CHAPTER V

CONCLUSION

Summary

Transgenic Rescue of Drosophila Arrestin1 Mutant Olfactory Phenotype with *Anopheles gambiae* Arrestin1 Homologue.

A previous report from the Zwiebel lab demonstrated that transgenic overexpression of DmArr1 in neurons of *arr1*^{1}*cn* mutant flies rescues the *arr1* olfactory deficit phenotype observed in mutant flies (Merrill et al., 2005). Similarly, rescue of function has been observed in *arr1*^{1}*cn* mutant flies when transgenic AgArr1 was expressed in antennal neurons (Walker et al., 2008). These results suggest homologous function of DmArr1 and AgArr1 within the peripheral insect olfactory system.

While this rescue of function with AgArr1 was observed for physiological olfactory responses under the EAG paradigm, no such rescue was observed in the olfactory-based larval motility assay. This observation, while inconsistent with a previous report pertaining to rescue of the *arr1* mutant larval motility phenotype with transgenic DmArr1 (Merrill et al., 2005), may result from broad overexpression of the AgArr1 transgene in all neurons. In this regard, the EAG system reflects summed electric potential in ORNs and should not be impacted by expression of arrestin transgene in non-olfactory neurons, whereas the larval chemotaxis behaviour may be impacted by arrestin transgene expression in both olfactory and non-olfactory neurons. It is worth noting here that the rescue of the larval behavioural phenotype was observed for one odorant at one concentration with a relatively small sample size (n=3). In order to more fully address this discrepancy, a thorough analysis of the ability of transgenic DmArr1 to rescue the

arr1^{1} mutant phenotype over a broad spectrum of odorants and dilutions. However, it is the author's opinion that it would be a more relevant endeavor to pursue examination of arrestin mutant olfactory-based behavioural phenotypes in adults. In the Zwiebel laboratory, attempts have been made to adapt adult fly olfactory-based behavioural assays to study of the arrestin mutants, but success in obtaining consistency with these assays has been lacking.

Additionally, rescue of the DmArr2 mutant olfactory-deficit phenotype was not observed under conditions of transgenic overexpression of either DmArr2 or AgArr2 in antennal neurons. For these negative results, no definitive statements can be made regarding the arr2 transgenes. Further analyses of these genes in the olfactory system are necessary to more fully characterize the underlying nature of the olfactory deficit phenotypes.

Spatial Expression Patterns of Drosophila Sensory Arrestins in the Antenna

Are the sensory arrestins coexpressed in antennal neurons? In order to more fully characterize expression patterns of DmArr1 and DmArr2 in the 3rd antennal segment, FISH experiments were performed. The broadly expressed Or83b gene was used as a control, and both DmArr1 and DmArr2 were independently compared to Or83b. While Or83b expression was robust, DmArr1 and DmArr2 were not detected in the antennae. In initial experiments, the DmArr1 and DmArr2 ISH RNA probes were tested in the fly eye, and both indicated robust expression of these genes, as expected. Moreover, the DmArr1 ISH probes were successfully utilized to detect expression of DmArr1 driven by the Gal4/UAS system. Together, with the failure to detect endogenous DmArr1 and DmArr2, and the aforementioned positive controls, it can be concluded that the expression levels of DmArr1 and DmArr2 are below the threshold of detection sensitivity for the available ISH system in the fly antenna.

In a related vein, the Gal4/UAS system was utilized in an attempt to characterize DmArr1 and DmArr2 promoter activity in the fly antenna. For these genes, 2kb of sequence immediately upstream of the transcription start site were cloned upstream of the Gal4 transcription factor. These Arrestin-Gal4 constructs were used to drive expression of transgenic GFP, positioned downstream of Gal4 binding sites. While DmArr1-driven expression of GFP was observed in subsets of antennal neurons, widespread expression was not detected. For DmArr2 promoter-driven expression of GFP, no reporter gene expression was observed in any cells in antennal sections. Similarly, in a previous report, anti-DmArr2 antibodies failed to detect DmArr2 protein in the 3rd antennal segment (Merrill et al., 2002). Regardless, lacking ISH data reflecting endogenous expression patterns of DmArr1 and DmArr2, the physiological relevance of the arrestin promoter-driven gene expression patterns cannot be determined.

With regard to the ISH and arrestin promoter-reporter expression studies, no decisive statements can be made pertaining to the relative expression patterns of these genes in the insect olfactory system. In order to address this issue, further experimentation utilizing more sensitive detection technology is warranted.

Temporally Induced Expression of Transgenic Arrestin

Temporally restricted induction of DmArr1 transgene studies have been designed and carried out to examine the effects of this gene on olfactory function in the antennae of *arr1* mutant flies. Data generated in this initial pilot study are inconclusive. Further experimentation is necessary to fully address this issue. Two expression systems, both variations of the Gal4-UAS paradigm, have been utilized to address the hypothesis that the *arr1* mutant olfactory deficit phenotype manifests as a result of chronic downregulation of olfactory signal transduction components. The Gene Switch system relies on flies having access to a hormone to activate induction, while in the TARGET

system induction is dependent on ambient temperature. In as much as the available data is inconclusive, inferences can nonetheless be made to guide future research in this project. Complications have been observed with the Gene Switch system, suggesting that the TARGET system may be preferable to carry out further investigations. Whatever means are employed, a more thorough analysis, incorporating an array of varying induction conditions, test-odorants used, and appropriate control groups, is required to determine the merit of the proposed hypothesis.

Future Directions

In *Drosophila*, analysis of arrestin mutant flies has resulted in the identification of an olfactory role for arr1 and arr2 in the primary chemosensory organs, the antennae and the maxillary palps (Merrill et al., 2002; Merrill et al., 2005). Importantly, functional rescue studies carried out via transgenic expression of DmArr1 in the *arr1* mutant background have conclusively linked the *arr1*^{1} mutant phenotype to the arr1 locus. However, at this point, many questions pertaining to the precise functional role for these sensory arrestins in the olfactory appendages of *Drosophila* remain unanswered.

Are Arr1 and Arr2 coexpressed in cells of the antennae, as they are in the eye (Dolph et al., 1993)? Are they (co)expressed in ORNs? If they are expressed in ORNs, do they interact with ORs? What other proteins might they interact with? If they are coexpressed do they serve redundant or disparate roles? If they are not expressed in ORNs, how might they impact olfactory sensitivity, as seen in the EAG assays? Generally speaking, what is the function of the sensory arrestins in the antenna? What mechanisms underlie the two unexpected aspects of the arrestin mutant phenotype (as described in Chapters III and IV). Elucidation of these questions will go a long way towards facilitating a greater understanding of internal olfactory mechanisms this insect model organism.

Spatial Expression Patterns of the Sensory Arrestins in the Antennae

Regarding the spatial expression patterns of the sensory arrestins within the antennae, an alternative to the ISH technique that may facilitate the detection of arrestin expression with a higher degree of sensitivity is a technique known as *in situ* reverse transcription-PCR. This technique combines reverse transcription-PCR and ISH to amplify mRNA in a gene-specific fashion in endogenous tissue and then label the amplicon message via standard ISH protocol. *In situ* reverse transcription-PCR has been widely used since its inception in the mid 1990s. A cursory PubMed search identifies scores of reports utilizing this technique. Yet, to date, no reports have demonstrated the viability of *in situ* reverse transcription-PCR in the fly antenna. Needless to say, thorough empirical experimentation would be necessary to evaluate the feasibility of this approach.

Alternatively, isoform specific antibodies could be generated to determine expression patterns of sensory arrestin protein in the antenna. Previous work in the laboratory identified arr1 but not arr2 expression in the antennae with monoclonal antibodies generated against arrestin protein (Merrill et al., 2002). Clarification of this issue is necessary. It may be the case that in the antennae, arr1 is the predominant isoform, in contrast to the eye, wherein arr2 is expressed at significantly higher levels than arr2 (Dolph et al., 1993).

Olfactory Physiology Refinement

The EAG has been vital in the identification of a role for the sensory arrestins in olfactory signal transduction. However, to this point, only eleven odorants have been examined in this context. Expansion of the odorant panel to a broader array of compounds, including identified natural odorant ligands (Stensmyr et al., 2003), may shed further light on the dynamics of the roles of Arr1 and Arr2 in the antennae.

Moreover, valuable information may yet be inferred from the refinement of physiological data to the level of the single ORN. Utilizing the SSR technique, ORN responsiveness to a wide panel of odorants has been characterized, leading to the classification of stereotypical sensilla and ORN subtypes (de Bruyne et al., 2001; Yao et al., 2005). A molecular correlation to this model has linked OR responsiveness to ORN subtypes for many receptors (Dobritsa et al., 2003; Hallem et al., 2004b). Analysis of arr1 and arr2 mutant responses via a comprehensive SSR analysis may yield valuable information in this regard. In this regard it could thus be possible to add another level of detail to the makeup of the olfactory code in the antennae, relating ORNs, and thus ORs, to arrestins based upon ORN specific phenotypes, should such a distinction exist. Additionally, a hypothesis has been posited that the reduction of EAG response amplitudes in *arr1* and *arr2* mutants result from the chronic down-regulation of olfactory signal transduction components as a means to compensate for unregulated signaling in the absence of wild-type levels of arrestin protein. In reference to this hypothesis, one might predict that background levels of neuronal activity in the ORNs, which are highly dependent upon the expressed OR (Hallem et al., 2004b), would be diminished in arrestin mutant flies relative to wild-type. This prediction could be tested with the SSR technique, as well.

Arrestin Biochemical Interactions

Another important point to consider is whether the sensory are directly interacting with OR-mediated olfactory signal transduction cascades. In light of recent data suggesting that the ORs may not be canonical GPCRs (Benton et al., 2006; Lundin et al., 2007), one must at least consider the possibility that they are not. For example, it may be entirely plausible that the manifestation of the sensory arrestin mutant phenotypes is solely the result of a role for these genes in the development of the adult antennae. While it has been demonstrated that Arr1 and Arr2 interact with rhodopsin in

photoreceptor cells (Alloway and Dolph, 1999; Satoh and Ready, 2005), there is no knowledge of arrestin protein interaction partners in the antennae. Recently, cell culture systems have been utilized to examine *Drosophila* OR function *in vitro* (Neuhaus et al., 2005; Benton et al., 2006). It may possible to incorporate sensory arrestin expression into these systems to spearhead investigations of arrestin protein-protein interactions with ORs and other molecules.

Closing Words

Malaria continues to be a morbidly detrimental problem to human inhabitants of sub-Saharan Africa and other mostly tropical parts of the world. One promising approach aimed at reducing the worldwide malaria burden is centered on the elucidation of the internal mechanisms underlying olfactory sensation in a prominent malaria mosquito vector, *An. gambiae.* The rationale for doing this has basis in the fact that wide-spread transmission of the malaria parasite by *An. gambiae* is largely mediated by an olfactory-based preference for taking blood meals specifically from human hosts. Many physiological and behavioural discoveries pertaining to the olfactory system of *An. gambiae* have been made. However, molecular and genetic studies in this important species have, until recently, been lacking, in this context.

The dipteran insect genetic model organism, *D. melanogaster* holds great potential for breakthroughs in this specific arena. Recent analyses of ORs, arrestins and other putative olfactory signal transduction components in the olfactory organs of both *Drosophila* and *An. gambiae* may indeed lead to significant advances in the fight against malaria. Undoubtedly, a comprehensive multi-faceted approach, in which olfactory-based methodologies serve as but one component, will prove to garnish the greatest successes in this epic struggle of man versus microbe.