-3551.

Bastiani, M.J., Harrelson, A.L., Snow, P.M., Goodman, C.S. (1987). Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. Cell 48:745-755.

Bate, C.M. (1976). Pioneer neurones in an insect embryo. Nature 260:54-56.

Bauer, C.K., Gewecke, M. (1991). Motoneuronal control of antennal muscles in *Locusta migratoria*. J Insect Physiol 37:551-562.

Bausenwein, B., Nick, P. (1998). Three-dimesional reconstruction of the antennal lobe in the mosquito *Aedes aegypti*. Proc 26th Göttingen Neurobiol Conference. Stuttgart & New York: Georg Thieme. p 386.

Bello, B.C., Hirth, F., Gould, A.P. (2003). A pulse of the *Drosophila* Hox protein abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. Neuron 37:209-219.

Bentley, D., Guthrie, P.B., Kater, S.B. (1991). Calcium ion distribution in nascent pioneer axons and coupled preaxonogenesis neurons *in situ.* J Neurosci 11:1300-1306.

Bentley, D., Keshishian, H. (1982). Pioneer neurons and pathways in insect appendages. TINS 354-358.

Bentley, D., Keshishian, H., Shankland, M., Toroian-Raymond, A. (1979). Quantitative staging of embryonic development of the grasshopper, *Schistocerca nitens*. Development 54:47-74.

Bentley, D., O'Connor, T.P. (1992). Guidance and steering of peripheral growth cones in grasshopper embryos. In: The Nerve Growth Cone. Letourneau, C., Kater, S.B. (Eds.) Raven Press. pp. 265-282.

Bentley, D., Toroian-Raymond, A. (1989). Pre-axonogenesis migration of afferent pioneer cells in the grasshopper embryo. J exp Zool 251:217-223.

Benton, R., Vannice, K.S., Gomez-Diaz, C., Vosshall, L.B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. Cell 136:149-162.

Berlot, J., Goodman, C.S. (1984). Guidance of peripheral pioneer neurons in the grasshopper: adhesive hierarchy of epithelial and neuronal surfaces. Science 223:493-496.

Biffar, L., Stollewerk, A. (2015). Evolutionary variations in the expression of dorso-ventral patterning genes and the conservation of pioneer neurons in *Tribolium castaneum*. Dev Biol 400:159-167.

Blumberg, B., MacKrell, A.J., Olson, P.F., Kurkinen, M., Monson, J.M., Natzle, J.E., Fessler, J.H. (1987). Basement membrane procollagen IV and its specialized carboxyl domain are conserved in *Drosophila*, mouse, and human. J Biol Chem 262:5947-5950.

Booker, R., Truman, J.W. (1987). Postembryonic neurogenesis in the CNS of the tobacco hookworm, *Manducta sexta*. I. neuroblast arrays and the fate of their progeny during metamorphosis. J Comp Neurol 255:548-559.

Boyan, G.S., Ball, E.E. (1993). The grasshopper, *Drosophila* and neuronal homology (advantages of the insect nervous system for the neuroscientist). Progress in Neurobiol 41:657-682.

Boyan, G.S., Ehrhardt, E.E. (2015). Pioneer neurons of the antennal nervous system project to protocerebral pioneers in the grasshopper *Schistocerca gregaria*. Dev Genes Evol 225:377-382.

Boyan, G.S., Herbert, Z., Williams, J.L.D. (2010). Cell death shapes embryonic lineages of the central complex in the grasshopper *Schistocerca gregaria*. J Morphol 271:949-959.

Boyan, G.S., Liu, Y. (2014). Timelines in the insect brain: fates of identified neural stem cells generating the central complex in the grasshopper *Schistocerca gregaria*. Dev Genes Evol. 224, 37-51.

Boyan, G.S., Therianos, S., Williams, J.L.D., Reichert, H. (1995). Axogenesis in the embryonic brain of the the grasshopper *Schistocerca gregaria*: an identified cell analysis of early brain development. Development 121:75-86.

Boyan, G.S., Williams, J.L.D. (2000). Building the antennal lobe: *engrailed* expression reveals a contribution from the protocerebral neuroblasts in the grasshopper *Schistocerca gregaria*. Arth Struct Dev 29:267-274.

Boyan, G.S., Williams, J.L.D. (2004). Embryonic development of the sensory innervation of the antenna of the grasshopper *Schistocerca gregaria*. Arth Struct Dev 33:381-397.

Boyan, G.S., Williams, J.L.D. (2007). Embryonic development of the sensory innervation of the antenna of the grasshopper *Schistocerca gregaria*. Arth Struct Dev 36:336-350.

Boyan, G.S., Williams, J.L.D. (2008). Evidence that the peripheral brain commissure is pioneered by neurons with a peripheral-like ontogeny in the grasshopper *Schistocerca gregaria*. Arth Struct Dev 37:186-198.

Brand, A.H., Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401-415.

Buck, L.B. (2000). The molecular architecture of odor and pheromone sensing in mammals. Cell 100:611-618.

Bullock, T.H., Horridge, G.A. (1965). Structure and function in the nervous system of invertebrates. Freeman and Company, London.

Burrows, M. (1996). The neurobiology of an insect brain. Oxford University Press.

Butt, F.H. (1960). Head development in arthropods. Biological Reviews 35:43-91.

Casares, F., Mann, R.S. (1998). Control of antennal versus leg development in *Drosophila*. Nature 392:723-726.

Casares, F., Mann, R.S. (2001). The ground state of the ventral appendage in *Drosophila*. Science 293:1477-1480.

Caudy, M., Bentley, D. (1986). Pioneer growth cone morphologies reveal proximal increases in substrate affinity within leg segments of grasshopper embryos. J Neurosci 6:364-379.

Chapman, R.F. (1982). The insects: structure and function. Hodder and Stoughton, London.

Chapman, R.F. (2002). Development of phenotypic differences in sensillum populations on the antennae of a grasshopper, *Schistocerca americana.* J Morphol 254:186-194.

Chapman, R.F., Greenwood, M. (1986). Changes in distribution and abundance of antennal sensilla during growth of *Locusta migratoria* L. (Orthoptera: Acrididae). Int J Insect Morphol & Embryol 15:83-96.

Chen, H-H., Zhao, Y-X., Kang, L. (2003). Antennal sensilla of grasshoppers (Orthoptera: Acrididae) in relation to food preferences and habits. J Biosciences 28:743-752.

Chen, H-H., Zhao, Y-X., Kang, L. (2004). Comparison of the olfactory sensitivity of two sympatric steppe grasshopper species (Orthoptera: Acrididae) to plant volatile compounds. Science in China Ser. C: Life Sciences 47:115-123.

Comer, C., Baba, Y. (2011). Active touch in orthopteroid insects: behaviours, multisensory substrates and evolution. Phil Trans R Soc B 366:3006-3015.

Condic, M.L., Bentley, D. (1989a). Pioneer neuron pathfinding from normal and ectopic locations in vivo after removal of the basal lamina. Neuron 3:427-439.

Condic, M.L., Bentley, D. (1989b). Removal of the basal lamina *in vivo* reveals growth cone-basal lamina adhesive interactions and axonal tension in grasshopper embryos. J Neurosci 9:2678-2686.

Condic, M.L., Bentley, D. (1989c). Pioneer growth cone adhesion *in vivo* to boundary cells and neurons after enzymatic removal if basal lamina in grasshopper embryos. J Neurosci 9:2687-2696.

Cooke, J.E., Moens, C.B. (2002). Boundary formation in the hindbrain: eph only it were simple. TINS 25:260-267.

Couto, A., Lapeyre, B., Thiéry, D., Sandoz, J.-C. (2016) The olfactory pathway of the hornet *Vespa velutina*: new insights into the evolution of the hymenopteran antennal lobe. J Comp Neurol

Cui, X., Wu, C., Zhang, L. (2011). Electrophysiological response patterns of 16 olfactory neurons from the trichoid sensilla to odorant from fecal volatiles in the locust, *Locusta migratoria manilensis*. Arch Insect Biochem Physiol 77:45-57.

Davies, S.N., Kitson, D.L., Roberts, A. (1982). The development of the peripheral trigeminal innervation in *Xenopus* embryos. J Embryol exp Morph 70:215-224.

Denburg, J.L., Norbeck, B.A. (1989). An axon growth associated antigen is also an early marker of neuronal determination. Dev Biol 135:99-110.

Diamond, P., Mallavarapu, A., Shnipper, J., Booth, S., Park, L., O'Connor, T.P., Jay, D.G. (1993). Fasciclin I and II have distinct roles in the development of grasshopper pioneer neurons. Neuron 11:409-421.

du Lac, S., Bastiani, M.J., Goodman, C.S. (1986). Guidance of neuronal growth cones in the grasshopper embryo. II. Recognition of a specific axonal pathway by the aCC neuron. J Neurosci 6:3532-3541.

Edwards, J.S., Chen, S.-W., Berns, M.W. (1981). Cercal sensory development following laser microlesions of embryonic apical cells in *Acheta domesticus*. J Neurosci 1:250-258.

Ehrhardt, E.E., Liu, Y., Boyan, G.S. (2015a). Axogenesis in the antennal nervous system of the grasshopper *Schistocerca gregaria* revisited: the base pioneers. Dev Genes Evol 225:39-45.

Ehrhardt, E.E., Kleele, T., Boyan, G.S. (2015b). A method for immunolabeling neurons in intact cuticularized insect appendages. Dev Genes Evol 225:187-194.

Ehrhardt, E.E., Graf, P., Kleele, T., Liu, Y., Boyan, G.S. (2015c). Fates of identified pioneer cells in the developing antennal nervous system of the grasshopper *Schistocerca gregaria*. Arth Struct Dev. pii: S1467-8039(15)00126.7. doi:10.1016/j.asd.2015.11.001

Eisen, J.S., Pike, S.H., Debu, B. (1989). The growth cones of identified motoneurons in embryonic zebrafish select appropriate pathways in the absence of specific cellular interactions. Neuron 2:1097-1104.

Emerson, M.J., Schram, F.R. (2012). Theories, patterns and reality: game plan for arthropod phylogeny. In: Arthropod Relationships. Fortey, R.A., Thomas, R.H. (Eds.) Springer. pp. 67-86.

Ernst, K.-D., Boeckh, J., Boeckh, V. (1977). A neuroanatomical study on the organization of the central antennal pathways in insects. Cell Tissue Res 176:285-308.

Fischer, A.H.L., Scholtz, G. (2010). Axogenesis in the stomatopod crustacean *Gonodactylaceus falcatus* (Malacostraca). Invert Biol 129:59-76.

Flanagan, D., Mercer, A.R. (1989). An atlas and 3-D reconstruction of the antennal lobes in the worker honey bee, *Apis mellifera* L. (Hymenoptera : Apidae). Int J Insect Morphol Embryol 18:145-159.

Galizia, C.G., McIlwrath, S.L., Menzel, R. (1999). A digital three-dimensional atlas of the honeybee antennal lobe based on optical sections acquired by confocal microscopy. Cell Tissue Res 295:383-394.

Galizia, C.G., Rössler, W. (2010). Parallel olfactory systems in insects: anatomy and function. Annu Rev Entomol 55:399-420.

Gan, W.-B., Macagno, E.R. (1995). Developing neurons use a putative pioneer's peripheral arbor to establish their terminal fields. J Neurosci 15:3254-3262.

Ganfornina, M.D., Sánchez, D., Bastiani, M.J. (1995). Lazarillo, a new GPI-linked surface lipocalin, is restricted to a subset of neurons in the grasshopper embryo. Development 121:123-134.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493-501.

Garbe, D.S., Bashaw, G.J. (2007). Independent functions of Slit-Robo repulsion and Netrin-Frazzled attraction regulate axon crossing at the midline in *Drosophila*. J Neurosci 27:3584-3592.

Garcia-Lopez, P., Garcia-Marin, V., Freire, M. (2010). The histological slides and drawings of Cajal. Frontiers in Neuroanatomy 4:1-16.

Gellerer, A., Franke, A., Neupert, S., Predel, R., Zhou, X., Shanlin, L., Reiher, W., Wegener, C., Homberg, U. (2015). Identification and distribution of SIFamide in the nervous system of the desert locust *Schistocerca gregaria*. J Comp Neurol 523:108-125.

Gewecke, M. (1972). Bewegungsmechanismus und Gelenkrezeptoren der Antennen von *Locusta migratoria* L. (Insecta, Orthoptera). Zeitschrift zur Morphologie und Ökologie der Tiere 71:128-149.

Gewecke, M. (1996). Schwimmverhalten und seine Steuerung durch Wasserströmungs-Sinnesorgane beim Teichschwimmer *Colymbetes fuscus* (Coleoptera: Dytiscidae). Entomologica Generalis 20:203-220.

Gewecke, M., Heizel, H.-G. (1980). Aerodynamic and mechanical properties of the antennae as air-current sense organs in *Locusta migratoria*. I. Static characteristics. J Comp Physiol A 139:357-355.

Gibson, G., Gehring, W.J. (1988). Head and thoracic transformations caused by ectopic expression of *Antennapedia* during *Drosophila* development. Development 102:657-675.

Gong, Q., Shipley, M.T. (1995). Evidence that pioneer olfactory axons regulate telencephalon cell cycle kinetics to induce the formation of the olfactory bulb. Neuron 14:91-101.

Goodman, C.S., Doe, C.Q. (1993). Embryonic development of the *Drosophila* central nervous system. In: The Development of *Drosophila melanogaster*. Bate, M., Martinez-Arias, A. (Eds.), Cold Spring Harbor Press. pp..

Göpfert, M.C. (2007). *Drosophila*-Antenne gewährt Einblicke in grundlegende Mechanismen des Hörens. Neuroforum 4:122-126.

Göpfert, M.C., Robert, D. (2001). Turning the key on *Drosophila* audition. Nature 411:908.

Göpfert, M.C., Robert, D. (2003). Motion generation by *Drosophila* mechanosensory neurons. PNAS 100:5514-5519.

Göpfert, M.C., Stocker, H., Robert, D. (2002). *atonal* is required for exoskeletal joint formation in the *Drosophila* auditory system. Dev Dynamics 225:106-109.

Graf, S., Ludwig, P., Boyan, G.S. (2000). Lazarillo expression reveals a subset of neurons contributing to the primary axon scaffold of the embryonic brain of the grasshopper *Schistocerca gregaria*. J Comp Neurol 419:394-405.

Grenningloh, G., Rehm, E.J., Goodman, C.S. (1991). Genetic analysis of growth cone guidance in *Drosophila*: fasciclin II functions as a neuronal recognition molecule. Cell 67:45-57.

Guo, M., Krieger, J., Große-Wilde, E., Mißbach, C., Zhang, L., Breer, H. (2014). Variant ionotropic receptors are expressed in olfactory sensory neurons of coeloconic sensilla on the antenna of the desert locust (*Schistocerca gregaria*). Int J Biol Sci 10:1-14.

Gupta, B.P., Rodrigues, V. (1997). *Atonal* is a proneural gene for a subset of olfactory sense organs in *Drosophila.* Genes Cells. 2:225-233.

Halter, D.A., Urban, J., Rickert, C., Ner, S.S., Ito, K., Travers, A.A., Technau, G.M. (1995). The homeobox gene *repo* is required for the differentiation and maintenance of glial function in the embryonic nervous system of *Drosophila melanogaster*. Development 121:317-322.

Hansson, B.S., Anton, S. (2000). Function and morphology of the antennal lobe: new developments. Annu Rev Entomol 45:203-231.

Hansson, B.S., Ochieng, S.A., Grosmaitre, X., Anton, S., Njagi, P.G.N. (1996). Physiological responses and central nervous projections of antennal olfactory receptor neurons in the adult desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae). J Comp Physiol A 179:157-167.

Hassanali, A., Njagi, P.G., Bashir, M.O. (2005). Chemical ecology of locusts and related acridids. Annu Rev Entomol 50:223-245.

Heinze, S., Reppert, S.M. (2012). Anatomical basis of sun compass navigation I: the general layout of the monarch butterfly brain. J Comp Neurol 520:1599-1628.

Hidalgo, A., Brand, A.H. (1997). Targeted neuronal ablation; the role of pioneer neurons in guidance and fasciculation in the CNS of *Drosophila*. Development 124:3253-3262.

Hidalgo, A., Booth, G.E. (2000). Glia dictate pioneer axon trajectories in the *Drosophila* embryonic CNS. Development 127:393-402.

Hildebrand, J.G. (1996). Olfactory control of behavior in moths: central processing of odor information and the functional significance of olfactory glomeruli. J Comp Physiol 178:5-19.

Ho, R.K., Ball, E.E., Goodman, C.S. (1983). Muscle pioneers: large mesodermal cells that erect a scaffold for developing muscles and motoneurones in grasshopper embryos. Nature 301:66-69.

Ho, R.K., Goodman, C.S. (1982). Peripheral pathways are pioneered by an array of central and peripheral neurones in grasshopper embryos. Nature 297:404-406.

Homberg, U., Christensen, T.A., Hildrebrand, J.G. (1989). Structure and function of the deutocerebrum in insects. Ann Rev Entomol 34:477-501.

Honegger, H.W. (1981). A preliminary note on a new optomotor response in crickets: antennal tracking of moving targets. J Comp Physiol 142:419-421.

Isbister, C.M., O'Connor, T.P. (1999). Filopodial adhesion does not predict growth cone steering events *in vivo*. J Neurosci 19:2589-2600.

Jan, L.Y., Jan, Y.N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. PNAS 79:2700-2704.

Jarman, A.P. (2014). Development of the auditory organ (Johnston's organ) in *Drosophila*. In: Development of Auditory and Vestibular Systems. Romand, R., Varela-Nieto, I., (Eds.), Academic Press. pp. 31-63.

Jarman, A.P., Sun, Y., Jan, L.Y., Jan, Y.N. (1995). Role of the proneural gene, *atonal*, in formation of *Drosophila* chordotonal organs and photoreceptors. Development 121:2019-2030.

Jay, D.G., Keshishian, H. (1990). Laser inactivation of fasciclin I disrupts axon adhesion of grasshopper pioneer neurons. Nature 348:548-550.

Jiang, C., Baehrecke, E.H., Thummel, C.S. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. Development 124:4673-4683.

Jin, X., Brandazza, A., Navarrini, A., Ban, L., Zhang, S., Steinbrecht, R.A., Zhang, L. (2005). Expression and immunolocalisation of odorant-binding and chemosensory proteins in locusts. Cell Mol Life Sciences 62:1156-1166.

Kaissling, K.-E., Leal, W.S. (2004). Biologische Nanokapseln für Duftstoffe. Naturwissenschaftliche Rundschau 2:66-71.

Kamikouchi, A., Inagaki, H.K., Effertz, T., Hendrich, O., Fiala, A., Göpfert, M.C., Ito, K. (2009). The neural basis of Drosophila gravity-sensing and hearing. Nature 458:165-171.

Karlstrom, R.O., Wilder, L.P., Bastiani, M.J. (1993). Lachesin: an immunoglobulin superfamily protein whose expression correlates with neurogenesis in grasshopper embryos. Development 118:509-522.

Keil, T.A (1984). Reconstruction and morphology of silkmoth olfactory hairs: a comparative study of sensilla tichodea on the antennae of male *Antheraea polyphemus* and *Antheraea pernyi* (Insecta, Lepidoptera). Zoomorphology 104:147-156.

Keil, T.A. (1992). Fine structure of a developing insect olfactory organ: morphogenesis of the silkmoth antenna. Microsc Res Tech 22:351-371.

Keil, T.A. (1997). Comparative morphogenesis of sensilla: a review. Int J Insect Morphol Embryol 26:151-160.

Keil, T.A., Steiner, C. (1990). Morphogenesis of the antenna of the male silkmoth, *Antheraea polyphemus*. II. Differential mitoses of 'dark' precursor cells create the Anlagen of sensilla. Tissue and Cell 22:705-720.

Keshishian, H. (1980). The origin and morphogenesis of pioneer neurons in the grasshopper metathoracic leg. Dev Biol 80:388-397.

Keshishian, H., Bentley, D. (1983a). Embryogenesis of peripheral nerve pathways in grasshopper legs: I. the initial nerve pathway to the CNS. Dev Biol 96:89-102.

Keshishian, H., Bentley, D. (1983b). Embryogenesis of peripheral nerve pathways in grasshopper legs: III. development without pioneer neurons. Dev Biol 96:116-124.

Klose, M., Bentley, D. (1989). Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. Science 245:982-984.

Kolodkin, A.L., Matthes, D.J., O'Connor, T.P., Patel, N.H., Admon, A., Bentley, D., Goodman, C.S. (1992). Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. Neuron 9:831-845.

Kononenko, N.L., Pflüger, H.J. (2007). Dendritic projections of different types of octopaminergic unpaired median neurons in the locust metathoracic ganglion. Cell Tissue Res 330:179-195.

Kotrla, K.J., Goodman, C.S. (1984). Transient expression of a surface antigen on a small subset of neurones during embryonic development. Nature 311:151-153.

Kulkarni, R.P., Bak-Maier, M., Fraser, S.E. (2007). Differences in protein mobility between pioneer versus follower growth cones. PNAS 104:1207-1212.

Kutsch, W., Bentley, D. (1987). Programmed death of peripheral pioneer neurons in the grasshopper embryo. Dev Biol 123:517-525.

Lakes-Harlan, R., Pollack, G.S. (1993). Pathfinding of peripheral neurons in the central nervous system of an embryonic grasshopper *(Chorthippus biguttulus)*. Cell Tissue Res 273:97-106.

Lefcort, F., Bentley, D. (1987). Pathfinding by pioneer neurons in isolated, opened and mesoderm-free limb buds of embryonic grasshoppers. Dev Biol 119:466-480.

Lin, D.M., Auld, V.J., Goodman, C.S. (1995) Targeted neuronal cell ablation in the *Drosophila* embryo: pathfinding by follower growth cones in the absence of pioneers. Neuron 14:707-715.

Liu, Y., Boyan, G. (2013). Glia associated with central complex lineages in the embryonic brain of the grasshopper *Schistocerca gregaria*. Dev Genes Evol 223:213-223.

Lobbia, S., Nitsu, S., Fujiwara, H. (2003). Female-specific wing degeneration caused by ecdysteroid in the Tussock Moth, *Orgyia recens*: hormonal and developmental regulation of sexual dimorphism. J Insect Sci 3:11-18.

Loher, W., Dambach, M. (1989). Reproductive behavior. In: Cricket Behavior and Neurobiology. Huber, F., Moore, T.E., Loher, W., (Eds.), Ithaca, Cornell University Press, pp. 43-82.

Ludwig, P., Williams, J.L.D., Boyan, G.S. (2002). The pars intercerebralis of the locust brain: a developmental and comparative study. Microsc Res Tech 56:174-188.

Ludwig, P., Williams, J.L.D., Lodde, E., Reichert, H., Boyan, G.S. (1999). Neurogenesis in the median domain of the embryonic brain of the grasshopper *Schistocerca gregaria*. J Comp Neurol 414:379-390.

Ludwig, P., Williams, J.L.D., Nässel, D., Reichert, H., Boyan, G.S. (2001). The primary commissure pioneers in the brain of the grasshopper *Schistocerca gregaria*: development ultrastructure and peptide expression. J Comp Neurol 430:118-130.

Manton, S.M. (1960). Concerning head development in the arthropods. Biol Rev (Cambridge) 35:265-282.

Masante-Roca, I., Gadenne, C., Anton, S. (2005). Three-dimensional antennal lobe atlas of male and female moths, *Lobesia botrana* (Lepidoptera: Tortricidae) and glomerular representation of plant volatiles in females J Exp Biol 208:1147-1159.

Maynard, K.R., McCarthy, S.S., Sheldon, E., Horch, H.W. (2007). Developmental and adult expression of semaphorin 2a in the cricket *Gryllus bimaculatus*. J Comp Neurol 503:169-181.

McConnell, S.K., Ghosh, A., Shatz, C.J. (1989). Subplate neurons pioneer the first axon pathway from the cerebral cortex. Science 245:978-987.

Meier, T., Reichert, H. (1991). Serially homologous development of the peripheral nervous system in the mouthparts of the grasshopper. J Comp Neurol 305:201-214.

Meier, T., Therianos, S., Zacharias, D., Reichert, H. (1993). Developmental expression of TERM-1 glycoprotein on growth cones and terminal arbors of individual identified neurons in the grasshopper. J Neurosci 13:1498-1510.

Melançon, E., Liu, D.W.C., Westerfield, M., Eisen, J.S. (1997). Pathfinding by identified zebrafish motoneurons in the absence of muscle pioneers. J Neurosci 17:7796-7804.

Miguel-Allaga, I., Thor, S. (2004). Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic Hox gene activity. Development 131:6093-6105.

Millonig, G. (1961). Advantages of a phosphate buffer for osmium tetroxide solutions in fixation. J Appl Phys 32:1637.

Nardi, J.B. (1983). Neuronal pathfinding in developing wings of the moth *Manduca sexta*. Dev Biol 95:163-174.

Norbeck, B.A., Feng, Y., Denburg, J.L. (1992). Molecular gradients along the proximal-distal axis of embryonic insect legs: possible guidance cues of pioneer axon growth. Development 116:467-479.

Ochieng, S.A., Halberg, E., Hansson, B.S. (1998). Fine structure and distribution of antennal sensilla of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae). Cell Tissue Res 291:525-536.

Ochieng, S.A., Hansson, B.S. (1999). Responses of olfactory receptor neurones to behaviourally important odours in gregarious and solitarious desert locust, *Schistocerca gregaria*. Physiol Entomol 24:28-36.

O'Connor, T.P., Bentley, D. (1993). Accumulation of actin in subsets of pioneer growth cone filopodia in response to neural and epithelial guidance cues in situ. J Cell Biol 123:935-948.

Oland, L.A., Tolbert, L.P. (2003). Key interactions between neurons and glial cells during neural development in insects. Annu Rev Entomol 48:89-110.

Page, D.T., Olofsson, B. (2008). Multiple roles for apoptosis facilitating condensation of the *Drosophila* ventral nerve cord. Genesis 46:61-68.

Patel, N.H. (1994). Imaging neuronal subsets and other cell types in whole mount *Drosophila* embryos and larvae using antibody probes. In: Methods in Cell Biology vol 44 *Drosophila melanogaster*: Practical Uses in Cell Biology. Goldstein, L.S.B, Fyrberg, E. (Eds), Academic Press, New York, pp 445-487.

Patel, N.H., Snow, P.M., Goodman, C.S. (1987). Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. Cell 48:975-988.

Pike, S.H., Melancon, E.F., Eisen, J.S. (1992). Pathfinding by zebrafish motoneurons in the absence of normal pioneer axons. Development 114:825-831.

Pittman, A.J., Law, M-Y., Chien, C-B. (2008). Pathfinding in a large vertebrate axon tract: isotypic interactions guide retinotectal axons at multiple choice points. Development 135:2865-2871.

Rajan, I., Denburg, J.L. (1997). Mesodermal guidance of pioneer axon growth. Dev Biol 190:214-228.

Ramón y Cajal, S. (1890). A quelle epoque aparaissent les expansion des cellule neurveuses de la moelle epinere du poulet. Anat Anz 5:609-613.

Raper, J.A., Bastiani, M.J., Goodman, C.S. (1984). Pathfinding by neuronal growth cones in grasshopper embryos: IV. the effects of ablating the A and P axons upon the behavior of the G growth cone. J Neurosci 4:2329-2345.

Reichert, H., Boyan, G.S. (1997). Building a brain: insights from insects. Trends Neurosci 20:258-264.

Robertson, H.W., Wanner, K.W. (2006). The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not, gustatory, receptor family. Genome Res 16:1395-1403.

Rospars, J.P. (1983). Invariance and sex-specific variations of the glomerular organization in the antennal lobes of a moth, *Mamestra brassicae*, and a butterfly, *Pieris brassicae*. J Comp Neurol 220:80-96.

Rytz, R., Croset, V., Benton, R. (2013). Ionotropic receptors (IRs): chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. Insect Biochem Mol Biol 43:888-897.

Saager, F, Gewecke, M. (1989). Antennal reflexes in the desert locust *Schistocerca gregaria*. J exp Biol 147:519-532.

Sabry, J.H., O'Connor, T.P., Evans, L., Toroian-Raymond, A., Kirschner, M. (1991). Microtubule behavior during guidance of pioneer neuron growth cones in situ. J Cell Biol 115:381-395.

Sánchez, D., Ganfornina, M.D., Bastiani, M.J. (1995). Developmental expression of the lipocalin Lazarillo and its role in axonal pathfinding in the grasshopper embryo. Development 121:135-147.

Sánchez-Soriano, N., Prokop, A. (2005). The influence of pioneer neurons on a growing motor nerve in *Drosophila* requires the neural cell adhesion molecule homolog Fasciclin II. The Journal of Neuroscience 25:78-87.

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B., Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452:1002-1006.

Schneider, D. (1964). Insect antennae. Annu Rev Entomol 9:103-122.

Scott, K., Brady, R., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C., Axel, R. (2001). A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. Cell 104:661-673.

Seidel, C., Bicker, G. (2000). Nitric oxide and cGMP influence axogenesis of antennal pioneer neurons. Development 127:4541-4549.

Selzer, R., Schaller-Selzer, L. (1987). Structure and function of luminal neurons in the early embryonic antenna of the american cockroach, *Periplaneta americana*. Dev Biol 122, 363-372.

Shankland, M. (1981). Embryonic development of a sensory afferent projection in the grasshopper embryo. I. Growth of peripheral pioneer axons within the central nervous system. J Embryol Exp Morphol 64:169-185.

Simpson, J.H. (2009). Mapping and manipulating neural circuits in the fly brain. In: Genetic Dissection of Neural Circuits and Behavior, 1st Edition. Goodwin, S. (Ed.) Elsevier. pp. 79-143.

Singer, M., Nordlander, R.H., Egar, M. (1979). Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt: the blueprint hypothesis of neuronal pathway patterning. J Comp Neurol 185:1-22.

Sivan-Loukianova, E., Eberl, D.F. (2005). Synaptic ultrastructure of *Drosophila* Johnston's organ axon terminals as revealed by an enhancer trap. J Comp Neurol 491:46-55.

Slifer, E.H., Prestage, J.J., Beams, H.W. (1957). The fine structure of the long basiconic sensory pegs of the grasshopper (Orthoptera, Acrididae) with special reference to those on the antenna. J Morphol 101:359-397.

Slifer, E.H., Prestage, J.J., Beams, H.W. (1959). The chemoreceptors and other sense organs on the antennal flagellum of the grasshopper (Orthoptera; Acrididae). J Morphol 105:145-191.

Snodgrass, R.E. (1935). Principles of Insect Morphology. McGraw-Hill, New York.

Snow, P.M., Bieber, A.J., Goodman, C.S. (1989). Fasciclin III: a novel homophilic adhesion molecule in *Drosophila*. Cell 59:313-323.

Sönderström, K.-O., Parvinen, L.-M., Parvinen, M. (1977). Early detection of cell damage by supravital acridine orange staining. Experientia 33:265-266.

Soria, J.M., Fairén, A. (2000). Cellular mosaics in the rat marginal zone define an early neocortical territorialization. Cereb Cortex 10:400-412.

Sousa-Nunes, R., Yee, L.L., Gould, A.P. (2011). Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in *Drosophila*. Nature 471:508-513.

Spreij, T.E. (1971). Cell death during the development of the imaginal disks of *Calliphora erythrocephalia*. Neth J Zool 21:221-264.

Stainier, D.Y., Gilbert, W. (1990). Pioneer neurons in the mouse trigeminal sensory system. PNAS 87(3): 923-927.

Stevenson, P.A., Hofmann H.A., Schoch, K., Schildberger K.. (2000) The fight and flight responses of crickets depleted of biogenic amines. J Neurobiol 43:107-20.

Supèr, H., Martínez, A., Del Río, J.A., Soriano, E. (1998). Involvement of distinct pioneer neurons in the formation of layer-specific connections in the hippocampus. The J Neurosci 18:4616-4626.

Takagi, A., Kurita, K., Terasawa, T., Nakamura, T., Bando, T., Moriyama, Y., Mito, T., Noji, S., Ohuchi, H. (2012). Functional analysis of the role of *eye absent* and *sine oculis* in the developing eye of the cricket *Gryllus bimaculatus*. Develop Growth Differ 54:227-240.

Takizawa, T., Meshorer, E. (2008). Chromatin and nuclear architecture in the nervous system. Trends Neurosci 31:343-352.

Torto, B., Obeng-Ofori, D., Njagi, P.G.N., Hassanali, A., Amiani, H. (1994). Aggregation pheromone system of adult gregarious desert locust *Schistocerca gregaria* (Forskal). J Chem Ecol 20:1749-1762.

Ullmann, S.L. (1964). The origin and structure of the mesoderm and the formation of coelomic sacs in *Tenebrio molitor* L. (Insect, Coleoptera). Phil Trans R Soc B 747:245-277.

Ungerer, P., Geppert, M., Wolff, C. (2011). Axogenesis in the central and peripheral nervous system of the amphiod crustacean *Orchestia cavimana*. Integ Zool 6:28-44.

Ungerer, P., Scholtz, G. (2008). Filling the gap between identified neuroblasts and neurons in crustaceans adds new support for Tetraconata. Proc R Soc B 275:369-376.

Usui-Isihara, A., Simpson, P., Usui, K. (2000). Larval multidendrite neurons survive metamorphosis and participate in the formation of imaginal sensory axonal pathways in the notum of *Drosophila*. Dev Biol 225:357-369.

Varela, N., Couton, L., Gemeno, C., Avilla, J., Rospars, J.P., Anton, S. (2009). Three-dimensional antennal lobe atlas of the oriental fruit moth, *Cydia molesta* (Busck) (Lepidoptera: Torticidae): comparison of male and female glomerular organization. Cell Tissue Res 337:513-526.

Venken, K.J.T., Simpson, J.H., Bellen, H.J. (2011). Genetic manipulation of genes and cells in the nervous system of the fruit fly. Neuron 72:202-230.

Vogt, R.G., Riddiford, L.M. (1981). Pheromone binding and inactivation by moth antennae. Nature 293:161-163.

Voronezhskaya, E.E., Ivashkin, E.G. (2010). Pioneer neurons: a basis or limiting factor of Lophotrochozoa nervous system diversity. Russian J Dev Biol 41:337-346.

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., Axel, R. (1999). A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96:725-736.

Vosshall, L.B., Wong, A.M., Axel, R. (2000). An olfactory sensory map in the fly brain. Cell 102:147-159.

Whitlock, K.E., Westerfield, M. (1998). A transient population of neurons pioneers the olfactory pathway in zebrafish. J Neurosci 18:8919-8927.

Wicher, D., Schafer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., Hansson, B.S. (2008). *Drosophila* odorant receptors are both ligand-gated and cycli-nucleotide-activated cation channels. Nature 452:1007-1011.

Wigglesworth, V.B. (1956). The haemocytes and connective tissue formation in an insect, *Rhodnius prolixus* (Hemiptera). Quarterly J Micro Science 97:89-98.

Wigglesworth, V.B. (1973). Haemocytes and basement membrane formation in *Rhodnius*. J Insect Physiol 19:831-844.

Williams, J.L.D. (1975). Anatomical studies of the insect central nervous system: a ground-plan of the midbrain and an introduction to the central complex of the locust, *Schistocerca gregaria* (Orthoptera). J Zool (Lond) 176:67-86.

Williams, J.L.D., Boyan, G.S. (2008). Building the central complex of the grasshopper *Schistocerca gregaria*: axons pioneering the w, x, y, z tracts project onto the primary commissural fascicle of the brain. Arth Struct Dev 37:129-140.

Williams, D.W., Shepherd D. (2002). Persistent larval sensory neurones are required for the normal development of the adult sensory afferent projections in *Drosophila*. Development 129:617-624.

Wong, J.T.W., Yu, W.T.C., O'Connor, T.P. (1997). Transmembrane grasshopper semaphorin I promotes axon outgrowth in vivo. Development 124:3597-3607-

Yack, J.E. (2004). The structure and function of auditory chordotonal organs in insects. Microsc Res Tech 63:315-337.

Yang, Y., Krieger, J., Zhang, L., Breer, H. (2012). The olfactory co-receptor Orco from the migratory locust (*Locusta migratoria*) and the desert locust (*Schistocerca gregaria*): identification and expression pattern. Int J Biol Sci 8:159-170.

Younossi-Hartenstein, A., Ehlers, U., Hartenstein, V. (2000). Embryonic development of the rhabdocoel flatworm *Mesostoma lingua* (Abildgaard, 1789). J Comp Neurol 416:461-474.

Zacharias, D., Williams, J.L.D., Meier, T., Reichert, H. (1993). Neurogenesis in the insect brain: cellular identification and molecular characterization of brain neuroblasts in the grasshopper embryo. Development 118:941-955.

Zinn, K., McAllister, L., Goodman, C.S. (1988). Sequence analysis and neuronal expression of fasciclin I in grasshopper and drosophila. Cell 53:577-587.

# 8. List of Figures and Tables

## 9. List of Abbreviations

| | |
|---|---|
| A1-A4 | annuli 1, 2, 3, 4 of the embryonic antenna |
| AMMC | antennal mechanosensory and motor center |
| AO | acridine orange (3,6-bis(dimethylamino) acridine) |
| BP | base pioneer |
| BSA | bovine serum albumin |
| BrdU | 5-Bromo-2'-deoxyuridine |
| CCD | charge-coupled device |
| cGMP | cyclic guanosine monophosphate |
| CSP | chemosensory protein |
| Cy3 | cyanine 3 |
| dA1 | dorsal A1 |
| DAPI | 4,6-diamidino-2-phenylindole |
| dBP | dorsal base pioneer |
| DNA | deoxyribonucleic acid |
| dUTP | deoxyuridine triphosphate nucleotides |
| EdU | 5-ethynyl-2'-deoxyuridine |
| EGTA | ethylene glycol tetraacetic acid |
| Exd | Extradenticle |
| FCS | fetal calf serum |
| Fig. | figure |
| FITC | fluorescein |
| GABA | gamma-Aminobutyric acid |
| GC | guanylyl cyclase |
| GFP | green fluorescent protein |
| GPI | glycosylphosphatidylinositol |
| GR | gustatory receptor |
| h | hour(s) |
| HRP | horseradish peroxidase |
| *hth* | homothorax |
| IR | ionotropic receptor |
| K+ | potassium ions |
| Lach | Lachesin |
| Laz | Lazarillo |
| mAb | monoclonal antibody |
| min | minutes |
| NGS | normal goat serum |
| NHS | normal horse serum |
| NO | nitric oxide |
| No. | number of |
| NTA | nerve tract associated |
| OBP | odorant-binding protein |
| OR | olfactory receptor |
| ORco | OR coreceptor |
| OSN | olfactory sensory neuron |
| PBS | phosphate buffered saline |
| PBT | PBS with Triton-X |
| PFA | paraformaldehyde in PBS |
| PH3 | phosphohistone-3 |

| | |
|---|---|
| PIPES | piperazine-N,N'-bis(2-ethanesulfonic acid) |
| PIPES-FA | paraformaldehyde in 100 mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$ |
| RNA | ribonucleic acid |
| s | seconds |
| S phase | DNA synthesis phase |
| SOP | sensory organ precursor |
| TES | N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid) |
| Ti1 | tibial 1 |
| TUNEL | terminal deoxynucleotidyl transferase dUTP nick end labeling |
| UAS | upstream activation sequence |
| vA1 | ventral A1 |
| vBP | ventral base pioneer |
| °C | degrees Celsius |
| µ | micro |

# 10. List of Publications

Ehrhardt, E., Liu, Y., Boyan, G.S. (2015). Axogenesis in the antennal nervous system of the grasshopper *Schistocerca gregaria* revisited: the base pioneers. Dev Genes Evol 225:39-45.

Ehrhardt, E., Kleele, T., Boyan, G.S. (2015). A method for immunolabeling neurons in intact cuticularized insect appendages. Dev Genes Evol 225:187-194.

Boyan, G.S., Ehrhardt, E. (2015). Pioneer neurons of the antennal nervous system project to protocerebral pioneers in the grasshopper *Schistocerca gregaria*. Dev Genes Evol 225:377-382.

Ehrhardt, E., Graf, P., Kleele, T., Liu, Y., Boyan, G.S. (2015). Fates of identified pioneer cells in the antennal nervous system of the grasshopper *Schistocerca gregaria*. Arth Struct Dev. pii: S1467-8039(15)00126-7. doi: 10.1016/j.asd.2015.11.001

# Curriculum Vitae

## Education

**PhD, Neuroscience, Graduate School of Systemic Neurosciences, Ludwig-Maximilians-Universität**, expected April 2016. Thesis title: **Early development of a sensory system: the antenna of the grasshopper *Schistocerca gregaria*** (funded by the GSN). Thesis Advisory Committee: Prof G.S. Boyan, Dr Y. Liu and Dr O. Griesbeck.

**MSc, Neuroscience, Graduate School of Systemic Neurosciences, Ludwig-Maximilians-Universität**, October 2012. Thesis title: **Motion tracking by non-linear retinal processing** (funded by the DAAD). Thesis Advisor: Prof T. Wachtler.

**BA, Biochemistry and German, Bowdoin College**, May 2010. Awarded honors in biochemistry. Thesis: **The effect of myosuppressin on nitric oxide feedback in the heart of the lobster *Homarus americanus***. Thesis Advisor: Prof P. Dickinson.

## Other research and employment

*Freie Universität* Neurobiology Dept., Pflüger lab. Berlin. Germany. 2009. Student Researcher.
-Dissected locusts and performed Western blots to detect the enzyme tyramine ß-hydroxylase in the central nervous system of locusts.

*Center for Vision Research*. Barlow lab. Syracuse. New York. 2007. Student Researcher.
-Collected tissue samples from young mice, genotyped tissue samples using PCR and gel electrophoresis, tested blood glucose levels, tested the vision of mice.

*Hawthorne-Longfellow Library*. Maine. 2006-2007. Interlibrary Loan Lending Assistant.
-Processed lending requests, scanned and sent articles, shipped books.

*Fruit Valley Veterinary Clinic*. Oswego. New York. Summers 2002-04, 2006, 2008. Veterinary Assistant and Animal Handler.
-Restrained animals, helped with treatments and medical record-keeping.

## Scholarships and awards

- National Merit Scholarship
- NSF Research Experience for Undergraduates Fellowship Award
- IDeA Network of Biomedical Research Excellence Academic Year Supply Award
- DAAD Study Scholarship: extended for a second year

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Volume 71, 1972, p 128-149,
Dr Michael Gewecke,

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**Figure 2.2**
Cellular and Molecular Life Sciences.
Expression and immunolocalisation of odorant-binding and chemosensory proteins in locusts,
Volume 62, 2005, p 1156-1166.
X. Jin, A. Brandazza, A. Navarrini, L. Ban, S. Zhang, R.A. Steinbrecht, L. Zhang, P. Pelosi,

**License number**: 3797130511634

**Figure 2.2**
Journal of Biosciences,
Antennal sensilla of grasshoppers (Orthoptera: Acrididae) in relation to food preferences and habits,
Volume 28, 2003, p 743-752.
Hu-Hai Chen, Yun-Xian Zhao, Le Kang,

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Development, Volume 54, 1979, p. 47-74.

dev.biologists.org/content/54/1/47.short

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E.E. Ball, H. Gert de Couet, P.L. Horn, J.M.A. Quinn,
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dev.biologists.org/content/99/2/255.short
**License number**: 3797631386590

**Figure 2.5**
Reprinted from Journal of Morphology,
R.F. Chapman,
Development of phenotypic differences in sensillum populations on the antennae of a grasshopper, *Schistocerca americana*,
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**Figures 2.6, 2.7, 2.8, 2.10**
Reprinted from Arthropod Structure & Development, Volume 33,
G.S. Boyan, J.L.D. Williams,
Embryonic development of the sensory innervation of the antenna of the grasshopper *Schistocerca gregaria*,
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www.sciencedirect.com/science/article/pii/S146780390400026X
**License number:** 3797660401138

**Figures 2.11b**
From Science, Volume 245,
S.K. McConnell, A. Ghosh, C.J. Shatz,
Subplate neurons pioneer the first axon pathway from the cerebral complex,
Copyright 1989, reprinted with permission from AAAS.
science.sciencemag.org/content/245/4921/978.short
**License number:** 3797680419928

**Figure 2.11c**
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from Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of *Drosophila*,
A. Hidalgo, A.H. Brand,
Volume 124, 1997, permission conveyed through Copyright Clearance Center, Inc
dev.biologists.org/content/124/17/3253.short
**License number**: 3797681061650

**Figure 2.12a,c**
Republished with permission from Macmillan Publishers Ltd: Nature,
Pioneer neurones in an insect embryo,
C.M. Bate,
Volume 260, copyright 1976,
**License number**: 3800740087212

**Figure 2.12c,d,e**
Republished with permission from Macmillan Publishers Ltd: Nature,
Peripheral pathways are pioneered by an array of central and peripheral neurones in grasshopper embryos,
Robert K. Ho, Corey S. Goodman,
Volume 297, copyright 1982
**License number**: 3800740252857

**Figures 2.13**
Reprinted from Arthropod Structure & Development, Volume 33,
G.S. Boyan, J.L.D. Williams,
Embryonic development of the sensory innervation of the antenna of the grasshopper *Schistocerca gregaria*,
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www.sciencedirect.com/science/article/pii/S146780390400026X
**License number:** 3805410917061

**Figure 5.3, 5.4, 5.6, 5.7, 5.9**
Springer and Development Genes & Evolution, Volume 255, 2015, p. 39-45,
Axogenesis in the antennal nervous system of the grasshopper *Schistocerca gregaria* revisited: the base pioneers,
Erica Ehrhardt, Yu Liu, George Boyan,
© Springer-Verlag Berlin Heidelberg 2014. With kind permission from Springer Science and Business Media.
**License number**: 3750840211371

**Figure 5.11, 5.12, 5.13**
Springer and Development Genes & Evolution, Volume 255, 2015, p. 377-382,
Pioneer neurons of the antennal nervous system project to protocerebral pioneers in the grasshopper *Schistocerca gregaria,*
George Boyan, Erica Ehrhardt,
© Springer-Verlag Berlin Heidelberg 2015. With kind permission from Springer Science and Business Media.
**License number**: 3750830665698

**Figures 5.14, 5.15, 5.16, 5.17, 5.18, 5.19, 5.20, 5.21, 5.22**
Reprinted from Arthropod Structure & Development,
Erica Ehrhardt, Philip Graf, Tatjana Kleele, Yu Liu, George Boyan,
Fates of identified pioneer cells in the developing antennal nervous system of the grasshopper *Schistocerca gregaria,*
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**License number:** 3767550189995

**Figure 5.24, 5.25, 5.26**
Springer and Development Genes & Evolution, Volume 255, 2015, p. 187-194,
A method for immunolabeling neurons in intact cuticularized insect appendages,
Erica Ehrhardt, Tatjana Kleele, George Boyan,
© Springer-Verlag Berlin Heidelberg 2015. With kind permission from Springer Science and Business Media.
**License number**: 3750831476722

**Figure 6.1**
Republished with permission of Company of Biologists,
from Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of *Drosophila*,
A. Hidalgo, A.H. Brand,
Development, Volume 124, 1997, permission conveyed through Copyright Clearance Center, Inc
dev.biologists.org/content/124/17/3253.short
**License number**: 3801980656079

**Figure 6.2**
Republished with permission of Company of Biologists,
from Nitric oxide and cGMP influence axogenesis of antennal pioneer neurons,
C. Seidel, G. Bicker,
Development, Volume 127, 2000, permission conveyed through Copyright Clearance Center, Inc
dev.biologists.org/content/127/21/4541.short
**License number**: 3801991179604

**Figure 6.3**
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from Pioneer neurones use basal lamina as a substratum for outgrowth in the embryonic grasshopper limb,
H. Anderson, R.P. Tucker,
Development, Volume 104, 1988, permission conveyed through Copyright Clearance Center, Inc
dev.biologists.org/content/104/4/601.short
**License number**: 3798201053856

**Figures 6.4**
Reprinted from Tissue and Cell, Volume 22,
T.A. Keil, C. Steiner,
Morphogenesis of the antenna of the male silkmoth, *Antheraea pholyphemus*. II. Differential mitoses of "dark" precursor cells create the Anlagen of sensilla,
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www.sciencedirect.com/science/article/pii/0040816690090066I
**License number:** 3802000856983

**Figure 6.5**
Reprinted from Journal of Morphology,
Eleanor H. Slifer, James J. Prestage, Harold, W. Beams,
The fine structure of the long basiconic sensory pegs of the grasshopper (orthoptera, acrididae) with special reference to those on the antenna,
Copyright 1957, with permission from John Wiley and Sons.
**License number**: 380201081136

**Figure 6.5**
Reprinted from Journal of Morphology,
Eleanor H. Slifer, James J. Prestage, Harold, W. Beams,
The chemoreceptors and other sense organs on the antennal flagellum of the grasshopper (Orthoptera; acrididae),
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**License number**: 3802010566393

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Reprinted from Arthropod Structure & Development,
Erica Ehrhardt, Philip Graf, Tatjana Kleele, Yu Liu, George Boyan,
Fates of identified pioneer cells in the developing antennal nervous system of the grasshopper *Schistocerca gregaria,*
Copyright 2016, with permission from Elsevier.
www.sciencedirect.com/science/article/pii/S146780390400026X
**License number:** 3767550189995

**Figures 6.8**
From Science, Volume 223,
John Berlot, Corey S. Goodman,
Guidance of Peripheral Pioneer Neurons in the Grasshopper: Adhesive Hierarchy of Epithelial and Neuronal Surfaces,
Copyright 1984, reprinted with permission from AAAS.
science.sciencemag.org/content/223/4635/493.short
**License number:** 3802011483435

**Figures 6.10**
Reprinted from Journal of Insect Physiology, Volume 37,
Christiane K. Bauer, Michael Gewecke
Motoneuronal control of antennal muscles in *Locusta migratoria*,
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Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation **'Early development of a sensory system: pioneer neurons in the antenna of the grasshopper *Schistocerca gregaria*'** selbständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittle bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation **'Early development of a sensory system: pioneer neurons in the antenna of the grasshopper *Schistocerca gregaria*'** is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den/Munich, date Unterschrift/Signature

## List of author contributions

| primary author(s) | figure number |
|---|---|
| George Boyan | Fig. 5.1a-d; Fig. 5.11a-c; Fig. 5.12; Fig. 5.13; Fig. 5.20; Fig. 6.7 |
| Erica Ehrhardt | Fig. 5.1e,f; Fig. 5.2; Fig. 5.4; Fig. 5.5; Fig. 5.6; Fig. 5.7; Fig. 5.8; Fig. 5.10; Fig. 5.11d; Fig. 5.14; Fig. 5.15; Fig. 5.19; Fig. 5.21; Fig. 5.22; Fig. 5.23; Fig. 5.25; Fig. 5.26; Fig. 5.27; Fig. 6.6; Fig. 6.9 |
| Tatjana Kleele | cover image; Fig. 5.3; Fig. 5.24 |
| Michaela Güntner, Bertram Niederleitner, George Boyan | Fig. 5.9 |
| Yu Liu | Fig. 5.16 |
| Philip Graf | Fig. 5.17; Fig. 5.18 |

Signature of lab head.....

Signature of doctoral candidate.....