Chapter 1

Introduction

Olfaction is a molecular sense, in which information carried in air-borne chemicals is transformed into patterns of brain activity that underlie odor perception. It is probably the most important sense for survival of most animal species ranging from insects to mammals. Detection and localization of food, avoidance of toxins and predators, and communication with cohorts and mating partners through volatile pheromones are examples of the range of olfaction dependent behavior. In contrast to the visual system, where a handful of receptor genes are sufficient to cover the relevant range of the electromagnetic spectrum, animals require a large repertoire of receptors to detect and discriminate the structurally diverse array of odorant molecules important for survival. The interaction between odor molecules and receptors is complex and a large gap still exists in our understanding of how the specific interactions between odorants and a large repertoire of receptors drive the behavior. The odorant receptor genes that permit chemo sensation were discovered in 1991 through insightful use of molecular biology (Axel, 1991). Nineteen years later, the process of receptor gene discoveries and involvement of new signaling mechanisms continues because signaling mechanisms and large gene families underlying odor detection are highly diverse in different animals. It is therefore interesting to know to what extent these differences either by means of new odorants receptor family or utilizing new signaling mechanism contributes to the behavior that they drive.

1.1 Olfaction in Insects

Insects are the most successful inhabitants on our planet. A large part of their success comes from their remarkable sensory system that allows them to cope up with a variety of environmental conditions. Despite their small size, insects can discriminate a wide variety of odors with remarkable sensitivity and specificity. Its olfactory circuit is structurally and functionally analogous to mammals, suggesting that it represents an
extremely good model system to study some of the complex problems of olfaction. The principle elucidated in insects often applies to higher animals. In both insects and mammals, odorants bind to receptors in the cilia or dendrites of OSN, each of which express one or a rarely few receptor types. In both kinds of animals, OSNs that express the same odorant receptor, send their axons to the same glomerulli, a spherical structure that consist of OSN axon terminals and dendrites of second order neurons. The glomeruli form the antennal lobe in the insect brain or its mammalian equivalent, the olfactory bulb. The olfactory information in these centers is processed and relayed to higher brain centers. Olfaction in fruit fly gains popularity because of its anatomical simplicity and availability of genetic information than vertebrates. *Drosophila* genetic approaches have been used to investigate the mechanism underlying olfaction and olfactory learning and memory.

1.2 Organization of olfactory system in adult *Drosophila*

In *Drosophila*, volatile odorants are detected by two pairs of head appendages; the antennae and maxillary palps which are decorated with small sensory bristles called sensilla that house between one and four olfactory sensory neurons (OSNs). The olfactory sensilla fall into different morphological types known as basiconic, coeloconic and trichoid (Venkatesh and Singh, 1984). Whereas basiconic sensilla are found on both the antennae and maxillary palp in *Drosophila*, trichoid and coeloconic sensilla are located exclusively on the third antennal segment and may serve distinct chemosensory function. Based on the response profile of the OSNs housed in sensilla basiconica, de Bruyne et.al (2001) identified seven distinct types, designated ab1 through ab7. The ab1 sensillum contains four OSNs and other sensilla each contain two OSNs (de Bruyne et.al 2001). In a separate study, Stensmyr and associates classified sensilla basiconica into eight types. The three types: S1, S2 and S3 reported by Stensmyr et.al (2003) in the proximo-medial region of third antennal segment display similarities in the distribution as well as response patterns with large sensilla basiconica described by de Bruyne et.al (2001). Anil Gupta (1997) measured the sensillary response to six odorants and identified six functional types and found that each type responds to more than one odorants (Gupta,
It has been shown that basiconic OSNs respond to general odors whereas trichoid neurons respond poorly to most odorants but respond to pheromones (Clyne et.al 1997). Recent studies have classified trichoid sensilla into three subtypes as T1, T2 and T3 based on sensory neuron that they house. Coeloconic sensilla has four subtypes designated as ac1 through ac4 (Yao et.al 2005), each type contains at least two neurons with a total of at least seven distinct OSN classes. These OSNs express a distinct class of olfactory receptors that are likely to underlie the strong response of these neurons to a variety of amines and carboxylic acid (Benton et.al 2008, Yao et.al 2005). The axon of these OSNs project to the antennal lobe which in *Drosophila* contains ~46 spherical functional units called glomeruli (Laissue et.al 1999). The OSNs in the AL make synapses with projection neurons (PNs) whose axons establish the link between AL and higher brain centers such as mushroom body and lateral horn. Local interneurons (LI) which are of both excitatory and inhibitory types, provide extensive lateral connections within the antennal lobe and have been shown to play an important role in processing olfactory information.

### 1.3 Olfactory Receptors (ORs)

Different classes of sensilla respond to different odor types. The olfactory response is mediated by receptors of different classes present on the membranes of sensory dendrites emanating from the OSNs. The fruit fly, *Drosophila melanogaster* has 62 odorant receptors encoded by 60 genes. It has a membrane topology inverse to the conventional GPCRs (Benton et.al 2006). The second crucial difference is that the fly OR is an obligate heterodimer and does not function as a single receptor protein (Benton et.al 2006). While most ORs are expressed in subpopulation of OSNs, the Or83b receptor is a notable exception; it expresses in ~70% OSNs and is a heterodimer with conventional ligand binding ORs. This heterodimerization of Or/Or83b is involved in the localization of ORs to OSNs dendritic terminals (Benton R et.al 2006). In the absence of Or83b, ORs localize primarily to OSN cell bodies rather than dendrites. The question arises what type of odors these ORs detect. It has been studied most comprehensively in *Drosophila*, where a near complete ligand-receptor assignment has been obtained from John R
Carlson and co-workers (Hallem EA et.al 2004, Kreher et.al 2005, Hallem and Carlson 2006). ORs expressed in OSNs associated with basiconic sensillae in the antennae and maxillary palp respond to food odorants (Hallem and Carlson 2006), whereas those associated with coeloconic neurons respond to amines, water and other specific odors (Yao et.al 2005). The tuning profile of a given *Drosophila* OR is complex. Most respond to multiple odors and most odors activate multiple ORs. Some receptors such as Or82a are highly selective. It responds strongly to only one of 110 chemicals and weakly to five different chemicals (Hallem and Carlson, 2006). A special case of odor detection is the perception of gaseous CO2. Recent work in *Drosophila* revealed that a group of about 25 OSNs in the antennae respond selectively to CO2 (Suh et.al 2004). These neurons express two chemosensory receptors, Gr21a and Gr63a, which together comprise candidate CO2 receptor (Scott et.al 2001, Jone et.al 2007). In insects, narrowly tuned receptors have been shown to play a major role in specific behavioral tasks. Detection of CO2 in *Drosophila* by highly selective receptor mediates repulsion, is one such example.

1.4 Olfactory Signal Transduction

Olfactory signal transduction occurs in OSN where it is mediated by a distinct family of seven transmembrane containing odorant receptors. The signal transduction mechanism in insects is complex and surprisingly different from vertebrates. In vertebrates, the odorant signal primarily utilizes a cAMP second messenger mechanism. cAMP binds and opens cyclic nucleotide gated channels upon odor stimulation, allows sodium and calcium to enter the dendrite that in turn triggers a second phase of depolarization mediated by calcium activated chloride channels( Lowe and Gold, 1993). In contrast, insects utilize various system involved in olfactory signal transduction. Biochemical studies by Breer and colleagues indicated that pheromone induction in silk moth results in rapid production of 1, 4, 5-triphosphate (IP3) but found no evidence for the production of 3’5’ cyclic adenosine monophosphate (cAMP) (Breer H et. al 1990). These results were confirmed by Ziegelberger G et al., 1990, where he detected pheromone induced production of 3’5’ cyclic guanosine monophosphate (cGMP) on a slower time scale more consistent with a role in modulating OSN sensitivity. The
biochemical and electrophysiological studies implicating second messengers in insect olfactory signal transduction is prompted by the finding that G protein subunits G\textalpha_s, G\textalpha_q and G\textalpha_o were present in OSNs (Kain P et al, 2008, Miura et al 2005, Jacquin J et al 2002). G\textalpha_s and G\textalpha_q were found enriched in sensory dendrites implicating them in transducing mechanism, but G\textalpha_o was localized only to the olfactory axon bundles, making it less likely that G\textalpha_o signaling is directly involved in transduction. Functional studies on the relevance of these various signaling pathways has been carried out in fruit fly in early 1990s. Carlson and colleagues investigated the G\textalpha_q pathway and found reduced response in flies mutant for PLC and associated phosphoinoside components but the effects were subtle (Carlson J R et al., 1994, 1995). The strongest evidence that G protein signaling coupled to phosphoinoside is required for fly’s sensitivity towards odor came from the study by Kain et al 2008. The authors examined Drosophila G\textalpha_q (dgq) null OSNs and found a shift to lower sensitivity in the absence of dgq. This phenotype was enhanced when OSNs also lacked PLC\textbeta_21c or a DAG kinase encoded by the rdgA gene (Kain et al 2008). The authors concluded that G\textalpha_q is crucial for optimal sensitivity in fly. The idea that fly’s olfaction uses second messenger and the ORs function as GPCR has been questioned by two recent studies led by Leslie Vosshall and Bill S Hanson (2008) (Sato K et.al 2008 and Wiched D et.al 2008). These authors showed that Or83b is actually an ion channel that di-merizes with tuning receptor to form an odorant gated ion channel. Or83b confers novel cation conduction when expressed in heterologous tissue culture cells, further more when it is co-expressed with the receptor the conduction becomes odor dependent. The mutation in the pore forming regions of Or83b modulated the conductance suggesting that this protein acts as an ion flux (Wicher et al., 2008). It has been further observed that the fast ionotropic response persisted in the presence of an inhibitor of G protein signaling. This led the authors to conclude that odor evoked currents mediated by ORs are independent of the G protein signaling pathway. Taken together these results suggest that the fly must have evolved a non canonical mechanism to transduce olfactory signals. One obvious cost of an ionotropic mechanism is the loss of signal amplification provided by GPCRs. In contrast, one benefit is the speed of signaling permitted by direct activation independent of second messenger pathway. It is therefore
possible that both signaling modes are present, perhaps acting at different range of concentrations.

1.5 Electrophysiological Studies of ORs

Ever since Schneider made the first EAG recording from *Bombyx mori* antenna in 1957 this method has been used as a convenient screening procedure for odors that could be of biological significance to insects. EAG measures the summated receptor potential of many OSNs between the tip and the base of antenna. The antennal cuticle has a high resistance which allows a clear potential difference across when the current flows.

The limitation of EAG technique in terms of OR sensitivity and specificity has been overcome by the single sensillum recording technique which monitors the electrical events elicited in OSNs when stimulated by odors. This physiological measurement of OSN activity allows a direct assessment of odor evoked responses, suggesting its odor sensitivity, specificity and response dynamics (de Bryune et al 2001). Electrical events as a result of receptor excitation were first shown in insect mechano-receptors by Wolbracht, 1960. Graded potentials with negative polarity have been measured from side wall recordings of mechano sensory hairs. This potential is correlated with the generated potential, which arises as a result of stimulus induced conductance changes at the tip of the hair and spread to the cell body at the base. It has been shown that the negative phase of the spike propagating antidromically along the dendrites can be blocked by local application of anesthetics as well as tetrodotoxin (Wolbarsht 1964). This led the authors to conclude that the positive and negative phase of the impulse have independent origins and can be considered separately. The interpretation of the positive-negative polarity of the recorded spike is as follow: impulses are generated at or near the cell body and pass antidromically along the dendrites towards the tips of the hairs as well as propagate downwards along the axon to the central nervous system. The path of least resistance between the electrode at the tip of the hair and cell body is through the dendrite rather than dendritic lumen. The external resistance is considered to be due to a tight constriction of the cuticle in the region where the dendrites enter the shaft of the hair. The positive phase of the spike is generated on or near the cell body and spreads in both
directions. When the impulse passes the constriction and enters the dendritic lumen, the opposite situation prevails and the impulse appears negative. The transition from the positive to the negative phase is very rapid, reflecting the short time that the impulse takes to pass the small distance of the constriction. The chemoreceptor impulse also mimics this behavior and under the influence of anesthetics when impulse is prevented from invading the dendrites, monophasic spike is recorded. Spike height can change in parallel with spike frequencies. It has been shown that when the receptor potential is small, the spike shape is biphasic; but when the receptor potential gets increased, the spike becomes large and monophasic (Morita et al. 1959). Negative phase of the spike alters with spike frequency where as positive phase either increases or remains unchanged (Najoi 1984). The shape of a spike recorded from the sensillary lymph depends on many other factors such as dendritic length, density of Na⁺ channels and the electrical resistance and capacitance associated with the neuron. A sensillum that houses multiple neurons can produce distinct spikes. In such cases, individual neuron could be identified by the shape of the extracellular spike produced by them.

1.6 Odor representation in peripheral and higher centers

Odor evoked responses at the peripheral level can be monitored as changes in the frequency of action potentials of olfactory sensory neurons (OSNs). An individual odorant can elicit a response of short duration from some OSNs and long lasting response from others. Likewise, an individual OSN can give a short response to some odors and long response from others (Hallem et.al 2004). Odor induced reduction of basal OSN activity has also been reported in many insect species including the fruit fly (de Bruyne et.al 2001). The existence of two modes of odor evoked responses at the level of OSN might be important for odor coding and it needs further careful examination. The information about an odor is contained in the population response of OSNs. Antennal lobe reformats these OSN responses by increasing signal to noise ratio. A recent study in fruit fly compares the odor evoked responses between OSNs and PNs and found that PNs responded to a broader range of odors than their corresponding OSNs (Bhandawat et.al. 2007). These responses were stronger and had increased signal to noise ratio. The odorant that produces weaker response at OSN level results in an apparent broadening at the
projection neuronal level. It is surprising to note that this type of signal amplification in the antennal lobe only occurs when the OSN input is weak. No gain of signal was recorded when OSN input was strong (Bhandawat et al. 2007). Taken together these results suggest that the antennal lobe has developed a neural ‘gain control’ mechanism that alters the relationship between OSN and PN firing towards odor stimuli. Olfactory information from antennal lobe is then sent to mushroom body. Mushroom body in *Drosophila* consists of approximately 2500 kenyon cells (KCs) that receive inputs from PNs. The responses of KCs to odors are highly selective and thus sparse when compared with those of their direct inputs in the antennal lobe projection neurons (Glenn Turner et al. 2008). Glenn Turner and his associates did an extensive study on the odor evoked responses of KCs using whole cell recording in vivo (Turner GC et al. 2008). Previous studies of KCs odor responses properties using genetically encoded calcium sensor GCaMP, indicated that few KCs responded to a given odor (Wang et al. 2004) implying that KCs firing patterns towards odors are very different. It has been shown that 6 ± 12% of odors evoke a response in a given KC (Turner GC et al. 2008). PNs, by contrast are broadly tuned and 53 ± 39% of odors evoke an excitatory response to PNs (Turner GC et al. 2008). These results together suggest that KCs have very sparse odor representation and it responds to odors with very few spikes. The question arises whether this sparseness could potentially facilitate learning by reducing overlapping representation of odorants as observed in the antennal lobes. It has been found that the odor separability is greater in KCs than any other upstream layers (Turner GC et al. 2008) and this could help in better odor discrimination.

1.7 Olfactory learning in insects and *Drosophila*

Learning can be defined as acquiring information based on repeated experience gathered by an organism in attempts to cope with the immediate demands of the environment. It can modify an individual’s behavior and increase the chances of survival. The simplest form of learning is non-associative learning which include habituation and sensitization. Associative learning is a capacity that is wide spread among several invertebrates and it allows extracting the logical structure of the world. Two major forms
of associative learning are usually recognized: in classical conditioning (Pavlov 1927), animals learn to associate an originally neutral stimulus (conditioned stimulus, CS) with a biologically relevant stimulus (unconditioned stimulus, US) whereas in operant conditioning (Skinner 1938), they learn to associate their own behavior with the reinforcer. Both forms of learning therefore allow predicting the reinforcement and depend on the unambiguous link that relates specific events in an animal’s environment. Both these forms of learning allow different levels of complexity. Simple links between an event and reinforcement or its absence allow solving elemental problems such as absolute conditioning in which a single stimulus is reinforced. In differential conditioning animals learn to respond to one stimulus which is associated with reinforcement whereas the other stimulus is non-reinforced. Insects are proven to learn these associations in order to solve demanding tasks such as navigation during exploratory behavior, foraging and the search for new oviposition site. A large part of their success comes from their remarkable sensory system. Insects can smell and discriminate odor with high sensitivity and specificity. Our understanding about olfactory learning in insects is increasing. It is becoming evident that olfactory learning is more dynamic than previously thought. For example, phytophagous insects learn specific odor templates that predict their host occurrence and reduce their searching behavior. The experience plays an important role in insect’s life. The effect of experience may not only result in an enhanced preference for the particular odor but also may result in the decline of responsiveness to the learned odor. This was shown for mate recognition in halictine bees (Smith and Ayasse, 1987). Male bees were normally highly attracted to female odors, but after contact with a female, the frequency of contacts and mating attempts declined. The olfactory response only returned to the initially high level when an unfamiliar female was offered. Many advances towards understanding insect olfactory learning have been achieved by using the genetically tractable fruit fly, *Drosophila melanogaster*. The fly keeps a record of its experience which it uses to inform its actions. First evidence on olfactory learning in fruit fly came from Seymur Benzer’s lab in early 1970s. Flies were trained to avoid an odor associated with electric shock. Odor memory was tested at given times by allowing the flies to run into either a tube containing CS+ and CS- odor and in each case the number of flies avoiding CS+ was counted. The olfactory learning in fruit fly took a significant
step forward with the development of a classical conditioning assay that involves a binary T maze choice (Tully et.al 1985). In this assay odor memory was tested by calculating the fraction of flies trapped in either of the two tubes containing odorant that they experienced during conditioning. The memory can be tested immediately after training or flies can be transferred to vials and housed until being tested at later time points to assess different memory phases. Although training flies to avoid shock is effective, shock is not an ecologically relevant reinforcer. Temple et.al (1983) described olfactory learning with odorants paired with sucrose reward. They used light (and negative geotaxis) to attract food deprived flies into a training tube painted with a band of sucrose and odor. Flies have to be hungry in order to execute enhanced behavioral attraction towards learned odor in the sugar rewarded paradigm (Temple et.al 1983). Using these approaches we gained considerable knowledge about the basic mechanism underlying olfactory learning in fruit fly. But understanding how the neural circuits direct such behavior is a major question that needs to be properly addressed and studied. At some point we would like to know how experience of smelling turns into behavior.

1.8 Aim of the thesis

Experiments carried out in our laboratory showed that the olfactory response of fruit fly imago dramatically changes with odor experience during early adulthood. Flies kept on odorless sucrose agar medium exhibit minimal attraction to chemicals. We called this phenomenon imaginal conditioning (Punita Panchal and Firdos A Khan, unpublished). The neural mechanism underlying imaginal conditioning is not known. The aim of the thesis is to study the neurophysiological aspect of imaginal conditioning. Extracellular single unit recordings as well as functional imaging have been employed in order to decipher the neural correlates of conditioning.

Bilal Rashid and colleagues (2005) have isolated imaginal conditioning mutants (icon) after conditioning flies with a single odor. On the basis of their phenotypes, these mutants have been grouped in three different classes. Class I mutants show diminished conditioned response (icon\'); class II mutants exhibit enhanced response (icon''\') and
class III show increased unconditioned or constitutive response (icon^\textsuperscript{c}). Jawaid Ahsan has performed the molecular characterization of these mutants and the P-element insert point was localized by either plasmid rescue or inverse PCR or both (Ahsan J, 2009). Electrophysiological characterization has been carried out in these mutants for various odorants in order to relate behavior with neural activities.

Effect of starvation in the adult fruit fly has also been studied at both behavioral as well as neurophysiological level. It has to be determined how much of the enhanced attraction can be attributed to starvation alone and how much is due to imaginal conditioning. We tried to dissociate these two effects both at the behavioral as well as at the level of sensory physiology. The detailed accounts are given below.

1.9 Outline of the thesis

In the present study we have examined the neurophysiological effect of imaginal conditioning in *Drosophila* imago. Prior odor experience greatly enhances attraction to odors to which the fly was exposed soon after its emergence. The effect is odor specific. It can not therefore, be treated merely as habituation. The time course of imaginal conditioning is slow, in the order of hours to days and it leads to increased attraction to familiar odorants. The unfamiliar odorants on the other hand become more aversive.

The work presented in chapter 3 shows that imaginal conditioning is associated with increase in electrophysiological responses of sensilla basiconica. Sensitization involves odorants that acts on common olfactory receptor which in our case is Or59b housed in type II sensilla basiconica. A set of olfactory mutants showing defects in imaginal conditioning in our behavioral assay have also been characterized electrophysiologically. It has been found that in three out of eight mutants, the odor induced response at the olfactory sensory neuronal level is reduced. These results provide evidence that at least a part of the conditioning is therefore due to changes at the peripheral level.
In order to study whether increased sensitivity at the receptor neuronal level also affects the sensory threshold of the synapses in the antennal lobe we employed in vivo two photon functional imaging. Chapter 4 shows that flies conditioned with ethyl acetate exhibit hypersensitivity to EA in Or42b OSN terminals. No increment in Ca$^{2+}$ influx was observed when OSNs output is blocked. These results suggest that OSN output is required for olfactory conditioning. The effect of conditioning has also been investigated at the level of projection neurons (PNs). Flies conditioned with EA exhibits lower physiological threshold to EA at the dendrite of cognate projection neurons. It has been further noted that imaginal conditioning is also associated with morphological changes of the selected glomeruli in the antennal lobe and OSNs. Flies conditioned with EA show increased glomerular volume when compared with unconditioned. The early exposure to hexanol also increases the volume of olfactory cilia in type III sensilla basiconica. Taken together, these results in chapter 4 suggest that odor induction increases the sensitivity not only in OSNs but also its cognate synapses and projection neurons. In addition, it is also associated with morphological changes in the olfactory pathway of Drosophila imago.

Chapter 5 describes the effect of starvation on the sensitivity of OSNs and fly’s olfactory behavior to food odorants. The attraction behavior towards ethyl acetate increases with starvation. Effect of starvation is pronounced against 2-3 butanedione and geranyl acetate implying that behavioral response towards food odorants is prompted by starvation. The response to aversive odor, benzaldehyde is largely unaffected by starvation. It has been found that in addition to enhanced behavioral attraction, starvation results increased in antennal sensitivity to food odorants. Single unit recordings from type IB and type IIA show that the flies have higher firing frequency when starved. Similar to behavioral responses, the effect of starvation is pronounced at lower concentration. Both starvation and imaginal conditioning increase the firing frequency of olfactory sensory neurons but not to the same extent. However, maximum response was obtained in flies where starvation is coupled with conditioning. An attempt has been made to decipher the underlying mechanism of starvation. Recently a number of neuropeptides have been shown to be involved in starvation (Carlsson et.al. 2010). We showed that Drosophila
neuropeptide F (dNPF) is involved in starvation. Blocking these peptides cause reduced starvation and hence flies do not show increased attraction to ethyl acetate. Imaginal conditioning mutants have been also used to study the mechanism underlying sensitivity change as a result of starvation and conditioning.

Chapter 6 describes an information theoretic approach of OSNs firing dynamics as a result of post-eclosion odor experience in *Drosophila* imago. Jensen-Shanon divergence metric (\(D_{JS}\)) from information theory has been employed in order to access the differences between ensemble spike response patterns of different odors. Cumulative spike count and cumulative information has been calculated to represent the dynamic patterns of odor responses. The results show that flies raised on an odor less synthetic medium have lesser sensitivity when compared with conditioned. Prolonged exposure to EA increases sensitivity to EA and several other related esters but it does not improve the ability of the fly to distinguish between them. Flies exposed to cornmeal medium display varied sensitivity to these odorants and at the same time greater capacity to distinguish between odors. The \(D_{JS}\) used in these experiments contrasts the differences in the probability of spike occurrence along a response time course than the average firing rate or total spike count. We have found that substantial distinction between odor dilutions occur before the peak firing rate, implying that the initial phase of the odor response contributes maximally to dilution discrimination. Taken together these results enable us to understand how post-eclosion odor experiences change the single unit response at the level of sensory neurons.