Differential modes of Eph signaling in olfactory dendrite targeting of *Drosophila*

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To my Parents…

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Abbreviations

ad	anterior-dorsal
AL	Antennal lobe
APF	After Puparium Formation
ato	atonal
BSC	Bloomington Stock Center
Bnl	Branchless
Btl	Breathless
CAMs	Cell Adhesion Molecules
CNS	Central nervous system
DH	Dextrin Homology domain
DIG	Digoxigenin
EGFR	Epithelial growth factor recpetor
Exn	Ephexin
ey	eyeless
F-actin	Fibrillar actin
FGFR	Fibroblat Growth Factor Receptor
Flp	Flippase
FRT	Flippase Recognition Target
GAP	GTPase Activating Protein
GEF	Guanine Nucleotide Exchange Factor
GPCRs	G-Protein Coupled Receptors
Gr	Gustatory Receptor
hs	heat shock
Htl	heartless
la	lateral

LI	Learning Index
LNs	Local Interneurons
MARCM	Mosaic Analysis of Repressible Cell Marker
MT	Microtubule
NMJ	Neuro Muscular Junction
O/E	Overexpression
OR	Odorant Receptor
ORNs	Odorant Recpetor Neurons
PBS	Phosphate Buffer Saline
PBT	Phosphate Buffer Saline triton
PBL	Phosphate Buffer Lysine
PH	Plextrin Homology domain
PI	Performance Index
PLP	Periodate Lysine Paraformaldehyde
PNs	Projection Neurons
ROI	Region Of Interest
RT	Room Temperature
RTKs	Receptor Tyrosine Kinases
SH3	SRC Homology 3 domain
SMART	Simple Modular Architecture Research Tool
syt	synaptotagmin
TEM	Transmission Electron Microscopy
UAS	Upstream Activating Sequence
WT	Wild Type
ve	ventral

Summary

Deciphering the mechanisms of sensory neural map formation is a central aim in neurosciences. Failure to form a correct map frequently leads to defects in sensory processing and perception. Since decades, scientists are engaged in identifying and characterizing specific molecules involved in neuronal circuit formation. The olfactory system of *Drosophila* has proven to be an amenable system to identify mechanisms for map formation in general as well as for development of olfactory maps in particular. The olfactory map discriminates a large number of odorants using precisely wired neuronal circuits. It develops in subsequent steps, initially forming a rough and later a precise map of glomeruli mainly consisting of olfactory receptor neuron (ORN) axons, projection neuron (PN) dendrites and local interneuron (LN) dendrites and axons. The mechanisms underpinning in particular the later stage of class-specific glomerulus formation are not well understood. Hence this system is of particular interest for attacking the problem of neural circuit formation.

Here, the role of the important system of guidance receptors, Eph and ephrin, and their downstream signaling molecule Ephexin (Exn) in a specific subset of olfactory and projection neurons is analyzed. This work reveals differential signaling mechanisms downstream of Eph signaling during olfactory map formation. Series of experiments show that the Eph-specific Rho-GEF Exn is required to fine-tune PN dendrite patterning within specific glomeruli. It provides the first report showing an *in vivo* neurite guidance defect in an *exn* mutant. Interestingly, the quality of the phenotypes is different between *eph* and *exn* mutants; while loss of Eph leads to strong misprojections of DM3/Or47a

neurons along the medial-lateral axis of the antennal lobe (AL), loss of Exn induces ventral ectopic innervation of a neighboring glomerulus. Genetic interaction between Eph and Exn suggests that differential signaling of the small GTPases Rac1 and Cdc42 mediated by Exn-dependent and -independent Eph signaling fine-tunes spatial targeting of PN dendrites within the olfactory map.

Taken together, these results support the hypothesis that the precise connectivity of an individual neuron can depend on different modes of signaling downstream of a single guidance receptor.

1. Introduction

A neuron is the basic working unit of a brain. Neurons never work in isolation: they always connect to each other in many remarkably specific patterns to form circuits. These circuits are required to process varied information that is crucial for an animal. They help an animal to interpret the world around it through its senses. Not all circuits have same kind of structure, since they have to perform different functions: but all have to be precisely assembled for the correct processing of the information. The basic wiring map is formed during the early development of an animal. This process involves several molecular mechanisms that regulate the developmental events.

Since decades scientists are investigating how a neural circuit is assembled. Why do neurons appear to never make mistakes while forming a neural circuit? Which external cues have the capacity to steer the extending neuronal processes? To answer some of these questions, this PhD thesis was initiated to decipher the role of Eph/ephrin signaling in forming the olfactory circuitry of *Drosophila melanogaster.*

The central question in this thesis is which factors are required for the correct patterning of the olfactory system.The first part of the introduction will describe in detail the mechanism of neuronal circuit formation, focusing mainly on the axon guidance and dendritic patterning. This will be followed by explaining why the olfactory system *of Drosophila melanogaster* is chosen to understand the mechanism of neuronal wiring. And finally the choice of working on Eph/ephrin signaling will be justified in this system.

1.1 Neuronal Wiring

Neuronal wiring can be broadly divided into two parts: 1) Activity dependent and 2) Activity independent (Goodman and Shatz, 1993). During the embryonic development of the central nervous system (CNS), the initial process of neuronal wiring is activity independent. The pioneer axons navigate through the environmental cues and target the area of interest. Then the follower axon navigates their way by interacting with the environmental cues and also taking the pioneer axons as the cue for their correct targeting. Studies have shown a difference between the growth cone dynamics and complexity, of the pioneer and follower axons (Bak and Fraser, 2003; Kulkarni et al., 2007). On the other hand the activity dependent wiring is taking place throughout the life of the animal. When the neuronal circuits become active (after initial wiring), the process of refinement and remodeling of these circuits occurs in an activity dependent manner. Here more focus is on the activity independent initial wiring of the brain.

Determining how neuronal connectivity is established during neural development and regulated during adult life is dependent on identifying the molecules and signaling events underlying neuronal guidance. Three kinds of experimental approaches have identified a variety of guidance molecules: 1) Biochemistry and *in vitro* tissue culture assays to detect proteins with either attractive or repellent properties (Keleman and Dickson, 2001; Kruger et al., 2005; Raper and Kapfhammer, 1990); 2) Forward genetics approaches to identify mutations that affect axon guidance *in vivo* (Berdnik et al., 2012; Hong et al., 2009; Medina et al., 2006; Schmitz et al., 2007); or 3) Genetic and tissue culture approaches to characterize the functions of molecules with structures that make them candidate guidance cues (Bossing and Brand, 2002; Brennan et al., 1997).

Overall these approaches made it possible to understand the process of neuronal wiring in terms of underlying molecular mechanism that leads to cytoskeletal dynamics and finally growth cone motility.

1.1.1 Axonal and dendritic guidance

Axon guidance is a process that is divided into several steps. Many of the neurons target their axons over a long distance, which is, generally broken into short range targets or choice points (Tessier-Lavigne and Goodman, 1996). This breaks down the process into series of decision making events. In a way, axon targeting can be defined as a simple linear extension process with some incorporated choice points leading to switch from one path to the other. Ramon y Cajal was the first one to visualize growing tips of axons and he noticed that they extend towards their target with high efficiency. He hypothesized that axon tips are driven by chemotactic cues.

Neuronal growth cones, fan shaped structure with many long and thin spikes, navigate through a series of intermediate choice points to find correct targets. A huge number of guidance cues in the extracellular environment are encountered at each choice point in the targeting path by the growth cone (Yu and Bargmann, 2001). A specific expression pattern of various guidance cues, receptors and ligands lead to the appropriate target choice of the axon. The reason why different axons choose different paths at the same choice point is due to the tailored expression pattern of various receptors and ligands. Guidance cues associated with particular intermediate or final targets can be chemo attractive or chemo repulsive (Figure 1.1.1).

The first axons (pioneers) in the central nervous system of both vertebrates and invertebrates grows along stereotyped routes to form a scaffold that provides basic framework for the follower axons (Wilson et al., 1990). These pioneer axons are actively guided to their targets by the information available in the environment of the developing nervous system. Many of the later developing axons travel along pre-existing axon

tracts (or fascicles) for at least some of their trajectory, switching from one fascicle to another at specific choice points (Raper et al., 1983). This "selective fasciculation" strategy simplifies the assembly of large nervous systems.

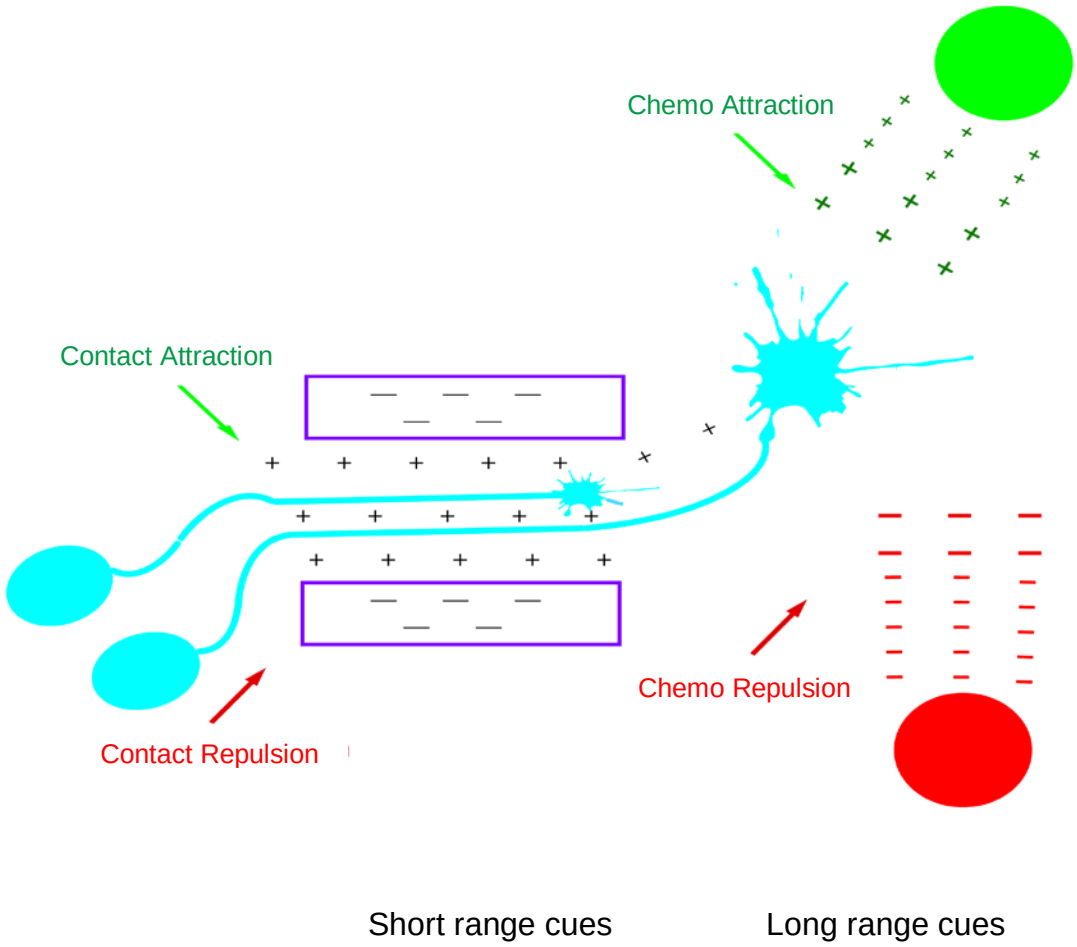


Figure 1.1 1 : Growth cone guidance divided into four different mechanisms.

Four types of guidance forces: contact attraction, contact repulsion, chemo attraction and chemo repulsion. Attraction in this context is the range of permissive and attractive cues whereas repulsion is the range of inhibitry and repulsive cues. Axon navigates through a corridor of attractive cues (short range cues) provided by other axons (selective fasciculation) and inhibited by other axons presenting the inhibitory cues (contact repulsion). Individual growth cones can be pushed by chemo repellant (shown in red) and attracted to their target by chemo attractents (shown in green). Push, pull and hem are the forces that that act together to ensure correct guidance. Figure adapted from (Tessier-Lavigne and Goodman, 1996)

Although these pioneer axons are quite important for guiding the follower axons, it is not the only cue available for the follower axons. Results from the studies leading to the ablation of pioneer axons have shown delayed or rerouted follower axons (Hutter, 2003). But it mostly doesn't prevent the followers from reaching the target (Pittman et al., 2008; Whitlock and Westerfield, 1998).

Similar to axons, dendrites also need proper guidance for correct targeting and positioning within the brain. The axons and dendrites from each neuron have disctinct physiological properties and despite their same cellular origin, they target separate synaptic regions. This suggests a regulatory system that seggregates axons from dendrites. Many different hypothesis can explain these differences: 1) Dendrites develop later than axons and hence can account for independence observed (Dotti et al., 1988). 2) Specific control of molecular localization via different targeting sequences or motors leads to differences in axons and dendrites (Shi et al., 2003). 3) Targeting of alternatively spliced forms, with different properties to different compartments. All in all, where a denrite targets is as integral to the neuronal circuit as is the targeting of an axon. Studies have shown that overlapping classes of guidance molecules are involved in both axonal and dendritic targeting (Kim and Chiba, 2004). Guidance molecules present in both axonal and dendritic growth cones mediate neuronal responses to extracellular cues thereby ensuring correct neurite path finding and development of the nervous system.

In the past literature, several neuronal circuits have been chosen for their best adequacy to study a particular step in the formation of a functional neuronal circuit. The study of neuronal circuit development in such models highlights some constants in the

programming of neuron connectivity. Two types of neuronal circuits that have been extensively studied for the neuronal guidance process in *Drosophila* are the CNS midline (Bossing and Brand, 2002; Kolodziej et al., 1996; Kuzin et al., 2005) and the visual system (Clandinin and Zipursky, 2002).

The CNS of bilateral animals is divided into two halves by a midline. These two halves have to be coordinated for the functioning of the brain of these bilateral animals. This coordination between the left and the right sides of the brain is mediated by the projections from various neurons located around the midline. These axons have two choices, either to cross the midline or not to cross the midline. This system is extremely good to study the axonal targeting process, and specifically to study how an axon makes a direction choice. The most studied animal for this direction choice by the axons is *Drosophila melanogaster*. The embryonic abdominal CNS consists of hemi segments which constitute segments A2-A7. Each hemi segment contains 342 neurons. Out of these neurons 34 neurons exit the CNS and target 30 abdominal muscles (Schmid et al., 1999). At the midline, the crossing of neuron projections (both axons and dendrites) is depicted as ipsilateral and contralateral, connected by commissures. At this midline there are many attractive and repulsive pathways working which leads to contralateral and ipsilateral presence of particular projections respectively. Several mutations have been shown to be associated with either a thickened commissure or a much thinner commissure showing either loss of repulsion or loss of attraction respectively (Tessier-Lavigne and Goodman, 1996). These results show that how axons depend on their extracellular environment for correct navigation. The most important cells for these neurons are the midline glia that acts as the organization center for CNS projections.

Molecules shown to be involved in this process are: Netrins and their receptors Frazzled and Unc5 (Keleman and Dickson, 2001; Kolodziej et al., 1996), Roundabout family of receptors and their ligands – slit, that are secreted by the midline glial cells (Battye et al., 1999; Seeger et al., 1993), Commisureless which causes degradation of Roundabout by diverting it to the endosome pathway (Keleman et al., 2002), etc.

In addition to the CNS midline, another extensively studied neuronal network is the visual system (Clandinin and Zipursky, 2002; Furrer and Chiba, 2004). In *Drosophila*, the eye consists of 800 ommatidia that contains 8 different photoreceptor cells, R1-R8. They send their projections in a sequential manner to the different layer of the optic lobe, that mimics the brith order of R cells. Among these R cells, R1-R6 target to the lamina and synapse with the target cells forming structures called catridges (Meinertzhagen and O'Neil, 1991). On the contrary, R7 and R8 axons target the deeper layer of medulla, R7 synpases deeper than R8. Screening for mutations that affect R cells targeting have identified several molecules invovled in the process. Examples include: brakeless (Senti et al., 2000), Flamingo (Usui et al., 2003), N cadherin (Lee et al., 2001), ephrin (Dearborn et al., 2002) , etc. Once the R cells axons have reached their specified layer, they are confronted by the choice of a specific target inside the layer which forms a precise topographic map. This process involves a set of cell adhesion molecules like N-cadherin (N-cad). Flamingo, a member of N-cad gene family, is involved in the target selection of R1-R6 and R8 axons.

Both these examples of neuronal circuits show that at each time point in their development, neurons are capable of integrating external information into their processes (axons or dendrites or sister branches) and respond appropriately.

Identification of moleules that control the differentiation and integration of neurons in a function circuit suggests that neurons are capable of integrating complex information provided by the external cues. Following section gives a detailed overview of the major classes of external cues that a neuron is exposed to while the neuronal circuit is formed.

1.1.2 Signaling guidance molecules.

To this date the role of many molecules have been established in neuronal wiring. Broadly there are four major families of guidance cues involved in neuronal wiring: Netrins, Slits, Semaphorins and Ephrins. In addition to these clases, other molecules known to play a role in neuronal circuit formation are: cell adhesion molecules (CAMs), immunoglobulin (Ig) family of proteins and cadherin superfamily (Figure 1.1.2).

Netrins can be either attractive or repulsive cues, hence they can attract and repel axons from the midline. In *Drosophila,* Netrins provide an attractive cue for the axons to the midline (Brankatschk and Dickson, 2006). The attractive effects of Netrins are mediated by receptors of the DCC (Deleted in Colorectal Carcinomas) family whereas the repulsive effects are mediated by the Unc5 family. In *Drosophila*, Frazzled is reported to lead to attraction (Kolodziej et al., 1996) and Unc5 leads to repulsion in response to Netrin (Keleman and Dickson, 2001). In addition netrins can also function as a “long range” as well as a “short range” cue.

Slits are large secreated molecules which are implicated in axon repulsion. The receptors of slit that mediate the repulsive response are Robo (roundabout) family of receptors (Brose et al., 1999). Similar to DCC and Unc5, Robo also belongs to the immunoglobulin superfamily. Slits are also known to mediate branching of both axons and dendrites mediated by Robo family (Brose and Tessier-Lavigne, 2000).

Semaphorins are large protein family that include secreated as well as transmembrane guidance cues. *In vivo* studies show Semaphorins to be invloved in neuronal development and present a key repulsive cue. Semaphorins signal through multimeric

receptor complexes. All Semaphorin receptor complexes include a Plexin protein. Plexin family are the major receptors for Semaphorins. Membrane bound Semaphorins bind to Plexins directly, but secreted vertebrate Semaphorins bind to the co-receptors Neuropilin-1 or Neuropilin-2 (He and Tessier-Lavigne, 1997; Winberg et al., 1998). Semaphorins under some circumstances act as both attractive and repulsive cues (Kruger et al., 2005). Transmembrane Semaphorins are capable to act as receptors. In *Drosophila*, they are known to regulate the dendritic targeting in the *Drosophila* olfactory system (Komiyama et al., 2007) and photoreceptor guidane in the *Drosophila* visual system (Cafferty et al., 2006).

Ephrins are a large family of cell surface signaling molecules (Pandey et al., 1995; Tuzi and Gullick, 1994). There are broadly 2 classes in vertebrates. They are always membrane bound and cleaved forms are not known to activate the receptor. Hence, they most probably act as short range cues. They bind to the receptor tyrosine kinase-Eph family (Tuzi and Gullick, 1994). They are implicated as short-range attractants and repellants in the guidance of many central and peripheral axons. They also lead to reverse signaling, involved in topographic mapping and axon guidance (Dearborn et al., 2002; Drescher et al., 1997). More details are explained in section 1.3.

Cell adhesion molecules (CAMs) regulate outgrowth or attraction by functioning as signaling molecules. Important members include DsCAM (down syndrome cell adhesion molecule), well known example from *Drosophila* (Hummel et al., 2003; Schmucker et al., 2000). It has over 19,000 isoforms that can bind to itself and mediate repulsion. It is known to be invloved in axonal and dendritic self-avoidance. In addition **cadherins** are

also involved in guidance process. In *Drosophila*, N-cad regulates targeting of axons in the lamina as mentioned above.

All the mentioned guidance molecules lead to attraction or repulsion by regulating cytoskeletal dynamics. This cytoskeletal dynamics lead to either growth cone extension or collapse depending on attractive or repulsive cue, respectively.

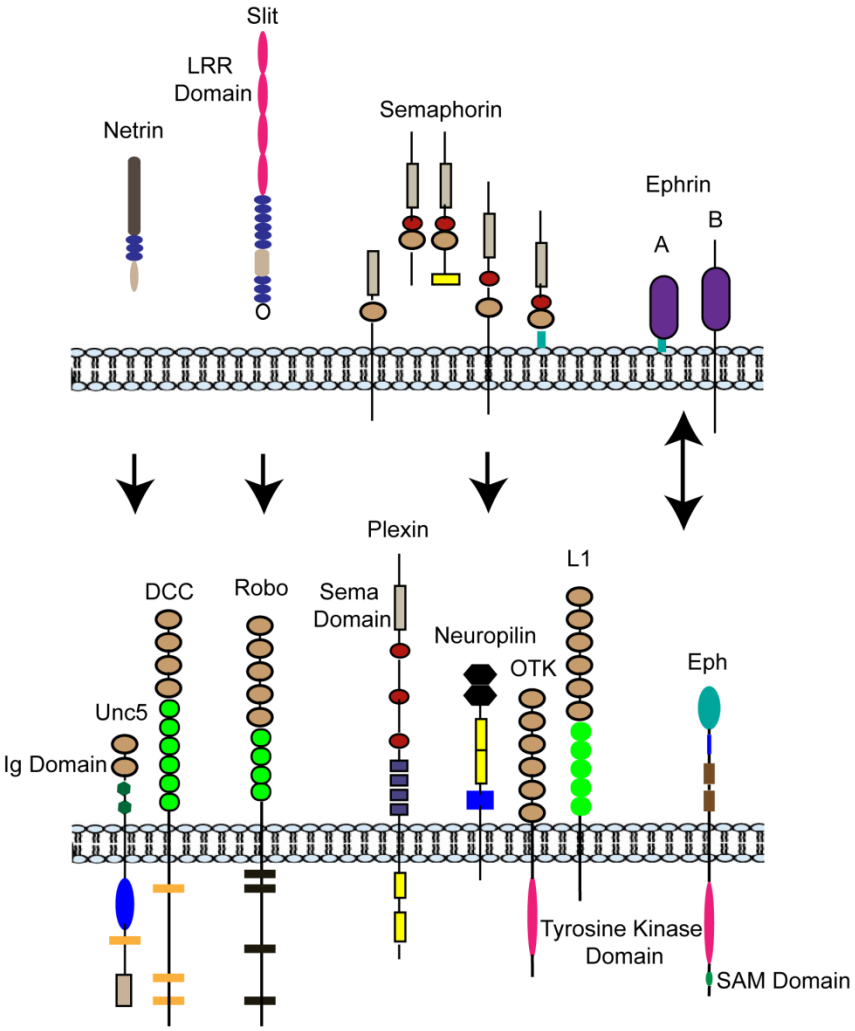


Figure 1.1 2 : Conserved family of guidance molecules.

Conserved family of guidance cues and their receptors known to play a role in axon guidance. Direction of arrow indicates the direction of signaling. Conserved tyrosine Kinase, SAM and immunoglobulin (Ig) domain of the receptors are indicated.

1.1.3 Cytoskeletal dynamics

Growth cone turning is a complex process in which actin-based motility is harnessed to produce persistent and directed microtubule (MT) advance (Myers and Baas, 2011). All the signaling molecules mentioned above and many more have the ability to transduce extracellular signals into structural changes in cell morphology (Dubreuil and Vactor, 2011). This results in either growth cone extension, steering or retraction. Changes in cytoskeletal dynamics steer growth cones so as to attract or repel them from the cue source. The arrangement of actin and the MT acts in a way that fibrillar actin (F-actin) is in the peripehral domain of growth cone, bundled MT is in the axon shaft and in the central doamin of the growth cone. Attractive and repulsive cues influence the mechanisms underlying growth cone behavior, including F-actin and MT assembly or disassembly. The assembly and disassembly of F-actin is an active process that requires the transfer of phosphate groups. This is mediated by small guanine nucleotide triphosphatases (GTPases).

The Ras superfamily of small GTPases consisting of almost 200 proteins, can be subclassified into six families: Rho, Ras, Rab, Arf, Sar and Ran (Colicelli, 2004). The Ras family of GTPases are activated by growth factor receptors and adhesion receptors and leads to signal transduction including ERK, MAP kinase, and PI3-kinase (Hall and Lalli, 2010). On the other hand, the major role of Rho family of GTPases is to control the assembly, disassembly, and dynamics of actin cytoskeleton (Symons, 1996). Several guidance cues are known to regulate members of Rho family of small GTPases: Rho, Rac and Cdc42. These GTPases cycle between active GTP-bound state to inactive GDP-bound state. This cycling between these two states is regulated by the action of

GTPases activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) respectively. Rho and ROCK (Rho associated protein kinases) often appear to work antagonistically to Rac/Cdc42. The former is associated with repulsive cues while the later with attractive cues (Govek et al., 2005).

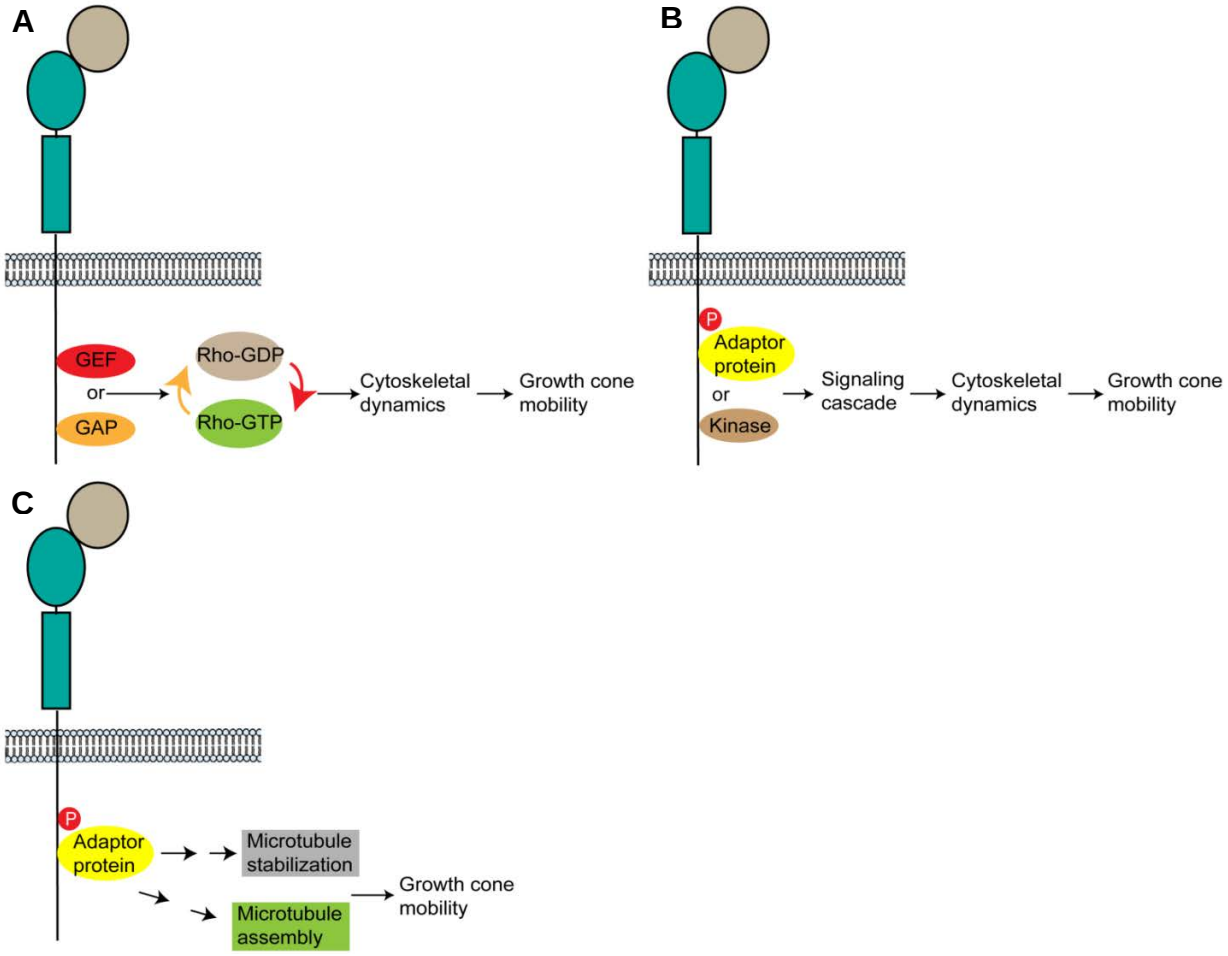


Figure 1.1 3 : Guidance cue receptor signaling strategies.

A) Rho GTPase activity is modulated by GEFs and GAPs, downstream of activated guidance receptors, which in turn leads to cytoskeletal dynamics. B) Activated guidance recpetors lead to signaling cascade, including kinases and adaptor proteins that regulate cytoskeletal dynamics. C) Direct regulation of MT dynamic by affecting MT binding proteins leading to growth cone motility.

Various signaling mechanisms are capable of regulating actin dynamics by three different ways (Figure1.1.3). 1) Activation of guidance cues modulates Rho GTPases activities by action of multiple GEFs and GAPs . 2) Activation of guidance cues leads to signaling events, like phosphorylation, triggering signaling cascades which regulate actin dynamics. 3) Activation of guidance cues leads to direct regulation of microtubule (MT) dynamics by effecting MT binding proteins (Hall and Lalli, 2010). Taken together, the range of events downstream of the guidance receptors provide multiple avenues for regulation of neuronal cytoskeleton and hence growth cone steering.

1.2 Drosophila olfactory system

Out of all the sensory modalities, olfactory system is the least studied one in the vertebrates. The most probable reason for this is the numerical complexity of odorant receptors (Buck and Axel, 1991; Parmentier et al., 1992). On the other hand numerical simplicity of the olfactory repotoire of insects made it extensively studied for its structure, development and function (Fan et al., 2011; Hansson and Stensmyr, 2011; Martin et al., 2011).

The olfactory system discriminates a large number of odorants using precisely wired neuronal circuits. It offers an excellent opportunity to study mechanisms of neuronal wiring specificity at the synapse level. *Drosophila melanogaster* has a very sophisticated olfactory system that permits it to discriminate hundreds of odorants. The perception of these odorants is essential for it to identify relevant food sources, mate and suitable oviposition sites. *Drosophila melanogaster* has been of particular interest for the study of olfaction because of the opportunity for genetic manipulation, availability of a complete genome sequence (Myers, 2000), and its ability to learn in olfactory-based associative learning paradigms (Busto et al., 2010). The adult olfactory system of *Drosophila* has similar, but numerically simplified, sensory and synaptic specificity as compared to vertebrates. All in all, *Drosophila melanogaster* is a good model system to study different aspects of development and functioning of an olfactory neuronal circuit. It presents a unique opportunity for precise genetic labeling and manipulations to test the requirement of specific cell types and molecules for correct wiring.

Drosophila melanogaster has two types of bilaterally symmetrical peripheral olfactory appendages: antenna and maxillary palp (Figure 1.2.1 A). These peripheral structures

have the olfactory sensory structures called the sensilla. These sensilla are divided into three categories based on its morphology: trichoidea, basiconica and coleoconica. Trichoidea contain one or three olfactory neurons, basiconica contain two or four olfactory neurons and coleoconica contain two or three olfactory neurons. The olfactory system is characterized by a large number of speciliazed neuronal cell types. In both insects and mammals, olfactory receptor neurons (ORNs) expressing specific olfactory receptors (ORs) converge their axons onto specific glomeruli, creating a spatial map in the brain (Mombaerts et al., 1996; Vassar et al., 1994). Each OR consists of seven transmembrane domains which transduces odor recognition into neuronal activation through G-protein-coupled signaling pathways (Clyne et al., 1999; Vosshall et al., 1999). Each ORN extends an axon that synapses with second-order neurons - Projection neurons (PNs) and Local interneurons (LNs) in the the antennal lobe (AL) (Wong et al., 2002). From this first olfactory synapse, information is relayed to higher brain centers that drive appropriate behaviors (Figure 1.2.1 B,C).

The adult antenna and maxillary palp develops from the eye-antennal discs. The differentiation of the eye-antennal disc is established by the action of patterning genes like *engrailed*, *wingless*, *decapentaplegic* and *hedgehog* (Cohen and Di Nardo, 1993). In addition two major transcription factors: *amos* and atonal (*ato*), are responsible for defining the olfactory progenitors (Goulding et al., 2000; Gupta and Rodrigues, 1997). Loss of *ato* leads to lack of coleoconica whereas loss of amos display a lack of both trichoid and basiconic sensilla on the antenna. Senisilla on maxillary palp are defined by *ato*.

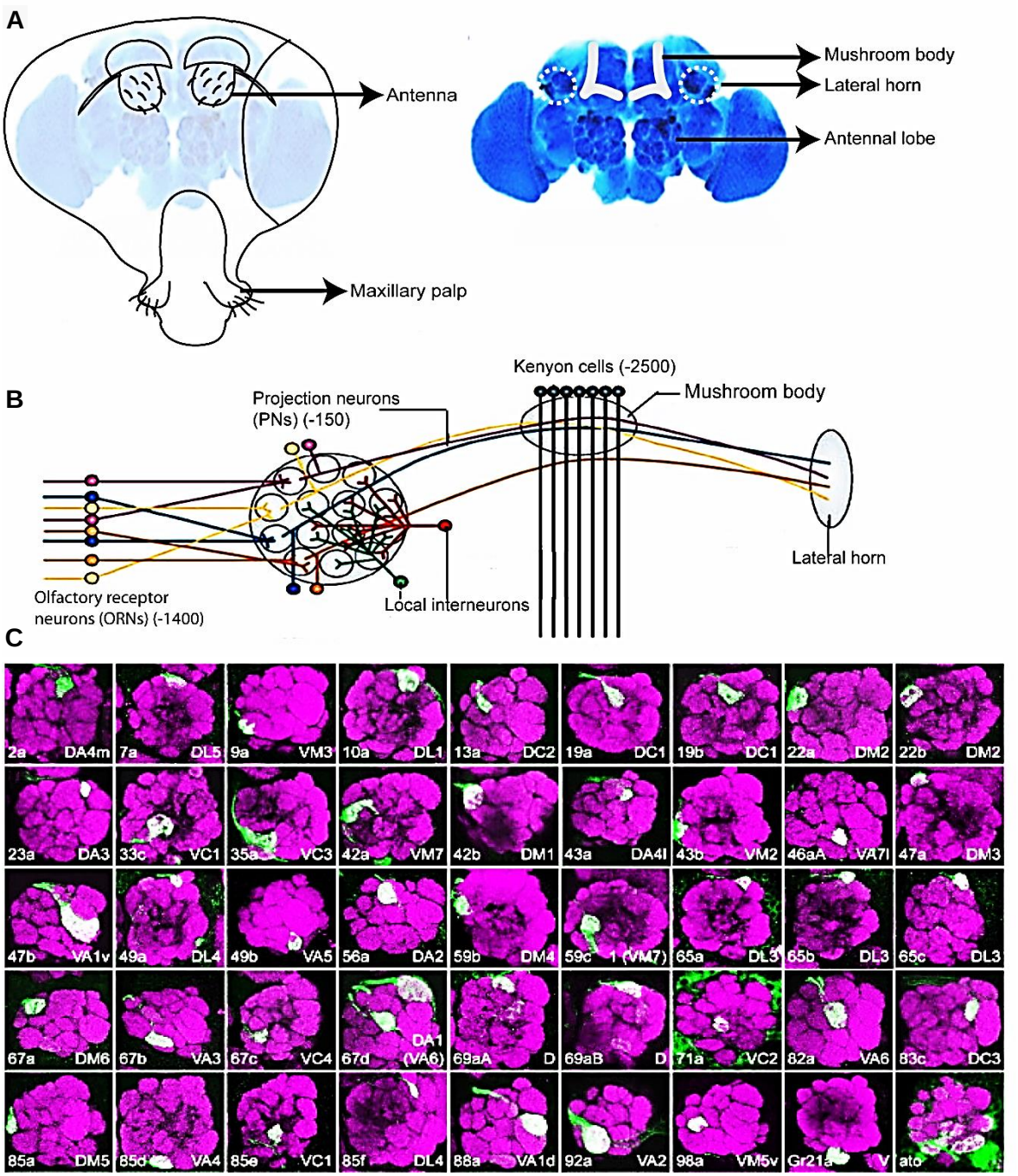


Figure 1.2 1 : Olfactory connectivity in *Drosophila.*

A) Cartoon of a *Drosophila* head showing peripheral olfactory organs: antenna and maxillary palp. The brain on right panel showing antennal lobe, mushroom body and lateral horn. B) Schematic of *Drosophila* olfactory circuit. ORNs expressing the same receptor (same colour) project their axons to the same glomerulus in the antennal lobe. They synapse with at least three classes of interneuron: uniglomerular PNs (same color code as their partner ORNs), multiglomerular inhibitory LNs (red) and multiglomerular

excitatory LNs (green). PNs form synapses with Kenyon cells in the mushroom body calyx en route to terminating in the lateral horn. Some PNs bypass the mushroom body calyx and project only to the lateral horn. Adapted from (Keene and Waddell, 2007). C) Antennal lobes of various *Or-mCD8-GFP* reporter lines were stained with anti-GFP to visualize the ORN axons (green), and counterstained with nc82 to visualize the glomerular structure of the antennal lobe (magenta). “ato” indicates *ato-GAL4*, *UAS-mCD8-GFP* animals. Adapted from (Couto et al., 2005).

The neuronal cells of the olfactory system of *Drosophila* develop and target their area of interest in a sequential manner. Projections neurons (PNs) are derived from three neuroblast lineages: anterior-dorsal (ad), lateral (la) and a ventral (ve) neuroblast. ad and la cluster send dendrites to mutually exclusive sets of glomeruli (Stocker et al., 1997).In larval stage, embryonic born PNs target a single glomerulus in the AL and persists in adult olfactory circuit. These neurons prune their dendrites and axon terminals during metamorphosis (Marin et al., 2005).

PN dendrite development starts with the extension of dendritic processes into different regions of the AL and by about 20 h after puparium formation (APF) most of the PNs occupy the early AL.The axon branches of PNs are evident in the lateral horn (LH) by 24-30 h APF. In parallel, ORN axons target the AL along the antennal nerve at about 18-20 h APF(Jefferis et al., 2004). PNs form the intial prototypic map of the AL and ORN axons follow their cognate PN partners for the correct targeting (Hong et al., 2009, 2012). ORN axons begin to merge with PN dendrites at about 35 h APF and most of the glomeruli start to emerge by 50 h APF (Hummel and Zipursky, 2004; Jefferis et al., 2004).

The whole process of wiring of ORNs and PNs is precisely regulated by various guidance cues (Figure1.2.2). Several molecular mechanisms have been implicated in PN and ORN targeting leading to a multistep targeting model involving different classes

of molecules. An initial coarse map of PN dendrites is established with the help of graded Semaphorin – 1a expression in the dendrites, which thereby react to Sema ligands expressed along the axes of the AL (Komiyama et al., 2007). Subsequently, the map is refined by receptors such as Dscam and the class-specific action of Capricious (Caps) (Hong et al., 2009). Caps provide PN dendrite repulsion between dendrites of specific neighboring glomeruli. Subsequently, ORNs invade the AL as axon bundles that sort out to form a spatial map by using similar players as PNs.

Unlike vertebrates, in *Drosophila* ORs do not instruct the targeting of ORN axons (Dobritsa et al., 2003). ORNs axons start targeting in three main fascicles to form the antennal nerve (AN) (Jhaveri and Rodrigues, 2002). This bundling process involves intra- and inter-class adhesion factors to form fascicles (Komiyama et al., 2004). These ORN axon fascicles also merge with the processes of other types of cells like the auditory neurons from the Johnston's organ. Repulsive cues come into action as soon as the axons start to segregate from the AN in order to target to different parts of the AL (Hummel and Zipursky, 2004; Hummel et al., 2003; Oland et al., 2008). At this point the ORNs split into three main projection routes – a dorsolateral, central and ventromedial route (Ang, 2003). This whole process of ORN targeting is a sorting process. Same classes of ORNs are bundled together by adhesion cues to target same glomerulus, whereas intra-class adhesion ensures that axons from the same ORN class converge on the same glomerulus. At the same point different classes of ORN axons sort out from each other using repulsive cues.

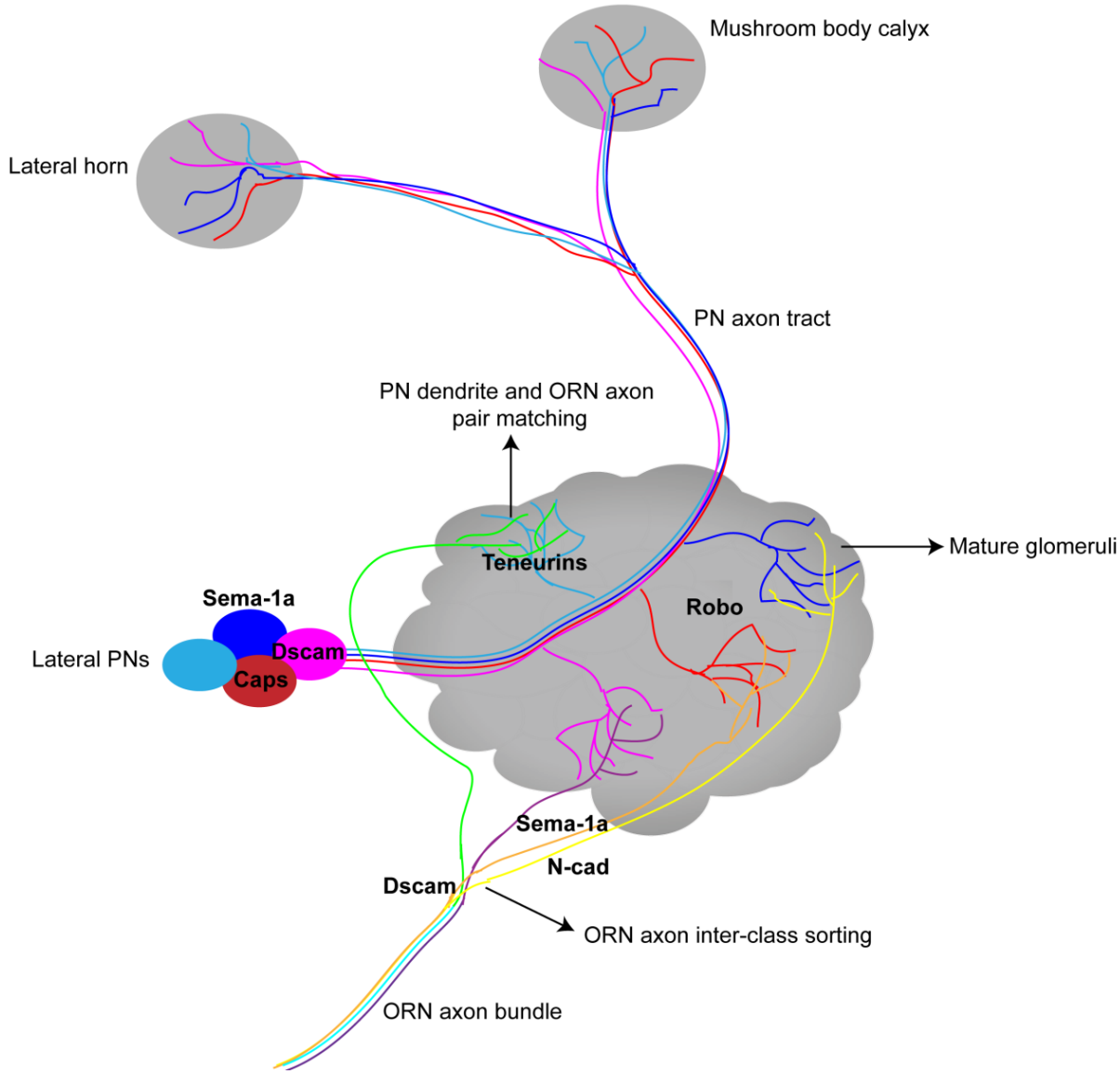


Figure 1.2 2 : Molecules involved in olfactory wiring.

Schematic showing the molecules required during various steps of olfactory map formation of *Drosophila*. Firstly, Semaphorin is required for the coarse dendritic map of PNs and Dscam and Caps are required for fine tuning of this map. Subsequently, Dscam, N-cad and semaphorin are required for interclass repulsion and intra class attraction of ORN axons. Whereas Robo regulates the positioning of ORN axons once they enter the AL. Finally Teneurins are required for PN dendrite and axon pair matching.

Examples of the attractive and repulsive cues involved in this process are shown in Figure 1.2.2. Examples include: 1) Dscam, plays a key role in inter- and intra- class sorting process , 2) Robo, regulates the positioning of ORN axons once they enter the AL (Jhaveri et al., 2004), 3) N-cad, required at later stages for ORN axon innervation into the proto-glomerulus formed by the PNs (Hummel and Zipursky, 2004), 4) Sema-1a, mediates repulsion between inter-class ORNs and helps in the sorting process along with the receptor (Lattemann et al., 2007; Sweeney et al., 2007). Finally, ORNs form synapses with their respective partner PNs by employing molecules such as N-cad and Teneurins (Hong et al., 2012).

While fundamental mechanisms have been identified, the molecular code required for the precise convergence of ORNs and PNs into class-specific glomeruli has not been solved.

1.3 Eph/ephrin signaling

As explained in the earlier section, olfactory neurons target their correct glomeruli by sorting their neurites from each other. Hence, a signaling process which is bi-directional and repulsive represents a good candidate guidance cue in this system. The Eph family of receptor tyrosine kinases (Hirai et al., 1987) and their membrane-bound ephrin ligands (Cerretti et al., 1995; Shao et al., 1994) represent a bidirectional signaling system of repulsive molecular cues for axon-axon and axon-target interactions (Brueckner et al., 1997; Henkemeyer et al., 1996). This signaling has been implicated in many axon guidance processes in vertebrate species (Brambilla, 1995; Kullander et al., 2001; Orioli D and Klein, 1997).

Eph receptors form the largest subfamily of receptor tyrosine kinases (RTKs). They interact with cell surface bound ligands: ephrins (Shao et al., 1994). Structurally ephrins are divided into two classes: 1) ephrin A (A1-A6) are tethered to the plasma membrane via glycosyl phspotidyl inositol moiety and 2) ephrin B (B1-B3) span the plasma membrane and possess a cytoplasmic tail (Figure 1.3.1B). Similar to ephrin, Eph receptors are also divided into class A and B depending on the sequence similarity (Gale et al., 1996)(Figure 1.3.1A). Major features of Eph/ephrin signaling are that it can transduce a signaling cascade in both directions (Holland et al., 1996), ligand and receptor can interact in trans (usually leading to activation) or in cis (usually leading to inhibition). Eph/ephrin signaling is involved in many developmental processes like cell sorting in embryonic patterning (Mellitzer et al., 1999; Xu et al., 1999), cell migration,modulation of cell adhesion, etc. Mostly, this signaling has been studied in the context of development (Klein, 2012) but some studies also implicate its role in adults

for higher brain functions like synaptic plasticity (Grunwald et al., 2001, 2004; Henderson et al., 2001).

A well-known example of the importance of Eph/ephrin signaling is the formation of the retinotectal map (Brennan et al., 1997; Marcus et al., 1996). The graded expression of Eph receptors and their membrane-bound ephrin ligands along two orthogonal axes in the tectum and retina repels Eph-expressing axons from ephrin-expressing target cells. By now it is clear that several Eph/ephrin gradients of different members of the large Eph/ephrin family play complex roles in the formation of this and other maps. A key concept emerged from these studies is the differential levels of repulsion guide the relative position of axons within their target area. The strength of repulsion is regulated by many different mechanisms: 1) amount of receptor and ligand on the cell surface, 2) parallel signaling mechanisms and 3) differential signaling downstream of the receptor and the ligand.

As the field of Eph/ephrin signaling has expanded a lot, the idea that Eph/ephrins do not work in isolation is established. Eph/ephrins work as a part of a complex network of regulatory pathways that control many different biological functions. Eph/ephrin signaling act in concert with other signaling systems including: 1) Fibroblast growth factor receptor (FGFR) signaling (Yokote et al., 2005), 2) Ret/GDNF signaling (Kramer et al., 2006), 3) RYK kinases (Halford and Stacker, 2001) and 4) Semaphorins (Ikegami et al., 2012) etc. In addition to parallel signaling mechanism, another mechanism with which Eph/ephrins work in a wide variety of biological processes are differential downstream signaling molecules depending on cell type and availability of signaling molecules (Jørgensen et al., 2009). Small GTPases including RhoA, Rac1, and Cdc42

are major players downstream of guidance receptors including Eph/ephrins. These molecules regulate different aspects of actin dynamics leading to growth cone collapse or growth cone extension. Studies have shown that attractants act through Cdc42 and Rac while repellants activate Rho (Noren and Pasquale, 2004; Sahin et al., 2005). Rho regulates growth cone collapse by activating Rho kinase that inhibits myosin light chain phosphatase which in turn leads to increase in myosin activity and contractility of acto-myosin network at the growth cone. Rho GTPases are modulated by the activity of positive regulators- GEFs and negative regulators- GAPs. They regulate small GTPases by promoting the GTPase activity and by exchanging the hydrolyzed GDP for a GTP (see section1.1.3). In general, the importance of GEFs in regulating axon guidance has been underscored. One of the few examples is the mutant of Unc-73 GEF in *Caenorhabditis elegans* and its *Drosophila* homolog Trio. Trio is shown to genetically interact with signaling components like Abl, Pak and Dock, but it hasn't been placed downstream of any specific signaling mechanism (Debant et al., 1996; Newsome et al., 2000).

Ephexin was identified as a direct interactor and RhoGEF downstream of EphA receptors (Shamah et al., 2001)(Figure 1.3.1D). In mammals, five family members comprise the Exn family (Exn1-5) compared to only one Exn in *Drosophila*. Exn is a Dbl family of GEF which contains N terminal DH/PH domain (Dbl homology and plextrin homology domain) and SH3 domain (Shamah et al., 2001)(Figure 1.3.1C). Neither ephrin binding nor kinase activity of Eph is responsible for binding to Exn to the receptor; instead, Exn is constitutively bound to Eph via its DH/PH domain. Mouse Exn1 enhances the relative activities of RhoA versus Rac1 and Cdc42 GTPases leading to an

ephrin-induced, phosphorylation-dependent growth cone collapse in cultured neurons (Sahin et al., 2005)(Figure 1.3.1D). It has been shown that Exn enhances the activity of RhoA and inhibits Pak in an Eph dependent manner, leading to growth cone collapse. Nevertheless, none of the mouse and/or fly mutants of *exn* family members are reported to show axon guidance phenotypes *in vivo*. In addition to its role in axon guidance, recent research in mice and flies indicated Exn-related functions in synapse development and maintenance downstream of Eph signaling. On the contrary to mouse, in *Drosophila melanogaster*, Exn is shown to interact with Cdc42 and not Rho1 (fly homolog of RhoA) downstream of Eph signaling in maintaining synaptic homeostasis at neuro-muscular junction (NMJ) (Frank et al., 2009). Although the specific mechanism by which Exn is controlled is not both in mouse and/or flies is not well understood.

Recent work showed that Eph/ephrin signaling is also required in invertebrates such as *Manduca sexta* and *Drosophila melanogaster*. In contrast to vertebrates, *Drosophila* has only one Eph receptor and one membrane-spanning ephrin ligand and one Exn GEF (Figure 1.3.1A, B, C). Eph/ephrin was found to be important for axon guidance of mushroom body Kenyon cells as well as neurons in the visual system (Boyle et al., 2006; Dearborn et al., 2002).It was shown that in Eph mutants, kenyon cells showed altered bifurcation of the axonal branches as compared to the wild type (WT) controls. Thus, suggesting an important role of Eph/ephrin signaling in guiding a subset of mushroom body branches to their correct synaptic targets.

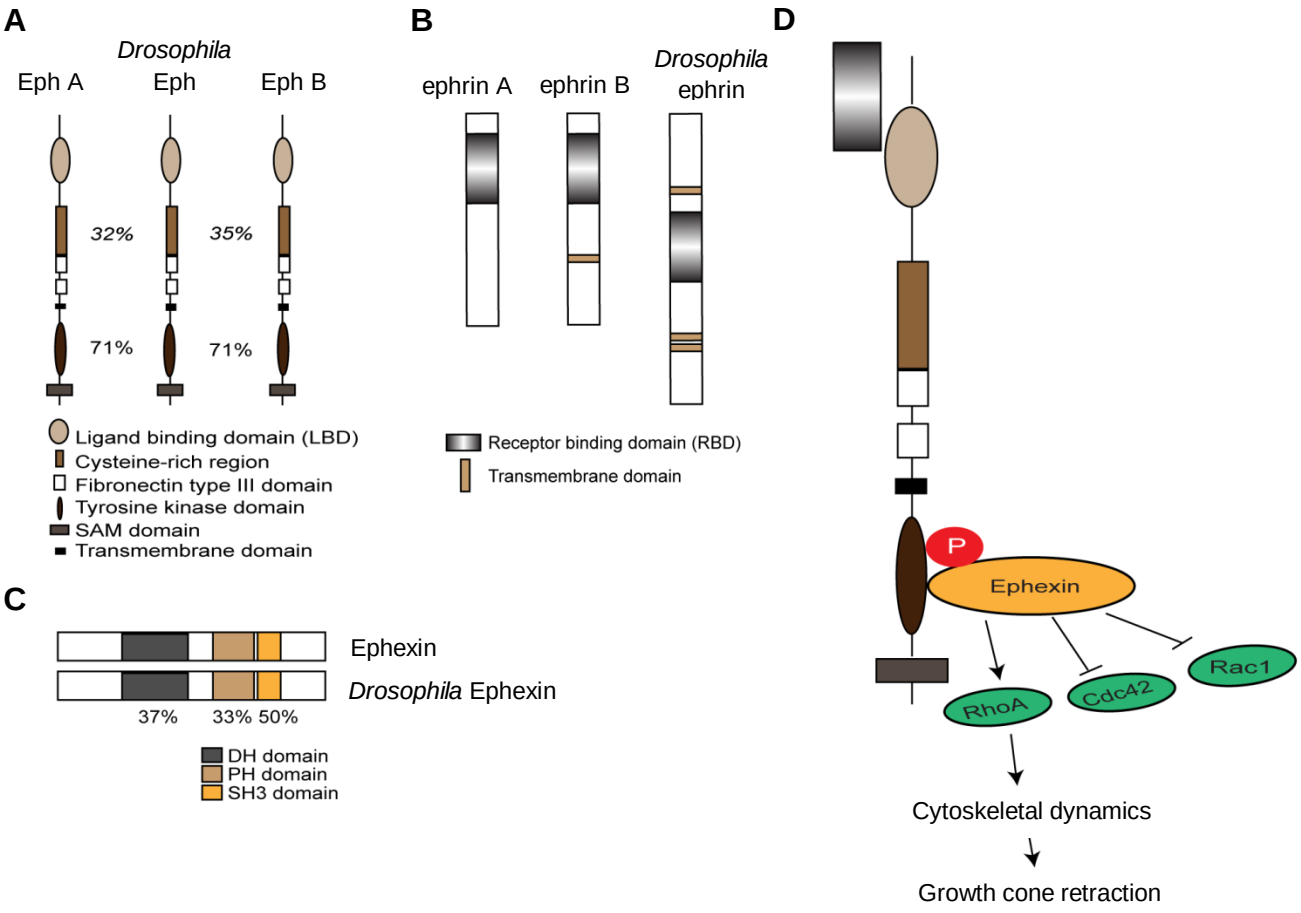


Figure 1.3 1 : Eph/ephrin signaling mechanism.

A) *Drosophila* Eph receptor shows equal homology compared to vertebrate Eph A and Eph B receptors. Different domains are indicated and the percent homology between Drosophila Eph and vertebrates Ephs is shown. Adapted from (Dearborn et al., 2002). B) *Drosophila* ephrin is a trans membrane ligand and hence shows more similarity to vertebrate ephrin B than ephrin A. Adapted from (Bossing and Brand, 2002). C) Drosophila Exn has conserved domains as the vertebrate Exn. D) Forward Eph signaling via Exn, in case of vertebrates. Activation of Eph phosphorylates Exn and leads to activation of RhoA GTPases downstream and hence leading to growth cone retraction. This growth cone retraction results in repulsion from the ligand expression cell.

In addition to *Drosophila melanogaster,* role of Eph/ephrin signaling in olfactory map formation is indicated by study in *Manduca sexta* (Kaneko and Nighorn, 2003). Here, Eph and ephrin were shown to be differentially expressed in ORN axons and glomeruli respectively. Experiments on cultured olfactory explants showed the repulsion of neurites expressing Eph from ephrin expressing substratum. Given these already published reports and the bidirectional and repulsive nature of Eph/ephrin signaling, it represented an attractive candidate for studying its role in olfactory map formation of *Drosophila melanogaster.* Hence, the work outlined here, is focused on the analysis of Eph/ephrin signaling including the downstream targets such as Exn and small Rho GTPases, in olfactory map formation.

Two recent reports were published during the final stages of the completion of this thesis, that also implicated Eph/ephrin in two different aspects of olfactory map formation in *Drosophila*: The first study showed that the enzyme Meigo controls ephrin glycosylation levels within PNs. Lack of Meigo reduces ephrin levels and leads to changes in PN targeting (Sekine et al., 2013). In another study it was shown that Eph in ORNs determines the arborization pattern of a large ephrin-expressing serotonergic neuron within the AL (Singh et al., 2013). Yet, the role and the molecular components of Eph/ephrin-mediated signaling in this system remain unclear.

2. Aim of the thesis

Fundamental mechanisms of olfactory map connectivity have been studied, although less is known about the molecules that lead to class specific neuron connectivity. To this aim, the role of Eph/ ephrin signaling and its downstream GEF, Ephexin was analyzed for class specific olfactory connectivity. In addition, small Rho specific GTPases were analyzed to decipher differential molecular mechanisms operating in this system.

Three specific questions asked in this thesis are as follows:

1. Is Eph/ephrin signaling required for olfactory map formation?
2. Which neurons require Eph, ephrin and Exn in this system?
3. What is the molecular mechanism downstream of Eph/ephrin signaling involved in this system?

These questions are addressed and answered by various experiments mentioned in the results section.

3. Results

A central question in the field of neurosciences is how the wiring specificity of various sensory maps is achieved. This work aimed at identifying molecules required for the formation of the olfactory sensory map of flies. Since the targeting of olfactory neurons involve the sorting of different axons (Lattemann et al., 2007), a repulsive bidirectional signaling system emerge as a potential player in the targeting process. This candidate approach to identify the potential molecular players suggests a potential role of Eph/ephrin signaling. Also the activity of various Rho GTPases is essential downstream of most of the guidance receptors, a Rho GTPase specific guanine nucleotide exchange factor (GEF) – Ephexin (Exn) was analyzed, along with Eph receptor and ephrin ligand.

In flies*, eph* and *ephrin* are present on the fourth chromosome. This chromosome is not accessible by genetic methods such as mosaic analysis; hence more detailed genetic analysis was done on *exn*, located on the third chromosome of the fly. Various genetic approaches were taken to decipher the role of Eph signaling in olfactory map formation.

In addition, Eph signaling was already shown in a previous study to play a role in establishing MB Kenyon cell projection pattern (Boyle et al., 2006), hence, its role in the functioning of mushroom body was speculated. To this aim Exn mutants were tested for any kind of learning memory defects. All these experiments and their results are explained in detail in the following sub-sections.

In a separate project, the role of Exn was analyzed in maintaining synapses at higher brain centers. These experiments were driven by already known studies that

established the role of Exn in synapse formation and maintenance in flies as well as mice (Frank et al., 2009; Shi et al., 2010). Hence to test the possibility of Exn being involved in maintaining synapses in olfactory system, quantitative and qualitative analyses of synapses was carried out by electron microscopic experiments.

3.1 Eph and Ephexin are required for correct targeting of the ORNs.

To assess the role of *eph*, *ephrin* and *exn* in ORN targeting, we used already published mutants for these genes (see materials and methods- fly stocks). Various ORNs labeled with GFP were analyzed for correct targeting in the background of *eph* mutation (eph^{x652}), *ephrin* hypomorphic mutation ($ephrin^{KG09118}$) and two *exn* mutations ($exn^{EY01953}$ and $exn^{EY\text{-}\Delta 23}$). Out of 11 different ORNs analyzed for targeting defects, only 3 showed mistargeting in the background of *eph* and *exn* mutations, whereas no mistargeting was observed in the background of $ephrin^{KG09118}$ (Figure 3.1.1). The possible explanation for this result is the hypomorphic nature of the ephrin mutation which leads to incomplete loss of ephrin protein and hence no obvious defect in ORN targeting. Loss of Eph receptor showed targeting defect in two classes of ORNs - Or47a and Gr21a. Whereas loss of Exn lead to the targeting defect in one additional class of ORN – Or47b, in addition to Or47a and Gr21a.

Or47a neurons target their axons to the DM3 glomerulus in the AL of a WT fly. In eph^{x652} homozygous mutant flies Or47a neurons, in addition to the DM3 glomeruli, target laterally. On the contrary for *exn* mutants - $exn^{EY\text{-}\Delta 23}$ and $exn^{EY01953}$, Or47a neurons target into and ventrally to the DM3 glomerulus.

Or47b neurons target their axons to the VA1lm glomerulus in the AL of a WT fly. These neurons show no targeting defect in case of eph^{x652} homozygous mutants, whereas *exn* mutants show targeting defect. The Va1lm glomeruli lose its crescent shape and encircle the neighboring VA1d glomeruli in *exn* mutants.

Gr21a neurons target their axons to the V glomerulus in the AL of a WT fly. In *eph*^{x652} homozygous mutant flies these neurons target in and dorsal to the V glomeruli. Similar phenotype is seen in *exn*$^{EY\text{-}\Delta 23}$ homozygous mutants whereas *exn*^{EY01953} homozygous mutants show a milder form of this targeting defect. In addition *exn* mutants also show a spreading of Gr21a neuron axons in the medial part of the AL. The difference in targeting defect seen in two types of mutants of exn could be attributed to the fact that *exn*^{EY01953} is a P element insertion mutant and hence is not a complete null. Thus, the spreading phenotype is a milder targeting defect whereas dorsal innervation by Gr21a neurons is a stronger targeting defect seen for both *eph*^{x652} and *exn*$^{EY\text{-}\Delta 23}$ mutants.

As shown, overlapping classes of ORNs were affected in both *eph* and *exn* mutants but both showed qualitative differences in the targeting defects. These qualitative differences are highlighted in a model shown in Figure 3.1.2.

In addition to the above mentioned classes of ORNs, many other classes of ORNs were screened for any targeting defects in *eph* and *exn* mutants (Figure 3.1.3). Surprisingly, both *eph* and *exn* mutants showed the targeting defect in very specific classes of ORNs (Or47a and Gr21a for eph mutants and Or47a, Or47b and Gr21a for exn mutants).

These data suggest that *eph* and *exn* are required for correct targeting of the ORNs. Whether *eph* and *exn* are required in the same pathway, is discussed in the following sections. In addition, these data show that Eph signaling is required in very specific classes of ORNs for their correct targeting.

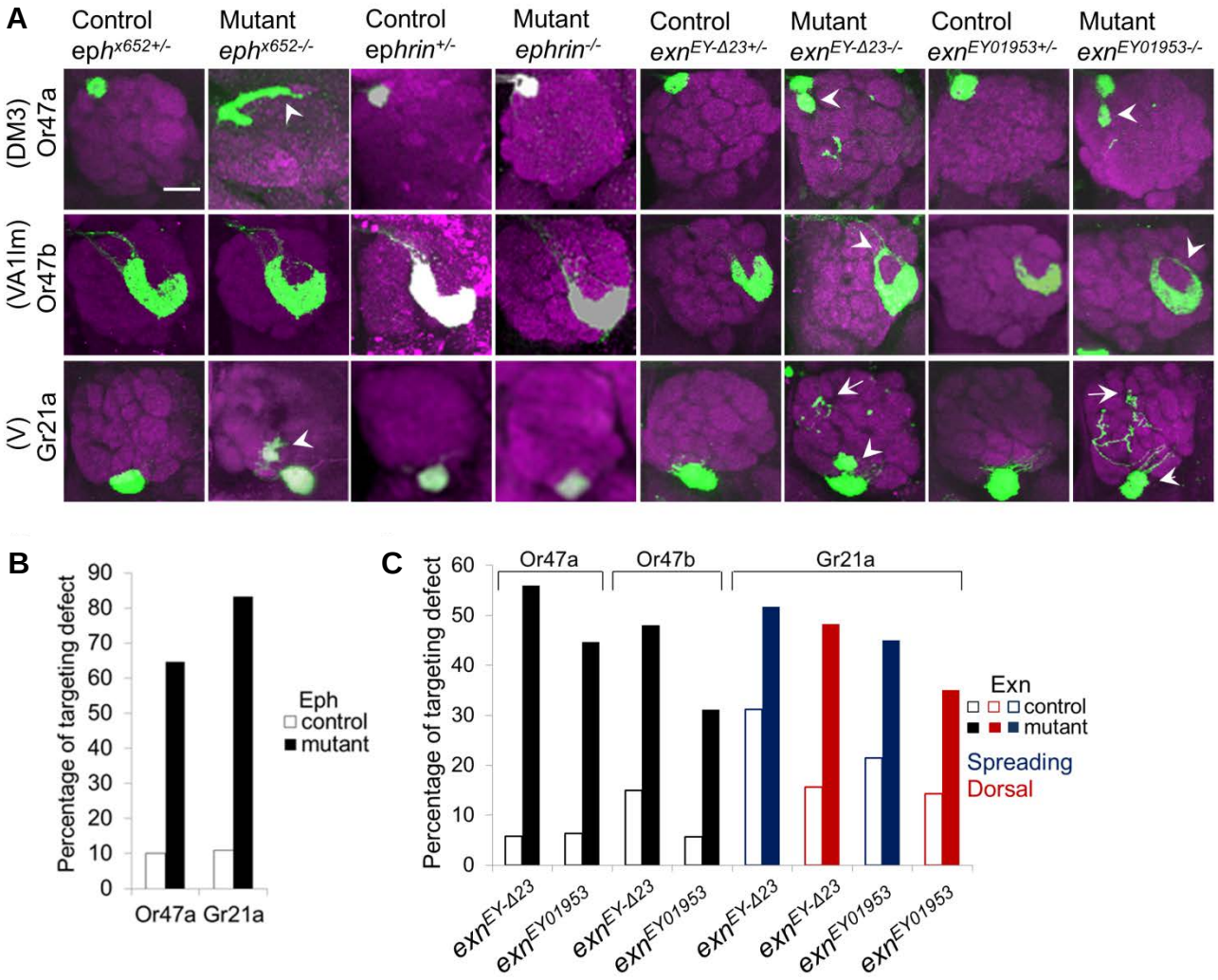


Figure 3.1 1 : Targeting defect of ORNs in *eph*, *ephrin* and *exn* mutants. .

A) Glomerular targeting of three different classes of ORNs in heterozygous control and homozygous mutant background is shown. Targeting defect is indicated by arrowhead. ORNs are labeled with GFP (green) and neuropil is labeled in magenta with anti-discs large antibody. Glomeruli innervated by these ORNs are mentioned in parentheses. For the analysis an average of 25 brains were analyzed for each genotype except *ephrin* mutant where a minimum of 10 brains were analyzed. The scale bar is 20 µm. *eph* null mutant (eph^{x652}) leads to lateral targeting defect for Or47a neurons, none for Or47b neurons and dorsal targeting defect for Gr21a neurons. *ephrin* null mutant ($ephrin^{KG09118}$) showed no targeting defect for all the mentioned three classes of ORNs. Two different null alleles of *exn* ($exn^{EY-Δ23}$ and $exn^{EY01953}$) show ventral targeting defect for Or47a neurons, rounding of glomeruli for Or47b neurons and spreading (indicated by arrow) and dorsal targeting defect (indicated by arrowhead) for Gr21a neurons. B) Quantification of the targeting defect seen in *eph* null mutants in OR47a and Gr21a

neurons. White bars indicate the heterozygous background and black bars indicate the homozygous mutant background. Y axis is the percentage of targeting defect (percentage of brains showing the targeting defect). X axis is the type of ORN analyzed. C) Quantification of the targeting defect seen in *exn* mutants in the three classes of ORNs- Or47a, Or47b and Gr21a. Here, X axis shows the genetic background- $exn^{+/-}$ (empty bars) or $exn^{-/-}$ (filled bars). Quantification of targeting defect for both the alleles of exn is shown. For Gr21a neurons blue bars (both empty and filled) show the spreading phenotype whereas red bars (both empty and filled) show the ectopic dorsal innervation of Gr21a axons.

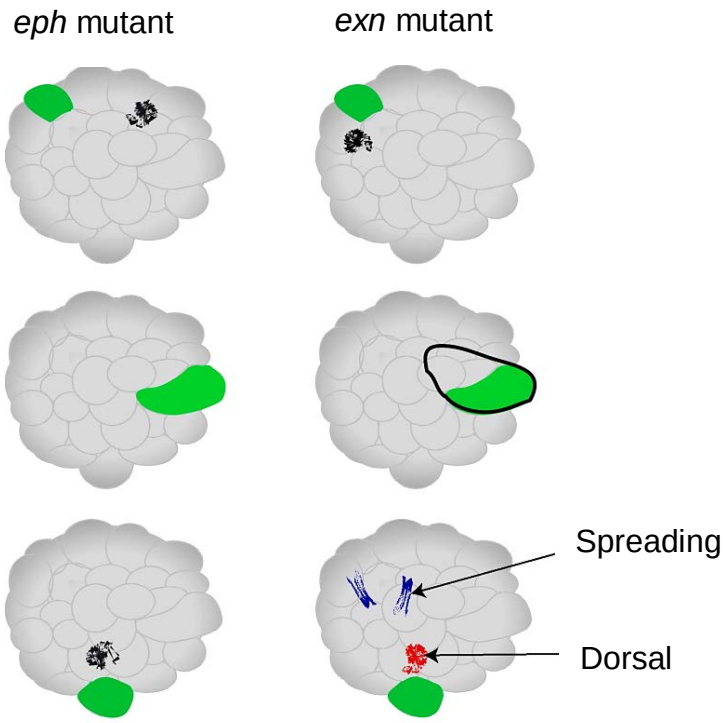
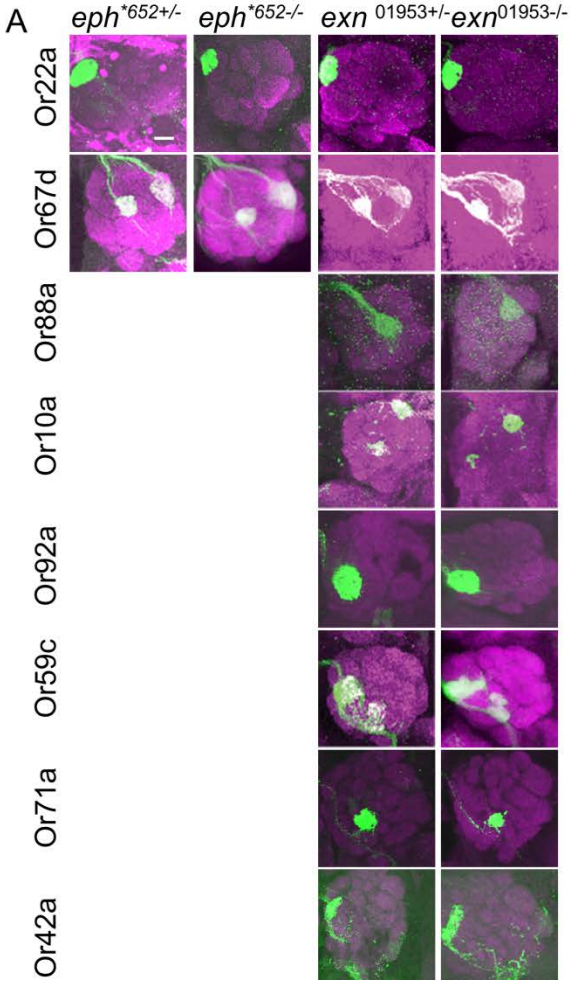
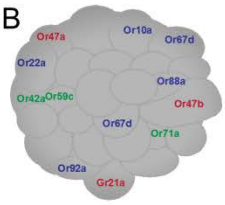


Figure 3.1 2 : Differential targeting defects of *eph* and *exn* mutant.

The targeting defect is depicted in the schematic. The schematic on the left shows normal innervation pattern of the ORN axons in green and black indicates the ectopic targeting pattern caused by loss of *eph*. The schematic on the right shows normal innervation pattern of the ORN axons in green whereas ectopic innervation is in black for Or47a and Or47b neurons in *exn* mutants. For Gr21a neurons two different targeting defects (spreading and dorsal innervation) are indicated in blue and red respectively. Both the schematics highlight the qualitative differences in the targeting defect caused by *eph* and *exn* in same classes of ORNs.





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ORN class	Olfactory organ	Glomerular innervation	eph*652-/-	exn$^{\Delta 23-/-}$
Or10a	Antenna	DL1	n.d	no
Or22a	Antenna	DM2	no	no
Or42a	Maxillary palp	VM7	n.d	no
Or47a	Antenna	DM3	lateral	ventral
Or47b	Antenna	VA1lm	no	rounding
Or59c	Maxillary palp	VM7	n.d	no
Or67d	Antenna	DA1(VA6)	no	no
Or71a	Maxillary palp	VC2	n.d	no
Or88a	Antenna	VA1d	n.d	no
Or92a	Antenna	VA2	n.d	no
Gr21a	Antenna	V	dorsal	Medial + dorsal

Figure 3.1 3 : Specific ORNs affected in *eph* and *exn* mutants.

A) Glomerular targeting of 8 different classes of ORNs in the background of *exn* mutant (*exn*$^{EY-01953}$) and two different classes in case of *eph* mutant. None of the mentioned classes of ORNs show targeting defect in the mutant. B) Schematic of the antennal lobe showing the positions of the glomeruli innervated by various ORNs (color coded) analyzed in *eph* and *exn* mutants. C) Table showing the analyzed ORN classes with their peripheral cellular location, glomerular innervation and presence or absence of the targeting defects in *eph* and *exn* mutants respectively.

3.2 Eph, ephrin and Ephexin expression in fly brain.

To assess the spatial and temporal requirement of *eph*, *ephrin* and *exn*, a detailed developmental expression analysis was carried out. Already available peptide antibodies and Fc fusion peptides were used to carry out the expression analysis of *eph* and *ephrin* (Bossing and Brand, 2002; Dearborn et al., 2002). Although the earlier studies show the expression of these molecules using these peptide antibodies, it did not show any specific signal at the stages that were analyzed in this study. Due to the absence of any working antibody for all the three molecules, other approaches were carried out to analyze the expression pattern. These included *in-situ* hybridization, GFP tagging of fosmid and use of Gal4 insertion lines. These experiments were done in collaboration with Dr. Laura Loschek.

For analyzing the Eph expression pattern two approaches were taken: *in-situ* hybridization and GFP tagging of the Eph fosmid. The Eph-GFP tag expression pattern was confirmed by *in-situ* hybridization. The differences in the expression between the two techniques could be attributed to the fact that Eph-GFP tag shows the expression of GFP where Eph is localized (membrane) whereas *in-situ* hybridization shows nuclear mRNA levels. For analyzing ephrin expression, *in-situ* hybridization was carried out for the same stages of development as Eph *in-situ* hybridization.

Finally for Exn expression analysis, a Gal4 insertion line was used and its expression pattern was confirmed by comparing it with *in-situ* hybridization. In addition to these techniques, Exn fosmid was tagged with V5 tag but the injection of this construct was unsuccessful and hence the Gal4 insertion line was used for analyzing the expression.

3.2.1 Eph, ephrin and Ephexin expression using *in-situ* hybridization analysis.

In-situ hybridization was carried out for *eph*, *ephrin* and *exn* at two developmental stages: 6 h and 24-30 h APF. This showed that both *eph* and *ephrin* are present in the developing brain but not in the antennal disc (6 h APF developing antenna) (Figure 3.2.1). On the other hand, *exn* is expressed in optic discs of 6 h APF and very faint expression is seen in the brain lobes. High level of expression is seen in the optic lobes of 24-30 h APF brain in case of all the three molecules. In addition expression was also seen in the brain and notably around the developing antennal lobe. This overlaps with the position of the cell bodies of interneurons that target the AL.

It is already reported that the olfactory receptor neurons (ORNs) are born at around 5-10 h APF and their axons start to target the AL after 18h APF. In addition, the projection neurons (PNs) start targeting the antennal lobe from larval to pupa transition. By 20 h APF more or less all the PNs have extended their dendritic fields to the AL (Jefferis et al., 2004). The presence of *eph, ephrin* and *exn* in the developing brain both at 6 h APF and 24-30 h APF and their absence in the 6 h APF antennal disc indicated that these molecules might be required in interneurons in the brain rather than the peripheral ORNs. Although the low resolution achieved with *in-situ* hybridization and the ubiquitous presence of Eph, ephrin and Exn at 30 h APF brain, cannot depict the presence of these molecules in the PNs. Thus an alternate ways were used for a high resolution and detailed expression analysis of Eph and Exn (section 3.2.2 and section 3.2.3).

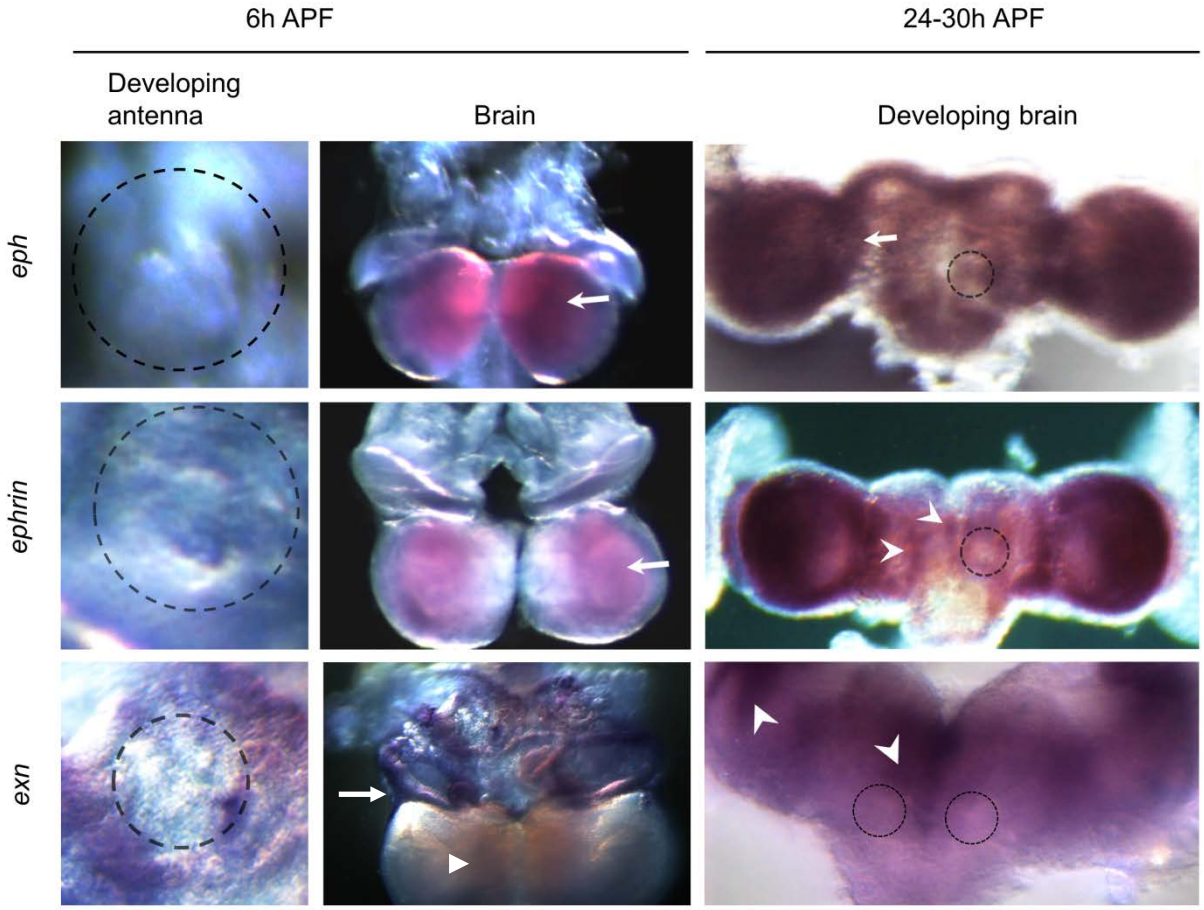


Figure 3.2 1 : *eph*, *ephrin* and *exn* expression by *in-situ* hybridization.

The left most panel shows the developing antennal discs (surrounded by dotted circle) depicting no *eph*, *ephrin* and *ephexin* expression. 6 h APF developing brain (middle panel) shows expression for both *eph* and *ephrin* (arrow). For *ephexin*, expression is seen in developing optic disc (arrow). Faint expression is also seen in the brain at 6 h APF (arrowhead). Right most panels show the *eph, ephrin and ephexin* expression in the brain at 24-30 h APF. High level of *eph* expression in the optic lobe is observed as indicated by the arrow. Also *eph* expression is seen in and around the developing AL (the dotted circle). *ephrin* is expressed in the optic lobe at 24-30 h APF stage along with the expression around the AL as indicated by arrowheads. *ephexin* expression is observed in the optic lobes and around the developing AL (arrowheads) at 24-30 h APF.

3.2.2 Eph expression analysis using fosmid tagging strategy.

Although *in-situ* hybridization analysis shows the presence of *eph* and *ephrin* in the developing brain but cannot resolve the expression pattern to specific cell types. In this regard, a fusion of Eph and GFP was created (Eph-GFP tag) by tagging a ~ 43kb Eph fosmid clone with a GFP tag (see material and methods). Using the tagged fosmid, Eph expression was observed at 6 h, 18 h, 24 h and 48 h APF brain (Figure 3.2.2). A sharp signal was detected in the developing AL at 18 h and 24 h APF. At these stages, the presence Eph in the neuronal cells (as shown by co-localization with elav), targeting to the AL, is observed (Figure 3.2.2 A). On the contrary no expression was seen in the developing antenna of the above mentioned stages. This data is consistent with the *in-situ* hybridization analysis for Eph. A decline in the Eph expression in the brain was seen at 48 h APF (Figure 3.2.2 A). This suggests that Eph is required during early stages of development in the fly brain. This early stage overlaps with the olfactory system wiring in general and PN dendrite targeting in particular. For a direct evidence of Eph expression in PNs, a co-localization experiment between Eph-GFP tag and PN driver (*GH146-Gal4*) was carried out. At 24 h APF, Eph-GFP tag showed co-localization with the GH146 positive PNs (Figure3.2.2 B). For this experiment *UAS-cd2* was driven by *GH146-Gal4* and visualized along with Eph-GFP tag. Together, these experiments suggest that Eph is expressed in the brain, specifically in the developing PNs, and not expressed in the developing antenna. Hence, Eph is most probably required in the PNs and not in the ORNs for correct targeting of ORNs.

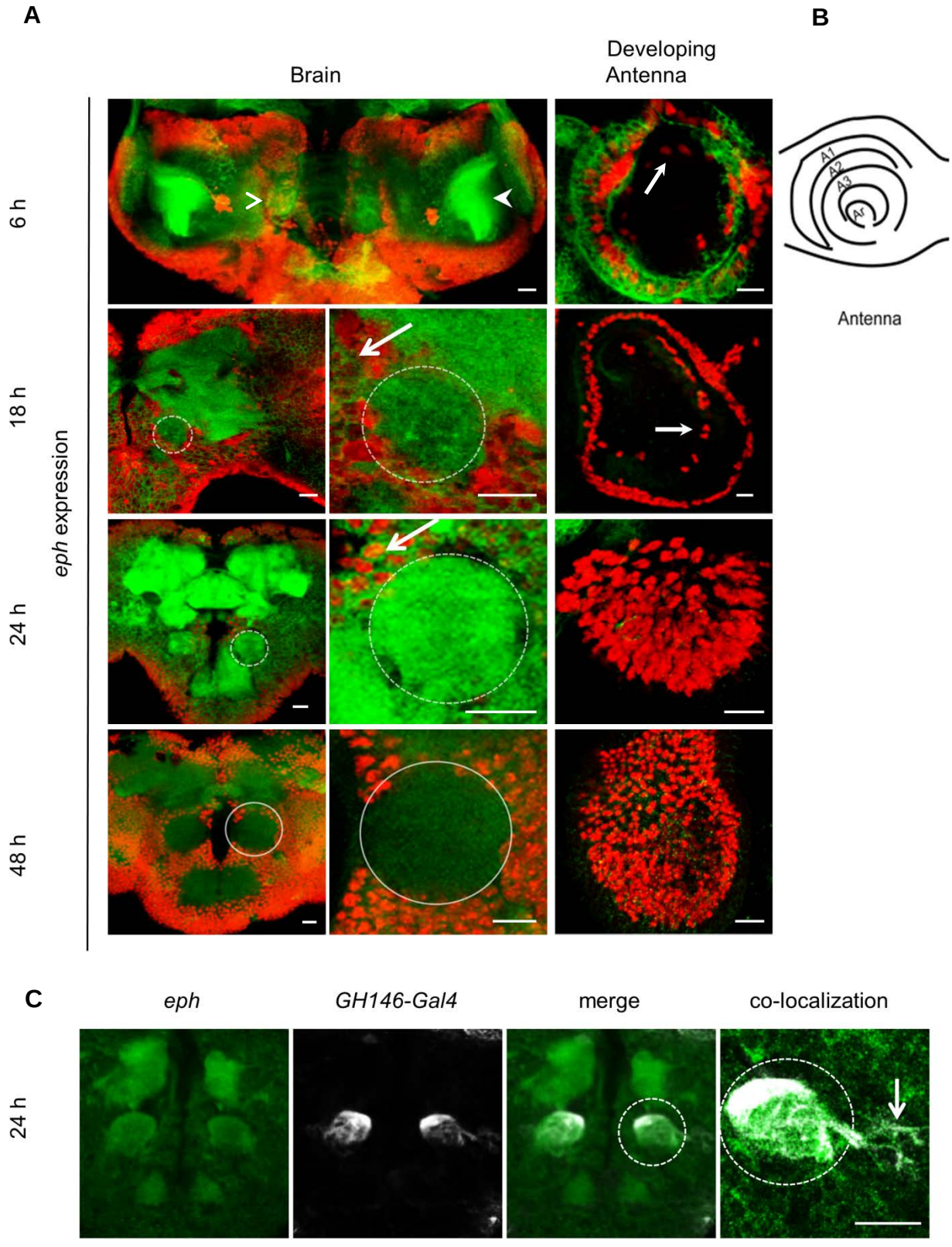

A
B
Brain
Developing Antenna
6 h
18 h
24 h
48 h
eph expression
A1
A2
A3
Antenna
C
eph
GH146-Gal4
merge
co-localization
24 h

Figure 3.2 2 : Eph expression in the brain specifically in the PNs.

A) Eph-GFP tag expression at 6 h, 18 h, 24 h and 48 h APF developing brain and antenna. Neuronal cells labeled with anti-elav antibody in red. Scale bar is 20 µm. No expression is seen in the developing antenna at any of the shown stages. Lot of expression is seen in the developing brain. At 6 h APF, expression is observed in the optic lobe (arrowhead) and in the potential antennal lobe area (empty arrowhead). At 18 h APF and 24 h APF, expression is seen in the developing antennal lobe (dotted circle) and also neuronal cells co-localize with Eph-GFP tag (empty arrow). At 48 h APF, Eph-GFP signal goes down and very less expression is observed in developing antennal lobe (circle). B) Schematic of the developing antennal disc. The third inner ring of the disc gives rise to the third segment of the antenna, as indicated in the schematic and developing antenna of 6 h and 18 h APF (arrow). C) Co-localization of Eph-GFP tag with GH146 positive neurons in the lateral cluster is seen at 24 h APF. GH146 positive PNs are labeled with *UAS-CD2* (anti CD2 antibody labeling in grey). Co-localization is observed in the developing antennal lobe (dotted circle) and in the developing lateral cluster of PNs (arrow). Scale bar is 20 µm.

3.2.3 Ephexin expression analysis using a *Gal4* insertion line.

Similar to *eph* expression analysis, *exn* expression was initially analyzed by *in-situ* hybridization (Figure 3.2.1). Expression was observed in brain and optical disc at 6 h APF but no expression was observed in the antennal disc. This again indicated that *exn* is required in the brain and not in the developing antenna. To get more insight into the expression of *exn* at various stages of development, a *Gal4* line was used. In this fly the *Gal4* transgene is inserted in the first intron region of the *exn* locus. We named this *Gal4* line *exn-Gal4*. The expression pattern of *exn-Gal4* line was analyzed by expressing GFP using three copies of *UAS-mCD8 GFP*. The expression pattern of the *exn-Gal4* line was validated with the *in-situ* hybridization for *exn*. As shown in Figure 3.2.3 A, C, both showed similar expression pattern in 6 h APF brain, optical and antennal disc. Hence, *exn-Gal4* was further used to analyze the expression of *exn* for 12 h and 48 h APF. At both these stages significant expression was seen in developing brain but very less expression in the developing antenna. Specifically, *exn-Gal4* expression was observed in the developing antennal lobe at 48 h APF. In order to characterize the cell types in which *exn* is expressed, GH146 positive PNs were labeled using *GH146-QF* and *QUAS-mtd tomato* and observed for co-localization in the *exn-Gal4* positive cells in an adult fly brain. As shown in Figure 3.2.3 D, *exn-Gal4* indicate the presence of *exn* in some specific GH146 positive PNs. Although, *in-situ* hybridization analyses indicate that *exn-Gal4* expression is indeed similar to Exn expression, the possibility of it only partly overlapping with Exn expression pattern can't be ruled out. *exn-Gal4* needs further validation to claim that it shows complete Exn expression.

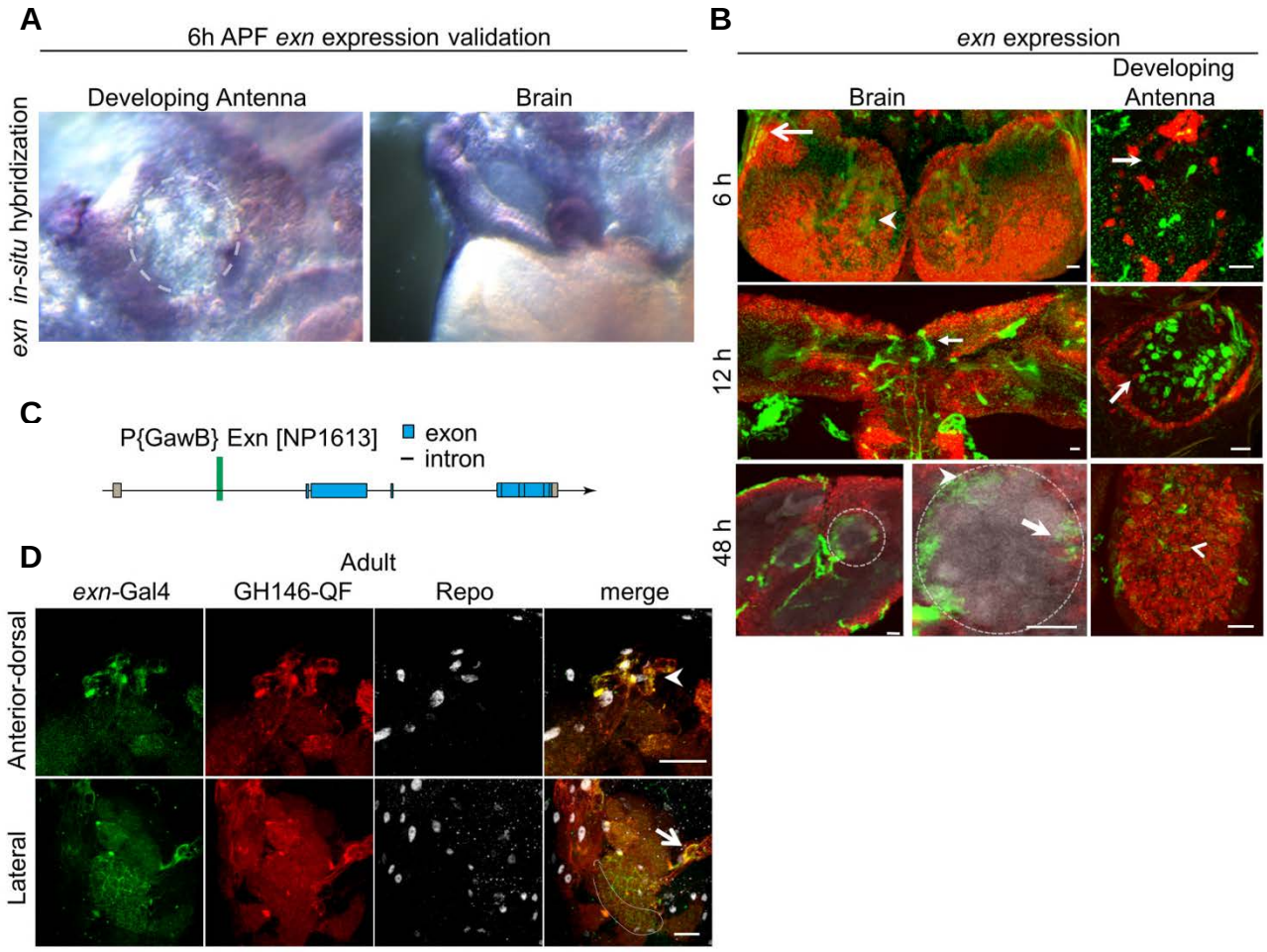


Figure 3.2 3 : Expression analysis of Exn.

A) Exn expression analysis in developing brain and eye antennal disc at 6 h APF by *in-situ* hybridization. As shown by the dotted circle no significant expression is seen in the developing antenna (antennal disc). On the contrary lot of expression is observed in the optic lobe as indicated by the arrowhead. Similar expression is observed in *exn-Gal4 UAs-mcd8GFP* as shown in B). B) Exn expression is analyzed by *exn-Gal4; UAS-mcd8GFP* (green). Neurons are labeled with anti-elav (red) and neuropil with nc82 (grey). At 6 h APF, expression is seen in optic nerve (empty arrow) and AL (arrowhead). At 12 h APF, expression is seen in the brain including the mushroom body (arrow). Magnified view of the developing brain at 48 h APF shows Exn expression in dorsal-medial glomeruli (arrowhead) and ventral-medial glomeruli (arrow) (encircled AL). *exn-Gal4* doesn't co-localize with the neurons of the third segment of antennal disc (arrows) at 6 and 12 h APF. At 48 h APF few cells are co-localized with neurons (empty arrowhead). C) Schematic of the Gal4 insertion in the intronic region of the *exn* locus. D) In adult brain *exn-Gal4* co-localizes with *GH146-QF*; *QUAS- mtd tomato* (red), in the anterior-dorsal cluster of PNs (including DM3) (arrowhead) and in the lateral cluster (including VA1lm indicated by dotted line and empty arrowhead for cell body). Exn is not expressed in glial cells (grey) stained with anti-repo antibody. Scale bar is 20 µm.

Taken together, the expression of Eph, ephrin and Ephexin in developing and adult brain relative to their levels in developing antenna suggest that Eph signaling is required in PNs rather than ORNs. In order to get a better insight into the spatial requirement of Eph and Exn, three different approaches were taken: 1) cell-type specific rescue, 2) cell-type specific RNAi knockdown, and 3) mosaic analysis. Results of these experiments are described in the following section.

3.3 Eph, ephrin and Ephexin are not required in ORNs.

In order to carry out the rescue experiment *SG18.1-Gal4* was used to drive the expression of *UAS-eph*^{wt} and *UAS-exn* in developing ORNs in *eph*^{x652} and *exn*$^{EY\text{-}\Delta 23}$ mutants, respectively. This driver line is known to be expressed in 70% of the developing ORNs (excluding for instance Gr21a neurons) (Shyamala and Chopra, 1999). Since this driver is not expressed in developing Gr21a neurons, rescue experiments were only intended in Or47a and Or47b neurons. As Or47b neurons show no targeting defect in *eph*^{x652} mutants, only Or47a neuronal rescue was conducted for *eph*. The re-expression of *UAS-eph*^{wt} in SG18.1 positive neurons in *eph*^{x652} mutants didn't rescue the lateral phenotype in Or47a neurons (Figure 3.3 A, B).

In accordance with these results, knocking down *eph* in developing ORNs (SG18.1 positive neurons) didn't lead to any significant targeting defect of Or47a neurons as compared to the controls. This experiment was conducted by expressing *eph* RNAi using *SG18.1-Gal4* driver. Similarly, knocking down *ephrin* in SG18.1 positive ORNs didn't lead to any significant targeting defect as compared to controls (Figure 3.3.1 E,F). The control used for RNAi experiments was a *3L attP2* background control flies. These flies were used to insert the UAS-RNAi construct and hence provided the most genetically similar background control for these experiments. The controls showed a lateral targeting defect in *SG18.1-Gal4* flies as shown in Figure 3.3.1 E,F. Hence, these results suggest that Eph signaling is not required in ORNs for their correct targeting.

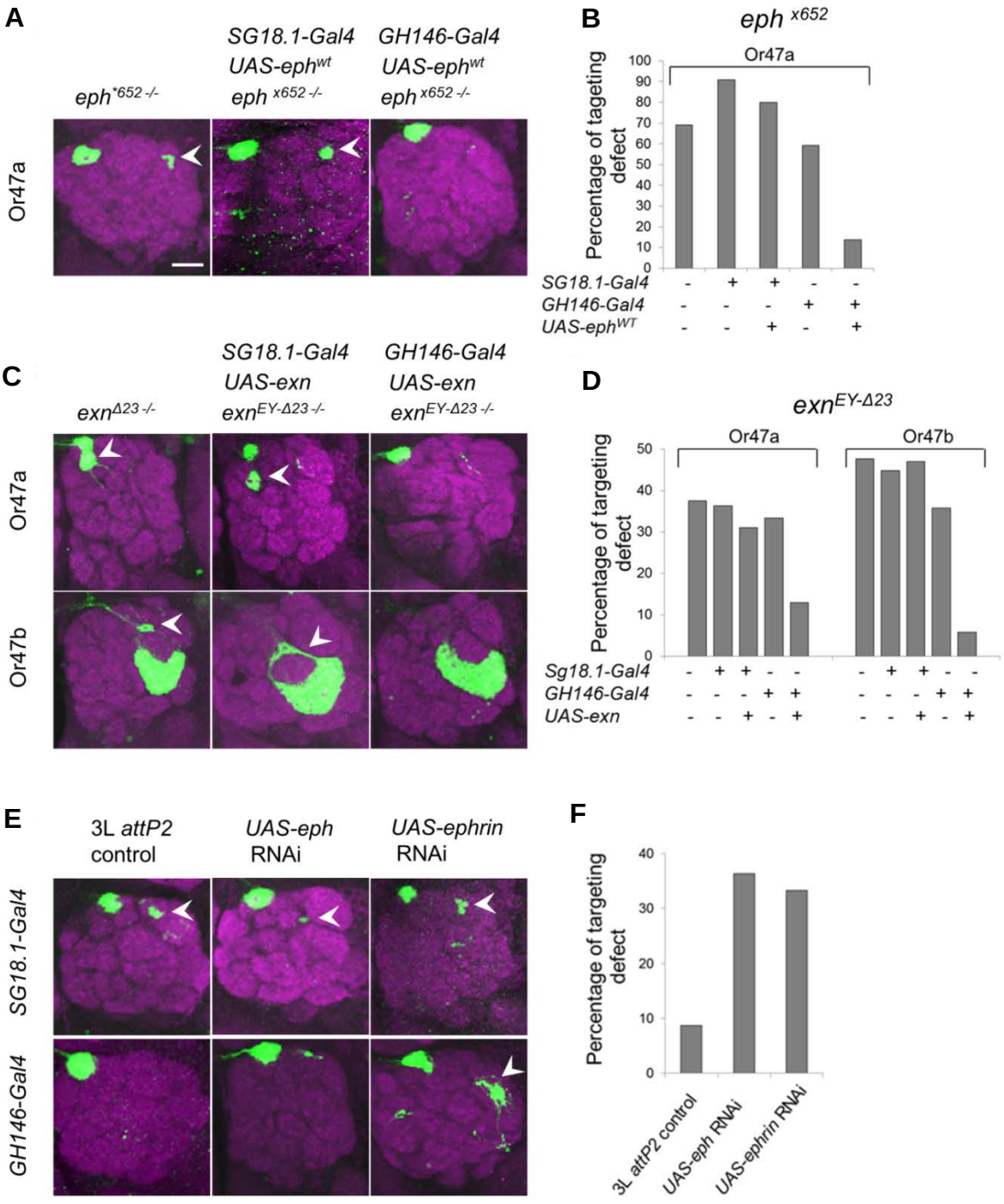


Figure 3.3 1 : Eph, ephrin and Exn are required in PNs rather than ORNs.

A) Lateral targeting defect of Or47a neurons (green) in *eph* mutant is rescued by re-expressing WT *eph* construct (*eph*^{wt}) in PNs using *GH146-Gal4*. Re-expressing *eph*^{wt} in majority of ORNs using *SG18.1-Gal4* driver shows similar lateral targeting defect as *eph* mutant (arrowhead). Scale bar is equal to 20µm in all the images. Magenta is the neuropil staining. B) Quantification for the rescue of targeting defect for *eph* mutants.

Percentage of targeting defect (Y axis) was calculated as explained in Figure 1.1 1. X axis indicates the genotype of the flies that were analyzed. On average 20 brains were analyzed for each genotype. Re-expression of *eph* in GH146 positive PNs shows significant reduction in targeting defect. C) Split phenotype for Or47a neurons in *exn* mutant shows rescue by re-expressing *exn* in PNs (using *GH146-Gal4*) but not in ORNs (using *SG18.1-Gal4*) as shown by arrowheads. Similarly the rounding phenotype for Or47b neurons is rescued by re-expression of *exn* in PNs but not in ORNs (shown by arrows). D) Quantification for the rescue of targeting defect for *exn* mutants. Re-expression of *exn* in GH146 positive PNs shows significant reduction in the targeting defect of both Or47a and Or47b neurons. E) Knockdown of *eph* and *ephrin* by expressing RNAi (inserted in 3L attP2 flies) in GH146 positive PNs show lateral targeting defect for Or47a neurons as indicated by arrowhead. Control flies (*GH146-Gal4*; 3L attP2) showed normal targeting of Or47a neurons. F) Quantification of the lateral targeting defect by knocking down *eph* and *ephrin* in PNs show a significant increase in lateral mistargeting of Or47a neurons as compared to the control.

Similar to *eph*, no rescue of ventral targeting defect of Or47a neurons was observed by re-expressing *UAS-exn* in $exn^{EY\text{-}\Delta 23}$ mutant by using *Sg18.1-Gal4* driver. In addition, rescue of Or47b neuron targeting defect, was also intended for $exn^{EY\text{-}\Delta 23}$ mutants (Figure 3.3.1 C, D). No rescue was observed for Or47b neurons targeting defect. In addition to the rescue experiment, MARCM (mosaic analysis of repressible cell marker) experiment was also conducted with *exn*. Eph is on the fourth chromosome of *Drosophila* and this chromosome cannot be manipulated by MARCM experiments. By contrast, *exn* is on the third chromosome of *Drosophila* and hence is accessible for mosaic analysis. To this aim, *eyflp* (Flp recombinase under the control of the eyeless promoter) mediated mosaic analysis was used to remove the *exn* gene selectively from ORNs in an otherwise heterozygous animal. Brains of *eyflp;FRT80B* $exn^{EY01953}$ animals showed no targeting phenotype in Or47a, Or47b, or Gr21a neurons compared to *eyflp;FRT80B* WT controls (Figure 3.4.1 A, C). These data confirm that *exn* is also not required in ORNs for olfactory map formation.

3.4 Eph, ephrin and Ephexin are required in PNs.

In addition to the ORNs, rescue of the targeting defect was intended by re-expressing *UAS-eph*^{wt} and *UAS-exn* in GH146 positive PNs. The *GH146-Gal4* driver is known to be expressed in around 70% of developing PNs, including DM3 / Or47a and VA1lm / Or47b specific PNs (Stocker et al., 1997). Contrary to re-expression in ORNS, re-expression of *UAS-eph*^{wt} in developing PNs resulted in the rescue of the lateral targeting defect of Or47a neurons in case of *eph*^{x652} mutants (Figure 3.3.1 A, B). Similarly, re-expression of *UAS-exn* in developing PNs resulted in the rescue of ventral targeting defect and rounding phenotype in Or47a and Or47b neurons in *exn*$^{EY-\Delta 23}$ mutants, respectively (Figure 3.3.1 C, D).

Furthermore, RNAi analysis was done in developing PNs using *GH146-Gal4* driver. Eph and ephrin RNAi showed a significant lateral targeting defect for Or47a neurons (36.3% and 33.3%, respectively) when expressed in developing PNs as compared to the controls (8.7%) (Figure3.3.1 E, F). None of the RNAi lines used against *exn* showed any targeting defect for Or47a neurons, irrespective of its expression in developing ORNs or PNs.

Finally, we obtained the direct evidence for the role of Eph signaling in PNs by analyzing the PN dendritic pattern in *exn*$^{EY-\Delta 23}$ mutants. Since there is no driver available that is expressed specifically in DM3 / Or47a or VA1lm / Or47b PNs, we used UAS Flp-out system to label these neurons specifically (see Materials and Methods). These experiments were done in collaboration with Cristina Organisti.

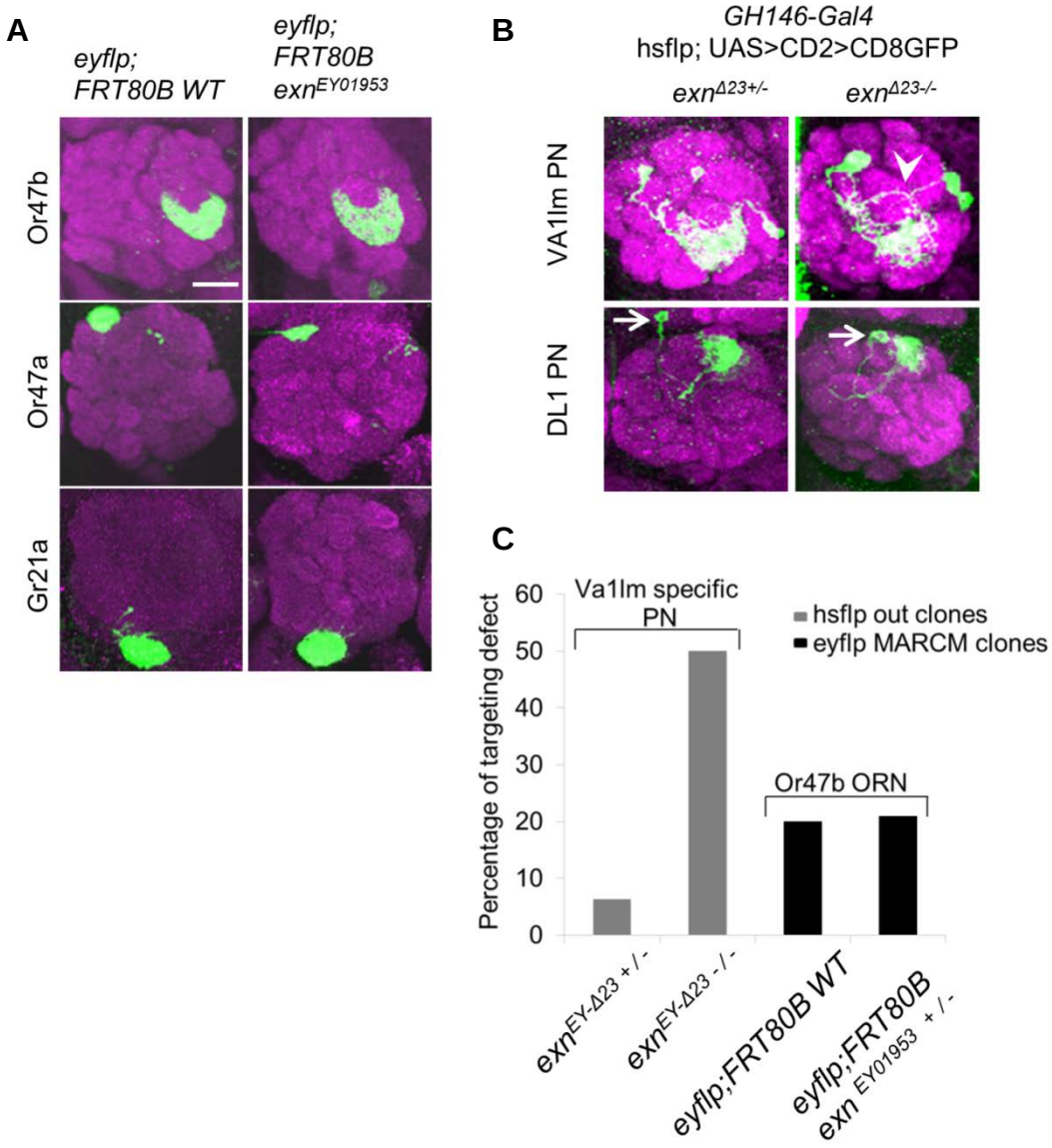


Figure 3.4 1 : Exn leads to mistargeting of PN dendrites.

A) *eyflp* induced MARCM clones (green) for exn mutant allele (*exn*^{EY01953}) show no targeting defects for all the three ORN classes (Or47b, Or47a and Gr21a) in comparison to the WT MARCM clones. B) *hsflp* out clones for VA1lm specific PN (upper panel) (green) show rounding phenotype (arrowhead) in exn homozygous mutant (*exn*$^{EY\text{-}\Delta23\text{-}/\text{-}}$, right panel, number of flies = 8) as compared to the heterozygous control (*exn*$^{EY\text{-}\Delta23+/\text{-}}$, left pane, number of flies = 16). The lower panel shows *hsflp* out clones for DL1 specific PN. Arrow indicates the cell body. No targeting defect is observed (number of flies = 6 for mutants, and 11 for heterozygous controls). C) Quantification of the *hsflp* out clones and *eyflp* MARCM show targeting defect in VA1lm specific PNs but not in Or47b ORNs respectively. Grey bar indicate the *hsflp* out PN clone targeting defect and black bar indicates the *eyflp* induced MARCM clone targeting defect quantification. Scale bar is equal to 20µm for all the images.

To this aim hsflp (Flp recombinase expressed under the control of a heat shock promoter) was used to get single or small subset of PNs and were analyzed for the pattern of PN dendrites innervating the VA1lm / Or47b in heterozygous controls and homozygous $exn^{EY\text{-}\Delta 23}$ mutants. In mutants, a significant increase (50%) in targeting defect of VA1lm specific PN was observed as compared to heterozygous control (6.25%)(Figure 3.4.1 B, C). In addition, DL1 specific PN dendritic innervation was analyzed. No targeting defect was observed in mutants as compared to controls. This result is in accordance with no targeting defect in DL1 glomerulus specific ORN axon (Or10a) targeting (see Figure 3.1 3).

Taken together, these data suggests that Eph, ephrin and Ephexin are required in PNs for the correct targeting of their dendrites and subsequently, the axons of matching ORNs. Although the direct evidence is only shown for *exn* mutants due to the lack of genetic tools for the fourth chromosome, rescue of the $eph^{-/-}$ mistargeting phenotype by a PN driver supports the hypothesis.

3.5 Different modes of Eph signaling in PNs.

As shown earlier in the loss of function analysis, both Eph as well as Exn show a requirement in olfactory map formation (Figure 3.1.1). Exn was placed specifically downstream of Eph in several previous studies. Interestingly, in our study the quality of targeting defect in both the mutants differed (Figure 3.1.2). While *eph* mutants lead to a clear lateral targeting defect of Or47a neurons, *exn* mutants led to a ventral targeting defect of the same. In addition, no targeting defect was seen for Or47b neurons in case of *eph* mutant while *exn* mutants showed Or47b neuronal mistargeting. These observations led to two hypotheses: 1) Exn acts downstream of other receptor signaling, or 2) Eph signaling works via many different signaling mechanisms and Exn controls only one of those mechanisms.

To test the first hypotheses candidates were selected to check their potential interaction with Exn. These candidates were either from vertebrate studies known to interact with Exn or they contain the putative binding domain for Exn same as in the Eph receptor. To test the alternate hypothesis, downstream signaling mechanism was dissected by performing genetic interaction and overexpression experiments. These interaction experiments were performed with small RhoGTPases that are shown in vertebrates to be working downstream of Eph and specifically Exn. Both these hypotheses were tested and are explained in the following sections.

3.5.1 Exn does not genetically interact with tested candidate receptors.

Genetic interaction experiments were conducted for Exn with candidate receptor mutants. These candidates were reported previously to either interact with Exn directly (as per vertebrate studies) or contain a potential domain like Eph receptor to interact with Exn. One of the appealing candidates was Fibroblast Growth Factor (FGF) receptor. Earlier reports from mouse study revealed that Eph is trans-phosphorylated by FGF receptor (Yokote et al., 2005).Same group later found out through a series of *in-vitro* experiments that FGF receptor can phosphorylate Exn as well. Considering these previous reports, Exn and FGF receptor genetic interaction in flies was analyzed. There are two types of FGF receptors in flies: Heartless (Htl) and Breathless (Btl). The ligand for them is called Branchless (Bnl) in flies. Trans- heterozygous experiments were conducted with loss of function alleles for *htl*, *btl* and *bnl* with $exn^{EY\text{-}\Delta 23+/-}$ in the background (Figure3.5.1 A). No targeting defect comparable with $exn^{EY\text{-}\Delta 23-/-}$ mutants was observed in these trans-heterozygous flies. Instead, both *htl* and *btl* showed a medial targeting defect which was independent of the $exn^{EY\text{-}\Delta 23+/-}$ background. Epidermal Growth Factor (EGF) receptor has similar tyrosine kinase domain as FGF receptor and hence was speculated to interact with Exn. Hence, interaction with EGF receptor was analyzed by over-expressing $UAS\text{-}egfr^{dn}$ in GH146 positive PNs (Figure 3.5.1B). Although, no targeting defect was observed by over-expressing $UAS\text{-}egfr^{dn}$ in PNs, over-expressing another potential candidate *ret* ($UAS\text{-}ret^{wt}$) in PNs led to both a mild lateral and medial targeting defect for Or47a neurons. Ret is known to interact with Eph (Kramer et al., 2006) and contains a similar tyrosine kinase domain as Eph receptor (interacts with Exn). Interestingly, when ret is over-expressed in PNs in $exn^{EY\text{-}\Delta 23+/-}$

background, lateral targeting defect of Or47a neurons is increased than the one observed in the WT (*exn*$^{EY\text{-}\Delta 23+/+}$) background (Figure3.5.1 B,C). This enhancement of mistargeting can be attributed to the interaction of ret with Eph signaling.

In addition of analyzing candidates having an Eph like domain to interact potentially with Exn, candidate molecules which can interact with Eph receptor and modulate the downstream signaling along with Exn were also analyzed. Examples include Vav (another Rho-GTPases specific GEF known to be working downstream of Eph receptor) and Src kinase (known to phosphorylate molecules downstream of Eph signaling). None of these candidates showed targeting defect both in heterozygous and trans-heterozygous condition (Figure 3.5.1 A).

Collectively, all these data do not support the hypotheses that Exn can interact with other receptor signaling in addition to Eph receptor signaling. Hence the alternate hypothesis was tested. Detailed experimental tests of the alternate hypothesis are described in the following sections.

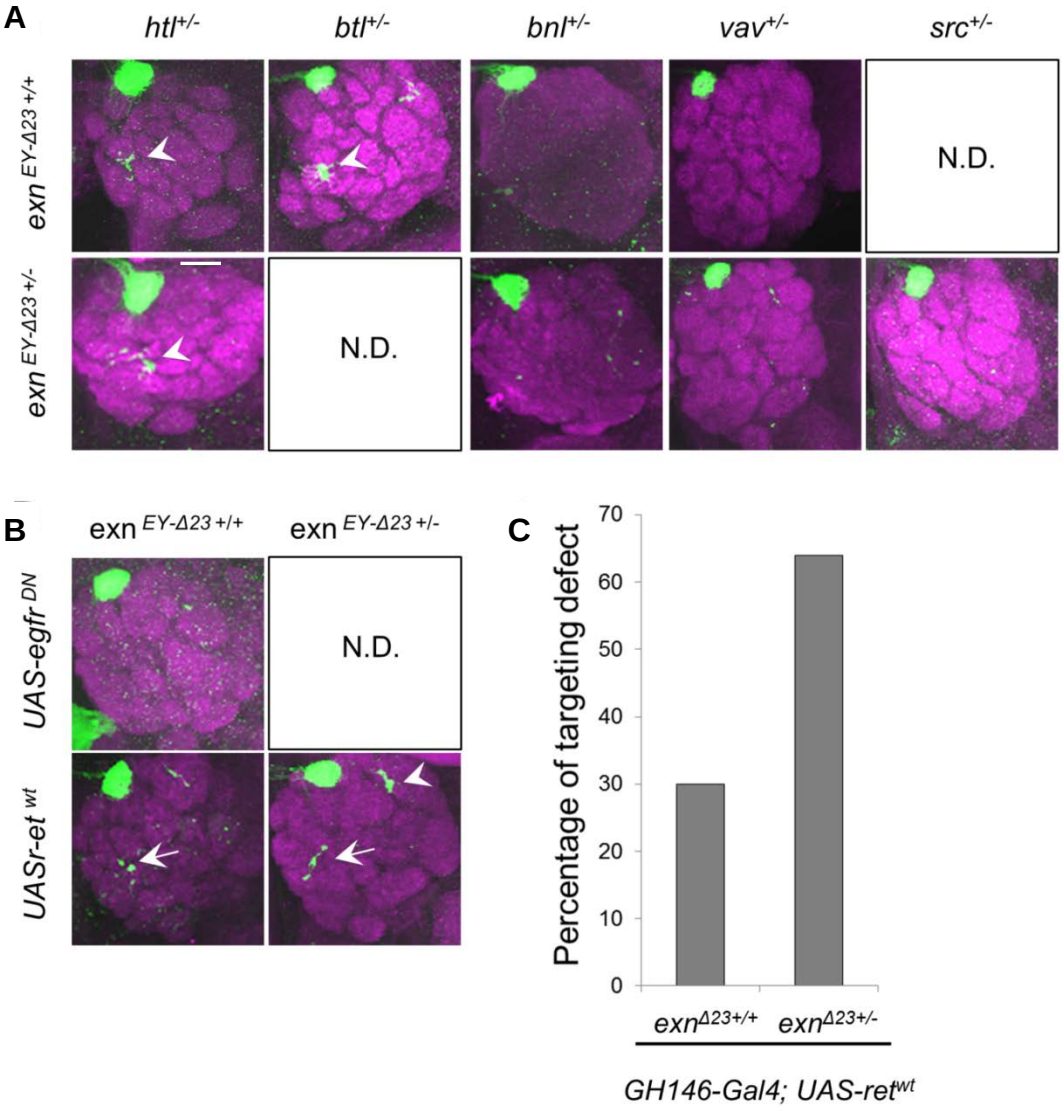


Figure 3.5 1 : Fly Exn do not show same interactions as vertebrates.

A) Trans-heterozygous analysis of *exn* with candidate genes. No increase in the targeting defect of or47a neurons was seen when mutant of candidate genes were in heterozygous *exn* mutant background. B) Overexpression of the dominant negative form of EGF receptor in PNs shows no defect in ORN targeting. Overexpression of ret^{wt}, lead to an increase in the medial and lateral targeting defect of Or47a neurons in $exn^{EY\text{-}\Delta23+/-}$ background. C) Quantification of the increase in both medial and lateral targeting defect of Or47a neurons in overexpressed ret^{wt} in $exn^{EY\text{-}\Delta23+/-}$ background. Scale bar is 20 µm for all the images.

3.5.2 Exn is required downstream of Eph receptor in the PNs.

To more directly assay if Exn acts downstream of Eph, potential genetic interactions between Eph, ephrin, and Exn were tested. To this aim, WT and truncated versions of Eph and a truncated ephrin in WT and *exn*$^{EY\text{-}\Delta 23}$ heterozygous flies were analyzed using *GH146-Gal4* as the driver (Figure 3.5.2). Overexpression of *UAS-eph*^{wt} in the wildtype or heterozygous *exn* background showed little effect on Or47a targeting (Figure 3.5.2 A). By contrast, overexpression of a truncated version of Eph (*UAS-eph*$^{\Delta C}$) in wildtype and heterozygous *exn* mutant flies resulted in equal lateral targeting defect (29% and 32%; Figure 3.5.2 B). This UAS construct of *eph* is reported to act as a dominant-negative on Eph forward signaling and the observed phenotype was reminiscent of the loss of function phenotype seen in *eph* mutants (see Figure 3.1.1). In addition, *UAS-eph*$^{\Delta C}$ might over- or mis-activate ephrin reverse signaling. Nevertheless, this possibility can be ruled out that ectopic ephrin reverse signaling is responsible for the phenotype observed by *UAS-eph*$^{\Delta C}$ overexpression, because overexpression of *UAS-eph*^{wt}, which also will activate ephrin reverse signaling, did not show the same effect.

Overexpression of *UAS-ephrin*$^{\Delta C}$ in the heterozygous mutant background of *exn* only, but not in WT led to Or47a ventral targeting defect in 28% of brains analyzed (Figure 3.5.2 A, B). Interestingly, here no lateral instead only the ventral targeting defect is seen in *exn* homozygous mutants (see Figure 3.1.1). These results indicate that overexpression of ephrin$^{\Delta C}$ and subsequent over-activation of Eph forward signaling changes the balance of Eph signaling such that Exn becomes a limiting factor. Hence, if Eph signaling is active and there is less Exn in the background, it mimics the *exn* mutant situation.

These results are consistent with our second hypothesis that Eph signaling works via different signaling mechanisms and Exn is a part of one of these mechanisms. Conclusively, Eph signaling is required in an Exn-dependent as well as an – independent mode and thus, loss of *exn* and loss of *eph* result in two different phenotypes.

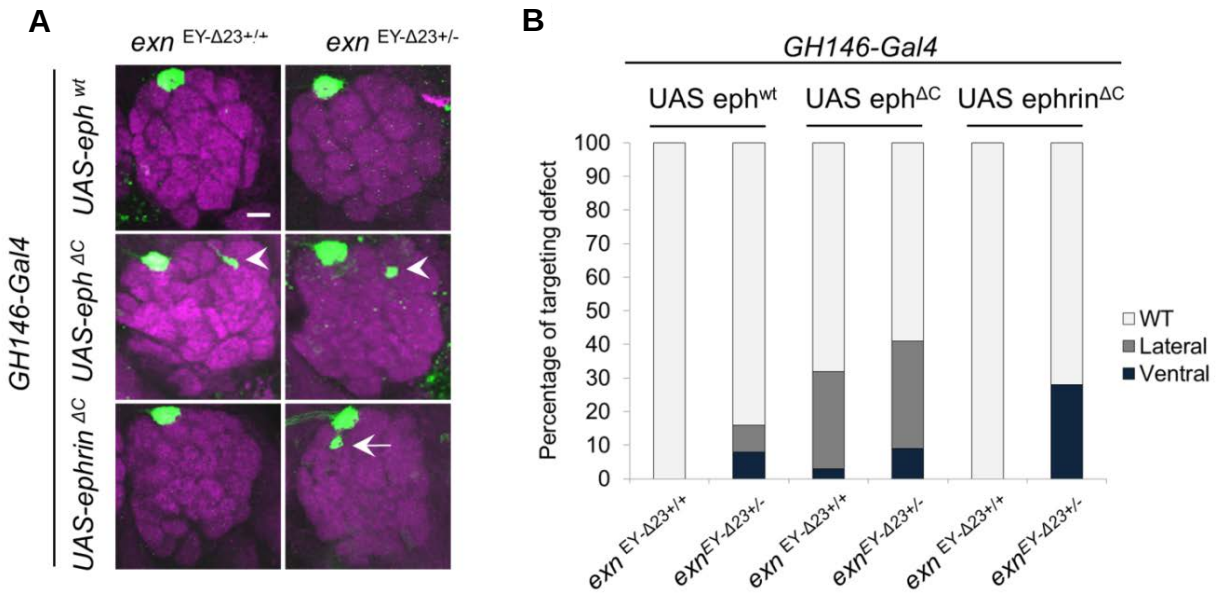


Figure 3.5 2 : Exn mutation reveals a differential signaling mechanism downstream of Eph.

A. Over-expression of *eph*^{wt}, *eph*$^{\Delta C}$ and *ephrin*$^{\Delta C}$ using *GH146-Gal4* (PN driver) in WT and *exn*$^{EY\text{-}\Delta 23+/-}$ background. *eph*^{wt} over-expression in PNs in WT and *exn*$^{EY\text{-}\Delta 23+/-}$ flies lead to no major targeting defect for Or47a neurons. *eph*$^{\Delta C}$ over-expression show lateral targeting defect in both WT and *exn*$^{EY\text{-}\Delta 23+/-}$ flies (arrowheads). Over-expression of *ephrin*$^{\Delta C}$ show no targeting defect in WT but show ventral targeting defect in *exn*$^{EY\text{-}\Delta 23+/-}$ background (arrow). Scale bar is 20µm. On average 30 brains were analyzed for each genotype. ***B.*** Quantification for over-expression analysis shows that the lateral targeting defect of *eph*$^{\Delta C}$ over-expression in PNs is same irrespective of absence of *exn* in the background. Overexpression of *ephrin*$^{\Delta C}$ in PNs show significant ventral targeting defect in *exn*$^{EY\text{-}\Delta 23+/-}$ background as compared to WT. Light grey bar indicates WT, dark grey bar indicates lateral and dark blue bar indicates the ventral targeting defect. Scale bar is 20 µm for all the images.

3.5.3 Eph and Exn genetically interact to prevent ORN mistargeting.

In order to gain additional evidence for the requirement of Exn as a component of Eph signaling, another set of genetic interaction experiments were performed. *eph* and *exn* mutants were analyzed in all possible combinations ranging from trans-heterozygous to double mutants. Here the extent of lateral versus ventral targeting defect for Or47a neurons was analyzed. As shown in Figure 3.5.3 B, *eph* homozygous mutants display a lateral targeting defect in 69% and 50% brains in an *exn* WT and *exn* heterozygous background respectively. On the other hand *exn* homozygous mutants in any combination displayed significantly higher ventral targeting defect as compared to the lateral one (Figure 3.5.3). Complete removal of both *eph* and *exn* resulted in less lateral and more ventral targeting defect as compared to the *eph* homozygous mutants in *exn* heterozygous background (Figure 3.5.3.B). This shift can be explained by the likely dominant ventral targeting defect as soon as both alleles of *exn* were non-functional. This result can be explained by already known nature of Exn as a Rho GTPases specific GEF. Exn is known to be active without being phosphorylated by Eph and this minimally active Exn regulates the downstream Rho GTPases differently than a fully active Exn (phosphorylated Exn). A similar process could happen in flies. Hence when Exn and Eph both are missing then *exn* mutant phenotype is dominant over the *eph* mutant phenotype. Double homozygous mutants not only showed a quantitative but also a qualitative increase in ventral targeting defect: 39% of the $exn^{-/-}$; $eph^{-/-}$ show a complete shift of DM3/Or47a glomerulus innervation to the ventral target site was observed (Figure 3.5.3 B). This shows that *eph* and *exn* do interact genetically and lead to a stronger targeting defect as compared to the individual homozygous mutants.

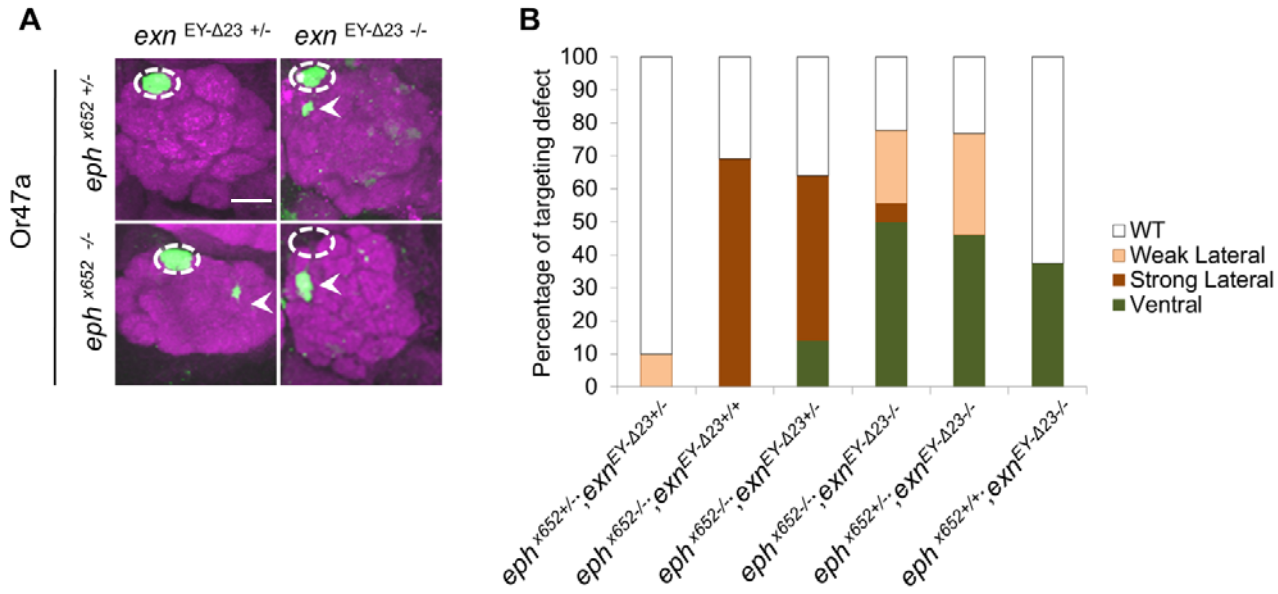


Figure 3.5 3 : Genetic interaction between Eph and Exn.

A) Genetic interaction between *eph* and *exn*. Trans-heterozygous mutants ($eph^{x652+/-}$; $exn^{EY-\Delta23+/-}$) show no targeting defect for Or47a neurons. Lateral targeting defect (arrowhead) is observed for $eph^{x652-/-}$ mutants in $exn^{EY-\Delta23+/-}$ heterozygous background. On the other hand ventral targeting defect for Or47a neurons appear in $exn^{EY-\Delta23-/-}$ mutants in both $eph^{x652+/-}$ heterozygous and $eph^{x652-/-}$ mutant background (arrowhead). The ventral targeting defect is stronger in double mutants of *eph* and *exn* as the DM3 glomerular innervation is missing (shown by dotted circle without any GFP labeling). B) Quantification for the genetic interaction between *eph* and *exn* mutants. White bar indicates WT targeting pattern, peach bar indicates weak lateral phenotype, orange bar indicates strong lateral phenotype and green bar indicates split glomerulus. The lateral targeting defect in $eph^{x652-/-}$ mutants in $exn^{EY-\Delta23+/+}$ and $exn^{EY-\Delta23+/-}$ background is comparable. The ventral targeting defect is comparable in $exn^{EY-\Delta23-/-}$ mutant irrespective of *eph* in the background. On an average 15 brains were analyzed for each genotype for calculating the percentage of targeting defect. Scale bar is 20 µm.

In summary, these data show that Eph genetically interacts with Exn during olfactory map formation. In addition, they suggest that Exn indeed acts downstream of Eph forward signaling. Finally, these results strengthen the hypothesis that different signaling pathways downstream of Eph, dependent and independent of Exn, fine-tune glomerulus targeting of a small subset of PN classes.

3.6 Exn-dependent and -independent role of small Rho GTPases downstream of Eph signaling.

Important molecular players downstream of axon guidance receptors include small Rho GTPases that regulate actin dynamics in different ways. Since Exn is known to regulate the activity of these small Rho GTPases, the possibility of a differential requirement of the three most important Rho GTPases: RhoA, Rac1 and Cdc42, was investigated. To this aim, a series of genetic interaction experiments were conducted using already published mutants for these RhoGTPases (Ji et al., 2002; Lajeunesse et al., 1997; Ng et al., 2002; Strutt et al., 1973).

First, a tans-heterozygous analysis was conducted for all the RhoGTPase mutants with *eph* and *exn* mutants respectively. The loss of function allele for *rac1* (*rac1*^{J11}) was the only RhoGTPase mutant that showed a lateral targeting defect by itself in a heterozygous situation (Figure3.6.1 A, B). This lateral mistargeting was a weaker version of the mistargeting observed in *eph* null mutants. Strikingly, removal of a copy of *eph (eph*$^{x652+/-}$*)* in the background of *rac1*$^{(J11)+/-}$ showed a strong lateral targeting defect in 100% of the brains analyzed. In some extreme cases, *rac1*$^{(J11)+/-}$ *; eph*$^{x652+/-}$ flies showed a very strong version of the lateral targeting defect: the DM3 glomerulus was no longer innervated by the Or47a neurons, instead, all neurons mistargeted to the lateral side. Similarly, in *rac1*$^{(J11)+/-}$ *; exn*$^{EY-\Delta23+/-}$ flies lateral targeting defect was observed but to a lesser extent as compared to *rac1*$^{(J11)+/-}$ *; eph*$^{x652+/-}$. No ventral targeting defect was observed in any interaction with *rac1*. These results suggest that *rac1* acts downstream of *eph* and to a lesser extent interacts with *exn* to maintain the DM3 specific neurons along the medial-lateral axis. On the contrary, *cdc42* heterozygous flies showed

different targeting defects in *eph*$^{x652+/-}$ and *exn*$^{EY\text{-}\Delta23+/-}$ background. Both, hypomorphic (*cdc42* $^{(2)}$) and null mutant (*cdc42*$^{(4)}$) of cdc42 was analyzed. Removal of one copy of *eph* in the background of heterozygous mutants of two different alleles of *cdc42* (*cdc42*$^{(2)}$ and *cdc42*$^{(4)}$) resulted in a significant number of brains with the lateral mistargeting phenotype (Figure 3.6.1 A,C). By contrast, heterozygous double mutants of *cdc42* and *exn* showed the ventral mistargeting phenotype in 35% and 27% of the brains analyzed for the two different alleles of *cdc42*, and very little lateral mistargeting was observed (Figure 3.6.1 A,D).

From the vertebrate studies, *rhoA* was implicated as the major RhoGTPase interacting with *exn* (Sahin et al., 2005). Surprisingly, no targeting defect was observed for *rhoA* heterozygous mutant in *eph* and *exn* heterozygous background, respectively. Taken together, with the trans-heterozygous genetic experiments, we conclude that Cdc42 but not Rac1 is required downstream of Exn to prevent ventral targeting defect. As opposed to vertebrate studies, this data suggests a different molecular mechanism downstream of Eph receptor in *Drosophila* olfactory system. In vertebrates, Exn downstream of activated Eph receptor leads to activation of RhoA but no activation of Cdc42 and Rac1. Here, instead, Exn and Cdc42 together maintain DM3 neurons in the dorsal-ventral axis. This result is also consistent with already published data showing the interaction between Exn and Cdc42 in maintaining synaptic homeostasis at NMJ in *Drosophila* (Frank et al., 2009).

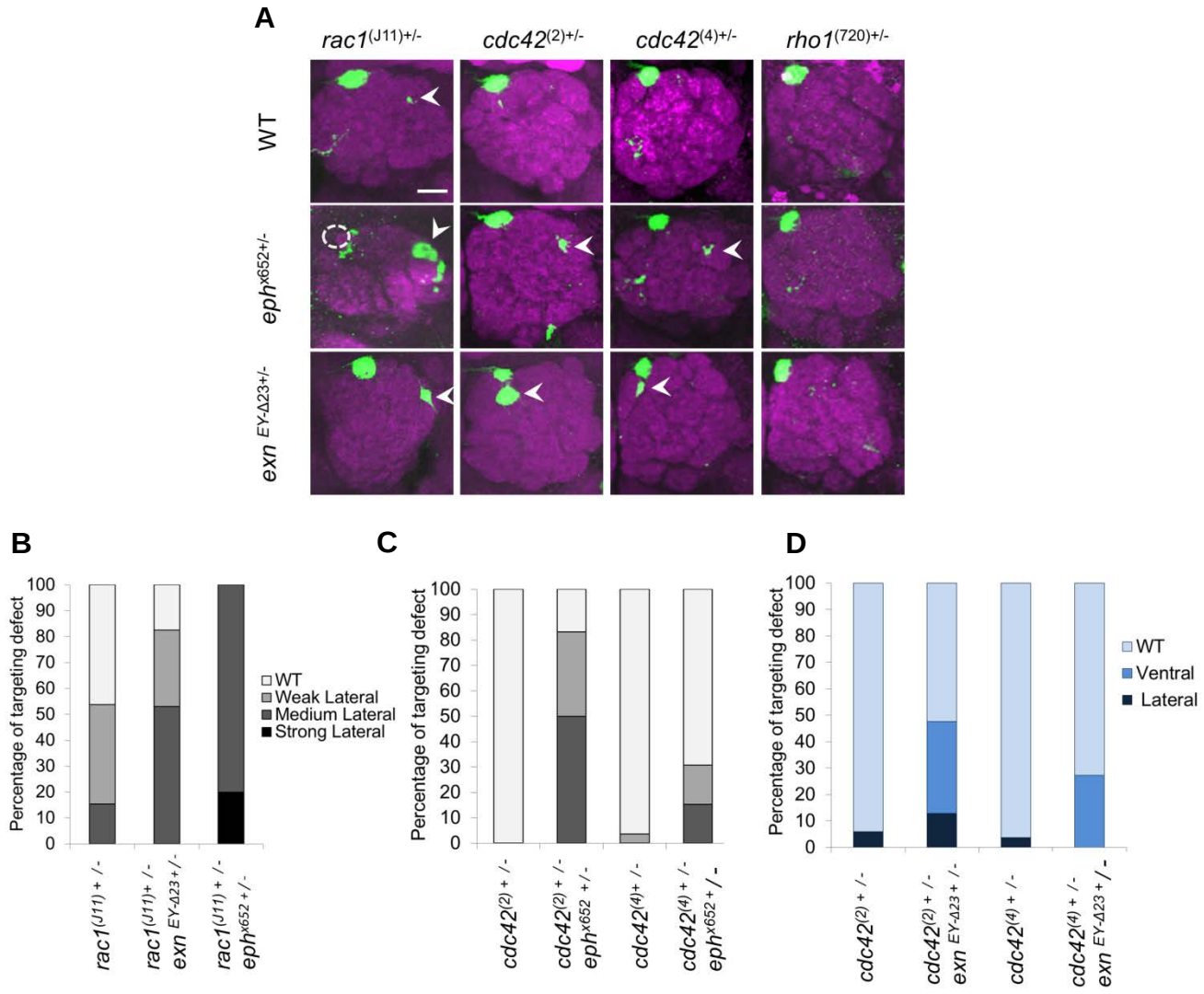
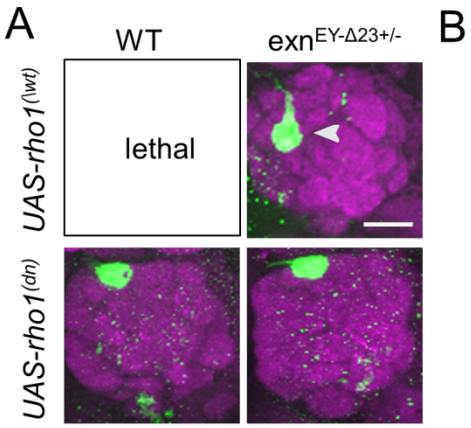


Figure 3.6 1 : Small GTPases, Rac1 and Cdc42 regulate olfactory map formation downstream of Eph and Exn

A) Rho GTPases heterozygous mutants in WT, *eph*$^{x652+/-}$ and *exn*$^{EY-\Delta23+/-}$ background show different targeting defects for Or47a neurons. On average 15 brains per genotype were analyzed. Heterozygous rac1 mutants show mild lateral targeting defect for Or47a neurons (arrowheads) which is enhanced in *eph*$^{x652+/-}$ and *exn*$^{EY-\Delta23+/-}$ background. In extreme cases the DM3 glomerulus is not labeled and only lateral is seen (dotted circle and arrowhead). Hypomorph (*cdc42* $^{(2)}$) and null (*cdc42* $^{(4)}$) allele for *cdc42* show no targeting defect in heterozygous condition. Although in *eph*$^{x652+/-}$ background they show lateral targeting defect, whereas in *exn*$^{EY-\Delta23+/-}$ background, they show ventral targeting defect for Or47a neurons (arrowheads). Heterozygous *rho1* mutant in WT, *eph*$^{x652+/-}$ and *exn*$^{EY-\Delta23+/-}$ background show no targeting defect. Scale bar is 20 µm for all the images. B), C) and D). Quantification of the genetic interaction between various Rho GTPases in WT, *eph*$^{x652+/-}$ and *exn*$^{EY-\Delta23+/-}$ background are shown. B), C) Show quantification for

lateral targeting defect in trans-heterozygotes: *rac1*$^{(J11)+/-}$;*eph*$^{x652+/-}$, *cdc42*$^{(2)+/-}$;*eph*$^{x652+/-}$, *cdc42*$^{(4)+/-}$;*eph*$^{x652+/-}$ and *rac1*$^{(J11)+/-}$;*exn*$^{EY-\Delta23+/-}$. D) Quantification for ventral targeting defect seen in Or47a neurons in *cdc42*$^{(2)+/-}$;*exn*$^{EY-\Delta23+/-}$ *and cdc42*$^{(4)+/-}$; *exn*$^{EY\Delta23+/-}$. Mild lateral targeting defect is seen in *cdc42* $^{(2)+/-}$ flies.

Next, over-expression analysis was conducted for various RhoGTPases to gain further insight into their role in olfactory map formation. WT, dominant negative and dominant active forms of all the three Rho GTPases were over-expressed in GH146 positive PNs in a WT and *exn*$^{EY-\Delta23+/-}$ background, respectively. These experiments were conducted both at 25 °C and 18 °C temperature, in order to increase the survival rate. Nevertheless, *rac1* and *cdc42* over-expression in GH146 specific PNs was lethal irrespective of the WT and exn$^{EY-\Delta23+/-}$ background (Figure3.6.2 B). On the contrary, flies over-expressing WT and dominant negative forms of *rhoA* in GH146 positive PNs survived (Figure3.6.2 A,B). *GH146-Gal4;UASrho1*$^{(wt)}$ flies survived at 18 °C but not at 25 degrees. Whereas, *GH146-Gal4; UAS-rho1*$^{(dn)}$ flies survived at 25 °C. These data points towards that both *cdc42* and *rac1* are extremely crucial for the PNs and change in their levels either by knocking down (in trans-heterozygous analysis) or over-expression leads to severe defects. As far as *rhoA* is considered, knocking down *rhoA* (trans-heterozygous experiments and over-expression of dominant negative form) leads to no defects while over-expression of *rhoA*$^{(wt)}$ leads to severe targeting defects (Figure3.6.2A, B). *GH146-Gal4; UAs-rho1*$^{(wt)}$ leads to a severe form of ventral targeting defect in *exn*$^{EY-\Delta23+/-}$ background. This suggests that less Exn overcomes the lethality induced by overexpression of Rho1.



B

		WT	exn EY-Δ23+/-
UAS rac1	WT	lethal	lethal
	DN	lethal	lethal
	DA	lethal	lethal
UAS cdc42	WT	lethal	lethal
	DN	lethal	lethal
	DA		
UAS rho1	WT	lethal	77% ventral defect
	DN	No phenotype	No phenotype
	DA	lethal	lethal

Figure 3.6 2 : Rho1 GTPase shows minor interaction with Exn in flies.

A) Over expression of rho1^{wt} is lethal in GH146 positive PNs but with heterozygous *exn*$^{EY\text{-}\Delta23+/-}$ ventral targeting defect of Or47a neurons is observed. This ventral phenotype is stronger than *exn*$^{EY\text{-}\Delta23-/-}$ as in this case the DM3 glomerulus is completely missing while ventral glomeruli is innervated. On the contrary no targeting defect is seen when rho1^{dn} is overexpressed in the GH146 positive PNs, both in WT and *exn*$^{EY\text{-}\Delta23+/-}$ background. Scale bar is 20 µm for all the images. B) Table showing the overexpression analysis of WT, DA and DN versions of Rac1, Cdc42 and Rho1. Both Rac1 and Cdc42 are lethal when overexpressed. The percentage of targeting defect seen by Rho1^{wt} overexpression in *exn*$^{EY\text{-}\Delta23+/-}$ background is shown.

Taken together, this data show Cdc42 and Rac1 as major downstream players of Eph signaling involved in maintaining the olfactory neurons along two different axes in an Exn-dependent and –independent mode (Figure 4.2.1). On the other hand RhoA seems to interact with Exn but plays a minor role in olfactory map formation. These results indicate that RhoA is inhibited by Exn and this inhibition prevents lateral mistargeting of DM3 innervating neurons.

3.7 Ephexin shows no defect in aversive olfactory learning and memory.

From all the experiments mentioned until now, role of Eph signaling has been established in setting the olfactory map in a very specific way. In addition, *exn* is proven to be a molecule that maintains a crucial balance downstream of Eph signaling. Hence the next question asked was whether this signaling plays any role in higher brain functions such as learning and memory formation. Since, Eph signaling is already known to be involved in setting mushroom body neurons targeting (Boyle et al., 2006), it can be speculated that it is involved in the most important function of mushroom body that is learning and memory. Although mutating *eph* is not lethal, the mutant flies are sick and difficult to amplify, hence memory experiments were conducted with *exn* mutant - $exn^{EY10953}$. In this study *exn* mutant flies were analyzed for aversive olfactory learning and memory. To this aim, *exn* mutants were tested for both short term and middle term (2 h) memory (see methods). These experiments were conducted and analyzed in collaboration with Dr. Stephan Knapek. Since $exn^{EY10953}$ allele had *yw* background, flies with *yw* background were chosen as the genetically closest controls. In addition w^{-} and CantonS (CS) flies were used as additional controls. Electric shock was reciprocally paired with two aversive odors: 4-methylcyclohexanol and 3-octanol. The leaning index was calculated as described in the method section.

As shown in figure 3.7.1, no defect was observed in both short term and middle term (2 h) memory in case of *exn* mutants as compared to the controls. It indicates that Exn is not required for aversive olfactory learning and memory. Although these results don't exclude the possibility that *exn* -independent role of *eph* might be required in the olfactory learning and memory.

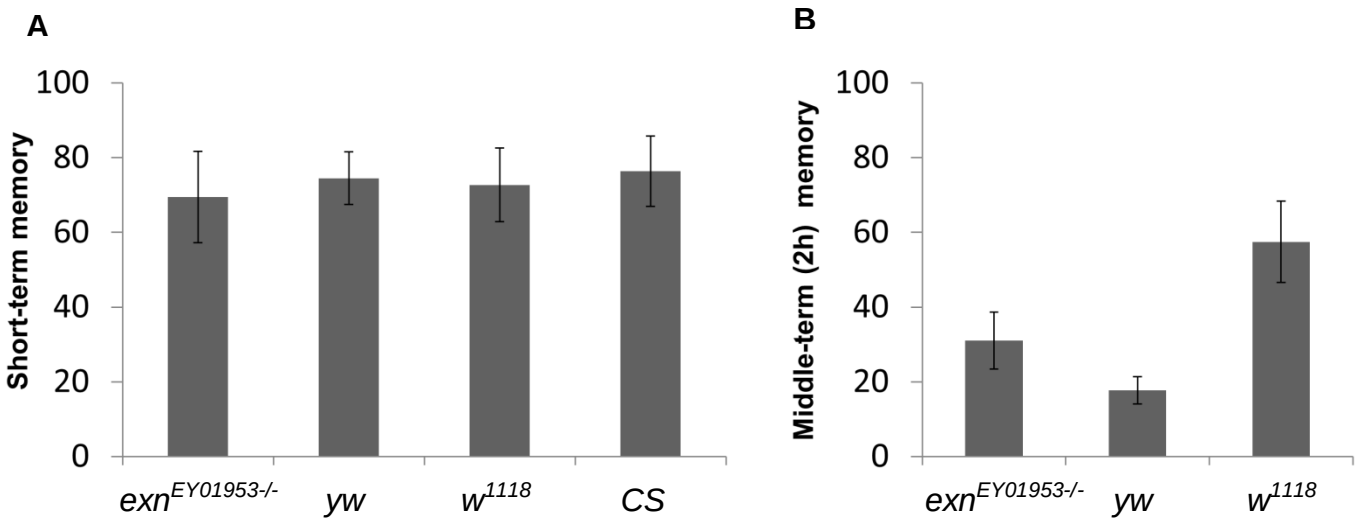


Figure 3.7 1 : Exn is not involved in aversive olfactory learning and memory.

A) Quantification of the short term memory. Y axis shows the Learning index (LI), which is the mean of two reciprocal preference indices (PIs). X axis shows the genotype of the flies analyzed. No significant change in the LIs of the mutant vs. controls is observed. B) Quantification of the Middle term (2 h) memory. Again the Y axis and the X axis are the same as A). No significant difference in the LI of mutant vs. control is observed.

From sections 3.1-3.6, the role of Eph signaling is established in olfactory system development and its requirement in PN dendrite targeting. Above mentioned results present the first report for an *in vivo* axon guidance defect in an Exn mutant. Furthermore, it shows that the relative balance of Exn-dependent and –independent Eph signaling within the same type of PN class restrict its dendrites to a particular glomerulus along the two main axes of the olfactory map (see section 4 for detailed discussion).

Till date, lots of studies have focused on the role of Eph signaling in the development of neuronal maps in vertebrates (Brennan et al., 1997; Klein, 2012; Kramer et al., 2006; Marcus et al., 1996; Mombaerts, 2006; St John and Key, 2001). On the contrary only a handful of data is available for the role of Exn downstream of Eph signaling (Margolis et al., 2010; Sahin et al., 2005; Shamah et al., 2001; Zhang et al., 2007). One idea that emerged from the reports in flies and mouse is the involvement of Exn in maintaining synapses and synaptic homeostasis (Frank et al., 2009; Margolis et al., 2010; Shi et al., 2010). Taking into consideration these reports, the role of Exn in synapses was analyzed in the olfactory system in a separate project. To address this, electron microscopic analysis was carried out in order to look for any synaptic defects in *exn* mutants as compared to the WT controls. The following section will explain the experimental details and results of this study.

3.8 Ephexin is not required for maintaining synapse number in the fly olfactory system.

In addition to the requirement in targeting process and map formation, the role of Exn in synapse formation was studied. Already published data is available which establishes the role of Exn in maintaining synapses. In this study, requirement of Exn in maintaining synapses was addressed by performing electron micrographic (EM) analysis in collaboration with Dr. Marianne Braun (Figure 3.8.1). The structure analyzed is *Drosophila* mushroom body calyx (the input center for the mushroom body). This structure was chosen, firstly because the axons of the PNs target this area and secondly, it has a defined structure loaded with synaptic connections at the posterior most side of the brain and hence amenable to sectioning for EM analysis.

Calyx, from *exn* mutant flies and WT (w1118) flies, was compared in terms of number of synapses (Figure 3.8.1 A1, B1 and D). The number synapses were counted from the EM images obtained from calyx sections of mutant and control flies. No significant difference in synapse numbers was observed between control and *exn* mutant (n=3). Along with counting the number of synapse, an interesting observation was the difference in the number of dense micro glomeruli. Micro glomeruli are the small claw like structures where axons of PNs and dendrites of Kenyon cells synapse. It is also the target of many extrinsic neurons. These micro glomeruli are filled with lot of synaptic vesicles as it is the activity center of the calyx. The numbers of normal and dense micro glomeruli were counted in both mutant and control samples. The number of dense micro glomeruli were significantly higher in *exn* mutants as compared to the controls (Figure 3.8.1 A2, A3, B2, B3 and C).

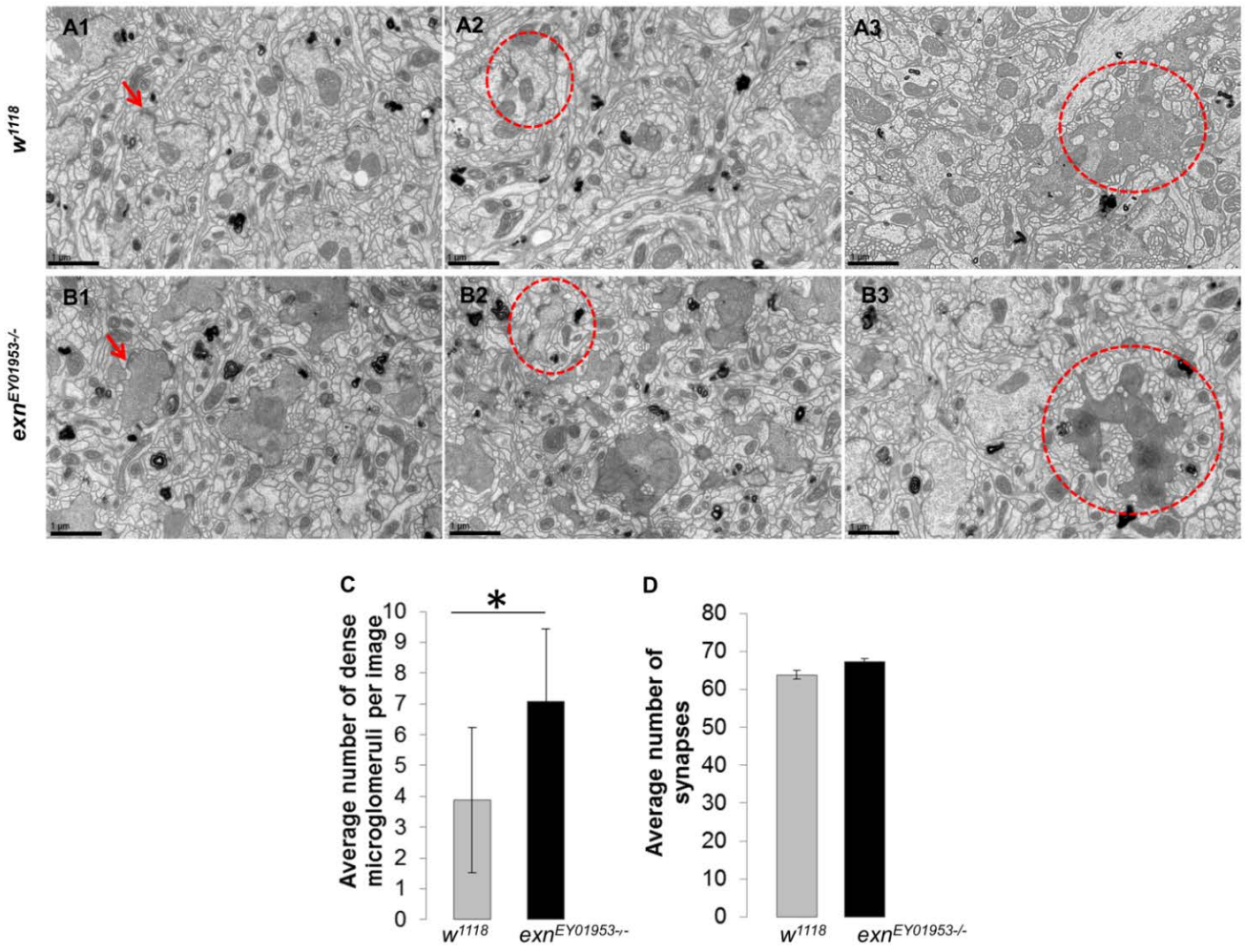


Figure 3.8 1 : Exn do not regulate the synapse number in MB calyx.

A1-A3) EM image of the calyx section of adult control (w^{1118}) fly brain.A1) Red arrow indicates the synapse and red dotted circle indicating the microglomeruli (activity centers of calyx where Projection neurons axons and kenyon cells dendrites make synapses). Scale is 1 µm. A3) Red dotted circle showing a denser (darker) microglomeruli. B) EM image of the calyx section of *exn* mutant. B1) Red arrow indicates the synapse. B3) Red dotted circle indicates the dense microglomeruli. C) Quantification of number of dense (dark) microglomeruli in control vs. *exn* mutant. The numbers of dense microglomeruli are significantly more in *exn* mutant as compared to the control at the p value of 0.0395. D) Quantification of number of synapses in control vs. *exn* mutant. The numbers of synapses in the control and *exn* mutant are not significantly different. Number of animals analyzed per genotype are 3.

These data show that Exn is most probably not required for maintaining synapse number, although more experiments are required to prove this result. On the other hand there is an increase in the number of dense micro glomeruli in *exn* mutants. There could be two major reasons for this observation: 1) These dense micro glomeruli are formed due to less synaptic vesicle re-cycling in *exn* mutants or 2) These dense micro glomeruli are the degenerating micro glomeruli and they are more in number in *exn* mutants. The first hypotheses fit with already published data regarding Exn and its involvement in maintaining synaptic homeostasis (Frank et al., 2009). Frank et al. (2009), has shown that *exn* mutants have decreased quantal content (synaptic vesicle release). Although more evidence is required to prove this hypothesis that indeed the phenotype observed is due to less vesicle recycling. This study was not carried forward due to lack of technical expertise to address this question.

4. Discussion

Regulation of neural circuit formation is the focus of research in developmental neurobiology. The role of Eph and ephrins during neuronal development, in particular, is studied in many different systems from worm to mouse. Recent research in the field of axon guidance is focused on the interplay of different guidance receptors for the connectivity of single neuron types (Dudanova and Klein, 2013). The presented work is an important contribution to the field, because it demonstrates that a single guidance receptor can use differential signaling to navigate neurons precisely within a multi-dimensional neural map in addition to what is shown earlier regarding the cross talks between different receptor signaling.

The olfactory system offers a very attractive system to study the mechanism of axon guidance. The remarkable feature of olfaction is the extraordinary diversity of odor molecules that exist and that need to be encoded by the brain. Hence, a precisely wired neural system is required for odor detection and discrimination. During the last two decades, experiments understanding the molecular logic of olfaction have led to major insights about detecting and discriminating a vast variety of odorants (Axel, 1995). There are different layers of organization required for such a precise function: First, the presence of a large number of ORs dedicated to olfaction. Second, combinatorial detection of odors, where one OR can detect many odorants and one odorant can be detected by many ORs (Hallem and Carlson, 2006). Third, ORNs expressing the same receptor converge their axons to the same glomerulus in the brain to form a map of ORs in the brain (Bhalerao et al., 2003; Gao et al., 2000). Fourth, ORN axons synapse with

various interneurons that modulate the olfactory signal and pass on the information to higher brain center (Wilson et al., 2004). In the presented work, the role of Eph/ephrin signaling is addressed at some of these different layers of organization.

Till date several molecules are known to play a role in the wiring process of olfactory map formation of *Drosophila*. Examples of molecules involved in olfactory neuron targeting include: Sema 1a (Komiyama et al., 2007; Sweeney et al., 2007), Dscam (Hummel et al., 2003), Ncad (Hummel and Zipursky, 2004) and others (see introduction). In addition there are molecules known to play a role in synaptic partner matching during the wiring process, including Teneurins (Hong et al., 2012).

Most of the known molecules play a general role in the targeting of the majority of PNs and/or ORNs. Hence, the basic molecular logic behind coarse olfactory map formation has been deciphered; however, our understanding of how specific classes of neurons navigate and precisely control where to form synapse is incomplete. One of the rarest examples of a class specific molecule for correct targeting is Capricious (Caps) (Hong et al., 2009). A subset of PNs that express Caps, mistarget their dendrites when Caps is knocked out from these neurons. It leads to innervation of these dendrites to glomeruli which are normally innervated by Caps negative PNs. Hence this study presented, as opposed to a graded expression of molecules involved in a coarse olfactory map formation, a specific expression pattern of a molecule leading to a discrete map for a specific class of PNs. In addition to Caps, more molecules need to be deciphered for their role in the fine-tuning of coarse olfactory map to a more discrete one. Through a series of results presented in this thesis Eph/ephrin signaling is proven to be required in

this fine-tuning process of specific classes of PNs, and subsequently ORNs, leading to a precise olfactory map formation.

Prior to this work, Eph/ephrin signaling had been demonstrated to regulate ORN axon targeting in moth and mouse (Cutforth et al., 2003; Kaneko and Nighorn, 2003; Serizawa et al., 2006; St John and Key, 2001). In moth, it has been shown to be involved in sorting of ORN axons in olfactory explant culture. In the case of vertebrates, it is required for neuronal activity dependent fine-tuning of the olfactory map. In these studies it has been shown that neuronal activity regulates adhesive molecules Kirrel2/Kirrel3 and repulsive molecules EphA5/ephrinA5. EphA5 expression is down-regulated and ephrin A5 expression is up-regulated in CNGA2 (cyclic nucleotide gated ion channel) knockout mice indicating that these genes are regulated in an activity dependent manner (Serizawa et al., 2006). ephrinA5 was shown to be expressed in an OR-specific manner leading to a mosaic pattern of glomeruli with different ephrin expression levels (Cutforth et al., 2003). On the contrary, EphA5 is expressed in the olfactory bulb; therefore changes in ephrinA5 expression levels in different ORNs lead to differential targeting in the olfactory bulb. Hence these studies established the role of Eph/ephrin signaling in vertebrate olfactory map formation. Nevertheless, the signaling and cellular mechanisms, in particular, in the formation of the insect olfactory map formation remained elusive. Importantly, although the form of olfactory maps of vertebrates and insects are strikingly conserved, they are formed using somewhat divergent mechanisms. Vertebrates and insects differ in the mapping at the level of primary olfactory center. Unlike mouse, flies have no relationship between the peripheral position of ORNs and their target in the antennal lobe. In addition,

vertebrates and insects show a lot of variation in the wiring process of the olfactory system, as well. The most striking difference is the requirement of vertebrate ORs in setting ORN projections (Mombaerts, 2006). OR proteins are detected not only in the cilia and soma but also in the axonal termini of the ORNs. On the contrary, in insects there is no evidence of OR localization in the axon termini of ORNs. Also, ORs are not involved in the targeting of ORNs (Dobritsa et al., 2003). Another significant difference is that insect ORNs do not regenerate throughout the life of the animal. Antennal lobe connections formed during development remain fixed, hence, there is no evidence for neuronal activity dependent axon guidance, fine tuning and maintenance of olfactory map (Larsson et al., 2004). Considering these differences, insect olfactory system provides an example of a non-spatial and OR independent wiring system. Hence, it provides an excellent system to study the requirement and detailed molecular mechanism involved in the wiring process which, in turn, should give insights in the formation of other sensory maps of higher organisms. Therefore this work on Eph/ephrin signaling in olfactory map formation of *Drosophila* might also provide insights in the formation of other sensory maps of higher organisms. Furthermore, the finding that Eph signaling acts via differential downstream mechanism in the process of olfactory map formation is interesting and might be used during axon guidance in vertebrates.

This thesis demonstrates that unlike what is shown in mouse and moth, the formation of the olfactory map in *Drosophila,* Eph receptor signaling is required in specific subset of PNs but appears to be dispensable in ORNs. Importantly, this study also unravels the downstream mechanism by which Eph signaling leads to the fine tuning of the

projections of specific subset of PNs. These data establish that the Rho-GEF Exn is required downstream of Eph in the formation of olfactory map. It presents the first report for an *in vivo* guidance defect of an *exn* mutant. Exn, downstream of Eph prevents dorsal-ventral mistargeting of olfactory neurons in particular glomeruli in the antennal lobe. By contrast, Eph also prevents lateral mistargeting of the same neurons through an Exn-independent pathway. Finally, genetic interaction experiments with putative downstream effectors of Eph and Exn indicate that two important Rho-GTPases act downstream of Eph on the two different axes of the AL in PN and henceforth ORN targeting. Rac1 genetically interacts with Eph to efficiently prevent mistargeting along the medial-lateral axis. Conversely, Cdc42 acts in an Exn-dependent manner to prevent dorsal-ventral mistargeting phenotypes. In summary, this work suggests a model in which the relative activity of Rho-GTPases Rac1 and Cdc42 and their effects on the cytoskeleton downstream of Eph and Exn defines the exact position of specific PN dendrites and subsequently of the respective glomerulus (Figure 4.2.1).This demonstrates how a single receptor modulates the targeting of a particular neuron by using differential modes of downstream signaling which are represented as Exn-dependent and -independent Eph receptor signaling in specific subsets of PNs in this system.

Following sections discuss these results and develop a model for the requirement of differential Eph signaling in olfactory map formation. Also, analysis of Exn for its role in higher brain center is discussed in detail in the following sections.

4.1 Role of Eph signaling in instructing the olfactory map

The olfactory map in the AL comprises several cell types including PNs, ORNs, LNs, as well as modulatory neurons and glia. Previous studies concentrating on the targeting of PNs and ORNs discovered several fundamental concepts of olfactory map formation as well as cell-surface molecules implementing them. PNs use Semaphorin-1a to arrange their dendrites along two main axes of the initial olfactory map. Cell-surface molecules including Dscam and N-cadherin shape this map and help to position and form all glomeruli by providing repulsive and adhesive forces, respectively (Hummel and Zipursky, 2004; Hummel et al., 2003). Subsequently, Caps is responsible for the refinement of a subset of PN dendrites into discrete glomeruli (Hong et al., 2009). Where do Eph receptor and Exn fit in and what are their precise roles during map formation?

A large body of work on the role of Eph and ephrin in mouse showed that differential or graded expression of these partner molecules results in distinct signaling outcomes ranging from repulsion to attraction of two axons or axon and target cell. As mentioned above, in the mammalian olfactory system, axon-axon sorting of olfactory sensory neurons is mediated by neural-activity regulated expression levels of EphA/ephrinA leading to repulsive interactions between neurons of different ORN classes (Serizawa et al., 2006; St John et al., 2002). Similarly, a previous *in vitro* study in moth using antennal explants suggested Eph/ephrin-dependent ORN axon sorting (Kaneko and Nighorn, 2003). Contrary to these reports, no evidence for Eph/ephrin-dependent ORN axon-axon sorting was found in *Drosophila*. Instead, the work in this thesis show that these molecules regulate PN dendritic patterning in the AL. Loss of Eph signaling

affects only a few and specific glomeruli such that ectopic glomeruli along either one of the two main axes of the map (dorsal-lateral and ventral-medial) became innervated by very specific classes of ORNs. This ectopic innervation was rescued by re-expressing Eph receptor in developing PNs but not in developing ORNs. In addition, similar ectopic innervation was observed when Eph and ephrin are knocked down in developing PNs using RNAi strategy. Complementing these analyses is the expression study which shows higher expression of both Eph and Exn in the developing brain as compared to the developing antenna. Hence, through a series of experiments, it's established that Eph and Exn are required in PNs for the correct olfactory map formation.

These results are supported by a previous report (Sekine et al., 2013) which showed that a protein resident in the endoplasmic reticulum (ER) – meigo leads to N-glycosylation of ephrin, which in turn is required for the correct targeting of PNs. The molecule meigo, which stands for medial glomerular, leads to a medial shift of the lateral glomeruli in the AL. This medial shift is rescued by over-expressing ephrin in PNs. Hence, ephrin signaling was shown to be involved in maintaining particular PN dendrites along the medial-lateral axis. In line with this report, the work in this thesis shows that Eph mutants lead to a shift of medial glomeruli (e.g., DM3) to a more lateral one. Hence, Eph signaling is also involved in maintaining specific PN dendrites along the medial-lateral axis. In contrast, Sekine et al. showed that knocking down ephrin in PNs leads to DL1 PN dendrite targeting defect, which in this study showed no targeting defect for Exn mutants. Since Eph mutant was not analyzed for PN targeting defect, it could very well be that Eph might have an Exn-independent role in the targeting of DL1 PNs. Nevertheless, comparing these two studies one can speculate that there are

differential levels of Eph or ephrin leading to the fine tuning of PN dendrite targeting. Since meigo glycosylates and thereby modulates the levels of ephrin, this could lead to differential activation of Eph signaling. This differential activation of signaling could possibly be the reason behind a specific requirement of Eph signaling in few classes of PNs for its correct targeting. Hence Eph signaling provides a mechanism for the formation of discrete olfactory map by fine tuning the targeting of specific class of neurons.

Since *eph* and *exn* null mutant ORN targeting defects are rescued by re-expressing these molecules in PNs but not in ORNs, a non-cell autonomous requirement of these molecules in ORNs is suggested. This indicates that PN dendrites do not target properly in the AL of these mutants and hence ORN targeting defect is observed. PN dendrite targeting in the AL involves targeting to the correct glomerulus and it's exclusion from other glomeruli. This involves dendrite-dendrite interaction and sorting. Hence, differential Eph signaling via ephrin ligand (as mentioned above) presents an attractive mechanism for correct PN dendrite targeting in the AL. Nevertheless this study does not fully establish that Eph/ephrin signaling is indeed repulsive and not attractive, and hence, responsible for sorting of different classes of PN dendrites. Loss of Eph leads to innervation of ectopic glomeruli but also frequently to a spilling-over of dendrites into neighboring glomeruli. Both of these phenotypes could be attributed to either lack of adhesion between dendrites of the same type or lack of repulsion between dendrites of different type. Interestingly, another recently published report on *Drosophila* olfactory system suggested that Eph/ephrin signaling acts as repulsive guidance cue for a specific serotonergic neuron (Singh et al., 2013). Here, ephrin is expressed and

required in a central serotonergic deutocerebral neuron (CSD) for its correct targeting in the AL. This neuron targets a widespread area of the AL and its dendrites are excluded from the glomeruli which show high expression of Eph. Hence it supports the hypothesis that Eph represents a repulsive cue and leads to the exclusion of ephrin expressing dendrites from the Eph expressing regions. They further showed that the expression of Eph in these specific glomeruli comes from the axons of ORNs, which repel ephrin expressing CSD neuron dendrites. The expression of Eph is shown to be in the ORNs at later stages of development which happens after the targeting of both PN dendrites and ORN axons is mostly over. In this thesis, it's shown that Eph is expressed in the brain and not in the developing ORNs. The stages analyzed are early during development, which are more related to the PN dendrite and ORN axon targeting time line. Unfortunately, the tools used and generated to address the question of differential expression of Eph, ephrin, or Exn did not yield enough cellular resolution to establish whether PNs express relatively higher or lower levels of these proteins. However, together with the presented genetic evidence and the previous publications on Eph and ephrin in the fly olfactory system are consistent with a role of Eph and ephrin as repulsive guidance cues. More detailed analysis of their expression as well as additional genetic experiments with single cell PN clones are needed to answer this point satisfactorily.

Eph mutant analysis showed the requirement of this signaling in very specific classes of neurons. Hence, it presents an example of second layer of organization which is involved in fine tuning of the olfactory map after formation of a coarse map. A similar kind of role is suggested for caps (Hong et al., 2009). Caps is shown to be involved in

the fine tuning of PN dendrites by tight regulation of differential expression in some PN classes, similarly, Eph signaling is speculated to be regulating very specific PN dendrite targeting by differential activation (possibly by the regulated levels of ephrin ligand, as discussed above). Hence, the presented work proposes that Eph signaling plays a similar role as Caps and acts on a few PN classes during the refinement of the initial into the final glomerular map.

Both of the above mentioned, previously published reports (Sekine et al., 2013; Singh et al., 2013) establish a role of Eph/ephrin signaling in the developing olfactory system of *Drosophila*. However, none of these reports addresses the molecular mechanism involved in this process. The following section discusses in detail the results which establish the molecular mechanism operating downstream of Eph signaling for correct targeting of PN dendrites and hence ORN axons.

4.2 Mechanism of Eph signaling during PN dendrite targeting

In genetic experiments presented in this thesis, both Eph and Exn showed an ectopic targeting of specific classes of ORNs, but the kind of ectopic innervation was different in Eph compared to Exn mutants. Loss of Eph led to the lateral targeting defect for DM3 innervating ORNs, while Exn mutant displayed ventral targeting defects for the same class of ORNs. Hence many questions arise: how do two molecules, which are supposedly part of the same signaling lead to different targeting defects when missing from the system?

To date, none of the mouse mutants of *exn* family members are reported to show axon guidance phenotypes *in vivo* in spite of the functional evidence provided by in vitro studies (Shamah et al., 2000). The presented work provides first mutant evidence for the involvement of Exn in neuronal guidance. Surprisingly, loss of *exn* did not mirror the *eph* mutant phenotype. To solve the mechanism involved for such a differential targeting defects, two hypotheses were tested. First hypothesis addressed the interaction of any other signaling system with Exn. And the second hypothesis addressed differential signaling downstream of Eph signaling leading to Exn dependent and in-dependent pathways.

To address the possibility of Exn being downstream to another receptor system in addition to Eph, the interactions with several candidate receptors was analyzed. These candidates were selected based on previously known interactions either with Eph/ephrin signaling or with Exn itself. One of the appealing candidates was Fibroblast Growth Factor (FGF) receptor. It has been shown in mouse that Eph receptor is trans-phosphorylated by FGF receptor and further FGF receptor phosphorylates Exn *in-vitro*

(Yokote et al., 2005). In addition, Epidermal Growth Factor (EGF) receptor has similar tyrosine kinase domain as FGF receptor and hence was speculated to interact with Exn. Another potential candidate tested is Ret. It has been shown to interact with Eph (Kramer et al., 2006). Also, Ret was selected as a putative candidate as it has a tyrosine kinase domain that can potentially interact with Exn. Considering these previous reports, Exn and candidate receptor genetic interaction in flies was analyzed. Although the contribution of another receptor system cannot be ruled out completely, none of the candidate receptors showed any effect on Exn mistargeting phenotypes in the olfactory system. One of the exceptions was Ret, which when overexpressed in less Exn background showed enhancement of Eph like phenotype. This enhancement can be attributed to cross-talks between Eph and Ret, rather than Ret and Exn interaction. Hence, it appeared potentially more likely that Exn was indeed specific to Eph receptor signaling in the olfactory system. To further explain the difference in *eph* and *exn* mutant targeting defects, second hypothesis was tested.

Despite of different targeting defects seen in Eph and Exn mutants, genetic experiments supported an interaction between the molecules. Overexpression in PNs of EphΔC led to a similar targeting defect of ORNs as Eph mutant irrespective of the presence of Exn in the background, supporting that Eph is involved in maintaining the medial-lateral axis of the AL. On the other hand overexpression of ephrinΔC in PNs led to no targeting defect in ORNs in WT but a ventral targeting defect in a background with less Exn. This showed that Exn downstream of Eph signaling maintains the ventral-lateral axis of the AL. These experiments supported the second hypothesis that differential signaling

downstream of Eph signaling leads to Exn dependent and in-dependent pathways. To prove this hypothesis, a careful analysis of Exn as a Rho-GEF was carried out.

As a RhoGEF, Exn enhances the activity of RhoGTPases. The grade of enhancement was shown to be regulated by the phosphorylation state of Exn, which in turn is defined by the activity of Eph signaling. Prior work has shown that mouse Exn1 in its role as RhoGEF enhances the relative activities of the small GTPases: RhoA versus Rac1 and Cdc42 leading to ephrin ligand induced, phosphorylation-dependent growth cone collapse in cultured neurons (Sahin et al., 2005). Only one study analyzed the function of Exn in *Drosophila* so far and found that Exn was required for the maintenance of synaptic homeostasis at the neuromuscular junction (NMJ) (Frank et al., 2009). Here, Exn was shown to interact with Cdc42, but in contrast to its function in mouse, no genetic interaction was observed for Rho1 (fly homolog of RhoA) and Exn downstream of Eph signaling. In mammals, phosphorylation of Exn by activated Eph receptor, strongly enhances selectively the activity of RhoA relative to Rac1 and Cdc42 (Sahin et al., 2005). And while non-phosphorylated Exn tends to promote axon outgrowth, phosphorylated Exn triggers growth cone collapse consistent with a role of Eph as repulsive guidance cue. Accordingly in the fly olfactory system, loss of function of Exn is different from loss of Eph-mediated phosphorylation of the same. Hence, resulting phenotypes are likely to be different and are consistent with what is shown. More specifically, loss of Eph results in a non-phosphorylated Exn and thus putatively results in reduced adhesion of similar neurites. This reduction might then result in the overshooting of these neurites and lead to formation of synapses with ORN axons in a more lateral but not medial glomerulus. In contrast to this scenario, loss of Exn results in

reduced repulsion. Dendrites reach the correct glomerulus, but fail to keep within the glomerulus borders and instead innervate neighboring glomeruli as observed for instance for the DM3 glomerulus.

In support of the above mentioned scenario, genetic interactions with the three main RhoGTPases proposed to be downstream of Exn were analyzed. It was found that Rac1 enhances the Eph lateral targeting defect, while Cdc42 enhances the Exn-like ventral targeting defect. On the other hand, Rho1 showed no defects in the targeting process in genetic interaction experiments. Only when Rho1 was over-expressed in PNs in a less Exn background, a very strong split phenotype was observed. Also lowering Exn levels rescued the lethality caused by over-expression of Rho1 in PNs. This shows that Rho1 might be an effector downstream of Exn but plays a minor role. Nevertheless, these results suggested that Rac1 is required to prevent the lateral mistargeting of Eph-dependent PN dendrites, while Cdc42 helps to keep dendrites from innervating neighboring ventral or dorsal glomeruli. They further indicated that fly Exn is interacting with Rho GTPases differently as compared to vertebrates. Nevertheless, how exactly Eph signaling activates Exn-dependent (Cdc42 and Rac1 mediated) and –independent (only Rac1 mediated) pathways remains unclear at this point. Given that the same PN dendrites are affected at least in two examples by both of these pathways, differential expression levels of Eph or Exn are less likely explanations. A much more plausible explanation, instead, is the difference in expression or activity of the ligand ephrin in different PN dendrites within the olfactory map. Data showing that overexpression of ephrinΔC in the majority of PNs enhances the ventral targeting defect supports an ephrin level dependent regulation of downstream Eph signaling.

Importantly, the recent study showing that Meigo glycosylates ephrin and thereby modulates its levels offers an attractive mechanism for a differential activation of Eph signaling by the ephrin ligand as it is described here. In fact, Meigo mutants display in part similar phenotypes compared to Eph mutants i.e., along the medial-lateral axis of the AL. This in turn supports the role of Eph/ephrin signaling in PN dendrite targeting and this is mechanistically mediated by the activity of small Rho GTPases which further lead to cytoskeletal dynamics.

Taken together, differential signaling downstream of Eph receptor regulates the activity of different small Rho-GTPases and results in maintaining the targeting of a subset of olfactory neurons along the two axes in the AL. Hence it presents an attractive model for a very specific regulation of neuronal map formation. Why Exn interaction with Rho GTPases is different in flies and vertebrates remains still unclear and could be different for different targeting requirements and systems. One could speculate that unlike vertebrates, flies have only one set of Eph receptor, ephrin ligand and Exn molecule and hence, they show a completely different mechanism downstream of Eph signaling for regulating signaling outcome in different neurons. During evolution this receptor family divided into various types of Eph receptors and hence the downstream signaling also diverged to carry out different biological functions. In vertebrates, genetic studies have indicated that multiple overlapping Eph receptors expression gradients and their combined signaling control axon path finding (Flanagan, 2006; McLaughlin and O'Leary, 2005). In addition, a global phospho-proteomic analysis suggested coincident regulation of EphA receptors in EphB2-activated cells (Jørgensen et al., 2009). Also reports have shown that different classes of Eph receptors cluster together and trans-activate each

other leading to the downstream signaling which regulates biological processes (Janes et al., 2011). Additionally, reports have shown interaction of Eph receptors with other classes of receptors which are important for development (Dudanova et al., 2010; Kramer et al., 2006). Overall these studies have shown that there are cross-talks between different Eph receptors and also between Eph receptor and other class of receptors. In addition to the cross talks between inter-class Eph receptors in case of vertebrates, a differential signaling downstream of a single type of Eph receptor could be potentially regulating different developmental processes. And that is exactly what is observed in this study. Since there are no different types of Eph receptors in flies, differential downstream signaling is the potential way to regulate different biological processes. Although what is explored in this study is only a component of this complex signaling, there could be a differential regulation of downstream signaling at various levels.

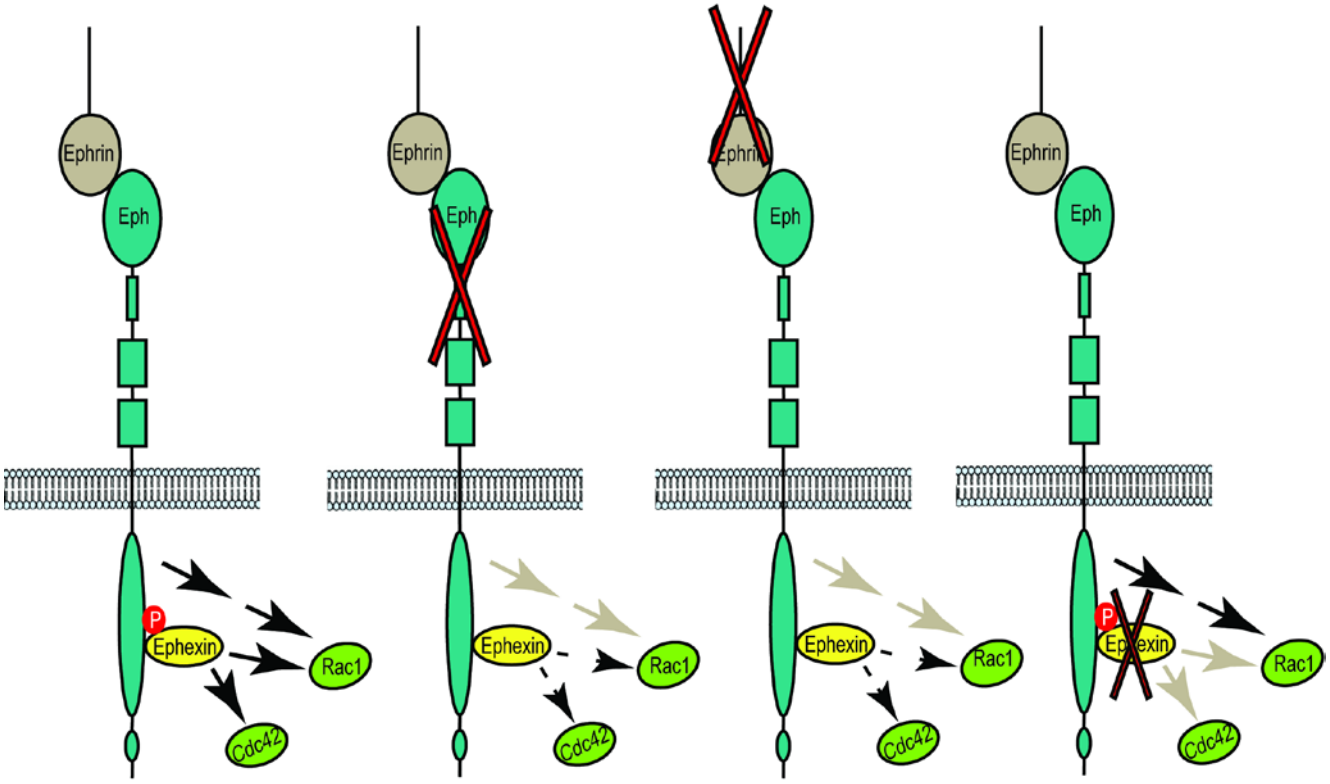


Figure 4.2 1 : Differential modes of Eph signaling in the olfactory dendrite targeting of *Drosophila*.

This model indicates the differential signaling downstream of Eph receptor during dendrite targeting of the PNs. The left panel shows the wild type situation, where Eph signaling is activated upon ephrin ligand binding: downstream to the receptor, Exn-dependent and independent interaction with Cdc42 and Rac1 GTPases are shown. The second panel displays the *eph* mutant condition (shown with a red cross), where Eph signaling is not functional but some signaling via Cdc42 and Rac1 is maintained via the activity of non-phosphorylated Exn. A similar situation is seen in the third panel where ephrin is knocked down via RNAi in PNs. The right panel illustrates the situation in the *exn* mutant. Here, Eph signals via Rac1 exclusively in an Exn-independent manner.

4.3 Role of Exn in higher brain center

PN dendrites innervate the AL while PN axons form synapses with Kenyon cells, the intrinsic cell type of the mushroom body, the brain center required for olfactory learning and memory. To address whether Exn was required for synapse formation also in the PN axon, genetic analysis of PN-KC synapses was carried out. In addition, the role of Exn was analyzed for aversive olfactory learning and memory. These experiments were in part inspired by the already published role of Eph signaling in setting MB Kenyon cells axonal projections (Boyle et al., 2006) and by the finding that Exn is required for synapse maintenance at the NMJ (Frank et al., 2009). In addition, studies in vertebrates show that Eph/ephrin signaling is required for long term potentiation (LTP) in the hippocampus and thus is shown to be required for hippocampus mediated tasks (Grunwald et al., 2001, 2004; Henderson et al., 2001). Hence, it is intriguing to check if Eph signaling is required in flies for learning and memory. In the scope of this thesis, the role of Eph signaling in olfactory based learning and memory was analyzed. Since *eph* mutant flies are not healthy enough to be amplified to do memory experiments with a high number of flies, most of the experiments were conducted with *exn* mutant flies. No significant defect was observed in the *exn* mutants for the paradigm tested for olfactory learning and memory. These data suggest that Exn is not involved in the tested paradigm for learning and memory. But these results do not rule out the possibility of the Exn-independent role of Eph signaling in this process. Hence, to address the role of Eph signaling in olfactory learning and memory formation, Eph mutants should be analyzed. Also, only short term and middle term (2 h) learning and memory experiments

were performed in this project. Hence, other olfactory learning and memory paradigms can help to address the role of Eph signaling in this process.

Furthermore the role of Exn was also analyzed in maintenance and formation of synapses between PNs and KCs. These experiments were inspired by already published reports, both in flies and mouse, showing involvement of Exn in maintaining synapses and synaptic homeostasis (Frank et al., 2009; Margolis et al., 2010; Shi et al., 2010). Taking into consideration these reports, the role of Exn in synapses was analyzed in the olfactory system. To look for synaptic defects in *exn* mutant flies, electron microscopic analysis was carried out. Results from these experiments indicate that *exn* mutants do not show any defects in synapse number as compared to wild type controls, but potential differences in the density of the activity centers (microglomeruli, (Leiss et al., 2009) in the calyx of *exn* mutants were found. The increase in the number of dense microglomeruli in *exn* mutant flies can be due to a defect in synaptic release. As *exn* mutant flies are already shown to have a synaptic defect in fly NMJ, this is one of the possible explanations for the presence of dense microglomeruli in fly mushroom body calyx. Although results indicate an increase in dense microglomeruli, to better analyze this result, a 3D EM analysis for MB calyx is required to show the net difference in the number of the dense microglomeruli in the *exn* mutants vs. controls. While no conclusion can be drawn from the present results at this point, it can be suggested that Exn might be involved in vesicle recycling in central synapses similarly to its role at the NMJ. In *exn* mutants this release deficit leads to dense microglomeruli which are packed with these synaptic vesicles. Thus, the numbers of dense microglomeruli are more in these mutants. To further prove this hypothesis, one needs to perform

electrophysiological experiments that could show if there is a synaptic vesicle release defect in *exn* mutants. It has been shown earlier that synapto-pHluorin (pH sensitive synaptic GFP) can be used to trace the release of synaptic vesicles in the MB calyx (Christiansen et al., 2011) by functional imaging. This experiment can give a better insight for Exn being involved in synaptic vesicle release. It could also, in principle, show if the dense microglomeruli are more or less active as compared to normal microglomeruli. Hence more characterization of this phenotype is required to prove the hypothesis that Exn is involved in synaptic vesicle release at the activity zones in the calyx.

4.4 Concluding remarks

In summary, the work in this thesis suggests a model in which the relative activity of Rho GTPases Rac1 and Cdc42 and their effects on the cytoskeleton downstream of Eph and Exn defines the exact position of specific PN dendrites and subsequently of the respective glomerulus. Furthermore, the relative balance of Exn-dependent and –independent Eph signaling within the same type of PN restricts its dendrites to a particular glomerulus along the two main axes of the olfactory map. Thus, this study proposes a molecular mechanism explaining how a single receptor can exactly position neurites within the dimensions of a sensory neural map. Since Eph/ephrin signaling is shown to affect only a specific subset of ORNs, this study supports the hypothesis of neuron specific molecular code for correct targeting of class specific neurons.

This work can be further strengthened by investigating a detailed role of small Rho-GTPases by doing various biochemical analyses to elaborate on the specific role of GTPases in context of Eph, ephrin, and Exn. Also, due to technical limitations most of the study was conducted in ORNs which were taken as the readout of PNs phenotype. Hence, more experiments with PNs as a direct readout would potentially give better insights of the proposed mechanism.

Future investigations of other neural maps will elucidate whether the proposed mechanism is of general importance and also employed in other model systems. Results included in this thesis might help to disentangle the details of the differential signaling mechanisms operating downstream of various signaling processes for neural circuit formation.

5. Materials and Methods

Following section will give an elaborate overview of the chemicals, antibodies, fly strains etc., used in various methods for the completion of the project on which this thesis is based.

5.1 Materials

5.1.1 Common buffers and solutions

Phosphate buffered saline (1xPBS): 137 mM Na_2HPO_4, pH 7.4, 1.5 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl.

Phosphate buffered saline + 0.5% Triton-X 100 (1xPBT): 0.5% (v/v) Triton-X 100 in PBS.

Phosphate buffer saline + 0.1% Tween®20 (1xPBST): 0.1% (v/v) Tween ®20 in PBS.

Phosphate buffered lysine (PBL) (200 ml): In 3.6 g Lysine add 0.1 M NA_2HPO_4 (until pH reaches 7.4) then add 0.1 M NaH_2PO_4 until volume reaches 200 ml. Filter sterilize afterwards and store at 4 ^oC for not more than 3 months.

Periodate lysine paraformaldehyde (PLP): 10 ml of 16% (v/v) paraformaldehyde (PFA) in 40 ml of PBL (final concentration of 4% PFA).

Blocking Solution: 10% (v/v) Donkey Serum in 1xPBT, 10% (v/v) Goat Serum in 1xPBT (both for immunohistochemistry), 0.2% Roche blocking reagent (for in-Situ hybridization) in MABT.

Solution A: 0.1 M Tris HCl (pH9.0), 0.1 M EDTA (pH 8.0) and 1% SDS in water.

TAE (50X) (2000 l): 484 g Tris base, 50 mM EDTA (pH 8.0), and 114.20 ml glacial acetic acid (pH 8.5).

Fly water: 8 ml Propionic acid in tap water.

Luria Bertani Medium (LB medium) (1000 ml): 10 g NaCl, 10 g Bacto - tryptone, 5 g yeast extract, 20 g agar (pH 7.5). 1.5% Agar added for making LB plates. Desired antibiotics were added after autoclaving and cooling down the media.

Fly food (50 l): 585 g of agar dissolved in 30 l of water by heating the mixture until boiling point. To this a homogenous mixture of 5 kg corn flour, 925 g yeast, 500 g soy flour, 4 kg molasses in water, was added. The volume of the mixture was made up to 50 l and heated at 96 ^{o}C for one and half hrs. After cooling the mixture down to 60 ^{o}C, 315 ml propionic acid, 120 g methylparaben, 125 g niparsin/methylparaben, 1 l of 20% ethanol and 500 ml of 10% phosphatidic acid were added.

Blue food and yeast paste (baby food): Instant dry yeast (Femipan Inc.) and Blue Drosophila food (Fischer Scientific) made into a paste with water.

Prehybridization solution: 50% Formamide deio, 0.2% Tween 20, 0.5% Chaps, 5 mM EDTA (pH 8.0), 50 mg/ml Heparin, 50mg/ml t-RNA (SIGMA R-5636; Lot 082K9135) and 5x SSC (pH 4.5).

Solution I (50 mL): 50% Formamide deionized, 5x SSC (pH 4.5), 0.2% Tween 20, and 0.5% Chaps. Always prepared fresh.

Solution II (50 mL): 50% Formamide deio, 2x SSC (pH 4.5), 0.2% Tween 20, and 0.1% Chaps. Always prepared fresh.

Solution III (50 mL): 2x SSC (pH 4.5), 0.2% Tween 20, and 0.1% Chaps. Always prepared fresh.

5x Maleic acid buffer (MAB) (1 L): Mix 58 g of Maleic acid 44 g NaCl. Adjust pH to 7.5, using 25-30 g NaOH pellets and then 5 N NaOH. Stored at 4°C.

MAB-Tween (MABT): 1x MAB, 0.1% Tween®20. Stored at RT.

Blocking solution (w/v): 1x MABT and 0.2% Blocking Reagent (#1096 176, Roche) Blocking reagent dissolved while rocking at 70° and kept on ice. Always prepared fresh.

NTMT (200 ml): 5 M NaCl (4 ml), 1M Tris-HCl (pH 9.5) (20 ml), 1M Mg Cl2 (10 ml), Tween®20 (200µl).

Developing solution for *In-situ* Hybridization (10 ml): BCIP (11 µl), NBT (14 µl) and NTMT (10 ml).

Fixative for TEM sample: 2.5% Glutaraldehyde (Electron Microscopy Sciences), 2.5% Paraformaldehyde (0.1M), Sodium Cacodylate 0.2M.

Osmication solution: 1% OSO4 (2% Osmium Tetraoxide) in 0.1M Sodium Cacodylate (Sodium Cacodylate 0.2M) pH 7.4.

5.1.2 Antibiotics

Table 5.1.2 1 : List of Antibiotics and their concentrations.

Name	Concentration
Ampicillin	100 µg/ml
Kanamycin	50 µg/ml
Chloramphenicol	25 µg/ml
Hygromycin	20 µg/ml

5.1.3 Antibodies

Table 5.1.3 1 : List of primary antibodies for immunohistochemistry.

Name	**Dilution**	**Source**
anti-GFP (rabbit)	1:1000	Clontech (USA)
anti-GFP (chicken)	1:200	Abcam
anti-DsRed (rabbit)	1:200	Clontech (USA)
anti- discs large (mouse)	1:50	Developmental Studies Hybridoma Bank (DSHB)(USA)

Table 5.1.3 1.: Continued

Name	Dilution	Source
anti-bruchpilot (nC82) (mouse)	1:50	DSHB (USA)
anti- elav (rat)	1:50	DSHB (USA)
anti-prospero (mouse)	1:20	DSHB (USA)
anti-DIG-AP	1:1000	Roche
anti-repo (mouse)	1:50	DSHB (USA)

Table 5.1.3 2 : List of secondary antibodies for immunohistochemistry.

Name	Dilution	Source
anti- mouse (cy3)	1:200	Dianova (Germany)
anti- mouse (cy5)	1:200	Dianova (Germany)
anti- rabbit (Alexa Flour 488)	1:200	Dianova (Germany)

Table: 5.1.3.2: Continued

Name	Dilution	Source
anti- chicken (Alexa Flour 488)	1:200	Dianova (Germany)
anti- rat (cy3)	1:200	Dianova (Germany)

5.1.4 Commercial kits

The following kits were used from the specified manufacturer. The kits were used according to the manufacturer instructions.

- QIAprep Spin Miniprep Kit
- QIAGEN Plasmid Maxi Kit
- QIAquick Gel Extraction Kit
- QIAquick PCR Purification Kit
- Roche DIG RNA labeling Kit (SP6/T7)

5.1.5 Enzymes and DNA standards

Following enzymes and DNA standards were used for all molecular biology experiments. All the DNA polymerases were used in standard quantity for the polymerase chain reaction (PCR). Also, the restriction enzymes were used as indicated by the manufacturer.

Table 5.1.5 1 : List of enzymes for molecular biology experiments.

Name	Source
Taq DNA polymerase	New England Biolabs(NEB) (USA)
PFU DNA polymerase	Promega
Phusion DNA polymerase	New England Biolabs(NEB) (USA)
SP6 reverse transcriptase	Roche
T7 reverse transcriptase	Roche
Restriction Enzymes	New England Biolabs (USA)
1kb DNA ladder	New England Biolabs (USA)
100bp DNA ladder	New England Biolabs (USA)

5.1.6 Chemicals

All chemicals were used of analytical grade.

Table 5.1.6 1 : List of Chemicals.

Name	Source
Agarose, high electro endosmosis	Biomol
Arabinose	Sigma Aldrich
Gel Loading Dye blue (6X)	New England Biolabs
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Ethanol Absolute	Sigma Aldrich
EDTA	Sigma Aldrich
EMbed-812 (Epon Kit)	Science services
Glycerol	Merck
Hydrochloric Acid (HCl)	Merck
Isopropanol (2-Propanol)	Sigma Aldrich
Methanol	Sigma Aldrich
4-Mthylcyclohexanol	Fluka
3-Octanol	Fluka
Osmium sulphate	Science services
Propylene oxide	Serva

Table 5.1.6.1: Continued

Name	Source
Tris Base	Sigma Aldrich
Tween 20	Sigma Aldrich
Ultrostain 1 (0.5%Uranyl acetate)	Leica
Ultrostain 2 (3% Lead acetate)	Leica
dNPTs	New England Biolabs
Mounting media (vectashield Fluorescence H-1000)	Vector Laboratories Inc.
Type F Immersion Liquid	Leica Microsystems CMS GmbH

5.1.7 Plasmids

For all molecular analysis following plasmids were used. The source and donors of these plasmids are mentioned below.

Table 5.1.7 1 : List of ESTs and Fosmids.

Plasmid	Source
RE61046 (Eph cDNA_clone)	Drosophila Genomics Resource Center
LD11109 (Ephrin cDNA_clone)	Drosophila Genomics Resource Center
GH03693 (Ephexin cDNA_clone)	Drosophila Genomics Resource Center
Eph fosmid (FlyFos015198)	Paval Tomancak
Ephexin fosmid (FlyFos025216)	Paval Tomancak

5.1.8 Primers and Oligonucleotides

Various oligonucleotides were used in this study to screen for recombinants as well as for analyzing genotypes.

eph EST TOPO cloning primer

Forward: AATGACGTTATAAAAGTTATCGATACC

Reverse: ATCTACATCGGAGTTCTGATTGAAG

ephrin EST TOPO cloning primer

Forward: TTTTTTGTTCCAGCTCTAAACAAG

Reverse: CAATCCGAAATATACTGTTCGATG

exn^{EY01953} mutation screening primer

Forward: CCTCCATGCGAGTGACAGTA

Reverse: CAAGCAAACGTGCACTGAAT

exn$^{EY-\Delta 23}$ mutation screening primer

Forward: CTGAAACAGAACTAACTGCTGTCCATTC

Reverse: CAGCGTCATCAAGATGAGGTTCTTG

exn fosmid tag primer

Forward: TCGGCGTTGAATCGCAGCAATGGCCAGCGCCTCAGCTGCTCCGGCAGT

CTGAAGTGCATACCAATCAGGAC

Reverse: CGACCTCGCCACGAGCCCGAGGTCGCCGTTTCCGGGGCAAGGTCACC

GGACTCCATCGTGGTCTTTATAATC

UAS sequence specific primer

Forward: TGTTTAGCTTGTTCAGCTGCGCTTG

Reverse: AGTACTGTCCTCCGAGCGGAGACTC

YFP sequence primers

Forward: GCACGACTTCTTCAAGTCCGCCATGCC

Reverse: TACATAACCTTCGGGCATGG

5.1.9 Fly stocks

For all the genetic analysis, required for the commencement of the project, various fly stocks were used. All these fly stocks were reared on standard fly food at 25 ^{o}C with 70% relative humidity, unless mentioned for specific experiments. The genetic makeup of these stocks is listed below. All the stocks mentioned below are the original stocks which were then crossed / recombined to produce a final fly used for analysis of the specific experiment.

Table 5.1.9 1 : Fly stocks used for various experiments.

Stock	Source	Experiments
Or-Gal4	Bloomington stock center (BSC)	Visualization of ORNs
Or syt GFP	BSC	Visualization of ORNs.
SG18.1-Gal4	BSC	Overexpression (O/E), rescue and RNAi analysis.
GH146-Gal4	BSC	O/E, rescue and RNAi analysis
NP1613-Gal4	Kyoto stock center	Expression analysis
Gh146-QF	BSC	Expression analysis
UAS-mdc8 GFP	BSC	Expression analysis
QUAS-mtdtomato	Maria Spletter	Expression analysis

Table 5.1.9.1: Continuation

Stock	Source	Experiments
Eph Fosmid tag	Frank Schnorrer	Expression analysis
UAS-eph$^{\Delta C}$	Richard Dearborn	O/E
UAS-eph^{WT}	Richard Dearborn	Rescue and O/E Analysis
UAS-ephrin$^{\Delta C}$	Andrea Brand	O/E Analysis
UAS-YFP-exn^{FL2}	Graeme Davis	Rescue and O/E analysis
UAS-YFP-exn^{FL10}	Graeme Davis	Rescue and O/E analysis
UAS-cdc42^{WT}	BSC	O/E Analysis
UAS-cdc42^{DN}	BSC	O/E Analysis
UAS-cdc42^{DA}	BSC	O/E Analysis
UAS-rac1^{WT}	BSC	O/E Analysis
UAS-rac1^{DN}	BSC	O/E Analysis
UAS-rac1^{DA}	BSC	O/E Analysis
UAS-rho1^{WT}	BSC	O/E Analysis
UAS-rho1^{DN}	BSC	O/E Analysis
UAS-rho1^{DA}	BSC	O/Analysis
FRT 80B	BSC	MARCM analysis
FRT 80, tub Gal80, GmRKO Cl	Takashi Suzuki	MARCM analysis

Table 5.1.9.1: Continuation

Stock	Source	Experiments
FRT 80 Cl	**T**akashi Suzuki	MARCM analysis
FRT 79	BSC	MARCM analysis
FRT 79, tub Gal80, Cl	BSC	MARCM analysis
heat shock flp	BSC	flap-out experiment
eyeless flp	BSC	MARCM analysis
$exn^{EY01953}$	BSC	Mutant analysis
$exn^{EY-\Delta 23}$	Graeme Davis	Mutant analysis
eph^{x652}	John B Thomas	Mutant analysis
$ephrin^{KG09118}$	BSC	Mutant analysis
$cdc42^{2}$	BSC	Mutant analysis
$cdc42^{4}$	BSC	Genetic interaction
$rac1^{J11}$	BSC	Genetic interaction
$rho1^{720}$	BSC	Genetic interaction
ephrin RNAi (P{TRiP.JF02365}attP2)	BSC	Knockdown analysis
Eph RNAi (P{TRiP.JF03131}attP2)	BSC	Knockdown analysis
(P{TRiP.HMS00246}attP2)	BSC	RNAi knockdown control

Table 5.1.9 2 : List of final genotypes of the flies analyzed for various experiments.

Genotype	Experiment
OrXX-Gal4,UAS-sytGFP; YY	Mutant analysis
OrXX-Gal4,UAS-sytGFP; Y/+	Heterozygous control
OrXX-sytGFP; YY	Mutant analysis
OrXX-sytGFP; Y/+	Heterozygous control
eph fosmid tag	Expression analysis
GH146-Gal4 / UAS-CD2; eph fosmid tag	Co-localization experiment
exn-Gal4 / UAS-mCD8 GFP	Expression analysis
UAS-mCD8 GFP, QUAS-mtd Tomato; GH146-QF; exn-Gal4	Co-localization experiment
UAS-eph^{wt}; GH146-Gal4, Or47a-sytGFP; eph^{*652}	Rescue experiment
GH146-Gal4,OrXX-GFP / UAS-YFP exn;exn$^{EY-\Delta 23}$	Rescue experiment
GH146-Gal4, ZZ; UAS-eph$^{\Delta C}$	O/E analysis
GH146-Gal4, ZZ; UAS-eph$^{\Delta C}$ / exn$^{EY-\Delta 23}$	O/E and genetic interaction
GH146-Gal 4, ZZ; UAS-eph$^{\Delta C}$, exn$^{EY-\Delta 23}$/ exn$^{EY-\Delta 23}$	O/E and genetic interaction
GH146-Gal4, ZZ; UAS-ephrin$^{\Delta C}$	O/E analysis
GH146-Gal4, ZZ; UAS-ephrin$^{\Delta C}$ / exn$^{EY-\Delta 23}$	O/E and genetic interaction
UAS-eph^{wt}; GH146-Gal4, ZZ	O/E analysis
UAS eph^{wt}; GH146-Gal4, ZZ; exn$^{EY-\Delta 23}$/+	O/E and genetic analysis

Table 5.1.9 2: Continued **Genotype**	**Experiment**
GH146-Gal4, ZZ; UAS-RNAi	RNAi analysis
ZZ, exn-Gal4 / UAS-RNAi	RNAi analysis
GH146-Gal4, ZZ	Control for RNAi
cdc42^{2} / +; Or47a-sytGFP	Control for genetic interaction
cdc42^{2} / +; Or47a-sytGFP; YY / +	Genetic interaction
cdc42^{4} / +; Or47a-sytGFP	Control for genetic interaction
cdc42^{4} / +; Or47a-sytGFP; YY / +	Genetic interaction
Or47a-sytGFP / rho1^{720}	Control for genetic interaction
Or47a-sytGFP / rho 1^{720}; YY / +	Genetic interaction
Or47a-sytGFP; rac1^{J11} / +	Control for genetic interaction
Or47a-sytGFP; rac 1^{J11} / YY	Genetic interaction
Or47a-sytGFP; exn $^{EY-Δ23}$ / +; eph^{x652} / +	Genetic interaction
Or47a-sytGFP; exn$^{EY-Δ23}$ / exn$^{EY-Δ23}$; eph^{x652} / +	Genetic interaction
Or47a-sytGFP; exn$^{EY-Δ23}$ / +; eph^{x652} / eph^{x652}	Genetic interaction
Or47a-sytGFP; exn$^{EY-Δ23}$ / exn$^{EY-Δ23}$; eph^{x652}/eph^{x652}	Genetic interaction
eyflp;OrXX-Gal4,UAS-sytGFP; FRT80B exn^{EY01953} / FRT80B tub-gal80, GMR-kushabire orange, CL	MARCM for ORNS
eyflp;OrXX-Gal4,UAS-sytGFP; FRT80B / FRT80B tub-gal80, GMR-kushabire orange, CL	Control for MARCM for ORNS
hsflp;GH146-Gal4/ UAS>CD2>CD8GFP; exn$^{EY-Δ23}$	Flp-out for labeling PNs in mutant
hsflp;GH146-Gal4/UAS>CD2>CD8GFP;exn$^{EY-Δ23+/-}$	Flp-out in heterozygous control

XX: one of the following OR markers: 10a, 21a, 22a, 42a, 47a, 47b, 59c, 67d, 71a, 88a and 92a. YY: *eph*^{x652} or *exn*$^{EY\text{-}\Delta 23}$ or *exn*^{EY01953}. ZZ: Or47a syt GFP. 1: WT or DN or DA

5.1.10 Equipment

Following are the various equipment and instruments used for carrying out different experiments for this thesis project.

Table 5.1.10 1 : List of equipment.

Equipment	Manufacturer
Confocal microscope Olympus FV-1000	Olympus
Light microscope stemi 2000	Zeiss
Ultramicrotome EM UC6	Leica
Fluorescence microscope M205 FA	Leica
Forceps (55)	Inox
Glass Slides and coverslips	Thermo Scientific
Thermo cycler (DNA engine tetrad)	MJ Research
Fly incubator	Percival
Bacterial incubator	Heraeus
Culture shaker incubator	Unitron
Nano-drop 1000	peQlab Biotechnology

5.2 Methods

5.2.1 *Drosophila* tissue dissection and Immunohistochemistry

Drosophila adult brain dissections were conducted in order to analyze the wiring in the brain. Also the developing brain and eye-antennal discs were dissected for expression analysis of various genes studied in this project. For analysis of various developing stages 0 h Pupae were collected and then aged to desired age.

Before adult brain dissection the fly was cleaned in 70% ethanol for maximum of 1min and then transferred to PBS on ice. For pupae dissection, these rinsing steps were skipped. Each fly was then dissected on the dissection pad in cold PBS and after dissection the brain was kept in 4% PBL on ice. All the flies of the same genotype were dissected and collected in the same tube. Afterwards this tube with the dissected brains was kept on a shaker at room temperature for 1 h maximum and then washed thrice with 0.5% PBT for 5 min each. Next, the brains were incubated with a desired concentration of the primary antibody for 5-6 h at room temperature or overnight at 4 ^{o}C on the shaker. Before putting the brain into secondary antibody, brains were rinsed thrice in 0.5% PBT for 5 min each. Finally, brains were again rinsed with 0.5% PBT twice for 15 min each followed once by PBS for 15 min and then mounted in mounting media for microscopic analysis.

For analyzing the wiring of the adult brain, all images were scanned with 40X objective. Different scanning objective lenses and zoom was used for brains analyzed for expression.

5.2.2 *In-situ* hybridization

RNA *in-situ* hybridization was carried out for analyzing the expression pattern of various genes studied in this project. All the samples (brains of various development stages) were dissected, fixed, washed and hybridized in RNAse free solutions. Following is the detailed protocol.

Day 1: Adult flies/ Pupae brain and eye-antennal discs were dissected in cold PBS and fixed in 4% PBL overnight at 4 ^{o}C.

Day 2: Samples were washed thrice in PBST for 5 min each. Subsequently all the samples were dehydrated by washing them in sequentially higher concentration of methanol. Each wash was for minimum of 5 min. Finally, the samples were stored in 100% methanol at -20 ^{o}C for a minimum of 2 h.

Day 3: Samples were taken out of -20 ^{o}C and then incubated at room temperature for 1 h in a solution made of 80% Methanol and 20% of 30% H_2O_2. Next, samples were rehydrated by subsequently lowering the concentrations of methanol to 20%. Samples were rinsed thrice in PBST for 5 min each followed by 1 h incubation in pre-hybridization solution on shaker at 55 ^{o}C. Dig labeled RNA probes were diluted in hybridization solution and samples were incubated with it for overnight at 55 ^{o}C.

Day 4: Roche blocking reagent was dissolved in MABT for several hours at 55 ^{o}C. Samples were rinsed thrice for 1 h each in solution I, II and III at 55 ^{o}C. Samples were rinsed in MABT thrice for 5 min each at room temperature and twice for 30 min each at 55 ^{o}C. Next, samples were blocked for 1.5 h at room temperature in blocking solution.

For detecting the dig labeled RNA probes, samples were incubated overnight with anti-DIG antibody conjugated with alkaline phosphatase at 4 ^{o}C.

Day 5: Samples were rinsed and washed with MABT at least 8-10 times for 30 minutes at room temperature before incubation in NTMT. Following equilibration in NTMT for 10-20 minutes developing solution was added. After the staining was developed, samples were rinsed in PBST and post fixed in 4% PBL. Images were taken at the Leica M205FA.

5.2.3 Fosmid tagging

Fosmids are huge genomic clones that can be modified by high throughput recombineering and targeted trans-genesis (Ejsmont et al., 2009). These tagged fosmids can be generally used for expression analysis by tagging desired fosmid with a reporter gene (for e.g. GFP). Specifically it could also be used for rescue and genetic analysis. In this study tagged fosmid is used for the expression analysis of *eph*. Also *ephexin* fosmid was tagged using the same protocol. A detailed published protocol was followed as mentioned in the published protocol (Sarov, 2009).

5.2.4 Transmission electron microscopic analysis

Fly brains were dissected and fixed in the mixture of glutraldehyde and paraformaldehyde (fixative for TEM) and kept at 4°C until sectioned. To analyze the region of interest (ROI), the brains were embedded in 4% agarose and cut into 30 µm section using vibratome. Next, sections were incubated in osmication solution for 40 min. Followed by three washes with distilled water, the samples were serially dehydrated. This included 10 min washes with 50%, 70% and 100% ethanol. This was followed by 100% propylene oxide wash for 10 min and then 24 h incubation. Finally sections were incubated with epon for 2 h and then were embedded in fresh epon. It was allowed to polymerize for 48 h at 60°C. Then the samples were cut in 1 µm thin sections using Ultramicrotome. ROI was localized for these sections and then ultrathin sections of 50 nm were cut. Sections (from now on called as grids as they are embedded) were finally counterstained with Ultrostainer 1 and 2.

After the grid preparations, images were acquired with JEOL JEM-1230 transmission electron microscope. 2.5 K magnification was used for overview image of the calyx (ROI) and 25 K magnification was used for getting images for counting synapses and microglomeruli.

For each genotype (w^{1118} and $exn^{EY01953}$) three female flies were dissected for sample preparation. For analyzing the images, a blind manual quantification was conducted for number of synapses and number of normal vs. dense microglomeruli. Statistical analysis was done by calculating the mean of number of synapses or the number of dense microglomeruli for both control and mutant respectively (standard error of mean was calculated).

5.2.5 Analysis of aversive olfactory learning and memory formation

These experiments were conducted in the same way as described before by Knapek et al., 2011 (Knapek et al., 2011). Before conducting the memory experiments, all flies were collected and transferred to fresh food vials at least 24 h before the experiment. Four different genotypes were analyzed for this experiment: *yw*, w^{1118}, CantonS (CS) and $exn^{EY-01953}$ Experiments were performed at room temperature (25°C) with a relative humidity of 75%. Mixed populations of males and females of 1-8 days of age were used to conduct the experiment. Standard aversive olfactory conditioning with two odorants was performed in a T-maze. For all memory experiments 4-methylcyclohexanol (Fluka) diluted 1:80 and 3-octanol (Fluka) diluted 1:100 were presented in odor cups with a diameter of 14 mm for 1 min. In order to associate punishment with one of these ordors, twelve pulses of electric shock (90V direct current for 1.2s each) were applied with an inter-pulse interval of 5 s. For short term memory retention experiments, flies were immediately tested after the training. For middle-term (2 h) memory retention, flies were kept in empty vials in darkness between training and test for 2 h. In order to avoid any learning-independent preference to any one of the odors, reciprocal experiments were conducted. In one experiment, odor 1 was electrified whereas in another set of experiment odor 2 was electrified. A learning index (LI) was then calculated as the mean preference of these two reciprocally trained groups. From each experiment, the number of flies choosing the punished (electrified) odor (N_+) and the non-punished (non-electrified) odor (N_-) were counted, and preference index (PI (avoidance) was calculated as the difference of N_- and N_+ divided by the total number of flies. A positive

value indicates avoidance from the shock, and PI of zero indicates no response. Flies were given 2 min to choose between the punished and non-punished odors.

The LI was calculated as a mean of the PIs for the reciprocally trained flies and a standard error of mean was calculated.

5.2.6 Genomic DNA isolation

Fly genomic DNA was isolated for various recombination experiments. In this protocol few flies were collected in an eppendorf 1 ml tube and kept on ice. Solution A was added to theses flies – 100 µl for 1-5 flies per eppendorf 1 ml tube, 200 µl for 6-10 flies and 400 µl for more than 10 flies. This volume of solution is referred to as one volume. These flies were then crushed and homogenized in solution A and then kept at 70 ^{o}C for 30 min. After that 14 µl of 0.8M potassium acetate (KAc) was added per 100 µl of solution A. This was followed by an incubation of 30 min on ice. The tube was then centrifuged and the supernatant was transferred to a new tube. It was then mixed with one volume of 1:1 mixture of phenol and chloroform. This mixture was again centrifuged and the upper aqueous phase was carefully transferred to a new tube, half volume of isopropanol was added to this mixture and again centrifuged. This time the pellet was retained and washed with 70% ethanol solution. After the ethanol wash the pellet was dried and finally dissolved in 100 µl of distilled water.

The isolated DNA was then subjected to PCR to check the genetic composition and confirm recombination event in the fly.

5.2.7 Gal4/UAS system

The Gal4/UAS system is a yeast derived repressible binary expression (Brand and Perrimon, 1993) used for studying the gene expression in some model organisms like Drosophila in a spatial and temporal manner. This system consists of two components. Frist, transcription activation protein - Gal4 and second one is an enhancer sequence - Upstream activator sequence (UAS). The Gal4 protein specifically binds to the UAS sequence and leads to the transcription of the gene downstream of the UAS element (shown in Figure 5.1). This system has been well exploited in Drosophila. There are lots of Gal4 lines which express Gal4 under a promoter element expressed in a subset and cells. These lines are specifically called the driver lines. Similarly, there are lots of UAS lines (better known as reporter lines) which have the UAS element upstream of the transgene, for e.g. green fluorescent protein, channel rhodopsin, shibire etc. These Gal4 lines, in general, are used mostly for reporter gene expression but specially, can be used for expressing a gene of interest for a particular study.

In this study Gal4/UAS system is used for various experiments. First, for visualizing the Olfactory Receptor Neurons (ORNS), using either syt GFP (synapse associated) or mcd8 GFP (membrane bound) and looking at targeting defects. Second, for expression analysis of *exn,* by using a specific Gal4 line inserted in *exn* locus (NP1613) and UAS mcd8GFP. Third, for the overexpression analysis of all the signaling molecules studied, either in the WT or $exn^{+/-}$ genetic background respectively. Fourth, for expressing RNAi (explained in the following section) against specific genes in a subset of PNs and ORNs. Last, for the rescue experiments by re-expressing the respective gene in its mutant background.

An extension to this genetic system is a protein called Gal80. This protein binds to UAS element and leads to inhibition of binding of Gal4. This whole Gal4/UAS – Gal80 system is exploited to develop a very useful genetic technique called MARCM (see below). Also, other parallel genetic system used in Drosophila is Q system derived from *Neurospora crassa* (Potter et al., 2010). In this system QF is the transcription activation protein, QUAS is the upstream activating sequence and QS is the transcription repressor. This system was used for the co-localization study of Ephexin and GH146 positive Projection Neurons (PNs).

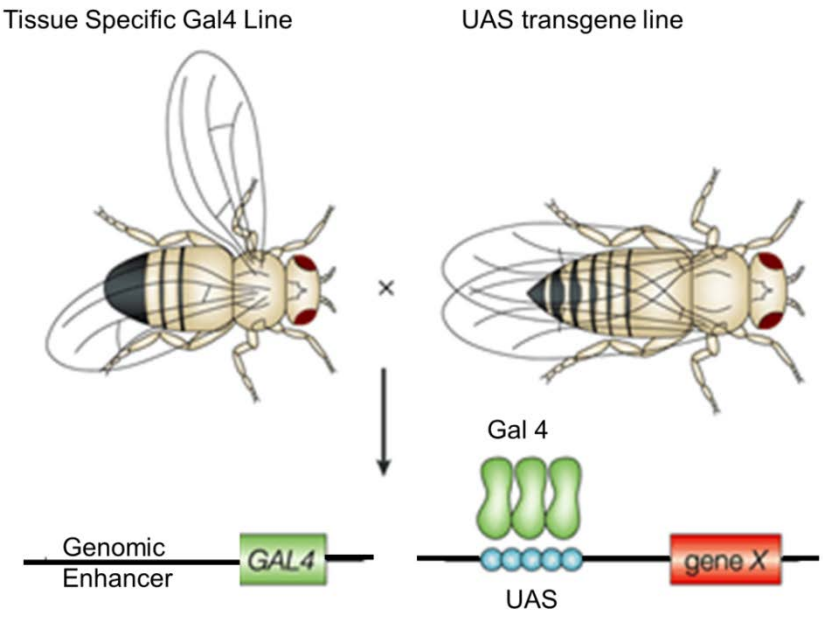


Figure 5 1 : Gal4/UAS system.

Schematic explaining the fly genetics behind the Gal4/UAS system. A fly with Gal4 insertion under a tissue specific promoter is crossed with a fly having a UAS element upstream of a transgene. The F1 progeny resulting from the cross expresses the transgene in the specific tissue which expresses Gal4. (Figure is modified from (St Johnston, 2002).

5.2.8 FLP/FRT system

Flippase is a yeast derived enzyme that induces a recombination event between two flippase recognition target (FRT) sites (Golic and Lindquist, 1989). This recombination tool has been used to develop various genetic techniques.

First is for labeling cells by inducing cis-recombination between two FRT sites on the same chromosome (shown in Figure 5.2 A). In this technique a gene or a stop codon sequence is placed between two FRT sites which are called- the FRT cassette. This cassette is then placed in between the ubiquitous promoter and a reporter gene. This reporter gene is only expressed when a flipping event is induced. This flipping can be induced by expressing flippase under a heat shock promoter. This process is called as flp-out event as the gene or stop codon in between the FRT sites is flipped out. Another approach is to put FRT sites between the UAS and reporter gene. This system is called the *UAS-flp* out system. This system could be combined with any of the Gal4 lines which are expressed in the desired tissue. Depending on whether the recombination occurs in the neuroblast or a post-mitotic cell, the UAS flp out system leads to a single or a subset of cells labeled from the Gal4 line. This system was used in this study to label specific GH146 positive neurons in the *ephexin* mutant background and hence their targeting was analyzed.

Second variation of this FLP/FRT system is trans-recombination system. In this system a recombination event occurs mitotically. This system is known as Mosaic analysis with repressible cell marker (MARCM) (Lee and Luo, 2001). This system works with Gal80 (mentioned in the above section). Gal80 suppresses the expression of reporter downstream to the UAS and hence Gal4 can't activate UAS sequence. A flipping event

removes Gal80 in mitotic cells and hence the reporter is expressed (shown in figure 5.2 B). In an extension to this method, a mutation is present on the FRT chromosome which is heterozygous over a FRT Gal80 chromosome. Hence Gal4 positive cells are not labeled in this heterozygous situation. When a flipping event is induced in the mitotic cell, then the mutation becomes homozygous in half of the daughter cell population produced. This system helps to study the requirement of a particular gene cell autonomously. One could also study its requirement cell non-autonomously by doing Reverse MARCM: mutation is present on the FRT Gal80 chromosome in this case. For this study MARCM analysis was done using eyeless flp to study the requirement of *ephexin* in the ORNs.

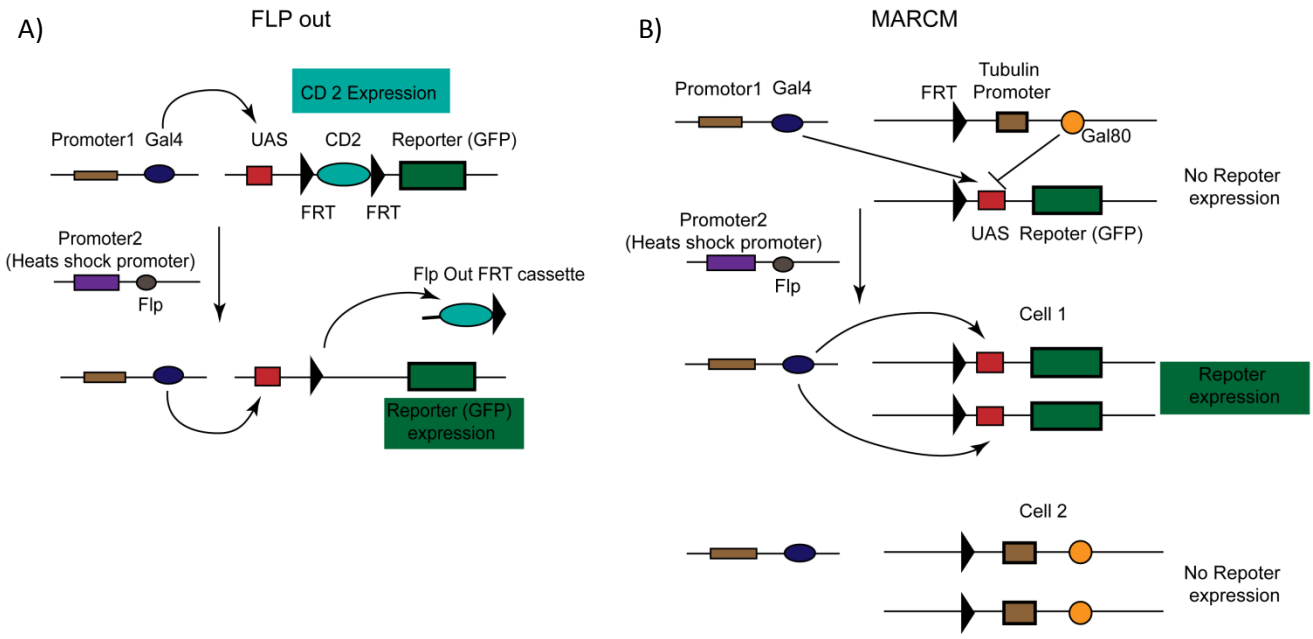


Figure 5.2 : FLP/FRT system.

A) UAS Flp-out system, showing the reporter gene expression in a flipped out cell. Flipping is carried out by the flippase enzyme expressed under a promoter. Flipping event removes CD2 and UAS drives the expression of reporter gene. B) MARCM technique, showing flipping event leading to recombination between two FRT sites and hence making one daughter cell homozygous for the mutation/ reporter.

5.2.9 RNAi Knockdown analysis

RNAi transgenic library is available for many of the *Drosophila melanogaster* genes. The Trip *UAS-RNAi* lines from Bloomington stock center were used in this study for analyzing the knockdown of the *eph* and *ephrin*. These RNAi lines are called TRiP (Transgenic RNAi Project). To confirm the phenotype a control line was used from the same stock center. This control line is the fly having the genetic background that was used to make the *UAS-RNAi* line. The detailed protocol of RNAi analysis is shown in Figure 5.3.

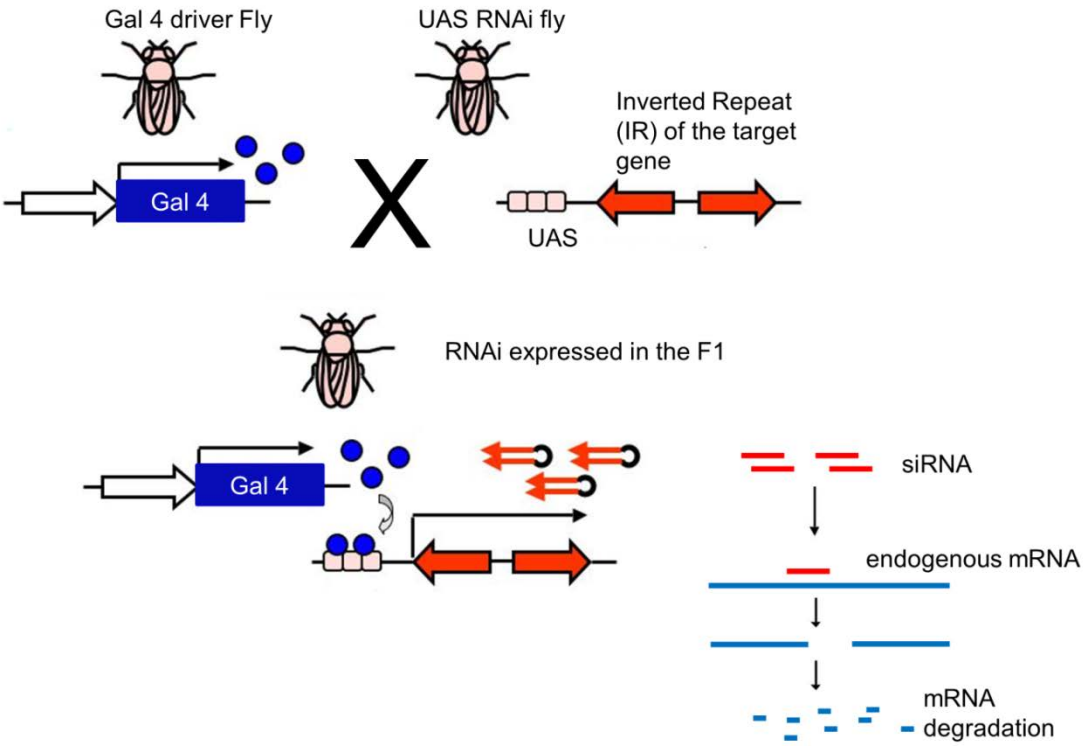


Figure 5.3 : RNAi mechanism in *Drosophila melanogaster.*

A fly having a Gal4 insertion under the driver of interest is crossed to a *UAS-RNAi* fly. The F1 progeny expresses the RNAi in the tissue where the driver is expressed, leading to the tissue specific knockdown of the gene of interest. Figure adapted from GlycoScience Protocol online Database(http://creativecommons.org/licenses/by-nc-sa/3.0/us/)

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7. Curriculum Vitae

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Educational Qualifications:

Feb. 2009 to present	PhD in Biology, Sensory Neurogenetics Lab, Max Planck Institute of Neurobiology, Germany.
Oct. 2008 – Dec. 2008	Research Training, Sensory Neurogenetics lab Max Planck Institute of Neurobiology, Germany.
2006-2008	Masters in Genetics, University of Delhi South Campus (UDSC), India.
2003-2006	Bachelors in Microbiology, Ram Lal Anand College University of Delhi, India

Publications and Scientific communications:

- **Sardana J**, Organisti C, Loschek LF, Grunwald-Kadow IC.“ Ephexin-dependent and -independent signaling downstream of Eph receptor mediates olfactory dendrite targeting in *Drosophila”.* Under preparation for submission to Journal of Neuroscience.
- CSHL Asia meeting 2013: “Ephexin, downstream of Eph signaling regulates dendritic patterns of specific olfactory projection neurons in Drosophila”. Poster presentation.
- QBI-MCN meeting 2012: "Eph, ephrin and Ephexin instruct dendritic patterns of specific olfactory projection neurons in fly". Poster Presentation.
- SFB Funding subgroup meeting 2012: Oral presentation of the PhD project. The meeting was on topic "Assembly and Regeneration of Circuits".

- CSHL conference 2011: “Rho specific GEF, Ephexin, is required in Drosophila Olfactory neuronal targeting”. Poster presentation.
- EMBO conference 2011: "Ephexin, a Rho specific GEF plays a role in olfactory system development". Poster Presentation.

Research Experience:

- Doctoral Thesis entitled “Differential modes of Eph signaling in olfactory dendrite targeting of *Drosophila*” under the guidance of Dr. Ilona Grunwald-Kadow at the Max Planck Institute of Neurobiology.
- Training in 2007 under the guidance of Dr. Veronica Rodrigues at NCBS, India. Project was based on “Study of interneurons in the olfactory circuitry of *Drosophila melanogaster*”.
- Training in 2005 from “Indian Agricultural Research In0stitute” – IARI (PUSA) under Dr. Livleen Shukla, Department of Microbiology. Project was based on “Farm yard manure as a carrier of various micro-organisms.

Awards and Scholarships:

- Max Planck doctoral scholarship (February 2012-present) for Ph.D. at Max Planck Institute of Neurobiology, Martinsried.
- Boehringer Ingelheim fonds travel grant (October 2008-December 2008) for short term research training at the Max Planck Institute of Neurobiology.
- Junior Research Fellowship in Life Sciences by Council of Scientific and Industrial Research (CSIR), India.
- Eligibility for Lectureship in Life Sciences by National Eligibility Test (NET) conducted by CSIR.
- Monsanto Scholarship holder for the year 2007 – 2008. (Awarded to First two university rank holders).
- Summer research fellowship from May 2007 to July 2007 given jointly by Indian Academy of Sciences, National Academy of Sciences and Indian National Science Academy. (Awarded on the basis of academic profile and research proposal).

- VSRP2007 – DBS (Department of Biological Sciences) Fellowship for summer training in 2007 by TIFR (Tata Institute of Fundamental Research), India.
- South Campus Endowment Scholarship holder for the year 2006-2007. (Awarded for securing third position in MSc Genetics entrance examination and interview conducted at the national level).
