CHAPTER II

A FUNCTIONAL ROLE FOR *ANOPHELES GAMBIAE* ARRESTIN1 IN OLFACTORY SIGNAL TRANSDUCTION

Preface

The information presented in this chapter was submitted and accepted for publication in the *Journal of Insect Physiology*. The author's contribution to this body of work include all AgArr1 rescue of function and heterozygous-condition physiology assays, as well as all *in situ hybridization* and Real Time PCR experiments.

Introduction

Olfaction plays a significant role in mediating a variety of critical behaviors in insects (Hallem et al., 2006). This olfactory dependence is particularly relevant in host-seeking and other behaviors of the mosquito *An. gambiae,* which is the principal Afro-tropical vector for human malaria (Zwiebel and Takken, 2004). In *An. gambiae*, as in other insects, odorants first encounter the peripheral olfactory system through pores on sensory hairs, known as sensilla, which populate head appendages (the antennae, maxillary palps and proboscis). It is here that they contact the dendrites of ORNs, and the components of signal transduction pathways that translate chemical information from the environment into neuronal activity (Steinbrecht, 1996). Largely as a result of studies in the insect genetic model system *Drosophila melanogaster,* many of the key players presumed to be involved in OR activation and subsequent olfactory signal transduction have been identified. Indeed, a novel family of putative 7TM GPCRs have been identified in *D. melanogaster* (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999) and *An. gambiae* (Hill et al., 2002; Robertson et al., 2003). Many of these proteins

have been subsequently shown to function as *bona fide* ORs (Dobritsa et al., 2003; Hallem et al., 2004b; Lu et al., 2007).

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 G protein (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), cyclic nucleotide and voltage-gated ion channels (Dubin et al., 1998), and, from our own work, sensory arrestins (Merrill et al., 2002; Merrill et al., 2005). While the roles of these downstream elements have not been fully elucidated, they are all consistent with the overall paradigm of GPCR-mediated signal transduction. There are, however, emerging indications that insect olfactory transduction may not embrace canonical GPCR signaling (Benton et al., 2006), suggesting that a comprehensive model for olfactory signal transduction in *D. melanogaster* and other insects still remains undefined.

As crucial as receptor activation and primary signal transduction are to the facilitation of detection of olfactory cues, integration of the signal in space and time is reliant on the appropriate termination of the transduction cascade, a process known as deactivation. Homologous GPCR desensitization, which ultimately results in reduced receptor responsiveness was originally studied in vertebrate systems, and specifically occurs subsequent to receptor activation and involves the rapid uncoupling of a receptor from its partner G protein (Krupnick and Benovic, 1998). Under this paradigm, ligand binding is followed by activation-dependent receptor phosphorylation (Wilden and Kuhn, 1982; Kuhn et al., 1984). While this step slightly diminishes signaling, subsequent binding of arrestin proteins are necessary for full GPCR deactivation (Kuhn and Wilden, 1987). Arrestins, which display a high selectivity toward the activated, phosphorylated form of the receptor (Gurevich and Gurevich, 2004), mediate this process by functionally

competing with G proteins for binding sites within the receptor to prevent further signal transduction (Ferguson and Caron, 1998). The first arrestins to be identified were isolated from the vertebrate visual systems (Wilden et al., 1986), and have accordingly been characterized as visual arrestins.

A second class of vertebrate arrestins encompass the non-visual subtypes (Krupnick and Benovic, 1998) and are known as β-arrestins because they were first described according to their role in the desensitization of β-adrenergic receptors (Lohse et al., 1990; Attramadal et al., 1992). They have since been shown to also regulate vertebrate olfactory signal transduction cascades (Dawson et al., 1993; Mashukova et al., 2006). Since that time, a plethora of these and other studies have led to the well-accepted paradigm whereby arrestin proteins facilitate a diversity of processes pertaining to GPCR-mediated signal transduction. These include desensitization processes linked to endocytic pathways that function in receptor internalization as well as recycling and degradation (Prossnitz, 2004). Moreover, the β-arrestins have been shown to mediate other cellular processes via interactions with secondary signal transduction cascades through the recruitment and activation of MAPK and other effector proteins (Lefkowitz and Shenoy, 2005; Gurevich and Gurevich, 2006a).

Arrestins have also been identified and characterized in several other insect species, most notably the fruit fly *D. melanogaster*, in which two visual arrestins, DmArr1 and DmArr2, were originally identified based on sequence homology (Smith et al., 1990; Hyde et al., 1990; LeVine et al., 1990) and function (Dolph et al., 1993) to vertebrate visual arrestins. Not surprisingly, these arrestins have also been shown to play a role in the internalization of rhodopsin in *Drosophila* (Satoh and Ready, 2005; Orem et al., 2006). DmArr1 and DmArr2 are also expressed in *D. melanogaster* olfactory tissues, leading to their reclassification as sensory arrestins (Merrill et al., 2002).

More recently, a comprehensive evaluation of the *in vivo* physiological and behavioral roles of *D. melanogaster* sensory arrestins in olfactory function via mutant analysis revealed decreased responsiveness to a diverse panel of odorants in both single and double *arr1[1]* and *arr2[5]* mutants in a concentration- and odorant-specific manner (Merrill et al., 2005). Moreover, *arr1[1]* mutant phenotypes were definitively linked to the *arr1* locus through functional rescue via expression of a wild-type DmArr1 transgene in *arr1[1]* mutant backgrounds. The results of this work firmly established an odorant-dependent role for sensory arrestins as they act in peripheral olfactory signal transduction in this system.

These findings led us to extend our analysis of olfactory arrestins to economically and medically relevant insects, such as the malaria vector mosquito *An. gambiae*. In an initial examination of *An. gambiae* arrestins, we characterized AgArr1, which is homologous to DmArr1 and not surprisingly similarly expressed in both photoreceptors and olfactory tissues (Merrill et al., 2002). In a subsequent study, three additional *An. gambiae* arrestins were characterized. AgArr2 is highly homologous to DmArr2 and, likewise, is expressed in multiple sensory systems. AgArr3 is homologous to the non-visual *kurtz* arrestin gene in *Drosophila* (DmKrz) (Roman et al., 2000) and is similarly ubiquitously expressed, while AgArr4, which is also widely expressed, belongs to a divergent arrestin class and has an unknown function (Merrill et al., 2003). Accordingly, AgArr1 and AgArr2 have been preliminarily characterized as insect sensory arrestins, whereas AgArr3 and AgArr4 represent non-sensory and atypical insect arrestins, respectively.

As the first step in the direct functional characterization of sensory arrestins, we have examined the hypothesis that AgArr genes act as *bona fide* sensory arrestins and true DmArr1 and DmArr2 orthologs by asking whether transgenic expression of AgArr1 or AgArr2 in *Drosophila arr* mutant ORNs can functionally rescue the olfactory

37

phenotypes associated with each mutant background. The results reported here demonstrate that while AgArr1 can functionally replace DmArr1 by restoring olfactory physiology to wild-type levels, there is a general lack of functional complementarity between putative interspecific Arr2 orthologs as well as all Arr1 and Arr2 paralogs. This, therefore, is a *de facto* validation of the hypothesis that AgArr1 is able to function as a true olfactory arrestin, thereby leading to the logical conclusion that it serves similar roles in *An. gambiae* ORNs. Thus, further exploration of the role of AgArr1 and other sensory arrestins may be carried out in *An. gambiae* to provide insight into the mechanisms controlling olfactory perception in this important disease vector insect. This will likely foster more informed approaches toward devising olfactory-based, vector-control strategies that may be utilized to reduce the transmission of malaria and other vector-borne disease.

Materials and Methods

Fly cultures

The wild-type flies used as controls in this experiment were Oregon R, obtained from Dr. C. Desai (Vanderbilt University). Hypomorphic *arr1^1 cn* mutant flies were kindly provided by Dr. P. Dolph (Dartmouth College) and have been well described (Dolph et al., 1993). Flies were grown in Erlenmeyer flask-shaped plastic bottles on a standard cornmeal, molasses, agar and sugar medium with yeast. All flies were cultured at 25°C, on a 12-h: 12-h light: dark cycle.

Odorants

Five odorants that have been shown to elicit diminished responses in *arr1^1 cn* mutant flies (Merrill et al., 2005) were used for the physiological assays: 1-butanol,

heptanoic acid and octyl acetate (Aldrich, Milwaukee, WI), and 2-heptanone and 1-octanol (Sigma, St. Louis, Missouri). Odorants were diluted in mineral oil (Sigma, St. Louis, Missouri) to the concentrations indicated in figure legends.

Electroantennogram Physiology

The electroantennogram (EAG) responses of wild-type and arrestin mutant flies were recorded in a similar manner to those in previous studies (Alcorta, 1991; Riesgo-Escovar et al., 1994; Merrill et al., 2005). Briefly, 2- to 7-day-old adult female *Drosophila* were immobilized in the narrow end of a pipette tip, such that only the anterior portion of the head protruded. Flies were placed 5 mm from the tip of a constant stream of clean humidified air provided by a stimulus control device (CS-05, Syntech, The Netherlands). Odorants were delivered by passing 0.5-s pulses of air through a glass Pasteur pipette containing a 1.5-cm-diameter filter disk (VWR International, West Chester, Pennsylvania) saturated with 20 μl-diluted odorant into the constant air stream. The stimulus control device delivered a continuous flow rate of ~340 cc/min and a pulse rate of ~350 cc/min. The difference in air velocity between the continuous air stream and stimulus pulse did not produce a significant EAG response, as evidenced by the fact that odor-free (oil-alone) responses are consistently low and reflect only minor background noise. Glass microelectrodes filled with 0.1 M KCl transmitted electrical responses to odorant stimulation via silver-chloride wires to a signal acquisition system (IDAC232, Syntech, The Netherlands). Data were collected at 25 Hz, amplified 10X and converted from analog to digital, then displayed on a Gateway PC computer. EAG analysis was performed using EAG2000 software (Syntech, The Netherlands). The reference electrode was inserted into the back of the head while the recording electrode was placed on the anterior dorso-medial surface of the 3rd antennal segment to establish

electrical contact. The amplitude of response, which represents the peak voltage

deflection in response to odorant presentation, was recorded for analysis.

D. melanogaster **Germline Transformation and Generation of Transgenic Rescue Lines**

All techniques were performed as previously described (Merrill et al., 2005).

Strains of *D. melanogaster* with either mutant DmArr1 ($arr1^1$ *cn*) or DmArr2 ($arr2^5$ *veh*)

background were used as parentals to generate the appropriate rescue lines. The Gal4-

UAS yeast transcriptional system, originally described by Brand and Perrimon (Brand

and Perrimon, 1993), was utilized to drive expression of the AgArr1, AgArr2 and DmArr2

transgenes in the DmArr1 mutant background; expression of the AgArr1, AgArr2,

DmArr1 and DmArr2 transgenes was also driven in the DmArr2 mutant background.

The *arr1*^{1} and *arr2*^{5} mutations were crossed into the Gal4^{C155} (Lin and Goodman, 1994)

line, in which the yeast Gal4 transcription factor is expressed under control of the pan-

neuronal *elav* promoter (Robinow and White, 1988): *w, elavGal4*^{c155}; $arr1^1cn/arr1^1cn$,

henceforth denoted as C155/1^1, or *w, elavGal4*^{c155}; +; $arr2^5$ *ve h*. For these experiments,

a variety of UAS responder lines were used, in which the specific transgenes were

positioned downstream of five UAS Gal4 binding sites. The *arr1*^{1} *and arr2*^{5} mutations

were crossed into the AgArr1, AgArr2, DmArr1 and DmArr2 transgene backgrounds.

Rescue lines to be examined were generated by crossing the Gal4 driver line in the *arr1*^{1}

mutant background with the responder lines containing the AgArr1, AgArr2 and DmArr1

transgenes, also in the *arr1*^{1} mutant background. Similarly, the Gal4/*arr2*^{5} line was

crossed with responder lines containing the AgArr1, AgArr2, DmArr1 and DmArr2

transgenes, which were also in the *arr2*^{5} mutant background. A specific genotypic

description of the UAS-AgArr1 line used in this report is as follows: *w/w; arr1*^{1} *cn/arr1*^{1}*cn;*

p*[UAS-AgArr1*^{1A}*]/p[UAS-AgArr1*^{1A}*]*, henceforth called 1^1/1A. Crossing virgin female *w,*

elavGal4^{c155}; arr1^1cn with male *w; arr1^1cn; p[UAS-AgArr1^{1A}]* generated progeny that expressed Gal4 and AgArr1 neuronally in the *arr1^1cn* background, henceforth C155/1^1/1A.

In Situ Hybridization

Fluorescence *in situ* hybridization (FISH) was performed using a modified version of previously reported methodology (Vosshall et al., 1999; Vosshall et al., 2000). Briefly, digoxygenin (DIG)-labeled AgArr1 and DmArr1 and fluorescein isothiocyanate (FITC)-labeled DmOr83b sense and antisense riboprobes were generated using standard kit reagents and protocol (Roche Applied Sciences, Indianapolis, Indiana). For each experiment, antennae from approximately 25 4- to 8-day-old female flies were dissected directly into Tissue-Tek O.C.T. compound-embedding medium (Sakura Finetek U.S.A, Torrence, California). Cryo-sections of 15 μm were generated and applied to Superfrost Plus VWR Microslides (VWR International, West Chester, Pennsylvania), then allowed to air dry for 3 h. A 10-min fixation in 4% paraformaldehyde [4% paraformaldehyde/1X phosphate buffered saline (PBS)] was followed by three 5-min washes with 1X PBS, a 10-min acetylation application and then three additional 5-min washes with 1X PBS (all washes performed at room temperature). Pre-hybridization and hybridization steps were carried out with hybridization solution as follows: 50% formamide, 5X SSC, 5X Denhardt's solution, 250 μg/ml salmon sperm DNA, 50 μg/ml heparin, 2.5 mM EDTA, 0.1% Tween-20. Pre-hybridization was carried out for 2 h at 55°C, and hybridization for 21 h at 55°C.

Subsequently, one 10-min 5X SSC wash (55°C), three 20-min 0.2X SSC washes (55°C) and one 10-min 1X PBS-tw wash (1X PBS, 0.1% Tween) were sequentially carried out prior to blocking and antibody labeling. Blocking was carried out for 2 h at

room temperature with B2 sheep solution [10% Normal Sheep Solution (Jackson ImmunoResearch, West Grove, Pennsylvania), 1X PBS-tw]. For antibody labeling, sheep anti-DIG-peroxidase (POD) (1:200) and sheep anti-FITC-alkaline phosphatase (AP) (1:500) (both Roche Applied Sciences, Indianapolis, Indiana) were diluted in B2 sheep solution, and applied to slides for 21 h at 4°C.

Five 5-min 1X PBS-tw washes were performed at room temperature. For visualization of DmArr1 and AgARR1 label, diluted tyramide-FITC reagent (Perkin Elmer, Waltham, Massachusetts) was applied for a 10-min incubation at room temperature. For visualization of DmOr83b, two 10-min equilibration washes with 0.1 M Tris-HCl (pH 8.2) were followed by a 30-min incubation with applied Fast Red tablet (Roche Applied Sciences, Indianapolis, Indiana) dissolved in 2 ml 0.1 M Tris-HCl (pH 8.2) and three subsequent 5-min 1X PBS-tw washes. Sections were mounted with Vectashield reagent (Vector Laboratories, Burlingame, California) viewed on a Zeiss Inverted LSM510 Confocal Microscope (Carl Zeiss Microimaging, Inc., Thornwood, New York) and analyzed with Zeiss LSM Image Browser Software (Carl Zeiss Microimaging, Inc., Thornwood, New York).

RNA Extractions

Antennae were dissected by hand and placed into 1.5-ml eppendorf tubes on dry ice. For each genotype, 120–150 antennae from female flies were dissected for RNA preparation. Total RNA was prepared using RNeasy Mini reagents, as described in the supplier's protocol (Qiagen Inc, Valencia, California), and then treated with DNase according to the DNA Free protocol (Ambion, Austin, Texas) to eliminate potential genomic DNA contamination. The total RNA preparation was then used for oligo-dT cDNA first-strand synthesis using Superscript III Reverse Transcriptase (Gibco/Invitrogen, Carlsbad, California), according to the manufacturer's instruction. For

each genotype, 500 ng total RNA was used for each reverse transcription reaction. For each genotype, three independent RNA extractions were conducted, in parallel, for subsequent use in comparison of relative expression levels (see *2.8. and 3.3.)*.

Primer Design

Real Time PCR (RT-PCR) primers were designed to span exon–intron boundaries, where appropriate, and were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa). In all cases, the following oligonucleotide primers were selected with an ideal annealing temperature of 60°C and optimized to minimize hindrance on account of hairpin formation and primer dimerization: Ribosomal protein rp49 (forward) GTCGCACAAATGGCGCAAGC and rp49 (reverse) TGCTGCCCACCGGATTCAAG produce a 133-bp cDNA product; DmArr1 (forward) ACTCAGGTGGAACCCATTGATGGAA, and (reverse) TTTCCGATATGGGCGCGAGG yield a 109 bp product; AgArr1 (forward), CGATCGATGGTATCGTCGTCCTC and (reverse) CGAAGAGGACGAGGTGATGGG yield a 112 bp product.

Quantitative Real Time PCR

For these experiments, an ABI 7300 Real Time PCR Instrument was used (Applied Biosystems, Foster City, California) together with the Qiagen QuantiTect SYBR Green PCR kit (Qiagen Inc, Valencia, California). For each reaction, 11.5 µl of 1X master-mix, containing 0.5 µM forward and reverse primers diluted with RNase Free water, was added to individual wells of a 96-well plate to which 1 µl cDNA was added as a template for the reaction. The ABI 7300 experimental protocol used was—activation (50°C, 2 min; 95°C, 10 min), amplification (95°C, 15 s; 60°C, 1 min) repeated 40 times, and a melting curve (60–95°C at 1°C/s). For each genotype and primer set, reactions were run in triplicate, and average fluorescence C_t values were obtained. Amplification

efficiencies were calculated as described previously (Bohbot and Vogt, 2005), and

relative gene expression ratios were determined using the Pfaffl method of analysis

(Pfaffl, 2001).

Results

The objective of the studies reported here was to validate a functional role for the

An. gambiae sensory arrestins AgArr1 and AgArr2 in peripheral olfactory processes in

an insect system. In order to address this, and in the absence of appropriate genetic

tools (e.g., mutants and a robust system for germline transformation) in *An gambiae*, we

have taken advantage of previously characterized olfactory phenotypes in *D.*

melanogaster arr1[1] and *arr2*[5] mutants, of which *arr1*[1] *mutants* can be functionally

rescued via transgene expression of the endogenous DmArr1 arrestin-encoding gene

(Merrill et al., 2005). We tested the hypothesis that the *D. melanogaster arr* mutant

phenotypes could also be rescued by AgArr1 and AgArr2 transgenes, which would

demonstrate true functional orthology for these genes, thereby providing support for a

similar role for these sensory arrestins in olfactory signaling in *An. gambiae*. Amino acid

sequence analysis supports this hypothesis, because AgArr1 has been shown to be

67% identical to DmArr1 (Merrill et al., 2002) and AgArr2 74% identical to DmArr2

(Merrill et al., 2003) (Figure 7). It is worth noting here that the sensory arrestin family of

proteins, consisting of DmArr1, AgArr1, DmArr2 and AgArr2, share two conserved

elements—four of five charged polar core residues (Hirsch et al., 1999) and two amino-

terminal lysine residues (Vishnivetskiy et al., 2000)—both of which are demonstratively

essential in receptor-phosphorylation-state recognition (Gurevich and Gurevich, 2004).

Of the two putative Src kinase Homology 3 (SH3) domains (Boxes, Figure 7), the

presence of which suggests potential interactions with downstream MAPK signaling

pathways (Luttrell et al., 1999), only one is conserved among all four insect sensory

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DmArr1   MVVNFKVFKKCSPNNMITLYMNRRDFVDSVTQVEPIDGIIVLDDEYVRQNRKIFVQLVCNFRYGREDDEMIGLRFQKELTLVSQQVCP-PQKQDIQLTKMQ 100
AgArr1   MVYNFKVFKKCAPNGKVTLYMGKRDFVDHVSGVEPIDGIVVLDDEYIRDNRKVFGQIVCSFRYGREEDEVMGLNFQKELCLASEQIYPRPEKSDKEQTKLQ 101
DmArr2   MVVSVKVFKKATPNGKVTFYLGRRDFIDHIDYCDPVDGVIVVEPDYLK-NRKVFGQLATTYRYGREEDEVMGVKFSKELILCREQIVP-MTNPNMEMTPMQ 99
AgArr2   MVVAVKVFKKSAPNGKLTVYLGKRDFIDHTDYCDPIDGVIVLDEEYLR-GRKVFGQLITTYRYGREEDEVMGVKFSKEMVLTKEQIYP-MENANMEMTPMQ 99
         **   .*****.:**. :*.*::*:***:*       :*:**::*:: :*:: .**:* *:  .:*****:**::*:.*.**: *   :*: *    : : : * :*


DmArr1   ERLLKKLGSNAYPFVMQMPPSSFASVVLQQKASDESQPCGVQYFVKIFTGDSDCDRSHRRSTINLGIRKVQYAPTKQGI-QPCTVVRKDFLLSPGELELEV 200
AgArr1   ERLLKKLGSNAIPFTFNISPNAFSSVTLQQGEDDNGDPCGVSYYVKIFAGESETDRTHRRSTVTLGIRKIQFAPTKQGQ-QPCTLVRKDFMLSPGELELEV 201
DmArr2   EKLVRKLGSNAYPFTFHFPPNSESSVTLQQEGDDNGKPLGVEYTIRAFVGDSEDDRQHKRSMVSLVIKKLQYAPLNRGQRLPSSLVSKGFTFSNGKISLEV 200
AgArr2   ERLVKKLGANAFPFTFHFPSMAPSSVTLQAGEDDTGKPLGVEYAIKAHVGEDESDKGHKRSAVTLTIKKLQYAPVSRGRRLPSSLVSKGFTFSQGKINLEV 200
         *:*::***:** **.::.. :*:**.**   .* ..* **.* ::  ::*:.: *: *:**  :.* *:*:*:*:** .:*   *.::* *.* :* *:::.***


DmArr1   TLDKQLYHHGEKISVNICVRNNSNKVVKKIKAMVQQGVDVVLFQNGQFRNTIAFMETSEGCPLNPGSSLQKVMYLVPTLVANCDRAGIAVEGDIKRKDTAL 301
AgArr1   TLDKQLYLHGERIGVNICIRNNSNKMVKKIKAMVQQGVDVVLFQNGSYRNTVASLETSEGCPIQPGSSLQKVMYLTPLLSSNKQRRGIALDGQIKRQDQCL 302
DmArr2   TLDREIYYHGEKTAATVQVSNNSKKSVKSIKCFIVQHTEITMVN-AQFSKHVAQLETKEGCPITPGANLTKTFYLIPLAANNKDRHGIALDGHLKDEDVNL 300
AgArr2   TLDREIYYHGEKIAANIVVTNNSRKTVKSIKCFVVQHCEVTMVN-AQFSKHIASLETREGCPITPGASFTKSFFLVPLASSNKDRRGIALDGHLKEDDVNL 300
         ***::* ***: ...: : ***.* **.**.:: *  ::..: ..: :* :** ****: **:.: * ::* *    * :* ***::*.:* .*


DmArr1   ASTTLIAS-QDARDAFGIIVSYAVKVKLFLGALGGELCAELPFILMHPKPS--RKA---QLEAEGSIEA----------------------------- 364
AgArr1   ASTTLLAQ-PDQRDAFGVIISYAVKVKLFLGALGGELSAELPFVLMHPKPG--TKAKVIHADSQADVETFR------QDTIDQQASVDFE--------- 383
DmArr2   ASSTMVQEGKSTGDACGIVISYSVRIKLNCGTLGGEMQTDVPFKLLQPAPGTIEKKRSNAMKKMKSIEQHRNVKGYYQDDDDNIVFEDFAKMRMNNVNMAD 401
AgArr2   ASSTLISEGKCPSDAMGIVISYSLRVKLNCGTLGGELQTDVPFKLMNPAPGSVERERVNALKKMKSIERHRYENSHYADDDDNIVFEDFARLRMNEPE--- 398
         **:*.*:: .    ** *::*:**:::** *:**** :::** *::* *.   .        . .:*
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Figure 7. Sensory arrestin protein sequence alignment. *D. melanogaster* Arr1 (DmArr1) protein sequence (GenBank accession no. NP_476681), *An. gambiae* Arr1 (AgArr1) protein sequence (GenBank accession no. AAG54081), *D. melanogaster* Arr2 (DmArr2) protein sequence (GenBank accession no. NP_523976), *An. gambiae* Arr2 (AgArr2) protein sequence (GenBank accession no. DAA00888). Shaded boxes indicate conserved phosphorylation detection residues (Vishnivetskiy et al., 2000; Gurevich and Gurevich, 2004). Open boxes indicate conserved putative SH3, PxxP recognition domains (Luttrell et al., 1999). Underlined residues are putative AP2 interaction domains (Laporte et al., 2000), which while conserved in arr2 proteins are lacking in the Arr1 orthologs.

arrestins. Moreover, a putative C-terminal AP-2 interaction domain, which has been shown to facilitate interactions between arrestin proteins and cellular endocytic machinery (Laporte et al., 2000), is conserved among the Arr2 proteins but absent from the Arr1 proteins, which overall are 46% and 53% identical, respectively, in *D. melanogaster and An. gambiae.*

Recent studies demonstrating the functionality of AgORs within the context of the olfactory system of *D. melanogaster* (Hallem et al., 2004a; Jones et al., 2005) lend credence to the notion of compatibility between the olfactory systems of *An. gambiae* and *D. melanogaster*. Indeed, successful rescue of function in these studies provides strong support for the experimental paradigm that is tested here, i.e., whether the mosquito sensory arrestins, AgArr1 and/or AgArr2, are capable of functionally restoring peripheral olfactory responses in the context of arrestin deficits in *D. melanogaster*. Validation of function in this regard would lend strong support to the view that these AgArrs are likely to function in the same manner in mosquito olfactory pathways. This result would foster the rationale that *in vivo* targeting of AgArr1 or AgArr2 function in *An. gambiae* should result in olfactory deficits in the mosquito, and would be expected to impact its vectorial capacity.

 Electrophysiology Data

As in previous studies (Merrill et al., 2005), we utilized the bipartite Gal4/UAS system (Brand and Perrimon, 1993) to drive expression of a wild-type copy of the AgArr1 gene across all ORNs in *arr1[1]* mutant *D. melanogaster*. This is accomplished by crossing fly lines expressing the yeast Gal4 transcription factor under regulatory control of the pan-neuronal *elav* promoter (Campos et al., 1987; Robinow and White, 1988) with lines containing a UAS-AgArr1 transgene in a *arr1[1]* mutant background. Olfactory signaling was then assayed within the context of EAG analysis of Oregon R wild-type

and rescue flies, as well as the parental mutant lines which, on their own, should not express the AgArr1 transgene. In these assays, we have examined olfactory responses to several representative class-I and class-II odorants previously observed to elicit diminished responses in *arr1[1]* mutant flies, and for which rescue of function was observed when tested in the DmArr1 transgenic flies (Merrill et al., 2005).

In these studies, class-I odorants, which include 1-octanol and 2-heptanone, elicited diminished responses in arrestin mutant fruit flies at all concentrations examined (Figure 8). In the case of 1-octanol (Figure 8A), rescue of function was apparent for C155/1[1]/1A flies at the three highest concentrations (10^{-1} through 10^{-3} dilutions) when compared with wild-type flies, while both parental Gal4 and UAS *arr1[1]* mutant lines responded at significantly lower amplitudes, consistent with our previous studies (Merrill et al., 2005). At the lowest concentration examined (10^{-4} dilution), wild-type and rescue responses were statistically indistinguishable from mutant responses, and all responses at this dosage were no different from those to the mineral oil solvent. For 2-heptanone (Figure 8B), wild-type responses at all concentrations were significantly higher than the mutant counterparts, and rescue flies responded at an intermediate level relative to wild-type and mutant flies at the three highest concentrations, indicative of a partial rescue. Only at the lowest concentration (10^{-4} dilution) were responses observed to be statistically indistinguishable from wild-type responses.

Class-II odorants, which include 1-butanol, heptanoic acid and octyl acetate, generally elicit diminished EAG responses in arrestin mutant flies only at the highest odorant concentrations (Merrill et al., 2005). For 1-butanol (Figure 9A), olfactory sensitivity in AgArr1 transgenic rescue flies was restored to wild-type levels at the two highest concentrations examined, while parental Gal4 and UAS lines consistently responded at significantly lower levels. At the two lower concentrations, olfactory responses were indistinguishable from the oil solvent and across all genotypes. For
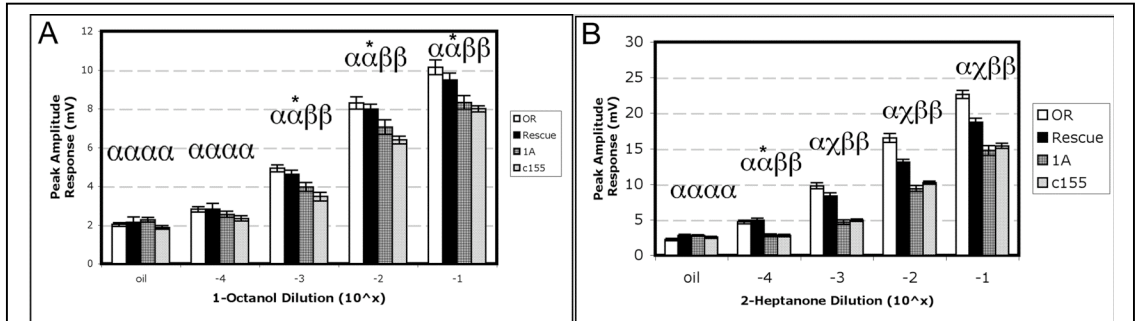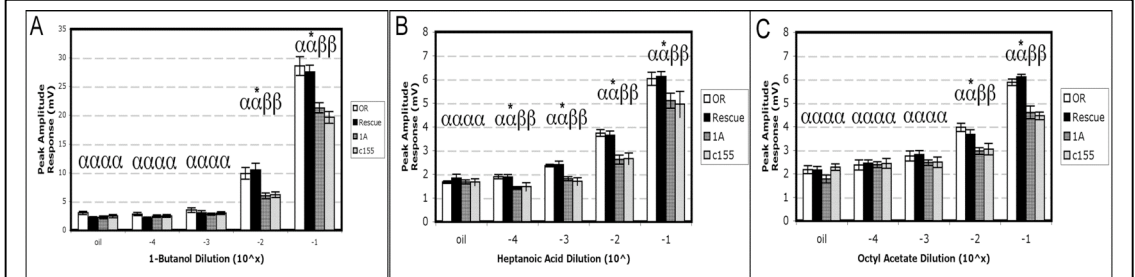
47

Figure 8. AgArr1-mediated rescue of function observed for class-I odorants.
A) Restoration of olfactory sensitivity to 1-octanol. B) For the odorant 2-heptanone, at all but the lowest concentration, partial rescue of function is observed wherein olfactory responses are intermediary to those of wild-type and parental Gal4 and UAS mutant strains. Genotypes: OR: wild-type Oregon R, c155: *w, elavGal4^{c155}; arr1^1*, 1A: *w; arr1^1;* UAS-AgArr1^{1A}, Rescue: *w, elavGal4^{c155}; arr1^1;* UAS-AgArr1^{1A}/+. α, β, and χ represent statistically distinct groups at each dilution. Where differences exist, p < 0.05. For all genotypes, n= 10–15 flies. One-way ANOVA was used for statistical analyses. Error bars represent standard error. Asterisks denote concentrations for which complete functional rescue is observed.

Figure 9. AgArr1-mediated rescue of function observed for class-II odorants.
A) Restoration of olfactory sensitivity to 1-butanol. B) Heptanoic acid: at all concentrations, olfactory sensitivity is restored for rescue flies. C) For the odorant octyl acetate, full rescue of function is observed at the two highest concentrations. Labels and genotypes are as presented in Fig. 8. For all genotypes, n= 10–15 flies. One-way ANOVA was used for statistical analyses. Error bars represent standard error. Asterisks denote concentrations for which complete functional rescue is observed.

heptanoic acid (Figure 9B), AgArr1 rescue flies' responses were statistically indifferent from wild-type flies at all concentrations tested. For octyl acetate (Figure 9C), rescue flies expressing AgArr1 responded at wild-type levels at the two highest concentrations examined, whereas responses at the two lowest concentrations were statistically indistinguishable across all fly lines as well as from responses to the mineral oil solvent. Together, these data provide strong support of AgArr1's capacity to nearly completely rescue the *arr1[1]* mutant olfactory phenotype when transgenically expressed in *D. melanogaster arr1[1]* background.

To further explore this phenomenon, we considered the possibility that, given the usage of the Elav-Gal4 promoter, transgenic expression levels of AgArr1 may not precisely mimic expression levels of endogenous DmArr1 in wild-type flies. To that end and to assess the phenotypic effects of alterations in DmArr1 levels, EAG response levels were examined in *arr1[1]cn* heterozygous flies. Here, Oregon R virgin females were crossed with *w; arr1[1]cn* mutant males, and responses were recorded for 2-heptanone and other odorants (described above). In all cases, wild-type response levels that were indistinguishable from those of Oregon R flies were observed (data not shown). In consideration of this, we suggest that a threshold be established wherein the heterozygous condition (i.e., approximately 50% of wild-type DmArr1 expression levels) sufficiently confers wild-type levels of olfactory sensitivity to the fly. This model however does not address the possibility that over-expression of AgArr1 may in fact be selectively responsible for the phenotype observed in response to olfactory stimulation with 2-heptanone.

Previously, we utilized a simple larval mobility assay to examine the ability of DmArr1 transgenes to functionally rescue defects in olfactory-mediated behavior in *Drosophila arr1[1]* mutant's responses to 1-octanol (Merrill et al., 2005). In a similar manner, we used these behavioral paradigms to examine the ability of transgenic

AgARR1 to rescue $arr1^1$-linked larval olfactory deficits when presented with 1-octanol and several other odorants towards which arr1[1] mutants displayed reduced attraction as compared to wild-type counterparts (Merrill et al., 2005). Behavioral assays across multiple AgArr1 transgenic lines were carried out to test larval responsiveness to multiple odorant concentrations, and in all cases a statistically significant level of functional rescue was not observed (data not shown).

In order to fully understand the functional relationships between *An. gambiae and D. melanogaster* sensory arrestins, we examined the ability of AgArr2 and DmArr2 transgenes to rescue the olfactory-deficit phenotype associated with the $arr2^5$ mutant allele in *D. melanogaster* (Merrill et al., 2005). Furthermore, similar efforts were undertaken to rescue the $arr1^1$ mutant phenotype with DmArr2 and AgArr2 transgenes. In all of these scenarios, involving multiple independent lines of inserts for each transgene, restoration of olfactory sensitivity to wild-type levels was never observed (data not shown).

Localization of Transgenic AgArr1 in the Antennae of Rescue Flies

In order to confirm the neuronal expression of the AgArr1 transgene in ORNs as an underlying basis for transgenic rescue, we applied the *in situ* hybridization (ISH) technique (Figure 10) in an examination of the Gal4/UAS rescue flies. In these studies, the broadly expressed co-receptor, Or83b, which plays an obligatory role in olfactory sensory physiology, acts as a *de facto* ORN marker for the majority (70–80%) of these cell types (Larsson et al., 2004). For these experiments, coexpression of AgArr1 and Or83b is observed in a majority of Or83b-positive olfactory sensory neurons, indicative of the presence of transgenic AgArr1 in the ORNs (Figures 10A-10C). Furthermore, these results are comparable to expression patterns of transgenic DmArr1, being driven by the same *elav-Gal4* construct, for which we have previously demonstrated rescue of
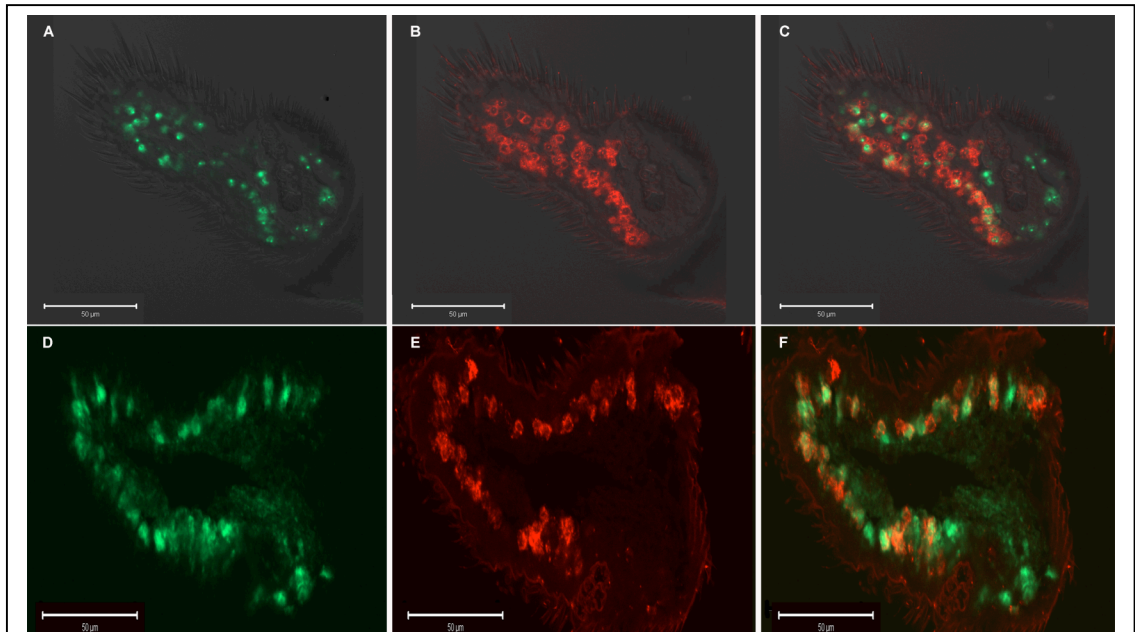
Figure 10. Transgenic AgArr1 expressed in Or83b positive olfactory sensory neurons. A) Fluorescence image of antisense DIG labeled AgArr1 probe in *arr1[1]* mutant antennae. B) Fluorescence image of antisense FITC labeled Or83b probe. C) Merge of A and B, indicating coexpression of Or83b and AgArr1 in most Or83b positive olfactory sensory neurons. D) Fluorescence image of antisense DIG-labeled DmArr1 probe in Gal4/UAS-DmArr1 flies. E) Fluorescence image of antisense FITC labeled Or83b probe. F) Merge of D and E, indicating coexpression of Or83b and transgenic DmArr1.

function in the *arr1[1]* mutant background (Merrill et al., 2005) (Figures 10D-10F). ISH control studies were also carried out in parental UAS-AgArr1 and UAS-DmArr1 lines, in which transgenic AgArr1 and DmArr1 expression is precluded, respectively. In both these instances, expression of transgenic AgArr1 or DmArr1 was undetectable in these flies (data not shown).

Real Time PCR Analysis of AgArr1 mRNA Levels in Transgenic Rescue Animals

In order to further validate transgene expression levels, we employed quantitative RT-PCR to assess antennal AgArr1 expression levels relative to both endogenous and transgenic DmArr1 in the antennae of Gal4/UAS-DmArr1 rescue flies. In these studies, where expression levels of the ribosomal protein gene *rp49* were examined to serve as a calibrator for total RNA levels (Figure 11), both DmArr1 and AgArr1 transgenes were expressed at markedly higher levels than the endogenous DmArr1 gene in wild-type Oregon R flies. Furthermore, we observed only an overall modest difference in levels of AgArr1 transgene expression (1.5-fold increase) relative to similarly expressed DmArr1 transgenes in *arr1[1]* mutant backgrounds, which is consistent with the similar patterns of phenotypic rescue in these lines. In all cases, endogenous levels of DmArr1 were undetectable in the *arr1[1]* mutant background (data not shown), consistent with its characterization as a hypomorphic arrestin allele (Smith et al., 1990).

Discussion

In light of our observations that both DmArr1/DmArr2 and AgArr1/AgArr2 are expressed in the antennae of *D. melanogaster* and *An. gambiae*, respectively (Merrill et al., 2002) where they may be reasonably assumed to be active in olfactory processes, we initially focused on a broad examination of the potential combinations for transgenic
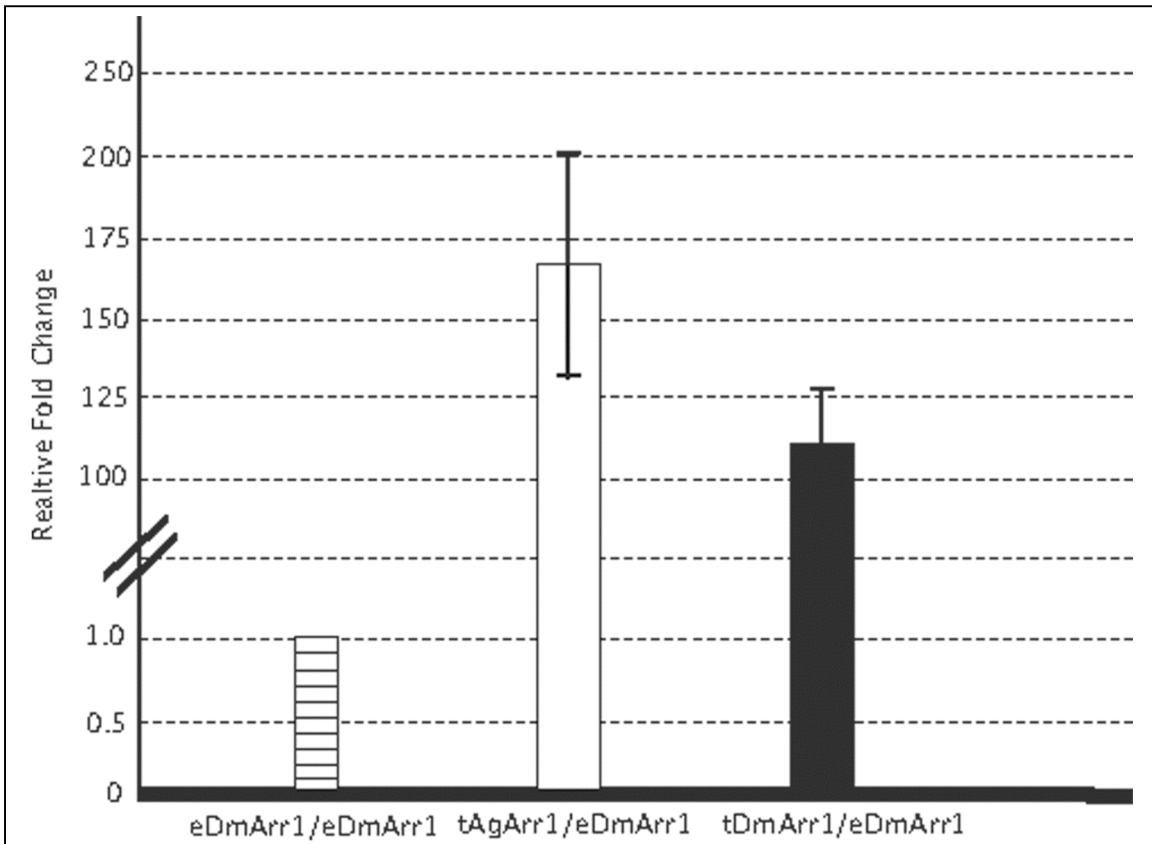
Figure 11. Expression of transgenic arrestin is quantitatively greater than endogenous wild-type arrestin. Relative expression levels of transgenic AgArr1 (tAgArr1) and transgenic DmArr1 (tAgArr1) in Gal4/UAS rescue flies, normalized to endogenous *rp49* expression levels, compared with expression levels of endogenous DmArr1 (eDmArr1) in wild-type Oregon R flies. Error bars are indicative of standard error, calculated from variability of relative expression levels for three independent experiments.

rescue of olfactory *arr* mutant phenotypes. Accordingly, DmArr1, AgArr1, DmArr2 and AgArr2 transgenes were assayed for the ability to restore olfactory sensitivity in *arr1*[1] and *arr2*[5] mutant fruit flies under the control of the pan-neuronal elav-Gal4 (Lin and Goodman, 1994) driver line. This driver was chosen rather than other ORN-biased Gal4 lines, such as those based on DmOr83b promoters, based on its ability to act over the complete spectrum and not just a simple majority of ORNs. In electrophysiological assays both DmArr1 and AgArr1 transgenes were able to robustly rescue *Drosophila arr1*[1] mutations insofar as peripheral olfactory signal transduction while none of the *Drosophila* or Anopheles transgenes were able to restore olfactory sensitivity associated with *arr2*[5] mutations. In addition, DmArr2/AgArr2 transgenes fail to rescue the *arr1*[1] olfactory deficit phenotype, consistent with the hypothesis that Arr1 and Arr2 play distinct and non-complementary roles in olfactory signal transduction. Furthermore, in contrast to electrophysiological studies, AgArr1 transgenes failed to functionally rescue larval behavioral deficits linked to the *arr1*[1] mutation.

While intriguing, the ability to restore peripheral olfactory sensitivity while failing to rescue behavioral phenotypes, may, in part, reflect uncharacterized aspects of transgenic expression as well as the complex nature of the behavioral output being considered. In the first assay, the electrophysiological responses to odorants recorded in the antennae in the form of EAGs are specifically linked to signal transduction in the antennal ORNs that respond to the odorants (Alcorta, 1991). While utilization of the pan-neuronal *elav*-Gal4 driver to mediate expression of transgenic arrestins in non-ORN neurons should not impact the electrophysiological responses of the ORNs to the odorants it is reasonable to speculate that ectopic over-expression of transgenic AgArr1 in all of the peripheral as well as central processing neurons of the Drosophila larvae may have confounding effects on the olfactory behavioral output. Thus, a lack of behavioral rescue in *arr1* mutant larvae must not be necessarily misconstrued to have

bearing on the impact of physiological rescue of peripheral ORN olfactory signal transduction in *arr1* mutant adult flies.

While both DmArr1/AgArr1 and DmArr2/AgArr2 protein pairs display significant conservation relative to each other, there is considerable divergence at the carboxy terminus between Arr1 and Arr2 (Figure 7). In light of the inability of cross-arrestin complementarity, this likely reflects significant divergence in the functional roles of these sensory arrestins. Indeed, recent reports have suggested a similar divergence of function of Arr1 and Arr2 within the context of the *Drosophila* visual system (Satoh and Ready, 2005; Orem et al., 2006). Elucidation of the relative expression patterns of endogenous DmArr1 and DmArr2 over antennal ORNs in *D. melanogaster* may shed further light on the specific roles that these two arrestin sub-types play in olfactory sensation.

Nevertheless, the results of this study confirm one of the central elements of our initial hypothesis in that we demonstrate that AgArr1 is a functional ortholog to DmArr1. Rescue of function of the physiological olfactory deficit phenotype observed in *arr1*[1]*cn* mutant flies was observed for four of five odorants examined, the lone exception being 2-heptanone, for which a partial rescue was observed for high-end concentrations. In this instance, intermediate EAG amplitude response levels between the responses of the wild-type Oregon R and parental arrestin mutant flies were observed. This is especially pertinent, because it relates to our previously reported physiological rescue of function of olfactory phenotypes in *arr1*[1] mutant flies with DmArr1 transgene, where a complete rescue was observed in response to 2-heptanone at all concentrations examined (Merrill et al., 2005).

AgArr1 transgene localization studies suggest there is not a strict one-to-one correspondence in the expression of the endogenous DmOr83b non-conventional OR and the transgenic AgArr1 protein. We interpret this in light of the observation that not all

antennal neurons display the Or83b marker (Larsson et al., 2004), such that it is

expected that AgArr1 under the control of the pan-neuronal *elav* promoter should be

present in a number of cells lacking the Or83b marker. While this is precisely what was

observed, we also noted the presence of a subpopulation of ORNs (15–20%) that

display the Or83b marker and paradoxically lack the transgenic AgArr1 message.

Without postulating as to the mechanism underlying this observation, it is reasonable to

suggest this lack of AgArr1 expression in Or83b-positive ORNs may also contribute to

the partial rescue observed in response to the odorant 2-heptanone. We additionally

examined mRNA expression levels of the AgArr1 and DmArr1 transgenes in relation to

endogenous wild-type DmArr1 and observed a significant overexpression of the mRNA

expression levels of each transgene relative to the endogenous wild-type DmArr1

transcript. This is consistent with numerous studies utilizing elav-Gal4 driver lines and

apparently does not cause a phenotype in and of itself, as olfactory sensitivity is restored

to wild-type levels when these transgenes are expressed in the $arr1^1$ mutant

background. However, only a modest difference was observed between the expression

levels of the AgArr1 and DmArr1 transgenes relative to each other. With all this in mind,

we favor the view that a variety of factors, including differential mRNA or protein stability

leading to subtle alterations in expression levels and/or spatial patterns, account for the

data, rather than just a true functional divergence between DmArr1 and AgArr1. Such

differences would reasonably be expected to be reflected in a broader contrast in

phenotypic rescue, rather than a partial rescue in response to one specific odorant.

In conclusion, this study supports the view of both a broad requirement for

sensory arrestins and a specific functional role for AgArr1 in insect olfaction. That said, a

mechanistic understanding of the precise role of sensory arrestins within the context of

insect olfactory signal transduction still remains unclear. Inasmuch as several studies

(Dolph et al., 1993; Satoh and Ready, 2005; Orem et al., 2006) provide evidence that

arrestins facilitate desensitization and receptor internalization in the *Drosophila* visual system, it has been assumed that Dm/AgArr1 and other sensory arrestins function in a similar manner within GPCR-based olfactory signal transduction paradigms in insect ORNs. Interestingly, it has recently been suggested that because *Drosophila* ORs manifest an inverted membrane topology when compared with conventional GPCRs (Benton et al., 2006), they may not act as canonical GPCRs in olfactory signal transduction. Therefore, insect sensory arrestins may affect peripheral olfactory sensitivity via cryptic interactions with other GPCRs or indeed other signaling pathways in ORNs.

Conversely, as the vertebrate β-arrestins are known to interact with classes of receptors unrelated to GPCRs (Lefkowitz et al., 2006), it may indeed be the case that the insect sensory arrestins play an essential role in the regulation of the functionality of insect ORs, irrespective of whether they are *bona fide* GPCRs. This study opens the possibility that the goal of disrupting proper olfactory signaling necessary for host-seeking and other behaviors underlying the vectorial capacity of *An. gambiae* may indeed be accomplished through targeting AgArr1 or other olfactory proteins. While still speculative, these efforts may ultimately help provide the basis for the design of novel approaches for anti-malarial programs that target olfactory-based mosquito behaviors.