CHAPTER I

INTRODUCTION

The detection of chemical cues in the environment, a process known as chemosensation, is crucial to all forms of life. Specifically, the chemosensory modality of olfaction, or the sense of smell, is of great underlying significance to most animals with regards to food detection, mate selection, predator avoidance and other vital behaviors (Hildebrand and Shepherd, 1997). Importantly, olfaction plays a key role in mediating the host seeking behaviours (Takken, 1991) of several disease vector mosquitoes that are responsible for the transmittance of malaria, yellow fever, dengue fever, West Nile virus, and other maladies to humans. In light of this, a plethora of laboratory and field research programs are in progress to investigate the internal mechanisms of olfaction in the major mosquito vector, *Anopheles gambiae* (henceforth *An. gambiae)*, bearing the most deadly of the mosquito borne diseases, malaria. *Drosophila melanogaster* is a dipteran genetic model organism, and serves as an excellent means to investigate common insect olfactory processes. The focus of this dissertation is an investigation of *D. melanogaster* and *An. gambiae* arrestin proteins within insect olfactory systems.

Olfaction in the Mosquito

Malaria is one of several mosquito borne diseases afflicting human populations, yet in terms of sheer impact it remains the most devastating. It is estimated that each year, there are roughly five hundred million clinical attacks of malaria, and over one million people die as a result of malaria infection (WHO, 2007). Most of these deaths occur in the developing world, resulting in an excruciating economic toll on affected countries (Takken and Knols, 1999), hindering their development in a vicious cycle that

travels along the road to nowhere. In Africa, where the majority of malaria cases occur (Snow et al., 2005), the primary mosquito vector is the species, *An. gambiae*, which is one of seven species of the *An. gambiae sensu lato* complex (Takken and Knols, 1999).

Malaria transmission requires the dynamic interplay among three organisms: humans, plasmodium parasites and the mosquito vector. A plasmodium-infected mosquito must take a blood meal from a human host to transfer sporozoites to the human host. The disease transmission cycle of plasmodium from an infected mosquito to a non-infected human, and from an infected human to a non-infected mosquito produces great disease potential in regions endogenous to both suitable anopheline mosquito vectors and plasmodium parasites.

Further compounding the prevalence of malaria transmission is the strong preference for human hosts (White, 1974) inherent to the *An. gambiae* mosquito. This trait, known as anthropophily, has been shown to be strongly mediated by the mosquitoes' sense of smell (Takken, 1991) (Figure 1). Moreover, additional *An. gambiae* characteristics, including preferences for feeding and resting indoors (endophagy and endophily, respectively) strongly elevate the vectorial capacity, or the sheer quantitative efficacy in transmittance of disease, of *An. gambiae* as a mosquito vector for human malaria (Takken and Knols, 1999).

Numerous electrophysiological and behavioural studies, both in the laboratory and in the field, have been conducted to improve our understanding of the anthropophilic nature of *An. gambiae*. Early studies demonstrated that host preference in *An. gambiae* is strongly mediated by olfactory cues (Takken, 1991). Furthermore, a reference for human hosts over other animals, such as cattle, was also evident (Coluzzi et al., 1975; Pates et al., 2001). The olfactory nature of the attraction towards human hosts was confirmed in laboratory studies demonstrating that the mosquitoes were responsive to

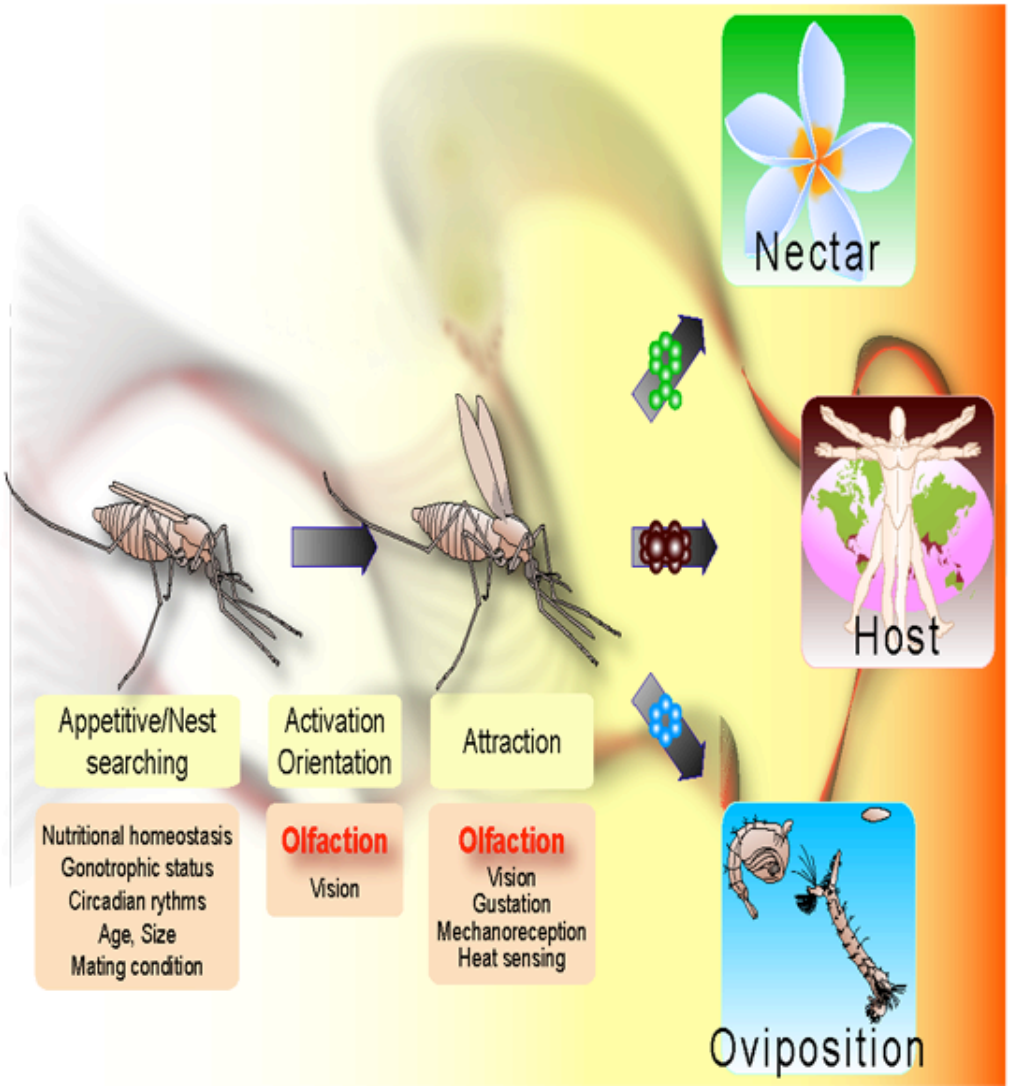

Figure 1. Host seeking behaviour mediated by olfaction. In *An. gambiae,* important behaviours are influenced by a myriad of factors. The mosquito sense of smell plays a prominent role in host-seeking behaviour. Specifically, *An. gambiae* displays a strong preference for taking blood meals from humans. These facts have dramatic consequences leading to the spread of malaria. Figure designed by Dr. Jonathan Bohbot.

isolated human volatiles in the absence of a human subject (Costantini et al., 1993; Mboera et al., 1997).

It has since been clarified that *An. gambiae* mosquitoes are attracted to emanations from human sweat (Braks and Takken, 1997) and human skin (Takken and Knols, 1990), and in particular, from the foot and ankle region (De Jong and Knols, 1995b), but not from human breath (De Jong and Knols, 1995a). Interestingly, aliphatic fatty acids are important components responsible for the mosquitoes' attraction to skin emanations (Knols et al., 1997), and these chemicals are actually metabolic byproducts of resident microflora on human skin (Nicolaides, 1974). Other human volatiles implicated in the attraction of *An. gambiae* include ammonia (NH_3) and lactic acid (Braks et al., 2001; Dekker et al., 2002). Moreover, a recent report identifies significant attraction of *An. gambiae* to a synergistic blend of NH_3, lactic acid, and carboxylic acids (Smallegange et al., 2005). Finally, while it has been established that many mosquitoes, including *An. gambiae*, respond to carbon dioxide (CO_2) both behaviourally (Knols et al., 1994; Dekker and Takken, 1998; Dekker et al., 2002) and electrophisiologically (Grant et al., 1995; Lu et al., 2007), its role in mediating human host seeking behaviours of *An. gambiae* is less than certain. Clearly, CO_2 cannot be a strict determinant of anthropophily for *An. gambiae,* as it is a common exhalent of all possible blood meal hosts (Takken and Knols, 1999; Zwiebel and Takken, 2004).

Until recently, the mechanisms underlying mosquito responsiveness to human odors had not been given much consideration. However, sequencing of the complete genome of *An. gambiae* (Holt et al., 2002) and subsequent identification of a large family of candidate odorant receptors (ORs) (Hill et al., 2002), have facilitated study of the molecular mechanisms mediating olfaction at the level of the odorant receptor neuron (ORN). Special consideration has been given to olfactory signal transduction cascades and the connectivity of ORNs to higher olfactory processing centers. It is at the level of

the ORN where the interface between the mosquito olfactory system and the environment lies. Thus an integration of knowledge concerning the external olfactory cues with internal olfactory processing mechanisms is necessary for a complete understanding of the most prominent features responsible for olfactory based behaviours including host seeking, sugar feeding and ovipositing.

It is this researcher's hope, for the benefit of the entire world, that investigations of the olfactory system of *An. gambiae,* coupled with existing knowledge of behaviourally relevant human odorants known to mediate host-seeking behaviour, will foster the development of better and safer mosquito attractants and repellents. These products, when coupled with numerous other strategies that are being considered to combat malaria, may go a long way towards dramatically reducing, if not ending, the immense human suffering and financial burden experienced by Africa and other regions as a result of malaria infestation. This is especially pertinent in light of the observations that, in recent years, drug-resistant forms of plasmodium, immune to more conventional methodologies of treatment of the malaria disease, have become prevalent (Marshall, 2000).

General Principles of Olfaction

The planet earth consists of a multitude of environments, most of which are occupied by various forms of life. The complex interactions between the abiotic and biotic components of an ecosystem result in an unyielding bombardment of chemical stimuli on the various life forms. Chemosensation, or the ability to detect chemicals in the environment, is a prominent sensory modality inherent to virtually all forms of life. From the simplest bacterium to the most complex multicellular organism, mechanisms exist to detect, identify and respond to chemicals. In invertebrates and vertebrates alike, detection of volatile compounds, or odorants is a matter of life or death. In that regard,

diverse behaviours including, but not limited to, food seeking, mate choice, predator avoidance, host seeking and ovipositing are known to have a central, if not predominant olfactory component (Hildebrand and Shepherd, 1997; Takken and Knols, 1999).

Inherent similarities as well as differences are apparent in the olfactory systems of invertebrate and vertebrate model organisms. The similarities imply that efficient strategies are employed to make sense of potentially thousands of chemical cues that an organism might encounter during its lifetime. The differences underscore the great importance of olfaction, such that complex olfactory systems would emerge multiple times during the history of evolution of life. Within the context of this dissertation, these similarities and differences will be examined at the cellular and molecular level with emphasis on the vertebrate and invertebrate model systems of the rat (*Rattus norvegicus)*, mouse (*Mus musculus)* and fruitfly (*Drosophila melanogaster)*. Examples from other species will be called upon where appropriate, and references to the mosquito vector, *An. gambiae* will be made where relevant information is available.

Cellular Organization of the Olfactory System

In both vertebrate and invertebrate model organisms, odorants interface with the organism through specific interactions with their ORs, which are present on the membranes of dendritic extensions of ORNs. In mammals, bipolar ORNs project their dendrites from the main olfactory epithelium (MOE) tissue, where the ORNs reside, into the nasal cavity in the form of many ciliated extensions; these olfactory cilia are covered with a thin protective layer of mucus. At the other end, these ORNs project their axons bilaterally into the forebrain to a specialized region known as the olfactory bulb, where they synapse with projection neuron dendrites in discrete spherical neuropil structures known as glomeruli. Interactions with modulatory interneurons also occur in the olfactory

bulb. Projection neurons then send their axons to higher olfactory centers in the brain (Firestein, 2001).

In most mammals, a second olfactory organ – known as the vomeronasal organ (VNO) – resides within the nasal cavity at a physically separate location from the main olfactory epithelium. The VNO is known to function in the processing of conspecific chemical cues (Wang et al., 2007), known as pheromones, and project their axons to the accessory olfactory bulb, which is contiguous with the main olfactory bulb (Wang et al., 2007). In human beings, the VNO is present, but its function in pheromone detection remains debatable (Meredith, 2001).

In insects, ORNs are also bipolar and reside in the lumen of specialized olfactory appendages that protrude from the head of the animal. In *Drosophila* the "insect nose" is known to consist of two types of appendages, the antennae (and specifically the 3rd antennal segment) and maxillary palps (Stocker, 1994). In *An. gambiae,* a recent report describes an olfactory role for the proboscis, or mouthpart, as well (Kwon et al., 2006). In all cases, the ORNs send their dendrites into the shafts of tiny porous hair structures known as chemosensory sensilla, which cover the surface of the olfactory appendages (Steinbrecht, 1996). Non-chemosensory sensilla, such as those involved in mechanosensation are also present on these appendages. ORNs project their axons bilaterally, in most cases, to the antennal lobe of the brain (Stocker, 1994), where they synapse with projection neurons in glomeruli, similar to those of the mammalian olfactory system (Ache and Young, 2005). The projection neurons then send their axons to higher brain centers such as the mushroom bodies and lateral horn (Stocker, 1994).

While pheromone sensation is an essential form of communication in the insect world, specialized olfactory structures analogous to the VNO are not known to exist. However, male bees, moths and cockroaches display sexual dimorphism with the presence of a macroglomerulus or macroglomerular complex (MGC), which are known

to process pheromonal information (Anton and Homberg, 1999). Strikingly, no MGC equivalent has been identified in the antennal lobe of the fruitfly (Laissue et al., 1999) or the mosquito (Ignell et al., 2005; Ghaninia et al., 2007).

Odorant Receptors

Odorants are known to interact with ORs that are expressed on the dendrites of ORNs. In mammals, early research suggested that ORs might be guanine nucleotide-binding protein (G protein) coupled receptors (GPCRs). This speculation was based upon observations that odorant stimulation of the olfactory epithelium led to measurable cellular elevations of cyclic adenosine monophosphate (cAMP) (Pace et al., 1985; Sklar et al., 1986), produced by adenylyl cyclase (AC) enzymes coupled to G-protein signal transduction pathways. In a landmark report it was confirmed that putative ORs expressed in the olfactory epithelium of the rat, are, in fact, seven-transmembrane (7TM) GPCRs (Buck and Axel, 1991).

Since then, putative 7TM GPCR ORs have been identified and characterized in numerous vertebrates (Mombaerts, 1999; Dryer, 2000) and invertebrates (Hallem et al., 2006). With the advent of genome-sequencing, 1200-1400 putative ORs have been classified in the model rat and mouse species (Ache and Young, 2005). In humans, over 800 putative ORs have been identified in the genome, but over half of these represent pseudogenes, based upon the presence of premature stop codons and other deleterious mutations within the coding sequence (Niimura and Nei, 2003). In comparison, the rat and mouse OR families display a much lower prevalence of pseudogenes, on the order of 20-25 percent (Ache and Young, 2005).

Insects have far fewer putative ORs than their mammalian counterparts. In 1999 a trio of scientific articles reported the identification of candidate ORs in the fruit fly, *D. melanogaster* (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999).

Subsequently, a superfamily of chemosensory receptors has been described of which 62 genes belong to the OR family (Robertson et al., 2003). Similarly, in *An. gambiae,* the OR gene family is proposed to consist of 79 members (Hill et al., 2002). In contrast to ORs from mammals, and even the nematode, *C. elegans,* which displays a rate of pseudogenesis in the OR family at roughly one-quarter of OR genes (Robertson, 1998), there are few if any known pseudogenes in the OR families of insects (Robertson et al., 2003; Robertson and Wanner, 2006).

Interestingly, a highly conserved (Krieger et al., 2003; Pitts et al., 2004) and broadly expressed putative OR, known as Or83b, has been identified as being expressed in the olfactory organs of *Drosophila* (Vosshall et al., 2000). It has been noted that this "receptor" is non-responsive to broad arrays of odorants used to stimulate olfactory responses (Dobritsa et al., 2003; Elmore et al., 2003), suggesting that it does not function as an independent OR. Rather, Or83b has been identified as a dimerization partner for functionally responsive ORs (Neuhaus et al., 2005) and further, is required for localization of other ORs to the membrane (Larsson et al., 2004)

Despite the fact that the gene families representing ORs in both mammals and insects have been characterized as 7TM resident membrane proteins, there are stark differences between the two. Mammalian OR genes lack introns within the coding sequence (Buck and Axel, 1991). Additionally, genomically linked subfamilies of ORs are prevalent, and ORs within these subfamilies display high degrees of homology to each other relative to other ORs (Buck and Axel, 1991). Insect OR genes, on the other hand, contain introns and genomic subfamily clusters are uncommon (Clyne et al., 1999; Hill et al., 2002), and do not share significant levels of homology with their mammalian counterparts (Clyne et al., 1999); that is to say, there is a strong indication that the insect ORs represent a novel OR gene family. Most importantly, while insect OR gene families have traditionally been classified as 7TM GPCRs, recent evidence in *Drosophila* strongly

suggests that they are not canonical 7TM GPCR proteins. Specifically, fruit fly ORs display an inverted membrane topology, as compared to all other GPCRs, wherein the N-terminal tail of the protein is intracellular and the C-terminal tail is extracellular (Benton et al., 2006; Lundin et al., 2007).

In consideration of these differences, it would be reasonable to conclude that the OR gene families of mammals and insects are the products of convergent evolutionary events. Strikingly, however, there exist remarkably similar organizing principles observed in the olfactory systems of mammals and insects with great emphasis given to the OR as a determinant functional unit.

Cellular and Molecular Determinants of Odor Coding

There is strong evidence in both mammals (Vassar et al., 1993; Malnic et al., 1999; Ressler et al., 1993) and insects (Vosshall et al., 2000; Couto et al., 2005; Fishilevich and Vosshall, 2005) that one ORN expresses one functional OR. However, in both mammals (Rawson et al., 2000), and insects (Goldman et al., 2005; Couto et al., 2005; Fishilevich and Vosshall, 2005) there are exceptions to this rule. This concept has great impact on the discriminatory power of olfactory coding, wherein the brain would interpret discrete neuronal activity to signify presence of specific odorants that have activated specific receptors. Notably, this rule is not universal across all taxa. In the nematode, *C. elegans,* for example, it is well known that chemosensory neurons typically express multiple functional ORs as the rule and not the exception (Troemel et al., 1995).

A further point to consider is the mechanism involved in ORN gene choice. In mammals, recent reports have identified a stochastic mechanism of OR selection (Serizawa et al., 2003; Lomvardas et al., 2006), whereas in insects a deterministic model has been presented (Ray et al., 2007). Evidence demonstrates that mammalian OR expression is regulated by positive random selection, mediated by a trans-acting

chromosomal regulatory locus (known as the H-enhancer), which acts upstream of the expressed OR (Serizawa et al., 2003; Lomvardas et al., 2006). This process is not entirely random, however, as OR expression is known to be restricted to one of four distinct zones in the MOE (Ressler et al., 1993; Vassar et al., 1993). In conjunction, negative feedback elements presumably function to strengthen the positive interaction thereby preventing coexpression of additional ORs (Serizawa et al., 2003; Shykind et al., 2004). Conversely, in *Drosophila,* a combination of *cis*-acting regulatory sequences, either upstream or downstream of the OR open reading frame, and *trans*-acting transcription factors have been identified to control OR gene choice in the maxillary palp. In this system, independent *cis* regulatory sequences have been reported to promote expression in the palp and prohibit expression in the antennae (Ray et al., 2007).

Moreover, in addition to the one ORN/one OR rule, observation of monoallelic exclusion has been confirmed in the mammalian olfactory system for the OR gene expressed in each neuron (Chess et al., 1994; Mombaerts et al., 1996). This regulatory mechanism controlling gene expression further refines the expression of ORs to one allele of one gene per one ORN. It is not currently known if a similar mechanism is at play in insect ORNs, however one report provides evidence that monoallelic exclusion is not occurring for ORs (Elmore and Smith, 2001).

It is clear that within the mammalian and insect systems there exists a constraint on OR spatial expression patterns. In rodent models, the olfactory epithelium can be broadly divided into four physically distinct OR expression zones (Ressler et al., 1993; Vassar et al., 1993). With some exceptions, individual ORs are expressed in a subset of ORNs restricted to one of the four zones. Similarly, in *Drosophila* distinct ORN classes, each defined by the receptor they express, are localized to a particular physical region of the antenna in a stereotypical fashion, where a particular OR is expressed in many ORNs throughout the region (de Bruyne et al., 2001; Hallem et al., 2004b).

Functionally, ORs have been deorphanized in both the mammalian and insect systems, that is to say, activating odorant response profiles have been characterized. A major breakthrough in *Drosophila* has been the development of the endogenous "empty neuron" system (Dobritsa et al., 2003). This expression system takes advantage of a chromosomal deletion line, termed Δ-*halo,* (Gross et al., 2003) wherein a particular OR locus (OR22a/b) has been deleted. This renders the OR22a class of ORNs nonresponsive to odorants. Utilization of the Gal4/UAS expression system (Brand and Perrimon, 1993) to ectopically drive expression of a wide array of Drosophila ORs using an OR22-Gal4 driver line (Dobritsa et al., 2003) has resulted in the deorphanization, via single sensillum recordings (SSRs) of a majority of *Drosophila* ORs (Dobritsa et al., 2003; Hallem et al., 2004b). Many of these ORs display response profiles that are similar to those of previously defined ORN classes (de Bruyne et al., 2001), suggesting a molecular correlate to the cellular classifications. Additionally, a recent report has demonstrated utilization of the "empty neuron" system to identify ligands of *An. gambiae* ORs (Hallem et al., 2004a), suggesting that this system may be suitable for the widespread deorphanization of ORs from the mosquito.

Other insect olfactory appendages have been functionally mapped, as well. In *Drosophila,* the maxillary palp has been well defined (de Bruyne et al., 1999; Goldman et al., 2005). In *An. gambiae,* olfactory coding maps have been described for the antennae (Qiu et al., 2006) and the maxillary palps (Lu et al., 2007). There has been a preliminary description of the olfactory characteristics of the labellum of the proboscis in *An. gambiae* (Kwon et al., 2006). In mammalian systems, there have been reports of OR deorphanization under heterologous and homologous conditions (Zhao et al., 1998; Raming et al., 1993b; Krautwurst et al., 1998; Malnic et al., 1999). However, these reports have been limited in scope, and one major hindrance to widespread functional

characterization of mammalian ORs has been an observed lack of surface expression in heterologous cell culture expression systems (McClintock et al., 1997).

Recently however, a series of reports have identified OR binding partners that facilitate trafficking of the OR to the plasma membrane. For one subfamily of ORs, dimerization with other GPCRs, such as the β_2-adrenergic receptor (β_2AR) (Hague et al., 2004) and a class of purinergic receptors (Bush et al., 2007) enables translocation of ORs to the membrane. Moreover, members of a family of single-pass membrane accessory factor proteins, the receptor transporting proteins, have been implicated in OR trafficking to the membrane (Saito et al., 2004; Zhuang and Matsunami, 2007). With a more reliable system to facilitate functional expression of mammalian ORs, high throughput deorphanization of these receptors is within reach, and will soon provide for a greater understanding of the breadth of the olfactory code.

Projection of Olfactory Information

It has been established that, generally, one ORN expresses one functional OR in mammals and insects (Smith, 2007). This property has dramatic consequences for olfactory coding in the brain. How is this olfactory code maintained from the level of the ORN to the first relay center in the brain? In both mammals (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996) and insects (Gao et al., 2000; Vosshall et al., 2000; Couto et al., 2005; Fishilevich and Vosshall, 2005), it has been thoroughly confirmed that ORNs that express the same OR project their axons to the same glomeruli (Figure 2), such that each OR is represented in a unique set of glomeruli. In this way, the glomerulus can be said to function as a basic unit of the topographical odor map for which the brain is able to process olfactory information.

It is evident that there exist ORs that are generalists (which are activated by many odorants) and ORs that are specialists (activated by relatively few odorants).

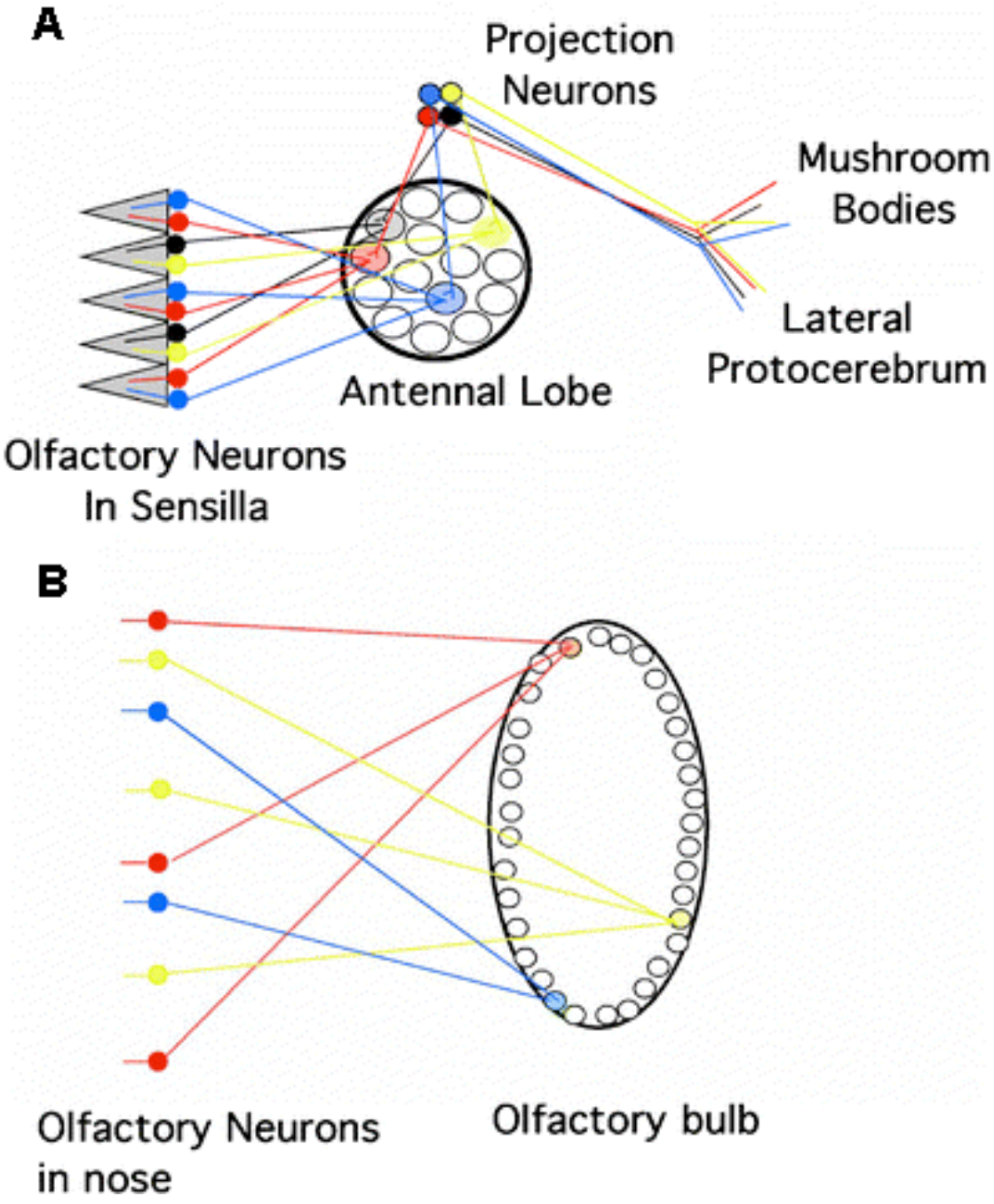

Figure 2. Projection of olfactory information. In both insects (A) and mammals (B), ORNs that express the same OR, represented by the same color sets, project their axons to the same glomeruli. (Figure from Smith, 2007)

Moreover, multiple receptors can be activated by the same odorant. Furthermore, higher concentrations of odorants activate larger numbers of ORs than do lower concentrations. These characteristics of OR activation applies to both mammalian (Malnic et al., 1999; Duchamp-Viret et al., 1999; Kajiya et al., 2001) and insect (Hallem and Carlson, 2006) olfactory systems, and is indicative of a combinatorial code mechanism. Within this paradigm, OR activation by specific concentrations and blends of odorants leads to specific patterns of glomerular activation, which thus relays to the brain the specific information (*i.e.* chemical cues) that was responsible for the activation of the receptors to begin with.

In mammalian olfactory systems, a role in axon guidance for ORs has been identified, although they are not the sole determinants for proper targeting (Mombaerts et al., 1996; Wang et al., 1998), and other signaling guidance cues have been identified as well (Chen and Flanagan, 2006). In *Drosophila,* it is evident that ORs do not function similarly, as onset of OR expression has been reported to occur relatively late in development, after ORN axonal projections have been initiated (Elmore and Smith, 2001). Furthermore, observations that deletion of endogenous ORs in classified ORNs does not alter axon guidance to the appropriate glomerular targets (Elmore et al., 2003; Dobritsa et al., 2003). Other axon guidance factors have been identified, including cell signaling molecules, transcription factors and cell adhesion proteins (Hallem and Carlson, 2004; Lattemann et al., 2007).

Olfactory Signal Transduction Cascades

Olfactory signal transduction begins when a ligand odorant molecule binds to a cognate receptor. In mammals, the core components of the olfactory signal transduction cascade have been elucidated with a high degree of confidence. In insects, on the other hand, a comprehensive model for olfactory signal transduction remains elusive. Before

considering the olfactory signal transduction components downstream of the receptor, a soluble class of proteins believed to function upstream of the receptor must be mentioned, namely, the odorant binding proteins (OBPs). OBPs exist in both mammalian and insect olfactory system. As their name implies, they were discovered to have odorant binding properties. Mammalian and insect OBP families comprise distinct gene families and several hypotheses have been outlined pertaining to their specific biological function (Pelosi, 2001; Rutzler and Zwiebel, 2005). However, to date, no definitive conclusions can be made in this regard.

Downstream of the receptor, the model for mammalian olfactory signal transduction will first be considered (Firestein, 2001). Subsequent to binding of the GPCR by a cognate odor, a conformational change in the receptor recruits and activates an olfactory specific $G\alpha_s$ variant known as G_{olf} (Jones and Reed, 1989), which, when activated, stimulates the activation of the effector enzyme, AC, specifically the ACIII variant (Pace et al., 1985; Sklar et al., 1986). ACIII catalyzes the formation of cAMP molecules from adenosine triphosphate, which bind to and induce the opening of cyclic nucleotide gated (CNG) ion channels (Nakamura and Gold, 1987; Dhallan et al., 1990). This results in the influx of calcium and sodium, to depolarize the membrane. Moreover, in a depolarization amplification step, calcium gated chloride channels are subsequently opened and chloride anions, which are maintained at relatively high levels inside the ORN, outflux from the cell, further strengthening the depolarization effect (Restrepo, 2005). In this manner, chemical information – the odor stimulus – is transformed into electrical information, as the action potentials generated by membrane depolarization relay the signal to the brain. The central importance of this pathway has been demonstrated by the fact that knockout mice for the G_{olf} (Belluscio et al., 1998), ACIII (Wong et al., 2000) and CNG (Brunet et al., 1996) loci display functional anosmia, meaning they lack responsiveness to odorant cues.

Additionally, there is evidence that other signal transduction pathways facilitate ORN sensitivity to odorants in mammals through phospholipase C (PLC) (Barry, 2003) and cyclic guanine monophosphate (Juilfs et al., 1997) mediated activity. However, in light of the aforementioned anosmic knockout mice, it has been suggested that the role of these pathways may be modulatory with regards to the G_{olf} –ACIII-CNG pathway (Paysan and Breer, 2001; Breer, 2003).

In insects, several components downstream of odorant-activated OR signaling pathways have been implicated as playing a role in *Drosophila* olfactory signal transduction. These include genes encoding: a Gα protein subunit (Kalidas and Smith, 2002), phospholipase C (Riesgo-Escovar et al., 1995), phosphatidylinositol transfer protein (Riesgo-Escovar et al., 1994), cAMP phosphodiesterase (Gomez-Diaz et al., 2004) and cyclic nucleotide (Baumann et al., 1994) and voltage-gated ion channels (Dubin et al., 1998). While the roles of these downstream elements have not been fully elucidated, they are consistent with the overall paradigm of GPCR-mediated signal transduction. Moreover, these data are indicative of multiple signaling pathways that activate AC and PLC cascades. Indeed, these dual signaling pathways have been observed to function in ORNs of the lobster (Hatt and Ache, 1994) and other animals (Ache and Zhainazarov, 1995). However, as previously mentioned, there are emerging indications that insect olfactory signal transduction may not embrace canonical GPCR signaling (Benton et al., 2006). Thus a comprehensive model for olfactory signal transduction in *D. melanogaster* and other insects remains undefined (Figure 3).

In sum it can be said that the olfactory systems of mammals and insects display profound similarities in terms of gross organizational principles as well as the logic of olfactory coding. On the other hand, the specific underlying mechanisms and molecular players involved are seemingly quite different. Ultimately, this suggests that the olfactory systems of mammals and insects are examples of convergent evolution, wherein the

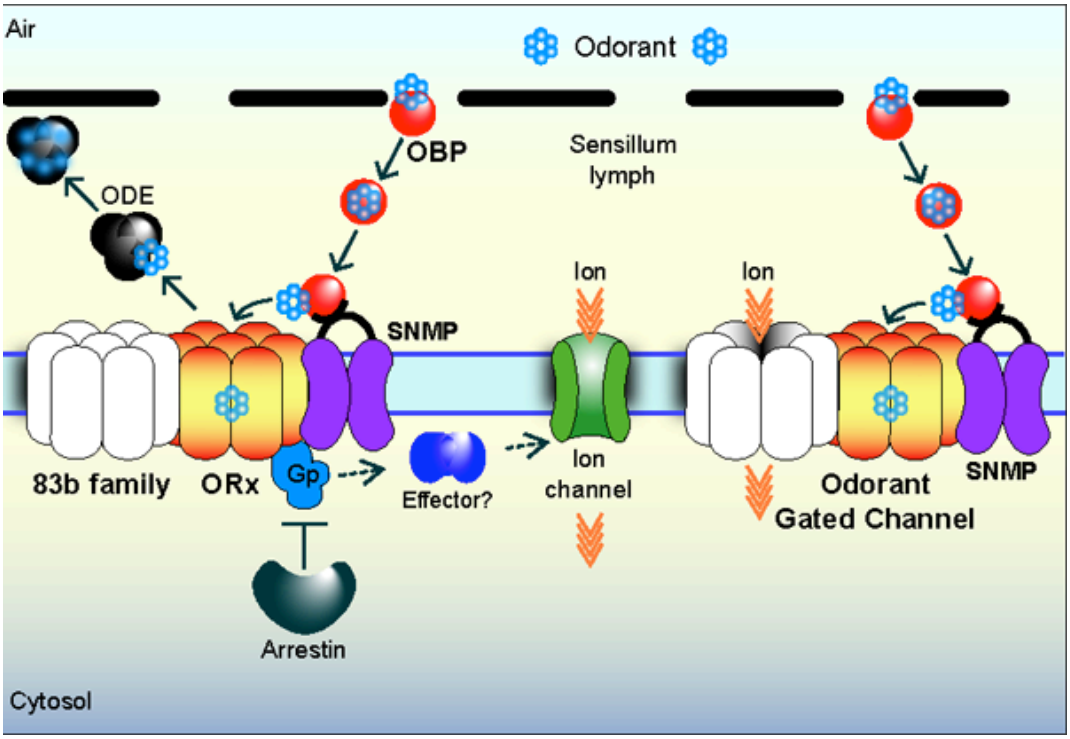

Figure 3. Proposed models of insect olfactory signal transduction. Two models have been proposed. Under both models, odors a presumed to be delivered to the OR through interactions with OBPs. On the left, OR activation is coupled to G protein signaling cascades. On the right, OR activation is directly coupled to the relevant ion channel. Odorant degrading enzymes (ODEs) have been implicated in clearance of active odorants from the sensilla lymph. Sensory neuron membrane proteins (SNMPs) are membrane proteins of unknown function (Rutzler and Zwiebel, 2005). Figure designed by Dr. Jonathan Bohbot.

basic principles of chemosensation have been adapted to fit the vital needs of these diverse forms of life.

The Arrestins

Deactivation of Olfactory Signal Transduction

Up to this point, the focus on the cellular and molecular mechanisms of olfaction has pertained to activation. In this regard, ligand binding to the OR initiates olfactory signal transduction cascades within the cell, ultimately resulting in the firing of action potentials to relay the signal to the brain. The story does not end here, however, as unregulated signaling in the face of a continuous presence of chemical stimulation presents problems for the cell, both in terms of taxation of cellular resources and signal to noise dynamics pertaining to the facilitation of precise spatial and temporal integration of olfactory signals being sent to the brain. In fact, cellular mechanisms exist that address these very problems.

Sensory adaptation, which is a diminishment of sensory response in the presence of a constant stimulus, results from modulatory effects upon the molecular components of the signal transduction cascade (Zufall and Leinders-Zufall, 2000). Indeed, for the canonical mammalian olfactory pathway, it is the very same event responsible for mediating the activating depolarization effect, an influx of calcium ions, that confers the adaptation effect on the cell (Leinders-Zufall et al., 1999). Much less is known about mechanisms underlying olfactory adaptation in insects, but reports have identified various ion channels in this process (Stortkuhl et al., 1999; Deshpande et al., 2000).

Olfactory adaptation is not, however, strictly limited to effects directly mediated by calcium ions (Zufall and Leinders-Zufall, 2000). One specific form of adaptation

occurs at the level of the receptor, and is known as homologous desensitization. It has been demonstrated that a class of proteins known as the arrestins serve as a crucial lynchpin in the process of homologous desensitization, and are ultimately responsible for deactivation of the receptor. As such, arrestin function is crucial for the regulation of sensory signaling. Accordingly, the focus of the remainder of this introduction will be on the arrestin family of proteins, with great emphasis on functional and structural characteristics of vertebrate and insect arrestins. Indeed, the central aim of the research for this dissertation seeks a greater understanding of the role of insect sensory arrestins in peripheral insect olfaction.

Introduction to the Arrestins

The arrestin family encodes a unique class of proteins conserved across a broad scope of life. Found primarily in the animal kingdom, arrestins have been identified in worms, insects, and mammals (Gurevich and Gurevich, 2006a). Arrestins were first identified within the context of the visual system of vertebrates, where they are essential in the regulation of rhodopsin-mediated signal transduction (Wilden et al., 1986). Since then, the scope of arrestin function has been expanded to the point that arrestins are believed to function in all cell types and mediate a plethora of cellular functions. This review will first examine arrestins in vertebrates. To be sure, a great majority of what is known about arrestins pertains to the vertebrate arrestins. Consideration will then be given to the insect arrestins, which were first described in the visual system of *D. melanogaster*.

Vertebrate Visual Arrestins

A rod-specific visual arrestin (arrestin1) acts downstream of the 7TM GPCR rhodopsin, subsequent to the activation of the receptor. Phosphorylation of specific

residues on the receptor is mediated by activation-dependent G-protein receptor kinases (GRKs), resulting in diminished signaling (Wilden and Kuhn, 1982; Kuhn et al., 1984). This phosphorylation is also responsible for recruitment of visual arrestin to the receptor (Kuhn et al., 1984). Arrestin1 binds and effectively blocks further signaling by the G protein, transducin (Wilden et al., 1986). It has been established that there is an inherent competition between arrestin and G-alpha protein for binding sites on the receptor (Krupnick et al., 1997). This process, which ultimately results in receptor deactivation, is known as homologous GPCR desensitization (Figure 4). Rhodopsin, however, represents one of the many GPCR family members, and visual arrestin expression is largely restricted to the retina of the eye. How then might other non-visual GPCRs be deactivated?

The answer to this question lies in the identification of additional arrestin family members first characterized for their role in homologous desensitization of β_2ARs. These proteins, known as β-arrestin1 (arrestin2) (Lohse et al., 1990) and β-arrestin2 (arrestin3) (Attramadal et al., 1992) are ubiquitously expressed and interact with a multitude of GPCRs, as well as with other non-GPCR receptors and non-receptor proteins (Lefkowitz et al., 2006). Additionally, in the retina, a cone-specific arrestin (arrestin4) has been described (Craft et al., 1994). In all, there are four vertebrate arrestin family members involved in the deactivation of GPCRs that signal the presence of light, odorants, neurotransmitters, hormones, and other compounds (Gurevich and Gurevich, 2008).

Structure/Function Relationships in the Vertebrate Arrestins

High resolution crystal structures have been determined for arrestin1 (Hirsch et al., 1999), β-Arrestin1 (Han et al., 2001), and arrestin4 (Sutton et al., 2005); in each case for the protein in the inactive, unbound state. Structurally, arrestins are globular multi-

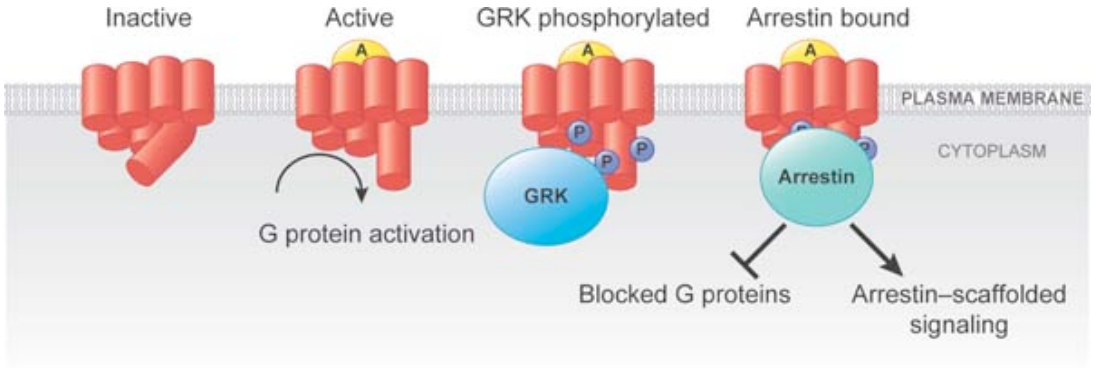

Figure 4. Diagram of arrestin-mediated receptor deactivation. Agonist activation of a GPCR facilitates activation of a G protein and also GRK, which phosphorylates the receptor in an activation dependent manner. Arrestin, which has high affinity for the active, phosphorylated form of the receptor, effectively blocks further G protein signaling. (Figure from Premont and Gainetdinov, 2007)

domain proteins, with two major domains, each consisting of 7-stranded β sheets and functionally regulatory regions located near the N and C termini and throughout the protein (Figure 5A). In coupling the structural data with elegant mutational and domain swapping-based experiments, inferences can be made regarding the mechanisms of action of these proteins pertaining to their role in binding to activated GPCRs.

Early work examining structure/function relationships revealed that visual arrestin displays a strong preference, or selectivity, for the activated and phosphorylated form of rhodopsin (Gurevich and Benovic, 1993). Arrestin displays virtually no binding to the inactive-unphosphorylated form of rhodopsin. Visual arrestin bound the activated-phosphorylated form of rhodopsin at a level far greater than the sum of binding to the inactive-phosphorylated and active-unphosphorylated forms. This suggested that a simple two-step interaction between phosphorylation and activation domains between the arrestin and receptors would not explain the nature of the binding of arrestins to rhodopsin. Rather, a model was proposed whereby coincident triggering of the phosphorylation and activation sensors on the arrestin would result in conformation changes of the protein to facilitate the engagement of multiple high-affinity binding sites (Gurevich and Benovic, 1993).

Structural analysis of the arrestins has shown that “free” unbound arrestin is maintained in its basal conformation state by three distinct intra-protein interaction sites (Gurevich and Gurevich, 2006b). While the two β strand sandwich domains are structurally self-confined, interactions between the hydrophobic residues at the interface of the two sandwich domains are prevalent (Figure 5A). Secondly, there is a shielded, solvent-excluded, polar-core, (Figure 5B) which structurally consists of a complex interaction between positively and negatively charged residues (Hirsch et al., 1999). Finally, a three-element interaction (Figure 5C) module consisting of hydrophobic

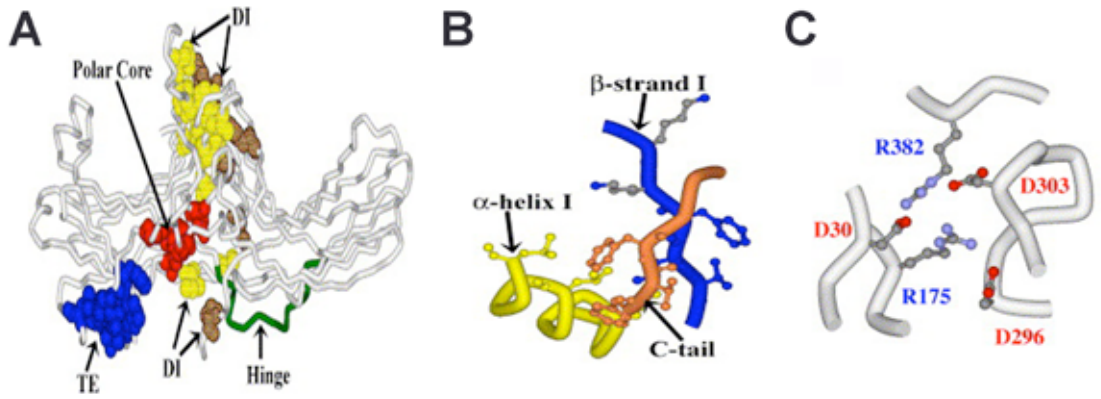

Figure 5. Basal arrestin structure. A) Model of unbound arrestin highlighting various motifs involved in maintaining the basal structure of the protein. DI- Interdomain hydrophobic residues; TE- three element interaction module see B; Polar Core- see C; hinge- flexible interdomain residues, required for global conformational changes between domains. B) The Three element interaction module. K14, K15 located on β-strand-I align with phosphorylation motifs on the receptor resulting in the disassociation of this structure and release of the C-tail. C) Polar Core. With disruption of the three element interaction, phosphorylation motifs access the arginine residues in the polar core and displace the aspartic acid residues. This releases constraints on the two major domains to facilitate high affinity receptor binding. (Figures from Gurevich and Gurevich, 2006b)

interactions between residues from β-strand-I and α-helix-I of the N domain and β-strand-XX of the C-terminus tail of arrestin functions at the surface of the protein.

Early experiments in the field identified the importance of multiply-phosphorylated receptor residues in the mediation of arrestin-binding to the receptor (Gurevich and Benovic, 1993; Gurevich et al., 1995). The functional phosphorylation sensor on the arrestin protein can be found in both the three-element interaction module as well as the buried polar core. The current model proposes that the interactions of two lysine residues, located at the N-terminus of the protein on the β-strand-I of the three-element module, with the receptor phosphorylation motifs on the receptor contort the components of the three-element module. This results in the dislocation of the C-terminal tail, which partially disrupts the polar-core. At the same time, this primary phosphate sensor unit guides the highly negatively charged phosphates to the polar-core, where they disrupt the core via interactions with the remaining positively charged arginine residue (Gurevich and Benovic, 1995; Vishnivetskiy et al., 1999; Vishnivetskiy et al., 2000).

Meanwhile, activation-sensor motifs, which are known to exist on the concave sides of both major domains (Vishnivetskiy et al., 2004), interact with activated-receptor sites and presumably disrupt arrestin inter-domain hydrophobic residues. Thus, in the process of arrestin binding to the receptor, the three interaction sites responsible for holding arrestin in its basal conformation have been disrupted. The net result of this is the facilitation of global conformational changes that carry to completion the interaction between necessary high-affinity binding sites that mediated the selectivity of arrestin for the active-phosphorylated receptor. Consistent with this model, an inter-domain hinge motif (Vishnivetskiy et al., 2002) has proven to be a crucial mediator of the movability of the two sandwich domains, relative to each other.

Importantly, it must be noted that while this model is largely based upon experiments with the visual arrestin, structural resolutions of the non-visual arrestin

isoforms imply that a consistency with this model is held across all members of the arrestin family (Gurevich and Gurevich, 2006b). Notably however, the β-arrestins do not display nearly the level of selectivity, as seen with the visual arrestin, for the activated-phosphorylated form of the receptor, although a binding preference for this form does exist. Indeed, the β-arrestins are quite capable of binding the inactive-phosphorylated form of the receptor as well (Gurevich et al., 1995).

The Vertebrate β-Arrestins

In order to appreciate the role that β-arrestins play in regulating cellular processes, one must recognize the fact that these proteins do much more than simply deactivate the GPCR. Ranging from the facilitation of internalization and recycling or degradation of GPCRs to similar interactions with non-GPCR receptors to the recruitment and activation of extra-GPCR signaling factors to a direct role in gene transcription, the β-arrestins are evidently quite essential to proper function of the cell and the organism. These β-arrestin mediated processes will be considered in the next section of this review.

β-Arrestins and Internalization, Recycling and Degradation

GPCR internalization is a common step directly following arrestin-mediated GPCR desensitization. Internalization can then lead to recycling of receptor proteins back to the plasma membrane, known as resensitization. Alternatively, internalized receptors may be targeted for lysosomal degradation. In early research in the study of β-arrestins, receptor-bound β-arrestins were implicated in mediating β_2AR internalization (Ferguson et al., 1996). Subsequently, it was observed that arrestin-receptor complexes can be internalized in clathrin-coated pits (Zhang et al., 1996; Zhang et al., 1997) and

that β-arrestins facilitate localization of clathrin (Goodman et al., 1996) and the clathrin adaptor protein, AP-2 (Laporte et al., 1999; Laporte et al., 2000) to arrestin-bound desensitized β_2AR via the direct interaction of these proteins with arrestin (Figure 6A). It has since been observed that arrestins are a requirement for clathrin mediated endocytosis of a variety of GPCRs (Ferguson, 2001). Additionally, β-arrestins are able to directly recruit several other components of the endocytic machinery to the receptor (Marchese et al., 2003). It is worth noting here that regulation of the β-arrestins is necessary for arrestin-mediated internalization of GPCRs. Specifically, a site-specific dephosporylation of β-arrestin1 (Lin et al., 1997) and ubiquitination of β-arrestin1 and β-arrestin2 (Shenoy et al., 2001) are prerequisite for this process to commence.

In a related vein, Oakley et al. (2000) defined two classes of GPCRs based upon the nature of their interactions with the β-arrestins. "Class-A" GPCRs bind β-arrestin2 with higher affinity than β-arrestin1, whereas "Class-B" receptors interact with both β-arrestins equally well. Moreover, Class-B receptors, in general, form interactions with arrestins that are relatively more stable than those of the Class-A receptors, which form transient complexes with arrestin. A consequence of this is that Class-A receptors are observed to recycle at faster rates the Class-B receptors (Oakley et al., 2000). Another report (Kohout et al., 2001) indicated that while β-arrestin1 and β-arrestin2 are equally effective at mediating receptor activation, differences arise between the two arrestins in terms of their ability to facilitate GPCR internalization. Additionally, it has been observed that β-arrestin2 contains a nuclear export signal (Scott et al., 2002) and is located in the cytoplasm of cells. β-arrestin1 lacks a nuclear export signal and is found in both the cytoplasm and the nucleus (Oakley et al., 2000; Scott et al., 2002). These reports clearly indicate a potential for the β-arrestins to display distinct functional roles in cells.

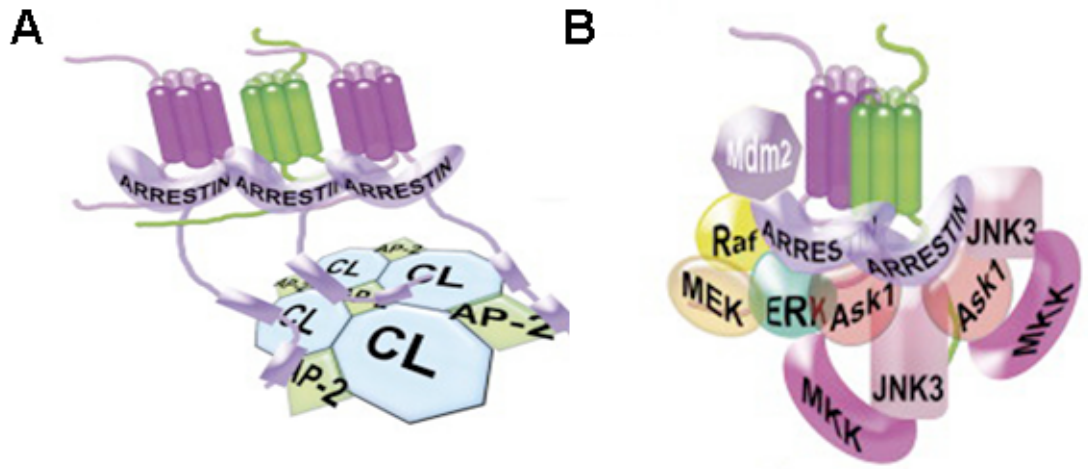

Figure 6. β-arrestin functional capabilities. In addition to their central function in GPCR deactivation, the β-arrestin proteins serve as scaffolding adaptors for various cellular factors. A) The β-arrestins facilitate clathrin-mediated GPCR internalization via recruitment of clathrin (CL) and adaptin2 (AP-2). B) Receptor-bound β-arrestins also recruit signaling molecules, including ubiquitin ligase (e.g. Mdm2) and MAP kinase (e.g. JNK3, ERK, MEK), which thereby initiate additional signaling cascades. These and other established roles for the β-arrestins are described in the text (from (Gurevich and Gurevich, 2008).

β-Arrestins and Ubiquitination

Receptor-associated β-arrestins also are known to recruit ubiquitination machinery (Figure 6B), specifically E3 ligases, to the receptor (Shenoy et al., 2001). This has consequences for ubiquitination of arrestins, which, as mentioned above, is required for arrestin mediated receptor internalization, as well as for ubiquitination of bound receptor (Shenoy et al., 2001; Martin et al., 2003; Girnita et al., 2005). Ubiquitination results in targeting of tagged receptors for lysosomal degradation. Strikingly, a correlation has recently been made between transient ubiquitination of β-arrestin associated with Class-A GPCRs (which transiently bind β-arrestin) and prolonged ubiquitination of β-arrestin associated Class-B GPCRs (which stably bind β-arrestin, respectively (Shenoy and Lefkowitz, 2005).

β-Arrestins and MAPK Signaling Complexes

Another class of molecule that can be recruited to the membrane by receptor-bound arrestins are the Mitogen-Activated Protein Kinase (MAPK) proteins (DeFea et al., 2000; McDonald et al., 2000; Luttrell et al., 2001). The arrestins serve as scaffolding proteins for MAPK phosphorylation cascades (Figure 6B), which in turn are activated, in a β-arrestin and GPCR-activation dependent manner. Thus, the β-arrestins can be said to deactivate GPCR signaling, as well as facilitate initiation of additional signaling cascades that affect myriad cellular processes, in a GPCR-dependent manner through the recruitment of MAPK components to the deactivated receptor.

β-Arrestins and Gene Transcription

There is also evidence that the β-arrestins can regulate gene transcription, both positively and negatively. Positively, β-arrestins may activate transcription factors

indirectly, via β-arrestin mediated signaling in response to GPCR activation (Wilbanks et al., 2004) , or directly, by binding to gene promoters in the nucleus to facilitate scaffolding of a multiprotein transcription complex in response to activation of certain GPCRs (Kang et al., 2005). Negatively, the β-arrestins have been shown to bind and sequester transcription factors, such as the NFκB complex in the cytosol, thus preventing translocation to the nucleus (Gao et al., 2004; Witherow et al., 2004).

β-Arrestins and Non-GPCR Receptors

Finally there is also evidence that the β-arrestins can also interact with different 7TM receptor-types that are not canonical GPCRs, as well as membrane-associated receptors that are not of the 7TM variety. Examples of these include “frizzled”, “smoothened”, and receptor tyrosine kinases, cytokine receptors, and ionotropic receptors (Lefkowitz et al., 2006; Gurevich and Gurevich, 2006a). In many of these cases, the arrestins’ role is not to mediate desensitization, but rather to facilitate receptor endocytosis and trafficking and additional signaling cascades. In other words, the β-arrestins serve many of the same functions for non-GPCR receptors as they do for the GPCR systems in which they were first described.

β-Arrestin Conclusions

The β-arrestins are known to interact with a variety of signaling molecules, including, but not limited to ubiquitin ligase proteins and MAPK enzymes, and there are indeed bigger-picture considerations that are reflected in this. For example β-arrestins mediate signaling crosstalk amongst diverse receptor signaling pathways (Lefkowitz et al., 2006). Moreover, elucidation of physiologically relevant functions affected by β-arrestin mediated activity include cellular chemotaxis, cancerous metastasis, and

programmed cell death (Lefkowitz et al., 2006). Incidentally, there have been recent reports that differential phosphorylation of presumably various receptor sites by different GRK proteins can tease out different functions mediated by the β-arrestin proteins (Kim et al., 2005; Ren et al., 2005).

Clearly, the β-arrestins, which are also known to regulate odorant induced desensitization (Dawson et al., 1993) and internalization of mammalian ORs (Mashukova et al., 2006), are indicative of the extent of the central importance of arrestins in cellular life. This is even more evident when one considers that β-arrestin1/β-arrestin2 double-knockout mice are embryonic lethal (Luttrell and Lefkowitz, 2002).

Insect Arrestins

Arrestins have been reported in *Drosophila* (Smith et al., 1990; Hyde et al., 1990), *An. gambiae* (Merrill et al., 2002; Merrill et al., 2003), and other insects (Raming et al., 1993a). The scope of this report will focus on *Drosophila*, as insect arrestins have been subjected to a great deal of investigation in this model system.

In *Drosophila*, as with the vertebrates, four arrestin-family genes have been identified (Merrill et al., 2003), with insect and vertebrate arrestins sharing a significant levels of homology (Smith et al., 1990; LeVine et al., 1990; Roman et al., 2000). Similar to their vertebrate counterparts, *Drosophila* arrestins were first identified within the context of the visual system. The first two insect arrestins to be discovered, DmArr1 and DmArr2, were originally characterized in the visual system, display widespread expression within the photoreceptors, and function in the deactivation of the visual light response (Dolph et al., 1993). DmArr1 and DmArr2 function similarly in that regard, although, as DmArr2 is expressed at a seven-fold higher level than DmArr1, DmArr2 serves a more prominent role in the deactivation of the light response (Dolph et al., 1993). On the other hand, recent reports have suggested distinct functions for these two

genes with regard to internalization of the rhodopsin receptor (Satoh and Ready, 2005; Orem et al., 2006).

A recent story has emerged wherein DmArr1 and DmArr2 have been shown to function in the olfactory appendages as well. DmArr1 and DmArr2 mutant fruitflies display olfactory deficit phenotypes in terms of both physiology and behavior (Merrill et al., 2002; Merrill et al., 2005). Thus, these two arrestins have been reclassified as sensory arrestins for their roles in the visual and olfactory systems, whilst lacking expression patterns in non-sensory tissues of the fly (Merrill et al., 2002).

A third arrestin has been characterized in *Drosophila,* and is known as *kurtz*. Highly homologous to the β-Arrestins, *kurtz* is similarly expressed ubiquitously. It was first described in having a developmental role, specifically in neurogenesis and fat body formation (Roman et al., 2000). Subsequent reports have indicated that *kurtz* also has an olfactory role (Ge et al., 2006), regulates dopaminergic signaling with consequences of effect on exploratory behaviour (Liu et al., 2007), and mediates ubiquitination of the "Notch" receptor, which also has implications with regards to development (Mukherjee et al., 2005). A fourth insect arrestin was originally described in *An. gambiae* that also displays nearly ubiquitous expression. A homologue has been identified in *Drosophila*, but remains uncharacterized (Merrill et al., 2003).

The sensory arrestins have known homologues in the malaria vector mosquito *An. gambiae* (Merrill et al., 2002; Merrill et al., 2003). These genes, AgArr1 and AgArr2, are also expressed in olfactory organs, suggesting that these genes may function in a similar fashion in the mosquito. While it has been presumed that DmArr1 and DmArr2 would function in the olfactory system as deactivators of OR signaling, recent evidence has cast some doubt on this hypothesis. *Drosophila* ORs display an inverted membrane topology inconsistent with that of all other known GPCRs (Benton et al., 2006; Lundin et al., 2007). DmArr1 and DmArr2 regulation of OR function cannot be ruled out, but in

lacking further evidence as to the underlying role of these arrestins in the olfactory appendages, no definitive statements can be made regarding the precise function of these genes in the olfactory system. This thesis has been devised to further investigate the specific roles that the sensory arrestins play in the regulation of olfactory signal transduction cascades in the 3rd antennal segment of the fly as well in the antennae of *An. gambiae.*