CHAPTER III

AN EXAMINATION OF SPATIAL EXPRESSION PATTERNS OF THE SENSORY ARRESTINS IN THE ANTENNAE OF *DROSOPHILA MELANOGASTER*

Preface

The experiments performed and results obtained in this chapter are unpublished. All RNA probe design and generation, as well as *in situ* hybridization experiments have been performed by the author. Analyses of DmArr/Gal4/UAS-GFP flies have also been performed by the author.

Introduction

In the insect genetic model organism, *D. melanogaster*, it has been determined that DmArr1 and DmArr2 are expressed in olfactory appendages (Merrill et al., 2002). Through the investigation of *arr1* and *arr2* single mutant and *arr1/arr2* double mutant fruit flies, a functional role in olfactory signal transduction has been described for these genes (Merrill et al., 2003; Merrill et al., 2005). In these studies, the EAG was utilized to record odor-evoked changes in extracellular voltage potentials in the antennae. For a broad spectrum of odorants, reduced voltage amplitude deflections were observed in arrestin mutant flies relative to wild-type (Merrill et al., 2005). One unexpected manifestation of the arrestin mutant phenotypes was that odorant-specific differences were observed. Specifically, when some odorants were presented, deficits were observed in either of the single mutant flies, as well as in the double mutants. For other odorant stimuli, however, deficits were not observed in either of the single mutants, but only in the *arr1/arr2* double mutants. These observations suggested that, in some cases, functional redundancy might exist within ORNs activated by specific odorants with regard to the activity of the arr1 and arr2 proteins.

One obvious hypothesis that can be tested to address the differential nature of the arrestin mutant EAG phenotypes is: DmArr1 and DmArr2 are broadly coexpressed in antennal neurons. To test this hypothesis, two approaches have been taken. First, the ISH technique has been utilized to examine RNA expression patterns in antennal sections. FISH has proved to be a reliable method for characterizing OR expression patterns in *Drosophila* (Vosshall et al., 2000; Fishilevich and Vosshall, 2005); in these studies, differentially labeled RNA probes have been used to determine coexpression patterns of two genes. Alternatively, a transgenic promoter/reporter-gene system has been employed, taking advantage of the bipartite Gal4/UAS expression system (Brand and Perrimon, 1993). In this case, plasmid constructs were generated containing sequences 2kb upstream of either the arr1 or arr2 transcription start site and positioned upstream of the Gal4 transcription factor. These constructs were then used to generate transgenic flies, such that the "Arr-Gal4" driver line would be crossed to a UAS-green fluorescent protein (GFP) reporter line, thus allowing for arrestin "promoter" activity to be assayed. While the Arr-Gal4/UAS-GFP experiments would strictly prohibit coexpression analyses of the arr1 and arr2 genes in this context, observations from the FISH experiments and the transgenic promoter/reporter assays could be compared to each other and definitive conclusions could be made about the spatial expression patterns of arr1 and arr2 in the antennae.

Materials and Methods

Fly Cultures

The wild-type flies used for the ISH experiments were Oregon R, obtained from Dr. C. Desai (Vanderbilt University). GFP-reporter flies, *w;* UAS-GFP: mCD8/Cyo, henceforth UAS-GFP, were obtained from the Bloomington Drosophila Stock Center

(Stock #5137, Bloomington, Indiana). Flies were grown in cylindrical 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.

In situ Hybridization

FISH was performed using a modified version of previously reported methodology (Vosshall et al., 1999; Vosshall et al., 2000). Briefly, DIG- and FITC-labeled Or83b, DmArr1 and DmArr2 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 Oregon R 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 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-POD (1:200) and sheep anti-FITC-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 then performed at room temperature. For visualization of Or83b-, DmArr1-, and DmArr2-DIG labeled probes coupled with the anti-DIG-POD antibody, diluted tyramide-FITC reagent (Perkin Elmer, Waltham, Massachusetts) was applied for a 10-min incubation at room temperature. For visualization of Or83b, DmArr1 and DmArr2 FITC-labeled probes coupled with the anti-FITC-AP antibody 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). Following either treatment, three subsequent 5-min 1X PBS-tw washes were performed. Sections were then mounted with Vectashield reagent (Vector Laboratories, Burlingame, California) and viewed on an Olympus BX60 Fluorescence microscope (Olympus America Incorporated, Center Valley, Pennsylvania) at 200X total magnification.

Generation of DmArr-Gal4 Constructs and Analyses of Transgenic Lines.

DmArr1 and DmArr2 upstream regulatory sequence fragments immediately upstream of the appropriate transcription start site were obtained via long-run PCR amplification of genomic DNA from Oregon R flies. PCR amplicons were generated with primers containing flaking Acc65I restriction digestion sites. For DmArr1 and DmArr2, PCR fragments were 2.0 Kb in length. These fragments were subcloned first into the pCRII-Topo vector (Invitrogen, Carlsbad, California) and then into the G4PN Gal4 vector via ligation into a unique Acc65I digestion site upstream of the Gal4 gene. DmArr1- and DmArr2-Gal4 constructs were sequence- and restriction digest-confirmed and then sent to Rainbow Transgenic Flies, Inc. (Newbury Park, California) for the generation and

establishment of multiple DmArr1-Gal4 and DmArr2-Gal4 homozygous fly-lines. Construct insertions were isolated on the 2nd and 3rd Chromosomes for DmArr1-Gal4, and X chromosome for DmArr2-Gal4.

For the analyses of Arr-Gal4/UAS-GFP flies, whole flies were cold-anesthetized at 4°C and viewed directly on the Olympus BX60 Fluorescence microscope. Alternatively, 15μM head plus antennae sections were generated as follows: 25 female heads were dissected into 4% paraformaldehyde/1X PBS/.01% Tween and held at 4°C for 30 minutes. Samples were then treated with three 5-min washes in 1X PBS at room temperature. An overnight 4°C treatment in 25% sucrose/1X PBS/.01% Tween was then carried out and followed by generation of 15μM cryo-sections. Sections were permitted to air-dry for one hour and then were post-fixed in 4% paraformaldehyde/1X PBS/.01% Tween for 30 minutes at room temperature. Finally three 5-min washes in 1X PBS washes at room temperature were performed and section slides were observed on the Olympus BX60 Fluorescence microscope.

Results

Fluorescence *in situ* Hybridization Studies

Multiple RNA FISH probes of varying lengths ranging from 600 to 1200 nucleotides, and containing labeled uridine triphosphate (UTP) nucleotides were generated to detect arr1 and arr2 mRNA specimen. Prior to attempting arr1 and arr2 coexpression studies, coexpression experiments were performed to compare expression of arr1 or arr2 to the broadly expressed Or83b gene (Vosshall et al., 2000; Larsson et al., 2004). In several independent experiments designed to examine various combinations of probes and antibodies, expression of Or83b was detected in cells

throughout the antennae while arr1 and arr2 mRNA were not detected anywhere in the antennae.

Transgenic Arrestin Promoter Assays

Multiple Gal4 driver lines were generated containing putative arr1 or arr2 upstream regulatory sequences cloned upstream of the Gal4 transcription factor and consisting of 2kb of sequence immediately upstream of the transcription start sites for these genes. These flies were crossed to UAS-GFP flies to drive expression of GFP in cells where arr1 and arr2 promoters would presumably be active.

Initially, single pair crossings were made between DmArr1-Gal4 virgins from multiple independent lines and UAS-GFP males, and whole flies were examined. GFP fluorescence was observed in the eye, ocelli, and legs, but not in antennae (data not shown). To examine reporter gene expression on a finer level of detail, antennae plus head cryo-sections from the Gal4/UAS flies were generated and GFP fluorescence was examined in the antennae at the cellular level. For all Arr1-Gal4 driver lines examined, GFP fluorescence was observed in a characteristic cluster of cells at the proximal-medial section of the 3rd antennal segment. GFP-positive cells were observed more distally, as well (Figure 12). For multiple DmArr2-Gal4 promoter lines, antennae plus head cryo-sections of Gal4/UAS-GFP flies were examined. In all cases, no GFP-positive cells were observed in the antennae, whereas GFP-fluorescence was observed in eye and ocelli tissue (data not shown).

Discussion

In order to potentially examine the underlying nature of the odorant-specific manifestations of the *arr1* and *arr2* mutant olfactory EAG phenotypes, consideration was given to the relative spatial expression patterns of these two genes in the antennae. To

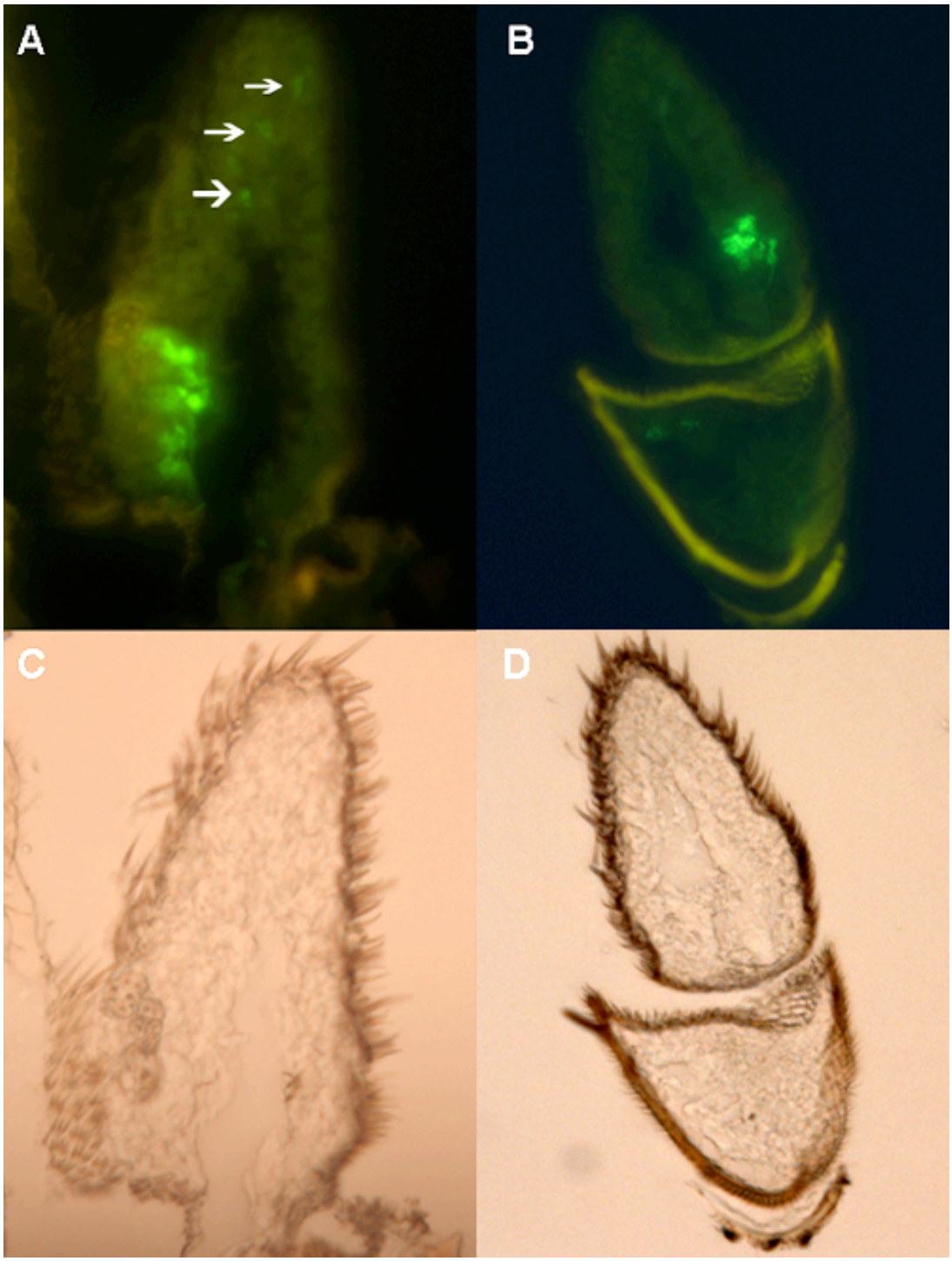

Figure 12. GFP expressed under transgenic control of DmArr1 promoter. A) A cluster of GFP-positive cells is observed in the proximal-medial section of the 3rd antennal section. Arrows demarcate GFP-positive cells falling outside this cluster. B) A horizontal mirror image of A) and derived from an independent DmArr1-Gal4 line, a proximal-medial cluster of GFP-positive cells is observed. C) and D) are brightfield images of A) and B), respectively.

that end, FISH experiments were employed to test the hypothesis that DmArr1 and DmArr2 are coexpressed in antennal ORNs.

The results of these studies indicate that, given the available technology, expression levels of DmArr1 and DmArr2 RNA are below the threshold of detection for FISH. Attempts were made to observe arrestin mRNA via hybridization with RNA probes containing either DIG- and FITC-labeled UTP nucleotides and visualized with either the horseradish peroxidase/tyramide signal amplification or alkaline phosphatase/fast red chromogenic systems. Under no conditions were signal detected for these genes.

These experiments were well controlled. The broadly expressed OR cofactor gene, Or83b, was used as a positive control, and Or83b expression was detected throughout the antennae in experiments in which DmArr1 and/or DmArr2 were not. All probes were subjected to dot-blot hybridization and gel electrophoresis to confirm the quality of the UTP-label and the probes, in general (data not shown). Prior to focusing on antennal tissues for these studies, head plus antennae dissections were subjected to the FISH protocol. In these experiments, arrestin RNA probes revealed robust arr1 and arr2 expression in the eye, but not the antennae, indicating ability of the probes to detect arrestin mRNA. Additionally, the DmArr1 probes were successfully used to detect transgenic DmArr1, overexpressed in neurons via the Gal4/UAS system, in the antennae, indicating ability of the arr1 probes to detect mRNA expressed in the antennae.

Alternatively, a genetic approach was taken, utilizing the Gal4/UAS system to drive reporter gene expression with putative arrestin upstream regulatory sequence. This approach was previously used, prior to the development of Gal4/UAS methodology, to directly drive Lac-Z expression in all photoreceptor cells in the eye with arr1 and arr2 upstream regulatory sequence (Dolph et al., 1993).

For DmArr1-driven expression of the GFP reporter gene, stereotypic expression patterns were observed for multiple Gal4 driver lines. These patterns, however, were not consistent with a previous finding of widespread expression of DmArr1 protein in the antennae (Merrill et al., 2002). For DmArr2-driven expression of a GFP reporter gene, no GFP-positive cells were observed in the antennae.

A previous report utilized 8kb and 4.7 kb of arr1 and arr2 upstream regulatory sequence, respectively, to driver reporter gene expression in the fly eye (Dolph et al., 1993). The regulatory sequence used in this report, roughly 2kb directly upstream of the transcription start site, for each arr1 and arr2, is well short of that previously used. However, it seemed reasonable that 2kb of upstream regulatory sequence would faithfully represent arrestin expression, as it has been recently reported that as little as 400 bp of upstream regulatory sequence was required to direct appropriate expression patterns of OR genes in *Drosophila*. One caveat to this, however, is that in some cases, crucial regulatory elements have been observed in the region downstream to the open reading frames (Ray et al., 2007).

An additional consideration that may confound the arrestin driver/reporter analyses is that the observations of GFP fluorescence in the antennal cryo-sections were examined without the inclusion of standard anti-GFP antibodies. While such augmentation may not be necessary for robustly expressed GFP, the ISH studies of endogenous arr1 and arr2 expression patterns indicate that these genes are not robustly expressed. As such, a weaker-than-expected arrestin promoter may not robustly drive expression of GFP. Therefore, reassessment of these promoter lines, in conjunction with the use of anti-GFP antibodies, may reveal enhanced GFP spatial patterns of expression. Alternatively, utilization of more robust UAS-GFP lines (Halfon et al., 2002) may yield similar results. In any case, it is difficult to accurately draw conclusions pertaining to the fidelity of transgenic promoter constructs without having knowledge of

the endogenous expression patterns of the genes in question. Thus, for the stated hypothesis to be fully addressed, endogenous expression patterns of arr1 and arr2 must be elucidated. This issue will be discussed further in the “Future Directions” section of Chapter V of this dissertation.