Abstract

GANGULY, INDRANI. Genetic analysis of odor-guided behavior in *Drosophila melanogaster*. (Under the direction of Dr. Robert Anholt and Dr. Trudy Mackay).

The ability to respond to and interact with the chemical environment is fundamental to the survival of many species. It governs predator-prey relationships, kin and mate selection, food localization, maternal behaviors and avoidance of environmental toxins. Olfactory behavior is determined by the concerted action of multiple genes that interact with one another and with the environment, be it external, genetic or sexual. Like a number of other quantitative traits, odor-guided behavior shows significant sex-specificity in its phenotypic expression. However, the molecular basis of sexual dimorphism remains poorly defined. This study provides the first example of an autosomal pleiotropic gene that undergoes sex-specific transcriptional regulation to provide the potential for sexually dimorphic olfactory behavior. The phenotypic and molecular characterization of a *P*-element tagged locus, *smi97B*, reveals that the multiple PDZ (*P*SD-95, *D*iscs-large, *Z*o-1) and LRR (*L*eucine-*R*ich *R*epeat) domain protein, Scribble (Scrib) is responsible for olfactory behavior in adult and larval stages of *Drosophila melanogaster*. In the adult, *scrib* is alternatively spliced to generate sex-specific transcripts that are correlated with sexually dimorphic olfactory phenotypes. Head-derived *scrib* splice variants differ in the number and positions of protein-interaction (PDZ and LRR) domains they encode. Since, Scrib is a synaptic scaffolding protein, these differences may direct the organization of sexually dimorphic synaptic signaling assemblies that contribute to odor-guided behavior.

GENETIC ANALYSIS OF ODOR-GUIDED BEHAVIOR IN

DROSOPHILA MELANOGASTER

By

INDRANI GANGULY

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment

of the requirements for the Degree of Doctorate of Philosophy.

GENETICS

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APPROVED BY:

Co-chair of Advisory Committee

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Dedication

I dedicate my doctoral dissertation to the memory of my darling father, who passed away during the course of this work. His sole source of unabashed pride and joy lay in every accomplishment of his two daughters.

He would have been a very proud papa!

Biography

I was born on the 11th of February (1975) on the shores of the Mediterranean Sea and bred in Mumbai, India. As a fifteen year old undergraduate in junior college, I came to the realization that although dance, theatre and physics were all incredibly enjoyable, the sole path to personal happiness lay in the study of Genetics. While my extra-curricular activities carried on unabated, my interest in studying biology was increasingly fuelled by the excellent faculty at St. Xavier's College, Bombay. In 1995, I graduated with a Bachelors degree in Life Sciences, securing the highest overall score in Bombay University. The following year, I enrolled as a graduate student in the Department of Genetics at North Carolina State University in Raleigh.

Acknowledgements

My graduate mentors, Dr. Robert Anholt and Trudy Mackay, for providing me with rare opportunities that greatly enhanced my growth as a scientist. Without Robert's signature tough love coupled with Trudy's encouragement, I would not have learned the valuable lessons that make a scientist out of a wide-eyed graduate student. I thank them for their patience and ongoing guidance throughout this work.

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The W. M. Keck Center for Behavioral Biology for providing a unique collaborative environment held together by a group of geneticists, physiologists, endocrinologists and ecologists that share an enthusiasm for wine, scientific argument and animal behavior. I will greatly miss the monthly soirees that had become such an enjoyable part of graduate school.

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Thanks to my aunt and uncle, Dr. Nita Ganguly and Dr. Ranjan Ganguly for their constant support and good advice. To all my friends both in the Departments of Genetics and Zoology as well as those unassociated with North Carolina State University for providing the many laughs we shared. To Bora Zivkovicz, for every one of those enjoyable but sometimes meaningless arguments during our "breaks" from the laboratory bench.

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It is difficult to express in words, my gratitude towards the two people that I shared my home and heart-ache with these last few years, my friend Ashish Gehani and my fiancé Eric Frey. They were unwitting participants in the day to day upheavals that forged my growth in the last six years, becoming experts in damage control. Despite never having worked a pipette, they were intimately associated with every aspect of my research. Our numerous discussions not only clarified aspects of my science to myself, but provided fresh perspectives that directly impacted the designs of some of my experiments. They bore the thin thread to my sanity and will forever remain my touchstones.

I believe it took my mother, the historian to wed my father, the electrical engineer-mathematician to blur the line between art and science for me. Monisha and Anjan Ganguly have taught me that scholarship is the *only* ideal and that a life spent seeking knowledge and appreciating its beauty will be a life well-spent. My every accomplishment, however little and insignificant is the sole product of their and my sister's parenting. Without ma's exactitude, papa's unabashed cheerleading and Tina's over-protective older-sister's wing, I could never have traversed this or any other path.

My fiancé, Eric Detlef Frey for spending many an uncomfortable night in a sleeping bag on my office floor to ensure my safety during the late night hours spent in Gardner Hall. I cannot thank him enough for his ever-present shoulder, ear and hand, but can confidently say that he has ensured a very special place for himself in my mother's heart.

Last, but by no means least, I would like to posthumously credit the millions of fruit flies that hold up the foundation of my work. It is my hope (and for the sake of my mother's and sister's peace of mind) that I have maintained them in almost decadent living conditions and given them free reign to express themselves through my work.

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CHAPTER 1:

Introduction

1.1 GENETIC ANALYSIS OF BEHAVIOR

The nature of animals is untutored –Hippocrates

All life forms interact with the environment. In animals, we observe this interaction as an emergent property called behavior. Since an animal's survival often depends on appropriate, context-dependent behavioral responses to its environment (such as avoidance of harmful agents or conditions) it is inherently advantageous for individuals to possess a genetic repertoire that affords them the phenotypic and genetic flexibility to survive environmental fluctuations. Indeed, in natural populations, most behavioral phenotypes are continuously variant along the phenotypic continuum. This is a hallmark of quantitative traits. Hence, like other quantitative traits, behaviors are determined by multiple interacting genes, as well as the environmental context in which they are expressed (Falconer and Mackay 1996).

(a) An old debate

Inquiries into the genetic basis of behavior can be traced back to the domestication of animals, but it was not until the late 1800s that Sir Francis Galton formalized the field of behavior genetics with his treatise on the inheritance patterns of mental capabilities in humans (Galton 1865; Galton 1869). Since then, research in behavior genetics has followed polarized approaches that in many cases reflect differences in guiding interests, *viz*. the quest for the biological relevance of a behavioral phenotype from an evolutionary perspective, versus the identification of the mechanisms that underlie its expression. At its core, genetics is the study of individual variation. A classical approach to behavior-genetic analysis involves applying quantitative and population genetics tools to the study of natural variation in behavioral phenotypes (Hirsch and Boudreau 1958; Hirsch and Erlenmeyer-Kimling 1961). This method of analysis provides information about the genetic architecture of complex, quantitative traits by revealing the effects of and interactions between the segregating polygenes that are statistically associated with observable, phenotypic variation in natural populations (Fernandez-Teruel *et al.* 2002; Guzman-Novoa *et al.* 2002; Hofstetter *et al.* 1995; Page *et al.* 2000). By estimating genetic parameters such as heritability, this approach also provides clues about the selective pressures that act on a behavioral trait, thus providing the background of biological context for a specific behavioral response. On the other hand, Seymour Benzer's isolation of single genes associated with defects in phototaxis (Benzer 1967), biological rhythms (*period*; Konopka and Benzer 1971) and learning behaviors (*dunce*; Dudai *et al.* 1976) in *Drosophila melanogaster*, has led to the popularization of a method that focuses on the identification and characterization of single genes which, when artificially mutated, lead to aberrations in the behavioral phenotype (for reviews, see Benzer 1973; Kyriacou and Hall 1994). This method uncovers piecemeal the molecular components that link gene expression to the manifestation of a behavior.

Depending on which aspect of behavior motivates an inquiry, scientists have been divided over the use of these two methods. Unfortunately, it is sometimes overlooked that the classical genetic and molecular genetic approaches to dissecting biological phenomena are complementary paths that lead to the comprehensive understanding of the basis of behavior (Dobzhanksy, 1967; Hirsch 1967).

(b) Modern approaches: QTL mapping, microarray analyses and mutagenesis screens

Modern quantitative-genetic analyses, such as quantitative trait locus (QTL) mapping, are powerful methods for identifying the multiple segregating loci that underlie variation in a behavioral trait, from a whole-genome perspective (for reviews, see Doerge 2002; Mackay 2001). Typically, a *mapping* population is generated by producing backcross, F_2 or advanced generational progeny between two inbred parental animal strains that are disparate with respect to the behavioral trait of interest. QTLs statistically associated with mean differences in the trait value between the two parental populations are identified using molecular markers within the mapping population (Figure 1A). This method has been very widely used to increase our understanding of the genetic architecture of a range of complex traits; for example, spatial (Milhaud *et al.* 2002) and language ability (Alarcon *et al.* 2002); learning (Valentinuzzi *et al.* 1998); anxiety (Fernandez-Teruel *et al.* 2002); circadian rhythms (Mayeda and Hofstetter 1999); foraging behavior (Sokolowski Page *et al.* 2000); ethanol (Browman and Crabbe 2000) and cocaine sensitivity (Jones *et al.* 1999); stinging and defensive behavior (Guzman-Novoa *et al.* 2002); and, exploratory behavior (Cohen *et al.* 2001). However, fractionating chromosomal regions within QTLs that comprise hundreds of genes to the level of a single gene associated with variation in a behavioral trait is undoubtedly a challenging task. It is limited by the number of genetic recombinants that are generated, as well as the density of the molecular markers in the genome. Furthermore, quantitative complementation tests to mutations in all positional candidate genes may significantly increase the resolution of this mapping technique, but remains dependent on the availability of these extant mutant stocks. As a result, there are few cases in which high resolution QTL mapping has been successful in resolving a QTL into a single behavioral gene with previously unknown functions (for an example in olfactory behavior, see Fanara *et al*. 2002).

The recent advances in genomic sciences and the development of cDNA microarray technology may subvert this problem while holding steadfast to the idea that study of the genetic basis of complex quantitative traits, like animal behavior, requires a genome-wide perspective. Complementary DNA (cDNA) molecules generated from single genes that represent all or most of the genome of a particular species, are spotted on microarray chips. These chips are then hybridized with purified RNA isolated from the animal populations that are differentiated with respect to their mean behavioral trait values. Relative differences in the intensity (or hue) of hybridization on a particular microarray spot reflect the differences in gene expression between the two populations under study. Toma and colleagues took advantage of this technology to identify and characterize some of the genes underlying naturally occurring variation in geotactic behavior between two divergent populations of *Drosophila melanogaster* (Toma *et al.* 2002; Figure 1B). An important extension that this approach provides to the analysis of behavior is that it allows for the identification of non-polymorphic loci that do not contribute to observable differences in trait values between two divergent populations, but nevertheless contribute to the development and manifestation of a behavioral trait. An important caveat however, is that the differential expression of non-polymorphic loci in divergent populations may result from their epistatic interactions with polymorphic loci, thus muddying the distinction between the causes and consequences of genes that are responsible for variation in a trait. Practical disadvantages to using the cDNA microarray technique is that only a fraction (50-70%) of the entire genome may be represented on the array chips, precluding the comprehensive analyses of all possible genetic loci that contribute to variation in a trait. Furtherrmore, the analysis is based on the competitive hybridization of RNA from two distinct populations and hence, each experiment requires large amounts of starting biological material. The development of high density oligonucleotide microarrays (example, Affymetrix chips) subverts these problems by giving greater genomic coverage and requiring smaller amounts of RNA for each replicate experiment.

Finally, microarray analyses are based on the assumption that differences in behavioral phenotypes are a result of large differences in gene expression levels (often a 2-fold difference). This severely limits the scope for identifying genes that display minor perturbations in expression levels (which may reflect molecular phenomena, such as RNA stability), yet substantially contribute to observable differences in behavioral phenotypes (Osborne *et al.* 1997; Sokolowski 2001).

In contrast to the genome-wide approaches described above that resolve naturally occurring variation in behavioral phenotypes by dissecting the multiple loci that underlie it, the single gene approach uses induced mutagenesis screens in laboratory raised animal strains to identify single genes that are required for the production of wild type behavior. Germ-line mutations are induced in single individuals of a laboratory parental strain by the administration of chemical mutagens, such as ethylnitrosurea (ENU) or ethylmethylsulfonate (EMS); or as in the case of *Drosophila*, by the introduction of artificially engineered non-autonomous *P*-element transposons. Each progeny isolated from a mutagenized parent represents a unique gametic event in the parental germ line that, in theory, results in a single gene mutation in its genome (Figure 1C). The number of single gene “hits” recovered from such a mutagenesis screen may virtually saturate the genome, thus exposing the phenotypic consequence of the many single genes that are responsible for the genesis of a behavior.

This method has an added advantage in that mutagenized genes affecting a behavioral trait may be molecularly tagged at the outset, which increases the ease with which they are recovered from the genome and further characterized. However, the artificial disruption of single genes in controlled laboratory settings often leads to severe mutations. Such a scenario may prove useful for investigations in developmental biology (where this approach is widely used) that seek to identify single master genes whose disruption lead to dramatic and qualitative phenotypes. Behavior traits, on the other hand, are determined by the action of many genes with small subtle effects on the overall phenotype. Hence, dramatic phenotypes arising from severe mutations of single genes are not always reflected in behavioral variants found in nature. This challenges their biological relevance. Moreover, an overwhelming majority of genes like *foraging* (de Belle *et al.* 1989; de Belle and Sokolowski 1987), *fruitless* (Ryner *et al.* 1996), *double-time* (Price *et al.* 1998) and *dunce* (Dudai *et al.* 1976) that contribute to a range of behaviors, display pleiotropic effects on sometimes unrelated vital cellular functions such hypoxia recovery, neuronal differentiation, imaginal disc overgrowth and female sterility, respectively (Sokolowski 2001). Severe mutations in such pleiotropic loci lead to lethality, thus precluding their detection in behavioral screens. It is only when mutations lead to subtle alterations of gene function (hypomorphic mutations) that the behavioral component of such loci are revealed (de Belle 2002; Greenspan 1997; Osborne *et al.* 1997).

An illustrative example of the pleiotropy displayed by behavioral loci comes from the case of *nightblind-A* (*nba*), a sub-unit of the voltage-activated calcium channel (Smith *et al.* 1996) originally identified by Heisenberg and Goetz (1975) in a screen for defects in optomotor responses in *Drosophila melanogaster*. It was subsequently identified in an independent screen for courtship behavior as *cacophony* (*cac*), a gene that caused specific defects in male-courtship song (Kulkarni and Hall 1987; Schilcher 1977; Von Schilcher 1976) followed by its identification as a lethal, impacting viability (Lefevre 1981). Intra-genic complementation between vision-related *nba* and courtship-related *cac* mutations (*nba*/*cac* heterozygotes display normal vision and courtship behaviors; Chan *et al.* 2002), which are alleles of the same gene, indicates segregation of the multiple functions of this gene as reflected in its two mutant alleles. Presumably, differences in the severity or nature of the mutations induced at this locus lead to disparate observable effects on the organism (Chan *et al.* 2002). It is thus to a behavior scientist's advantage to screen for subtle mutations causing *modulation* of gene function, as opposed to its complete disruption, in order to dissect the pleiotropic functions of a behavioral locus.

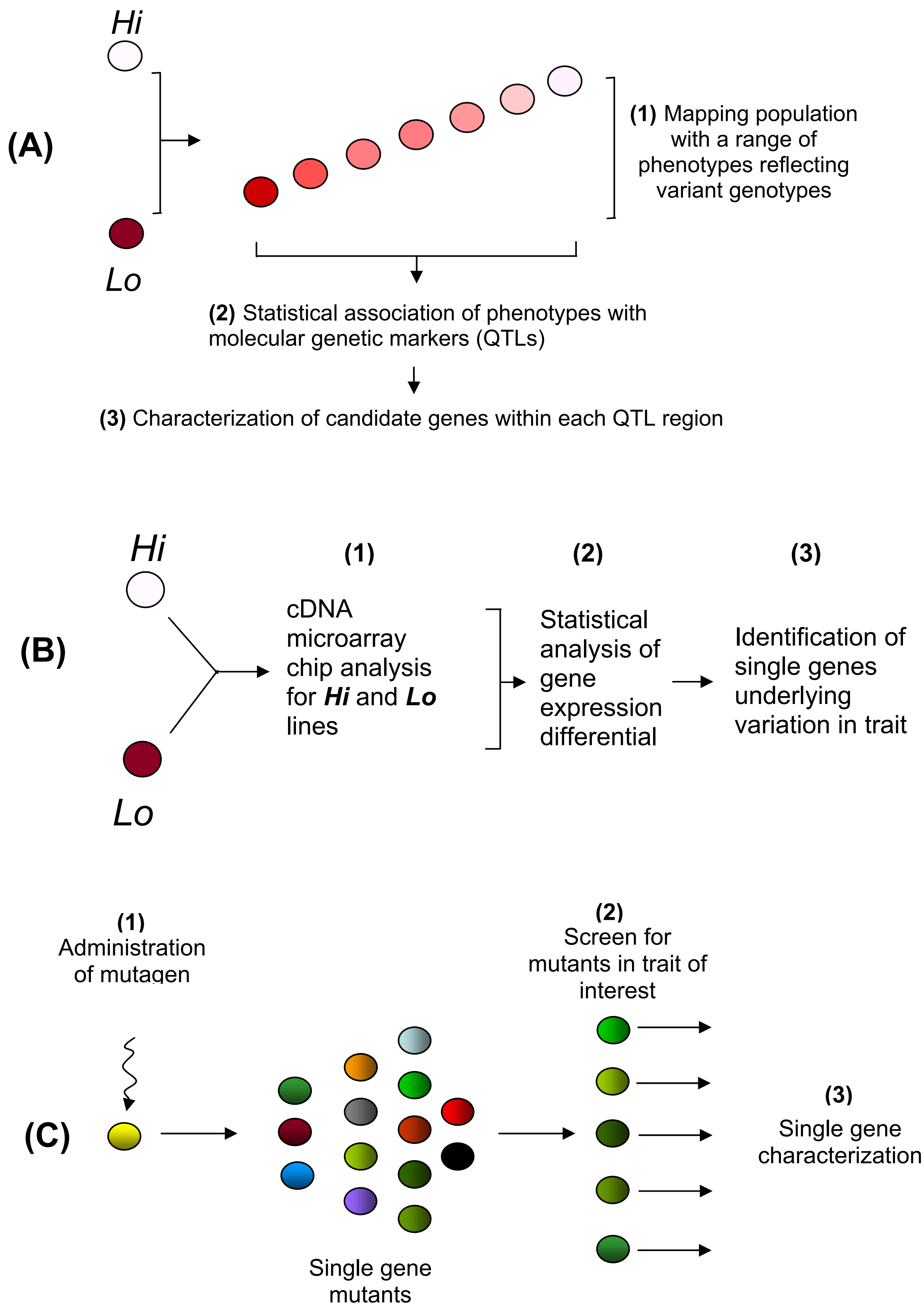


Figure 1: Schematic representation of behavior-genetic approaches (legend overleaf).

Figure 1 (A). QTL mapping approach: Two inbred highly differentiated populations are crossed to generate a mapping population with a range of phenotypic values. Associations are made between genotypic markers in the mapping population and statistically significant variations in the behavioral phenotype. Candidate genes are chosen for further characterization within a QTL region. **(B)** Microarray analysis: A statistical comparison is made between gene expression profiles of two highly differentiated strains. Genes that are differentially expressed in the two populations are further characterized to examine the effects on behavior. The red and white solid circles represent the two differentiated populations. **(C)** Genome-wide mutagenesis screens: A single individual from a highly inbred wild-type strain (yellow solid circle) is subjected to mutagenesis to derive progeny that each bear a single gene mutation (colored solid circles). Behavior screens isolate mutants showing defects in the trait of interest (green solid circles). Each mutation is cloned and characterized to identify single genes that contribute to the trait of interest.

The study of behavior encompasses most aspects of animal biological investigation; behavioral responses are the final end product of gene-environment interactions, anatomical development of receptor and effector organs and the neurobiology of the neural responses that underlie it. However, a distinction may be made between (1) *developmental* genes that contribute to the development of anatomical structures necessary for a behavioral response, i.e., development of receptor organs, structures in the peripheral and central nervous systems, and effector organs; and, (2) genes for *behavior* that encode molecular components in the dynamic process of reception, processing, integration and execution of the trait. For example, both *antennaless* and *smell-blind* mutants in *Drosophila* fail to respond to a variety of odorants (Begg and Hogben 1946; Lilly and Carlson 1990). However, *antennaless* is responsible for the loss of antennal organs in the fly, whereas *smell-blind* encodes a Na^+-channel expressed in the sensory neurons of the antenna and is involved in the propagation of an olfactory signal. Extensive work in the long history of sensory biology has established the role of specific organs in metazoans that are committed to the reception of different kinds of environmental signals. This provides an added impetus for modern day behavior geneticists to identify subtle mutations in observable phenotypes that uncover genes involved in the sensory physiological underpinnings of behavior.

In conclusion, it is likely that the ongoing pursuit of genes that determine animal behavior will resolve an old debate in behavior-genetic analyses (for a review see Greenspan 1997; Tully 1996), to give way to multi-disciplinary, intellectually broad strategies that combine quantitative genetic and genomic approaches with mutagenesis screens for adult viable mutants with subtle behavioral effects in controlled, genetically homogeneous backgrounds to provide a comprehensive analysis of behavior (Mackay 2001; Nadeau and Frankel 2000).

1.2 INFLUENCE OF THE SEX-ENVIRONMENT ON BEHAVIOR

Although behavior is a product of the genes that determine it, there has been a long-standing appreciation for the effect of the environment on the expression of behavioral traits (nature versus nurture). In fact, the first study in behavior genetics conducted by Sir Francis Galton in 1865, was the genetic dissection of mental capacity conducted by using identical twins that shared the same genetic material but raised in disparate home environments (and *vice versa* in the case of non-identical twin pairs). In 1909, Wilhelm Johannsen coined the terms *phenotype* and *genotype* to formally distinguish between an observable trait in an individual and the genes that presumably contributed to it. Hence, it was already clear during the early stages of genetic research that the phenotype was not always a faithful index of the genotype that generated it (McClearn and DeFries 1973).

Behavioral traits are notorious for tremendous within- and between-individual variation that arises from their complex genetic underpinnings as well as their susceptibility to both internal (sex-environment, ontogenic context or genetic background) and external environmental factors that influence their expression (Bucan and Abel 2002; de Belle and Heisenberg 1996; Sokolowski 2001). The influence of the sex-environment on the expression of morphological traits such as body size and shape are the more obvious examples of sexual dimorphism in animals. Advances in the field of quantitative and behavioral genetics have documented sex-specific differences in the phenotypic expression of a range of life history and behavioral traits. Examples include variation in complex traits, such as hypertension (Chen, 1996), obesity (Martin *et al.* 2002), immune function (Verthelyi, 2001), life span (Lio *et al.* 2002) and verbal ability in humans (Small and Hoffman 1994); drug response (Dow-Edwards *et al.* 2001), ethanol preference (Bowers and Wehner 2001) and metabolic gene expression (Sanchez *et al.* 2000) in rodents; the ability to vocalize species-specific songs in birds (Gahr *et al.* 1998; Nealen and Perkel 2000); and, sensory bristle number, life span and olfactory behavior in *Drosophila* (Mackay 2001; Mackay *et al.* 1996; Vieira *et al.* 2000). Although it is adequately clear that the phenotypic expression of most traits show sensitivity to the sex-environment, its molecular basis remains unclear.

Perhaps one of the best characterized models of sex-determination in animals is derived from research in *Drosophila melanogaster*. It involves a regulatory cascade that is initiated by the ratio of *X*-chromosomes to the set of autosomes in an individual. Sex-specific RNA-splicing regulators such as *Sex-lethal* (*Sxl*), *transformer* (*tra*), and *transformer 2* (*tra2*) lead to the generation of sex-specific transcription factors such as *doublesex (dsx)* and *fruitless (fru)*, which ultimately contribute to morphological and behavioral differences in the sexes that are required for reproduction (see review by McKeown 1994). This pathway may be of some relevance to other species since homologues of *Sxl* have been found in distant dipterans (Meise *et al.* 1998; Muller-Holtkamp 1995; Sievert *et al.* 2000), while *tra-2* has functionally conserved homologues in the mouse (Segade *et al.* 1996), rat (Daoud *et al.* 1999) and human (Beil *et al.* 1997). Although this well-characterized hierarchical cascade is responsible for establishing critical aspects of sexual dimorphism, its downstream targets remain largely unknown. Moreover, the identification of three regulatory genes, *intersex* (*ix*), *hermaphrodite* (*her*) and *dissatisfaction* (*dsf*) that also contribute to the regulation of sex-determination but, unlike *Sxl* and *tra*, are expressed in both males and females, suggest alternative pathways for the generation of sexual dimorphism. This notion is strengthened by the isolation of 46 sex-biased genes from the *Drosophila* head, of which three (*turn on sex-specificity, sex-specific enzyme1 and 2*) are reportedly dependant on *tra* and *dsx* for expression, while one (*female-specific independent of transformer*) is independent (Fujii and

Amrein 2002). This study localized the expression of these sex-biased transcripts in the fat cells that surround the *Drosophila* brain and the authors propose that the distinct physiological conditions set up by their sex-specific expression may contribute to the development of sexually dimorphic behaviors. The large number of sex-biased transcripts isolated in this screen also brings to focus the extent of sexual dimorphism in the brain of the fruit fly, which may elicit a host of sexually dimorphic behavioral responses that are not necessarily restricted to those required for courtship and reproduction (Fujii and Amrein 2002). In fact, transcriptional profiling in *Drosophila* using cDNA microarrays reveals that 60% of the variance in global gene expression levels is attributed to the effect of the sex-environment alone (Jin *et al.* 2001). These observations point to the pervasive influence of the sex environment on animal biology.

Sexual dimorphism at the level of the genome that results in quantitative differences in behavior implicates concomitant variability in neural organization. Sexually dimorphic neuroanatomical structures may be one way in which sex-specific differences in genomes may manifest themselves in the whole organism. For example, the macroglomerular complex in the brain of *Manduca sexta* is male-specific and responsible for processing female pheromonal blends (Hansson *et al.* 1991); the male-specific muscle of Lawrence, in *Drosophila*, whose development depends on innervations by male-specific neurons, is required for courtship (Gailey *et al.* 1997) and the regions that control vocalization in zebra finches (the high vocal center and archistriatum) are five to fourteen times larger in males compared to females (only the male of the species sings; Nottebohm and Arnold 1976). However, differences in gross anatomical structures of the nervous system between males and females may not entirely account for the basis of sexually dimorphic behaviors. An alternative, less visually overt mechanism whereby sex-dependent differences in the transcriptome may direct neural variability is by the organization of sexually dimorphic supra-molecular complexes at the synapses of neural circuits in males and females. Compositionally distinct sexually dimorphic neural circuits would likely transduce and regulate the processing of biologically relevant molecular signals in a sexually dimorphic manner, as will be exemplified in this dissertation.

1.3 THE BIOLOGY OF OLFACTION

"The truth of a man lies in his nose." –Ovid

Of environmental signals, none are more varied or prevalent than chemical cues; chemoreception is the fundamental example of organismal-environmental interactions displayed throughout the animal kingdom. In higher animals, specialized sensory modalities are committed to the reception, discrimination, processing and the execution of context dependant behaviors elicited by chemical cues. Gustation is the ability to respond to soluble chemical cues that are present in the immediate aqueous milieu of the organism, and olfaction is the response to odorants which, by the nature of their volatility, are carried over greater distances in space. In most species olfaction governs critical survival strategies, such as thwarting predation, avoidance of noxious agents, food localization, kin recognition and mate selection, and in some cases, parental behaviors that ensure progeny survival. Olfactory behavior is the emergent manifestation of one of the oldest sensory systems in the animal kingdom, and the exploration of its basis enhances our understanding of the genetic basis of a critical quantitative trait.

Chemosensory behavior is dependant on (1) reception of the olfactory signal by sensory neurons present in designated peripheral olfactory organs; (2) the transduction and propagation of the activating signal to central processing units of the nervous system; (3) the integration, decoding and processing of information in higher brain regions; and finally, (4) the direction of a context-dependant biologically relevant behavioral output from the organism. The binding of odorant ligands to transmembrane olfactory receptors (ORs) represent the first step in the serial transduction of an activating olfactory signal. Genome-wide analyses of the distribution and number of olfactory receptors in vertebrates and invertebrates reveal that an astonishing proportion of the genome is dedicated to them. This abundance of putative receptor genes translates into a preliminary estimation of 500 in the nematode model organism, *Caenorhabditis elegans* (reviewed by Troemel 1999); 59 in the arthropod, *Drosophila melanogaster* (Clyne *et al.* 1999; Vosshall *et al.* 1999; Gao and Chess, 1999) and 1000 olfactory receptor (OR) genes in mammals (Buck and Axel 1991; Freitag *et al.* 1998; Parmentier *et al.* 1992; Selbie *et al.* 1992), making them the largest gene family identified. A dedication of almost 1% of the genome to merely the reception of olfactory cues hints at the importance of the sense of smell in animals, as well as the multiplicity of genetic factors that underlie it. Not surprisingly, quantitative genetic studies reveal that the behavioral response to volatilic cues is continuously variant, as is evidenced by a normal distribution of the trait value in natural populations (Alcorta and Rubio 1989; Bartoshuk and Beauchamp 1994; Mackay *et al.* 1996; Maeda *et al.* 2001). Hence, olfactory behavior is determined by the expression of multiple genetic factors, their interactions with one another, as well as with the environment, be it genetic, sexual or “external” (with respect to the organism). Although ongoing research in olfaction continues to substantially contribute to our knowledge of its anatomical, physiological and biochemical aspects (see Rawson and

Gomez 2002; Prasad and Reed 1999; Vosshall 2000; Mombaerts, 2001), there remains a gap in our understanding of the genetic networks that drive the expression of this complex quantitative trait.

(a) Neuroanatomical layout of the olfactory system in animals

Comparative neuroanatomists have long since noted the structural and functional similarities between sensory systems across phyla, with the olfactory system in vertebrates and insects being one of the best examples (Figure 2; Delthier 1990; Hildebrand 1995).

Peripheral organs such as the nasal epithelium in vertebrates and antennal segments in insects are committed to the reception, collection and transmission of olfactory signals from the environment to central processing units. In the vertebrates, the nasal olfactory epithelium contains over a million bipolar olfactory receptor neurons (ORNs) that extend their knobular, ciliated dendrites into the peri-epithelial space, where they are bathed in mucus (reviewed by Anholt, 1993). Single-cell RT-PCR (reverse transcriptase-polymerase chain reaction) experiments in vertebrates reveal that each ORN expresses a single OR out of a total of 1000 OR genes (Chess *et al.* 1994; Malnic *et al.* 1999). ORNs expressing the same OR are widely dispersed within spatially restricted zones on the olfactory neuroepithelium (Ressler *et al.* 1994b; Qasba and Reed 1998; Vassar *et al.* 1994). The axons of ORNs form a nerve bundle called the olfactory nerve (ON) that travels through the cribiform plate at the roof of the olfactory organ to the olfactory bulb (OB). Here, the ORNs segregate such that ORNs expressing the same OR converge upon one or few of about 1800 structurally and functionally distinct spherical neuropil called glomeruli (Mombaerts *et* *al.* 1996; Mori *et al.* 1999; Ressler *et al.* 1994a; Treloar *et al.* 1996; Vassar *et al.* 1994). In the vertebrate OB, each glomerulus contains arborizations from (1) afferent ORNs expressing the same OR, (2) efferent projection neurons, such as mitral and tufted cells, and (3) peri-glomerular cells that establish communication with neighboring glomeruli. The olfactory bulb also contains granule cells that form sub-glomerular connections between dendrites of mitral cells (Boeckh and Tolbert 1993; Christensen *et al.* 1993; Kanzaki *et al.* 1989; Pinching and Powell 1971a; Pinching and Powell 1971b; Shepherd and Greer 1998; Treloar *et al.* 1996). Mitral cells and tufted cells are second order neurons that extend from the glomerulus to send their axons to distributed regions of the vertebrate brain. In particular, mitral cells send extensive arborizations to the piriform and entorhinal cortices (Ketchum and Haberly 1993; Shipley and Ennis 1996; Zou *et al.* 2001), which in turn sends projections to the frontal cortex (Broad *et al.* 2002; Datiche and Cattarelli 1996) and hypothalamus (Price *et al.* 1991).

The olfactory system in insects is remarkably similar in neuroanatomical organization to the vertebrate model, yet quantitatively simpler. In *Drosophila melanogaster*, there are about 1200 ORNs in the main olfactory organ, the third antennal segment, and about 120 ORNs in the maxillary palps. Each ORN is thought to express one or few of the 59 putative OR genes that have been identified by screening the sequenced *Drosophila* genome (Vosshall *et al.*1999; Clyne *et al.*1999; Vosshall 2001; Vosshall 2000). Dendrites of insect ORNs extend into cuticular projections called sensilla that decorate the surface of the peripheral olfactory organs. Each sensillum (of which there are three distinct types: trichoid, basiconic and coeloconic sensilla) houses 1-4 ORNs in the antennae and 2 ORNs in the maxillary palps that are bathed in sensillar lymph. Interestingly, the distribution of olfactory sensilla on the antennae of *Drosophila* display sex-specific differences: males possess 15-

20% more trichoid sensilla than females, while females have 12-24% more basiconic sensilla than males (Shanbhag *et al.* 1995; Shanbhag *et al.* 1999; Stocker and Gendre 1988; Stocker 2001; Stocker 1994). The functional significance of the sexually dimorphic distribution of sensilla remains unexplored. Electrophysiological recordings from single ORNs contained within the same sensillum when exposed to a panel of odorants, reveal 16 functional ORN classes in the antennae and 6 in the maxillary palps, with ORNs present in the same sensillum often belonging to different classes. However, the distribution of ORNs within sensilla in antennae and maxillary palps are not random; instead observing a strict pair rule, such that pairs of neurons responding to particular odorant spectra always occur together in the same sensillum, forming 7 functional sensillar classes in the antennae and 3 in the maxillary palps (de Bruyne *et al.* 2001; de Bruyne *et al.* 1999). Axons of chemosensory neurons in the antenna are collected as two bundles that converge at the base of the organ to form the antennal nerve that travels from the periphery to the central nervous system (CNS), where they segregate to arborize in glomerular structures in the antennal lobe (AL; analogous to the vertebrate OB; Stocker 1994; Stocker and Siddiqui 1983). Each glomerular structure (43 have been identified in *Drosophila*; Laissue *et al.* 1999) receives arborizations from (1) afferent ORNs, (2) projection neurons (PNs) that are equivalent to vertebrate mitral cells, (3) local interneurons (comparable for periglomerular cells) and (4) local neurons that provide connections between PNs (Boeckh and Tolbert 1993; Christensen *et al.* 1993). PNs have been shown to extend their axons to punctate nuclei in the superior lateral protocerebrum (the insect forebrain), the lateral horn and mushroom bodies (Homberg *et al.* 1988; Wong *et al.* 2002; Marin *et al.* 2002; Laurent *et al.* 1998). Hence the olfactory system in dipterans is remarkably similar in neuroanatomical and functional organization to the vertebrate system, yet is quantitatively vastly less complex than the latter.

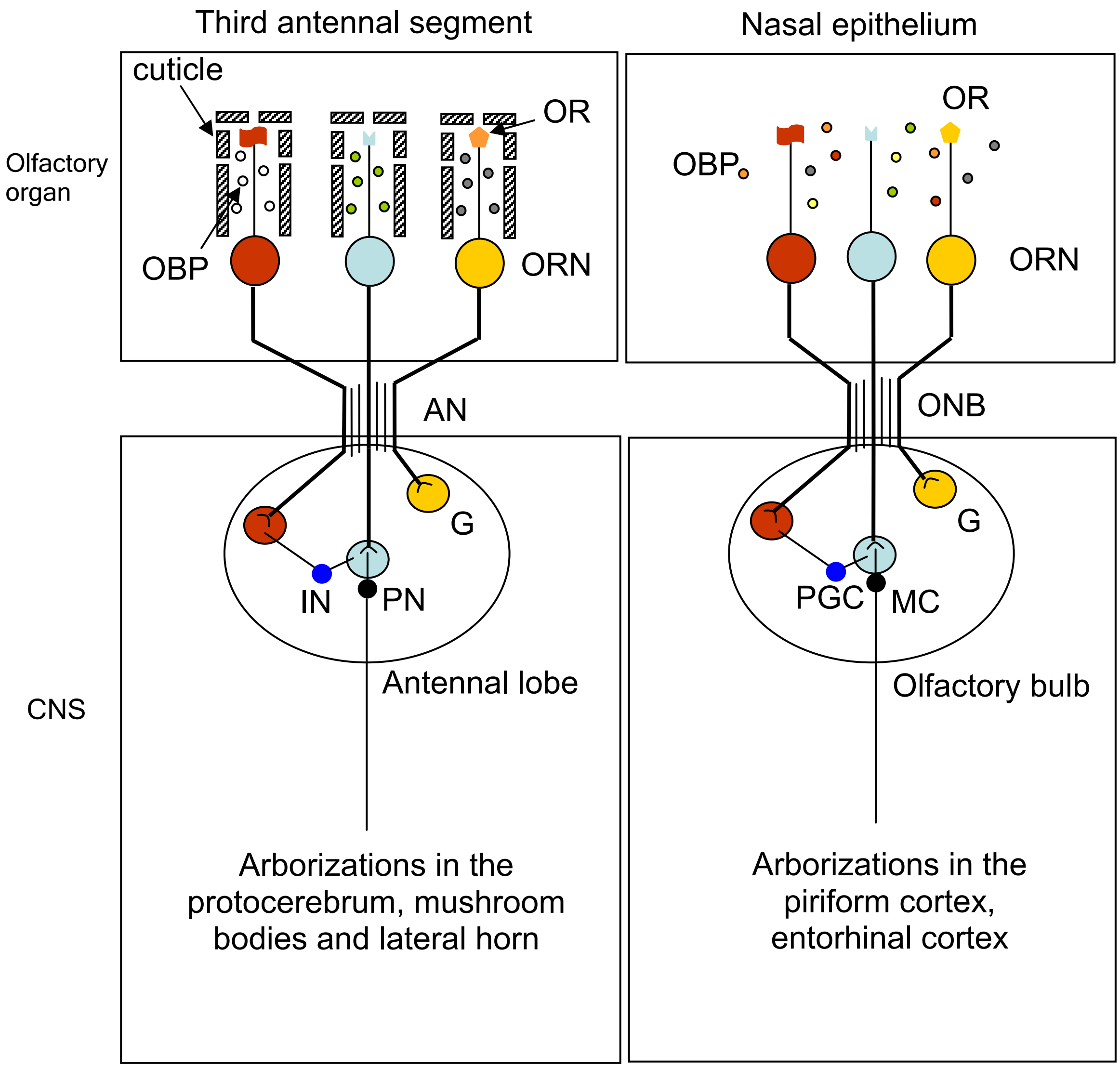
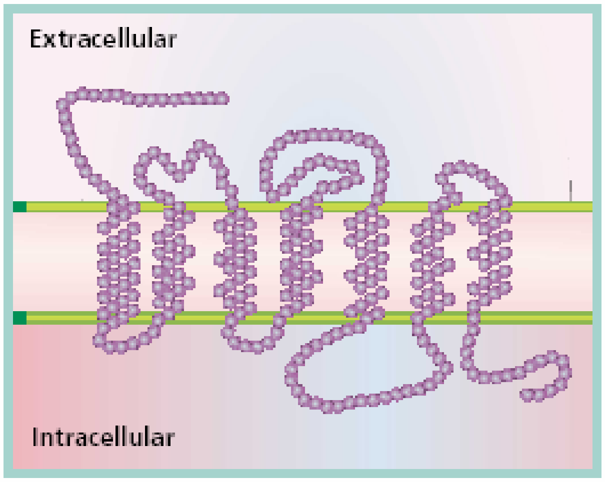


Figure 2: Schematic representation of the parallels in the neuroanatomical layout of insect and vertebrate olfactory systems. In insects (left panel), each olfactory sensory neuron (ORN) is encapsulated by cuticular projections on the third antennal segment, called olfactory sensilla. Each sensillum is filled with sensillar lymph that predominantly contains a single kind of odorant binding protein (OBP). ORNs expressing a single type of odorant receptor (OR) converge upon a single glomerulus (G) in the antennal lobe (AL). Projection neurons (PN) that synapse with the ORNs transmit the olfactory signal in a stereotypical manner to specific higher order regions of the central nervous system (CNS). In vertebrates, the nasal epithelium secretes mucus containing a diverse population of OBPs that surrounds the OR bearing cilia of vertebrate ORNs. ORNs bearing a single type of OR converge upon one of few stereotypically located glomeruli in the olfactory bulb. Second order neurons, the mitral cells (MC), synapse with afferent ORNs in the olfactory bulb and transmit the olfactory signal to the olfactory cortex in the brain. Interneurons (IN) in insects and peri-glomerular cells (PGC) in vertebrates may send arborizations to neighboring glomeruli.

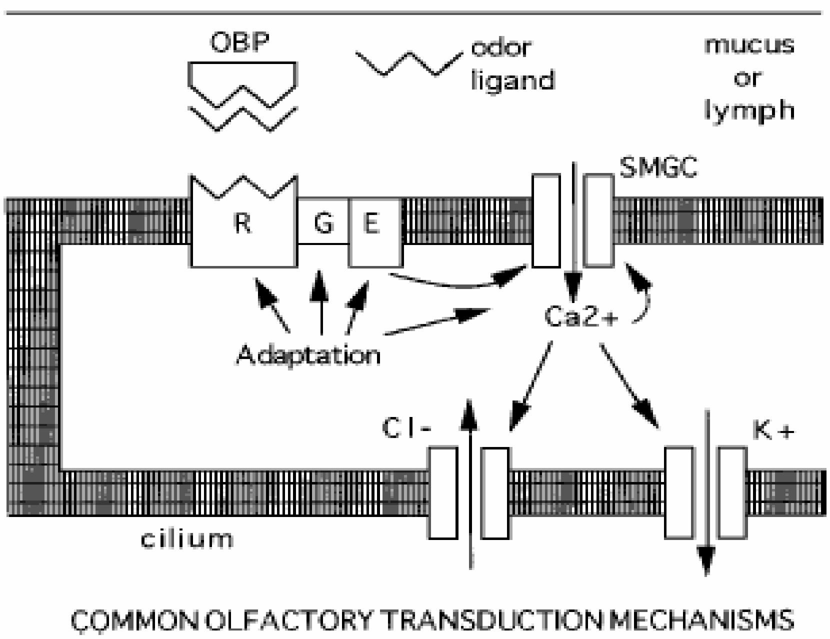
(b) Molecular basis of olfactory behavior

Most animals with differentiated olfactory systems are capable of detecting odoriferous compounds in homeopathic concentrations, to elicit context-dependant behavioral responses. This necessitates an underlying signal transduction pathway that amplifies the signal initiated by the binding of the odor ligand to chemosensory receptors. The search for ORs in animal genome was based on the information that ORNs exposed to odorants generated elevated levels of the second messenger, cyclic adenosine monophosphate (cAMP) by the activation of adenylyl cyclase (Boekhoff *et al.* 1990b; Breer *et al.* 1990; Pace *et al.* 1985; Sklar *et al.* 1986). Since adenylyl cyclase activation is dependant on guanidine triphosphate (GTP), ORs were likely to be GTP-binding protein coupled receptors (Figure 3). In 1991, Buck and Axel identified and cloned 18 G-protein coupled receptors containing 7 hydrophobic membrane-spanning domains from the rat and demonstrated that their expression was restricted to the olfactory epithelium. To date, ORs have been identified in 12 vertebrate species: the rat (Buck and Axel 1991; Drutel *et al.* 1995; Levy *et al.* 1991; Raming *et al.* 1993; Singer *et al.* 1998; Strotmann *et al.* 1994; Thomas *et al.* 1996), mouse (Nef *et al.* 1992; Ressler *et al.* 1993; Sullivan *et al.* 1996; Asai *et al.* 1996; Kubick *et al.* 1997; Mombaerts *et al.* 1996; Qasba and Reed 1998); human (Parmentier *et al.* 1992; Vanderhaeghen *et al.* 1997); catfish (Ngai *et al.* 1993); zebrafish (Barth *et al.* 1997; Byrd *et al.* 1996; Weth *et al.* 1996); dog (Issel-Tarver and Rine 1996; Issel-Tarver and Rine 1997; Parmentier *et al.* 1992); pig (Matarazzo *et al.* 1998); opossum (Kubick *et al.* 1997); mudpuppy (Zhou *et al.* 1997) and lamprey (Berghard and Dryer 1998). In addition, they have also been identified in invertebrate models, *C. elegans* (Bargmann and Kaplan 1998) and in *D. melanogaster* (Clyne *et al.* 1999). Genome comparison of the ORs identified reveal that they are members of very large families of highly divergent genes.

(A)



(B)



(Hildebrand and Shepherd, 1997)

Figure 3 : Schematic representation of, (A) a 7-transmembrane olfactory receptor; and, (B) the main sensory transduction mechanism identified in vertebrates and insects. Olfactory binding proteins (OBP) in the lymph may assist in odorant binding to the 7-transmembrane G-protein coupled olfactory receptor ®. G-proteins (G) associated with the ligand-boundreceptor activates downstream effector molecules such as, adenylyl cyclase or phospholipase C. The subsequent increase in second messengers (such as cAMP), opens second messenger gated channels (SMGC). This causes an influx of calcium ions that lead to the opening of calcium gated chloride and potassium channels, contributing to membrane depolarization.

In vertebrates, an odorant binding to its receptor activates adenylate cyclase type III (Anholt 1993; Pace *et al.* 1985; Sklar *et al.* 1986) by a G_s-like alpha-subunit, G_{olf} (Jones and Reed 1989). This leads to increased levels of cAMP, which in turn, triggers the opening of cyclic nucleotide non-selective cation channels (CNCs) causing a generator current (Firestein *et al.* 1991; Nakamura 2000; Nakamura and Gold 1987). Calcium entering through the CNCs (Frings 1997; Frings *et al.* 1992) is able to amplify the response by opening calcium gated chloride channels (Kleene and Gesteland 1991; Kurahashi and Yau 1993). It is also capable of inactivating responses by binding calmodulin kinase II to phosphorylate CNCs thereby lowering the affinity for the second messenger, cAMP (Chen and Yau 1994; Kurahashi and Menini 1997; Wei *et al.* 1998). Knockout mice deficient in functional sub units of CNCs are generally anosmic (Brunet *et al.* 1996), as are mice deficient in G_{olf} (Belluscio *et al.* 1998). Thus, cAMP appears to be the principal second messenger in vertebrate olfactory transduction. In addition to the cAMP pathway, the inositol-1,4,5-triphosphate (IP_3) pathway has also been implicated in olfactory transduction (Boekhoff *et al.* 1990a; Boekhoff *et al.* 1990b; Huque and Bruch 1986; Ronnett and Moon 2002). Functional analyses using calcium imaging demonstrate that a calcium transient induced by a known odorant, lilial, is completely blocked by a specific inhibitor of phospholipase C (Noe and Breer 1998). Finally, olfactory transduction in vertebrates may also involve guanylyl cyclases, which appear to be expressed along with cyclic guanidine monophosphate (GMP)-stimulated phosphodiesterase, in a subset of ORNs that converge upon glomeruli restricted to a specific region of the olfactory bulb, the “necklace” glomeruli (Juilfs *et al.* 1997; Juilfs *et al.* 1999).

The nematode, *C elegans* has been successfully used as an invertebrate model system for studies on olfaction (recently reviewed by Mori 1999). Behavior-genetic analysis of chemotactic behavior in the worm identified a 7-transmembrane, G-protein coupled receptor, encoded by *odr-10*, which was specific for the detection of diacetyl (Sengupta *et al.* 1996). Expression of Odr-10 in mammalian cultured cells results in diacetyl mediated activation (Zhang *et al.* 1997), making it the first OR to be functionally linked to an odorant ligand. *C elegans* differs from the mammalian system in that a single chemosensory neuron expresses many different odorant receptors (Troemel *et al.* 1995). In fact, introduction of the *odr-10* gene in *odr-10* mutants can elicit either attractant or repellant responses to diacetyl, depending on the chemosensory cell in which it is expressed (Troemel *et al.* 1997). This may suggest the possibility of multiple olfactory signal transduction pathways present in the worm. Genome-sequencing projects have identified a large number of G-alpha, G-beta and G-gamma subunits of heterotrimeric GDP-binding proteins (G proteins) involved in the G-protein coupled receptor activation of downstream targets such as adenylyl cyclase, cGMP-phosphodiesterase, and phospholipase C (Bargmann 1998; Jansen *et al.* 1999). Both *odr-3* and *gpa-2* encode G-alpha subunits of G-proteins that are expressed in the chemosensory neurons. Unlike *odr-3*, the *gpa-2* mutation does not cause olfactory deficits. However, the *odr-3/gpa-2* double mutant shows reduced sensitivity to butanone (Roayaie *et al.* 1998). It is of interest that two chemotaxis mutants, *tax-2* and *tax-4*, encode homologues of beta and alpha subunits of the vertebrate olfactory CNCs, suggesting similarities between olfactory transduction pathways in *C. elegans* and vertebrates (Coburn and Bargmann 1996; Komatsu *et al.* 1996). Defects in the *tax-2* or *tax-4* genes only impair olfactory function mediated by one of the two nematode chemosensory neurons, the AWC neuron, but not the AWA neuron, which expresses the Odr-10 receptor protein (Bargmann *et al.* 1993; Sengupta *et al.* 1996). These observations, taken together with the discovery of chemosensory mutant, *osm-9* (Colbert *et al.* 1997), which encodes a cation channel homologous to the capsaicin receptor (Caterina *et al.* 1997) and *Drosophila* TRP channels (involved in phototransduction; Colbert *et al.* 1997; Tsunoda *et al.* 1997) point to alternative olfactory signal transduction pathways in the worm. The most likely scenario is that *at least* two distinct signaling pathways are used in *C. elegans* sensory systems: one involving the novel Odr-3 G-alpha subunit (related to G_i/G_o) and the Tax-2 and Tax-4 channels; and the other involving Odr-3 and the Osm-9 cation channel (Colbert *et al.* 1997; Roayaie *et al.* 1998).

In contrast to nematodes, the olfactory system in *Drosophila* more closely resembles the vertebrate model with respect to overall organization. However, the molecular basis of olfactory transduction in insects is not as clear. Bioinformatic analyses of the completed genomic sequence of *Drosophila* (Adams *et al.* 2000; Rubin 2000) identified a large family of putative 7-transmembrane G-protein coupled *Drosophila* olfactory receptors (DORs), encoded by genes dispersed throughout the genome (Clyne *et al.* 1999; Gao and Chess 1999; Vosshall *et al.* 1999). Sequence analyses indicate that these receptors are highly divergent, with an average amino-acid identity of approximately 20%, and may be grouped into sub-families that share greater sequence identity. DORs are expressed in discrete subsets of ORNs in the antennae and maxiallry palps (Clyne *et al.* 1999; Vosshall *et al.* 1999), but not in the larval olfactory organ (the dorsal organ), or chemosensory neurons present in the proboscis, leg or wing (Vosshall 2000). This suggests that DORs are committed to the reception of volatiles only in the main adult olfactory organs. A total of 59 putative DORs have been identified (Adams *et al.* 2000; Rubin 2000), which is a significantly smaller number than the 1000 putative G-protein coupled ORs identified in the mouse (Zhang and Firestein 2002) and the 500 ORs in worms (Bargmann 1998; Troemel 1999). The high degree of sequence divergence between DORs and their smaller number may indicate that there is less functional redundancy in the fly olfactory system than the mammalian olfactory system. Recently, two landmark studies demonstrated ligand specificity for a single DOR (Stortkuhl and Kettler 2001; Wetzel *et al.* 2001), giving credence to the notion that the 7-transmembrane G-protein coupled receptors identified in the *Drosophila* genome by bioinformatics analyses are indeed functional chemosensory receptors. Stortkuhl and Kettler (2001) over-expressed the DOR gene, *Or43a*, in the fruit fly antennae and recorded increased electrophysiological responses to benzaldehyde, cyclohexanone, cyclohexanol and benzylalcohol. Wetzel *et al.* (2001) provided further strength to these results by expressing *Or43a* in a heterologous system, *Xenopus* oocytes, and demonstrating a concomitantly specific electrophysiological response to this same panel of odorants at very high concentrations.

ORNs that express DORs extend their dendrites into the lumen of olfactory sensilla that are present on the olfactory organs. Here, the ORNs are bathed in sensillar lymph that contains a very large number of small molecules called odorant binding proteins (OBPs). Since different OBP populations occur within each sensillum, each *Drosophila* ORN is surrounded by its own specific microenvironment with respect to these molecules. Currently 51 OBPs have been identified in *Drosophila* (Hekmat-Scafe *et al.* 2002) and it is speculated that their primary function may involve either the solubilization of non-polar volatiles that must traverse through the aqueous medium of the sensillar lymph to reach the ORs, or the termination of an odor-activated response by removing ligands from the ORs and the lymph. The only evidence for the role of OBPs in olfactory discrimination comes from the characterization of the *lush* mutant (Kim *et al.* 1998; Kim and Smith 2001). The *lush* gene encodes an OBP, and when mutated causes abnormally high tolerance in flies for concentrated ethanol, propanol and butanol (repulsive stimuli to wild-type flies). However, in light of the recent functional study of DOR, Or43a, it appears that OBPs are not absolutely required to mediate ligand-receptor activation. The results of Wetzel *et al* (2001) indicate that Or43a expression in *Xenopus* oocytes is capable of responding to odorants with high specificity and sensitivity, in the absence of OBPs.

The identification of a cyclic-nucleotide gated channel (Baumann *et al.* 1994) and a cyclic nucleotide potassium channel (Dubin *et al.* 1998) in the *Drosophila* antenna suggest that cAMP may play a role in insect olfactory transduction. Recent studies provide support for this notion by demonstrating that mutations in *dunce*, which encodes for cyclic adenosine monophosphate diesterase, and *rutabaga*, which encodes an adenylyl cyclase, disrupt olfactory responses in *Drosophila* (Devaud *et al.* 2001; Martin *et al.* 2001). On the other hand, mutations in *rdgB* (*retinal degeneration B*), which encodes a phosphatidyl inositol transfer protein (Vihtelic *et al.* 1993); *norp A* (*no receptor potential A*), which encodes a phospholipase C (PLC); and $dG_q\alpha$, which encodes a G protein subunit known to activate PLC, impair both vision (Ranganathan *et al.* 1995) and olfaction (Riesgo-Escovar *et al.* 1995; Woodard *et al.* 1992) in *Drosophila*. Furthermore, it has recently been shown that the inositol-triphosphate (IP_3) receptor is required for olfactory adaptation (Deshpande *et al.* 2000), suggesting that the IP_3 pathway is also involved in *Drosophila* olfactory transduction (Stortkuhl *et al.* 1999).

(c) Sub-cellular scaffolds for signal integration and propagation

Signal transduction is a dynamic process, requiring the interlinking of biochemical reactions mediated by physical interactions between cellular proteins (Kholodenko *et al.* 2000). Because many intracellular signaling cascades share common downstream targets, specificity of a signal transduction pathway that underlies sensory activation entails the organization and spatial distribution of multimeric signaling complexes of interacting proteins that form a *transducisome* in the cell (Chevesich *et al.* 1997; Huber *et al.* 1996; Shieh and Zhu 1996; Tsunoda *et al.* 1997). In situations where multiple pathways exist in the same cell,*molecular linkers* may also be required for either mediating or preventing cross-talk between two parallel pathways. In the olfactory system, there is evidence for the existence of parallel signal transduction pathways in the same ORN (Dionne and Dubin 1994; Spehr *et al.* 2002). Considering the high sensitivity of olfactory signaling in animals, it is possible that scaffolding molecules organize sub-molecular assemblies in the chemosensory neuron (Paysan and Breer 2001).

PDZ domain proteins

There is an emerging idea that PDZ (*Psd-95; Dlg; Zo-1*) domain containing proteins may be responsible for the wiring of complex biochemical circuits that govern cellular responses to external stimuli (Harris and Lim 2001). The term PDZ is derived from the three proteins in which these domains were first identified: Post-synaptic density-95, a protein involved in neuronal post-synaptic signaling; Discs large, a *Drosophila* tumor suppressor, and Zona occludens-1, which is responsible for maintaining epithelial polarity. PDZ proteins are widely distributed among metazoans; 0.2-0.5% of the open reading frames in the worm, fly and human represent genes that encode PDZ proteins (Schultz *et al.* 2000; Schultz *et al.* 1998a; Schultz *et al.* 1998b). These domains mediate protein-protein interactions in the cell by binding to short carboxy-terminal sequence motifs, typically 5 amino acid residues in length (Niethammer et al. 1998; Sheng and Sala, 2001). PDZ domains in proteins are divided into 3 classes based on the sequence motifs to which they bind. Those that bind to consensus amino acid sequence, X-S/T-X-L/V, belong to Class I; those that bind to, X-ϕ -X- ϕ, belong to

Class II; and those that bind, X-D-X-V, belong to Class III, where X stands for any amino acid and ϕ is a hydrophobic residue (Sheng and Sala 2001). Some identified PDZ domain proteins and the molecules they interact with are listed in Table 1.

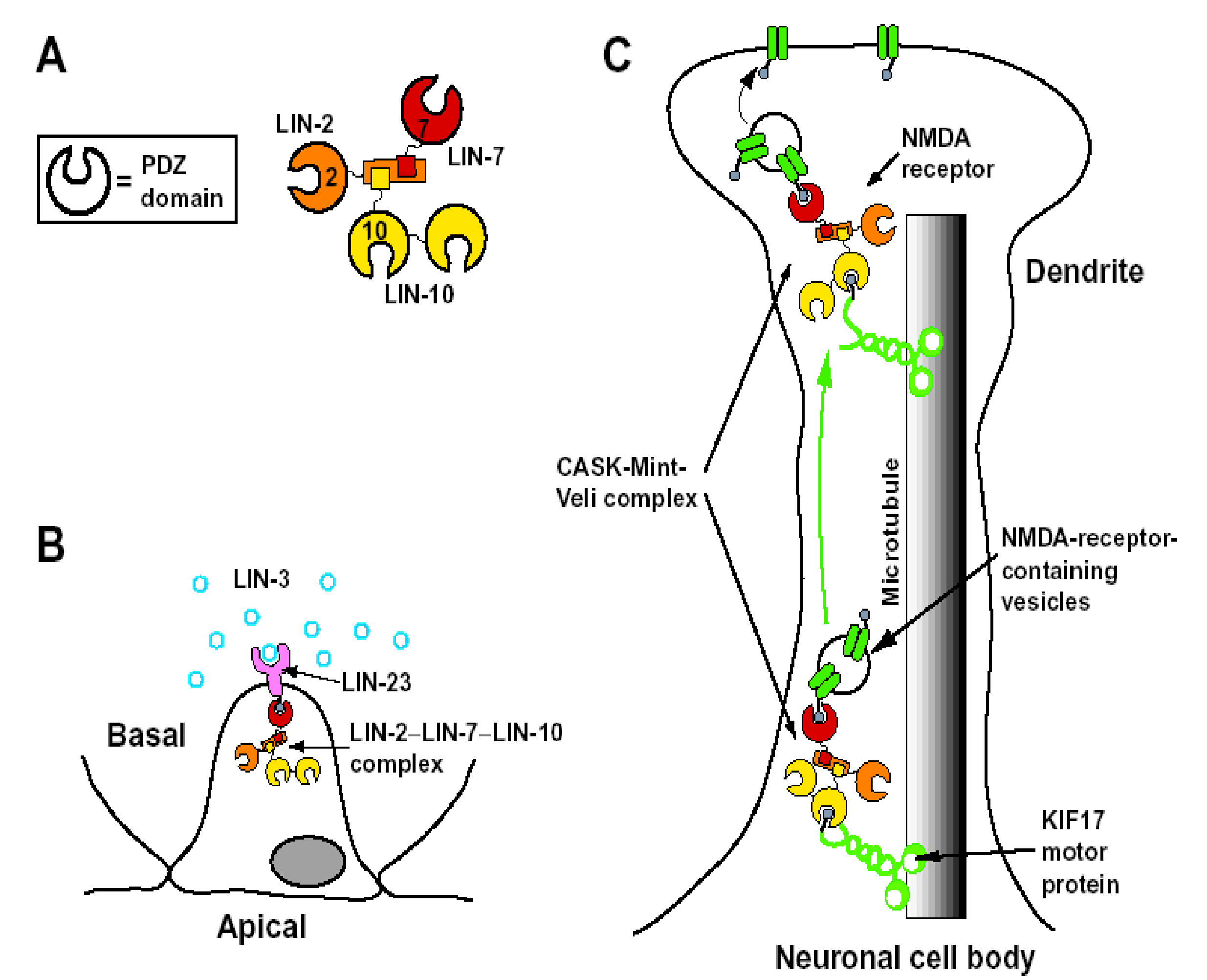
Class	C-term sequence	Interacting protein	PDZ domain	References
Class I				
X-S/T-X-V	E-S-D-V	NMDAR2A, B	PSD-95 (PDZ2)	Kornau et al 1995
	E-T-D-V	Shaker channel		Kim et al 1995
	Q-S-S-V	Citron	PSD-95 (PDZ3)	Zhang et al 1999
	Q-T-S-V	CRIPT		Niethammer et al 1998
	T-T-R-V	Neuroligin		Irie et al 1997
	E-T-S-V	PMCA4b	PSD-95 (PDZ1/2/3)	E Kim et al 1998
	E-S-L-V	Voltage-gated sodium channel	Syntrophin	Gee et al 1998
X-S/T-X-L	Q-T-R-L	GKAP	Shank	Naisbitt et al 1999
	S-S-T-L	mGluR5		Tu et al 1999
	D-S-S-L	β2-adrenergic receptor	NHERF (PDZ1)	Hall et al 1998
	P-T-R-L	GRK6A		Hall et al 1999
	D-T-R-L	CFTR		Wang et al 1998
Class II				
X-ϕ-X-ϕ	E-Y-Y-V	Neurexin	CASK	Hata et al 1996
	E-F-Y-A	Syndecan	CASK, syntenin	Hsueh et al 1998
	E-Y-F-I	Glycophorin C	p55	Grootjans et al 1997; Marfatia et al 1997
	S-V-K-I	GluR2	GRIP (PDZ5), PICK-1	Dong et al 1997 Xia et al 1998
	S-V-E-V	EphB2	GRIP (PDZ6), PICK-1	Torres et al 1998
	G-I-Q-V	EphA7	GRIP (PDZ6), PICK-1 syntenin	
	Y-Y-K-V	EphrinB1	GRIP (PDZ6), PICK-1 Syntenin	
Class III				
X-D-X-V	V-D-S-V	Melatonin receptor	nNOS	Stricker et al 1997

(Sheng and Sala, 2001)

Table 1: Classification of a few PDZ domain proteins and their binding partners

Clues about the functional significance of PDZ domain proteins in the organization of signaling cascades have been derived from the *Drosophila* visual system, the *C. elegans* Ras signaling developmental pathway and mammalian synaptogenesis. The *Drosophila* visual system responds to single photons with unitary membrane potentials that are generated by the organization of signaling components of the IP_3 cascade by a PDZ domain protein, InaD (Shieh and Zhu 1996; Tsunoda *et al.* 2001; Tsunoda and Zuker 1999). InaD contains 5 PDZ domains that can each interact in a modular fashion with PLC (PDZ domains 1 and 5); protein kinase C (PDZ domains 2 and 4) and the transient receptor potential (trp) channel (PDZ domain 3). These interactions bring individual components within close proximity of one another, thus increasing signaling efficiency in the visual system. The Ras pathway in *C.elegans* is involved in epithelial/vulval development and is similarly organized by the PDZ domain proteins Lin-2, Lin-7 and Lin-10 (Figure 4A and B). Mutations in any of the three genes encoding these proteins results in the mislocalization of the epidermal growth factor (EGF) receptor, Let-3, which would normally be localized to the basolateral region of the cell, leading to subsequent loss of Ras signaling (Hoskins *et al.* 1996; Kaech *et al.* 1998; Simske *et al.* 1996). In addition to a role in epithelial development, Lin-10 has also been shown to target glutamate receptors to neuronal synapses (Rongo *et al.* 1998). Interestingly, mammalian homologues of *lin-2*, *lin-7* and *lin-10* (Cask, Mals and Mint, respectively) show expression patterns restricted to the presynaptic (axonal) and post-synaptic (dendritic) compartments of neurons (Misawa *et al.* 2001; Setou *et al.* 2000), 2000). These proteins are involved in the transport of *N*-methyl-*D*-aspartic acid (NMDA) receptors (NMDAR) by linking the carboxy-terminal tail of the NR2B sub unit of NMDAR (Jo *et al.*1999) to the kinesin-related motor protein, KIF17 (Setou *et al.* 2000). Hence the complex of 3 PDZ domain proteins serve to transport NMDAR containing vesicles along microtubules to appropriate sub-cellular locations within the neuron (Figure 4C). The NR2B sub unit of

NMDAR is also bound by, Psd-95, one of the first PDZ proteins identified. Psd-95 is localized to the post-synaptic density of neuronal dendrites and links NMDA receptors to a calcium/calmodulin activated enzyme, nNOS, which leads to the efficient generation of second messengers (nitric oxide) in response to calcium influxes linked to NMDA receptor opening (Brenman *et al.* 1996; Harris and Lim 2001).



(Harris and Lim, 2001)

Figure 4: Schematic representation of the Lin-2, Lin-7 and Lin-10 group of interacting PDZ proteins and their mode of function in polarized cells. A. Lin-2, Lin-7 and Lin-10 interact with each other in a manner that leaves their PDZ domains free to interact with other proteins. B. In polarized epithelial cells they are responsible for the proper localization of the EGF receptor, Lin-3 to the basolateral membrane. C. In polarized neuronal cells, their mammalian homologs bind the kinesin-related motor protein, KIF17, to transport vesicles containing NMDA receptor subunits, along microtubules to the synapse.

Epithelial and neuronal cells share a common structural and functional property in that they are both polarized. Neurons, for example, have topologically distinct pre- (axonal) and post-synaptic (dendritic) domains that are analogous to the apical and basal regions of the epithelial cell. Polarity determination mediated by the distribution of proteins in appropriate subcellular domains is critical for cellular functions such as signaling and development. Recently, a new class of PDZ proteins called LAP proteins have been identified in the worm (Legouis *et al.* 2000), rat (Apperson *et al.* 1996), fly (Bilder and Perrimon 2000), mouse and human (Borg *et al.* 2000; Nagase *et al.* 1995). They derive this name on account of an additional modular protein binding domain comprising 16 leucine-rich repeats (hence, LAP stands for Leucine-rich repeats And PDZ) located at their amino-termini. LAP proteins function as molecular scaffolds by organizing the distribution of polarity determinants within appropriate cellular domains for the maintenance of cellular polarity (Bilder 2001; Bilder and Perrimon 2000). They also play a role as tumor suppressors that tightly regulate cellular growth and proliferation (Bilder *et al.* 2000). LAP proteins may carry out their functions via a dynamic process of vesicular trafficking that transports molecules to their appropriate subcellular destinations. In fact, the human homologue of Discs large (Dlg; a *Drosophila* PDZ domain protein), hDlg, interacts with a kinesin-like motor protein, Gakin that transports hDlg and its associated partners to appropriate regions of the cytoplasm of resting T lymphocytes (Jo *et al.* 1999; Misawa *et al.* 2001; Setou *et al.* 2000). The discovery of Gakin provides a link between scaffolding proteins and their means of transporting their cargoes to specific cellular compartments. In *Drosophila*, Dlg physically (Mathew *et al.* 2002) and genetically (Bilder *et al.* 2000) interacts with the LAP protein, Scribble (Scrib), which in turn plays a role in mediating short term plasticity of the larval neuromuscular junction (Roche *et al.* 2002). An additional role for LAP proteins in the nervous system comes from the characterization of Densin-180 (Apperson *et al.* 1996), a post-synaptic density protein, found to be associated with glutamatergic neurons in the rat forebrain, a region involved in learning.

(d) Olfactory coding

One of the most intriguing mysteries of animal sentience involves the basis of sensory perception. In the case of olfaction, some of the central questions are, how does an animal respond to and discriminate between the near infinite number of odorants in their milieu? What is the biological and evolutionary significance of an animal's ability to respond in predictable manners to some odorants, and not others? How is the overwhelmingly diverse chemical environment represented in the brains of animals? Of what relevance is the biological context of an odorant signal? To address these questions, researchers in the field of olfaction have invested resources into cracking the neural code of olfaction. ORNs expressing a single type of OR converge upon one or few specific glomeruli in the OB of vertebrates (Mombaerts *et al.* 1996; Vassar *et al.* 1994) or the AL of insects (Vosshall *et al.* 1999). Mitral cells in vertebrates and projection neurons in insects receive pre-synaptic inputs from the afferent ORNs in a single glomerulus and send their axons to higher order brain structures where olfactory signals are processed and integrated. By studying activation patterns in the glomeruli in response to activating signals from the periphery (carried in by ORNs), insights into the neural coding of olfactory signals may be gleaned. Some of the latest discoveries using this approach are highlighted below.

First, human perception studies as well as receptor-ligand binding studies suggest that most odorants are coded by a combination of olfactory receptors. Conversely, a single odorant receptor may bind with varying specificity to many odorants (Malnic *et al.* 1999;

Polak 1973). Exposure to small, relatively simple molecules like methanol causes highly focal glomerular activation patterns, while the activation patterns for larger molecules such as hexanoic acid are more distributed over the olfactory bulb (Ho *et al.* 1995; Johnson and Leon 2000b). This suggests that olfactory receptors may each recognize/bind different molecular features of an odorant, giving rise to a combinatorial olfactory code both at the level of receptors as well as the glomeruli the ORNs innervate in the bulb (Bozza and Kauer 1998; Korsching 2002; Figure 5A, B). Next, odorant molecules of the same size elicit distinct spatially segregated patterns of glomerular activity that are determined by their functional groups (Fried *et al.* 2002; Johnson and Leon 2000b; Meister and Bonhoeffer 2001; Rubin and Katz 1999; Uchida *et al.* 2000). If, however the odor compounds vary in molecular length, but contain the same functional group, then molecular length gains precedence for discrimination, as reflected by distinct molecular-length-specific glomerular activation maps in the brain (Johnson and Leon 2000b; Meister and Bonhoeffer 2001). Third, spatially recognizable patterns of activity in glomerular sets may be used as a tool to predict whether an animal is able or unable to discriminate between odorants (Figure 5C, D). Hence, enantiomers of carvone evoke statistically different glomerular activation patterns and are readily distinguished in cross-habitation behavioral paradigms. On the other hand, rats are unable to spontaneously discriminate between the enantiomers of limonene, both of which activate the same pattern of glomerular activity in the brain (Linster *et al.* 2001).

The ability to discriminate between olfactory noise and relevant signal is of particular importance for animals, since they are usually exposed to odorant bouquets and composites in their natural habitat. As the concentration of a particular odorant increases relative to volatilic contaminants in a mixture, its spatial map in the OB or AL increases concomitantly (Guthrie and Gall 1995; Rubin and Katz 1999). Since homologous olfactory receptor neurons with closely related olfactory OR converge upon neighboring glomeruli, (Conzelmann *et al.* 2001; Tsuboi *et al.* 1999), it is conceivable that an increase in concentration of a particular odorant may recruit other ORN types that bear receptors with lower binding specificities to send activating signals to the brain (Guthrie and Gall 1995; Stewart *et al.* 1979). In some cases, the increase in concentration of a particular odorant may recruit new foci of glomerular activation that may cause variation in the perception of that odorant at different concentrations (Figure 5E). For example, in biophysical studies carried out in humans, pentanal is one of a number of compounds that are perceptually different depending on the relative concentration of the odor; at low concentrations, pentanal is a pleasant odor, but quickly turns unpleasant as its concentration increases (Arctander 2000; Dravnieks 1985). In animal models it has been demonstrated that aldehyde response can be visualized in the dorsal aspect of the OB only when presented to animals at high concentrations (Johnson and Leon 2000a; Uchida *et al.* 2000).

Cleland *et al.* (2002) computed the statistical correlation between the neural representation of odorants and behaviors elicited in the animal, and found it to be very high (0.93). However, it is interesting to note that the potential difference in glomerular activation patterns in the brain may not always translate to predictable discriminatory behavior in animals. Differences in glomerular activation patterns in response to two odorants may be behaviorally ignored by rats when tested for spontaneous discrimination between them. However, if the same test is coupled with differential reinforcement, such as a sweet food reward, rats learn to use the differences in neural activation to discriminate between the same pair of odorants (Linster *et al.* 2002). Furthermore, reinforced learning in young animals lead to alterations in glomerular activity patterns by enhancing it (Johnson *et al.* 1995; Sullivan and Leon 1986). Plasticity in the neuroanatomical structure of glomeruli as well as learning induced changes in glomerular activation maps in insect brains, have also been documented (Devaud *et al.* 2001; Faber *et al.* 1999).

These observations hint at the adaptive value of olfactory perception in animals and the biological significance of an odor. Hence, extensive reproducible glomerular patterns of activation are evoked in the OB of rats exposed to conspecific pheromones such as pentanoic acid and 2-methylbutyric acid; while exposure to aliphatic acids that are never encountered in nature show dispersed activation patterns with discontinuous patterns (Johnson and Leon, 1999).

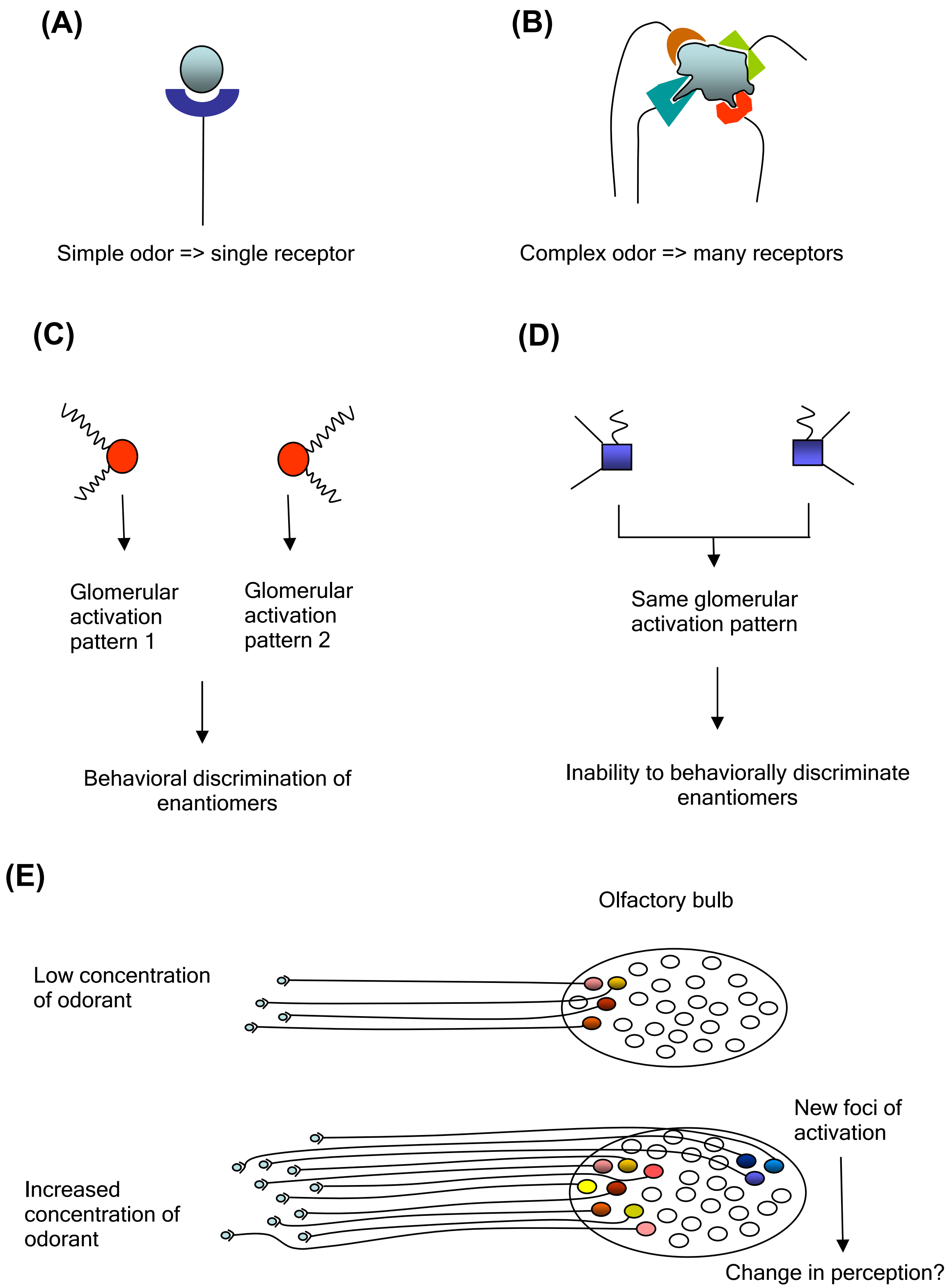


Figure 5: Olfactory coding (legend overleaf)

Figure 5: **(A)** Simple odorants such as methanol are thought to be coded by a single receptor, since they show restricted glomerular activation patterns, often with single foci of activation. (**B**) Complex molecules are coded by multiple ORs that show binding specificities to specific molecular moieties. **(C)** Enantiomers of carvone evoke different glomerular activation patterns and elicit olfactory discriminatory behavior in rats. **(D)** Enantiomers of limonene display similar glomerular activation patterns are not readily discriminated in behavioral assays. **(E)** With an increase in the concentration of odorant presented, the glomerular activation map is extended and perception is enhanced. In some cases new foci may be recruited that may in turn change sensory perception of the odorant from attractive to repulsive sensations.

1.4 *Drosophila melanogaster*, A MODEL ORGANISM

Am not I
A fly like thee
Or are not thou
A man like me?

- William Blake, "The Fly"

Drosophila melanogaster has sustained a long standing love affair with geneticists since the day in the fall of 1905, when it strayed into a fly-trap set up at the laboratory of T. H. Morgan at Columbia University. Pioneering work from T.H. Morgan's laboratory revealed the obvious advantages to using a model system that (1) had a short generation time of 10-14 days; (2) possessed a small genome comprising 4 chromosomes that were mutable in the presence of artificial agents; (3) displayed easily observable non-lethal phenotypes arising from single gene mutations (such as, *white*, which was the first spontaneous mutant observed in Morgan's laboratory); (4) could be used to generate balancer chromosomes with inversions bearing observable genetic markers, which allowed for the stable maintenance of lethal mutations as heterozygotes (Muller 1918) and, (5) was economical to rear in large numbers.

Since Muller's first genetic screens in *Drosophila melanogaster* conducted in the laboratory of T. H. Morgan, increased understanding of fruit fly biology as well as the sequencing and annotation of the *Drosophila* genome (Adams *et al.* 2000; Rubin 2000) has led to its popularization as a model organism for developmental, ecological, biochemical, evolutionary and behavioral research. The fruit fly genome has been completely sequenced and annotated and reveals the presence of over 61% cognates with mammalian genomes, spawning investigations that use *Drosophila* as a model for a range of human conditions such as Alzheimer's and Parkinson's diseases (Bier and Reiter 2002; Muqit and Feany 2002; Reiter *et al.* 2001). Moreover, highly inbred, isogenic *Drosophila* strains can be easily generated owing to their short generation time and relative robustness under homozygous conditions. This enhances the resolution with which single gene effects may be detected by reducing the genetic variance due to multiple segregating loci in a heterozygous genetic background. Furthermore, the availability of balancer chromosomes in *Drosophila* allow for the propagation of null mutations and large deficiencies (covering almost 70% of the Drosophila genome) that are routinely used in functional analyses and complementation mapping.

It is interesting to note that over half a century before Benzer and Konopka isolated the clock mutant, *period*, and revolutionized the field of behavior genetics (Konopka and Benzer 1971), Morgan's student; Fernandus Payne had published the first study on *Drosophila* as a model for the inheritance of a behavioral trait, phototaxis (Payne and Morgan 1910). In recent times, standardization of behavioral patterns coupled with sophisticated molecular and quantitative genetic tools for mutagenesis and analysis has thrust *Drosophila* to the forefront in behavior genetic research. The fruit fly has emerged from its humble origins as a cheap alternative to studying the basis of inheritance in animals (Weiner 1999), to a premiere model organism that elucidates the genetic basis of behaviors such as courtship (Hall 1978), phototaxis (Markow and Merriam 1977), geotaxis (Toma *et al.* 2002), olfaction (Rodrigues and Sidiqi 1978), gustation (Balakireva *et al.* 2000), learning and memory (Tully *et al.* 1994), foraging (de Belle *et al.* 1989), aggression (Chen *et al.* 2002), alcoholism (Moore *et al.* 1998) and more recently sleep (Hendricks *et al.* 2000; Shaw *et al.* 2000) and consciousness (Gamo 2002).

(a) Quantifying odor-guided behavior in *Drosophila*

The response to odorants may be divided into two qualitatively distinct behavioral phenotypes displayed by animals: attraction or repulsion. In *Drosophila*, attractive olfactory responses in adults may be quantified using the olfactory trap assay (Woodard, 1989) which involves recording the number of individuals that will localize into an "olfactory trap" (a 1.5 ml microcentrifuge tube) in response to an attractive odorant within it. This assay was developed by Carlson and colleagues and led to the identification of a number of olfactory trap abnormal (*ota*) mutants, of which *rdgB* is an example (Woodard, 1992). On the other hand, the Y- (Ayyub, 1990; Alcorta, 1991) and T- maze (Helfand, 1989) assays may be employed to test attractive, repulsive or discriminative behavior in populations of fruit flies. Two odorant stimuli, or one coupled with a control are presented at the distal arms of the Y- or T-maze, to which, a population of flies beginning at the stem of the apparatus, in the case of the Y-maze and the junction of the arms in the T-maze, respond. Subsequently, at the end of one replicate, the number of individuals present in either arm are retrieved and counted. Using this behavioral paradigm, some of the first olfactory mutants were identified; for example, *smell-blind / olfD / paralytic*; *swisscheese / olfE* and *olfC / myospheroid* (Ayyub, 2000).

It is rather an interesting phenomenon that flies of the Canton-S wild-type strain stereotypically "jump" in response to an increase in the concentration of the odorant presented to them. Hence, flies climbing the walls of the test chamber jump to the bottom when presented with an odor puff (Carlson, 1996). Flies with normal olfactory function may be separated from those with olfactory defects, called abnormal chemosensory jump (*acj*) mutants that remain undisturbed along the walls of the chamber. This unusual jump response is primarily mediated through the antennae and thus may be considered an olfactory mediated response. One of the mutants isolated using this behavioral paradigm, is the *acj6* mutant, which encodes a POU domain transcription factor, critical for regulating the expression of ORs in antennal ORNs (Ayer and Carlson 1991; Ayer and Carlson 1992; Carlson 1993).

Anholt *et al.* (1996) developed a simple "dipstick" avoidance assay to quantify olfactory avoidance behavior in adult *Drosophila*. Upon the introduction of a repellant odorant into the test vial, flies immediately migrate away and tend to spend more time in a subsection of the vial that is distal from the odorant source. The statistical average of the number of individuals present in the distal compartment of the vial, recorded at 30 s intervals over a period of 1 min, is calculated as the "avoidance score" for a single replicate. By using a genetically homogeneous, inbred parental strain of flies, genetic variance in behavioral responses are minimized. Thus, the resolution of mean differences in behavioral response between genotypes that differ with respect to a single gene mutation is only limited by the number of replicates performed. Each test lasts for 1 min, during the course of which 10 observations are made, hence sample sizes can be fairly large. This increases the statistical power to resolve both dramatic as well as subtle differences in olfactory avoidance responses. These aspects of the dipstick avoidance assay makes it a very versatile tool in the study of behavior; it has been successfully used to study the genetic basis of variation in olfactory behavior in natural populations of *Drosophila melanogaster* (Fanara *et al.* 2002; Mackay *et al.* 1996), as well as to screen for single gene mutants in an isogenic background strain (Anholt *et al.* 1996; Kulkarni *et al.* 2002).

1.5 PROJECT OVERVIEW AND AIMS

One of the main efforts in the study of behavior is directed towards the demonstration of a mechanistic link between genes, their regulated expression and the nervous system they shape to produce the end-point phenotype, displayed as behavior. This study uses olfactory avoidance behavior in *Drosophila melanogaster* as a model to provide evidence for the sex-specific transcriptional regulation of a single autosomal locus, *smi97B* that is correlated with sexually dimorphic mutant behavioral phenotypes.

Anholt *et al.* (1996) devised a simple, rapid and reproducible assay for testing behavioral responses of *D. melanogaster* to repellant odorants. By testing flies derived from isogenic, genetically homogenous backgrounds within-strain genetic variance is reduced, thus increasing the statistical power to detect differences in behavior between genotypes. Furthermore, males and females are tested separately, allowing for the resolution of sex-specific behavioral differences. This assay was also employed to study the genetic basis of variance in olfactory avoidance responses in natural populations (Mackay *et al.* 1996). 43 *X* chromosome and 35 *third* chromosomes were extracted from a natural population obtained from the Raleigh Farmer's market and substituted into the controlled isogenic background of a laboratory strain, *Samarkand;rosy*^{506} (*Sam;ry*^{506}). Analysis of avoidance responses to the repellent odorant, benzaldehyde, showed that the genetic correlation in avoidance behavior between males and females was extremely low. Hence, genes affecting variation in avoidance behavior have sex-specific effects; this was especially pronounced for loci on chromosome 3. This notion was further elaborated by the identification and characterization of *Vanaso*, a chromosome *3* quantitative trait locus (QTL) found to be associated with variation in *female-specific* olfactory avoidance behavior (Fanara *et al.* 2002). Sex-specific effects in olfactory behavior were also found in a genome-wide mutagenesis screen conducted by Anholt *et al.* (1996). This screen was based on the disruption of single genes by *de novo* insertion of a non-autonomous *P*-element, *P{lArB}*, in the autosomal genome of the homozygous *Sam;ry*^{506}strain. Of the 14 novel smell-impaired (*smi*) lines detected, 4 (*smi45E*, *smi21F*, *smi51A* and *smi97B*) displayed sexually dimorphic olfactory deficits to a panel of 3 unrelated repellants (benzaldehyde, 2-n-propylpyrazine and 2-isobutylthiazole). Since none of the mutants identified displayed gross morphological defects in their olfactory organs, it seems likely that the genes disrupted in these lines are involved in behavior and not morphological alterations. Quantitative analyses of the underlying epistasis (non-allelic interactions) between these loci revealed that 8 out of the 12 *smi* loci showed extensive non-additive pair-wise interactions as double heterozygotes.

One of the mutants isolated from this screen is *smi97B*, which contains the 18.5 kb *P{lArB}* insertion in cytological location 97B of chromosome *3* (determined by *in situ* hybridizations to the *P{lArB}* element in larval salivary gland-derived polytene chromosomes). In addition to displaying the most dramatic deficits in olfactory avoidance behavior, *smi97B* was found to be consistently sexually dimorphic in the expression of its mutant phenotype: *smi97B* females were more severely affected than males, and appeared anosmic to high concentrations (100% and 1%) of all three repellants tested. In addition, epistatic analyses revealed that *smi97B* interacted to suppress and enhance the mutant phenotype of *smi51A* and *smi21F*, two other loci with sex-specific mutant effects.

These observations set the foundation for the work described in this dissertation, which is a study of the role of *smi97B* in olfactory avoidance behavior in *Drosophila melanogaster*. The main text is divided to present the observations obtained from the genetic, molecular and neurohistological analyses of the gene (*scribble*) disrupted by the *P{lArB}* insertion in the *smi97B* locus. First, behavior and genetic analyses demonstrate that *smi97B* is a *P{lArB}*-induced allele of *scribble* (*scrib*) that causes generalized olfactory impairment in larvae and adults. Previous characterization of *scrib*, which encodes a scaffolding protein, reveals that its pleiotropic functions include polarity determination in embryonic epithelia, tumor-suppression and synaptogenesis (Bilder *et al.* 2000; Bilder and Perrimon 2000; Li *et al.* 2001; Mathew *et al.* 2002; Roche *et al.* 2002). Hence, hitherto identified mutant alleles of *scrib* were recessive lethals in embryos and larvae. Identification of the first homozygous viable allele of *scrib* attributes a novel adult role for this gene in olfactory behavior. To genetically dissect this locus, four homozygous lethal alleles of *scrib* were scrutinized for olfactory functions in the adult by quantifying their effect on avoidance behavior, when crossed to *smi97B*. The results indicate that null alleles of *scrib* failed to complement the behavioral defects in *smi97B*, signifying its role in olfaction. However, hypomorphic *scrib* alleles displayed intragenic complementation of the olfactory phenotype of *smi97B*, suggesting pleiotropy of *scrib* function. Molecular characterization garners support for the functional pleiotropy of *scrib* with the identification of alternatively spliced variants. Moreover, deficits in olfactory behavior are shown to correlate with the reduction of *scrib* expression caused by the *P{lArB}* insertion in *smi97B*.

A theme that runs through the course of this investigation is the effect of the sex-environment on the expression of olfactory behavior. Differences in phenotypic values between males and females of the same genotype, as well as the evidence for sex-specificity in the regulation of *scrib*, demonstrate an over-reaching influence of the sex-environment even on the autosomal genome, thus providing the potential for sexually-dimorphic differences in olfactory behavior. Furthermore, sexual dimorphism at the level of the genome that results in subtle quantitative differences in behavior implicates concomitant subtle variability in neural organization. The molecular components that link sex-dependent differences in the transcriptome to variation in neural function are unknown, but likely involve variations in synaptic organization. Scrib is a scaffold protein composed of protein-binding modules that mediate the organization of supra-molecular assemblies at the cell membrane (Bilder 2001). Homologous to vertebrate postsynaptic density proteins, it has recently been localized also to glutamatergic synaptic boutons of the larval neuromuscular junction, suggesting a role in synaptic organization (Mathew *et al.* 2002; Roche *et al.* 2002). To provide anatomical evidence for the function of *scrib* in olfaction, immunochemical localization of its gene products in olfactory organs and the brain is described. The studies described suggest that Scrib may provide a molecular basis for the organization of sexually dimorphic neuronal circuitry by recruiting different ensembles of proteins to synapses, thus giving rise to sexually dimorphic effects in the *smi97B* mutant.

This study sets out to conduct the analysis of a complex behavior by demonstrating the genetic, molecular and neurobiological underpinnings of odor-guided behavior in *Drosophila melanogaster*.

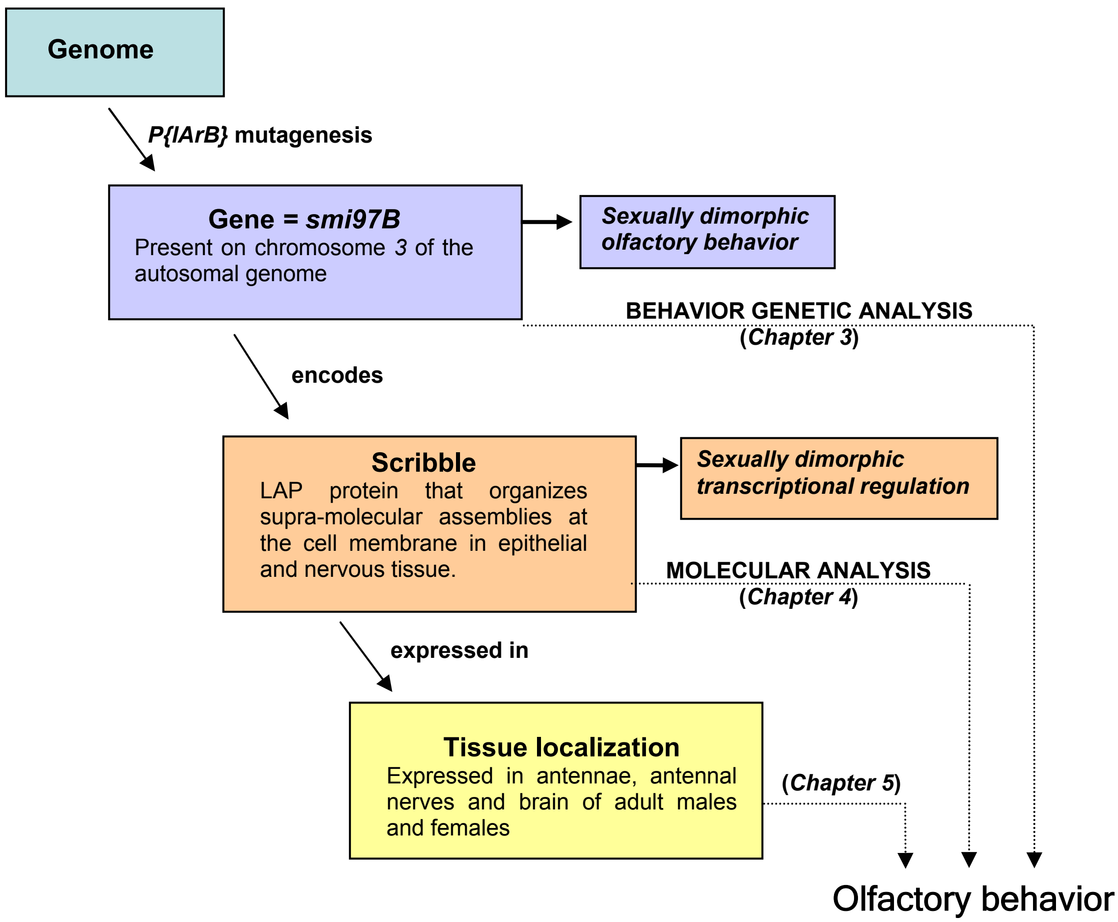


Figure 6 : Schematic representation of project rationale.

CHAPTER 2:

Experimental Methodology

2.1 BEHAVIOR-GENETIC ANALYSIS OF *smi97B*

(a) Fly stocks and husbandry

Sam;ry^{506} (hereby abbreviated as *Sam*): An isogenic, *P*-element free (M) strain, maintained in the laboratory by full-sib inbreeding for over 200 generations. It was derived by substituting chromosome *3* of the isogenised *Samarkand* background with one from an unrelated strain that carries the *rosy^{506}* eye-color marker and is wild-type for olfactory behavior (Anholt *et al.* 1996).

smi97B: Generated by the insertion of a single non-autonomous *P{lArB}*-element transposon in 97B on the cytological map; otherwise co-isogenic with *Sam;ry^{506}*(Anholt *et al.* 1996).

Stocks for the reversion experiment: ***Sam1; Sam2; Sb,P{ry^{+}, Δ2-3}/TM6,Ubx*** contains a modified *P*-element bearing a deletion of one of its termini and the 3rd intronic region (*Δ2-3*), that results in the expression of high levels of transposase, as well as its stable insertion within the genome (Laski *et al.* 1986; Robertson *et al.* 1988). ***Sam1; Sam2; TM3, Sb ry^{RK} / H*** contains a chromosome *3* bristle marker, *Stubble* (Karess and Rubin 1984; Rubin *et al.* 1985).

scribble **stocks:** ***Df(3R)Tl-X;97B-D2***: The deficiency breakpoints are 97B; 97D2 and uncovers *scrib*. Ethylmethylsulphonate (EMS) point mutations, ***w;FRT2AFRT82B scrib^{1} / TM3, Sb*** and ***w;FRT82B e scrib^{2} / TM6c, Sb*** (null alleles). *P{lacW}*-insertion lethal mutants, ***scrib^{j7B3}*** **and** ***scrib^{S042405}***. Since all *scrib* alleles are lethal mutations, they were maintained as heterozygotes over balancer chromosomes.

Stocks for the transgenic rescue: ***FM4/Sam1***; ***Sam2***; ***ry^{506}*** the *X*-chromosome balancer contains the dominant eye-marker, *Bar.*; ***Sam1; Sam2; TM3, Sb ry^{RK}/H. CyO,TM6/Xa*** which contains the translocation, *Xa*, which is homologous to both chromosomes *2* and *3* and hence responsible for their co-segregation during meiosis. *P{hs-scrib}; +; +*, a transgenic stock containing a 6 kb full-length cDNA *scrib* clone (Bilder, 2000), under the regulation of a heat-shock promoter, *Hsp70*.

Descriptions of abovementioned gene markers and chromosomes are available in *The Genome of Drosophila melanogaster* (Lindsley and Zimm, *Academic Press, Inc,* 1992). All fly stocks were maintained on 10 ml molasses-yeast-agar media in clear plastic vials (Capitol vial, Inc. Fultonville, NY) at 25° C, 70% humidity, on a 12 hour light-dark cycle; excepting the transgenic stock that was reared at 18° C, to prevent activation of *Hsp70*.

(b) Behavioral assays:

Repellants and controls: Benzaldehyde, 2-n-propylpyrazine, 2-isobutylthiazole and isoamyl acetate are obtained from Sigma-Aldrich, and stored under argon after breaking the seal. Benzaldehyde, 2-n-isopropylpyrazine and isobutylthiazole were applied at concentrations of 1% (v/v in distilled water) and 100% for the initial screen (Anholt *et al.* 1996). Benzaldehyde was subsequently used at a 0.3% concentration (v/v in distilled water) for the adult avoidance assay. For the larval test, undiluted benzaldehyde and isoamyl acetate were used. Distilled water was used as the control for both adult and larval assays.

Adult olfactory assay: Adult olfactory behavior is quantified as avoidance responses to repellant odorants within a 1 min time period (Anholt, *et al.* 1996). 3-5 day old adults are sexed, divided into single sex sets of 5 individuals and placed in empty fly vials (Capitol vial, Inc. Fultonville, NY) plugged with cotton wool, for 3 h, at 25° C. A cotton-swab saturated with the appropriate concentration of the repellant or the water control is introduced into the vial through the cotton plug. The transparent vials are visually divided into two chambers by laying it on its side on a white base with lines that demarcate the 3cm and 6cm point from the bottom edge of the 9cm long vial. The tip of the swab extends into the vial up to the 6cm mark (Figure 9). The sealed end of the vial abuts a white surface to standardize the visual effect of the white cotton plug at its other end. The flies are given 15 seconds to acclimatize to the experimental setup, following which, the number of individuals observed in the chamber that is distal from the cotton-swab is recorded every 5 s.

Statistical analysis:

The average of 10 recordings (at 5 s intervals) is the avoidance score for one replicate. The mean avoidance score for a genotype and sex is calculated by averaging the avoidance scores obtained from the replicates. Differences in mean avoidance scores between genotypes, or sexes, are assessed using Student's *t*-test.

Third-instar larval collection: Third instar feeding larvae are scooped out of food medium and immersed very briefly in 15% (w/v) sucrose solution to release adhering food particles. They are then extensively washed in distilled water to remove sucrose.

Larval chemotactic assay: Larval olfactory responses are quantified by modifying the larval plate assay described by Heimbeck *et al.* (1999). Petri-dishes (85mm) overlaid with 10ml of 1.2% (w/v) agarose in distilled water are allowed to air-dry, following which, filter paper discs saturated with 2 ul of undiluted odorant or water are set diametrically opposite each other on the agarose surface. To prevent odorant diffusion through the agar (which could result in larval gustatory responses), the filter paper discs are first set on severed lids of 1.5 ml micro centrifuge tubes. The lid is replaced and the number of larvae within an arc of radius 10 mm from the odorant source is recorded every 30s (Figure 10).

Statistical analysis: 11 observations for each time point are averaged over replicates and differences in olfactory responses between *smi97B* and *Sam;ry*^{506} larvae assessed by 2-way analysis of variance (ANOVA) with Genotypes and Time as fixed effects.

Larval motility assay: Larval motility is quantified by modifying the locomotion assay described by Wang *et al.* (2002). Single individuals are placed in a petri-dish overlaid with

1.2% agarose, in the absence of odorant stimuli. Path lengths are traced on the lids of the dish over a 60 s time period for each replicate.

Statistical analysis: Wild-type and mutant recordings are compared using Student's *t*-tests.

(c) Derivation and analysis of *smi97B* revertants and excision lines

Crossing scheme: Mobilization of the non-autonomous *P{lArB}*-element in *smi97B* can be effected by providing an external source of transposase (Laski *et al.* 1986; Robertson *et al.* 1988). *Sam1; Sam2;* ry^{506}*, P{lArB}smi97B* virgin females are crossed to *Sam1/Y; Sam2; Sb (Δ2-3)/Ubx* males (that provides the transposase) in small mass matings of 10 pairs. F1 males of genotype *Sam1/Y; Sam2; Sb(Δ2-3)/* ry^{506} *P{lArB}smi97B* have the transposase source present in the same genome as the *P{lArB}*-element, which causes mobilization of the *P*-element at high frequencies in the germ line. These "jumpstart males" are singly mated with *Sam1; Sam2; TM3, Sb* ry^{RK}*/H* females (G1) to generate males of genotype *Sam1/Y; Sam2; TM3, Sb* ry^{RK} */*ry^{506}*,* P^{minus}*.* Each G2 male is derived from a single unique pre-meiotic gametic event that involves the mobilization of *P{lArB}* in the male G1 parent. G2 males are singly mated with females from the *TM3, Sb/H* stock to generate *Sam1; Sam2; TM3, Sb/* ry^{506}, P^{minus} males and females (G3). Homozygous individuals of genotype *Sam1; Sam2;* ry^{506}*,* P^{minus} are obtained from small mass matings between G3 males and females (Figure 7). Excision lines are maintained as homozygotes wherever possible, or as heterozygous against the *TM3, Sb* balancer, from which homozygous individuals are selected for characterization and assayed for olfactory avoidance behavior.

Molecular characterization:

Southern blot: 5µg genomic DNA isolated from each genotype by homogenizing ~100 individuals is *Hin*dIII digested overnight. Digested fragments are size-fractionated via (1X) Tris-acetate-EDTA buffered gel electrophoresis (60mV) and blotted onto nylon membranes

(Hybond-N, Amersham Radiochemicals, Arlington Heights, IL). Blotted membranes are probed in series with digoxigenin-labeled (Roche Applied Science, Indianapolis, IN) rescued fragment (585 bp; genomic fragment immediately downstream from the *P{lArB}* site of insertion), pBluescript (3kb) and *lacZ* (3kb). Hybridization is visualized by the chromogenic conversion of X-phosphate (5- chloro-4-bromo-3-indolyl phosphate) by alkaline phosphatase-conjugated anti-digoxigenin secondary antibodies, in the presence of NBT (nitro blue tetrazolium chloride).

(d) Complementation mapping

Olfactory avoidance responses of heterozygous F1 progeny generated by individually crossing lethals, *scrib*^{1}, *scrib*^{2}, *P{lacW}scrib*^{j7B3}, *P{lacW}scrib*^{S042405} and *Df(3R)Tl-X* to *smi97B*, were compared to the responses of F1's generated by crossing to *Sam* using Student's *t*-test.

(e) Transgenic rescue of *smi97B*

Crossing scheme: The genetic background of *P{hs-scrib}; +; +* was standardized by introgressing it's *X*-chromosome into *Sam* and then crossing it into the *smi97B* homozygous background. Chromosomes *1* and *3* were followed through the experiment by crossing *FM4/Sam1; Sam2; ry*^{506} virgin females to the isogenic stock *Sam1; Sam2; TM3, Sb/H* in small mass matings of 10 pairs, which generated heterozygous females with *Bar*-eyes (*Bar* carried on *FM4*) and *Stubble* (*Sb*) bristles, *FM4/Sam1; Sam2; Sb/ry*^{506} (G1). Virgin G1 females were crossed to *Sam1; Sam2; ry*^{506}*, P{lArB}smi97B* and *Bar*-eyed, *Stubble*-bristled *FM4/Sam1; Sam2; TM3, Sb/ry*^{506}*, P{lArB}* females were selected in generation G2 (Figure 8). In tandem, *FM4/Sam1; Sam2; ry*^{506} females are mated to males of the *CyO;TM6/Xa* stock (G0). The *T(2;3)ap*^{Xa} translocation (causing serrated wing margins) recruits both chromosomes *2* and *3* during meiosis, thus generating F1 progeny of genotype, *FM4/+; Xa/Sam2; ry^{506}*, that are crossed to the transgenic stock *P{hs-scrib}; +; +* (G1). *Bar*-eyed, *Xa*-winged females of genotype *FM4/P{scrib}; Xa/+;+* crossed to homozygous *smi97B* males generated *P{scrib}/Y; Xa/Sam2; ry^{506}, P{lArB}* males (G3) containing the *scrib* transgene-bearing chromosome in the *Sam* isogenic background. These males are mated with the bar-eyed, *Sb*-bristled females, *FM4/Sam1; Sam2; TM3, Sb/ry^{506}, P{lArB}* (G2) selected from the other set of crosses (G3). *P{scrib}; Sam2; ry^{506}, P{lArB}* progeny with wild-type eyes, wings and bristles in generation G4 are collected and maintained as the transgenic stock. *Bar*-eyed or *Sb*-bristled females from G4 are once again crossed to *P{scrib}/Y; Xa/Sam2; ry^{506}, P{lArB}* males to generate *P{scrib}; Sam2; ry^{506}, P{lArB}* males and females. This stock, *P{hs-scrib}; smi97B* contains *P{hs-scrib}* on chromosome *1* in the homozygous *smi97B* background.

Heat-shock protocol: *P{hs-scrib};P{lArB}smi97B* was reared at 18° C to prevent leaky *scrib* expression resulting from a moderate activation of the *Hsp70* promoter at 25° C. Heat shock was provided by placing flies in vials with 10ml of 1.5% (w/v in distilled water) non-nutritive agarose, in a 37° C incubator for 45 minutes. Following heat-shock, flies were returned to food media at 25° C for 24 h, before being tested for olfactory avoidance responses.

Experimental design: The wild-type *Sam*, *smi97B* mutants and *P{hs-scrib}; smi97B* transformants were tested for olfactory avoidance responses at 25° C. Each set, comprising all three genotypes, was tested without prior heat-shock as well as following heat shock. Avoidance scores of mutants and transformants were each compared to those of *Sam* using Student's *t*-test.

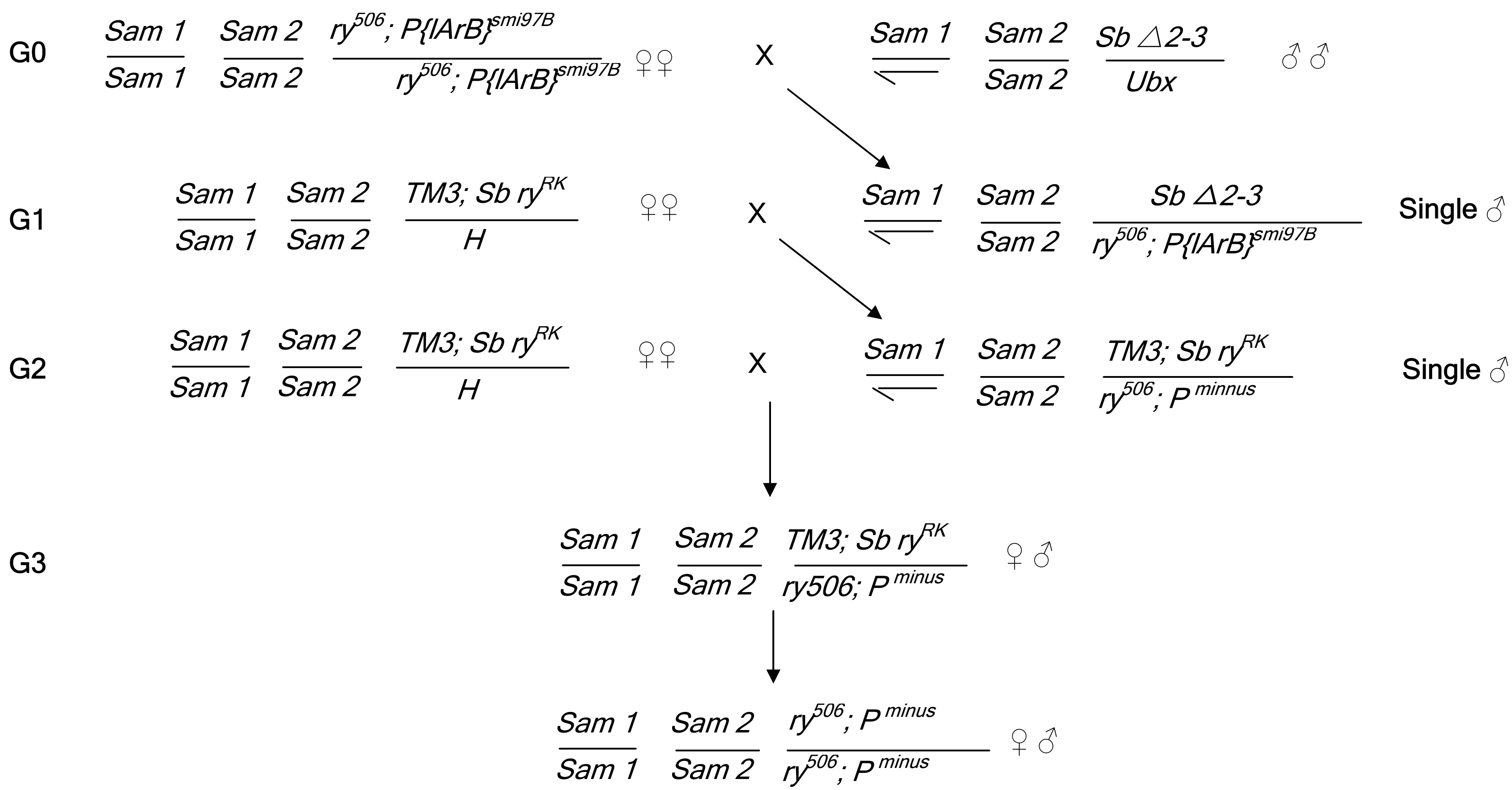


Figure 7: Crossing scheme followed to generate *smi97B* excision lines by mobilizing *P{lArB}*.

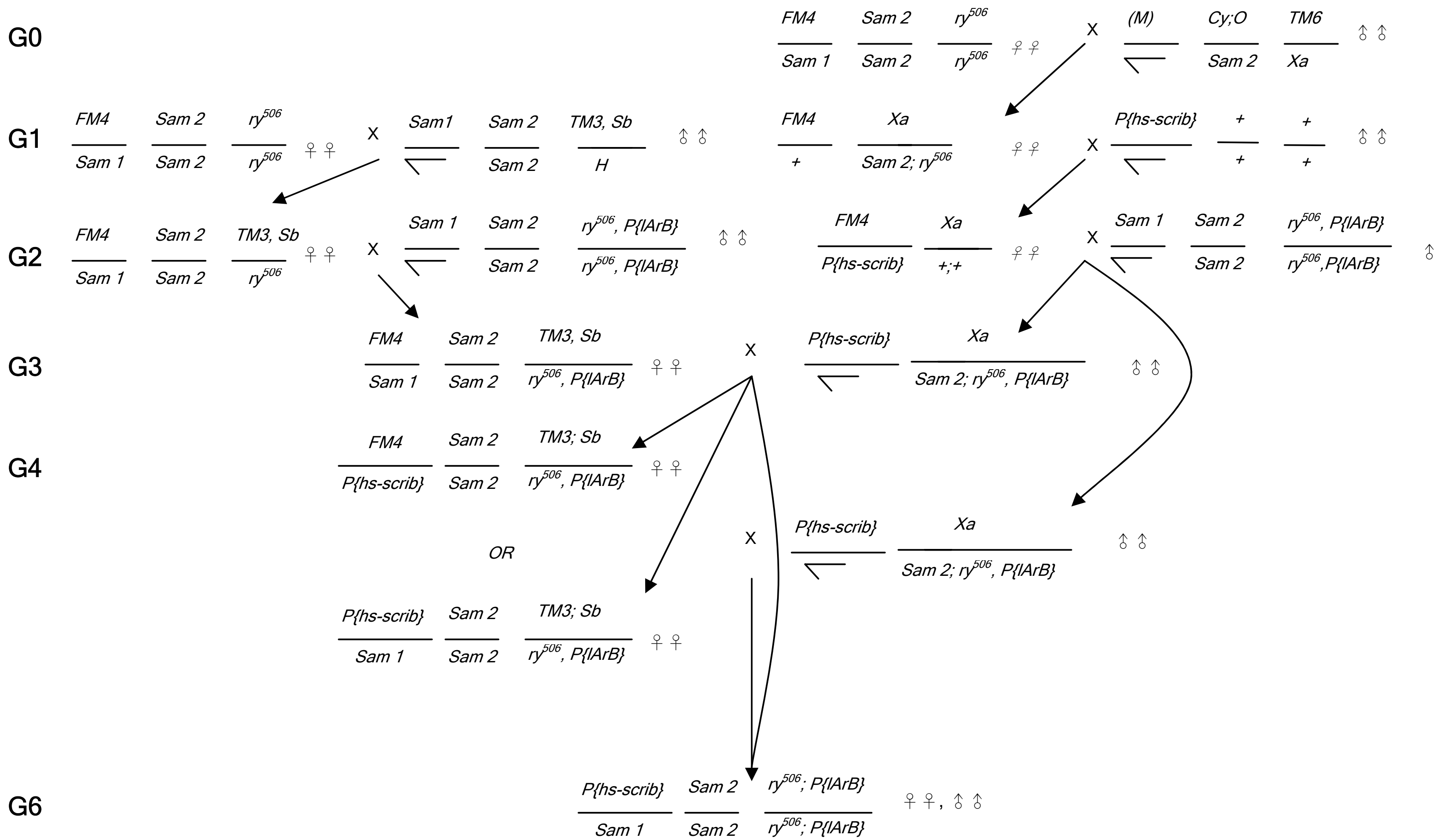


Figure 8: Crossing scheme followed to introgress *P{hs-scrib}* into the *smi97B* homozygous background

2.2 MOLECULAR-GENETIC ANALYSIS OF *smi97B*

(a) Tissue collection:

Flies were CO_2-anaesthetised, sexed and collected in eppendorf micro centrifuge tubes for manual homogenization with plastic pestles. Fly heads were dissected from anaesthetized adults using DEPC (Diethylpyrocarbonate)-treated needles. Larvae were washed and cleaned off the food medium in culture vials and also collected in 1.5 ml eppendorf tubes for homogenization.

(b) DNA, RNA and protein isolation

Genomic DNA isolation was carried out by homogenizing ~100 whole flies (males and females) in an ice-cold buffer containing 1% SDS (Sodium dodecyl sulfate), followed by phenol-chloroform RNA extractions. Extractions were conducted using 0.05% DEPC treated apparatus to minimize RNase contamination. Total cellular RNA from ~100 flies of each sex and ~30 third instar-larvae was isolated using TRIzol reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's directions. Protein extracts from each sex and genotype were isolated by homogenizing 5 whole flies in boiling 2 X Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8; 25% glycerol; 2% SDS; 0.01% Bromophenol blue; Bio-Rad, Hercules, CA) and β-mercaptoethanol, according to the manufacturer's directions. An alternate method of protein extraction involved homogenization of whole flies in triple lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% SDS; 0.5% deoxycholate, 1% Nonidet NP-40 as detergents, 0.02% Na-azide, and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF); and aprotinin, leupeptin, pepstatin A and iodoacetamide to control for proteolytic degradation of protein isolates).

(c) Cloning the *P{lArB}* insertion site in *smi97B*

The *P{lArB}* construct in *smi97B* is an engineered pUC*hsneo* plasmid containing the *P-lacZ-hsp70* fusion gene, *Adh*$^+$ and *ry*$^+$ marker alleles and the pBluescript plasmid vector (Figure 21). 10 µg of *smi97B* genomic DNA was digested overnight with 10 units of *Hin*dIII in a total volume of 25 µl at 37°C. 0.2 µg of purified recombinant fragments containing the pBluescript plasmid vector and a piece of genomic DNA immediately flanking its 3' end were self-ligated in a total volume of 50µl with 7.5 units of T4 DNA ligase (Promega, Madison WI). Incubation was carried out at 15°C for 4 hours. 3µl of purified ligated mixture was transformed into Epicurean coli® XL1-Blue Competent cells (Stratagene, Cedar Creek TX), which were plated on LB-ampicillin (50µg/ml) plates overlaid with isopropyl-beta-D-thiogalactopyranoside (IPTG) and X-gal. Plasmid preparations of single white colonies were carried out using the QIAquick mini-prep system (Qiagen, Valencia CA). Plasmid inserts were sequenced using T7 and T3 primers that flank the pBluescript vector (Figure 21). A 585 bp insert was obtained (rescued fragment) whose sequence matched a region in band 97B of chromosome *3* in the Berkeley Drosophila Genome Project database.

(d) Molecular analysis of *scrib* gene expression:

***a..* Northern blot analysis:** 60-100 µg of total RNA was size-fractionated by 1% formaldehyde gel-electrophoresis in 1X MOPS buffer (200mM 3-(N-morpholino) propanesulfonic acid, 80mM sodium acetate, and 10 mM EDTA, pH 8.0). The gels were denatured in 0.5N NaOH for 20 min, and blotted onto nylon membranes (Hybond-XL, Amersham Radiochemicals, Arlington Heights, IL) in DEPC-treated 20X standard saline citrate (SSC; 3M NaCl, 300mM tri-sodium citrate, pH 7.0) by capillary action, for 16 h at ambient temperature.

Hybridization and detection: Blotted and UV-cross linked membranes were prehybridized for 1 h at 60° C in Northern Max Prehyb/Hyb buffer (Ambion, Austin, TX). They were subsequently probed in series with full-length (6kb) *scrib* and *β-actin* cDNA clones that were nick translated using [^{32}P]-labeled dCTP (Prime-a-gene nick translation kit; Promega, Madison WI) and purified using G50 Sephadex columns. Hybridizations are performed at 60° C for 16h, followed by stringency washes: 2X SSC; 0.1% SDS for 10 min at ambient temperatures and 0.1X SSC; 0.1% SDS for15 min at 60° C. Radioactivity was detected by exposure in a phosphoimager (Molecular Dynamics, Sunnyvale, CA) and images were processed using the Imagequant program.

b. **Western blot analysis:** 60-100μg of protein was subjected to SDS-polyacrylamide gel-electrophoresis (25mM Tris, 192 mM glycine, 0.1% SDS buffer) in precast 4-15% gradient Ready mini-gels (Bio-Rad, Hercules, CA) and electrophoretically blotted onto nitrocellulose membranes (Osmonics).

Generation of anti-Scrib antibodies*:* 166μg of keyhole limpet haemocyanin (KLH)-conjugated synthetic C-terminal peptides, DMRNPLDEIEAVFRS and EPLISEVTISTSRIH (translated from the 6kb and 4.6kb *scrib* transcripts, respectively; obtained from Genemed Synthesis Inc) was individually suspended in a 1ml mixture of 1:1 saline and Freund's complete adjuvant. The suspension was injected into four healthy female rabbits (two replicates) that were previously bled to obtain ~3 ml of blood for preparation of pre-immune sera that were used as controls for immunochemical analyses. The injections were followed up with two booster injections administered 14 days apart, and antiserum was prepared from the second bleed. In addition, antiserum raised against a mixture of N-and C-terminal peptides of Scrib, RYSRTLEELFLDANHIRDLPKNF and VDAEDMRNPLDEIEAVEFRS, respectively (translated from the 6kb transcript) was also used for immunochemical analyses (a kind gift from D. Bilder).

Enzyme-linked immunosorbent assay (ELISA*):* 0.2μg of pure unconjugated peptide (diluted in 1ml of 100 mM $NaHCO_3$, pH 9.6) was coated onto microtitre plate wells at 4° C for 16 h. The wells were washed in 1X phosphate buffered saline with 0.05% Tween-20 (1X PBS-T20) and blocked for 1h in 1% w/v non-fat dry milk in 1X PBS-T20, at ambient temperature. Preimmune sera and antisera were serially diluted in 10 mM PBS and applied to the peptide coated plates for 16h at 4° C. Following incubation with horseradish peroxidase (HRP) conjugated secondary antibodies, immunoreactivity was quantitated by the colorimetric analysis of the chromogenic substrate, ABTS (2-2'-Azino-bis(3-ethylbenothiazoline sulfonic acid)) in the presence of 0.03% hydrogen peroxide.

Scrib antibody incubation: Blots were blocked in 1% (w/v) non-fat milk in 1X PBS-T20, for 1 h at ambient temperatures or overnight at 4° C. Then washed and incubated with polyclonal rabbit-antisera or preimmune sera at 4° C for 16 h. All washes were conducted in 1X PBS-T20. Immunoreactivity was detected using chemiluminescence of HRP-conjugated goat secondary antibodies, exposed to Xo-Mat AR film (Kodak Eastman Co.); or by chromogenic detection of HRP-conjugated secondary antibodies in the presence of H_2O_2 and 3,3-diaminobenzidine.

Controls: Controls for Western analysis of Scrib expression levels were carried out by (1) incubating blots with rabbit pre-immune serum, and/or (2) incubating Scrib antibodies with the purified poplypeptides they were raised against for 1hr prior to their incubation with Western blots.

(e) Isolation of alternatively spliced scrib variants:

Construction and screening of head cDNA library: cDNA libraries were constructed by cloning reverse-transcribed adult head poly$(A)^+$ RNA into the ZAP Express vector (Stratagene, Cedar Creek, TX). The library was screened by plating it at a dilution of 5 X 10^4 plaque forming units (pfu) per culture plate. Four plates were screened with a digoxigenin-labeled full-length (6kb) *scrib* cDNA (Boehringer Mannheim DIG-labeling kit). Eleven hybridizing plaques were identified, the inserts of which were sequenced using T7 and SP6 primers.

Bioinformatics: Similarity searches were carried out by querying the nucleotide sequences of inserts against known sequences entered into the Genbank and Berkeley Drosophila Genome Project databases, using Blast (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/) and Fly Blast (http://www.fruitfly.org/blast/), respectively. Sequence translation and alignment were carried out by using the Expasy Translate tool (http://us.expasy.org/tools/dna.html) and Blast 2 Sequences (http://www.ncbi.nlm.nih.gov/gorf/bl2.html; Tatusova 1999).

2.3 LOCALIZATION OF Scrib IN ADULT OLFACTORY TISSUE

(a) Enhancer trap staining of *lacZ* gene: Adult flies were sexed, anaesthetized under CO_2 and fixed for 5 min in 0.5% glutaraldehyde dissolved in 1X PBS (phosphate buffered saline) supplemented with 1mM $MgSO_4$. They were extensively washed in PBS and stained for β-galactosidase (*lacZ*) activity by incubating in an equimolar solution of 35 mM $K_3Fe_9(CN)_6$ and $K_4Fe(CN)_6$ containing 1 mg/ml of the substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside). Incubation was carried out at 37° C for 16 h. Antennae were dissected and staining was observed under a Zeiss microscope. Images were stored as .jpeg files.

(b) *In situ* hybridization of *scrib* probes: *In situ* hybridization was performed on 12 μm-thick formalin-fixed and paraffin-embedded sections through adult heads with heat-denatured digoxigenin-labeled *scrib* riboprobes (Boehringer-Mannheim). The 6 kb *scrib* cDNA (obtained from D. Bilder) was digested separately with *Hin*dIII and *Not*I enzymes, and digoxigenin sense and anti-sense riboprobes were synthesized using SP6 and T7 RNA polymerases, respectively. Slides with paraffin-embedded sections were heated to 60° C for 15 min in an air-incubator and then deparaffinized in xylene. Following rehydration in graded alcohols, they were fixed in 4% paraformaldehyde for 20 min. They were then washed in PBT (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, pH 7.4) and treated with 200 mM HCl for 10 min. Between each subsequent step, slides were washed in PBT. To reduce background staining, the sections were treated with acetic anhydride and triethanolamine-HCl for 10 min. Proteinase K digestion was carried out for 1 h at 37° C (5 μg/ml in PBT), following which the slides were dehydrated through graded alcohols and rinsed in chloroform. Hybridizations with sense and anti-sense probes were carried out at 55° C in hybridization buffer (50% formamide, 5 X SSC, 100 μg/ml tRNA, 50 μg/ml heparin and 0.1% Tween 20, containing heat-denatured digoxigenin probes). Slides were washed for an additional hour at the same temperature with hybridization buffer without the riboprobes, followed by 20 min sequential washes with 75% hybridization buffer-25% PBT, 50% hybridization buffer-50% PBT and 25% hybridization buffer-75% PBT at 55° C. Hybridization products were visualized with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals, Indianapolis, IN), using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as substrates. Images were digitally captured and processed with Adobe Photoshop.

(c) Immunohistochemical staining of Scrib

***a.* Chromogenic detection:** 12 μm-thick paraffin-embedded sections of 3-10 day old male and female heads fixed overnight in 10% buffered formalin were deparaffinized and rehydrated through graded alcohols (100% - 50%). Endogenous peroxidases were deactivated by treatment with methanol and hydrogen peroxide for 20 min. The sections were then blocked with 1% (w/v) bovine serum albumin in PBT for 30 min at room temperature. Incubation with anti-Scrib antibodies: anti-Scrib1 (raised against the C-terminal polypeptide sequence, DMRNPLDEIEAVFRS) diluted 500 fold in blocking buffer; anti-Scrib 2 (raised against the polypeptide sequence, EPLISEVTISTSRIH) diluted 200-fold and orig-Scrib (raised against a mixture of N- and C- terminal peptides, RYSRTLEELFLDANHIRDLPKNF and VDAEDMRNPLDEIEAVEFRS, respectively) diluted 1000-fold in blocking buffer, were carried out for 16 h at 4° C. Immunoreactive products were visualized using a HRP-labeled secondary goat-antibody with H_2O_2 and 3, 3-diaminobenzidine, as the chromogenic substrate (staining could be enhanced in the presence of NiCl). Sections were dehydrated through graded alcohols and mounted in Permount. Images were digitally captured and processed with Adobe Photoshop.

b. **Immunofluorescence detection:** 12 μm-thick paraffin-embedded sections of 3-10 day old male and female heads fixed overnight in 10% buffered formalin were deparaffinized for 16 h and subsequently rinsed for 3 X 30 min in xylene. Sections were rehydrated through graded alcohols (100%-50%) and peroxidase treated with methanol and hydrogen peroxide for 45 min at room temperature. Blocking was carried out in 1% (w/v) bovine-serum albumin in PBT for 2 h at room temperature. Primary antibody incubations were carried out at 4° C for 16 h with mouse anti-ELAV, mAb 22C10, mAb 8C3 (anti-syntaxin), rAb Scrib1 and rAb Scrib2 (100 fold dilutions). Cy3 fluorophore-conjugated goat anti-mouse secondary antibodies and Cy5-conjugated donkey anti-rabbit antibodies were used at 500 fold dilutions in 5 % non fat milk in PBS-T20. Incubations with secondary antibodies were carried out at room temperature for 2 h. Slides were washed 3 X 10 minutes in PBS-T. Sections were dehydrated in graded alcohols (30%- 100%) and mounted in the dark using the Slowfade Antifade kit (Molecular Probes, Eugene, OR). Immunofluorescence was captured at 860 nm (Cy3) and 720 nm (Cy5), using a confocal microscope.

(d) Immunolabeling of Scrib in whole mounts of adult brains: Adult flies were sexed and anaesthetized in CO_2. Whole brains were dissected in 4% formaldehyde-PBS and incubated in the same solution for 2 h on ice at 25°C in severed lids of 1.5 ml micro centrifuge tubes. Fixative was allowed to infuse the tracheal network around the brain by incubating the tissue in 4% formaldehyde in PBS with 0.25% Triton under vacuum. Air was let in and out of the vacuum chamber every 30 min in a 2 h period. The tissue was washed in PBS containing 2% Triton X-100 for 3 X 5min and blocked in 10% normal goat serum containing 1% Triton X-100, 0.02% NaN3 for 2 days at 4° C. Primary incubation was carried out with rabbit anti-Scrib1 (10 fold dilution) in PBS containing 1% Triton X-100, 0.25% normal goat serum, 0.02% NaN3 and 0.1 mg/ml ribonuclease, for 24 h at 4° C. Brains were subsequently washed in PBS containing 1%

Triton X-100 and 0.3% NaCl for 3 X 20 min to reduce background staining of secondary antibodies. Cy3-conjugated donkey anti-rabbit secondary antibody incubations were carried out at 50 fold dilutions for 16 h at 4° C, in the dark. Tissue was washed 3 X 20 min and incubated in NBD-ceramide and Rnase (0.1 mg/ml) in 0.5% Tween 20 for 16 h at room temperature. Brains were washed in PBS-T, cleared and mounted in FocusClear TM (Pacgen, Vancouver, Canada). Fluorescence was captured at 860 nm, using a confocal microscope.

CHAPTER 3:

Behavior genetic analysis of *smi97B*

3.1 *smi97B* causes generalized, sexually dimorphic smell-impairment

(a) Adult olfactory deficits

The primary behavioral screen of *P{lArB}*-element insertion lines revealed generalized olfactory deficits in *smi97B* in response to high concentrations of odorants (Anholt *et al.* 1996). However, even at a concentration of 0.3% benzaldehyde (Figure 11), olfactory responses were significantly lower in *smi97B* homozygotes, compared to the *P*-element free co-isogenic strain, *Sam* (P < 0.0001). Furthermore, avoidance responses of *Sam/smi97B* heterozygotes were indistinguishable from wild-type (P = 0.56), indicating that the*smi97B* mutation is recessive (Figure 11A). The manifestation of olfactory deficits in *smi97B* homozygotes cannot be attributed to unrelated locomotor defects, since previous tests revealed that their responses to distilled water were not significantly different from the wild-type strain, *Sam* (Anholt *et al.* 1996)). The avoidance assay measures behavioral responses in the two sexes separately, thus allowing a statistical comparison between male and female mutant phenotypes. The magnitude of the mutational effect of *smi97B* was found to be sexually dimorphic (P = 0.019); males were hyposmic, while females were anosmic to 0.3% benzaldehyde (Figure 11B).

(b) Larval olfactory deficits

To investigate the developmental consequences of the *smi97B* mutation, olfactory responses of third-instar feeding larvae were quantified. Since larvae are attracted to most odorants (even those that elicit avoidance behavior in adults), the kinetics of odor-guided responses of wild-type and *smi97B* larvae towards benzaldehyde and isoamyl acetate were measured. Ten wandering larvae were cleaned and placed between the test odorant and the water control on a Petri-dish overlaid with non-nutritive agarose (see Methods). The number of individuals that traveled to within a 10 mm radius of the odorant source was recorded at 30 s intervals and averaged over 11 time points for each replicate (see Figure 10 for assay set up). A two-way analysis of variance with Genotypes and Time as fixed effects revealed significant effects of Genotypes for both odorants ($P < 0.001$). Time and Genotype x Time interaction terms were not significant for either odorant. Differences in olfactory responses between *smi97B* and *Sam* larvae do not arise due to differences in larval motility, which was indistinguishable between the genotypes in the absence of an odor-cue ($P = 0.57$; Figure 12 A and B).

DISCUSSION

Drosophila larvae respond to olfactory cues primarily via their dorsal organ (Python and Stocker 2002; Singh and Singh 1984), which is histolysed during metamorphosis to give rise to adult olfactory organs, such as the antennae and maxillary palps. Interestingly, despite this disparity in developmental origins, a number of studies have revealed an overlap between genes responsible for olfactory acuity in both developmental stages (Ayyub and Siddiqui, 1990; Lilly and Carslon, 1990; Meckenna, et al. 1989; Ayer and Carlson, 1991; Aceves-Pina and Quinn, 1979). Likewise, although the direction of the response is opposite in adults and larvae, these data suggest that the *smi97B* mutation causes generalized olfactory deficits in both. This raises the possibility of developmental pleiotropy of *smi97B*.

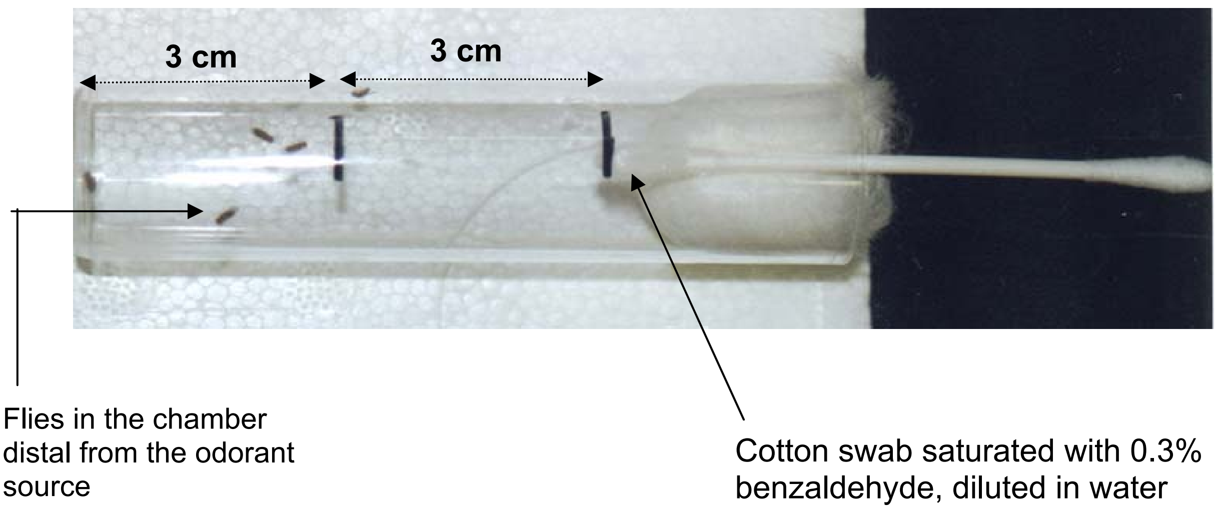


Figure 9: Adult olfactory behavior assay. Wild-type flies in the test chamber will migrate away from the repellant odorant, benzaldehyde, presented on the Q-tip. Avoidance behavior is quantified by averaging the number of individuals present in the chamber distal from the odorant source at 30 s intervals, over a 1 min period.

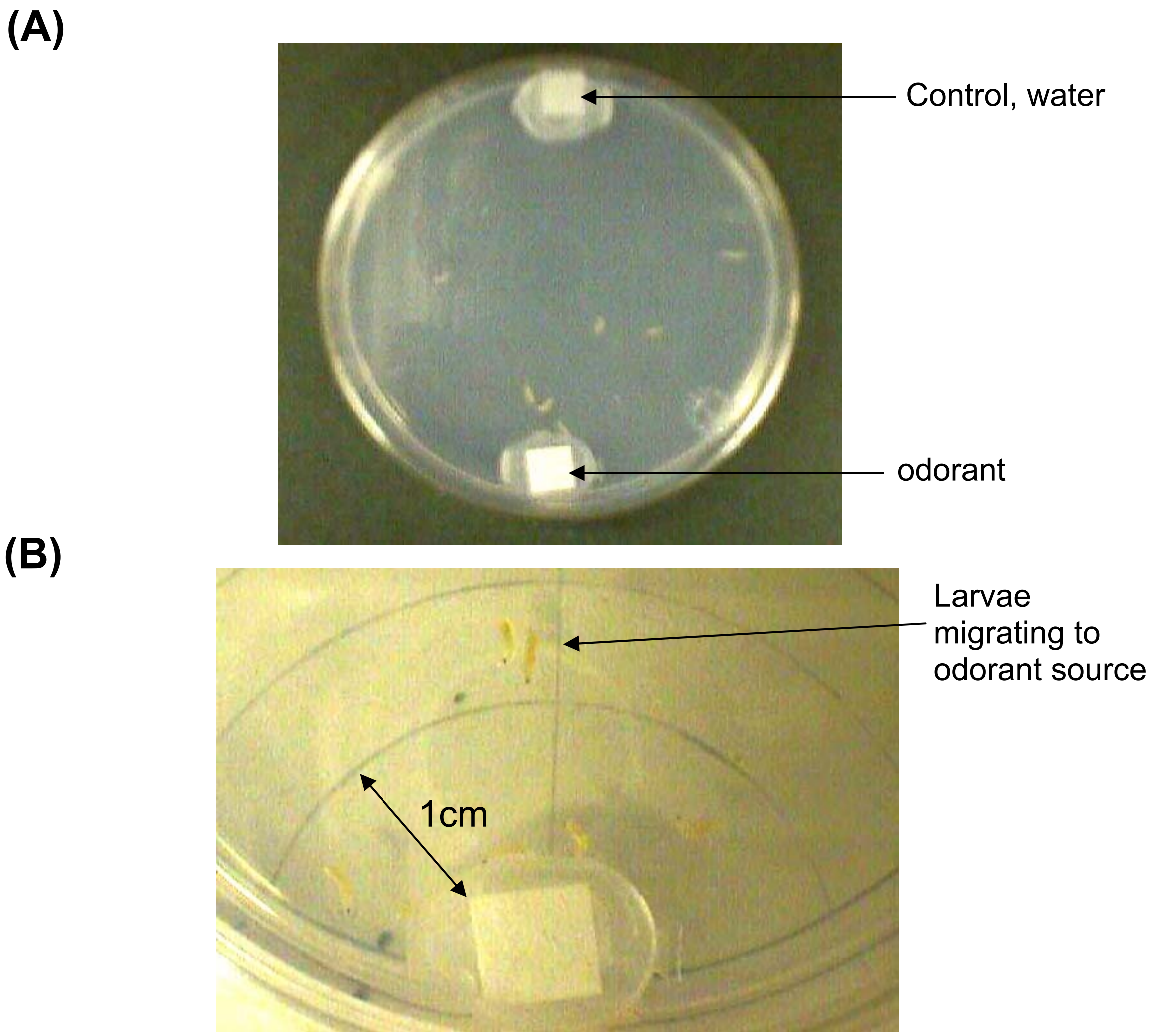


Figure 10: Larval olfactory assay. In a choice assay between distilled water and odorants, benzaldehyde and isoamylacetate, larvae will localize towards the filter paper saturated with the odorants. **(A)** Wild type larvae, starting from the center of the Petri-dish, quickly migrate towards the filter paper saturated with odorant. **(B)** The number of larvae that move at least 4-5 body lengths to arrive at a region that is within a 1 cm radius of the odor source is recorded at 30 s intervals.

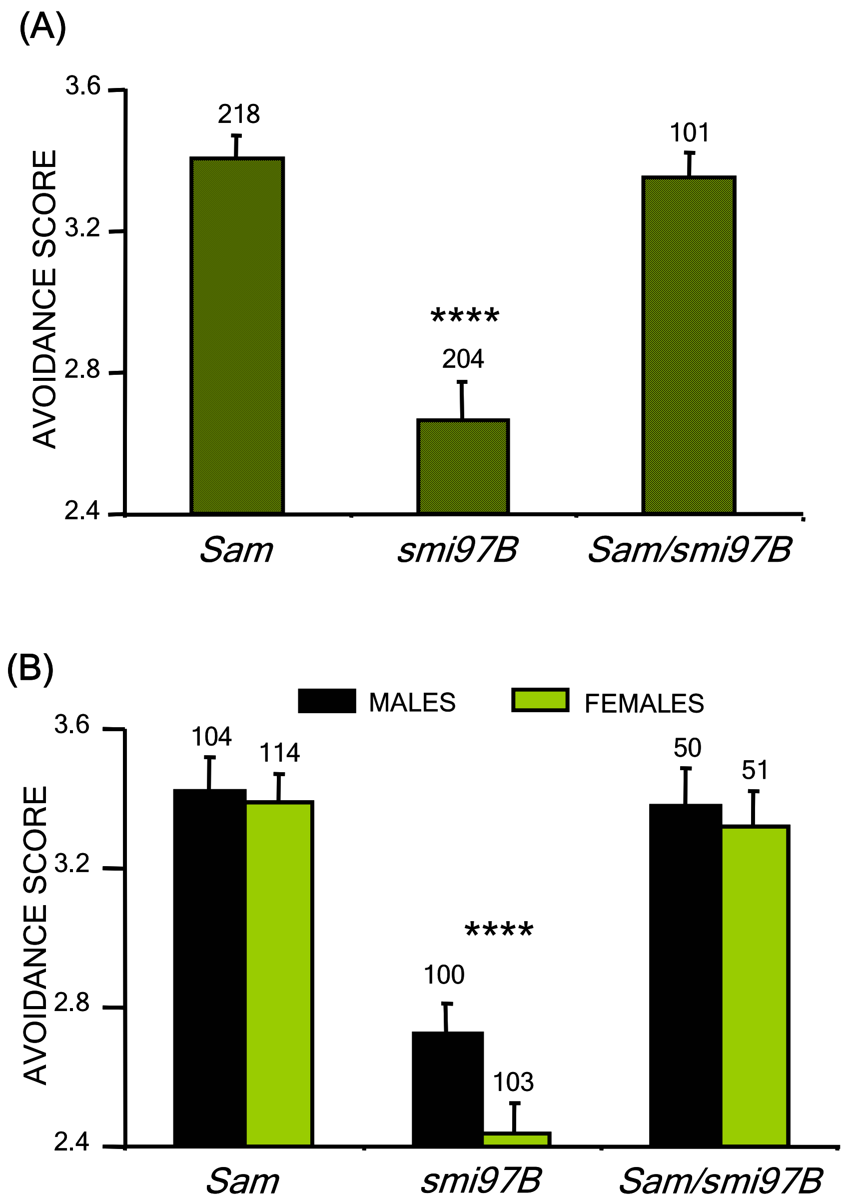


Figure 11: Adult olfactory responses to 0.3% benzaldehyde. (**A**) *smi97B* is a recessive olfactory mutant. Avoidance responses of *Sam, smi97B* and heterozygotes, *Sam/smi97B*. Males and females combined. (**B**) The smell-impaired phenotype of *smi97B* is sexually dimorphic. Avoidance scores of homozygous *smi97B* females were significantly lower than *smi97B* males ($P < 0.05$). (**A**) and (**B**) represent mean avoidance scores with error bars indicating the standard errors of the mean. Number of replicates for each data set is noted above the error bars. (**** $P < 0.0001$).

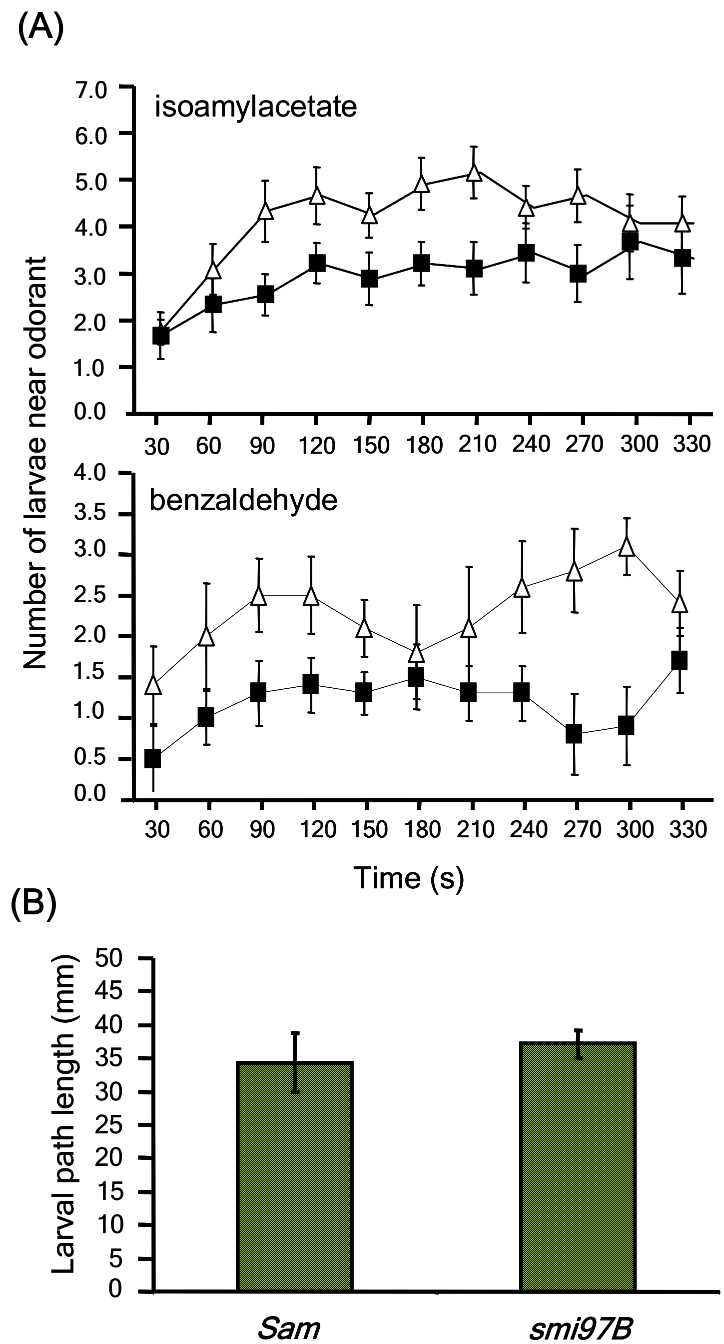


Figure 12: Characterization of larval olfactory behavior. (A)*smi97B* causes deficits in larval olfactory responses to isoamylacetate and benzaldehyde. Fewer *smi97B* larvae (■) respond to the odorant source compared to *Sam;ry506* (Δ) ($P < 0.0001$, for both odorants). Number of larvae present at the odorant source was averaged over 10 replicates for benzaldehyde and 11 replicates for isoamylacetate. Error bars indicate standard errors of the mean. B. Larval motility in the absence of odorants. Motility of *smi97B* and *Sam;ry506* larvae were not statistically different ($P = 0.57$). The bar graphs represent mean larval path lengths recorded over 1 min. Error bars indicate standard error of the mean.

3.2 *P{lArB}* excision restores wild-type olfactory phenotype

If indeed smell-impairment in *smi97B* arises from *P{lArB}*-element mediated gene disruption, and not an unrelated mutation in the third chromosome, it follows that the precise excision of *P{lArB}* would result in the restoration wild-type olfactory responses (Figure 13). *P{lArB}* is a non-autonomous *P*-element whose mobilization requires the introduction of an external source of transposase (see Experimental Methodology). Germ line cells in male progeny that contain the *P{lArB}*-element in the same genome as the introduced transposase source undergo transposition events that may result in the precise excision of the 18.5 kb *P{lArB}*-element. However, imprecise excisions may also occur that either result in the excision of genomic regions flanking the *P*-element, causing deletions, or partial excisions that leave behind remnants of unexcised *P{lArB}* fragments. Since each male undergoes a unique gametic event, lines generated from these males are genotypically distinct and can be scored for olfactory behavior. Avoidance responses of *smi97B* excision lines ranged from complete restoration of wild-type olfactory acuity to sex-specific deficits in olfaction (Figure 14). Following behavioral characterization, the excision lines were molecularly characterized by Southern blot analyses to map the excisions (Figure 15).

Most of the excision lines were phenotypic revertants displaying olfactory responses that were not significantly different from *Sam* (for example, $smi97B^{16A}$; $P = 0.07$). However, two lines displayed sex-specific deficits; $smi97B^{15A}$ males showed significantly reduced olfactory responses compared to *Sam* males ($P = 0.004$), while females were not impaired ($P = 0.19$); and $smi97B^{2A}$ females were hyposmic ($P = 0.017$), while the males were unaffected ($P = 0.86$). Molecular characterization using Southern blot analyses revealed the presence or absence of *P{lArB}* (or fragments thereof) in the genomes of the excision lines. Behavioral revertants such as $smi97B^{16A}$ had undergone no deletions as a result of the excision of *P{lArB}*, and were missing only the *P*-element, rendering them genotypically identical to *Sam*. However, imprecise excisions had resulted in a remnant 3.6 kb unexcised *P{lArB}* fragment in the genome of *smi97B*^{15A} and a 2.5 kb remnant in *smi97B*^{2A} (Figure 15).

DISCUSSION

These data suggest that olfactory phenotypes of *smi97B* can be correlated with the *P{lArB}*-element induced disruption of this locus. Moreover, the identification of *smi97B* alleles, *smi97B*^{15A} and *smi97B*^{2A} provides insights into the regulation of the olfactory phenotype of *smi97B*. The size of the *P{lArB}* element inserted in the *smi97B* locus is correlated with sex-specific olfactory deficits. Based on these observations, it is reasonable to propose that the 18.5 kb *P{lArB}* element insertion in this locus causes the sub-optimal binding of transcription factors to upstream regulatory regions, thus causing olfactory impairments in both sexes, with more severe effects in females. A reduction in the size of the insertion to 2.6 kb causes a less severe disruption and is reflected in the concomitantly milder behavioral phenotype detectable only in females. However, if the transcription factors binding to upstream regulatory regions of the *smi97B* locus are sex-specific, a difference in the size of these factors may dictate their binding specificities to upstream regulatory elements. Hence a 3.5 kb insertion in *smi97B*^{15A} may exclusively alter normal binding of transcription factors only in males, thus causing a lack of mutant phenotype in females. This notion may find moderate support from a recent study that demonstrated that almost 60% of the *Drosophila* transcriptome is sexually dimorphic (Jin *et al.* 2001). However, definitive proof lies in the identification and characterization of the regulatory factors that bind promoter-enhancer elements of this locus. Nevertheless, it is clear from these observations that *smi97B* undergoes complex sex-specific regulation.

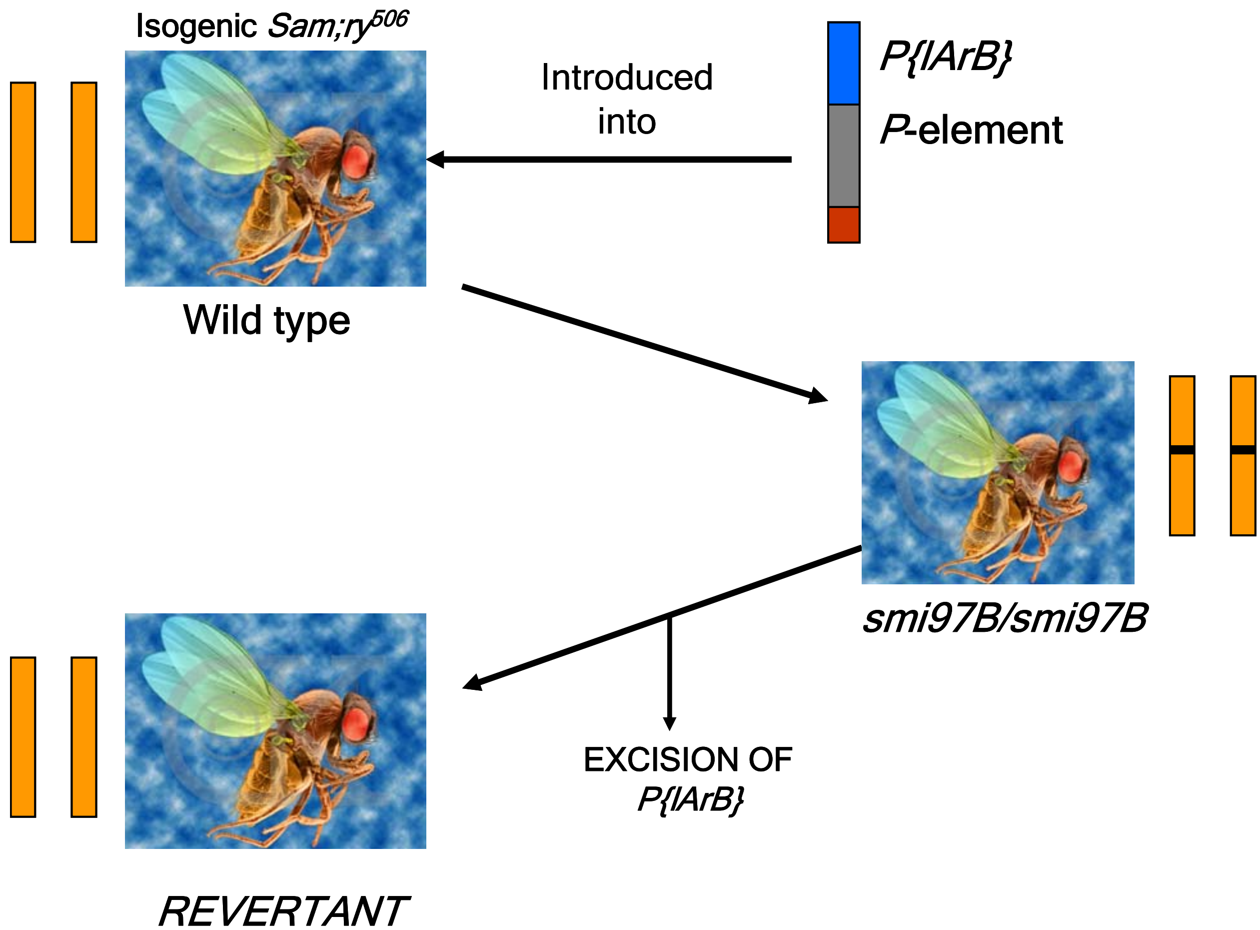


Figure 13: Rationale for reversion experiment

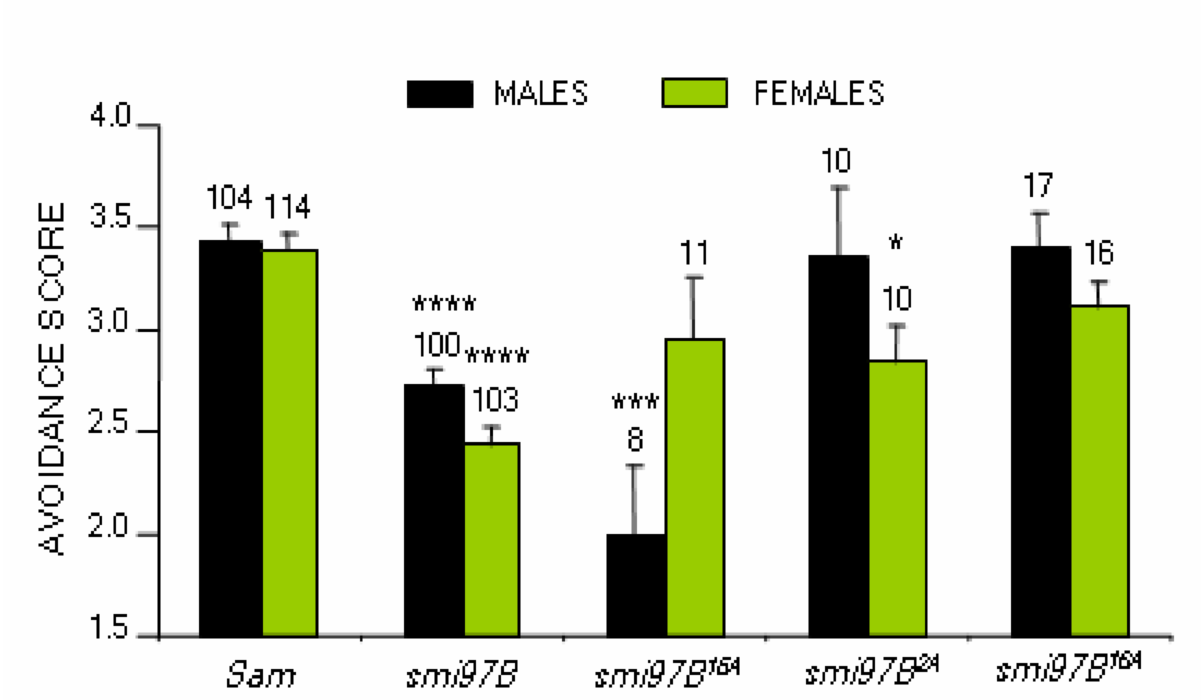


Figure 14: Avoidance responses of *smi97B* excision lines. Avoidance responses of *Sam*, *smi97B*, excision alleles *smi97B*^{15A} and *smi97B*^{2A}, and phenotypic revertant *smi97B*^{16A}. The bar graphs represent mean avoidance scores with error bars indicating the standard errors of the mean. The number of replicates for each data set is noted above the error bars. (* $P < 0.05$, *** $P < 0.005$, **** $P < 0.0001$).

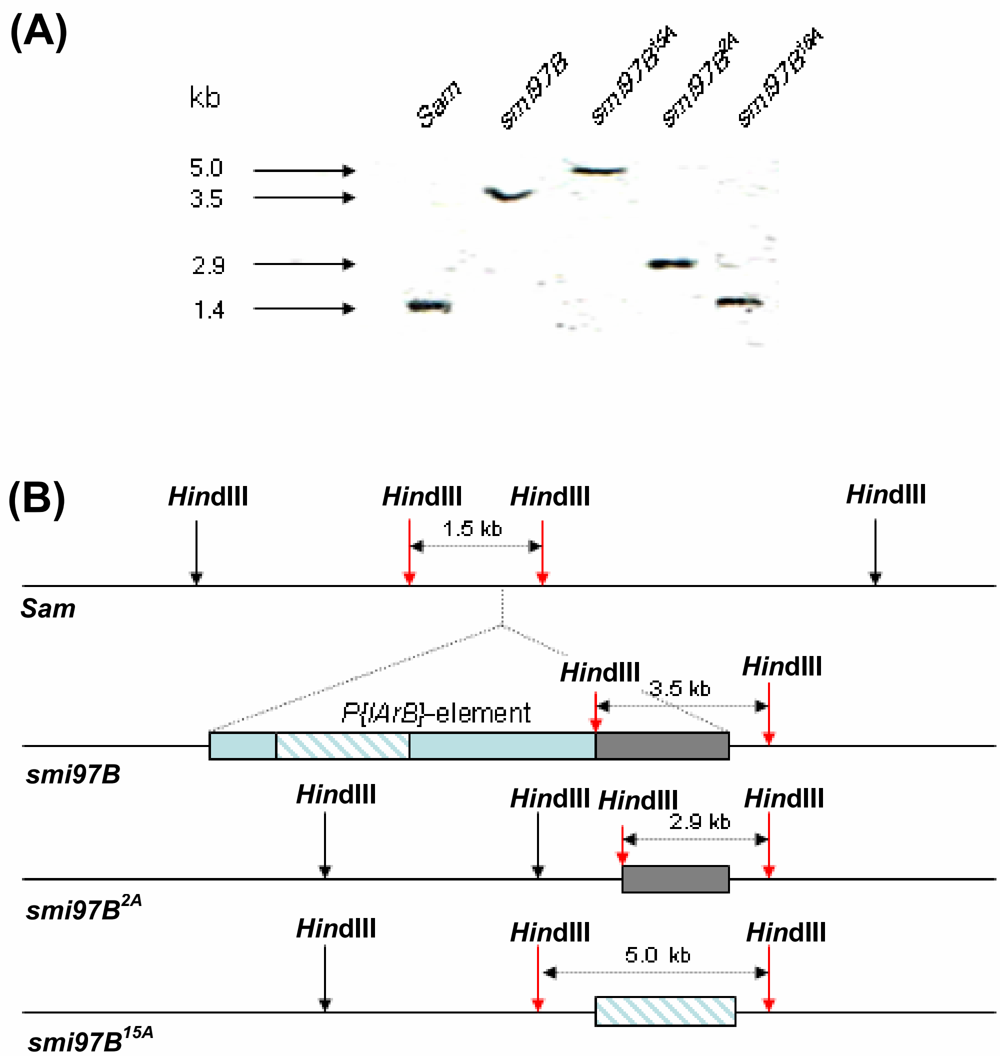


Figure 15: Molecular analysis of *smi97B* excision lines. (A) Southern blot of *Hin*d III digested genomic DNA from *Sam*, *smi97B, smi97B*^{15A}*, smi97B*^{2A} and *smi97B*^{16A}, probed with a [^{32}P]dCTP-labeled 0.54 kb genomic fragment adjacent to the 3′ end of the *P{lArB}*-element. (B) Schematic representation of the Hin*d* III digested fragments detected on the Southern blot. The region digested between the red arrows indicates the fragments that hybridize to the rescued fragment. The grey-shaded box represents the plasmid vector pBluescript and the blue hatched box, the *lacZ* gene; both are contained in the *P{lArB}*-element.

3.3 *scribble* null alleles fail to complement smell-impairment in *smi97B*

One of the obvious advantages of *P*-element mutagenesis is that it molecularly tags its site of insertion, which facilitates the identification of candidate genes in its vicinity. Sequence analysis of the region flanking the *P{lArB}*-element (see Experimental Methodology) revealed that the open reading frame of *scribble* (*scrib*) lies approximately 1kb downstream from its site of insertion. There were no indications from previous studies that *scrib* played a role in olfaction. However, it is highly pleiotropic; involved in epithelial polarity determination in embryonic development (Bilder and Perrimon 2000), in the suppression of neoplastic tumor genesis in a variety of cells (including larval nervous tissue; Bilder *et al.* 2000; Li *et al.* 2001), in immune responsiveness (Wu *et al.* 2001), and finally, in the modulation of synaptic growth and function in the larval neuromuscular junction (Mathew *et al.* 2002; Roche *et al.* 2002). To test the hypothesis that *scribble* is also involved in olfaction, a series of complementation tests between *smi97B* and *scrib* alleles were conducted. No adult phenotypes of *scrib* had been characterized thus far, presumably due to an inability to isolate viable adults from mutational screens that detected only the severest perturbations in the gene. Since *smi97B* is a recessive olfactory mutant, phenotypic characterization of heterozygotes generated by crossing *smi97B* to *scrib* alleles would yield information about the possible role for *scrib* in adult olfaction.

Functional complementation mapping of the *smi97B* phenotype was carried out using both null as well as hypomorphic mutations of *scrib*. Avoidance responses of heterozygotes generated by crossing *scrib* alleles to *smi97B* were compared to the responses of control heterozygotes generated by crossing the same alleles to *Sam*. The deficiency *Df(3R)Tl-X* is a null allele containing a deletion in chromosome *3* that uncovers *scrib* (cytological bands

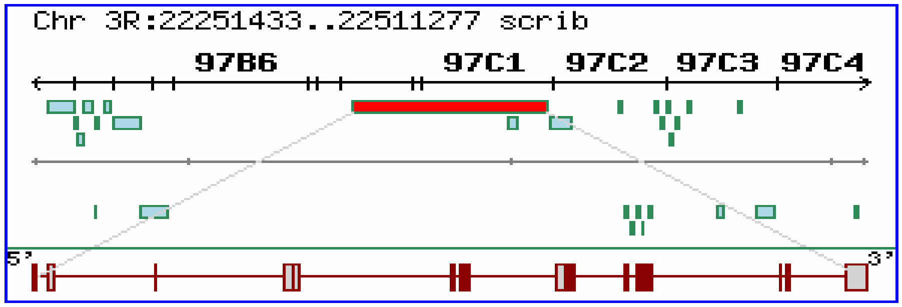
97B to D2). *Df/smi97B* hemizygotes displayed significantly reduced olfactory deficits when compared to the controls, *Df/Sam* (*P* < 0.0001 for both sexes; Figure 17A), with highly significant sex-specific differences (*P* < 0.0001); once again, females were more severely affected than males. Ethylmethylsulphonate (EMS) induced null mutations, *scrib*^{1} and *scrib*^{2} also failed to complement the olfactory deficits of *smi97B*; avoidance responses of *scrib*^{1}*/smi97B* and *scrib*^{2}*/smi97B* were significantly reduced compared to controls (*P* = 0.009 and *P* < 0.0001, respectively; Figures 17B and C). However, sexual dimorphism in the smell-impaired phenotypes of the heterozygotes was not observed. This was possibly due to background genetic effects arising from the *scrib* parental stocks that led to a slight modification of the smell-impaired phenotype.

P{lacW}-induced mutations *scrib*^{S042405} and *scrib*^{j7B3} contain single *P*-element insertions in the 5' untranslated region of *scrib* (approximately, 1.5 kb downstream from the *P{lArB}*-element in *smi97B*) and in the second intron of *scrib*, respectively (Figure 16). Although they are hypomorphic, both mutations cause lethality due to malformation of embryonic epithelia. When crossed to *smi97B*, viable heterozygotes are recovered that can be assayed for olfactory behavior. Avoidance responses of *scrib*^{S0421405}*/smi97B* females were significantly lower than *scrib*^{S0421405}*/Sam* females (*P* < 0.0005), while male responses were not significantly different from control males (*P* = 0.383; Figure 18A). Hence, *scrib*^{S0421405} failed to complement the olfactory deficit caused by *smi97B*, but only in females. On the other hand, *scrib*^{j7B3} fully complemented the smell-impaired phenotype of *smi97B*; i.e. avoidance responses of *scrib*^{j7B3}*/smi97B* flies were the same as *scrib*^{j7B3}*/Sam* controls (*P* = 0.96; Figure 18B).

DISCUSSION

Complementation mapping of *smi97B* provides evidence for the allelic relationship between *smi97B* and *scrib*. All previously identified *scrib* alleles are homozygous embryonic lethals, which can be rescued by generating heterozygotes with *smi97B*. However, null alleles of *scrib* fail to complement the smell-impaired phenotype of *smi97B*, which suggests that in addition to a developmental role, *scrib* is also responsible for odor-guided behavior in adults. The $scrib^{j7B3}$ mutant allele complements the olfactory phenotype of *smi97B*, while $scrib^{S042405}$ displays female-specific failure to complement the smell-impaired phenotype. This implies that the *P{lacW}*-$scrib^{j7B3}$ insertion in the second intron of *scrib* (Figure 15) affects embryonic development, but not olfactory behavior, whereas the $scrib^{S042405}$ insertion disrupts both embryonic development, as well as female-specific olfactory behavior. Hence, intra-genic complementation observed with *P{lacW}*-lethals, $scrib^{j7B3}$ and $scrib^{S042405}$ further strengthens the idea of pleiotropy in *scrib* function and hints at the functional compartmentalization of alternatively spliced *scrib* variants.

(A)



(B)

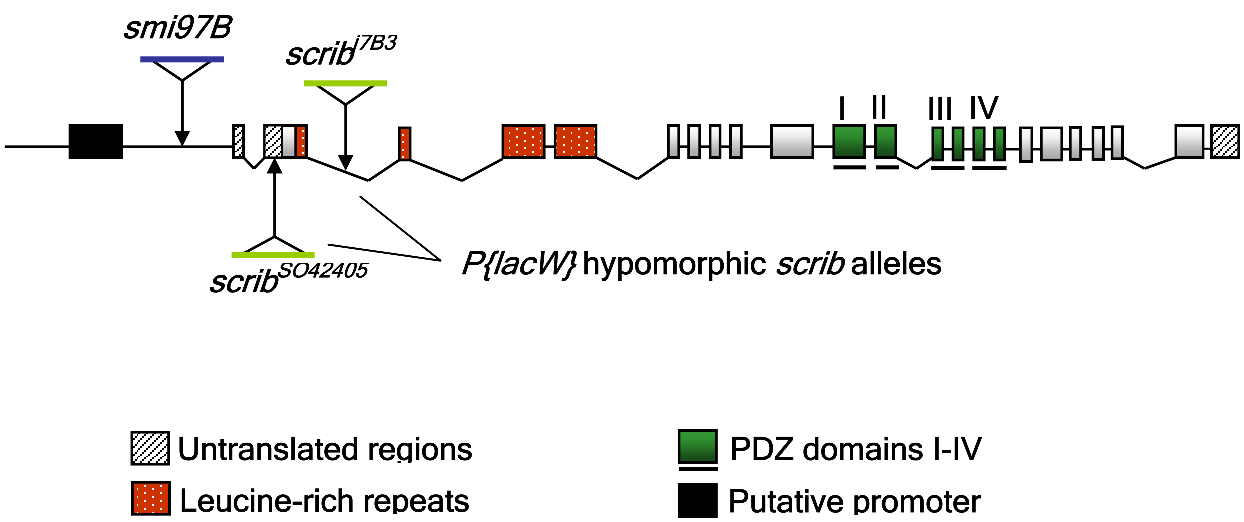


Figure16: Molecular map of *scrib*. (A) Schematic representation of the region in chromosome 3R that contains *scrib*. The deficiency, *Df(3R)Tl-X* spans cytological bands 97B to 97D2. The *P{lArB}*-element insertion in *smi97B* is within band 97B6. **(B)** Schematic representation of *scrib*, showing *P{lArB}* and *P{lacW}* insertions in *smi97B* and *scrib* alleles, respectively.

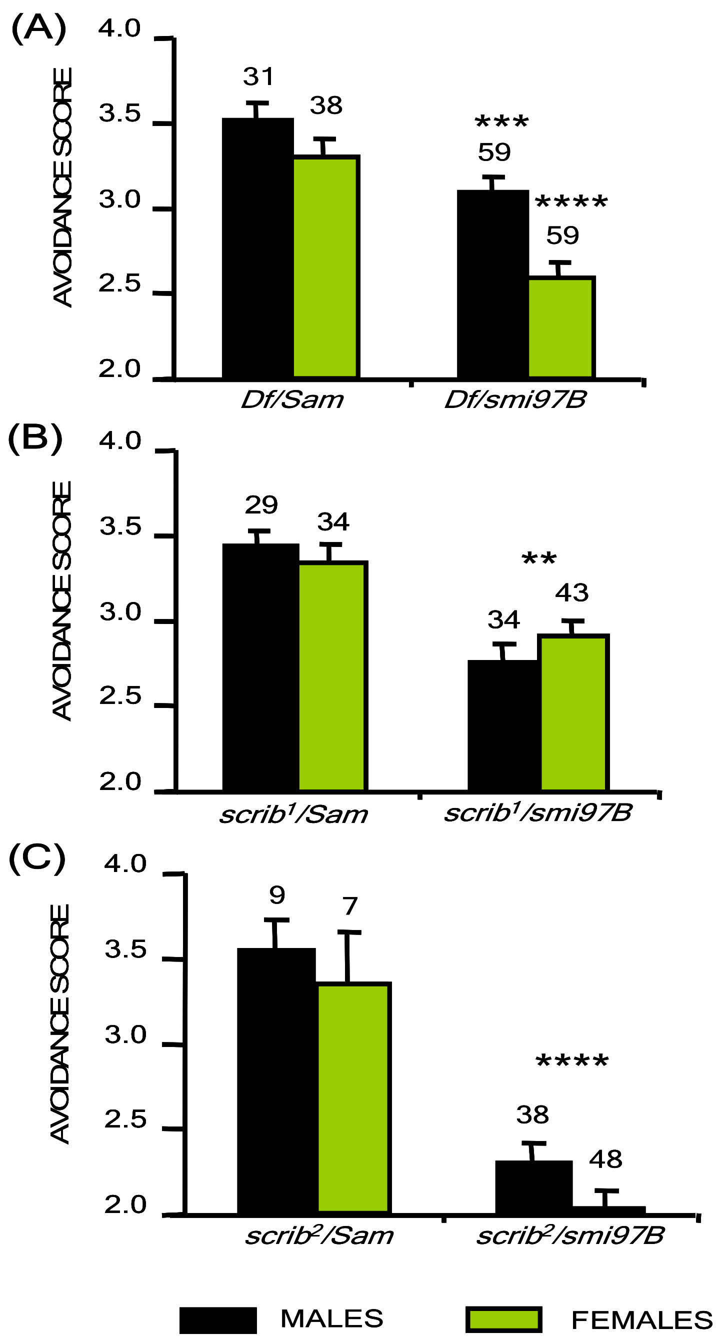


Figure 17: ***Df(3R)Tl-X*** **and** ***scrib*** **null alleles fail to complement the olfactory phenotype of** ***smi97B*****.** Avoidance responses of F1 progeny generated by crossing *Df(3R)Tl-X* (A), *scrib*^{1} (B) and *scrib*^{2}(C), to *Sam* and *smi97B*. Avoidance scores of *smi97B* heterozygotes are compared to *Sam* heterozygotes for each cross. The bar graphs represent mean avoidance scores with error bars indicating standard errors of the mean. Number of replicates for each data set is noted above the error bars. (** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$).

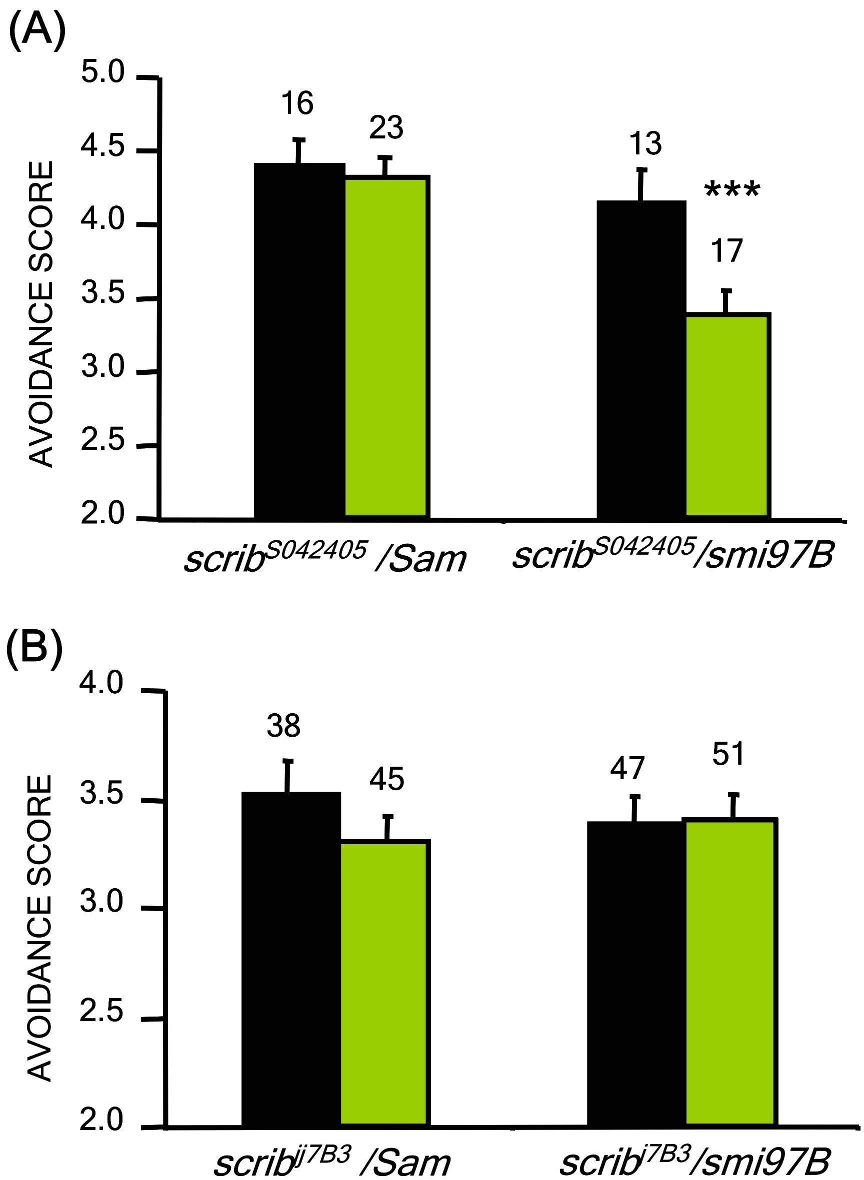


Figure 18: Inter-allelic complementation between *scrib* hypomorphic alleles and *smi97B* Avoidance responses of F1 progeny generated by crossing, *scrib*^{S042405} (A) and *scrib*^{j7B3} (B) to *Sam* and *smi97B* reveal that the smell-impaired phenotype of *smi97B* is complemented by *scrib*^{S042405} only in males, and by *scrib*^{j7B3} in both males and females. Avoidance scores of *smi97B* heterozygotes are compared to *Sam* heterozygotes for each cross. The bar graphs in (A) and (B) represent mean avoidance scores with error bars indicating standard errors of the mean. Number of replicates for each data set is noted above the error bars. (*** $P < 0.005$).

3.4. Transgenic expression of *scrib* rescues olfactory deficits in *smi97B*

Presumably, olfactory deficits in *smi97B* reflect a disruption in gene expression due to the *P{lArB}*-insertion. This implies that impairment in *smi97B* may be reversed by restoring normal gene expression. Moreover, if the introduction of a functional copy of *scrib* into the *smi97B* background restores wild-type olfactory behavior, it provides definitive evidence for the allelic relationship between *smi97B* and *scrib* (Figure 19).

A functional copy of the *scrib* gene was introduced into the *smi97B* background in the form of a transgene on the *X*-chromosome under the regulation of the heat-shock *Hsp70* promoter. The stock containing the *scrib* transgene was obtained from Bilder and Perrimon (2000) and introgressed into the *smi97B* background (Figure 7) to avoid confounding background genetic effects on behavior. The derived transgenic stock, *scrib^{hs};smi97B* was reared at 18° C to prevent activation of the *Hsp70* promoter and subsequent expression of *scrib*. *scrib^{hs};smi97B* flies tested in the absence of heat-shock displayed mean avoidance responses that were significantly lower than *Sam* (P = 0.007), but the phenotype was sexually dimorphic (P = 0.023). Although *scrib^{hs}*;*smi97B* females were significantly smell-impaired (P = 0.003), male responses were not statistically different from *Sam* males (P = 0.53; Figure 20A). When heat-shocked at 37 °C avoidance responses of *scrib^{hs}*;*smi97B* flies were restored to those of the parental strain, *Sam* (P = 0.68 ; Figure 20B). This proved that expression of *scrib* in the *smi97B* background rescues olfactory deficits, suggesting that *smi97B* is a homozygous viable allele of *scrib.*

DISCUSSION

Female-specific smell-impairment of *scrib*^{hs};*smi97B* flies at 25° C may be an indication of sex-specific differences in the penetrance of the smell-impaired phenotype. From a molecular perspective this suggests that *smi97B* females require quantitatively higher levels of gene transcription in order to reverse olfactory deficits, compared to males. Once again, these results in addition to those presented in the previous sub-sections allude to the sex-specific theme that marks the regulation of expression of *smi97B* in adults.

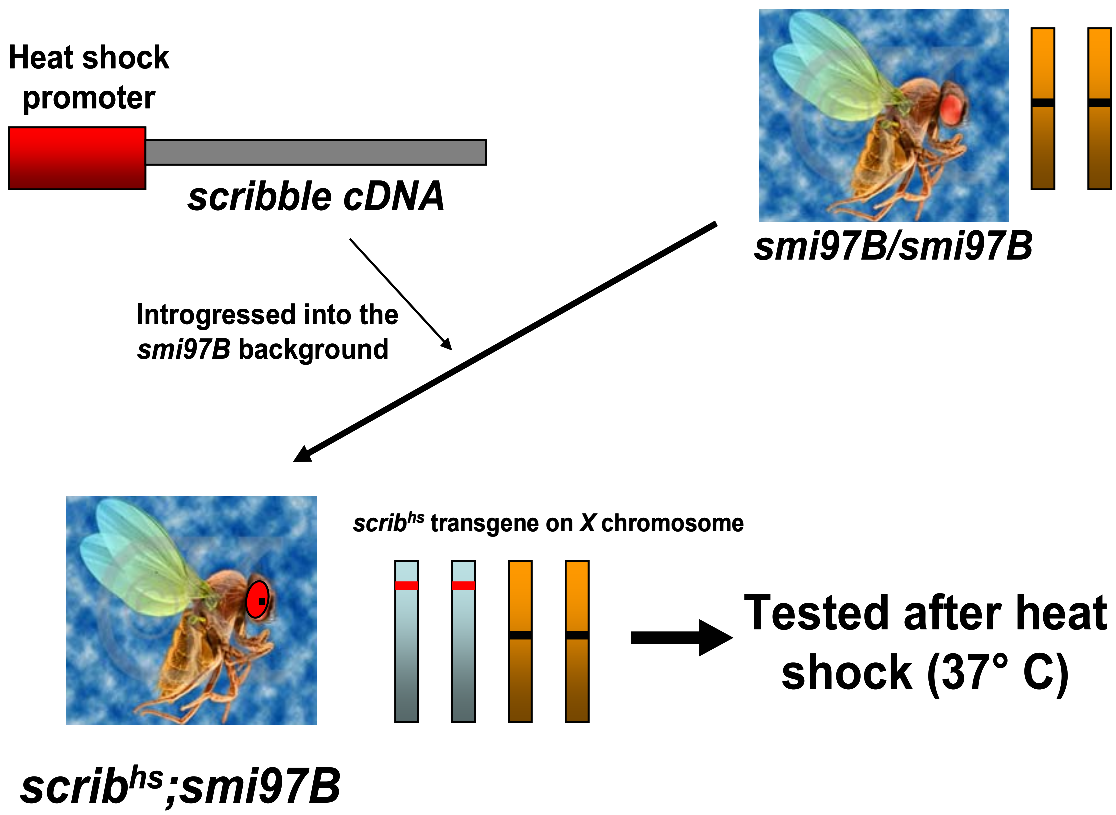


Figure 19: Outline for transgenic experiment.

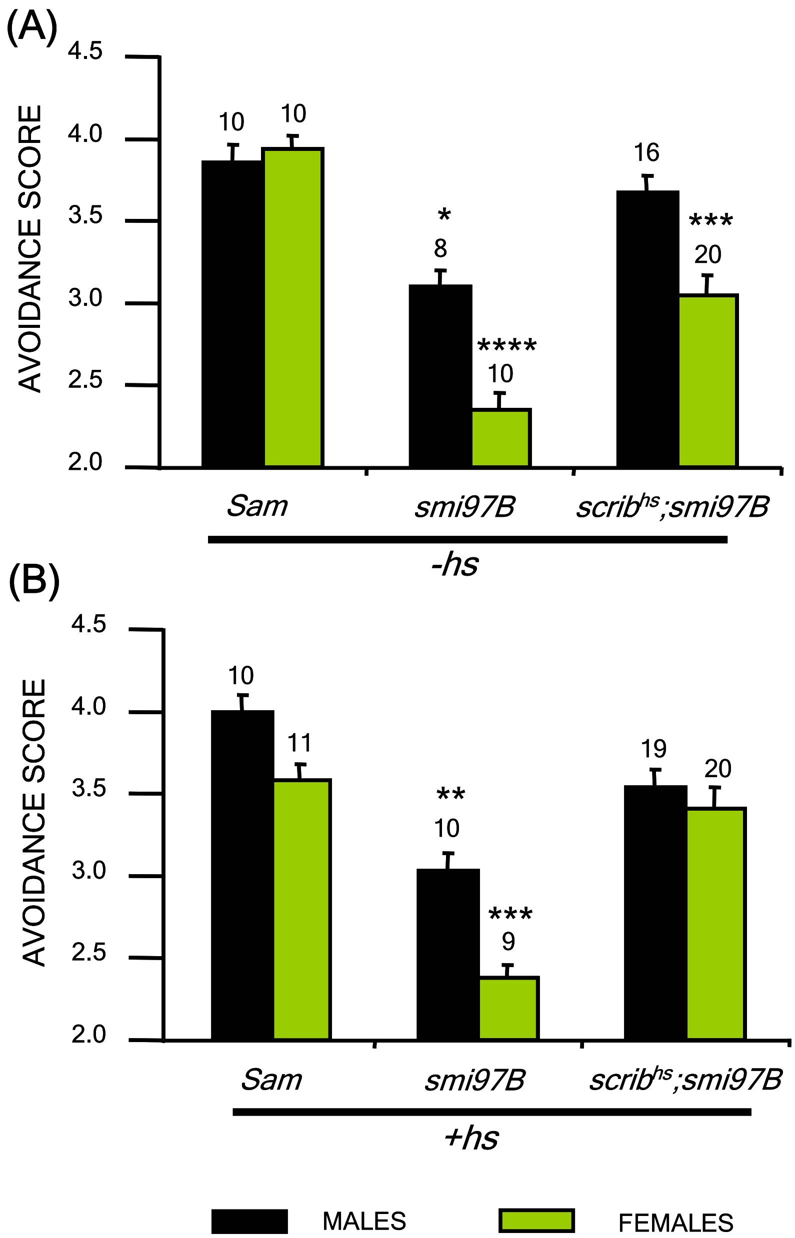


Figure 20: Behavioral rescue of *smi97B* by transgenic expression of *scrib*. Avoidance scores of *Sam*, *smi97B* and the *scrib*^{Hs}*;smi97B* stock (containing a *Hsp-70*-regulated full-length *scrib* cDNA in a homozygous *smi97B* background) recorded at 25° C, (A) and after heat-shock, (B). Partial (male-specific) rescue of the *smi97B* phenotype occurs in the absence of heat-shock due to the leaky expression of *scrib* at 25°. The *smi97B* smell-impaired phenotype is rescued after heat-shock. The bar graphs represent mean avoidance scores with error bars indicating the standard errors of the mean. Number of replicates for each data set is noted above the error bars. (−*hs*; absence of heat-shock; +*hs*; 37° for 45 min; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Conclusion

In conclusion, quantitative genetic analyses of the *smi97B* locus facilitated the identification and phenotypic characterization of the first homozygous viable allele of *scrib*, thereby revealing its role in mediating olfactory behavior in adults and larvae. Genetic dissection of this locus suggests an underlying sexual-dimorphism in the regulation and expression of the olfactory phenotype.

CHAPTER 4:

Molecular genetic analysis of *scrib*^{smi97B}

4.1 *scribble* lies 1 kb downstream from the *P{lArB}* insertion site

P-elements typically cause disruptions in gene expression by preferentially inserting within upstream regulatory regions. Hence, characterization of the genomic regions that flank their site of insertion reveals the identity of the gene/genes that are most likely affected by the insertion. The *P{lArB}*-element, at its 3' end, contains a bacterial plasmid derivative, which can be digested out in a way that it includes a fragment of flanking genomic DNA (Figure 21). This recombinant molecule containing a 584 bp genomic fragment as an insert in the plasmid vector of *P{lArB}* can be circularized, ligated and cloned into *Escherichia coli*. Sequence analysis of the genomic DNA insert (called the "rescued fragment") revealed the *P{lArB}* insertion site to be 1,084 bp upstream from the open reading frame of *scrib.* Presumably, *P{lArB}* had recruited the upstream promoter and enhancer-elements of *scrib* to direct the tissue-specific expression of its reporter gene, *lacZ*, in adult olfactory organs (Anholt, et al. 1996). The genomic organization of *scrib* reveals that it spans over 60 kb within cytological bands 97B6-C2 of chromosome *3*. Its open reading frame contains up to 23 exons that encode a 200 kD scaffolding protein. Scrib contains 16 LRRs (*l*eucine-*r*ich *r*epeats) and 2-4 PDZ domains (modules that are found in the *p*ost-synaptic density-95, *d*iscs-large and *z*ona occludens-1 proteins) that mediate protein-protein interactions within the cell (Figure 16).

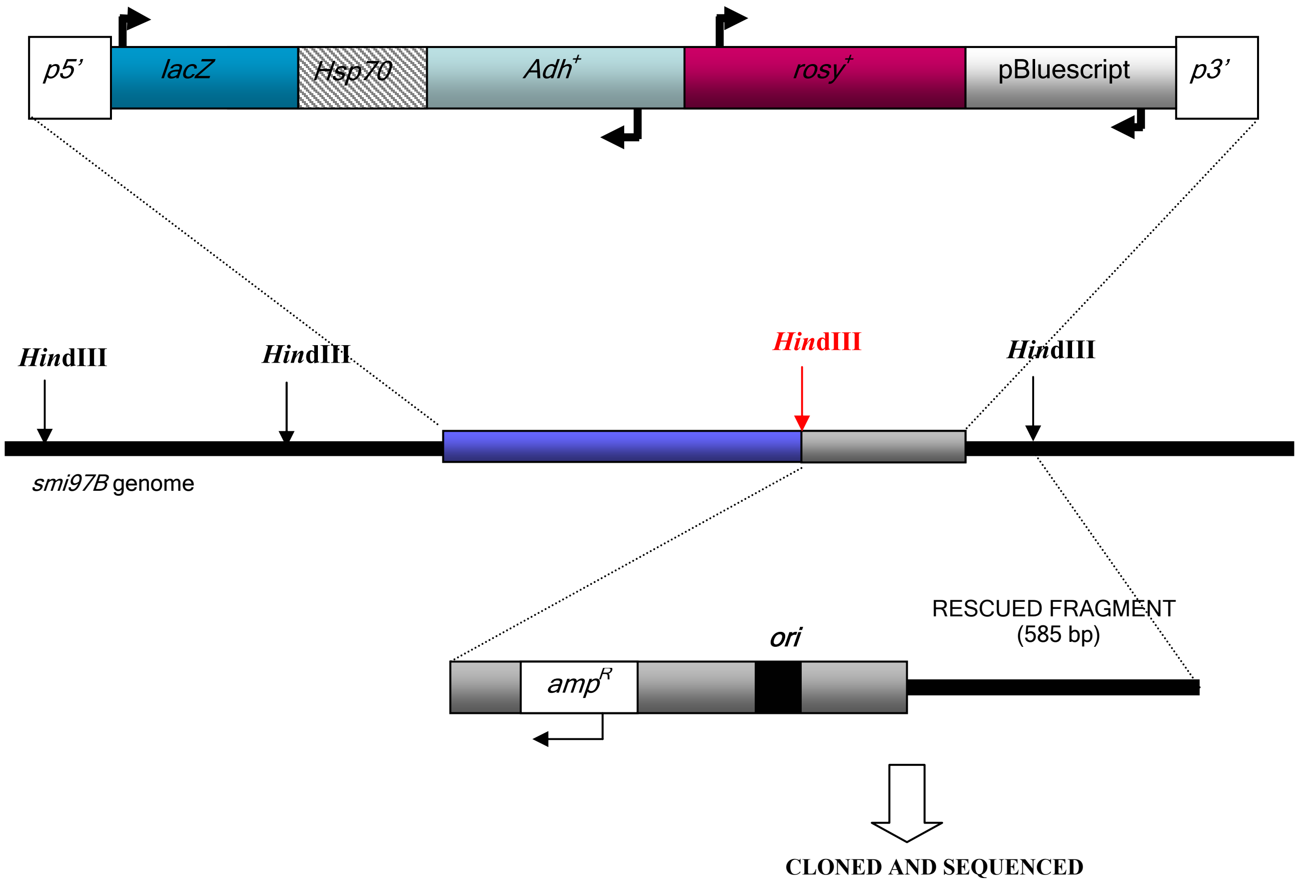


Figure 21: ***P{lArB}*** **rescue of** ***smi97B***: Schematic representation of the molecular map of *P{lArB}* and surrounding genomic region of *smi97B*. *P{lArB}* contains a *Hin*dIII digestion site at the 5' end of its pBluescript vector. Digestion of the genomic DNA of *smi97B* with Hin*d*III releases the pBluescript plasmid vector and a piece of flanking genomic DNA at a downstream Hin*d*III site.

4.2 *scrib* expression is disrupted by the *P{lArB}* insertion

Bilder and Perrimon (2000) had previously demonstrated that *scrib* expressed a single 5.9 kb transcript in embryos that encoded a 200 kD protein. Further studies by Li *et al.* (2001) revealed the expression of 2 Scrib isoforms in larvae, Scrib1 and Scrib2, which are expressed in the larval central nervous (CNS) and the peripheral nervous systerms, respectively. However, they did not detect immunoreactive polypeptides specific to *scrib* in adult tissues. The data reported in this study provide evidence for the sex-specific alternative splicing of *scrib* in the adult, which is correlated with sexually dimorphic olfactory deficits.

Three major RNA species were detected on Northern blots when whole fly extracts were probed by a full-length *scrib* cDNA clone (see Experimental Methodology). The 5.9 kb transcript that was originally reported by Bilder and Perrimon (2000) as being expressed in embryos, was expressed in adults, yet almost undetectable in larvae. On the other hand, a 7.1 kb transcript was expressed predominantly in males and larvae, but to a lesser degree in females. Finally, there was a 4.6 kb transcript that was female-specific (Figure 22).

To establish a link between the expression of these transcripts and the *P{lArB}* insertion, we compared *scrib* expression in adults and larvae of *Sam*, smell-impaired mutant, *smi97B* and a complete behavioral revertant allele, *smi97B*^{16A} . There was an overall reduction in *scrib* expression in *smi97B* compared to *Sam*, while the excision of the *P{lArB}*-element from *smi97B*^{16A} restored *scrib* expression levels to that of wild-type. Specifically, the 7.1 kb *scrib* transcript was reduced in *smi97B* larvae compared to *Sam* and *smi97B*^{16A}. Transcript levels in anosmic *smi97B* females were also decreased compared to controls. In *smi97B* males, however, the reduction in *scrib* expression was less obvious (Figure 22).

In the case of revertant allele *smi97B*^{15A}, which was generated by the imprecise excision of *P{lArB},* significantly smell-impaired males displayed a dramatic reduction in *scrib* transcript levels while behaviorally wild-type females expressed *scrib* at a level that was indistinguishable from *Sam* (Figure 23). Transcript levels were not obviously reduced in mildly hyposmic *smi97B*^{2A} females. Since the *smi97B* mutation in males and the *smi97B*^{2A} mutation in females caused olfactory phenotypes that were relatively less severe, it is possible that reduction in *scrib* expression was concomitantly slight. The inability to detect this reduction on Northern blots may reflect a limitation in the technique in resolving small quantitative differences in transcript expression.

Antibodies raised against a mixture of amino- and carboxyl-terminal peptides of Scrib detected at least 5 immunoreactive polypeptides on Western blots. These included a male-specific 58.5 kD polypeptide, a female-specific 54.0 kD polypeptide, and a common 42.5 kD immunoreactive band (Figure 24). In whole fly extracts, the 58.5 kD male-specific and 54.0 kD female-specific polypeptide bands were reduced in *smi97B* compared to *Sam*, but restored to wild-type levels in the behavioural revertant, *smi97B*^{16A}. The 42.5 kD isoform was unaffected by the *P{lArB}* insertion in *smi97B* and was present at levels that were the same as controls. The 200 kD and 180 kD bands characteristic of embryonic extracts were not detected in adult flies, which is in agreement with the same study that failed to detect these higher molecular weight isoforms beyond late embryonic stages (Li *et al.* 2001).

DISCUSSION

Previous studies have implicated the involvement of *scrib* transcripts in embryonic epithelial development (Bilder and Perrimon, 2000) and tumor suppression (Li, *et al.* 2001) of the larval nervous system. The molecular characterization of *scrib* expression in adult stages was made possible by the isolation the first homozygous viable allele of *scrib*, *scrib*^{smi97B}. The observations in this study reveal that *scrib* expression is reduced in smell-impaired adults, providing a role for *scrib* in olfaction. Furthermore, the identification of male-biased and female-specific transcripts that are correlated with sexually dimorphic olfactory mutant phenotypes suggests that *scrib* undergoes sexually dimorphic transcriptional regulation in adults to contribute to odor-guided behavior. Sex-specific expression profiles of Scrib polypeptides corroborate the results obtained from Northern blot analyses. However, the Scrib polypeptides detected on Western blots are smaller in molecular weight than those predicted by translating the known sequences of *scrib* transcripts. A possible explanation for low molecular weight immunoreactive bands of Scrib may be extensive proteolytic degradation in adults. However, repeated experiments with a panel of protease inhibitors (see Experimental Methodology) included in protein extracts failed to detect the predicted higher molecular weight peptides.

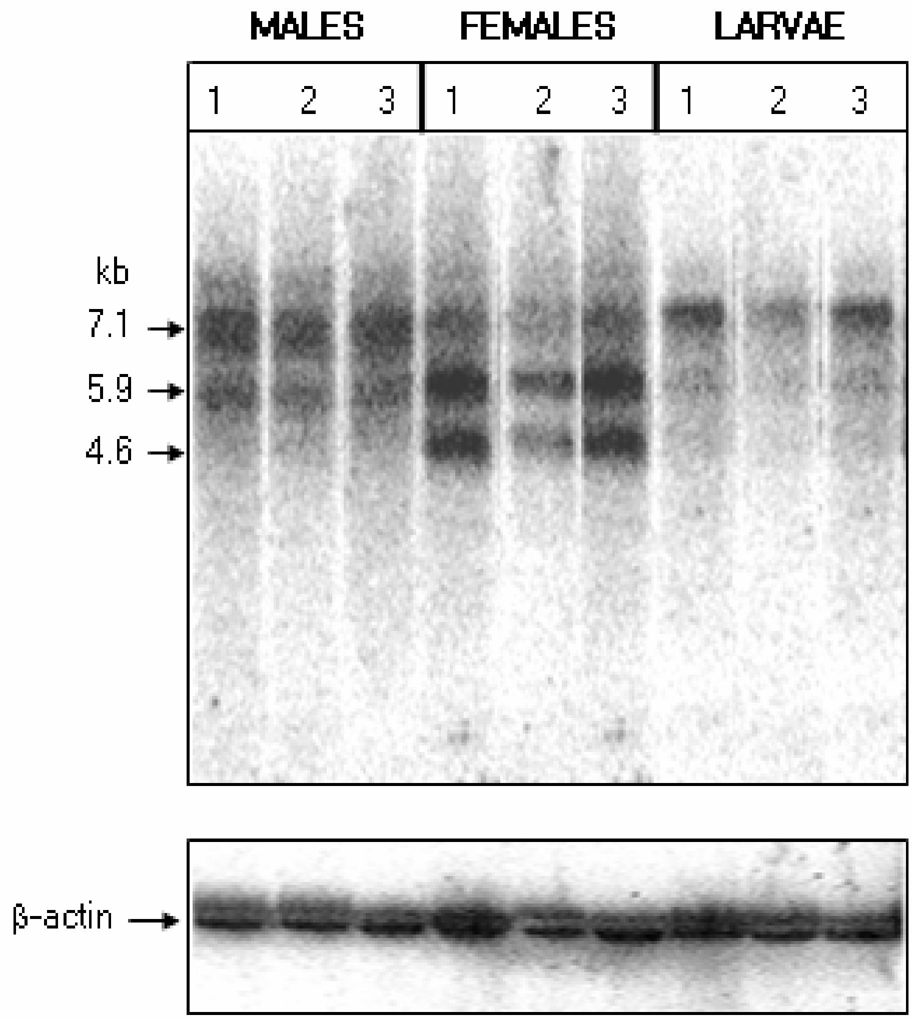


Figure 22: Sex-specific expression of *scrib* through alternative splicing. Northern blot of whole-fly extracts from *Sam* (lane 1), *smi97B* (lane 2) and the phenotypic revertant *smi97B*^{16A} (lane 3). probed with a full-length *scrib* cDNA (upper panel) detects three major transcripts (arrows). The same blot was probed with β-actin to control for equal loading of the lanes (lower panel). Note the reduction in message in the *smi97B* mutants.

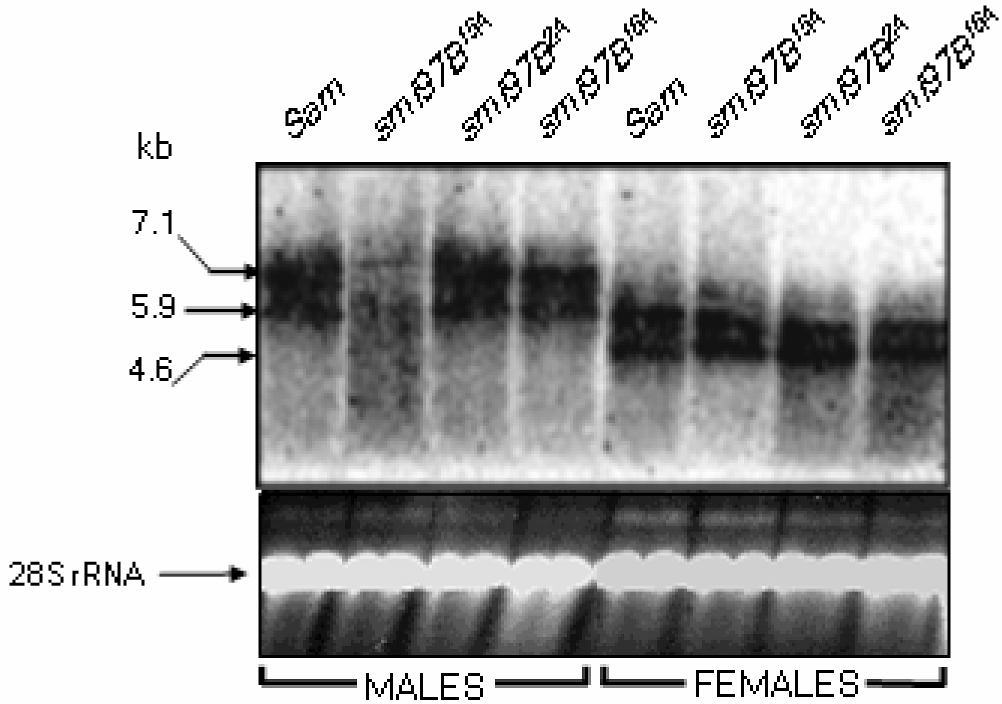


Figure 23: Analysis of *scrib* mRNA levels in males and females of excision alleles. A reduction in *scrib* levels is detected in smell-impaired males of *smi97B*^{15A}, but not in behaviorally wild-type *smi97B*^{15A} females. The lower panel shows ethidium bromide stained 28S rRNA bands to control for equal loading.

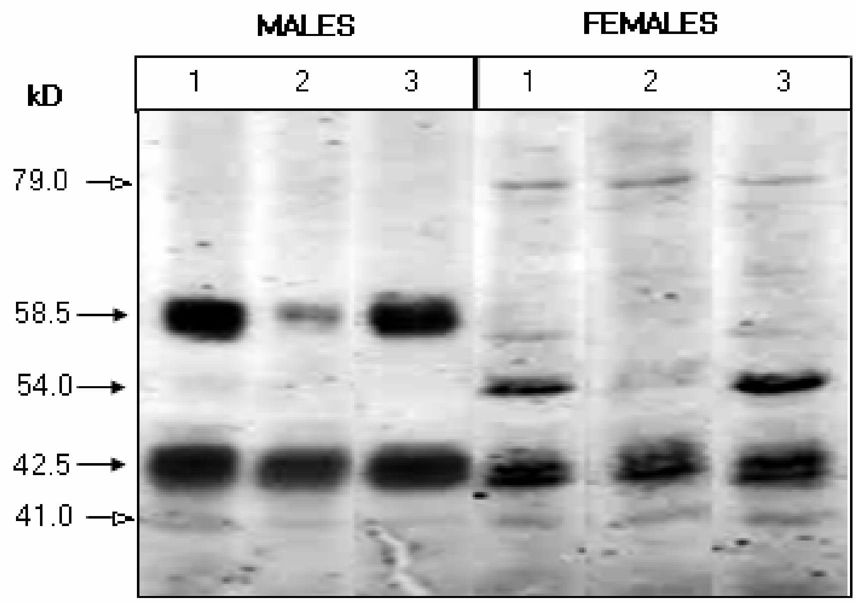


Figure 24: Western blot analysis with anti-sera raised against N- and C-terminal Scrib peptides. Protein isolates from *Sam* (lane 1), *smi97B* (lane 2) and *smi97B^{16A}* (lane 3) were incubated with Scrib antibodies. Three major polypeptide bands (closed arrows), along with minor immunoreactive bands (open arrows) are detectable. Note sex-specific expression of Scrib, and the reduction of the 58.5 kD and 54 kD bands in *smi97B* males and females, respectively.

4.3 The molecular correlates of transgenic rescue of *smi97B*

Behavioral characterization showed that the heat-induced transgenic expression of *scrib* in the *smi97B* homozygous background rescued smell-impairment (Figure 20). To correlate these observations with molecular data, whole fly extracts from *Sam*, *smi97B*, and *scrib*^{hs};*smi97B*, before and after heat shock, were incubated with antibodies against the carboxyl-terminal peptide of Scrib. Immunoreactivity was almost undetectable in whole-fly protein extracts from *scrib*^{hs};*smi97B* flies raised at 18 °C (a non-permissive temperature for the *Hsp70* promoter); as was the case for *smi97B* homozygous mutants. However, when *scrib*^{hs};*smi97B* flies were heat-shocked to induce Scrib expression, the intensity of immunoreactivity increased to levels that were comparable to those in *Sam* (Figure 25).

DISCUSSION

Interestingly, the antibodies used in this experiment were raised against the carboxyl-terminal peptides of Scrib (rAb Scrib1) and detected a 120 kD isoform in both sexes and a 80 kD female-specific isoform (both of which are significantly reduced in *smi97B* compared to controls). These did not match the immunoreactive polypeptides that had been detected by antibodies raised against a mixture of amino- and carboxyl-terminal peptides of Scrib (rAb Scrib), used in the experiment described in the previous section (Figure 24). Pre-incubation of the rAb Scrib1 antiserum with the synthesized Scrib carboxyl-terminal peptides against which it was raised, abolished its immunoreactivity. This taken together with the observation that immunoreactivity was reduced in *smi97B* and non-heat shocked transformants, but restored in phenotypic revertants and heat-shocked transgenic flies, establishes the specificity of its binding. Hence, differences in the immunoreactive bands detected by the two antisera may be attributable to the predominant antigenicity of the amino-terminal Scrib peptide compared to the carboxyl-terminal peptide, in the mixture of peptides used to raise the antiserum. It also suggests the possibility of amino- and carboxyl-terminal modifications in different subsets of Scrib peptides that preclude the detection of all peptides by any one of the two antisera.

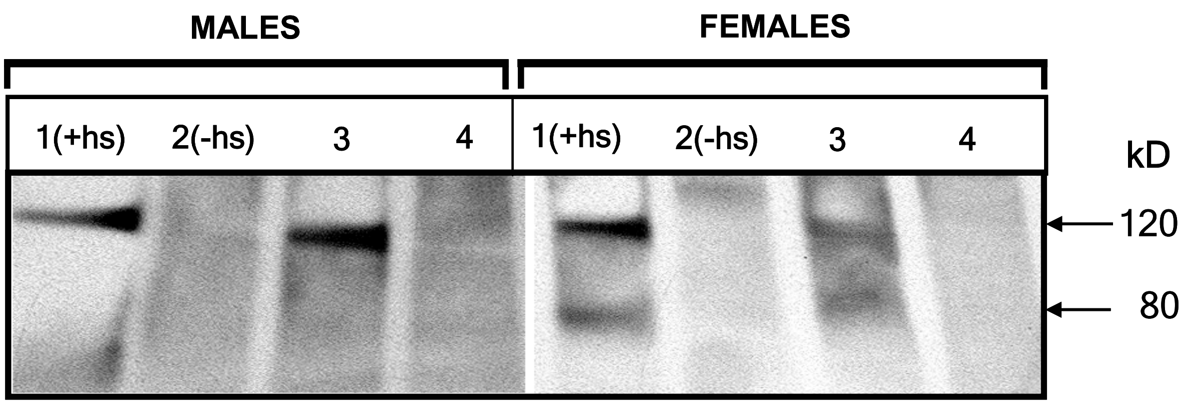


Figure 25: Molecular correlates of transgenic rescue. Immunochemical analysis of Scrib expression in *scrib^{hs};smi97B* following heat shock 1(+hs), *scrib^{Hs};smi97B* raised at 18° C 2(-hs), *Sam* (3) and *smi97B* (4). The antibody used in this experiment was raised against a C-terminal polypeptide of Scrib and detects a 120 kD band in both sexes, in addition to an 80 kD female-specific band.

4.4. A diversity of hitherto undetected *scrib* splice variants: a case for pleiotropy

The variety of protein isoforms detected by antibodies raised against Scrib peptides raised the possibility of additional splice variants of *scrib*. Since Northern blots are primarily used to obtain gross estimations of transcription, it was possible that rare transcripts had not been detected using this technique. Hence, a *Drosophila* head cDNA library screen was conducted in order to isolate additional, less prominent *scrib* transcripts.

Out of a total of 2 X 10^5 phage recombinants plated, eleven clones in the head phage library hybridized to a full-length *scrib* cDNA probe. Of these, seven had unique inserts, which were sequenced and translated *in silico* (Figure 26 and 27). The sizes of three inserts corresponded with splice variants detected on Northern blots (Figure 26 *a, b* and *c* and figure 22), but the remaining four are presumably rare, since they had escaped detection. cDNA clone *a* is 7.1 kb in length and corresponds with the transcript detected in males and larvae on Northern blots. It encodes a putative protein with 16 LRRs and 4 PDZ domains and contains a unique 3' untranslated exon. A 5.9 kb clone (clone *b*), identical to clone *a*, but missing the 3' untranslated exon, corresponds with the transcript detected in both sexes and larvae. Although the proteins encoded by both these cDNAs are identical, the 3' untranslated sequence of the male and larval biased 7.1 kb transcript may be responsible for tissue-specific or temporal expression of the protein. Finally, there is a 4.6 kb cDNA clone encoding 16 LRRs, 2 PDZ domains (only I and II) and a unique coding exon at its 3' end. This clone (clone *c*) corresponds with the female-specific transcript detected on Northerns. The four additional clones (*d*, *e*, *f* and *g*) share the first 285 bases of the last coding exon of clone *c*. Clones *d* and *e* are identical and encode 4 PDZ domains, but differed in their number of

LRRs; *d*, coding 16 and *e*, 13 (Figure 26 and 27 *d* and *e*). In contrast to the 4.6 kb female-specific transcript that only encodes for PDZ domains I and II, clones *f* and *g* encode only PDZ domains III and IV (Figure 26 and 27 *f* and *g*); with *f* lacking the LRR region altogether and *g* encoding only 13. In addition, *f* encodes a unique stretch of amino acids at its carboxyl-end (Figure 27 *g*).

DISCUSSION

The diversity of cDNA clones isolated from *Drosophila* heads suggests an array of functions effected by *scrib*. LRRs and PDZ domains mediate protein-protein interactions that lead to the assembly, trafficking and localization of proteins in sub-cellular regions (see Introduction). Sub-cellular scaffolds are critically important for the normal functioning and development of metazoan cells. This is especially the case for polarized cells that must necessarily be divided into sub-cellular domains that have restricted macromolecular assemblies mediating specific cellular functions.

Functional analysis of the multi-PDZ domain proteins, CAP70 (Cystic fibrosis transmembrane conductance regulator-associated protein, 70 kD) and NHERF (Na+/H+ exchanger regulatory factor) suggests that the function of PDZ-domain proteins are not restricted to passive protein-assembly, but may also be involved in the modulation of activity of the various channels and receptors they may be associated with (Bezprozvanny and Maximov 2001; Moyer *et al.* 2000; Raghuram *et al.* 2001). Furthermore, the stereo-conformational positioning and tandem combinations of PDZ domains within these proteins were also found to be critical for normal CFTR (Cystic fibrosis transmembrane conductance regulator) channel activity (Raghuram *et al.* 2001; Wang *et al.* 2000). The splice variants of *scrib* isolated from adult head libraries, in this study, primarily differed with respect to the number and position of its protein binding modules. It is likely that these differences may contribute to functional differences between the various *scrib* products by determining both the nature of the binding partners they recruit, as well as their effects on these interacting proteins.

The unique combination of 16 canonical LRRs (23 amino acids each) coupled with 1-4 PDZ domains, places Scrib within an emerging family of LAP (*L*RRs *a*nd *P*DZ) proteins that have only been isolated in metazoans. In addition to these protein domains, LAP proteins also contain a conserved LAP-specific domain (LAPSD) at the carboxy-terminal of their LRRs. Densin-180 was the first member of the LAP protein to be characterized and was found to be tightly associated with the post-synaptic density of glutamatergic neurons expressed in the rat forebrain, where it is phosphorylated by the Ca^{++} sensitive enzyme, CAMKII (Apperson *et al.* 1996). Studies on the *Drosophila* neuromuscular junction reveal that Scrib is involved in mediating short term plasticity by rapidly recruiting synaptic vesicles into the readily releasable pool of vesicles in the pre-synaptic cell (Roche *et al.* 2002). The diversity of *scrib* splice variants isolated from the adult head cDNA library suggests that multiple isoforms of Scrib may be responsible for the organization of a variety of protein assemblies at the pre- and post-synaptic regions of neurons. Furthermore, sex-specific differences in expression of Scrib isoforms may provide the molecular basis of sexually dimorphic neuronal circuitry that contributes to odor-guided behavior in *Drosophila melanogaster*.

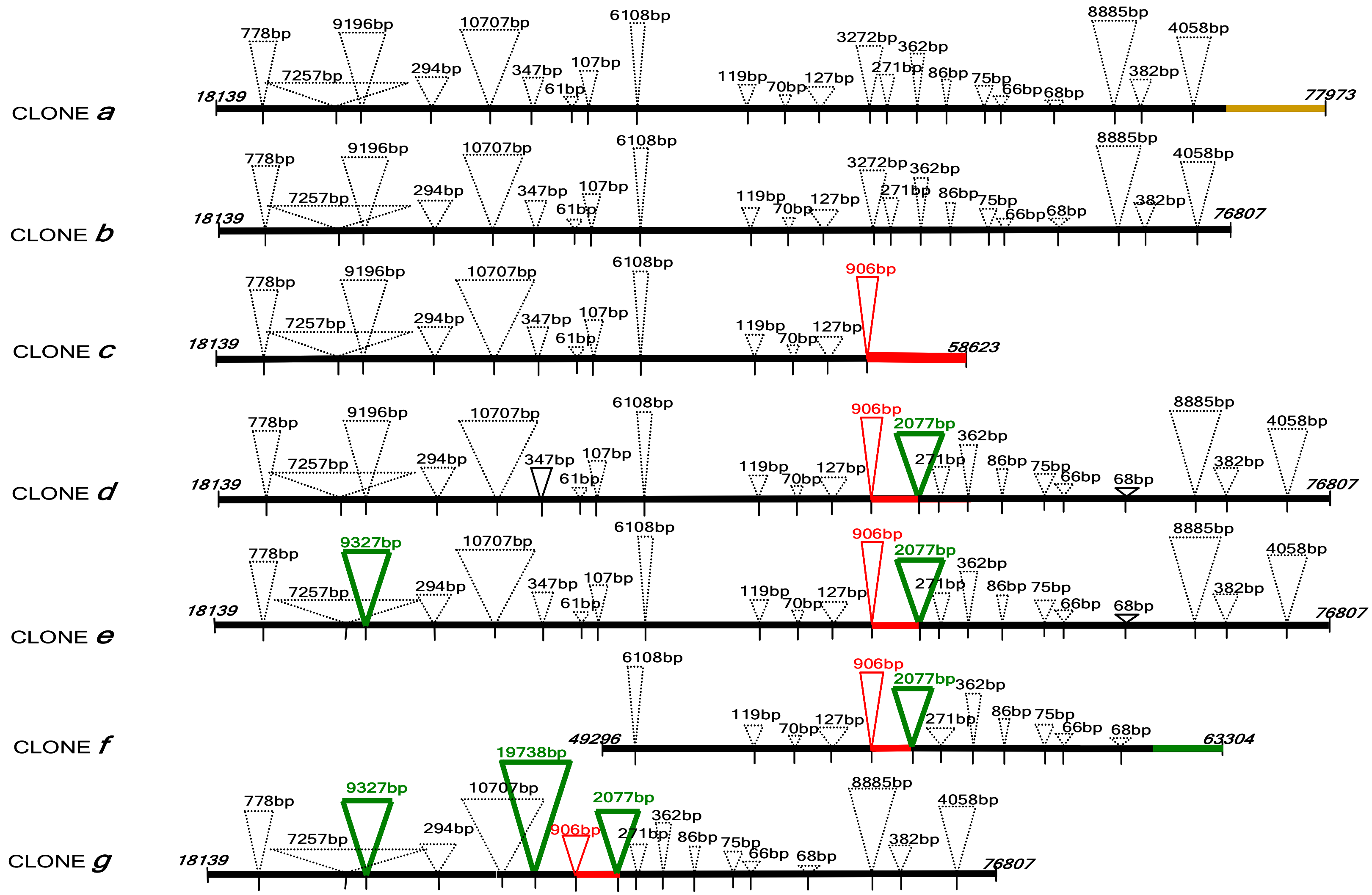


Figure 26: Alternatively spliced variants of *scrib* isolated from a head cDNA library. Genomic organization of *scrib* cDNA clones, *a-g*. Loops above solid black line represent spliced out intronic sequences and the heavy horizontal line indicates exonic sequences. Mustard, green and red lines represent introns and exons that are common to just one (clone *a*) or a subset of clones.

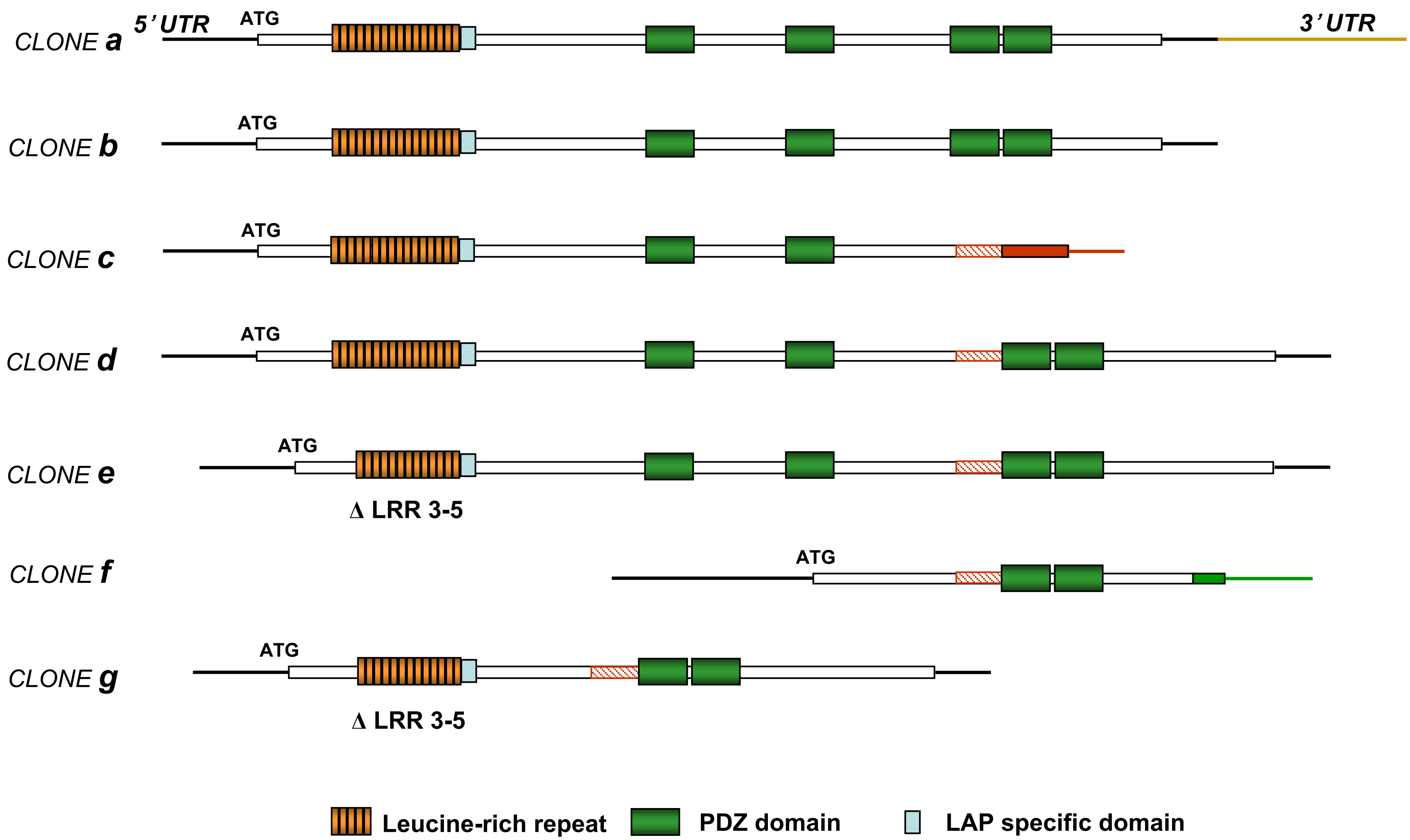


Figure 27: Schematic representation of polypeptides translated from *scrib* cDNA sequences

Conclusion

Molecular analysis of *scrib* in the adult demonstrates that sexually dimorphic olfactory deficits in *smi97B* and its excision alleles arise from a reduction in *scrib* expression. Conversely, the introduction and transgenic expression of *scrib* in the *smi97B* mutant background restores olfactory function. Perturbations in expression levels in only a subset of *scrib* products that are specifically associated with olfactory deficits corroborate observations from the behavior-genetic experiments and suggests pleiotropy in *scrib*. The identification of sex-specific *scrib* products in the adult suggests that it undergoes developmental regulation of expression required for sexually dimorphic olfactory behavior in adult ontogenic stages.

These observations provide a link between the sex-specific transcriptional regulation of *scrib* and sexually dimorphic deficits in olfactory behavior in adult flies.

CHAPTER 5

Expression of *scrib* in the adult nervous system

5.1 Reporter gene (*lacZ*) expression in adult antennae

Behavior-genetic characterization provides evidence for the requirement of *scrib* in olfactory behavior. To further support these observations, the expression patterns of *scrib* in adult tissues, especially in the organs involved in olfaction, were investigated.

Enhancer trap technology (O'Kane and Gehring 1987) enables the visualization of endogenous gene expression patterns of a locus disrupted by a *P*-element insertion, by proxy. Since *P*-elements display an insertion site preference for non-coding regions of genes (Kelley *et al.* 1987), a reporter gene spliced into a *P*-element construct may recruit *cis*-acting promoter enhancer elements of a flanking genomic locus to direct its own expression in a tissue-specific manner. In this case, the *lacZ* reporter gene contained within the inserted *P{lArB}*-element in *smi97B* and the *P{lacW}*-element in *scrib* alleles, $scrib^{j7B3}$ and $scrib^{S042405}$ is under the regulation of a weak (*Hsp*) promoter (Figure 21), but may recruit *cis*-acting promoter and enhancer elements of *scrib* to direct its own expression in a manner that mimics endogenous *scrib* expression.

Staining for *lacZ* can be visualized by assaying for β-*galactosidase* activity, which is the enzyme it encodes. Enhancer trap staining in whole flies revealed diffuse *lacZ* expression in the third antennal segment of *smi97B* (Figure 28A, C, E). Previously, at higher magnification, it was demonstrated that *lacZ* staining in the third antennal segment of *smi97B* contained a few foci of intense staining that corresponded to cells present at the base of the arista (Anholt, *et al.*1996), a feather-like projection on the antennae, required for mechanosensory responses (Gopfert and Robert 2002; Figure 28G). The excision allele, $smi97B^{15A}$ retains a portion of the *P{lArB}*-element that comprises the *lacZ* gene (Figure 15). Staining for *lacZ* expression in $smi97B^{15A}$ revealed diffuse reporter gene expression localized to the third antennal segment (Figure 29). On the other hand, *smi97B*^{2A} is missing the *lacZ* reporter gene from the 2.5 kb *P{lArB}* remnant in its genome (Figure 15) and does not stain for reporter gene expression (Figure 29). *scrib*^{j7B3} and *scrib*^{S042405}alleles contain *P{lacW}*-elements inserted in the 5' untranslated region of *scrib* and it's second intron, respectively (Figure 16). Staining for *lacZ* in *scrib*^{j7B3} revealed no staining in the adult olfactory organs (Figure 29). However, *scrib*^{S042405}, which failed to complement the smell-impaired phenotype of *smi97B* in females, displayed intense *lacZ* staining in the third antennal segments of adult males and females (Figure 29). No sex-specific differences in *lacZ* expression patterns were observed for any of the lines.

DISCUSSION

lacZ expression in the third antennal segment of *smi97B*, *smi97B*^{15A} and *scrib*^{SO42405} suggests that the enhancers recruited by the *P*-element constructs in these lines direct *scrib* expression in adult olfactory organs. Multiple enhancer-promoter elements directing expression in different cell types may indicate the segregation of the multiple *scrib* transcripts identified in this study (Figure 26 and 27), in adult olfactory organs. Hence, the localized expression of *lacZ* in a few cells at the base of the arista in *smi97B* could represent a subset of *scrib* transcripts that are expressed in a cell-specific manner within the antennae. This supports the notion that multiple transcripts of *scrib* that organize compositionally variant protein assemblies may be restricted in their expression to specific regions of the olfactory organs in order to perform their separate functions.

The wild-type, *Sam* and *smi97B*^{2A} do not contain the *lacZ* reporter construct and hence were used as controls for this procedure. There is a conspicuous lack of *lacZ* staining in the *P{lacW}* mutant, *scrib*^{j7B3}. This may be explained in one of two ways: (1) by inserting into the second intron of *scrib*, the *lacZ* reporter in the *P{lacW}* element in *scrib*^{j7B3} is unable to recruit enhancers to direct its expression, or, (2) the *lacZ* reporter recruits enhancers of *scrib* that do not direct expression in the olfactory organs of the fly. Genome sequence analyses coupled with promoter-prediction bioinformatics tools reveal that the *P{lArB}*-element in *smi97*, which lies 1,084 bp upstream from the open reading frame of *scrib*, lies 2 kb downstream from a putative promoter (Figure 30). This promoter presumably directs diffuse expression of *lacZ* in the olfactory organs, as well as intense expression in a few cells at the base of the arista. The *P{lacW}* insertion in *scrib*^{SO42405} is in the 5' untranslated region of *scrib*. Although, its insertion site has not been molecularly characterized, sequence analyses tools reveal the presence of a second putative promoter that lies within the 5' untranslated region of *scrib* (Figure 30). Since, the *P{lacW}* insertion in *scrib*^{SO42405} disrupts olfactory behavior in a female-specific manner (See Section 3.3), it is possible that the promoter flanking this *P*-element directs the expression of female-specific *scrib* transcripts throughout adult olfactory organs. Finally, in the case of *scrib*^{j7B3}, there is a third predicted putative promoter that lies 175 bp upstream from its *P{lacW}* insertion in the second intron of *scrib* (Figure 30). Olfactory responses of *scrib*^{j7B3}/*smi97B* heterozygotes were non-significantly different from wild-type (Figure 18), which suggests that the *P{lacW}* insertion in *scrib*^{j7B3} may not disrupt olfactory function in adults. This is consistent with a lack of *lacZ* expression in olfactory organs of *scrib*^{j7B3} mutants (Figure 29).

Enhancer trap analysis provides preliminary clues about the tissue-specific expression pattern of a gene that is mutated. However, the pattern of β-galactosidase activity may not always precisely coincide with the expression pattern of the flanking gene and thus, confirmatory tests require further investigation using molecular probes directed against *scrib* gene products.

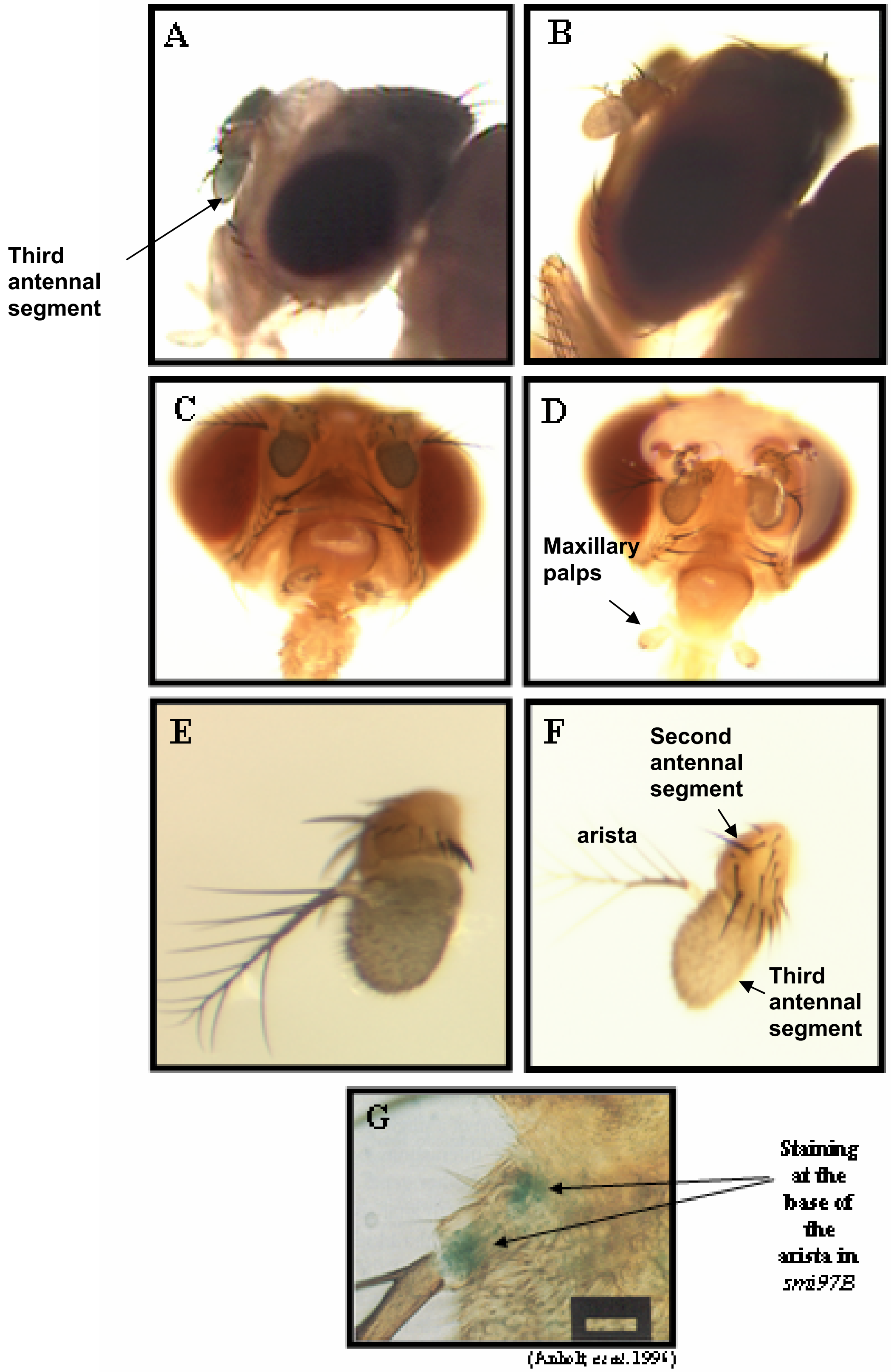

A
B
Third antennal segment
C
D
Maxillary palps
E
F
Second antennal segment
arista
Third antennal segment
G
Staining at the base of the arista in *smi97B*
(Anholt et al. 1996)

Figure 28: Enhancer trap staining of *smi97B*. Staining for *β-galactosidase* activity in whole flies reveals diffuse staining in the third antennal segment of *smi97B* (A, C, E), but no staining in wild-type, *Sam* flies that do not contain the *P{lArB}*-construct. (G) Magnified view of the third antennal segment of *smi97B* showing intense punctate staining at the base of the arista, which is a mechanosensory organ of the fly. Panel (G) is a reproduced image from Anholt, *et al.* 1996.

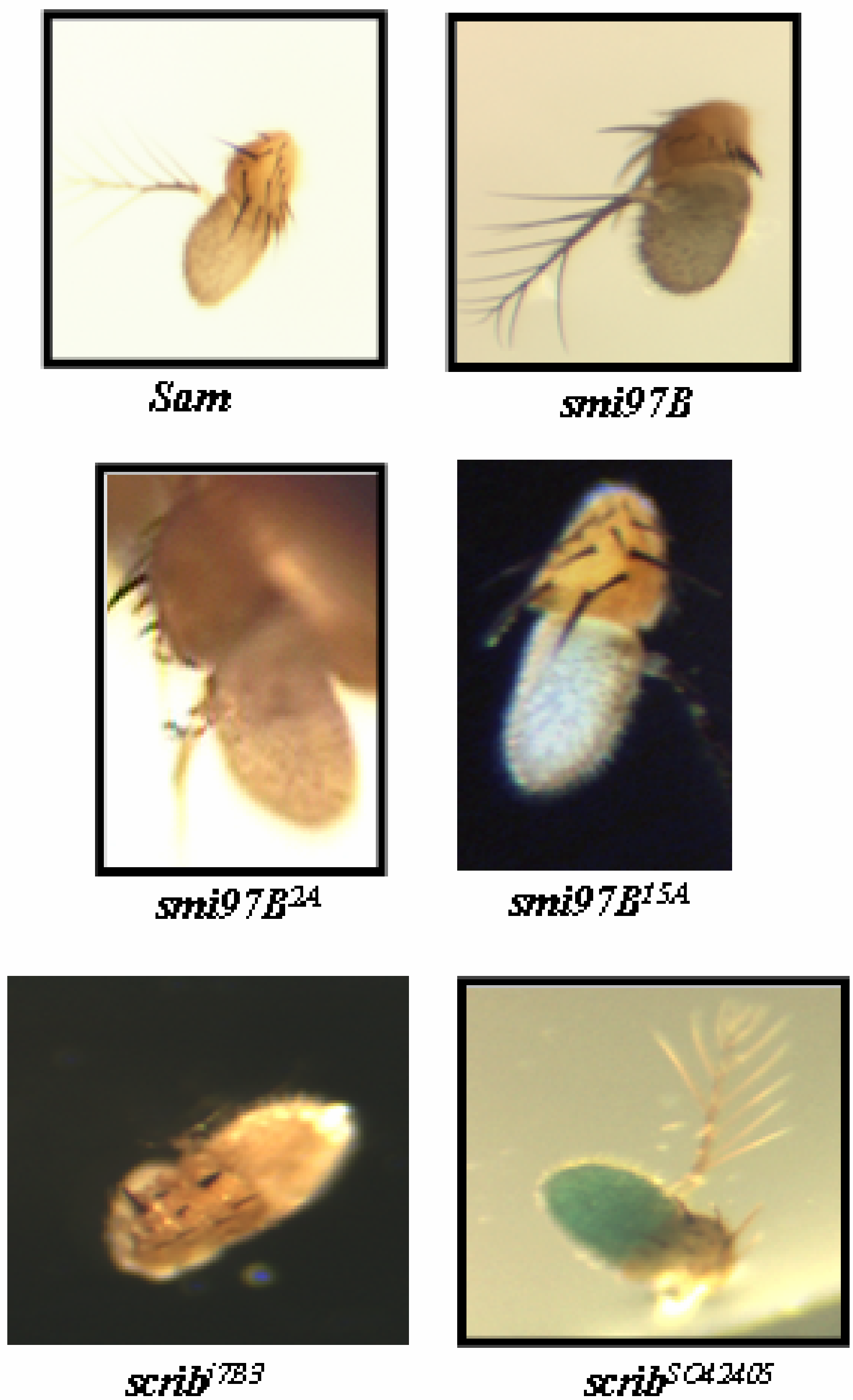


Figure 29: Enhancer trap staining for *lacZ* in antennae of *Sam*, *smi97B*, excision alleles, *smi97B*^{15A} and *smi97B*^{2A}, and *scrib* alleles, *scrib*^{j7B3} and *scrib*^{SO42405}.

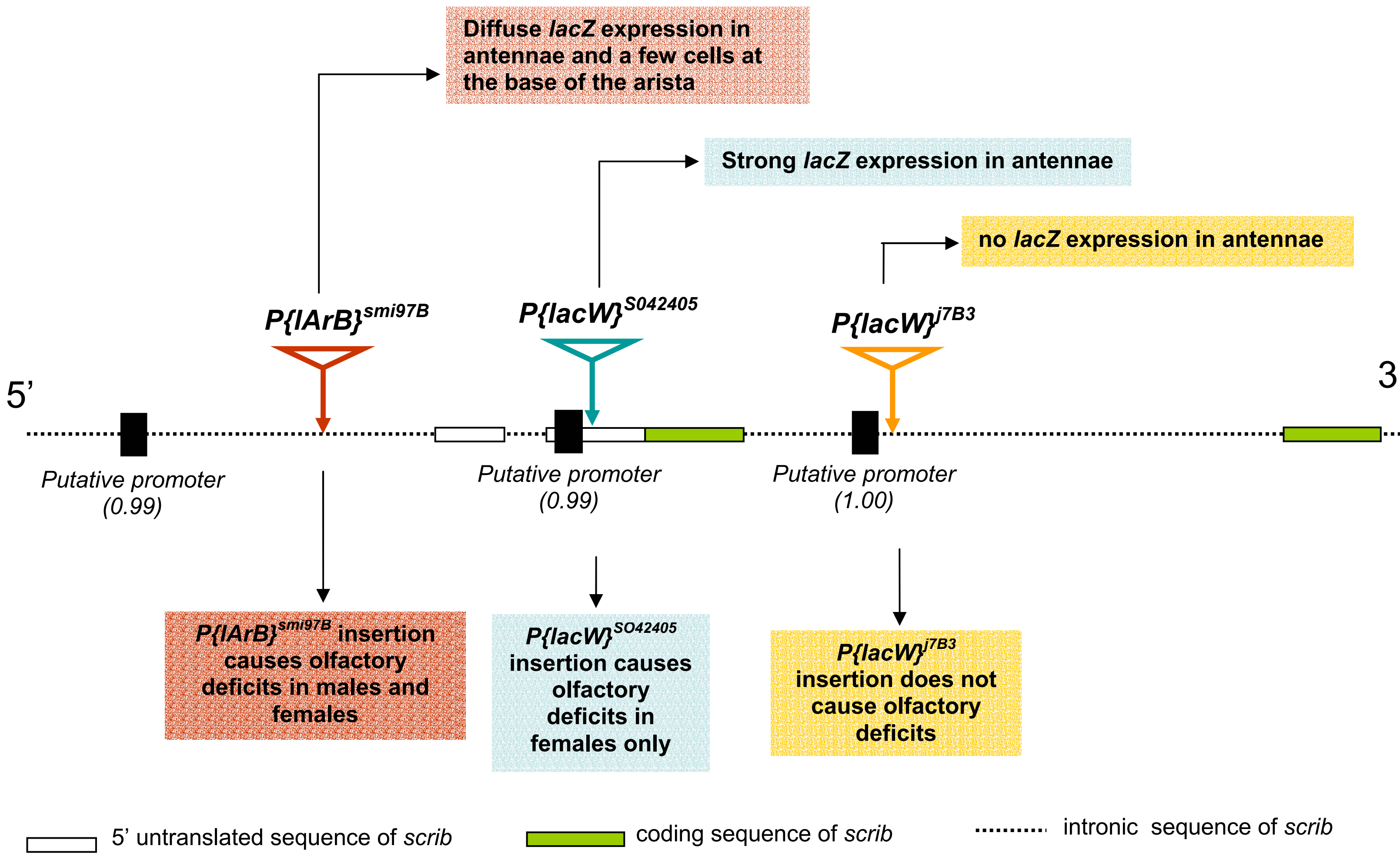


Figure 30: *P*-element insertion sites in the *scrib* locus, in relation to putative promoters that drive *lacZ* reporter gene expression in adult tissues. (Legend overleaf)

Figure 30: *P*-element insertion sites in the *scrib* gene, in relation to putative promoters that drive *lacZ* reporter gene expression in adult tissues. Putative promoter sequences were predicted using the Neural Network Promoter Prediction bioinformatics program (Reese and Eeckman 1995; Waibel *et al.* 1989). The numbers within brackets, represent promoter prediction scores computed by the program. The insertion site of $P\{lacW\}^{SO42405}$ has not been molecularly characterized to a single base, but lies within the 5' untranslated region of the *scrib*.

5.2 *In situ* hybridization of *scrib* in olfactory organs and central nervous system

Digoxigenin-labeled ribonucleic acid probes (riboprobes) complementary to a *scrib* messenger RNA (mRNA) representing the entire coding sequence of *scrib*, were used to identify tissues in wild-type heads that expressed *scrib* (see Experimental Methodology). Punctate staining was observed in the third antennal segment in regions that corresponded with cell bodies of ORNs as well as in the maxillary palps (Figure 31A, C). Intense staining was also observed in the second antennal segment which contains Johnston's organ, a tissue that contains the neuronal cell bodies of nerves that mediate the mechanosensation and audition (Gopfert and Robert 2002) in *Drosophila* (Figure 31A). In the central nervous system, staining was distributed in the cortical cell bodies in the brain (Figure 32A, B). There were no differences in tissue-specific *scrib* expression patterns in males and females. Riboprobes identical to the *scrib* mRNA sequence (called sense probes) did not hybridize to the sections, thus controlling for non-specific staining (Figure 31B, D; Figure 32C).

DISCUSSION

Previously, Li and colleagues (2001) localized Scrib expression to the larval central and peripheral nervous systems. Subsequently, it has been localized to the synaptic boutons in the larval neuromuscular junction (Mathew *et al.* 2002; Roche *et al.* 2002). These experiments reveal the extensive expression of *scrib* in ORNs of the adult olfactory organs and cortical cells of the CNS. Further, intense staining in the second antennal segment, corresponding to the position of Johnston's organ, as well as staining observed in the second order and third order neuronal cell bodies from the eye in the lamina and optic chiasma, respectively (Figure 32A and B) suggest that the scaffolding protein Scrib may not be functionally restricted to olfactory transduction.

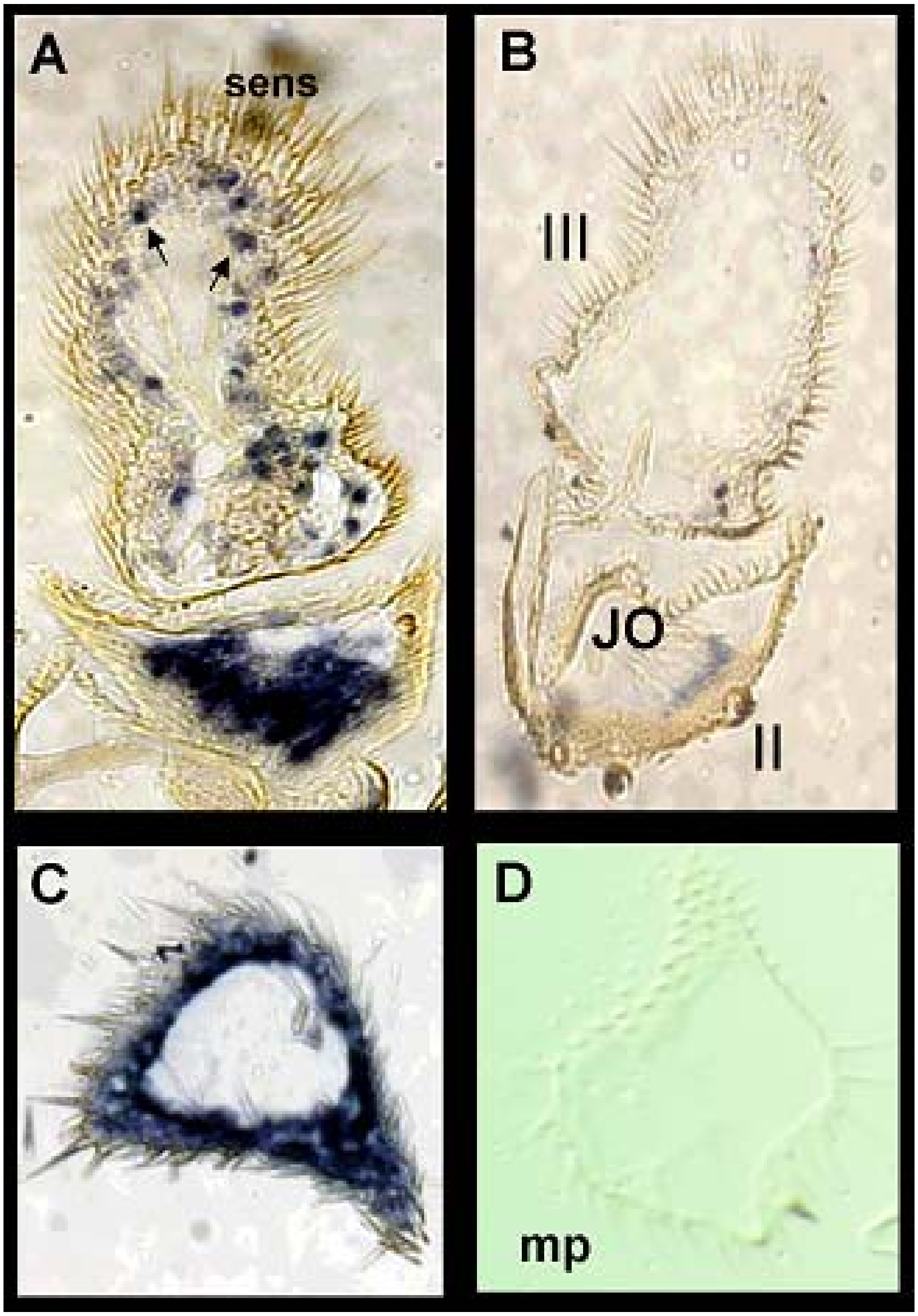


Figure 31: *In situ* hybridization of adult olfactory organs with riboprobes corresponding to the anti-sense (A and C) and sense strands of *scrib* (B and D). Hybridizations were carried out on adult *Sam* males and females. Abbreviations: **sens** designates the olfactory sensilla containing the dendrites of ORNs; **III** stands for the third antennal segment; **II**, the second antennal segment; **JO**, Johnston's organ present in the second antennal segment; and, **mp**, designates the maxillary palps. Arrowheads point towards the neuronal cell bodies of ORNs in the third antennal segment.

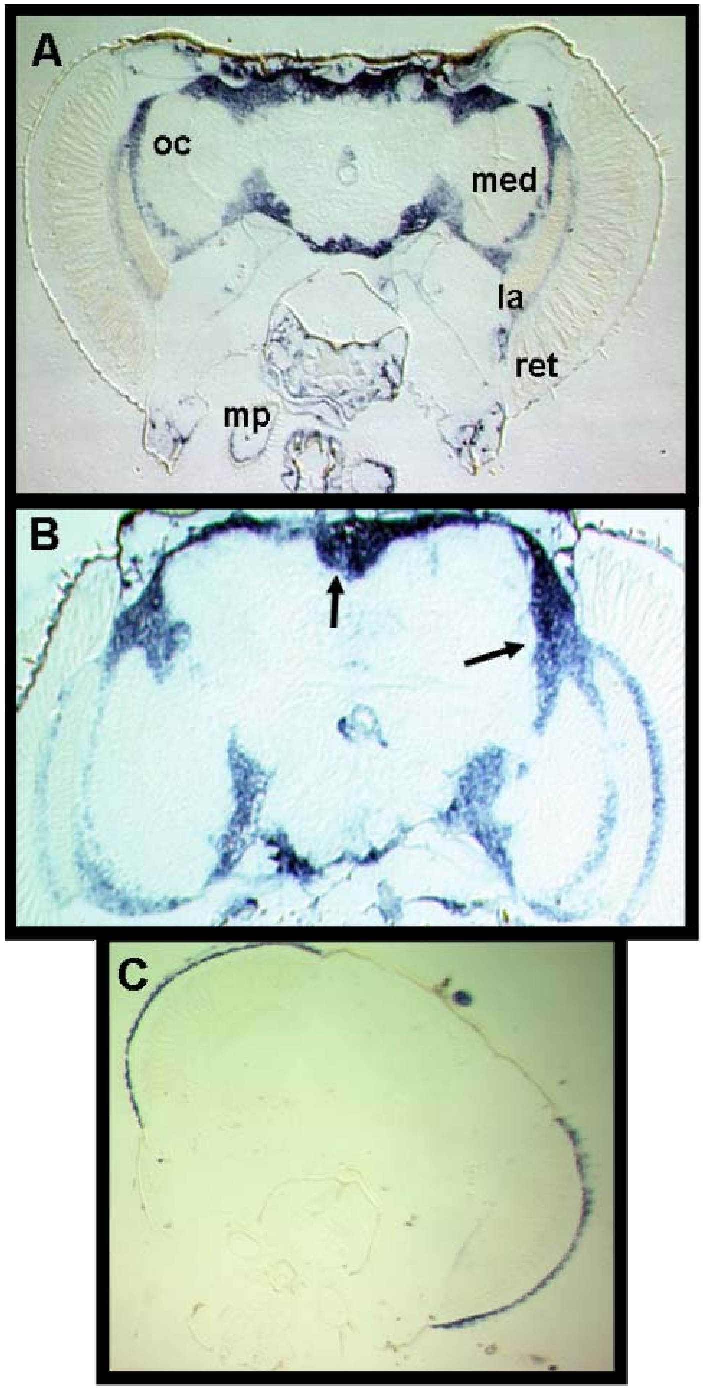


Figure 32: In situ hybridization of adult head sections with *scrib* anti-sense (A and B) and sense (C) riboprobes. Abbreviations: **med**, designates the medulla of the eye; **la**, the lamina; **oc**, the optic chiasma; **ret**, retina; and **mp**, designates the maxillary palps. Arrowheads point towards cortical neuronal cell bodies stained by *in situ* hybridization.

5.3 Immunolocalization of Scrib in olfactory organs and central nervous system

The *in situ* hybridization protocol uses molecular probes directed towards the detection of transcription products of *scrib*. In order to visualize the expression of the Scrib protein in neuronal projections in the olfactory organs and CNS of the adult fly, immunohistochemical staining was carried out. Towards this end, head sections were incubated with antibodies raised against the C-terminal peptides of Scrib; anti-Scrib1 was directed towards fifteen amino acid residues of the larger male isoform (corresponding to the 7.1 kb and 5.9 kb mRNA transcripts of *scrib*) and anti-Scrib 2 towards those of the smaller female-specific isoform (corresponding to the 4.6 kb mRNA transcript of *scrib*).

Immunochemical staining was observed throughout the antennae and maxillary palps. The neuronal nuclear protein, ELAV appeared to be expressed in cells that co-express cytosolic Scrib (Figure 33). In the brain, Scrib was localized to the axonal projections of neurons that were distributed through the tissue. However, specific enrichment was observed in axonal projections in the higher brain regions: the superior-medial protocerebrum and the ventro-lateral protocerebrum (Figure 34). In frontal sections through the brain, Scrib expression is particularly intense at the junction where the antennal nerves from the peripheral antennae entered the antennal lobes, as well as in the superior medial protocerebrum (Figure 35A, B). This pattern of tissue-specific enrichment was found to be consistent with the expression pattern of syntaxin, a pre-synaptic protein involved in regulated exocytosis and vesicular trafficking (Figure 35C). Staining of whole mounted adult brains for Scrib expression reveals a punctate pattern in the protocerebrum that suggests synaptic localization of Scrib in the brain (Figure 36). Incubations of head sections with anti-Scrib 1 and anti-Scrib 2 antibodies revealed that that there were no differences in tissue-specific expression of Scrib between the two sexes. Futhermore, both Scrib isoforms were detected in sections through male and female heads.

DISCUSSION

Immunohistochemical staining for Scrib in paraffin sections through the adult head provides insight into the expression of Scrib in the axonal projections of the ORNs and CNS neurons that express *scrib* transcripts. Scrib peptides are expressed throughout the antennae and maxillary palps. In the brain, Scrib expression appears particularly intense in regions that are associated with the transmission and processing of sensory information: the antennal nerves and the protocerebrum, respectively. Furthermore, expression of Scrib in the brain mimics the expression patterns of the synaptic vesicular trafficking protein, syntaxin. The notion that Scrib may play a synaptic role in the brain is also derived from its punctate pattern of expression in the protocerebrum, in whole mounts of adult brain tissue.

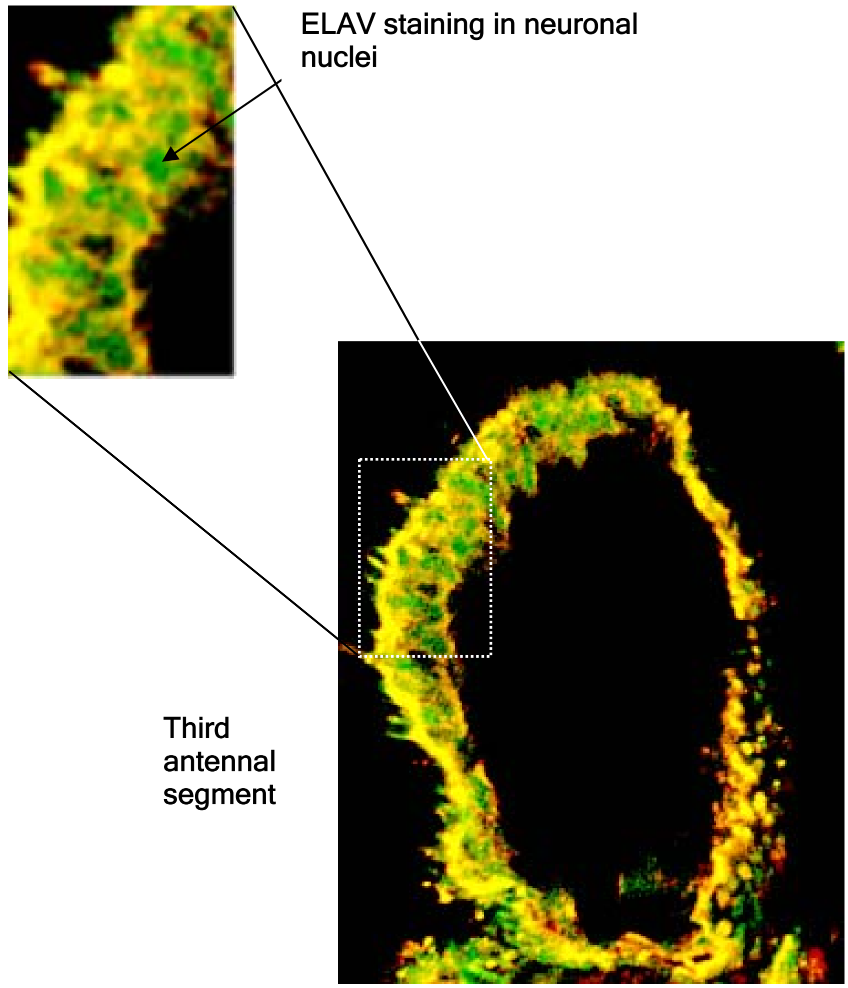


Figure 33: Localization of neuronal nuclear protein, ELAV and Scrib in the ORNs of the second antennal segment. ELAV expression is visualized as a green stain and Scrib as red. Regions of co-localization appear yellow. Yellow staining in the outer edges of preparation reflect non-specific immunoreactivity of both anti-sera to the antennal cuticle.

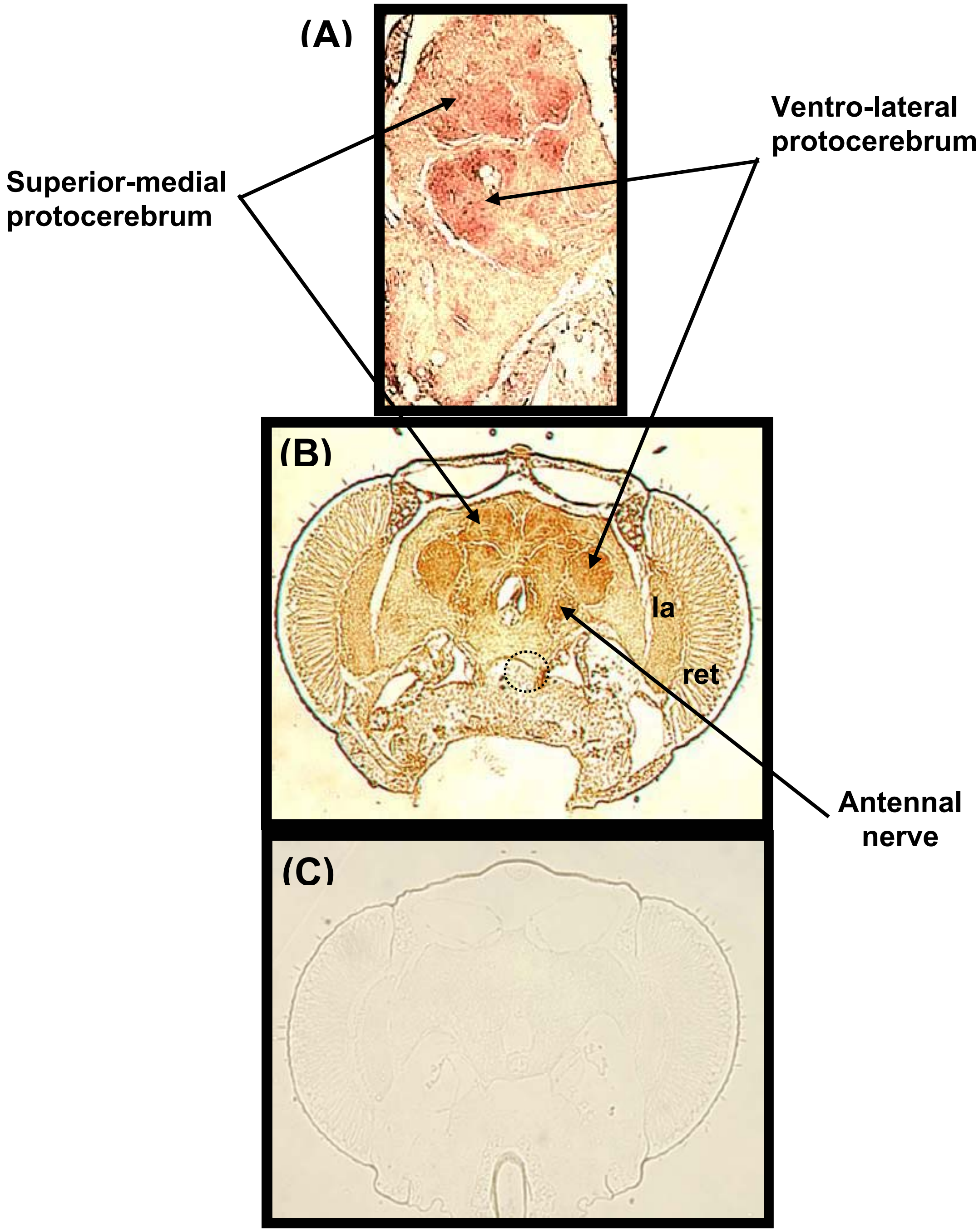


Figure 34: Immunohistochemical staining of Scrib in the adult brain. (A) Sagittal section and (B) horizantal section of the head incubated with anti-Scrib antibodies. (C) Head section incubated with rabbit pre-immune serum. Abbreviations: **la**, lamina; **ret**, retina.

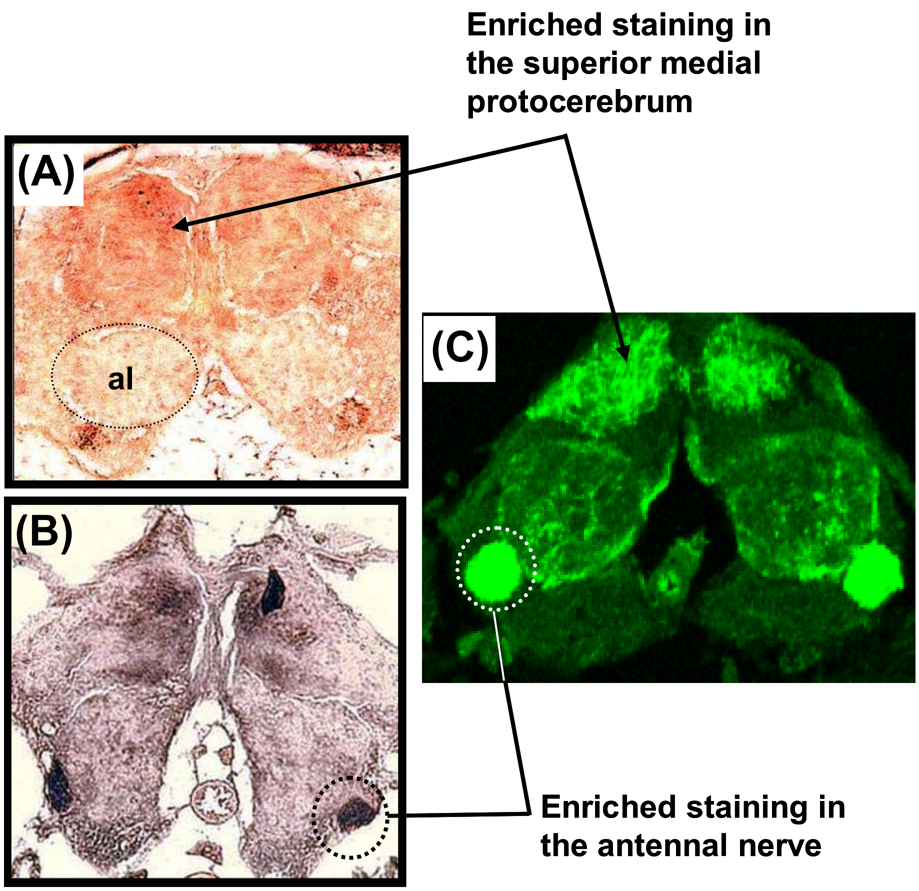


Figure 35: Expression patterns of Scrib in frontal head sections is similar to that of synaptic protein, syntaxin. Immunohistochemical staining with anti-Scrib antibodies (A and B) shows enrichment of Scrib expression in the antennal nerves (circles) and the superior medial protocerebrum. This pattern is similar to the expression patterns of syntaxin revealed by staining with anti-syntaxin antibodies, ©. Abbreviations: al, denotes the antennal lobes (also circled).

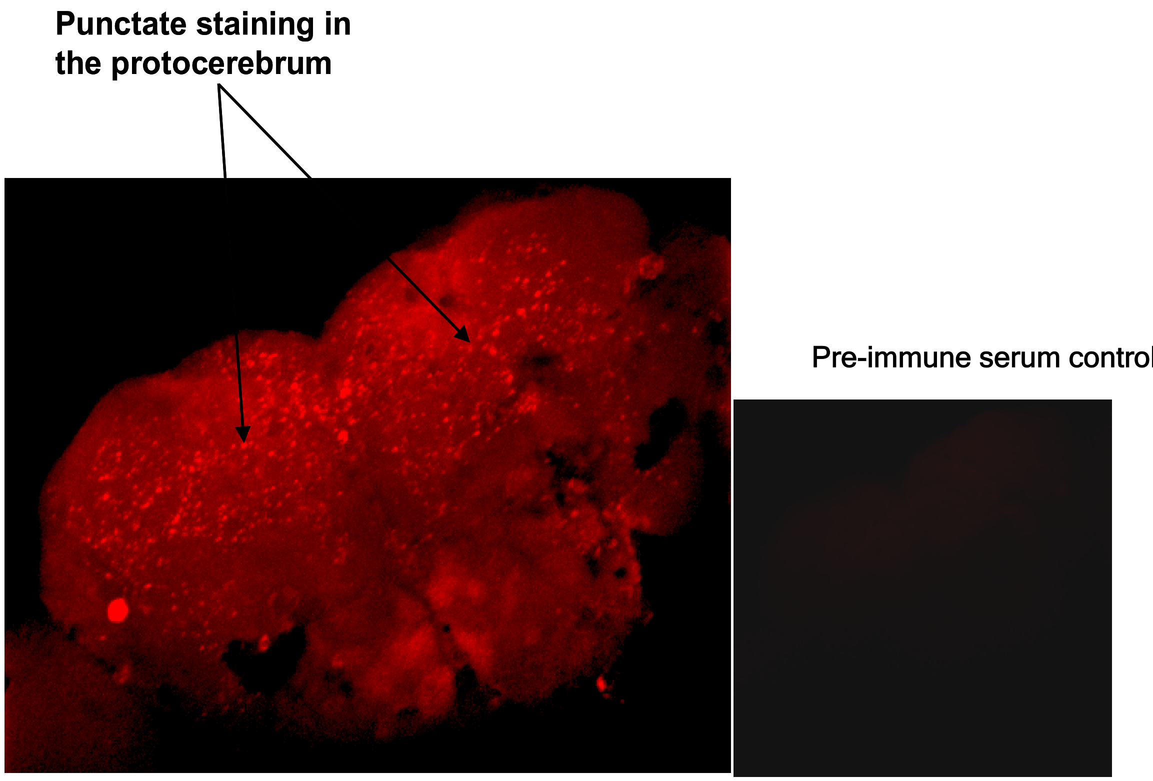


Figure 36: Scrib staining in whole brains. Puntate Scrib expression in the protocerebrum. Whole brains incubated with pre-immune serum did not show non-specific staining.

Conclusions

Behavior-genetic analyses using complementation tests, indicate that *smi97B* is an allele of *scrib*; thus implicating *scrib* in mediating olfactory behavior. Molecular characterization confirms this observation and extends it further to correlate deficits in olfactory behavior in *smi97B* to reduced levels of *scrib* expression in the fly. Enhancer trap technology, *in situ* hybridization and immunohistochemical procedures enable the visualization of *scrib* expression in tissues that are involved in olfaction: the third antennal segment, maxillary palps and the CNS. Enhancer trap staining of whole flies indicates that there is a position effect on *lacZ* expression in three *P*-element insertion lines, *smi97B*, $scrib^{j7B3}$ and $scrib^{SO42405}$. This may arise due to the presence of multiple enhancer elements in the *scrib* locus that each direct reporter gene (*lacZ*) expression in the tissue-specific manner of endogenous *scrib* trancripts. *In situ* hybridization and immunohistochemical staining revealed that *scrib* gene products were expressed throughout the adult olfactory organs and the CNS; but also, in organ systems that are involved in audition (Johnston's organ) and vision (optic chiasma and lamina). These results indicate a universal function for Scrib in sensory perception.

Scrib isoforms follow a pattern of expression in the brain that is similar to those of synaptically localized proteins (Figure 35 and 36). In the larval NMJ, Scrib is involved in synaptic organization (Mathew, *et al.* 2002) and synaptic vesicle dynamics that underlie plasticity (Roche, *et al.* 2002). The protocerebral region of the brain (considered the forebrain in the Dipteran insects) receives higher order arborizations from the various sensory centers as well as the mushroom bodies (a region involved in information integration; (Connolly *et al.* 1996; Heisenberg *et al.* 1985; Ito *et al.* 1998). It is likely that Scrib may perform a similar synaptic function in the adult brain. However, Scrib expression is not localized to the synapse and staining by the different methods visualizes its expression throughout the peripheral olfactory organs that contain the dendritic and axonal projections of the ORNs and in neuronal projections in the CNS.

Anti-Scrib 1 detects a Scrib isoform that is translated from a transcript expressed in both males and females, while the anti-Scrib 2 antibody detects C-terminal peptides translated from the female-specific 4.6 kb transcript. However, immunohistochemical staining was observed in tissue sections of male heads incubated with anti-Scrib 2 antibodies. This implies the existence of alternate Scrib isoforms expressed in both sexes that share the C-terminal peptides of Scrib 2. Hence, sex-specific isoforms, in addition to non-sex-specific Scrib isoforms may be expressed in overlapping regions of the olfactory system in males and females to modulate differences in behavioral outputs by organizing sexually dimorphic protein assemblies at the synapse.

CHAPTER 6:

Overall conclusions and future directions

OVERALL CONCLUSIONS

Genetic analyses of complex traits such as behavior necessitate the combination of a mutagenesis screen that isolates homozygous viable mutants with a rapid and highly reproducible assay that detects subtle as well as major effects on the trait value. The *P*-element mutagenesis of the autosomal genome of *Drosophila* and subsequent behavioral screening isolated 12 genetic loci that contributed to olfactory avoidance behavior in adults (Anholt, *et al.* 1996). Of these, the *smi97B* mutation contained a single *P{lArB}* *P*-element insertion in cytological band 97B of chromosome *3* and displayed profound olfactory deficits in avoidance response to high concentrations of benzaldehyde, 2-n-propylpyrazine and 2-n-isobutylthiazole. Furthermore, a statistical comparison of olfactory avoidance responses in males and females to the panel of repellants, revealed significant sexual dimorphism in the mutant behavioral phenotype. This study describes the characterization of *smi97B*, the molecular basis of its behavioral sexual dimorphism and evidence for the role of this gene in mediating odour-guided behavior in *Drosophila melanogaster*.

(a) Insights from behavior-genetic analysis of *smi97B*

The *P{lArB}*-element insertion in *smi97B* causes generalized olfactory deficits in *Drosophila* adults and larvae In adults, the mutant phenotype is sexually dimorphic, with females being more severely affected than males. Excision of the 18.5 kb *P{lArB}*-element transposon from the *smi97B* genome, eliminates olfactory deficits in revertants that are genotypically and phenotypically identical to the wild-type parental strain, *Sam*. This establishes the effect of the disruption of the *smi97B* locus by the inserted *P{lArB}*-element on olfactory behavior.

The correlation between the mutation in *smi97B* and smell-impairment is further strengthened by the observation that mutants containing remnant *P{lArB}* fragments in their genomes (due to imprecise excision events) also display olfactory deficits. Specifically, the *smi97B*^{15A} mutation contains a 3.5 kb remnant *P{lArB}* fragment and causes male-specific anosmia, while the *smi97B*^{2A} mutation contains a 2.5 kb *P{lArB}* disruption at the original site and causes mild female-specific hyposmia. This is the first insight into the existence of sex-specific upstream regulatory elements in the *smi97B* locus that mediate sex-specific olfactory phenotypes.

Molecular characterization of the genomic region flanking the *P{lArB}* insertion in *smi97B*, reveals that the *P*-element lies 1,084 kb upstream from the open reading frame of *scribble* (*scrib*); a gene previously implicated in embryonic development (Bilder and Perrimon 2000), tumor suppression (Bilder *et al.* 2000; Li *et al.* 2001) and synaptogenesis of the larval neuromuscular junction (Mathew *et al.* 2002; Roche *et al.* 2002). A chromosomal deficiency that uncovers the *scrib* locus on chromosome *3*, *Df(3R)Tl-X*, fails to complement the olfactory mutant phenotype of *smi97B*, thus mapping the gene disrupted by the *P{lArB}* insertion in *smi97B* to the region containing *scrib*. Complementation mapping of the *smi97B* olfactory phenotype with point mutations *scrib*^{1} and *scrib*^{2}, reveals that null mutations in the *scrib* gene causes smell-impairment in adults. Intra-genic complementation of the *smi97B* phenotype by hypomorphic scrib alleles, *scrib*^{j7B3} and *scrib*^{SO42405} provides further evidence for the allelic relationship between *smi97B* and *scrib*. The *scrib*^{SO42405} mutation disrupts olfactory function in females, but males are unaffected; while the *scrib*^{j7B3} mutation has no observable effect on olfactory behavior in adults. This demonstrates that *scrib* performs pleiotropic functions that are molecularly compartmentalized and amenable to genetic dissection (Table 2). The final confirmation that *smi97B* is an allele of *scrib* comes from the restoration of wild-type olfactory behavior in *smi97B* by the transgenic expression of *scrib* in the *smi97B* mutant background. Leaky expression of the *scrib* transgene rescues olfactory deficits of *smi97B* in males only, while females remain statistically smell-impaired. This indicates sex-specific differences in the penetrance of the mutant smell-impaired phenotype of *smi97B*. Quantitative analysis of *smi97B* reveals that mutations in the *scrib* gene result in sex-specific effects on odor-guided behavior in *Drosophila melanogaster*.

GENOTYPE	OLFACTORY PHENOTYPE Males	OLFACTORY PHENOTYPE Females	DEVELOPMENTAL PHENOTYPE
Sam	wild type	wild type	wild type
smi97B	hyposmic	anosmic	wild type
smi97B^{15A}	anosmic	wild type	partial viability
smi97B^{2A}	wild type	hyposmic	wild type
scrib^{1} *(null)*	ansomic	anosmic	lethal
scrib^{2} *(null)*	anosmic	anosmic	lethal
scrib^{SO42405}	wild-type	hyposmic	lethal
scrib^{j7B3}	wild type	wild type	lethal

Table 2: Summary of *scrib* alleles. Shaded green boxes represent phenotypes observed in heterozygotes over *smi97B*.

(b) Insights from molecular genetic analyses of *smi97B*

Behavioral characterization provides evidence for the role of *scrib* in olfaction as well as insights into its complex regulation. These observations are confirmed by molecular analyses, which reveal that *scrib* is alternatively spliced to generate 3 observable transcripts on Northern blots; a 6 kb transcript primarily expressed in both males and females, and to a lesser degree in larvae; a female-specific 4.6 kb transcript; and a 7.1 kb male- and larval-biased transcript. Furthermore, *scrib* expression is correlated with olfactory deficits; transcript levels are reduced in *smi97B* adult and larvae, compared to the wild-type parental strain and a genotypically wild-type revertant of *smi97B*, $smi97B^{16A}$. However, reduction in scrib expression caused by the *smi97B* mutation is greater in females than males, which correlates with the relative severity of the olfactory phenotype in *smi97B* females compared to males.

Protein analyses corroborate these results and anti-bodies raised against Scrib peptides detect sex-specific expression of mutliple isoforms on Western blots. A subset of these isoforms is reduced in *smi97B* mutants, but restores to wild type levels in the revertant, $smi97B^{16A}$. The lack of concordance in the sizes of the Scrib isoforms detected on Western blots and the predicted sizes of proteins translated from the *scrib* mRNA transcripts, allude to post-translational modification of the Scrib protein in adults. This adds another level of complexity in the regulation of expression of *scrib in vivo*. Finally, the phenotypic rescue of smell-impairment in *smi97B* by a *scrib* transgene is consistent with the molecular restoration of its expression levels to that of wild type.

The isolation of mutliple *scrib* cDNAs obtained from a wild-type head library circumvents the limitation of Northern blot technology in the identification of rare transcripts. This experiment indicates that *scrib* generates splice variants that vary in the organization of the protein-binding modules they encode. Since Scrib is a scaffolding protein, the number and organization of its protein binding modules determine the nature and composition of the proteins it interacts with. The largest transcript encodes 16 leucine-rich repeats and 4 PDZ domains affording it the maximum number of interactions with cellular proteins, while the shortest only encodes 2 PDZ domains. In addition, the presence of 5' untranslated regions may direct tissue-specific expression or stability in two of the transcripts characterized. It is likely that the sex- and tissue-specific expression of these transcripts may well determine the organization of molecular assemblies in the cell that are sexually dimorphic in nature and composition. The recent discovery of sex-specific proteins expressed in the fat bodies that surround the adult brain, strengthens the idea that there may exist considerable sex-specific micro-environments in and around the CNS that direct sexually dimorphic behaviors in the fly (Fujii and Amrein 2002).

(c) Insights from tissue localization of *scrib* expression

The fundamental role of *scrib* in olfaction is substantiated by the localization of its expression in organs that are involved in the reception, transmission and processing of olfactory information. Enhancer-trap technology reveals the promoter-specific expression of the *lacZ* reporter gene in the adult olfactory organs of *P*-element mutants, *smi97B*, $smi97B^{15A}$ and $scrib^{SO42405}$. Differences in staining patterns, as well as the lack of *lacZ* expression in the olfactory organs of $scrib^{j7B3}$ may be attributed to three functionally distinct putative promoters that flank the *P*-element insertions in these mutant lines.

Detection of *scrib* gene products in the olfactory organs, the eye, the auditory organ and the CNS hints at the universality of *scrib* function in sensory transduction and central processing. Although Scrib mimics the expression patterns of synaptic proteins in the adult central nervous system, it is also expressed in the axonal projections of neurons. Based on its function in the larval NMJ (Mathew *et al.* 2002; Roche *et al.* 2002) as well as in the distribution of polarity determinants in embryonic epithelia (Bilder 2001; Bilder and Perrimon 2000), Scrib may play a dynamic role in the transport and organization of synaptic vesicles along neuronal circuits in the adult.

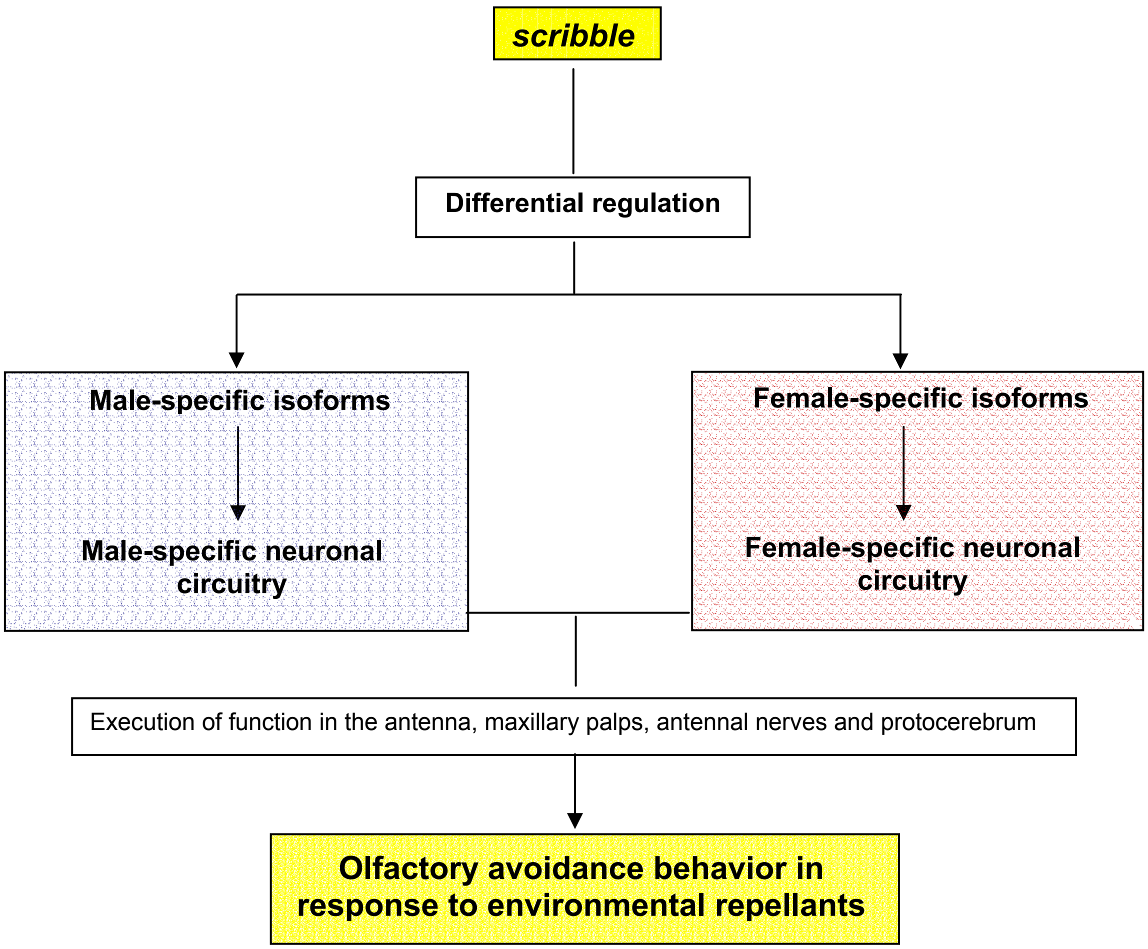


Figure 37: Overall conclusion

FUTURE DIRECTIONS

(a) Identification of genes that interact with *scrib* to contribute to olfactory behavior

Complex biological processes are the end products of extensive molecular networks in the cell that functionally direct emergent phenotypes, such as animal behavior. Hence, a perturbation in the expression or function of any one component may have correlated consequences in the expression and function of other components in the network. A single *P{lArB}* element insertion in *scrib* causes disruption of olfactory avoidance behavior in flies. By virtue of its role as a cellular scaffolding protein, as well as its pleiotropic functions in different tissues and organ systems, the disruption of the *scrib* locus may well lead to correlated perturbations in other genomic loci.

Preliminary data from high density oligonucleotide microarrays reveals that 98 genes (see Table 3 for a subset of co-regulated genes) of a total of 14,000 *Drosophila* genes represented on Affymetrix microarray chips show statistically significant deviations in expression levels in *smi97B* compared to the *Sam* transcriptome ($P < 0.001$). A comparison of expression profiles of *smi97B* males and females reveals that the single *P{lArB}*-element insertion in the *smi97B* locus caused perturbations in expression levels of a larger number of loci in the female genome versus the male genome (Figure 38). This may explain the severity of the olfactory deficits in *smi97B* females compared to males.

Microarray analyses reveal the identity of genes that are co-regulated with *scrib* to contribute to deficits in olfactory behavior. Genome-wide mutagenesis screens (using *P*-elements or chemical mutagens like EMS) and deficiency screens may be carried out to identify and characterize loci that genetically interact with *scrib* to generate perturbations in olfactory behavior. The functional characterization of the genetic elements identified using these schemes will serve to increase our understanding of the role of *scrib* in mediating odor-guided behavior in *Drosophila melanogaster*.

UP REGULATED GENES IN *smi97B*

In males and females:

Phosphoenolpyruvate carboxylase (dephosphorylation of GTP); *ankyrin-like protein* (membrane cytoskeleton organization, cellular polarity, vesicular trafficking and intracellular transport); *hydrogen-transporting ATPase subunit* (receptor-mediated endocytosis, associated with clathrin coated vesicles); *ade2; CG18107; CG10924; CG8215; CG1910; CG1548;*

Males only:

Sed5 (vesicle targeting protein); *E2f; CG11426; CG7300; CG4355* (protein tyrosine phsphatase-like protein); *CG5784*

Females only:

CG8715; CG5966 (triacylglycerol lipase); *CG10192* (translation initiation factor 4G-like protein); *CG3699*; *ATPα* (Na+/K+ transporting ATPase, α-subunit); *CG9297*; *ade3* (phosphoribisylamine-glycine ligase); *sgg* (protein serine/threonine kinase); Cry (crystalline); *CG14630; CG4396; CG7590; CG4779; CG11797; CG11218* (antennal binding protein); *CG14153*; *CG9299* (cuticle protein); *CG11909*; *Nmdmc* (NAD-dependant methylenetetrahydrofolate dehydrogenase); *CG6128* (tissue α-L-fucosidase); *CG6385* (sarcosine dehydrogenase); *CG10383; CG12238; CG11089; CG7958; fax* (*failed axonal connections*); *BG:DS05899.1 (l*ong-chain-fatty-acid-CoA-ligase); *CG1583*

DOWN REGULATED GENES IN *smi97B*

In males and females:

CG7722, serpin; CG17531 (glutathione transferase); *CG9953* (lysosomal pro-X carboxypeptidase); *CG15081*

Males only:

CG7255 (cationic amino-acid transporter); *CG8202; CG17530* (glutathione transferase); *CG14027; CG15661*(UDP-glycosyltransferase, endogenous control of receptor ligands, also expressed in vertebrate huippocampal cells); *CG7496* (peptidoglycan recognition protein); *CG13305; CG4306; CG1793; CG8613; EG:25E8.2* (ubiquitin conjugating enzyme-like); *CG1057*

Females only:

CG4526; CG10622; Vm34Ca (vitelline membrane protein); *CG9170* (nicotinic acetylcholine-gated receptor channel); *CG8109; CG1497; CG7296; CG17124; CG10187; CG14132; CG8818; scrt* (*scratch*, transcription factor expressed in the sensory organ mother cell) ; *Odc1* (ornithine decarboxylase); *CG3239* (membrane-anchored zinc metalloendopeptidase*); CG9370; Paps* (PAPS synthetase, involved in biotransformation of xenobiotics, also expressed in the vertebrate cerebral cortex)

Table 3: List of up and down regulated genes in the *smi97B* genome.

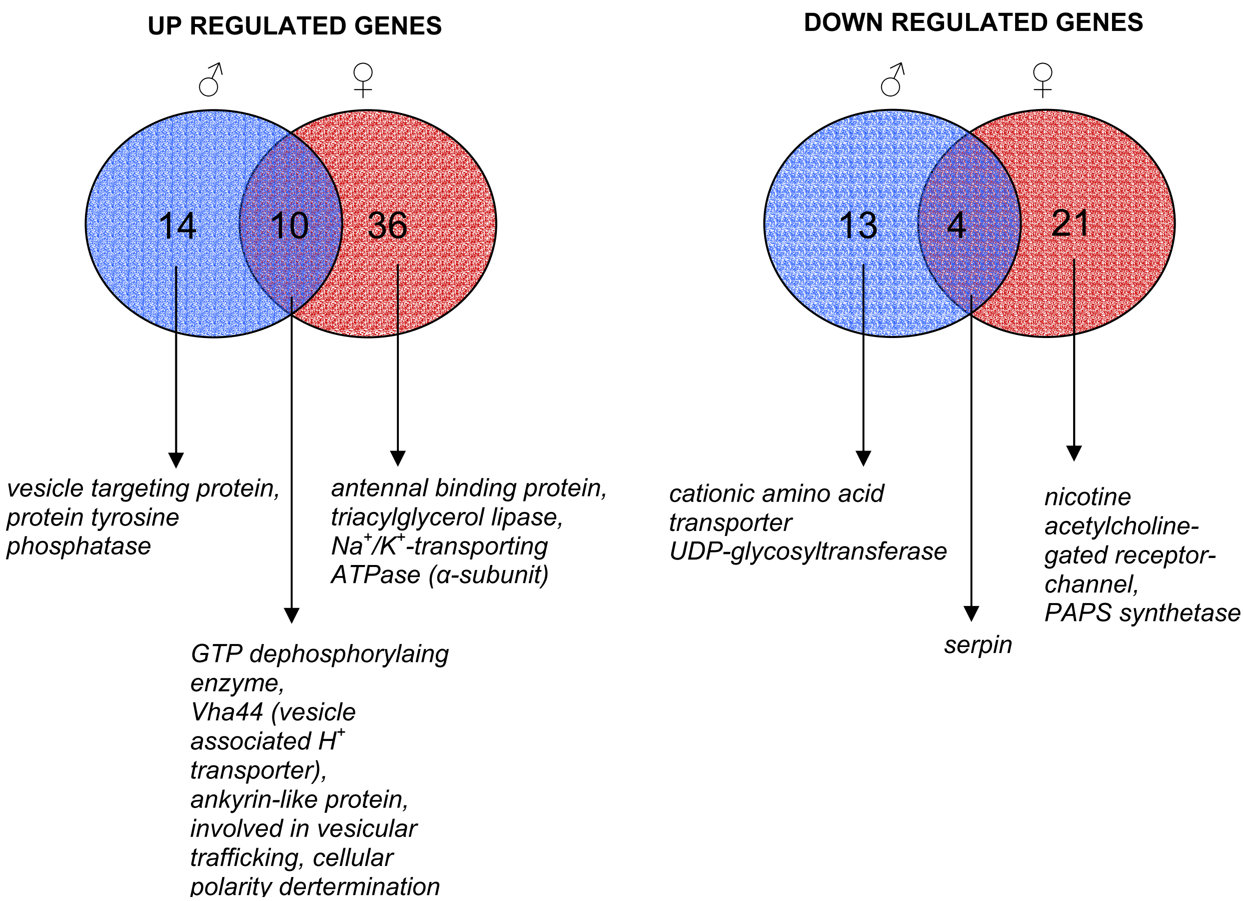


Figure 38: Venn diagrams representing genes up- and down-regulated in *smi97B*

(b) Functional characterization of Scrib

By virtue of its PDZ domains and LRRs, Scrib mediates the organization and possibly trafficking of a number of cellular targets that contribute to the neuronal underpinnings of odor-guided behavior. Biochemical analyses of the binding partners of Scrib will provide insights into the nature of molecular assemblies that determine neuronal function in olfactory behavior. Yeast two-hybrid screens and co-immunoprecipitation assays may be conducted to isolate proteins that physically interact with the Scrib protein.

The diversity of *scrib* transcripts isolated from the head may be functionally characterized by their targeted disruption using double stranded RNAi protocols. Effects of disruption of alternatively spliced *scrib* transcripts may then be phenotypically and molecularly characterized to distinguish their separate functions in the cell.

Sex-specific transcriptional regulation of *scrib* raises inquiries about its relation to the sex determination pathway in *Drosophila*. Although the upstream regulators in this hierarchy have been well characterized, their downstream targets are yet to be elucidated. Molecular assays that quantify *scrib* expression levels in *tra* or *dsx* double mutants over *scrib* alleles will provide evidence for the possible regulation of *scrib* by these sex-determination regulator genes.

Finally, genetic experiments using the *P*-elements inserted in the *scrib* locus may be conducted to generate partial excisions or deletions in the putative promoter regions of this gene. This will serve to extend the analyses provided in this study for the complex regulation of *scrib*.

(c) Identification and functional characterization of naturally occurring *scrib* polymorphisms

Mutations in the *scrib* gene result in sexually dimorphic olfactory phenotypes. To understand the ecological and evolutionary significance of this locus, quantitative investigations into the naturally occurring variation in the *scrib* locus may be pursued. Towards this end, single nucleotide polymorphisms in the promoter-enhancer regions of this large gene may be statistically associated with variation in olfactory behavior in natural populations of *Drosophila*.

In summary, the genetic analysis of *smi97B* provides a glimpse into the inter-connectivity of function (evidenced by the multiple olfactory alleles of *scrib*) and molecular elements (mediated by its differentially regulated transcripts) that underlie the manifestation of a complex trait. It also illustrates the underlying sexual dimorphism in the molecular elements of a biological process that provides the *potential* for evolutionarily relevant sexually dimorphic phenotypes.

Does wisdom perhaps appear on earth as a raven inspired by the smell of carrion?
-Nietzsche

CHAPTER 7: Bibliography

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