Chapter 3

Mechanism of long term olfactory habituation

3.1 Introduction:

Habituation, the progressive reduction in behavioral responsiveness to repeated similar stimuli involves experience dependent neuronal plasticity which modulates the synaptic strength and neuronal firing. It is the simplest form of learning, which had been studied in different model system starting from *Aplysia* to mammals in different modalities. Habituation in mice can be induced at different timescale based on different time duration of odor representation and also on inter-trial interval (ITI) (McNamara et al., 2008). Shorter duration and short ITI gives rise to short term memory which lasts for ~ 10 mins whereas longer duration and long ITI gives rise to long term memory which lasts for >30 mins. Mice habituated using the short timescale paradigm exhibited no cross-habituation to the most chemically similar odorants. In contrast, mice habituated at the longer timescale can be cross-habituated significantly to odorants differing in chain length by up to three carbon atoms (McNamara et al., 2008). These results demonstrate that the time scale of habituation alone can determine the stimulus specificity of olfactory habituation memory. This study also addresses different mechanisms underlying short and long term memory. Metabotropic glutamate receptors of the mGluR II/III family play a significant role in short term memory whereas NMDA type of glutamatergic receptors are necessary for the long term one. Similarly habituation to ethanol induced startle response in *Drosophila* lasts for >30 mins and can be reversed spontaneously (dishabituation) to naïve level after a brief mechanical stimulus representation (Cho et al., 2004). The mechanical stimulation which is independent of olfactory pathway is sufficient to reverse the attenuated state. This cross-modal dishabituation indicates that habituation is a central brain process where processes of different modalities interact to each other and the outcome can be seen in the form of behavioral change. This study tried to address the neuronal basis of olfactory memory, by demonstrating that inactivation of α and β lobes in mushroom body resulted in significantly reduced magnitude of habituation. This study also shows that loss of ‘rutabaga’, which is a Ca\(^{2+}\)-Calmodulin dependent adenylate cyclase enzyme, perturbs habituation more severely.
Following the search of new molecules, necessity of Glycogen synthase kinase-3β (GSK-3β) function in olfactory startle response habituation has been demonstrated (Wolf et al., 2007). GSK-3β is known to have significant role in cell fate specification, cell polarity, and in signal transduction pathways such as Wnt and insulin (Cohen and Frame, 2001) and also in circadian rhythms. But whether this molecule is specifically required for olfactory habituation, or it’s a general requirement for behavioral plasticity and its upstream signalling pathways are not known.

Many studies had also looked for structural change in the brain which is found to be associated with the behavioral modification. Glomerular neuropil in the antennal lobe of honey bee workers undergo modification during the course of aging and age related shift in behavior (Winnington et al., 1996). Changes in the antennal lobe are site specific. Specific glomerulus in the antennal lobe significantly increases in volume during the first four day of adult life with changing to foraging duties. Similarly, studies in *Drosophila* showed that several days of odor exposure causes stimulus dependent specific decrease in glomerular volume in the antennal lobe (Devaud et al., 2001). 4 days of benzaldehyde exposure causes volumetric decrease in V and DM2 glomerulus, whereas iso-amyl acetate exposure caused change in DM6 glomerulus. Their study also points out 30% synaptic loss after odorant exposure as a reason behind the volumetric reduction and requirement of cAMP signalling pathway for this morphological change. To go in further detail studies had focused on the dedicated circuit of CO₂ of *Drosophila* and tried to find out the neuronal correlates of neurophysiological and behavioral changes. These alterations are associated with stimulus dependent anatomical plasticity within olfactory glomeruli. Persistent stimulus (CO₂) induced activation of olfactory receptor causes behavioral attenuation in odor induced startle response and also volumetric increase in the odor specific glomerulus (V) (Sachse et al., 2007). As the GABAergic inhibitory local interneurons are thought to modulate odor evoked activity in the *Drosophila* antennal lobe, CO₂ exposure induced physiological change in certain populations of LNs had been examined in their study. This study found chronic CO₂ exposure for 4 days results in greater CO₂ evoked activity in LNs. According to their hypothesis, the enhanced CO₂ evoked activity in LNs may results in increased inhibition onto the CO₂ specific PNs, which in turn reduces their excitability and results in corresponding attenuation of behavior. But how specific subset of local interneurons physiological activity get
modulated by CO₂ sensitive ORNs and how those LNs increased inhibition can regulate activity of specific subset of PNs to bring about the behavioral habituation is still unknown.

Despite lot of studies have tried to address the neuronal and molecular mechanism of habituation but still there is a lack of understanding of general circuit mechanism underlying habituation and its cellular and molecular basis. Here we have thoroughly examined different characteristics of habituation in terms of their odorant selectivity, recovery using odor choice assay. Along with the behavioral plasticity, we have also found occurrence of odorant selective glomerular growth after prolonged exposure to two odors, CO₂ and ethyl butyrate. By performing functional imaging in PN dendrites in the antennal lobe we have shown that PN activity declines, followed by LTH induction which is also odorant specific. Our study indicates that excitatory neurotransmitter glutamate and inhibitory neurotransmitter GABA release from LN1 subset of local interneuron and NMDAR and GABAA receptor onto PNs is crucial for display of habituation (Das et al., 2011).

3.2 Result

3.2.1 Long term Habituation to CO₂ and EB:

Two odors, carbon di-oxide (CO₂) and ethyl butyrate (EB) have been used for the odor induced habituation in this study. There are specific sets of odorant receptor present in the *Drosophila* antennae or maxillary palps which perceive specific odors. For example, carbon di-oxide activates Gr21a and Gr63a odorant receptors (Jones et al., 2007) and ethyl butyrate activates Or9a, Or22a, Or35a, Or43b, Or67a, Or67c, Or85a and Or85b receptors (Hallem and Carlson, 2006). Flies respond to odor in a concentration dependent manner, where lower concentration of few odors acts as an attractive stimulus and higher concentration acts as a repulsive one. But CO₂ is unique in the sense that even in lower concentration it acts as a repulsive stimulus. When 2-3 day old flies naïve response been checked in upright Y- maze olfactometer to different dilution of CO₂, it was observed that with increasing concentration of CO₂ from 1%, 2.5% to 5%, flies repulsive response increases from 0.35± 0.02, 0.43± 0.03 to 0.57±0.02 (Fig 3.1a). Similarly with EB, flies shows increasing repulsive olfactory response of 0.071± 0.02, 0.3± 0.03 and 0.62± 0.03 to 10⁻⁴, 10⁻³.⁵ (1 mL of EB diluted
in 100ml and 3 ml of that dilution mixed to 97 mL of water) and 10⁻³ dilution of EB (Fig 3.1b).

Fig 3.1 Sensitivity of CS flies to CO₂ and EB odors. Naïve response of wild type flies has been checked with 1%, 2.5% and 5% concentrations of CO₂ (a) and 10⁻⁴, 10⁻³.⁵ and 10⁻³ concentrations of EB (b).

To induce habituation at a long term scale, 0-12 hrs old flies were exposed to either 20% EB or 5% CO₂ for four days long. After ~ 12 hrs of starvation when the flies were tested to either 10⁻³ dilution of EB or 5% CO₂ in the maze, the olfactory response comes down from 0.73 to 0.29 (100+/− 5.4 to 40.9+/− 3.7) (Fig 3.2b) for EB and 0.83 to 0.48 (100+/− 4.7 to 57.3+/− 3.8) for CO₂ (Fig 3.2a), respectively. All the behavioral data has been normalised with respect to its control (Paraffin oil exposed animals served as control for EB experiments and mock exposed animals for CO₂ experiments). It is been shown that more than ~24 hrs old flies were unable to show LTH (Sachse et al., 2007). Drosophila pupae have no sensory input during development and only after eclosion the functional set point of the olfactory system get adapted to local environment. This time period is known as ‘critical period’. So during this critical time period, i.e. early in the life the neural circuit would be more plastic to bring activity induced plasticity. To check whether LTH induced behavioral change is reversible or not, after four days of exposure to odor, flies were transferred and allowed to recover in food media without any odor exposure for several days. After LTH to CO₂ slow recovery has been observed at 2 day (76.6+/− 2.2) and 6 day
(86±1.6) (Fig 3.2a). Similarly EB habituated flies also show almost complete recovery when tested at 6 day (93.3±1.9) (Fig 3.2b). So these results follow the first criteria of habituation, i.e. the attenuation of behavioral response and it recovers over time.

To check another criterion of habituation, i.e. stimulus specificity, the odor specificity of habituation has been tested. Flies have been exposed to 5% CO₂ for four days and then tested to EB (10⁻³) and iso amyl acetate (IAA) (10⁻³). Flies show decreased behavioral response only when tested to CO₂ but not to EB (control 100±6.2, CO₂ exposed 86.03±4.8) or to IAA (control 100±3.1, CO₂ exposed 88.4±3.5) (Fig 3.3a). Similarly after EB exposure flies show reduced olfactory response only when tested to EB but not to CO₂ (Fig 3.3b).
Fig 3.3 Habituation is odorant specific. a) Flies have been exposed to 5% CO₂ for 4 days and checked their response to CO₂, IAA and EB. b) Flies have been exposed to 20% EB and then checked their response to EB and CO₂. Fig b) has been done by Madhumala.

Flies also demonstrate habituation in a short term scale, where 30 mins of exposure to 15% CO₂ or 5% ethyl butyrate make the flies’ response to attenuate in a level comparable to LTH (Das et al., 2011). This STH is also odorant specific and recovers in a half life of 45 mins. The odor induced short term habituation follows also another unique character of classical habituation, i.e dishabituation. 30 seconds vortexing of habituated flies (30 mins of odorant exposed) in a vial make the flies response instantly reverse back to the naïve level (Sudhakaran et al., 2012a). This observation indicates that this odor induced habituation is not a result of receptor adaptation or motor fatigue, but a central brain phenomenon.

3.2.2 LTH is associated with glomerulus selective growth in the antennal lobe

Previous studies have shown that behavioral change is also associated with morphological plasticity (Sachse et al., 2007). CO₂ specific modulation of V glomerulus volume occurs after 4 days of exposure to 5% CO₂. In order to confirm the finding in our case, wild type CsBz flies have been exposed to 5% CO₂ and keeping another set untreated for four days. Fig 3.4a is the schematic representation of V and DM5 glomerulus in the antennal lobe after AMIRA reconstruction. Following dissection and nc82 antibody staining, different glomeruli (V, DM5, DM2, DM6 and VM2) had been measured using AMIRA software and compared between the control
and CO₂ exposed animals. Specific and significant increase (control- 100+/- 7.18 and CO₂ exposed- 149.27+/- 11.78) had been seen only in V glomerulus but not in other glomeruli (Fig 3.4b and 3.5a). CO₂ being perceived only by specific CO₂ sensing ORNs (GR21a and Gr63a) which make synapse and relay olfactory information to LNs and V PNs innervating V glomerulus. CO₂ exposure had no effect on glomeruli that do not receive input from CO₂ sensitive OSNs.

This implies that long term habituation is associated with glomerulus selective structural plasticity in the olfactory lobe. Similarly LTH to EB is associated with volumetric increase in DM5, DM2 and DM6 but not in V and VM2 (Fig 3.4b and 3.5b). So odorant induced LTH is accompanied with growth in odorant selective glomerulus (Fig 3.5).

![Fig 3.4. Schematic representation of different glomeruli and their odorant induced volumetric change. A) AMIRA 3D reconstruction of V and DM5 glomeruli in the antennal lobe. B) Increase of DM5 and V glomerulus volume after LTH to EB and CO₂ respectively.](image)

![Fig 3.5. Long term Habituation is associated to odorant selective glomerular volume increase. Flies have been exposed to 5% CO₂ (a) or 20% EB (b) for 4 days and different](image)
glomerular volume has been measured and compared with the control flies. fig b) has been done by Madhumala.

3.2.3 Induction of LTH reduces PN activity in the antennal lobe

Habituation results in reduced behavioral response after a long term odor exposure. The fly central olfactory system is composed of three kinds of neurons, OSNs, LNs and PNs. PN is the only interneuron which conveys the olfactory information from the antennal lobe to the higher centre (Mushroom body and Lateral horn). Fly higher centre is known to play a critical role in olfactory associative learning (Akalal et al., 2006; Connolly et al., 1996), but not in habituation (as it is being a non-associative kind of memory). So it was predicted that the odorant evoked PN response in the dendrites might be reduced after odorant exposure which can bring about habituation. To test this prediction, GH146 Gal4> UAS GCaMP3 flies have been used where GH146 Gal4 marked EB responsive PNs would express one copy of GCaMP3. GCaMP3 is a genetically encoded calcium indicator, which senses the calcium level in a cell and changes its fluorescence instantaneously (Fig 3.6a). When expressed in a neuron it is able to monitor the neuronal activity and a single action potential triggers its fluorescence changes. With the use of two photon imaging the EB evoked responses were checked in the PN dendrites of EB responsive glomeruli DM2 and DM5 after 4 days of 20% EB exposure. Delivery of 0.5% EB for 2 sec produced a strong calcium flux in DM2 and DM5 glomeruli and the fractional increase in the GCaMP fluorescence was measured as $\frac{\Delta f}{f}$ (Fig 3.6b).
Fig 3.6. Odor induced LTH is accompanied with reduction in glomerulus selective PN activity. a) Left panel shows the EB and 3-octanol evoked functional activity in DM5 and DC2 glomeruli, respectively. Right panel shows the representative traces of EB and 3-Octanol evoked PN response ($\Delta F/F$) in DM5 and DC2 glomeruli respectively in paraffin oil and EB exposed animals. b) Quantification of change in EB evoked PN response in DM2 and DM5 glomeruli in P oil, EB and CO$_2$ exposed animals and in DC2 glomerulus after EB exposure. This figure has been contributed by Adrian Dervan in collaboration.

There was a significant reduction in EB evoked GCaMP response in DM2 (Paraffin oil exposed- 878 ± 106 and EB exposed- 410 ± 91) and DM5 (Paraffin oil exposed- 928 ± 115 and EB exposed- 456 ± 53) glomeruli in the four day EB exposed animals compared to the controls (Fig 3.6b). Similarly, it was also reported that four days of 5% CO$_2$ exposure causes reduction in CO$_2$ evoked response in V subset of projection neurons (Sachse et al., 2007). To check whether the reduced activity in PNs is also odorant specific or not, similar to the behavioral habituation, the GH146> GCaMP3 flies were exposed to 5% CO$_2$ for four days and checked for EB evoked response. There was no significant drop in the DM2 (Control- 940 ± 85 and CO$_2$ exposed- 1,265 ± 82) and DM5 (Control- 638 ± 68 and CO$_2$ exposed- 730 ± 99) glomeruli (Fig 3.6b). The effect seen in the previous experiment could be due to; these PNs are not at all receiving the CO$_2$ information as the GH146 Gal4 marked PNs do not include CO$_2$ responsive PNs. So one odor has been selected, 3-octanol where the odor activates DC2 (Dorsal central) glomerulus (Wang et al., 2003) and the PNs innervating this glomerulus is marked by GH146 Gal4 (Fig 3.6a). Interestingly EB exposure did not
affect the 3-octanol induced response in DC2 glomerulus (Paraffin oil exposed- 718 ± 117 and EB exposed- 923 ± 131) (Fig 3.6b). These data indicate that this physiological plasticity in terms of reduction in PN response is coupled with the long term habituation and it is odorant specific (Das et al., 2011).

3.2.4 Neurotransmission from LN1 but not LN2 is required for LTH

Reduced PN response followed by LTH may arise due to enhanced inhibition onto the PNs by the antennal lobe neurons. Among the ORNs, LNs and PNs, some population of local interneurons are known to be inhibitory in nature that release GABA as a neurotransmitter (Chou et al., 2010; Lai et al., 2008). But some populations of LNs can be excitatory (Shang et al., 2007) and cholinergic (Das et al., 2008) too. To find out whether LN induced GABAergic inhibition onto the PNs is necessary to drive habituation; Gal4-LN1 and Gal4-LN2 had been used for the following experiments. Gal4-LN1 marks ~20 cells, of which ~12 (60%) are GABA positive and ~7 are cholinergic; whereas GAL4-LN2 labels ~40 cells of which ~24 (60%) are GABA positive and ~18 cells are ChaT positive (Das et al., 2008). The neurotransmitter release from these LNs can be reduced conditionally by crossing these Gal4 to temperature sensitive dominant conditional dynamin, UAS Shi ts1 flies and shifting them to non-permissive temperature (32°C) for any period of time. Shibire is homolog of mammalian dynamin protein in Drosophila and is involved in vesicle recycling process (Vanderbliek and Meyerowitz, 1991). Shi ts1 is temperature sensitive mutation in shibire, where the fly shows a paralytic phenotype in restrictive temperature (32°C). Expression of UAS Shi ts1 under LN1 Gal4 will only block synaptic transmission from LN1 subset of local interneuron at 34°C. LN1 Gal4 flies were crossed to UAS Shi ts1 and kept in 18°C till eclosion period, so that the developmental period of the LN1> Shi ts1 flies will not be affected. 0-12 hrs old flies had been collected, for CO2 experiments half of the flies were mock exposed and the other half were 5% CO2 exposed.

The exposure and starvation was done in 18°C. When the flies were tested at 34°C (restrictive temperature for Shibire), the LN1> Shi ts1 flies failed to show habituation to CO2 in contrast to the same genotypic flies which show normal habituation when tested at room temperature (permissive temperature) (Fig 3.7a). The control flies +/-
UAS Shi^{ts1} shows habituation to CO\textsubscript{2} when the test done in both room temperature and 34°C (Fig 3.7a). These results imply that release of neurotransmitter from LN1 subset of neurons during retrieval period is necessary for display of habituation. Similar result was found when the same LN1\textsuperscript{> Shi^{ts1}} flies were checked for LTH to EB (Fig 3.7b). As LN1 is multiglomerular in nature blocking neurotransmission from LN1 subset of local interneurons blocks habituation to both CO\textsubscript{2} and EB. To check whether neurotransmission from other local interneuron is also necessary for LTH, LN2 Gal4 was used as it marks ~24 GABA +ve cells and similar experiments were performed. Strikingly LN2\textsuperscript{> Shi^{ts1}} flies habituates normally to CO\textsubscript{2} when the test was done at 32°C and at room temperature along with its control UAS Shi^{ts1}/+ (Fig 3.8). This result indicates that neurotransmitter release from LN2 subset of local interneurons is not required for the display of habituation.

Fig 3.7 Neurotransmitter release from LN1 subset of neurons during retrieval period is necessary to mediate LTH. UAS Shi^{ts1} has been driven under LN1 Gal4 to block neurotransmission for a precise duration by keeping the flies at higher temperature. Flies have been tested for LTH to CO\textsubscript{2} (a) and EB (b) at room temperature 24°C and at 34°C.
Fig 3.8 Neurotransmission from LN2 subset of neurons is not required to induce LTH.
UAS Shi$^{sl}$ has been driven under LN2 Gal4. Flies has been exposed to air and 5% CO$_2$ for 4 days and checked for their LTH behavior at 24°C and at 34°C.

Thus, our experiments suggest synaptic release from specific class of local interneurons drives habituation. Similar experiments when performed for STH more precisely blocking the neurotransmission during training and testing. Results obtained indicate requirement of LN1 neurotransmission during acquisition and recall period of habituation (Das et al., 2011). Necessity of LN1 mediated neurotransmitter release during exposure period has been discussed later.

As LN1 subset of local interneurons is known to be inhibitory as well as excitatory (Glutamatergic) (Das et al., 2011) in its neurotransmitter identity and the neurotransmission from this subset of neurons is necessary for LTH, it has been asked whether disruption of GABA release from these neurons affects LTH or not. To address this question UAS GAD RNAi had been crossed to LN1 Gal4. γ-amino butyric acid (GABA) is synthesized by the enzymatic activity of glutamic acid decarboxylase (GAD). Knock down of GAD in LN1 would block only the GABA synthesis in LN1. When checked for LTH, the LN1> GAD RNAi flies fail to show habituation compared to its control (LN1 Gal4/+ ) (Fig 3.9). This result indicates that
GABAergic transmission from LN1 subset of local interneuron is necessary to induce LTH. But when GABA synthesis was blocked in EB responsive PNs (GH146), flies show normal LTH (Fig 3.9).

**Fig 3.9 GABAergic transmission from local interneurons drives habituation.** UAS GAD RNAi has been driven under LN1 GAL4 and GH146 Gal4 to reduce GABA synthesis in LNs and PNs, respectively and looked for their LTH to EB.

To confirm that UAS GAD RNAi selectively blocks GABA synthesis, we have expressed UAS GAD RNAi under GH146 Gal4, UAS mCD8GFP. GH146 +ve PNs include ventral cluster (contains 6-8 multiglomerular projection neurons) (Das et al., 2008) and APL neuron (Wu et al., 2012) which are known to be GABAergic. In the control flies GH146 Gal4, UASmCD8GFP> UAS lacZ the strong GABA immunostaining was observed in the ventral cluster of neurons as well as in the APL neuron (Fig 3.10a). In the experimental flies GH146 Gal4, UASmCD8GFP> UAS GAD RNAi amount of GABA immunostaining noted to be reduced (Fig 3.10b). These observations confirm that UAS GAD RNAi selectively knock down GABA synthesis in the neurons of interest.
Fig 3.10 GAD RNAi expression in GH146 subset of PNs reduces GABA secretion from the GABAergic cells. a) In the control flies left panel shows GH146 expression in GFP and GABA immunostaining in the whole antennal lobe. In the inset GABA staining can be observed in the ventral cluster and in APL neuron marked with yellow arrow. b) In the flies after expressing GAD RNAi amount of GABA expression get reduced in the ventral cluster and APL neuron.

3.2.5 GABA_A receptor on the PNs is necessary to receive inhibitory input from LNs and for display of habituation

To find out whether the inhibitory output from LNs work onto PNs to mediate behavioral decrement, expression of GABA receptor on the odorant specific PNs had been knocked down in a RNAi mediated method and checked their effect on behavior. There are two kinds of GABA receptors present in the antennal lobe, the fast ionotropic GABA_A receptor and the slow metabotropic GABA_B receptor. In Drosophila three genes encode GABA_A receptors, resistance to dieldrin (Rdl), GABA and glycine-like receptor of Drosophila (Grd), and ligand-gated chloride channel homologue 3 (Lcch3). The GABA_A receptor is highly expressed in the Drosophila antennal lobes (ALs) and the MBs (Harrison et al., 1996). UAS Rdli^{8-10} transgenic flies had been used to knock down GABA_A receptor expression (Liu et al., 2007).
UAS Rdli\textsuperscript{8-10} has been driven in CO\textsubscript{2} specific V projection neurons and EB specific GH146 projection neurons. VPN\textgreater{} Rdli\textsuperscript{8-10} flies show block in LTH to CO\textsubscript{2}, but not affecting LTH to EB (Fig 3.11a & 11b). Similarly GH146\textgreater{} Rdli\textsuperscript{8-10} flies fail to show LTH to EB but normal LTH to CO\textsubscript{2} (Fig 3.11a & 11b). These observations confirm the previous result indicating that synaptic output from inhibitory LN1 neurons onto odorant selective projection neurons is necessary for the decrease in the olfactory avoidance behavior after habituation.

![Graph](image)

**Fig 3.11 GABA\textsubscript{A} receptors in the PNs are necessary to induce LTH.** UAS Rdli has been driven under CO\textsubscript{2} responsive V PN and EB responsive GH146 subset of PNs and looked for their LTH behavior to both CO\textsubscript{2} (a) and EB (b). fig b) has been done by Madhumala.

When V and DM5 glomerular volume been checked in those animals, interestingly usual increase in V and DM5 glomerular volume had been seen in V PN\textgreater{} Rdli and GH146\textgreater{} Rdli flies, respectively like its control after CO\textsubscript{2} and EB LTH (Fig 3.12a & 12b). So GABA\textsubscript{A} receptor is essential for expression or display of habituated behavior but not necessary for LTH-associated structural plasticity. These results interprets that the GABA reception by the GABA\textsubscript{A} receptor on the PNs is not required during the acquisition period but necessary during the later period of memory formation (retrieval period).
Fig 3.12 GABA<sub>A</sub> receptor knock down in PNs doesn’t affect odor induced volumetric change. GABA<sub>A</sub> receptor has been knocked down in PNs by driving UAS Rdli under CO<sub>2</sub> responsive V PN (a) and EB responsive GH146 (b) subset of PNs and looked for their odorant induced structural growth in V and DM5 glomerulus. fig b) has been done by Madhumala.

Fig 3.13 UAS Rdli<sup>8-10</sup> against GABA<sub>A</sub> receptors in PNs increases baseline electrical activity in projection neurons. In GC56/++; GH146/++ flies that express GCaMP in PNs (i), co-expression of an UAS Rdli<sup>8-10</sup> (ii) causes substantially increased levels of live GCaMP signal without any change in levels of GCaMP protein (iii and iv). v and vi) Quantification of fluorescence intensities of above images. This figure has been contributed by Adrian Dervan in collaboration.

The UAS Rdli<sup>8-10</sup> has been validated by looking at the odorant evoked activity in PN. UAS Rdli<sup>8-10</sup> has been expressed in GC56/++; GH146/++. The live GCaMP signal (Fig.
3.13 i, ii) and the GCaMP protein expression (Fig. 3.13 iii, iv) have been monitored. It was noted that Rdli expression in GH146 subset of PN increases baseline electrical activity by the observation that live GCaMP signal in the Rdli knock down animal increases (Fig 3.13v) without changing the level of GCaMP protein (Fig 3.13vi). These results imply that knocking down of GABA_A receptor in PNs make the PNs fail to receive GABA and as a result the baseline spontaneous activity increases (Das et al., 2011).

As local interneurons are multiglomerular in nature then it would be expected to inhibit most PNs, but the odorant selective behavioral habituation points to a mechanism which makes the GABAergic output from LNs to be synapse specific. So the need of specificity and also as LN1 is also known to include few glutamatergic cells among their cluster encouraged us to look for their function in the course of habituation. Knocking down glutamate neurotransmitter release from LN1 or blocking NR1 (NMDAR) in odorant selective PNs result in block in habituation. These observations suggest that glutamate release from LN1 subset of local interneurons is necessary which in turn activates the NMDA receptor present in the projection neurons (Das et al., 2011). Glutamate receipt and at the same time depolarization by the ORNs make the NMDAR active in the PNs and as a result that particular LN- PN synapse get potentiated or strengthened. The experiments showing requirement of glutamate release from LN1 and NMDAR in PN had been done by Rashi (Das et al., 2011). The potentiation of synaptic release from LN1 was again supported by a very important observation, the requirement of *rutabaga*, the adenylate cyclase function in LN1 subset of local interneurons during the adult time period to induce habituation (Das et al., 2011).

3.3 Conclusion and Discussion:

In this study we specifically show that the odorant induced habituation follows many criteria of habituation in terms of their stimulus specificity, recovery. We have chosen CO2 as one of the odor to induce habituation, because of its physiological relevance in nature; CO2 is a major component of *Drosophila* stress odor. So it mediates avoidance behavior in the fly. Flies generally feed onto rotten food, which produces a major amount of carbon di-oxide that in turn reduces the attraction behavior of the flies to the food source. Unless habituated to a certain level of CO2 it would be difficult for
them to locate the food source. Another reason is the circuit of CO₂ is unique in the sense that it activates only 2 classes of ORN (Gr21a and 63a) which innervates only one glomerulus (V) and make synapse with V projection neurons. Reagents are available which mark specifically one subset of ORN and PN and are helpful for genetic manipulation. Our results demonstrate that unreinforced odor exposure causes odorant selective habituation and this olfactory response attenuation is associated with specific structural and functional plasticity. The specific structural change associated with LTH also recovers with time (Sachse et al., 2007). This indicates that maintenance of long term memory may be a result of the structural plasticity. Our study explains how plasticity in specific synapse (LN-PN) level brings about the behavioral change. Among the three kind of antennal lobe neurons local interneurons are known to play a major role in fine tuning in odor perception by lateral excitation and inhibition (Olsen and Wilson, 2008). Our study indicates that neurotransmitter release and probably GABA release from LN1 which work onto the PNs and reduce its activity, is crucial for displaying habituation. The LN1 subset of local interneurons is multiglomerular in nature so the GABA released from the LNs can inhibit many PNs. But the odorant specificity in behavioral, physiological and structural level points towards a mechanism which brings about the ‘glomerulus selectivity’.

This urge for finding the reason behind selectivity led us to look for the role of glutamate release from LNs. It was observed that PNs harbor postsynaptic NMDAR and either NMDAR knock down in PN or blocking glutamate release from LNs blocks LTH (Das et al., 2011). This observation led to a model where NMDAR acts as a coincident detector where PNs get depolarized by ORNs and simultaneously receive glutamate from LNs. Other PNs may receive the glutamate but fail to get depolarized by the ORNs which make them as silent PNs. The active PNs may release some neurotransmitter cue which in turn can activate the LNs to release more
Fig 3.14. Proposed model for olfactory habituation. The diagram shows the detailed circuit of ORNs, LNs and PNs in V glomerulus in the antennal lobe. Acetyl choline release from ORNs activates LNs to release glutamate and GABA. After exposure to CO₂ the synapse between LN and PN strengthens by coincident activation of NMDAR onto the PNs by simultaneous receive of glutamate and depolarization by ORNs.

inhibitory neurotransmitter. Recent study (Sudhakaran et al., 2012b) has shown that activating PNs only by TRPA1 is sufficient to drive habituation and established the importance of recurrent/ feedback inhibition in the antennal lobe. But the identity of the kind of neurotransmitter released from the PNs and activate the LNs need to be characterized.

Calcium²⁺/calmodulin dependent adenylate cyclase, rutabaga enhances production of cAMP which in turn activate protein kinase A (PKA). The requirement of rutabaga in LNs (Das et al., 2011) is the only evidence which supports the idea of potentiation of
inhibitory neurotransmission and also gives a hint that may be some neuromodulators are also involved in the process of habituation which activates the GPCR and in turn activates *rutabaga* in LNs. But there is some lack of understanding that how the inhibitory LNs get activated, is there a direct activation from PNs or is it through the excitatory LNs.

**Acknowledgement:**

Fig 3.2b, 3.3b, 3.5b, 3.11b and 3.12b where the experiments were performed with EB were contributed by Madhumala K. Sadanandappa.

Figs 3.6 and 3.13 have been contributed by Adrian Dervan.