

Neuromodulation in the Chemosensory System of Mosquitoes - Neuroanatomy and Physiology

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Cover: A female *Aedes aegypti* gorging on a human host (Siju)
(Photo by Rickard Ignell)

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Abstract

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The impact of mosquito vectored disease on global public health is overwhelming. Mosquitoes depend on chemosensory capability for blood-feeding and as such have a highly developed chemosensory system, which consists of peripheral and central components. The repertoire of mosquito behaviors during their life cycle are modified by external and internal factors. The main goal of this thesis is to describe some factors that modulate odor-associated behavior, focusing on biogenic amines, neuropeptides, and physiological state change.

I generated a detailed distribution map of serotonin, one of the major biogenic amines in the central and peripheral chemosensory system of *Aedes aegypti*. The arborization pattern of serotonin-immunoreactive (SI) neurons in the central chemosensory neuropil included the antennal lobe (AL), subesophageal ganglion, tritocerebrum and higher brain centers. In addition, I found SI fibers in the peripheral chemosensory organs: the antennae, the maxillary palps and the labia. Furthermore, to investigate the affects of the gonotrophic and circadian state of mosquitoes on serotonin, dopamine and octopamine levels in the heads of female *Ae. aegypti*, I used high-performance liquid chromatography coupled with electrochemical detection. Changes in the titer level of these biogenic amines are correlated to flight activity and physiological status of this mosquito. The neuropeptides in the mosquito AL are a highly diverse class of neurochemicals described by matrix assisted laser desorption ionization time-of-flight mass spectrometry of a single AL. I isolated and identified a total of 26 neuropeptides belonging to 10 gene families. Additionally, the cellular distribution of four major families of neuropeptides revealed distinct localization patterns of each of these families in the AL and to other olfactory-associated neuropil areas in the brain. Finally, I employed single sensillum recording to investigate the modulation of odorant receptor neurons housed in the antennal *sensilla trichodea* after a blood meal. Three functional classes of short blunt-tipped type II sensilla displayed a higher sensitivity after blood feeding to the host and oviposition-associated cues, indole and three phenolic compounds. This study indicates modulation of olfactory behavior occurs at the peripheral level post blood-meal, which may be associated with the physiological status of the mosquitoes.

Keywords: olfaction, disease vector, biogenic amines, neuropeptides, neuromodulation, anatomy, electrophysiology

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Dedication

To my family, for being everything to me.
And in memory of my late grandfather, who taught me simplicity in life

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Siju, K.P., Hansson, B.S. & Ignell, R. Immunocytochemical localization of serotonin in the central and peripheral chemosensory system of mosquitoes. *Arthropod Structure and Development* 37, 248-259.
- II Siju, K.P., Gramsbergen, J.B., Hansson, B.S. & Ignell. Quantification of biogenic amines in female yellow fever mosquitoes, *Aedes aegypti*, throughout the first gonotrophic cycle. Submitted
- III Siju, K.P., Schachtner, J., Scheiblich, H., Neupert, S., Predel, R., Hansson, B.S. & Ignell, R. Neuropeptides in the antennal lobe of the yellow fever mosquito, *Aedes aegypti*. Manuscript
- IV Siju, K.P., Hill, S.R., Hansson, B.S. & Ignell, R. Influence of blood meal on the responsiveness of olfactory receptor neurons in antennal sensilla trichodea of the yellow fever mosquito, *Aedes aegypti*. Manuscript

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1 Objectives

The collective objectives of my thesis work were to explore aspects of neuromodulation in the chemosensory systems of the mosquito. This included neuroanatomical and biochemical studies of neuromodulators, as well as electrophysiological studies demonstrating neuromodulation at the peripheral olfactory system level.

2 Introduction

Insects are the most diverse group of organisms inhabiting our planet, and have in the course of evolution adapted to a variety of habitats. In doing so, they have developed various degrees of specialization of their sensory systems. The main task of the sensory systems is to read the external environment and display it accurately to the organism in the form of neural representations (Vosshall, 2003). Most insects depend heavily upon their chemosensory systems to survive in their environment (Ache & Young, 2005), and as a consequence of this the chemosensation is among their most highly developed sensory modalities (Strausfeld & Hildebrand, 1999). Interestingly, during the course of evolution the chemosensory systems have undergone modifications to be able to address the needs of different animals, without altering their basic properties, which are more or less conserved across the insect order (Eisthen, 2002; Strausfeld & Hildebrand, 1999). This warrants a question: how have insects adapted to their specialized habits and habitats without major changes in the basic structure and function of their chemosensory system? The work in this thesis is mainly focusing on these questions. These issues are related to the plasticity and modulation of the chemosensory systems, which enables insects to adapt to temporary changes in their internal and external environment. Investigations into these questions may provide us with an opportunity to learn and appreciate the mechanism of information processing and modulation in insect chemosensory system.

In exploring the modulatory aspects of insect chemosensory systems, I have chosen to work with mosquitoes, the most dangerous animals on earth. Mosquitoes are effective vectors for many socio-economically important diseases, and as such, have gained considerable attention. The behavioral

repertoire displayed by mosquitoes is astonishing, and all behaviors are influenced by external or internal factors. Although we have a general understanding of the behavioral changes triggered by these factors, the physiological and neurochemical processes eliciting these transformations are largely unknown. Based on the rather stringent behavioral changes exhibited by the mosquitoes, I believe that mosquitoes may be good model organisms to study the affect of modulators on specific behaviors, and more specifically chemosensory-driven behaviors. One major advantage of studying chemosensory systems is the recent developments in both understanding and technology within this field. Here, I focus primarily on the yellow fever mosquito, *Aedes aegypti*. Thus, most of the descriptions in this thesis mainly pertain to this species. However, where information is available and relevant, I mention other species of mosquitoes as well.

3 Mosquito

Mosquitoes have long maintained a special position in the way they have shaped human history. They are often referred to as the ‘most dangerous animal on earth’, which may sound strange considering their size, but the damage and trauma they can inflict on human lives aptly justify the title and belittle their small size. There are approximately 3500 identified species of mosquitoes, which belong to a single family, Culicidae. This family is further subdivided into three sub-families including Culicinae (Taxonomic serial number 126087), Anophelinae (TSN 125955) and Toxorhynchitinae (TSN 125931). Members of the Culicidae family span millions of years of evolutionary history, and considerable changes in their morphology, physiology and behavior have enabled them to adapt to the wide variety of environments in which they live in today. Both male and female mosquitoes feed on food source whose primary ingredient is sugar. However, during the course of evolution, structural features adapting female mosquitoes to hematophagy as well have developed. At present, most female mosquitoes (except Toxorhynchites) require a vertebrate blood-meal in order to complete a successful reproductive cycle.

Even though not all mosquitoes are capable of transmitting diseases, the small fraction of species that do act as vectors of several diseases are capable of causing devastating effects on human population around the world. Among all mosquito-vectored-diseases, malaria is the most devastating, and most publicly known. Although malaria affects all ages, malaria mortality is

most prevalent in children, pregnant women and the elderly, and kills around 1-3 million people annually.



Figure 1. A female *Aedes aegypti* engorging a blood-meal from a human host (Photo by Rickard Ignell)

In the present study, I have mainly worked with the culicine mosquito, *Ae. aegypti*, also known as the yellow fever mosquito (Figure 1). Yellow fever is a viral disease causing large epidemics in tropical regions of Africa and Americas: 500 million people are at risk every year (www.who.net). Apart from being the vector for yellow fever, this mosquito species also transmits other viral diseases such as dengue and chikungunya. Dengue fever is a viral disease which is endemic in at least 100 countries including about 40% of the world population. Cases of dengue hemorrhagic fever are the leading causes for childhood mortality in many Asian countries. In general, *Ae. aegypti* is best known for being an extremely effective vector for many arboviral diseases around the world. An estimate of the fatality caused by these mosquito-transmitted diseases exceeds several millions worldwide. Recent years have seen a resurgence of many of the diseases vectored by *Ae. aegypti* reported from around the world. The best examples would be the outbreaks of dengue, yellow fever and chikungunya (Gubler, 2004; Kaur *et al.*, 2006). These diseases are also invading new regions of the world: the first outbreak of chikungunya in Europe occurred in 2007 in the north-eastern Emilia Romagna region of Italy (www.who.int). The reason for these outbreaks are not fully understood, however several factors give us

indications: increases in population growth, global travel and global temperature increases might be some factors that caused the resurgence of the epidemics. Moreover, increase in urbanization and ineffective public health infrastructure have also added to the severity of these outbreaks (Gubler, 2004). In addition, climate change and subsequent rise in temperature in several countries, otherwise inhospitable to these diseases, have prompted many scientists to closely monitor the rise in mosquito population and potential outbreak of diseases (Gould & Higgs, 2009).

Although different species of mosquitoes are more or less specialized vectors for diseases, the best method to tackle the spread of any mosquito-borne disease is to implement effective control mechanisms to limit the spread of mosquito populations and thereby disease transmission. In the past, several diseases control measurements have been adapted. Many of these methods advocated the use of insecticides to kill off the mosquitoes. But, as it happened in other insect control programs, formation of insecticide resistance and the environmental toxicity of the insecticides drastically reduced the effectiveness of these methods. Even though these methods are still used in control strategies together with other methods, we still need to formulate novel safe and efficient ways to control mosquito populations. One highly supported initiative is to use odor-mediated repellents in conjunction with odor-baited traps to achieve a push-pull strategy. Another method that could be used for insect control is using chemicals that specifically target the signaling mechanisms at the nervous system level in mosquitoes (van der Goes van Naters & Carlson, 2006).

4 Behavior

The behavior of any organism is not a single and simple event – it is the culmination of several closely linked and beautifully orchestrated biological events involving external and endogenous factors. Mosquitoes have an extensive behavioral repertoire, and these behaviors are often plastic enabling the mosquito to adapt to changes in their environment as well as to changes of its physiological status. These behaviors include mate finding, location of energy-rich resources and blood hosts as well as oviposition sites.

In the life cycle of a mosquito, reproduction is, by far, the most important and at the same time the most demanding physiological process. In order to attain reproductive success and species survival, each female mosquito will go through a sequence of behavioral and physiological events that ultimately culminate in egg-laying. The reproductive phase in the life

cycle of a female mosquito is known as the gonotrophic cycle (Clements, 1999). A full gonotrophic cycle of most female mosquitoes is marked by two behaviorally and physiologically distinct phases. The initial phase includes host-seeking and blood-feeding behaviors. This phase is followed by a pre-oviposition behavior and ultimately oviposition. The number of gonotrophic cycles displayed by a single female mosquito is dependent on her life span and nutritive status; she may oviposit from one to thirteen times (Klowden, 1990).

Host-seeking and blood-feeding behavior

Mosquitoes utilize both physical and visual cues for driving their host-seeking and blood-feeding behaviors. However, the primary sensory cues used in host-seeking, identification and acceptance are chemosensory cues. The behavioral repertoire leading up to host acceptance and ultimately blood-feeding is triggered by host specific cues detected by olfactory and gustatory sensory hairs, called sensilla, that cover the peripheral chemosensory organs.

Freshly eclosed female mosquitoes, up to 24 h post emergence, generally do not respond to host cues. Their competence to detect chemical cues usually develops 30h post-emergence and the sensitivity, at least of the olfactory system, increases with age and reaches an optimal level by 14 days (Clements, 1999; Davis, 1984b). Based on behavioral observations we know that mosquitoes are differentially attracted to human and other vertebrate blood hosts based on the more or less distinct olfactory cues (signature) they release (e.g. McIver, 1968; Bosch, Geier & Boeckh, 2000; Steib, Geier & Boeckh, 2001; Ponlawat & Harrington, 2005). Thus, profiling the composition of volatiles released from single blood hosts is the primary step to take in order to establish an understanding of the behavioral processes that lead up to blood-feeding. The volatile cues comprise organic and inorganic compounds derived from human breath, sweat and urinary/fecal contamination from the body surface (Clements, 1999). Several studies have attempted to isolate and chemically identify behaviorally active compounds from various blood-host odor extracts (Geier *et al.*, 1999; Bernier *et al.*, 2007; Logan *et al.*, 2007; Ghaninia *et al.*, 2008). For example, GC-MS analysis of human skin emanates have revealed 300-400 chemical compounds that all may play an important role as host attractants for different species of mosquitoes (Bernier *et al.*, 2000). A wide range of physiological studies have consistently identified compounds in these

emanates, as well as in human breath, that all seem to be involved in the host attraction behavior of various mosquito species. These compounds include L-lactic acid (e.g. McIver, 1968; Bernier *et al.*, 2002; Bernier *et al.*, 2007;), 1-octenol-3-ol (e.g. Klein *et al.*, 1990), ammonia (e.g. Geier *et al.*, 1999) and CO₂ (Dekker *et al.*, 2005) Among these compounds, CO₂ is one of the most important cues, and has been shown to be involved in behavioral activation (Dekker *et al.*, 2005) of several species of mosquitoes. As a result, several species, particularly zoophilic ones, have developed a high sensitivity to detect CO₂. Most of the identified host compounds do not act individually. Instead, compounds are often required to act synergistically to trigger a behavioral response in the mosquito. For example, L-lactic acid together with ammonia or fatty acids trigger attraction in both *Aedes* and *Anopheles* mosquitoes (Geier *et al.*, 1999; Bosch, Geier & Boeckh, 2000; Bernier *et al.*, 2007).

Behavioral experiments in *Ae. aegypti* as well as in *An. gambiae* have shown that fatty acids are an important group of attractive compounds mediating host-seeking; in fact, these compounds have been suggested to represent the olfactory signature of humans. Attraction of these mosquitoes is influenced by carbon chain length, blend composition and also the addition of synergistic compounds such as L-lactic acid, ammonia and CO₂ (Bosch, Geier & Boeckh, 2000). Recent technological development in the field have further advanced our ability to identify novel behavioral attractants. These advances primarily involve gas chromatography coupled to electrophysiological investigations of the olfactory system of the mosquito (Logan *et al.*, 2007; Ghaninia *et al.*, 2008). Logan *et al.* (2007) and Ghaninia *et al.*, (2008), have reported several novel compounds from human sweat that have evoked physiological or behavioral response in *Ae. aegypti*. Those compounds includes octanal, nonanal, decanal, dodecanl, geranyl acetone and 2,6-dimethyl-2,6-octadien (Logan *et al.*, 2007; Ghaninia *et al.*, 2008).

Pre-oviposition and oviposition behavior

The pre-oviposition behavior of female mosquitoes includes active search for an oviposition site. Most females tend to lay their eggs in an open or enclosed aquatic habitat. The predominant oviposition sites are stagnant water in small pools, ponds or tree holes (Clements, 1999). In addition, many mosquito species, for example *Ae. aegypti*, prefer to oviposit in man-made habitats occurring with increased frequency near human habitation,

such as water-storage tanks, discarded containers and vehicle tires. The main criteria for selecting oviposition sites are the chances of the survival of their progeny, escape from predators and other environmental hazards such as changes in temperature and water condition. As in host-seeking behavior, several cues, including visual and physical cues, are believed to be involved in oviposition site selection (Bently & Day, 1989). However, chemical cues emanating from the oviposition site seem to be the predominant cues driving the mosquitoes to their preferred place for egg laying (Bently & Day, 1989; Millar *et al.*, 1992; Blackwell *et al.*, 1993; Isoe, Millar & Beehler, 1995; Du & Millar, 1999; Burkett-Cadena & Mullen, 2007).

In the past, several studies have demonstrated the chemical composition of the odor blends associated with oviposition-related sites (e.g. Blackwell *et al.*, 1993; Blackwell & Johnson, 2000; Rejmánková, Harbin-Ireland & Lege, 2000). Behavioral studies have also confirmed that different mosquito species are differentially attracted to these odorants (Mboera *et al.*, 1999; Mboera *et al.*, 2000; Ganesan *et al.*, 2006; Ponnusamy *et al.*, 2008.). However, many oviposition sites are shared by different species. By analyzing the odor profile of many of the oviposition-associated sites, we now have substantial information regarding the chemical cues used by different mosquito species while locating their preferred oviposition sites (Bently & Day, 1989; Clements, 1999). In several species of mosquitoes, substances of conspecific egg, larval and pupal origin have been found to be oviposition attractants (Bently & Day, 1989). Several chemical metabolites produced by different bacterial species from organic infusions also serve as potential oviposition attractants (Bently & Day, 1989). These chemicals include phenol, 3-methylphenol, 4-methylphenol, 3-ethylphenol, 4-ethylphenol, indole, 3-methylindole etc. Some of these chemicals have also been found to be associated with mammalian waste products (Kline *et al.*, 1990; Miller *et al.*, 1992; Cork, 1996). In a recent study, Ponnusamy *et al.* (2008) demonstrated that bacteria-associated carboxylic acids and methyl esters from bamboo and white-oak leaf infusion showed potential oviposition attraction activity in gravid *Ae. aegypti*. Oviposition is the final step in a gonotrophic cycle of all female mosquito in which eggs are deposited at the suitable sites.

Biological rhythm

An inherent property of all eukaryotic organisms is the demonstration of rhythmicity in their life with well-defined periodicities in their physiology and behavior. These periodicities or rhythmic cycles are known as biological rhythms, which are controlled by an internal biological or circadian clock. These rhythms are always synchronized to environmental cues such as light and temperature. However, even in the absence of these cues an organism still exhibit their rhythmic activity, regulated by the presence of their self-sustaining endogenous biological clock (Saunders, 1997). Biological rhythms provide several adaptive advantages to the organism by allowing them to anticipate the cyclic environmental conditions and also the changes in these cyclic events, such as the small daily changes in the duration of daylight (Clements, 1999).

Many mosquitoes express a strong daily periodicity according to their physiological and behavioral status. These cyclical behavioral events include: locomotion and flight activity; nectar/host-seeking; nectar/blood-feeding; and the entire gonotrophic cycle. The periodic and rhythmic nature of these events enable mosquitoes to adapt to their environment even when the external conditions are not favorable. Each species of mosquitoes has evolved different patterns of daily rhythms for most of their behavioral activities. *Aedes aegypti* is a diurnal species, by definition this means that this it is active during the day. In fact, this mosquito species displays heightened flight activity early and late in photophase (Jones, 1981). In the case of *Anopheles* and *Culex* species, which are nocturnal/crepuscular in nature, the host-seeking and blood-feeding activities are confined to the scotophase period (Clements, 1999).

Both exogenous and endogenous factors can modify the periodicities exhibited by mosquitoes. External factors such as light intensity, temperature and wind can influence the biological rhythm and modify the behavior accordingly (Clements, 1999). Similarly, endogenous factors contribute in modifying the biological rhythm in mosquitoes. It has been shown that both insemination and blood-feeding cause a decrease in the flight activity pattern of female mosquitoes (Jones, 1981). In addition, several neurochemicals present in the central nervous system (CNS) are also involved in modulating the circadian rhythm activities in mosquitoes (Yuan *et al.*, 2005).

Why is the periodicity in the behavior of each mosquito species so important? In mosquitoes, the activity patterns are species-specific and

directly influence their abilities to act as vectors of diseases; during high activity periods mosquitoes engage in host-seeking and blood-feeding and that in turn increases spread of the pathogen. The knowledge about the activity pattern of each mosquito species will thus help us in understanding the disease transmission dynamics (Lazzari, Minoli & Barrozo, 2004).

5 Chemosensory system of mosquitoes

The system that detects and processes chemical signals from the environment is known as the chemosensory system. The chemosensory system can be divided into two principle components: 1) the peripheral system, in which the chemical information is detected and transformed into electrical information; and 2) the central system, in which the information is processed.

5.1 The peripheral chemosensory system

Three appendages on the head serve as the principal chemosensory organs in mosquitoes: the paired antennae, the paired maxillary palps and the labium (Figure 2A). The antennae and the maxillary palps serve as the main and secondary olfactory organs, respectively, whereas the labium is the main gustatory organ. A recent study has also shown that olfactory receptors are expressed in olfactory receptor neurons in labial sensilla of mosquitoes (Kwon *et al.*, 2006).

The antennae

The antennae, or flagella, are filamentous and composed of 13 flagellomeres (Figure 2A). In all mosquito species the antenna is sexually dimorphic: in females the olfactory sensing hairs, or sensilla, are distributed throughout the flagellomeres, whereas in males these sensilla are confined to the distal few segments (McIver, 1982). The antennae are attached to the head capsule through the cup-shaped pedicel and the ring-shaped scape. The cup-shaped pedicel contains Johnston's organ (JO), the peripheral mechanosensory organ in mosquitoes (Boo & Richards, 1975).

Anatomical and functional classification of antennal chemosensory sensilla

Hair-like, cuticular structures present on the chemosensory organs are known as sensilla, and house olfactory receptor neurons (ORN). The sensilla are either single-walled or double-walled, and are further sub-divided into morphological classes based on their characteristic size and shape. Single-walled and double-walled olfactory sensilla bears numerous pores or slits, respectively, that facilitate the entry of odor molecules into the lumen of the sensilla, where the ORNs dendrites swim in viscous fluid, the sensillum lymph. The cuticular sensillum covers and protects the dendritic branches of the ORNs, whose cell bodies often are located at the base of the sensillum shaft. The axons of these ORNs project together towards the brain and form the antennal nerve bundle, which extends towards the primary olfactory centre of the brain, the antennal lobe (AL).

There are approximately 1000 sensilla present on each antenna of a female *Ae. aegypti* (McIver, 1978, 1982). These sensilla may be separated into five morphological types, *sensilla chaetica*, *sensilla ampullacea*, *sensilla coeloconica*, *sensilla trichodea* and grooved pegs (McIver, 1978, 1982). Among these, *sensilla chaetica*, *sensilla ampullacea* and *sensilla coeloconica* contain sensory neurons responsible for mechano-, thermo- or hygro-reception, constituting up to 10% of the total antennal sensillum population. The remaining population, consisting of grooved pegs and *sensilla trichodea* are tuned to olfactory cues.

The grooved peg sensilla, otherwise termed *sensilla basiconica*, are another class of olfactory sensilla present on the antennae. These sensilla are present small in number approximately 100 per each antenna of female *Ae. aegypti*. Each grooved-peg harbors three to five receptor neuron. These sensilla generally responds to short-chain carboxylic acids including L-lactic acid (Davis & Sokolove, 1976).

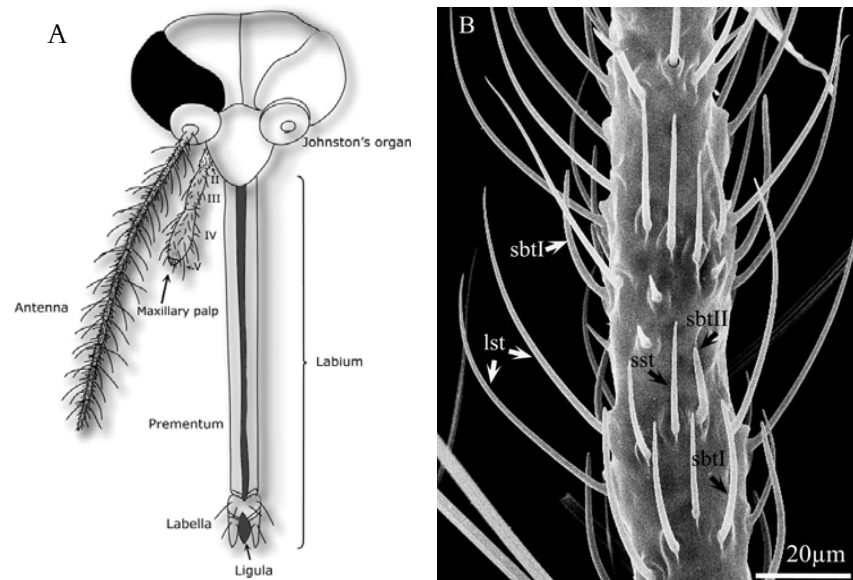


Figure 2. (A) Schematic representation of a female *Aedes aegypti* head showing various peripheral appendages (Siju, Hansson & Ignell, 2008). (B) Scanning electron micrograph of a single antennal flagellomere of female *Aedes aegypti* showing four sub types of *sensilla trichodea*: short-tipped (sst), short blunt-tipped I (sbtI), short blunt-tipped II (sbtII) and long sharp-tipped (lst) (Ghaninia, Ignell & Hansson, 2007)

Among the olfactory sensilla, *sensilla trichodea* are the most abundant ones: there are approximately 800 of these sensilla on each female antenna (Ismail, 1964; McIver, 1978). Several studies have been aimed at describing the structure and function of this class of sensilla in *Ae. aegypti* and other mosquito species (McIver, 1978; Ghaninia, Ignell & Hansson, 2007). According to their external structure, *sensilla trichodea* can be sub-divided into four morphological types, long sharp-tipped (lst), short sharp-tipped (sst), short blunt-tipped type I (sbtI) and short blunt-tipped type II (sbtII) (Figure 2B) (McIver, 1978; Ghaninia, Ignell & Hansson, 2007). In addition to these four morphological types there are trichoid-like sensilla, referred to as intermediate sensilla, whose function has not been explored extensively

(Ghaninia *et al.*, 2008). Based on transmission electron microscopy and single sensillum recording (SSR) analyses we know that these *sensilla trichodea* harbors two sensory neurons (McIver, 1978; Ghaninia, Ignell & Hansson, 2007). Systematic analysis of *sensilla trichodea* have revealed their functional properties. Based on the response spectra of individual ORNs housed in these sensilla to a set of behaviorally relevant odors it has been possible to identify discrete functional types of sensilla. From within the panel of behaviorally relevant compounds are those associated with plants, hosts, and oviposition sites. In all, 11 classes of *sensilla trichodea* have so far been described in the female *Ae. aegypti* (Ghaninia, Ignell & Hansson, 2007).

The maxillary palps

The maxillary palps in insects are considered as accessory olfactory organs, and often play a specialized function in olfaction. In mosquitoes the maxillary palps, similar to the antennae, are sexually dimorphic. In species of *Aedes* (Figure 2A) and *Anopheles* mosquitoes, the paired maxillary palps have five segments, where the distal or fifth segment does not contain any chemosensory sensilla. In *Aedes*, segment four and in *Anopheles* segment two, three and four harbor the majority of the chemosensory sensilla (McIver, 1972,1982; McIver & Siemicki, 1975). There are about 30 single-walled multiporous capitate peg sensilla on each maxillary palp of a female *Ae. aegypti*. Three ORNs are present in each of these sensilla (McIver, 1972). One of these is activated by CO₂ and a second one responds to 1-octen-3-ol; so far, no ligand has been identified for the third neuron in *Ae. aegypti* (Grant *et al.*, 1995; Grant & O'Connell, 1996).

The labium

The labium, the main gustatory organ, is a stout organ composed of three parts: a long prementum; a pair of labella, or labellar lobes, that articulate on the distal end of the prementum; and a terminal ligula (Figure 2A) (Clements, 1999; Ignell & Hansson, 2005). The paired labellar lobes contain uniporous hair sensilla (*sensilla trichodea*). These sensilla function as contact chemosensilla and sensory neurons in these sensilla respond to a water, salts and sucrose (McIver & Siemicki, 1978 a, b). As stated above, although labial palps are chiefly gustatory organ, a recent study identified olfactory sensilla on the labellar lobes of *Ae. aegypti* (Kwon *et al.*, 2006)

Olfactory receptors

Receptor proteins that span the dendritic membrane of ORNs in both vertebrates and invertebrates are known as the olfactory receptors (OR) (Buck & Axel, 1991; Clyne *et al.*, 1999; Gao & Chess, 1999; Vosshall *et al.*, 1999). Olfactory receptors are seven transmembrane domain proteins that are encoded by genes expressed in ORNs. The presence of ORs and genes encoding these receptors was initially reported from rats. In an elegant experiment, utilizing the power of molecular biology techniques, Linda Buck & Richard Axel (1991) identified the first large family of olfactory receptor genes that encode OR proteins expressed in the rat olfactory epithelium (Buck & Axel, 1991). The importance of this discovery was later recognized as Buck and Axel were awarded the Nobel Prize in Physiology or Medicine in 2004. Following this discovery, OR gene families from several other organisms, including many insect species have been reported. Most notable among these have been the identification of the complete repertoire of OR genes in *Drosophila melanogaster* (60; Clyne *et al.*, 1999; Gao & Chess, 1999; Vosshall *et al.*, 1999) and later also in several mosquito species, including *An. gambiae* (79; Fox *et al.*, 2002; Hill *et al.*, 2002), *Ae. aegypti* (131; Bohbot *et al.*, 2007) and *Culex quinquefasciatus* (180; SR Hill, personal communication).

Structural elucidations studies of vertebrate ORs revealed that they belong to a large superfamily of seven-transmembrane G-protein coupled receptors (GPCRs). In line with this finding, researchers assumed that insect ORs also belong to the same family of proteins. However, an intriguing finding that surfaced during the identification of insects ORs was that they showed no apparent homology in structure to vertebrate ORs (Vosshall *et al.*, 1999; Hill *et al.*, 2002). This hypothesis was recently validated through an observation of the membrane topology of two insects ORs in which it was concluded that insect ORs contain a novel family of seven-transmembrane domains with an inverted topology in which the N-terminal is inside the cell and the C-terminal is outside (Benton *et al.*, 2006; Wistrand, Kall & Sonnhammer, 2006). This orientation is in stark contrast to the classic GPCR membrane topology, such as that found in the vertebrate ORs, in which the N-terminal located outside the cell and the C-terminal faces the cytosol. With these experimental evidence it clear that ORs of insects are not structurally and functionally related to their counterparts in the vertebrate olfactory system.

In insects, apart from the conventional ORs, an additional and highly conserved insect receptor gene sequence was discovered and named as *Or83b* gene family during the search for OR genes in *D. melanogaster*

(Vosshall *et al.*, 1999; Larsson *et al.*, 2004). This special receptor gene was in most cases, if not all, found to be co-expressed with a regular OR and was found to be essential for the trafficking of regular ORs to the proper dendritic location (Larsson *et al.*, 2004; Benton *et al.*, 2006). Molecular and structural studies have later confirmed that Or83b, together with a conventional OR, facilitate the proper binding of odorant ligands during the first step in olfactory signal transduction, and thus, these ORs function together as a heterodimeric receptor (Benton *et al.*, 2006). Homologous of the OR83b receptor have since been identified in many insect species, including the mosquitoes, *An. gambiae* (Fox *et al.*, 2002), *Ae. aegypti* (Melo *et al.*, 2004) and *Cu. quinquefasciatus* (Xia & Zwiebel, 2006) in which it was designated as OR7.

In all vertebrates each functional type of ORN expresses only one particular OR. This phenomenon is conserved across all the vertebrates studied. In the case of insects, this rule does not hold. Or83b has been shown to be present in combination with a conventional OR in almost all ORNs studied in insects. However, there are exceptions to this rule as well, since there are a few cases in which multiple conventional ORs are co-expressed on single ORN along with Or83b in *D. melanogaster* (Dobrista *et al.*, 2003; Goldman *et al.*, 2005).

Perireceptor events and olfactory transduction mechanisms

The entry, exit and residence time of the odorants are referred to as perireceptor events (Stengl *et al.*, 1999). These events are triggered by the entry of the odorant molecules through the pores or slits of the sensillum. Due to the hydrophobic nature of most odorant molecules, it is hypothesized that these molecules must be actively transported by chaperones through the aqueous sensillum lymph to the appropriate ORs on the dendrites of the ORNs. In insects, odorant binding proteins (OBPs), in the sensillum lymph, have been shown to more or less selectively bind to odorant molecules (Vogt & Riddiford, 1981). These protein molecules are hydrophilic in nature and they are secreted in large quantities into the sensillar lymph by auxillary cells in the sensilla (Bloomquist & Vogt, 2003). Several studies have suggested that OBPs have multiple functions in olfaction including selective binding and transportation of the odorants from the pores through the hemolymph to the ORNs, filtering of odorants, etc. (e.g. Bloomquist & Vogt, 2003). Although the precise role of OBPs in olfactory signal transduction have been debated for several years, recent studies have given evidence to show the importance of these class of proteins in olfactory processes (Laughlin *et al.*, 2008). Odorant binding proteins in

mosquitoes have been described in *An. gambiae* (Zhengxi *et al.*, 2004), *An. stephensi* (Sengul & Tu, 2008), *An. arabiensis* (Zhengxi *et al.*, 2004) and *Ae. aegypti* (Bohbot & Vogt, 2005) and *Cu. quinquefasciatus* (Ishida, Cornel & Leal, 2002).

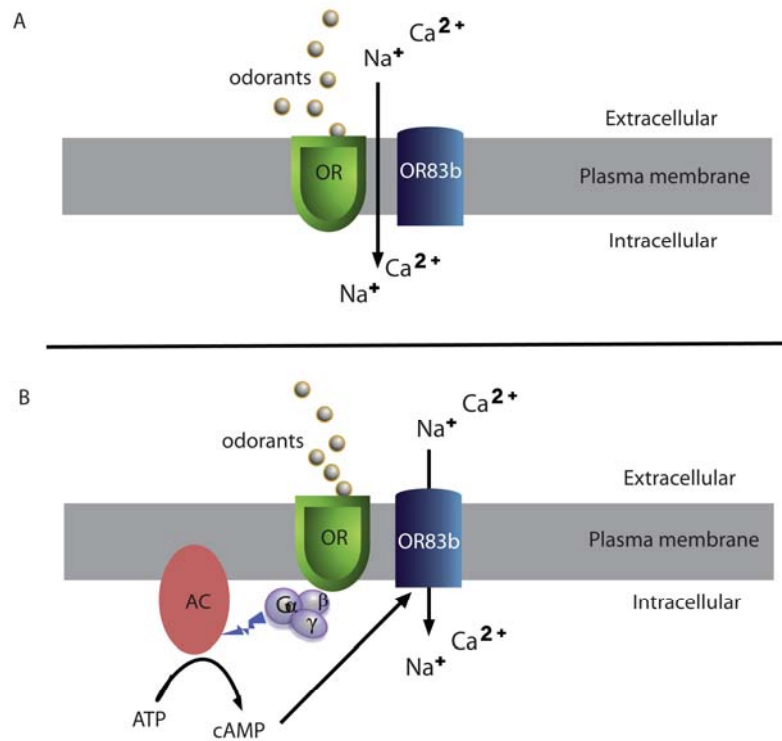


Figure 3. Two recently proposed models for olfactory transduction mechanism in insect. (A) Model proposed by Sato *et al.*, (2008), in which olfactory transduction occurs due to the activation of a OR-OR83b complex directly by odorants and form a direct non-selective cation channel. No second messenger system present in this model. (B) Dualistic model proposed by Wicher *et al.*, (2008), in which olfactory transduction occurs through two mechanisms including a non-selective cation channel and a second messenger system. AC, adenylyl cyclase; cAMP, cyclicAMP. Modified after (Ha & Smith, 2008).

After the identification of insect ORs a debate was initiated concerning the olfactory signal transduction mechanism (Vosshall *et al.*, 1999). In vertebrates, with a classical GPCR structure of the ORs, the signal transduction system mainly involves a G-protein coupled second messenger system (Reed, 1992; Hildebrand & Shepherd, 1997). Since insect ORs are

dissimilar to vertebrate ORs, the signal transduction mechanism may be through different pathways the two systems. In fact, the inverted topology of the insect ORs places the canonical GPCR region for G-protein coupling outside the cell, strongly suggesting a different transduction pathway. Recently two elegant studies gave an insight into the mechanism of the olfactory signal transduction mechanism in insects (Sato *et al.*, 2008; Wicher *et al.*, 2008). These studies unequivocally reported that olfactory signal transduction involves a non-selective cation channel conduction formed by the heterodimerization complex of a conventional OR and the ubiquitous receptor OR83b. In the study by Sato *et al.* (2008), a direct non-selective cation channel formation through a heterodimer complex of a conventional OR and OR83b was observed, which did not propose the involvement of a second messenger system activation (Figure 3A). In contrast, Wicher *et al.* (2008) proposed a dualistic model in which, apart from the direct formation of a non-selective cation channel by the coupling of a conventional OR and OR83b, there is an involvement of a metabotropic pathway. According to this model when an odorant binds to the conventional OR, it activates a Gs subunit bound to the OR, thus stimulating membrane-bound adenylate cyclase and result in the production of cAMP. Both the ionotropic and the metabotropic pathways activates the opening of a non-selective ion channel motif present in the OR83b protein that gate Na⁺ and Ca²⁺ ions and result in membrane depolarization (Figure 3B). This change in the membrane permeability causes the generation and propagation of action potentials along the ORN axon membrane to the antennal lobes. In this way the information regarding odor quality, quantity and spatio-temporal pattern will be translated from chemical signals into a code for the central nervous system in the form of neural information.

5.2 Central components of the chemosensory system

The central chemosensory system consists of neuropil structures associated with chemical information processing. These neuropil have been analyzed both anatomically and functionally in various insect species. With the availability of classical and redefined anatomical tools and the aid of molecular biological techniques we have recently been able to achieve a better resolution of the anatomical and functional properties of these neuropils. The main neuropils involved in chemosensory processes are: the primary olfactory centre, the antennal lobe and the primary gustatory centers in the subesophageal ganglion and tritocerebrum. Processed information from these neuropil is relayed to higher order brain centers.

The antennal lobe

The antennal lobes (ALs) are part of the insect deutocerebra, which are located on either side of the esophagus (Anton & Homberg, 1999; Schachtner, Schmidt & Homberg, 2005). The ALs are considered to be the anatomical equivalents of the olfactory bulbs in vertebrates (Strausfeld & Hildebrand, 1999). Each AL consists of spherical neuropilar compartments referred to as glomeruli that contain the synaptic contacts between the ORNs and AL interneurons. Glomeruli are arranged in one or more layers around a central fiber core (Anton & Homberg, 1999; Schachtner, Schmidt & Homberg, 2005). Each AL glomerulus may be separated from other glomeruli by a glial sheath, although the ALs of some insects have been reported to lack glia (Schachtner, Schmidt & Homberg, 2005). The ALs of a wide variety of insect species have been investigated anatomically. These studies generally reveal that the number, shape, size, and arrangement of glomeruli are species-specific and/or sex-specific (Anton & Homberg, 1999; Schachtner, Schmidt & Homberg, 2005). One of the better studied AL is that of *D. melanogaster*, which contains approximately 50 glomeruli (Lassiue *et al.*, 1999; Couto *et al.*, 2005; Fishilevich & Vosshall, 2005). Recently, high-resolution 3D maps of the ALs of two mosquito species have been described. These maps reveal that the ALs of male and female *An. gambiae* contain 61 and 60 AL glomeruli, respectively (Ghaninia, Hansson & Ignell, 2007), whereas the ALs of male and female *Ae. aegypti* contain 49 and 50 glomeruli, respectively (Ignell *et al.*, 2005).

Four main neuronal elements innervate the AL: the ORNs, the local interneurons (LNs), the projection neurons (PNs) and the centrifugal neurons (CNs). This ensemble of neurons and their synaptic connections make the AL a site of great neuronal complexity (Anton & Homberg, 1999; Vosshall & Stocker, 2007). As the first step in the olfactory processing the AL must functionally map different odor information relayed by different ORNs from the peripheral system. Once this task has been achieved, the next step is to sort, filter and, if necessary, modulate the signals in the glomerular structures. As the final step in the process, the filtered and modulated signals are relayed, via the PNs, to higher order olfactory processing centers in the protocerebrum, the calyces of the mushroom bodies and the lateral horn of the protocerebrum, where further sensory integration occurs.

Olfactory receptor neurons

Axons of ORNs originating from sensilla in the peripheral olfactory organs including antennae, maxillary palps and labia targets the AL. Axons of antennal ORNs originating from the antennae enters the AL through the antennal nerve (Anton & Homberg, 1999). Individual ORNs expressing the same ORs, and thus having the same function, converge onto a single AL glomerulus. The combination of all ORN functional types converging to their different glomeruli thus forms a functional glomerular map in the AL. In most of the insects examined, the projection pattern of ORNs from a single antenna follow an ipsilateral arborization in the AL. A similar ipsilateral arborization pattern has also been demonstrated in the mosquitoes *Ae. aegypti* and *An. gambiae* (Anton *et al.*, 2003; Ignell *et al.*, 2005). In contrast, in another dipteran insect, *D. melanogaster*, a large proportion of ORNs display a bilateral arborization pattern in the AL (Stocker, 2001). The axons of ORNs entering into the AL convey neural information regarding the odor quality and quantity by synapsing onto LNs and PNs of the AL. The principle neurotransmitter present in the ORNs is acetylcholine (Galizia, 2008).

Local interneurons

The LNs are amacrine cells, with cell bodies generally located in a lateral cell cluster of the AL. According to their arborization patterns within the AL, LNs may be classified as either oligoglomerular (those that ramify in only a few glomeruli) or multiglomerular (those that display homogenous or heterogenous ramification in many or all glomeruli). Local interneurons have a general presence in the ALs of all insects species studied (Anton & Homberg, 1999; Schachtner, Schmidt & Homberg, 2005). However, the number of LNs is species dependent and that varies from approximately 100 in flies up to 4000 in bees (Galizia, 2008). Local interneurons interconnect the glomerular array 'horizontally' and thus provide an anatomical network that facilitates cross-talk (internal communication within the AL) between glomeruli. Local interneurons receive sensory input from PNs, and may receive input from ORNs as has been demonstrated in *D. melanogaster* (Olsen & Wilson, 2008). Local interneurons are instrumental in the providing a filter through which relevant information from ORN inputs is passed forward through the PNs to higher brain centers. Local interneurons establish this filter using inhibitory, and in the case of *D. melanogaster*, excitatory circuits within the AL (Shang *et al.*, 2007). The LN network in the ALs mainly involves the synchronization of the activity in the PNs (Ng

et al., 2002). The principle neurotransmitter found in the LNs is gamma amino butyric acid (GABA) (Anton & Homberg, 1999; Galizia, 2008). Neuropeptides and biogenic amines have also been shown to be expressed in these neurons (Anton & Homberg, 1999; Nässel & Homberg, 2006).

Projection neurons

Projection neurons connect the AL ‘vertically’ with higher brain centers such as the calyces of the mushroom body and the lateral horn of the protocerebrum, through different anatomically distinct fiber tracts (Anton & Homberg, 1999; Vosshall & Stocker, 2007). The cell bodies of PNs are found in the periphery of the AL and the total number differs greatly among different species, ranging from 150 in *D. melanogaster* (Stocker, 2001) to 900 in *M. sexta* (Homberg, Christensen & Hildebrand, 1989). Differentiation of PNs is based mainly on their branching pattern in the AL, which is either uniglomerular or multiglomerular, and on the fiber tracts through which they relay information to the higher brain centers (Anton & Homberg, 1999). In total there are five major fiber tracts formed by PNs that connect the AL to the protocerebrum; the inner antenno-cerebral tract (IACT), the middle antenno-cerebral tract (MACT), the outer antenno-cerebral tract (OACT), the dorsal antenno-cerebral tract (DACT) and the dorso-medial antenno-cerebral tract (DMACT) (Anton & Homberg, 1999). However, the number and the projection route of these tracts may vary considerably among different insect species. In the mosquito *Ae. aegypti* there are only four major fiber tracts reported including IACT, DACT, MACT and DMACT (Ignell *et al.*, 2005). The PNs are mainly involved in conveying olfactory information to the higher brain centers and also act as a gain control in tuning the information (Anton & Homberg, 1999; Bhandawat *et al.*, 2007; Olsen & Wilson, 2008).

Centrifugal neurons

In contrast to LNs and PNs, centrifugal neurons (CN) appear to have their cell bodies outside the AL: in the SOG, in the ventral nerve cord or in the protocerebrum (Anton & Homberg, 1999; Schachtner, Schmidt & Homberg, 2005; Galizia, 2008). In insects, centrifugal neurons are found to be in small numbers. These neurons project their axons into the AL and often display extensive ramifications, mostly with varicose appearance. Centrifugal neurons connect to the AL with higher brain centers in which they often have wide-field ramifications. A prominent centrifugal neuron identified in the insect brain including mosquitoes is the serotonin-

immunoreactive (SI) centrifugal neuron which shows characteristic morphology in many insects (Dacks, Christensen & Hildebrand, 2006; Siju, Hansson & Ignell, 2008). Dacks, Christensen & Hildebrand (2006) described the morphology of AL SI centrifugal neurons in variety of insect species. Apart from the SI centrifugal neurons, there other types of centrifugal neurons having efferent inputs to the insect ALs including midline neurons of the SOG, ascending neurons of the ventral nerve cord and collaterals of descending neurons from the protocerebrum (Anton & Homberg, 1999). Several of these neurons are immunoreactive to different biogenic amines and neuropeptides (Anton & Homberg, 1999; Schachtner, Schmidt & Homberg, 2005). From the morphology and arborization pattern of centrifugal neurons, it has been suggested that most of these neurons are mainly involved in modulation of odor processing in the AL (Anton & Homberg, 1999)

The subesophageal ganglion and the tritocerebrum

In mosquitoes, the SOG, composed of the mandibular, the maxillary and the labial neuromeres, together with the tritocerebrum (TC) form the primary gustatory neuropil (Ignell & Hansson, 2005). These neuropil receive afferent input from gustatory receptor neurons housed in sensilla present on the gustatory organs. The principal neurons found in the SOG/TC include, beside the afferent neurons, local interneurons and projection neurons. The SOG/TC have been divided into seven anatomically distinct neuropil regions (Ignell & Hansson, 2005)

6 Neurochemicals in the Chemosensory Systems

The complexity in signaling mechanisms of the insect nervous system is due largely to the presence of myriads of neurochemicals. The diversity of neurochemicals enables the nervous system to function transduce a bewildering magnitude of signals and thus enables the insect to perform simple as well as complex behavioral tasks at an astonishing pace and accuracy. Profiling the neurochemicals in the chemosensory system enables us to begin to understand the diversity of signaling molecules and their importance in the integration of sensory information (Homberg & Muller, 1999).

Neurochemicals in the insect nervous system can be classified into three main classes according to their function: 1) Neurotransmitters, which are the principal neurochemicals that facilitate the communication between individual neurons. Neurotransmitters are released at the synapse between neurons and primarily act on the postsynaptic neuronal membrane to produce post synaptic current. The principle neurotransmitters in the insect chemosensory system are acetylcholine, GABA and nitrous oxide (NO) (Homberg & Muller, 1999; Hansson & Anton, 2000); 2) Neuromodulators, are neurochemicals that can modulate neuronal communication and are released synaptically. Neuromodulators modify the properties of the neuronal ion channels through mechanisms that differ from those used by the classical neurotransmitter at that synapse (Nässel, 2002; Farooqui, 2007). Neuromodulators are able to alter the properties of neuronal membranes both pre- and post-synaptically (Nusbaum *et al.*, 2001). Several classes of neurochemicals are considered to be neuromodulators, and among the more prominent ones are the biogenic amines and several of the neuropeptides (Nässel, 2002). 3) Neurohormones may be either local neurohormones or circulating neurohormones. The special feature of local neurohormones is that they only affect a small region near to their site of release. Local neurohormones are sometimes called non-synaptic neuromodulators since, although they are released from neurohaemal sites, their mechanism of action is that of a neuromodulator (Nässel, 2002). Circulating neurohormones are released into the haemolymph at a distant specific release sites, often diffusing long distances before they reach their effector organs. Circulating neurohormones are most often found to act on peripheral target tissues or organs (Nässel, 2002).

A broad spectrum of neurochemicals, including acetylcholine, GABA, biogenic amines and neuropeptides are present in the chemosensory system of all insect species studied so far (Homberg & Muller, 1999; Schachtner, Schmidt & Homberg, 2005; Nässel & Homberg, 2006). However, studies of these neurochemicals in the mosquito nervous system are limited and there are only few studies that have assessed the presence of neurochemicals in the chemosensory system. In the present thesis, I have studied some of the important biogenic amines and neuropeptides in the chemosensory system of mosquitoes. Below is a general overview of some of the major neurochemicals found in the chemosensory system of insects, including that of mosquitoes.

6.1 Biogenic amines

Biogenic amines are one of the most extensively studied groups of neurochemicals in the nervous system of both vertebrates and invertebrates. Over the years, several pharmacological studies have provided ample evidence for their activity in these systems (Roeder, 1994). In insects, there are five biogenic amines: serotonin, tyramine, octopamine, dopamine and histamine. All of them are derived from amino acids through the metabolic action of several specialized enzymes (Blenau & Baumann, 2001). Serotonin is a decarboxylation product of tryptophan; tyramine, octopamine and dopamine are decarboxylation products of tyrosine; histamine is derived from histidine (Figure 4). Serotonin and dopamine also occur in the vertebrate system, while octopamine and tyramine are considered as the insect equivalents of the adrenergic transmitters, epinephrine and norepinephrine, of the vertebrate system (Figure 4).

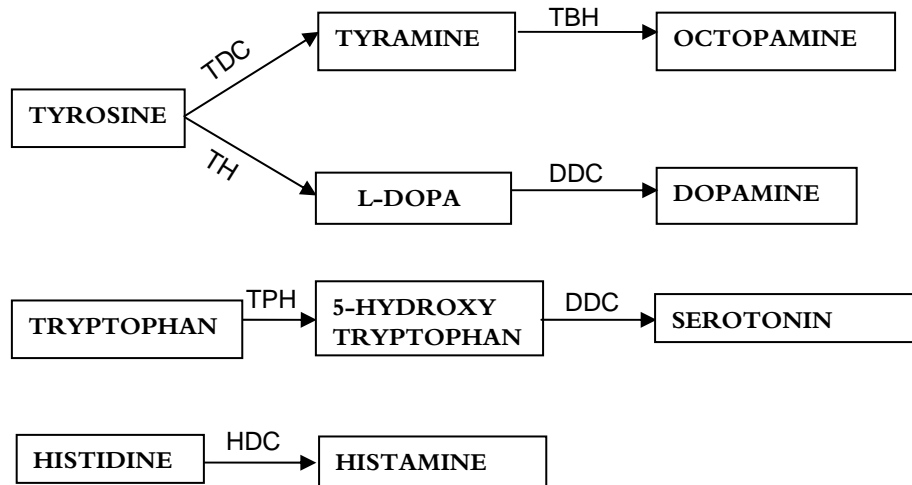


Figure 4. Biogenic amine biosynthesis in insects. TDC: tyrosine decarboxylase, TBH: tyramine β -hydroxylase, TPH: tryptophan hydroxylase, DDC: dopa decarboxylase, HDC: histidine decarboxylase (Monastirioti, 1999).

Serotonin

Serotonin, or 5-hydroxytryptamine, is an indolamine found in all vertebrate and invertebrate nervous systems (Monastirioti, 1999). It is synthesized from tryptophan by the enzymatic actions of tryptophan hydroxylase and DOPA decarboxylase. Several studies have demonstrated the presence of serotonin in the central and peripheral nervous system of insects (for a review see Nässel, 1988). In the primary olfactory center, immunocytochemical techniques have helped to identify and describe a variety of SI AL neurons have been identified and described in a number of insect species (Anton & Homberg, 1999; Dacks, Christensen & Hildebrand, 2006). In most insects examined, serotonin immunoreactivity in the AL arises from a single centrifugal neuron with a large cell body in the lateral cluster of the AL. However, considerable difference in the arborization pattern of this neuron has been observed in different insect species and has been postulated to have evolutionary implications (Anton & Homberg, 1999; Dacks, Christensen & Hildebrand, 2006). The arborization pattern of the SI centrifugal neuron found in mosquitoes resembles to that have been reported in the cockroach (Salecker & Distler, 1990; Siju, Hansson & Ignel, 2008). The arborization of the AL SI neurons shows a beaded appearance of varicose swellings along the fine process in deutocerebrum. In the protocerebrum, the SI neuron was found to have wide-field arborization in most part of the neuropil areas of the higher brain centers. Ultrastructural studies in two insects revealed that SI neurons in the AL predominantly make out put synapses (Salecker & Distler, 1990; Sun *et al.*, 1993). These results suggest that SI neurons in insects may have a role in local olfactory processing in the AL and also function as feed back neurons modulating the olfactory processing.

By acting as a neurotransmitter or neuromodulator, serotonin regulates and modulates several key physiological and behavioral processes in insects. These include learning and memory, motor activity, neural development and control of circadian rhythm (Menzel & Muller, 1996; Mercer *et al.*, 1996; Nässel, 1988; Yuan *et al.*, 2005). In insects, the neuromodulatory role of serotonin in the chemosensory system has been well-established by several studies. In general serotonin was found to influence structural and functional plasticity of the insect AL (e.g. Kloppenburg *et al.*, 1999; Mercer *et al.*, 1996). Apart from the central chemosensory effect, serotonin has been found to influence the peripheral chemosensory system in which they are involved in the modulation of the sensitivity of chemical signals (Dozler *et al.*, 2001; Gatellier *et al.*, 2004; Grosmaître, *et al.*, 2001). In addition, changes in serotonin concentration in the brain of several insects have showed an effect on pheromone perception, circadian rhythm activity and

feeding-related activity (Lange *et al.*, 1989; Linn *et al.*, 1992; Orchard, 2006).

Serotonin mediates its effect by acting on multiple GPCRs. Four serotonin receptor types have been characterized from *D. melanogaster*: 5HT1A, 5HT1B, 5HT2, and 5HT7 (Blenau & Baumann, 2001). These serotonin receptors have mammalian homologues of the same names. In *D. melanogaster* 5HT1B and 5HT2 are involved in modulating circadian rhythm entrainment (Yuan *et al.*, 2005; Nichols, 2007).

Dopamine

Dopamine is a catecholamine found in high concentrations in the invertebrate and vertebrate nervous systems. This biogenic amine is produced from the amino acid tyrosine by the action of the tyrosine hydroxylase enzyme (Monastirioti, 1999). Several studies have attempted to immunocytochemically localize dopamine in the chemosensory system of insects. Studies on *Calliphora vomitoria*, *Calliphora erythrocephala*, *D. melanogaster* and *Schistocerca gregaria* have failed to show any immunoreactivity in the ALs (Nässel & Elekes, 1992; Wendt & Homberg, 1992). However, dopamine immunoreactivity in the AL has been observed in other insects, including *Acheta domestica*, *Periplaneta americana*, *Apis mellifera* and *Manduca sexta* (Klemm, 1976; Schäfer & Rehder 1989; Distler, 1990). In general, dopamine immunoreactive neurons are found to be centrifugal in nature, with wide-field arborization patterns in both the AL and the protocerebrum, suggesting that dopamine acts as a neurotransmitter or neuromodulator in the CNS of insects (Blenau & Baumann, 2001; Schachtner, Schmidt & Homberg, 2005). So far no immunocytochemical studies have been available demonstrating the distribution of dopamine in mosquito brains.

Dopamine affects several behavioral mechanisms in insects, including those involved in arousal, locomotor activity and sexual behavior (Kume *et al.*, 2005; Menzel *et al.*, 1999; Neckameyer *et al.*, 2000, 2001; Pendlton, 2000, 2002; Keene & Wadell, 2005; Neckameyer, 1998). Also, in the honeybee, dopamine has been implicated in olfactory learning and memory (Mercer & Erber, 1983; Mercer & Menzel, 1982). In general, dopamine seems to act by influencing synaptic and behavioral plasticity in insects (Monastirioti, 1999).

Two subtypes of dopamine receptors have been identified in insects. Both of these have been identified in *D. melanogaster*, in which the first subtype is expressed in the entire nervous system (Gotzes, Balfanz & Baumann, 1994), whereas expression of the second subtype is restricted to the mushroom bodies (Han, *et al.*, 1996). It should be noted that these

receptors are pharmacologically distinct from the dopamine receptors found in vertebrates (Kokay *et al.*, 1999).

Octopamine

The monoamine octopamine is an analogue of norepinephrine found in the vertebrate nervous system and was first isolated from the salivary glands of the octopus, *Octopus vulgaris*; hence the name octopamine (Erspamer & Boretti, 1951). Octopamine is highly abundant in the nervous systems of many invertebrates, shown by immunocytochemical localization and biochemical detection methods (Pfluger & Stevansson, 2005).

Octopamine immunoreactive neurons have been observed in the ALs of several insect species including *S. gregaria*, *A. mellifera*, *M. sexta* and *D. melanogaster* (Monastirioti, 1999; Schachtner, Schmidt & Homberg, 2005). The octopaminergic neurons are characterized by blebby arborization pattern in the AL and other brain neuropils. Octopamine immunoreactivity in the ALs mainly originates from ascending dorsal unpaired medial (DUM) and ventral unpaired medial (VUM) neurons from the suboesophageal ganglion. These neurons also seemingly give rise to octopamine immunoreactive efferent fibers innervating several peripheral organs such as flight muscle and oviduct (Roeder, 2005). In mosquitoes, octopamine immunoreactive neurons have not yet been reported.

Due to its neurotransmitter, neurohormonal and neuromodulatory functions, octopamine plays a prominent role in influencing the physiology and behavior of several insects. Octopamine has a direct effect on both the central and peripheral nervous systems (Roeder, 2005). These effects have mainly been studied in the locust (Roeder, 2005) and a number of moth species (Linn & Roelofs, 1986; Linn, Campbell & Roelofs, 1992). Octopamine is an abundant biogenic amine: the levels of octopamine in the insect nervous system have major implications on the physiology and behavior of the insects. Several behaviors of the insects appear to be modulated by changing the level of octopamine in the CNS and/or hemolymph.

Octopamine receptors have been identified in several insect species, including *S. gregaria* (Roeder & Gewecke, 1990), *P. americana* (Nathson & Greengard, 1973), *D. melanogaster* (Han, Millar & Davis, 1998) and *Cu. pipiens* (Pratt & Prayor, 1986). These receptors are G-protein coupled receptors and occur in four pharmacologically different forms (Roeder, 1999, 2005). There are indications of octopamine having a role in modulating olfaction at the peripheral level since in lepidopteran species

including, *Heliothis virescens* and *B. mori* and *Mamestra brassicae*, octopamine receptors have been shown to be expressed in the antennae (von Nickisch-Rosenegk *et al.*, 1996; Brigaud *et al.*, 2009).

Since octopamine is only present in the invertebrate nervous system, developing specific target molecules for their receptors, in order to control octopamine signaling, has been suggested as a novel and highly specific control method for pest species (Homberg, 1994; Roeder, 2005)

6.2 Neuropeptides

In the nervous system of many organisms, neuropeptides are considered to be the most diverse signaling molecules in terms of structure, distribution and function (Nässel, 2002). Neuropeptides consist of few to many amino acids (usually 5 to 30) and often show great diversity in molecular structure and genetic relationship (Orchard, 2001; Nässel, 2002). Most neuropeptides have been found and/or isolated from nervous tissue, hence the name neuropeptide. Neuropeptides are produced in specific neuronal populations by gene transcription – at first they are encoded as larger precursors known as prepropeptides. These prepropeptides enter the secretory pathway in which maturation and further processing occurs due to cleavage by several enzymatic processes. The individual neuropeptides thus produced undergo post-translational modifications (most often amidation) and are later transported to and stored in vesicles close to their release sites (Nässel, 2002).

Neuropeptides were originally classified into different families based on similarity in amino acid sequences. Now in the post-genome sequencing climate neuropeptides are often classified according their precursor gene sequence similarity (Nässel, 2002). The majority of neuropeptides identified have been implicated in controlling or modulating most, if not all, physiological processes in insects. Immunocytochemical and biochemical studies have provided ample evidence for the presence of large families of neuropeptides in the AL of a wide variety of insect species. Although there are several families of neuropeptides present in the AL, the following four families of peptides are consistently described in the insect AL, including the FMRFamide-related peptides (FaRPs), the tachykinin-related peptides (TRPs), and the allatotropins and the allatostatins (Nässel, 2002; Schachtner, Schmidt & Homberg, 2005; Nässel & Homberg, 2006).

FMRFamide-related peptides

Among all neuropeptides, the FMRFamide-related peptide (FaRPs) superfamily stands out as the most diverse family of peptides in terms of structure and function. The first FMRFamide peptide was isolated from mollusc by Price & Greenberg (1977) as a cardioacceleratory peptide. This neuropeptide family has been classified into four main types namely, the extended FMRFamides (present only in the dipterans), the myosuppressins (decapeptides with a conserved structure of XDVTXHXFLRFamide) and the extended FLRFamides and the sulfakinins (the extended HMRFamides). All the four types share a conserved C-terminus (RF-amide). In such a diverse family of modulators it would be supposed that many functions would be ascribed to these neuropeptides, as is the case. The FaRPs have been shown to modulate skeletal and visceral muscle, stimulate enzyme secretion, stimulate hormone release and inhibit malpighian tubule secretion (Hill, 2005). Several immunocytochemical and peptide isolation studies have demonstrated the presence of FaRPs in the AL of variety of insect species (Schachtner, Schmidt & Homberg, 2005; Nässel & Homberg, 2006).

Tachykinin-related peptides

The tachykinin related peptide (TRP) family is diverse and related to the vertebrate tachykinin peptide family. Although they have low sequence similarity with vertebrate tachykinin peptides, the physiological actions appear conserved (Nässel, 2002). In vitro studies with TRPs on insect tissues have demonstrated a functional role in the modulation of, for example, muscle contraction, fluid secretion, rhythmic motor pattern generation and circadian rhythm (Nässel, 2002). In addition, from the distribution pattern of TRPs in the insect nervous system, several other modulatory roles have been suggested for these peptides (Nässel, 2002).

Several studies have mapped the distribution of TRPs in the olfactory system of insects immunocytochemically (Schachtner, Schmidt & Homberg, 2005; Nässel & Homberg, 2006). These studies have generally demonstrated the presence of TRPs in LNs of the AL (Schachtner *et al.*, 2005). Winther *et al.* (2006) showed that a lack of TRPs in the larval and adult nervous system of *D. melanogaster* results in a reduction in olfactory perception as well as in locomotor activity. A previous study by Meola *et al.* (1998) in the mosquito *Culex salinarius* has also reported the presence of TRP immunoreactive neurosecretory cells in the antenna that send projections into the AL suggesting a role for TRPs in modulating olfactory processing in the mosquito.

The allatotropin family

The allatotropin (AT) peptide family share a conserved C-terminal amino acid sequence (TARGFamide), and have been shown to modulate juvenile hormone biosynthesis in insects (Nässel, 2002; Audsley, Weaver & Edwards, 1999; 2000). Apart from this function, allatotropin has been shown to influence heart beat frequency and circadian rhythm activity (Petri *et al.*, 2002).

Allatotropin immunoreactivity has been studied in a number of species and has generally revealed large numbers of LNs innervating the AL (Schachtner, Schmidt & Homberg, 2005). In dipteran insects, allatotropin immunoreactivity in the AL has been shown in *D. melanogaster* and *Phormia regina* (Zintan *et al.*, 1993; Tu *et al.*, 2001). From the general staining pattern of allatotropin immunoreactivity in AL LNs, it has been suggested that this peptide may have a neuromodulatory role in the insect olfactory system, particularly at the level of primary signal integration (Nässel, 2002). Homberg *et al.* (2004) also demonstrated the presence of allatotropin immunoreactivity in the antennal nerve afferent fibers in the locust *S. gregaria*, suggesting that allatotropin might have a role in the modulation of sensory neurons.

Allatostatin family

This family of neuropeptides was originally identified as the peptides that inhibit juvenile hormone biosynthesis in the cockroach (Woodhead *et al.*, 1989). In several insect species, later studies have demonstrated that allatostatins have other functions, such as controlling heart rate, gut contractions, nutrient absorption and circadian rhythms (Sarkar *et al.*, 2003, Hernandez-Martinez, *et al.*, 2005; Petri *et al.*, 2002). This indicates that allatostatin may play a neuromodulatory or a hormonal role in the insect nervous system (Nässel, 2000). There are three structurally unrelated allatostatin families found in insects. These are classified according to the insects from which they were originally isolated: allatostatin type-A (AST-A; YXFGLamide; *Diptera* type), allatostatin type-B (AST-B; AWQDLNAGWamide; Cricket type), allatostatin type-C (AST-C; PISCF; *Manduca* type). In most insects, all three types of allatostatins have been identified (Nässel, 2002). The AST-A type is by far the most extensively studied.

Immunocytochemical localization of AST-A in the ALs of several insect species identify LNs, PNs as well as CNs as containing AST-A (Schachtner, Schmidt & Homberg, 2005). Although AST-A immunoreactivity is more

common in LNs and PNs, AST-A immunoreactive CNs have been found in locusts and moths (Schachtner, Schmidt & Homberg, 2005). The AST-A type of peptides have also been shown to colocalize with FaRPs and serotonin (Nässel, 2002). Presence of allatostatins in the chemosensory system of several insect species suggest that these peptides are involved in information processing in this neuropil. However, no conclusive evidence for the function of these neuropeptides in the olfactory system is yet available. The receptors mediating AST-A signaling have been characterized in the cockroach (Lungchukiet *et al.*, 2008) and in *D. melanogaster* (Lenz, Williamson & Grimmelikhuijzen, 2000a, b).

Neuropeptide Receptors

Neuropeptides mediate their biological effects through their interaction with specific receptors. Neuropeptide signaling generally occurs through GPCRs. However, there is always an exception to the rule as insulin-like peptides have been shown to interact with ion-gated channels (Claeys *et al.*, 2005). As a result of the genome sequencing efforts on several insect species, that which may be the entire peptide receptor encoding gene families have been identified, including those of *D. melanogaster* (Brody & Cravchik, 2000; Hewes & Taghert, 2001; Taghert & Veenstra, 2003), *A. mellifera*, (Hauser *et al.*, 2006) *An. gambiae* (Hill *et al.*, 2002; Riehle *et al.*, 2002) and *Tribolium castenatum*. (Hauser *et al.*, 2008) In insects, several studies have been aimed at characterizing the binding to the receptors of their endogenous neuropeptide ligands (ref). For example, FaRP receptors have been identified and characterized from three FaRP subfamilies in *D. melanogaster*: myosuppressin, sulfakinin, and FMRFamide receptors (Cazzamali & Grimmelikhuijzen, 2002; Meeusen *et al.*, 2002). Four TRP receptors have also been characterized in flies (Li *et al.*, 1991; Monnier *et al.*, 1992; Guerrero, 1997; Poel *et al.*, 2004). All three families of allatostatin receptors have been identified (Bendena *et al.*, 1999; Duve *et al.*, 1997), however, no allatotropin receptors have yet been characterized.

6.3 Signal transduction in the biogenic amines and neuropeptide pathways

As mentioned above, both biogenic amines and the majority of neuropeptides mediate their biological effects through GPCRs (Blenau & Baumann, 2001; Hewes & Taghert, 2001; Nässel, 2002; Claeys *et al.*, 2005). There are several types of GPCRs present in insects and the choice of the receptor largely depends on the individual neurochemical. In general, when these neurochemicals are released they bind to their specific receptors located on the cell surface of the effector tissue, which leads to a conformational change of the receptor that will trigger the associated G-protein subunit to dissociate and then activate the second messenger systems within the cell. Activation acts either through the adenylate cyclase or the phospholipase pathway and influences the production of second messengers including cyclic AMP, diacylglycerol, inositoltriphosphate or directly alter the intracellular Ca^{2+} concentration. This will result in the activation of protein kinases which can modify the properties of cytosolic proteins, ion channel activity and transcription factors to bring about an amplified and long term modulated cellular response.

7 Experimental techniques

Immunocytochemistry

For the past several decades immunocytochemistry has been the method of choice to study cellular and regional distribution of neurochemicals in the nervous system (Nässel, 1996). For immunocytochemical analysis of neurochemicals, a primary antiserum against a particular neuroactive substance, usually raised in a rabbit, goat, sheep or mouse, selectively binds to its specific antigen and forms an antigen-antibody complex. This complex can be visualized following the application of a secondary antibody detection system, which utilizes chemical tags, such as fluorophores, to label the complexes. In the case of fluorescent immunocytochemistry cells containing antibody-antigen complexes are visualized using epifluorescence or confocal

microscopy. In insects, the first application of immunocytochemistry was performed in the cockroach in which antisera purified from neurohemal organs were used to detect tissue specific antigens (Eckert, Gersch & Wagner, 1971). There are several steps involved in the immunocytochemical procedure. The procedure initially requires the production of specific antisera against the compound of interest; followed by tissue preparation and fixation. Application of the primary antibody to the fixed tissue of interest is followed by the addition of the secondary antibody conjugated to its chemical detection system. The secondary antibody recognizes the part of the primary antibody complex as its antigen as it has been raised against the mammal in which the primary antiserum was derived (e.g. rabbit, mouse, etc.). Optimization of each step in this process is usually required for every new primary antibody, tissue and insect species under investigation (Nässel, 1996). The choice of an appropriate secondary detection system for the tissue, species and antigen involved is important. The secondary detection system can include a fluorescently tagged secondary antibody of particular wavelength that can be excited using the appropriate lasers in a fluorescence/confocal microscope system, currently the most common immunocytochemical technique for neuroactive chemicals. Common fluorescent tags, known as fluorophores, are FITC, Texas Red and Alexa Fluor. There are, however, other secondary antibody detection systems; the most common of these is the 3,3'-Diaminobenzidine (DAB) system in which a biotinylated secondary antibody coupled with streptavidin-horseradish peroxidase that reacts with DAB to produce a brown chemical product which can be visualized with standard light microscopy.

High performance liquid chromatography coupled to electrochemical detection

Although immunocytochemical methods have been useful in the identification and mapping of neurochemicals in the nervous system, accurate quantitative information is not easily extracted from these studies. Several biochemical methods have been employed in order to quantify neuromodulators in the nervous system. One such method is the utilization of high performance liquid chromatography coupled with electrochemical detection (HPLC-ECD). This method is used effectively in analyzing those molecules which are not readily ionized, such as biogenic amines (Gramsbergen, 2002; Hardie & Hirsh, 2006; Andersen, 2006). Here, the biogenic amines are extracted from the tissue sample in perchloric acid

with the addition of antioxidants to protect the biogenic amines from degradation. This sample is then injected onto the HPLC column and subjected to a gradient of solvents in the mobile phase to separate the compounds within the extract. These separated compounds, including the biogenic amines will elute from the column at different times, thus they will be able to be singly detected using the ECD. It is the differential oxidation potentials are measured by a silver/silver chloridized electrode which describes the different compounds detected. Compounds are identified by the retention times and quantified by the peak height (voltage differential) of an external standard injected into the same column to quantify the biogenic amine (Gramsbergen, 2002)

Matrix assisted laser desorption ionization time-of-flight mass spectrometry

In recent years, many novel neuropeptides have been discovered due to the development of a highly sensitive mass spectrometric technique. With an increased interest in exploring peptide diversity in different organisms, the technique known as matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become a formidable tool. The identification of neuropeptides from insect the nervous tissues, and indeed even individual cells, has been greatly expanded through the use of MALDI-TOF-MS. In this technique, the tissue to be analyzed is embedded in a suitable solid matrix and bombarded with a laser exciting the matrix molecules thus ionizing the neuropeptides. These ions are activated and move away from the tissue sample according to a ratio of their mass and ionic charge. The ions are accelerated towards the MS detector where they are detected and identified based on the time elapsed between excitation and detection (i.e. time-of-flight; TOF; Nilsson *et al.*, 1998; Hummon, Amare & Sweedler, 2005). The main advantage of this technique is that peptides of known mass can be detected from single neurons, cell body clusters or neuropils. This techniques has been effectively employed in the identification of neuropeptides in the ALs of insects such as *D. melanogaster* (Winther *et al.*, 2003; Predel *et al.*, 2004) *Schistocerca gregaria* (Homberg *et al.*, 2004), *Manduca sexta* (Utz *et al.*, 2007), *H. virescens* (Berg *et al.*, 2007), *Tribolium castaneum* (Schachtner *et al.*, 2009) and *Ae. aegypti* (Siju *et al.*, in preparation).

Single sensillum recording

Single sensillum recordings (SSRs) are performed by inserting a sharp tungsten or glass electrode into the lumen of the chemosensory sensillum in order to establish an electrical contact with the sensory neurons present. The electrical activity is observed as waveforms, denoted spikes, believed to correlate directly with action potentials generated in the ORNs. The spikes are recorded, amplified and stored/analyzed via computer software. This method was developed for insect studies by Boeckh (1962). The SSR technique provides a highly sensitive assay to analyze the olfactory system of mosquitoes (e.g. Qiu *et al.*, 2006; Ghaninia, Ignell & Hansson, 2007; Hill, Hansson & Ignell, 2009). By using SSR, the coding characteristics of ORNs in individual sensilla can be analyzed enabling us to functionally classify the ORNs and generate peripheral coding maps.

8 Summary of results

8.1 Biogenic amine systems in the mosquito chemosensory system (Paper I and II)

Extensive studies have been reported in a variety of insects pertaining to their biogenic amine systems. The majority of these studies have been focused on e.g. the sensory systems and the peripheral systems. A detailed study of the biogenic amine system in mosquitoes is largely lacking. In order to begin to provide a detailed information concerning the distribution of biogenic-amine expressing neurons innervating the chemosensory system of mosquitoes, I decided to begin by focusing on an important member of the biogenic amine family, serotonin, with the assistance of immunocytochemistry.

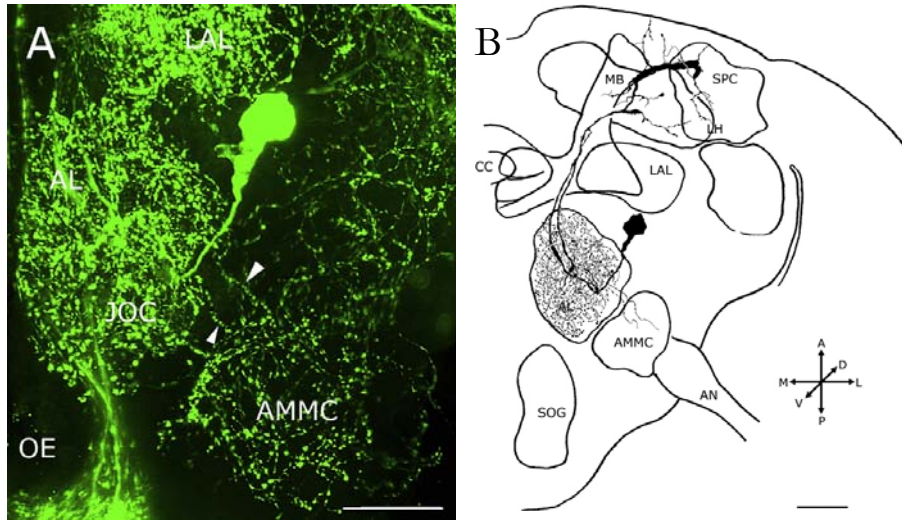


Figure 5. (A) Serotonin-immunoreactive neuron arborizing within the antennal lobe (AL) of a male *Aedes aegypti*. (B) Reconstruction of the AL serotonin-immunoreactive centrifugal neuron overlaid on a central brain outline. LAL, lateral accessory lobe; JOC, Johnston's organ center; OE, esophagus; AMMC, antennal motor and mechanosensory center. An, antennal nerve; CC, central complex; MB, mushroom body; SPC superior and inferior medial protocerebrum; LH, lateral horn; SOG, subesophageal ganglion; M,medial; P,posterior; D,dorsal; L,lateral; A, anterior. Scale bar= 25 μm . (Siju, Hansson & Ignell, 2008).

In this project, I revealed the detailed morphology of SI neurons in the central and peripheral chemosensory systems of two mosquito species, *Ae. aegypti* and *An. gambiae*. In the central chemosensory system, I identified the SI neurons innervating the AL, the subesophageal ganglion and the tritocerebral neuropil. I identified a single SI centrifugal neuron innervating the AL, which is characterized by its varicose synaptic swellings in the AL and wide-field arborizations in the protocerebrum (Figure 5). In contrast to what was previously reported in mosquitoes as well as other dipteran insects

(Dacks, Christensen & Hildebrand, 2006), the AL SI neuron showed an exclusively ipsilateral arborization. This morphology of the AL SI neuron closely resembled that which was previously reported in cockroaches (Salecker & Distler, 1990). The evolutionary significance of this morphological similarity is, however, not known at the current time.

Another novel finding was the identification of SI efferents innervating the peripheral chemosensory organs, including the antennae, the maxillary palpa and the labium. Moreover, I was able to describe SI efferents in Johnston's organ located at the base of the antennae. These findings have previously not been reported in any insect and indicate a modulatory role of serotonin in the peripheral chemosensory systems. In addition, by measuring the volume of the SI varicosities in the AL throughout a 24h day. I observed a correlation between the SI varicose volume and the mosquito activity levels suggesting that serotonin may play a role in the modulation of mosquito olfactory-related activity. Furthermore, I determined the effect of blood-feeding on the SI varicose volume. Here, I observed that the SI volume was significantly reduced in female mosquitoes 5 min after blood-feeding, compared to non-blood-fed mosquitoes suggesting serotonin is being released from these varicosities within 5 minutes of the onset of blood-feeding. Results from this study demonstrate that serotonin has a complex distribution pattern in the chemosensory systems of mosquitoes. Presence of serotonin in central and peripheral chemosensory system suggests that this biogenic amine plays a crucial role in sensitivity modulation involved in controlling chemosensory behaviors of mosquitoes

The second part of this project (paper II) was designed to study correlations between changes in the biogenic amine titer and behavior in the context of the physiological status of female mosquitoes. In this project I used HPLC-ECD analysis to quantify the major biogenic amines (serotonin, dopamine and octopamine) present in the head of these mosquitoes. To do so, I assayed levels of serotonin, dopamine and octopamine through out a single gonotrophic cycle of *Ae. aegypti* females. I measured the titer of these biogenic amines assesing: 1) rapid changes occurring immediately after a blood-meal; 2) circadian changes in biogenic amine levels during the flight-activity period of non-blood-fed and blood-fed mosquitoes; and 3) changes in biogenic amine levels occurring at the end of a complete gonotrophic cycle, i.e. post-oviposition.

In the case of serotonin there were no changes in the level within the first hour following a blood-meal. However, I observed a slight change in the levels of serotonin during the flight activity period of non-blood-fed mosquitoes. Here, lower levels of serotonin in the head indicate that

serotonin was released from the CNS during the flight activity period of this mosquito. Compared with non-blood-fed mosquitoes, blood-fed mosquitoes have lower serotonin levels throughout the gonotrophic cycle. This result supports the hypothesis that blood-fed mosquitoes may down-regulate the production of serotonin in response to blood-feeding.

I further found that blood-feeding induced significant changes in the level of dopamine in blood-fed compared to non-blood-fed mosquitoes. Higher levels of dopamine are observed in blood-fed mosquitoes following a blood-meal. This increase in the dopamine titer was evident immediately (within 5 min) after blood-feeding and was maintained until 72h post-blood meal. In other insects, a high level of dopamine is associated with increased flight activity; however, blood-fed mosquitoes in general reduce their activity following a blood-meal (Jones, 1981). Furthermore, a higher level of dopamine was found in the heads of mosquitoes that had completed a gonotrophic cycle compared to those of the same age which have not blood-fed. This may be related to the sexual receptivity of female mosquitoes towards male mosquitoes. A study has reported a similar phenomenon in *D. melanogaster* (Neckameyer, 1998).

Octopamine levels in blood-fed mosquitoes showed a rather complex pattern consisting of fast, intermediate and slow changes in titer. The fast and intermediate changes may be correlated with changes in the sensory state of the the mosquito, whereas the drastic decrease in the level during the scotophase may be attributed to the motionless postural effect observed in mosquitoes after blood-feeding. Postural effects of a reduction in octopamine has been demonstrated previously in insects and arthropods (Linn & Roelofs, 1986; Wood, 1995; Wood, Gleeson & Derby, 1995).

The results from these analyses indicate that stereotyped changes in the levels of biogenic amines in response to shift in physiological state may play a functional role in the manifestation of the different behavioral repertoires observed during the gonotrophic cycle of these mosquitoes.

8.2 Neuropeptides in the antennal lobe of mosquitoes (paper III)

Neuropeptides are the most versatile class of neuroactive compounds present in the nervous system of all organisms studied (Nässel, 2002). They are structurally and functionally diverse and found to act as neurotransmitters, neurohormones or neuromodulators in the nervous system. In insects, neuropeptides have been suggested to influence the physiology of several systems, including those involved in e.g. olfaction, circadian rhythms,

locomotion and feeding. Although several studies have previously dealt with the neuropeptides in the insect nervous system, particularly the sensory systems (Nässel, 2002; Nässel & Homberg, 2006) there has been no record of this in the chemosensory system of mosquitoes. The dearth of studies in this branch prompted me to look at the neuropeptides in the primary olfactory centers of the disease vector mosquito, *Ae. aegypti*

In this project, I used a systematic approach to identify all neuropeptides expressed in the AL. This was accomplished through MALDI-TOF-MS profiling of single ALs of male and female *Ae. aegypti*. A total of 26 neuropeptides, representing 10 neuropeptide gene families, were identified through this approach (Table 1). Having profiled the ALs for the presence of different neuropeptides, I then elucidated the cellular localization pattern of four of the major families of neuropeptides with the help of immunocytochemical techniques. These four families included FMRamide (FMRFa)-related peptides (FaRPs), tachykinin-related peptides (TRPs), allatotropin and allatostatin. The immunolocalization pattern of these neuropeptides confirmed the presence in the AL, thus corroborating the result obtained through MALDI-TOF-MS profiling. Each neuropeptide family showed a distinct immunolocalization pattern.

The first neuropeptide family analyzed was the FaRPs. FMRFa-immunoreactive (ir) neurons revealed varicose LNs that innervated all AL glomeruli. Interestingly, I observed that a few of the medial glomeruli were relatively denser innervated compared to other AL glomeruli. I also observed a single FMRFa-ir neuron that displayed wide-field arborizations in the AL and also in distinct areas of the protocerebrum. In the ALs, the thick varicose fiber of this neuron arborized in the non-glomerular neuropil area of posterior and lateral regions of the AL. In addition, these fibers wrapped around the maxillary palp-associated glomeruli in the medio-dorsal region of the AL. The axon of this neuron exited the AL antero-laterally and bifurcated at the level of the central complex (CC). One branch projected further into the superior protocerebrum, whereas the other branch projected into the fan-shaped body of the CC. Based on the arborization pattern of FMRFa-ir neurons in the AL of *Ae. aegypti* I believe that members this neuropeptide family may play a functional role in the modulation of olfactory processes. A novel finding in this study was the FMRFa-ir centrifugal neuron that connects the maxillary palp-associated glomeruli in the AL to protocerebral areas, such as the CC and the lateral accessory lobe, associated with motor control. Based on the observed morphological patterns, I speculate that FaRPs are involved in modulating odor perception and motor control in mosquitoes. Based on previous

reports, the FaRPs may also be involved in modulating circadian rhythm activity. (Soehler *et al.*, 2008, Stembrini & Villar, 2005).

Peptides	sequence	calculated	measured mean	mean deviation	male (n=20)	female (n=20) abundance [%]
A-type allatostatins						
AST-A 4	RVYDFGLa	868.4676	868.4693	0.0506	45	50
AST-A 5	LPNRYNFGLa	1092.5949	1092.592	0.0629	55	75
AST-A 3	ASAYRYHFGLa	1183.6007	1183.6056	0.0615	70	75
Allatotropin						
AT	APFRNSEMMTARGFa	1613.7675	1613.7527	0.0848	65	55
Corazonin						
Corazonin	pQTFQYSRGWTNa	1369.6289	1369.6527	0.0388	5	15
FMRFamides						
FMRFa-3	AGQGFMRFa	912.4514	912.3995	0.1521	25	10
FMRFa-7	GSGNLMRFa	880.4463	880.4472	0.0478	15	5
Myoinhibitory peptides						
Mip 2	AWNKINGGWa	1044.5379	1044.5608	0.0494	95	100
Mip 3	VNAGPAQWNKFRGSWa	1716.8723	1716.8263	0.1131	15	10
short neuropeptide F						
sNPF-1 (4-11)	SPSLRLRFa	974.5894	974.5885	0.0451	95	100
sNPF-2 (4-11)*	APQLRLRFa	999.621	999.6211	0.0456	100	100
sNPF-3	APSQRLRWa	1012.5805	1012.5812	0.0457	100	100
sNPF-1	AVRSPSLRLRFa	1300.7966	1300.8241	0.0906	15	25
Neuropeptide-like precursor 1						
NPLP-2	NLASARASGYMLNa	1366.6901	1366.6906	0.0634	100	100
NPLP-3	NIASLARKYELPa	1373.7905	1373.791	0.0625	100	100
NPLP-1	SYRSLLRDGFa	1384.7337	1384.7423	0.0676	65	65
NPLP (pp 177-193)	NIQSLLRGMLPSIAP-OH	1710.9576	1710.8134	0.0824	90	90
NPLP (pp 92-111)	NLGSLARAGLLRTPSTDYL-OH	2018.1034	2018.1173	0.0874	80	65
NPLP (pp 195-217)	NMQSLARDNSLPHFAGAAAQES-OH	2315.0838	2315.1206	0.1740	70	80
sulfakinin						
SK-1	FDDYGHMRFa	1186.5104	1186.5134	0.0735	25	55
SK-2	GGGEGEGQFDYGHMRFa	1857.7615	1857.7177	0.0819	30	25
Pyrokinin (PK-PBAN)						
PK-3	NLPFSPRLa	942.552	942.5965	0.0481	5	20
Tachykinin related peptides						
TKRP-1 (x2)	APSGFLGLRa	916.5363	916.5364	0.0420	100	100
TKRP-3	APSGFLGMRa	934.4927	934.4966	0.0429	95	95
TKRP-2	VPSGFTGMRa	950.4876	950.4918	0.0423	95	90
TKRP-4	VPNGFLGVRa	957.5629	957.5413	0.0665	60	75

Table 1 Calculated and measured monoisotopic masses [M+H]⁺ obtained by direct profiling of single antennal lobes and subsequently matching profiles with masses calculated from sequences of 26 predicted neuropeptides stemming from 10 different neuropeptide genes in *Aedes aegypti*.

Immunolocalization of the TRPs revealed a set of LNs that arborized throughout the AL. The varicose morphology of TRP-ir LNs in the AL of *Ae. aegypti* closely resembles that previously reported in *D. melanogaster*. (Winther *et al.*, 2003). This similarity prompted me to suggest that TRPs may have a similar function in the olfactory system of these two dipteran insects. A previous study has demonstrated the role of TRPs in the olfactory system of *D. melanogaster*, in which the lack of TRPs in larval and adult flies obstructed the olfactory perception and locomotor activity in response to olfactory stimuli (Winther *et al.*, 2006).

The third family of neuropeptides analyzed by immunocytochemistry was the allatotropins. I observed a uniform staining pattern of this neuropeptide in LNs of the AL with a small number of cell bodies in the lateral cell cluster. In other insects, allatotropin have been implicated in circadian rhythm activity. (Petri *et al.*, 2002).

The fourth family of neuropeptides investigated was the allatostatins. Allatostatin immunoreactivity was found through out the AL. I did, however, I observed an intense granular staining of this peptide in some of the antero medial glomeruli: similar to what was shown among the FaRPs. In addition, a thick fiber with varicosities was also found arborizing throughout the AL. The immunoreactivity to the AL glomeruli originated from 12-16 somata in the lateral cluster. The thick varicose fiber seemed to be originating from a single neurite in the tritocerebrum. Selective innervation pattern of this neuropeptide in a few glomeruli suggest a specific role of this peptide in those glomeruli. However, the exact role of allatostatin in the AL is not yet described

In summary, in this study I identified all neuropeptides expressed in the AL of *Ae. aegypti*; members of four families were cellularly mapped and identified. The results clearly indicate that, like in other insect species, the AL of mosquitoes contains a wide range of neurochemicals. The high diversity of neuropeptides suggest that a complexity of signaling mechanism are involved in shaping the olfactory processing in the AL of mosquitoes. Many of the identified neuropeptides may play a neuromodulatory role in the mosquito AL that potentially could influence the behavior of this disease vector.

8.2 Sensitivity modulation at the peripheral olfactory system (paper IV)

A blood-meal by a female mosquito induces a plethora of physiological changes. Often these physiological changes are reflected in behavioral patterns. The mechanisms triggering these behavioral changes are largely unknown. Considering that the olfactory system is involved in driving several important behaviors of mosquitoes, it is plausible that the observed behavioral changes are triggered by modulation of this system. The question I asked in this project was: does a change in the gonotrophic status of the mosquito influence the activity of ORN housed in the antennal sensilla? By using the single sensillum recording technique I assayed for differences in sensitivity of ORNs housed in *sensilla trichodea* in response to blood-feeding, 24h and 72h post-blood meal. In the majority of these sensilla, i.e. the long

sharp, short sharp and short blunt type I sensilla no changes occurred in response to blood-feeding. However, in the short blunt type II (sbtII) sensilla trichodea, I observed a change in sensitivity in three of the 5 functional subtypes after blood-feeding, in which the response to indole and phenols was significantly increased 24h post-blood meal, and were generally maintained 72h after blood-feeding.

9 Conclusion and future perspectives

Mosquitoes largely depend on the chemosensory systems to interact with their environment. An understanding of chemosensory information processing and modulation is of paramount importance in the development of novel safe, effective and efficient mosquito control methods. Although, there has been research into the neurochemicals present in the chemosensory system of several insect species, very little is currently known concerning neuromodulation in disease vector mosquitoes. Therefore, in the present study, I have investigated the modulation of the chemosensory system of mosquitoes.

In this thesis, I have gathered detailed information on some important aspects of the modulation of mosquito chemosensory behaviour including the identification and characterization of biogenic amines and neuropeptides present in the mosquito chemosensory system; and the effects of mosquito physiological state on peripheral sensitivity and neuromodulator levels in the olfactory system. To begin the characterization of the modulation of mosquito olfaction, I have isolated and identified putative neuromodulators from the heads of *Aedes aegypti* mosquitoes. I have isolated the biogenic amines dopamine, octopamine and serotonin, along with 26 neuropeptides from four families. For the biogenic amines, I used immunohistochemical techniques have generated a detailed map of serotonin distribution in the central and peripheral chemosensory systems, and with HPLC-ECD quantified serotonin, dopamine and octopamine titers throughout a female's first gonotrophic cycle, providing insight into modulatory function of these biogenic amines in mosquito olfaction and gustation.

As neuropeptides are the most diverse neurochemicals in insects, the profiling of neuropeptides in the AL of mosquito may give new insight into the complex signaling strategies adopted by these disease vectors. To begin the characterization of the mosquito neuropeptides in the chemosensory

system, I mapped distribution of the four neuropeptide modulatory families I isolated and identified from heads using immunohistochemistry.

In order to understand the role of physiological state in modulating the peripheral chemosensory system of mosquito, I studied the ORN sensitivity at the peripheral olfactory organ, antennae following a blood-meal. These studies indicate that there is indeed modulation of the peripheral olfactory sensory neurons in response to a change in physiological state.

In summary, the results presented in the thesis support the hypothesis that neuromodulators are working in both the peripheral and central olfactory systems in order to modulate the transduction of odor and gustatory signals, and ultimately the behavior of the yellow fever mosquitoes. This work thus lends support to those arguments which advocate the use of modulators, their agonists and antagonists in the development of novel mosquito control strategies.

Although the research in this thesis has provided a good beginning description of neuromodulation in the mosquito chemosensory system and its components, it is only a partial picture. Future work should be focused on profiling more of the neurochemicals in the chemosensory system identified in this study. With the help of more powerful immunohistochemical techniques and newly-derived specific antisera, we will be able to provide more detailed distribution pattern of several these putative neuropeptides. Co-localization studies of these neuropeptide should also be conducted to understand the co-transmitter role they play in chemosensory signaling. In addition, ultrastructural studies are needed to determine the synaptic connectivity of these neuropeptidergic neurons. It is also important to describe the functional roles of these neurochemicals in the chemosensory system, and ultimately the effect of these changes on mosquito vector-related behaviors. Molecular and pharmacological studies of these neurochemicals may provide an insight into the precise modulatory mechanisms of these chemicals. It is equally important to characterize the cognate receptors for most of these neurochemicals beginning with demonstrating their cellular distribution to identify the modulatory targets within the chemosensory systems. Considering that we have genome sequence available for many insects, and particularly three mosquito species, along with the availability of different genetic tools, it would be largely possible to achieve this goal. Here gene knock-down technology (i.e. RNA interference) should be conducted, which will compliment anatomical, physiological and behavioral methods discussed above. Manipulating the spatial and temporal pattern of expression of several of these neuroactive compounds in the chemosensory system may provide ample evidence to the

functional and behavioral significance. Finally characterizing the neurochemicals in different mosquito species may provide a clue regarding the evolution of different behavioral preference exhibited by mosquitoes.

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The best thing happened to me in my life without any doubt is my family- **Sibi**, my little brother, you are grown up and not a little brother anymore, but I missed seeing you growing up. Thanks for taking care of everything in my absence at home, love you. **Sili, Abhiyettan** and **Lakshmi**, the first little family. Abhiyetta, even though, we have been together only 24 hours, you gave me lot of support and encouragement during my struggle to complete, especially when I am really down, thanks for everything. Sili, my dear sister, thanks for being the sweet little sister you are, love you.

All what I am now, truly belongs two people, my **Amma** and **Achan**. I wouldn't have achieved anything in live without your love and support. With all limitations, you always kept your hopes in me and encouraged me to do what I like best. You both gave me freedom and let me do whatever I wanted to do. I hope one day I can make you proud and I always want you both with me rest of my life, Love you.