The Role of Serotonin-cAMP Mediated Signaling in *Drosophila* Central Synaptic Transmission and its Implications in Larval Olfactory Associative Learning

A dissertation presented to

the faculty of

the College of Arts and Sciences of Ohio University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy

Archan Ganguly

March 2012

©2012 Archan Ganguly. All Rights Reserved.
This dissertation titled
The Role of Serotonin-cAMP Mediated Signaling in Drosophila Central Synaptic Transmission and its Implications in Larval Olfactory Associative Learning

by
ARCHAN GANGULY

has been approved for
the Department of Biological Sciences
and the College of Arts and Sciences by

_______________________________________
Daewoo Lee
Associate Professor of Biological Sciences

_______________________________________
Howard Dewald
Interim Dean, College of Arts and Sciences
ABSTRACT

GANGULY, ARCHAN, Ph.D., March 2012, Biological Sciences

The Role of Serotonin-cAMP Mediated Signaling in Drosophila Central Synaptic Transmission and its Implications in Larval Olfactory Associative Learning

Director of Dissertation: Daewoo Lee

Changes in synaptic plasticity are thought to be the basis of information storage leading to memory formation. In both vertebrates and invertebrates, the cAMP signaling pathway has been shown to be critical for learning and memory. The combined use of advanced genetic tools and behavioral learning assays in Drosophila has helped define the role for cAMP signaling in olfactory associative learning. However, very little is known about how cAMP mediated changes in synaptic transmission, especially at central synapses, contribute to behavioral learning. In this thesis I have used a combination of electrophysiology on Drosophila primary neuronal cultures and behavioral larval learning assays to explore if cAMP mediated changes in synaptic plasticity can contribute to behavioral learning. I demonstrate that cAMP signaling activates PKA and thus leads to an increase in the frequency of excitatory cholinergic synaptic currents and a suppression of inhibitory GABAergic synaptic currents. I also observe that cAMP modulates the inhibitory GABAergic synapses in two distinct ways. It increases pre-synaptic GABA release and also decreases post-synaptic GABAA-receptor response possibly through PKA mediated phosphorylation, thus resulting in an overall reduction of GABAergic synaptic transmission. This regulation of post-synaptic GABAA-receptors by cAMP is altered in the mushroom body (MB) neurons of cAMP
mutants (e.g. dunce and rutabaga). These flies have physiologically opposite levels of cAMP and show defects in olfactory learning. I further demonstrate that a biogenic amine (5-HT) increases cholinergic EPSC frequency and decreases GABAergic IPSC frequency. My work shows that 5-HT acts through the d5-HT7 receptor to activate cAMP-PKA signaling as its effect on cholinergic and GABAergic currents is similar to that observed with cAMP alone. I further note that the d5-HT7 receptor is expressed in the mushroom body neurons, which is critical for both olfactory appetitive and aversive learning. In summary, my work suggests that the d5-HT7 receptor in the MB, acting through the cAMP-PKA pathway, is a key player in both the regulation of synaptic plasticity and olfactory associative learning in Drosophila.

Approved: _____________________________________________________________

Daewoo Lee

Associate Professor of Biological Sciences
DEDICATION

To my loving wife, Jeevisha
ACKNOWLEDGEMENTS

I would like to begin by thanking my advisor Dr. Daewoo Lee for his excellent mentoring during my entire time in his lab. The work in this thesis would not have been possible without his training to shape me as a good scientist, who is not scared of taking risks and pushing the limits of what can be done in our lab. I will always be indebted to him for his patience during teaching me electrophysiology; something which I believe will be an extremely valuable asset in my scientific career. Dr. Lee taught me during my initial years that scientific debates, without actual data are pointless; and, over the years I have realized this to be absolutely true. This thesis would not have been possible without his constant patience and support.

I would like to thank my committee members Dr. Ralph DiCaprio, Dr. Robert Colvin and Dr. Peter Jung for their support, advice and keen interest in the development of my thesis. I would also like to thank them for their enormous support during my hunt for a post-doctoral position. I would like to thank Dr. Colvin for the extremely entertaining rides during our trips to the annual SFN meetings.

I express my sincere gratitude to Dr. Soichi Tanda for his generous gifts of several fly strains, his enormous help with the P-element mobilization crosses and his invaluable suggestions about fly genetics. I would also like to thank Dr. Robert Colvin, Dr. Frank Horodyski, Dr. Bonita Biegelke, Dr. Karen Coschigano, Dr. Erin Murphy and Dr. Janet Duerr for their generosity with various reagents during the course of my Ph.D. A special
thanks to Michelle Pate at the FACS sorting facility in ARC for her help with sorting; and, to Jeff Thuma at the confocal facility for his help with confocal imaging.

I would like to thank Aeran Lee for all her efforts during these years for fly stock maintenance, which may seem trivial but I know by experience that they can be rather overwhelming. I would also like to thank HTC undergraduates Mary Dolan and Scott Varga for their sincerity and the long hours they spent helping me with the larval learning experiments. My labmate Lyle has been a good friend over the years and I thoroughly enjoyed all the discussions (scientific and non-scientific) with him and the parties at his place. The Lee lab has always been an interesting place thanks to some really colorful undergraduates including Kauroon Darya, Brad Schultz, Derek Bowden, Tyler Hanak, Mary Dolan, Teng Zhang and Scott Varga, with all of whom I enjoyed working and interacting.

My officemates and batchmates Jinu Abraham, Ying Shen, Renna Bhandare, Mohor Chatterjee, Jian Li, Nilesh Khade, Aditi Vyas were a constant source of several wonderful conversations. I would like to thank them all for making the office a nice place to forget the pains of a yet another failed experiment. I would particularly like to thank my batchmate Mohor for listening to me and empathizing with the pains of a final year graduate student.

OU gave me the opportunity to work along with some excellent teachers during my years as an anatomy TA. In particular, Dr. Robert Hikida and Dr. Mark Berryman were
both excellent teachers who taught me everything I know about anatomy. Their enthusiasm for teaching was phenomenal, and something that I never experienced earlier during my undergraduate years. I sincerely thank them for this.

All this would not have been possible without the unconditional love and support of my parents who supported me in my endeavors over the years, even when others doubted my abilities. I cannot thank them enough for all the sacrifices they made for me over the years to make this possible. I would also like to thank Arpan for all his love and for being a wonderful brother.

Last but by no means the least, my best friend, girlfriend and now my wife Jeevisha has been my biggest source of love and support during my entire Ph.D. Being a scientist herself, her critical comments and suggestions were extremely valuable. Moreover, her help with the FACS sorting and mRNA isolation were a life saver during the final months of my Ph.D. She also undertook the painful job of editing everything I wrote and converting all my figures into illustrator files, which I honestly did not have the patience to deal with. Above all, her unending faith, love and support in all my efforts, scientific or otherwise, make me the person I am. This thesis would not have been possible without her unending love.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Dedication</td>
<td>5</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>6</td>
</tr>
<tr>
<td>List of Tables</td>
<td>13</td>
</tr>
<tr>
<td>List of Figures</td>
<td>14</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>16</td>
</tr>
<tr>
<td>Chapter One: Introduction</td>
<td>17</td>
</tr>
<tr>
<td>1.1: The Origins of Studies on Mechanisms of Memory Formation</td>
<td>17</td>
</tr>
<tr>
<td>1.2: Various Forms of Synaptic Plasticity Involving the cAMP-PKA Pathway</td>
<td>21</td>
</tr>
<tr>
<td>1.3: Components of the cAMP-PKA Pathway: Molecules and Mechanisms</td>
<td>23</td>
</tr>
<tr>
<td>1.3.1 Neurotransmitters</td>
<td>24</td>
</tr>
<tr>
<td>1.3.2 NMDA, AMPA, GABA and ACh Receptors</td>
<td>24</td>
</tr>
<tr>
<td>1.3.3 Enzymes in the cAMP-PKA Pathway</td>
<td>25</td>
</tr>
<tr>
<td>1.3.4 Protein Kinase A (PKA)</td>
<td>25</td>
</tr>
<tr>
<td>1.4 Regulation of Synaptic Excitation and Inhibition by the cAMP-PKA Pathway: Implications in Learning</td>
<td>26</td>
</tr>
<tr>
<td>1.5: Biogenic Amines and their Receptors in Synaptic Plasticity</td>
<td>28</td>
</tr>
<tr>
<td>1.6: <em>Drosophila</em> as a Model System for Studies on Synaptic Plasticity</td>
<td>30</td>
</tr>
<tr>
<td>1.7: The <em>Drosophila</em> Adult Olfactory System</td>
<td>33</td>
</tr>
<tr>
<td>1.8: The <em>Drosophila</em> Larval Olfactory System</td>
<td>35</td>
</tr>
<tr>
<td>1.9: Structure, Development and Significance of the Larval Mushroom Body</td>
<td>36</td>
</tr>
<tr>
<td>1.10: The Role of Extrinsic MB Neurons in Olfactory Learning</td>
<td>39</td>
</tr>
<tr>
<td>1.11: Biogenic Amines and their Receptors Involved in <em>Drosophila</em> Learning</td>
<td>41</td>
</tr>
<tr>
<td>1.12: Learning Assay in the <em>Drosophila</em> Larvae: Principle and Design</td>
<td>43</td>
</tr>
<tr>
<td>1.13: Major Aims of this Thesis</td>
<td>44</td>
</tr>
<tr>
<td>Chapter Two: Materials and Methods</td>
<td>49</td>
</tr>
<tr>
<td>2.1: <em>Drosophila</em> Stocks</td>
<td>49</td>
</tr>
<tr>
<td>2.2: <em>Drosophila</em> Primary Neuronal Cultures</td>
<td>50</td>
</tr>
<tr>
<td>2.3: Pupal Neuronal Cultures</td>
<td>51</td>
</tr>
</tbody>
</table>
4.2.3 The Effect of 5-HT on Cholinergic EPSCs in a G-α-s Null Mutant Background.......................................................................................................................... 96

4.2.4 Identification and Characterization of a d5-HT7 Mutant (#24705) in Drosophila.......................................................................................................................... 98

4.2.5 The d5-HT7 Receptor Modulates the Effect of 5-HT on Excitatory Cholinergic EPSCs .................................................................................................................. 99

4.2.6 5-HT Modulates Cholinergic EPSCs Primarily through Pre-Synaptic Mechanisms ............................................................................................... 100

4.2.7 5-HT Causes a Decrease in the Frequency of GABAergic IPSCs ............ 101

4.2.8 The Effect of 5-HT on the Frequency of GABAergic IPSCs in the Presence of a PKA Blocker (H-89).................................................................................. 102

4.2.9 The d5-HT7 Receptor Mediates the Effect of 5-HT on GABAergic IPSCs.. 102

4.2.10 Effect of 5-HT on Miniature IPSC (mIPSCs) in Wild-type (w1118) Neurons103

4.2.11 5-HT Regulates GABAergic IPSC (sIPSCs) by Decreasing Post-Synaptic GABA_A Receptor Response ...................................................... 104

4.3 Discussion............................................................................................................. 105

Chapter Five: Drosophila 5-HT7 Receptor (d5-HT7) in the Mushroom Body Regulates Larval Olfactory Learning ................................................................. 121

5.1 Introduction......................................................................................................... 121

5.2 Results ................................................................................................................ 128

5.2.1 Appetitive Associative Learning: Pentyl Acetate versus Propionic Acid... 128

5.2.2 Appetitive Associative Learning in Wild-type and known Learning Mutants.............................................................................................. 129

5.2.3 The d5-HT7 Receptor is Required for Olfactory Associative Learning....... 130

5.2.4 d5-HT7 Expression in Drosophila Mushroom Body Neurons.............. 131

5.2.5 Appetitive Olfactory Learning Requires d5-HT7 Expression in the MB Neurons of Drosophila Larval Mushroom Body .......................... 132

5.2.6 Over-Expression of the d5-HT7 Receptor in the MB neurons Enhances Appetitive Learning Response......................................................... 133

5.2.7 Temperature Sensitive Down-Regulation of d5-HT7 Receptor Specifically in the MB Impairs Olfactory Associative Learning.......................... 134

5.2.8 Expression of the d5-HT7 Receptor in Mutant Background Rescues the Impairment in Olfactory Associative Learning.......................... 135

5.2.9 The d5-HT7 Receptor is Required for Aversive Olfactory Learning........ 137

5.3 Discussion......................................................................................................... 140
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.01: Fly Strains Obtained from Bloomington Stock Centre</td>
<td>61</td>
</tr>
<tr>
<td>Table 5.01: Gustatory Response Index of 3rd Instar Larvae of Different Genotypes</td>
<td>158</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.01</td>
<td>Pupal Cultures</td>
<td>46</td>
</tr>
<tr>
<td>1.02</td>
<td>The Larval Learning Assay Set-Up</td>
<td>47</td>
</tr>
<tr>
<td>2.01</td>
<td>Pupal Cultures</td>
<td>62</td>
</tr>
<tr>
<td>2.02</td>
<td>The Larval Learning Assay Set-Up</td>
<td>63</td>
</tr>
<tr>
<td>3.01</td>
<td>Suppression of Inhibitory GABAergic Post-Synaptic Currents by Forskolin (FSK)</td>
<td>81</td>
</tr>
<tr>
<td>3.02</td>
<td>Inhibitory Effects of cAMP on GABAergic Synaptic Transmission</td>
<td>82</td>
</tr>
<tr>
<td>3.03</td>
<td>Modulatory Actions of Forskolin (FSK) on Inhibitory GABAergic Post-Synaptic Currents (IPSCs) in Learning and Memory Mutants</td>
<td>83</td>
</tr>
<tr>
<td>3.04</td>
<td>cAMP Modulates GABAergic IPSCs through a Post-Synaptic Mechanism</td>
<td>84</td>
</tr>
<tr>
<td>3.05</td>
<td>Effect of Forskolin on the Frequency of Spontaneous Action Potentials in GABAergic Neurons</td>
<td>85</td>
</tr>
<tr>
<td>3.06</td>
<td>Effect of GABA Puffing in the Presence and Absence of Forskolin</td>
<td>86</td>
</tr>
<tr>
<td>3.07</td>
<td>Effect of GABA Puffing on Embryonic Mushroom Body Neurons</td>
<td>87</td>
</tr>
<tr>
<td>3.08</td>
<td>Effect of GABA Puffing on Wild-type Late Stage Pupal Mushroom Body Neurons</td>
<td>88</td>
</tr>
<tr>
<td>3.09</td>
<td>Effect of GABA Puffing on Mutant Late Stage Pupal Mushroom Body Neurons</td>
<td>89</td>
</tr>
<tr>
<td>4.01</td>
<td>Effect of 5-HT on Cholinergic EPSCs in Wild-type (w1118) Neurons</td>
<td>110</td>
</tr>
<tr>
<td>4.02</td>
<td>Effect of 5-HT on Cholinergic EPSCs in the Presence of a PKA Blocker (H89)</td>
<td>111</td>
</tr>
<tr>
<td>4.03</td>
<td>Effect of 5-HT on Cholinergic EPSCs in a Gas Null Mutant Background</td>
<td>112</td>
</tr>
<tr>
<td>4.04</td>
<td>Identification and Characterization of a d5-HT7 Mutant (#24705) in Drosophila</td>
<td>113</td>
</tr>
<tr>
<td>4.05</td>
<td>Effect of 5-HT on Cholinergic EPSCs in a d5-HT7 Mutant and in Wild-type Neurons in the Presence of a d5-HT7 Receptor Antagonist</td>
<td>114</td>
</tr>
<tr>
<td>4.06</td>
<td>Effect of 5-HT on mEPSCs in Wild-type (w1118) Neurons</td>
<td>115</td>
</tr>
<tr>
<td>4.07</td>
<td>Effect of 5-HT on Wild-type (w1118) GABAergic IPSCs</td>
<td>116</td>
</tr>
<tr>
<td>4.08</td>
<td>Effect of 5-HT on Wild-type (w1118) GABAergic IPSCs in the Presence of a PKA Blocker (H89)</td>
<td>117</td>
</tr>
<tr>
<td>4.09</td>
<td>Effect of 5-HT on GABAergic IPSCs in the d5-HT7 Null Mutant (#24705) Neurons</td>
<td>118</td>
</tr>
<tr>
<td>4.10</td>
<td>Effect of 5-HT on mIPSCs in Wild-type (w1118) Neurons</td>
<td>119</td>
</tr>
<tr>
<td>4.11</td>
<td>Serotonin Regulates GABAergic IPSCs by Decreasing Postsynaptic GABA&lt;sub&gt;A&lt;/sub&gt; Receptor Response</td>
<td>120</td>
</tr>
<tr>
<td>5.01</td>
<td>Response Index (3 minute) for Larvae Trained with Propionic Acid and Distilled water (DW) or 1M sucrose (SUC)</td>
<td>145</td>
</tr>
</tbody>
</table>
Figure 5.02: Response Index (3 minute) for Larvae Trained with DW + Odor or 1M SUC + Odor. ........................................................................................................................ 146
Figure 5.03: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 1M SUC + Pentyl Acetate for two Wild-type and two Learning Mutant Strains. 147
Figure 5.04: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 1M SUC + Pentyl Acetate for Wild-type and Two d5-HT7 Receptor mutant Strains........................................................................................................................................ 148
Figure 5.05: Expression of GFP Specifically in MB Neurons (Kenyon cells) Using Gal4-UAS Binary Expression System.......................................................... 149
Figure 5.06: Detecting d5-HT7 Expression in FACS Sorted GFP+ive MB Neurons...... 150
Figure 5.07: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 1M SUC + Pentyl Acetate for Wild-type and a MB Specific d5-HT7-RNAi Strain. ........................................................................................................................................ 151
Figure 5.08: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 1M SUC + Pentyl Acetate for Wild-type and a Strain Over-Expressing the Wild-type d5-HT7 Gene in the MB (Kenyon Cells)........................................................................... 152
Figure 5.09: Spatio-Temporally Restricted Expression of GFP in the Mushroom Body Neurons Using the Gal80/Gal4 Expression System................................................. 153
Figure 5.10: Response Index (3 minute) for Larvae Expressing d5-HT7- RNAi in a Temperature Sensitive Manner Specifically in the Mushroom Body ...................... 154
Figure 5.11: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 1M SUC + Pentyl Acetate for Wild-type and Strains Expressing MB-Gal80 and d5-HT7 Genes Specifically in the MB Neurons of d5-HT7-RNAi and d5-HT7 Null Backgrounds, Respectively. ........................................................................................................ 155
Figure 5.12: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 0.1% Quinine Hemisulphate (Bitter Compound) + Pentyl Acetate....................... 156
Figure 5.13: Olfactory and Gustatory Discrimination Controls ...................................................... 157

Figure 6.01: AMP Modulates Inhibitory GABAergic Transmission through Post-Synaptic GABA\textsubscript{A}-Like Receptors ........................................................................ 167
Figure 6.02: Serotonin (5-HT) Regulates Excitatory Cholinergic Transmission through an Increase in Pre-Synaptic Neurotransmitter Release ........................................... 168
Figure 6.03: Serotonin (5-HT) Regulates Inhibitory GABAergic Transmission by Decreasing Post-Synaptic GABA\textsubscript{A} Receptor Response ............................................. 169
Figure 6.04: d5-HT7 is Expressed in Mushroom Body Neurons (Kenyon cells) where it Regulates Olfactory Learning................................................................. 170
LIST OF ABBREVIATIONS

1. cAMP: cyclic Adenine Monophosphate
2. db-cAMP: dibutyl cyclic Adenine Monophosphate (a cAMP homolog)
3. PKA: Protein Kinase A
4. CREB: cAMP Response Element-Binding protein
5. GPCR: G-Protein Coupled Receptor
6. MB: Mushroom Body
7. AL: Antennal Lobe
8. PN: Projection Neurons
9. UAS: Upstream Activating Sequence
10. LTP: Long Term Potentiation
11. LTD: Long Term Depression
12. MB: Long Term Facilitation
13. IPSCs: Inhibitory Post-Synaptic Currents
14. EPSCs: Excitatory Post-Synaptic Currents
15. mIPSCs: Miniature Inhibitory Post-Synaptic Currents
16. mEPSCs: Miniature Excitatory Post-Synaptic Currents
17. FSK: Forskolin (activator of cAMP)
18. GABA: γ-Aminobutyric Acid
19. 5-HT: 5-Hydroxy Tryptophan (also known as Serotonin)
20. DA: Dopamine
21. OA: Octapamine
22. GFP: Green Fluorescent Protein
23. RFP: Red Fluorescent Protein
24. PBS: Phosphate Buffered Saline
25. PCR: Polymerase Chain Reaction
26. RT-PCR: Reverse Transcription Polymerase Chain Reaction
27. FACS: Fluorescence Activated Cell Sorting
1.1: The Origins of Studies on Mechanisms of Memory Formation

Edward Thorndike’s work on cats learning to escape from a cage (Thorndike, 1898) is the first documented evidence of animals being used as model systems to study learning behavior. He showed that with repeated trials the cats learned to press a lever which opened the cage door which allowing them to escape. Later in 1904, Ivan Pavlov’s work on classical conditioning demonstrated that a reflex behavior (salivation on food presentation) can be systematically associated with a conditioned stimulus (ringing of a bell) with learning (Pavlov, 1927). In the 1930’s, B.F. Skinner used the now popular ‘Skinner Box’ to show that animals can learn to associate and perform a particular action based on the outcome, e.g. pressing a lever for food reward (Skinner, 1930).

However, these purely behavioral studies could not provide any information on where memory is stored, or identify regions of the mammalian brain required for learning and memory. To study this, in the 1920’s Karl Lashley began to examine the effect of removing small regions of the rodent cerebral cortex on their maze learning ability (Lashley, 1931). He postulated that memory or information in the brain is not localized to a certain subset of neurons in a specific anatomical region but is the result of aggregate activity (bioelectric field) generated by a large cluster of neurons in the brain (Kandel, 2009). This theory, called the aggregate field approach, was in contrast to the
cellular connectionist approach proposed by Santiago Ramon y Cajal which stated that learning resulted from alterations in the strength of a synapse (Cajal, 1894). Cajal’s theory was adapted by Donald Hebb in 1949 to give rise to the now famous theory of Hebbian plasticity which states that a change in pre-synaptic excitation or input leads to a corresponding change in the post-synaptic target (Hebb, 1950).

The first evidence against the aggregate field approach came from studies in human subjects performed by Wilder Penfield in 1938. Penfield, a neurosurgeon, demonstrated that activating the temporal lobe of patients by electrical stimulus leads to recollection of images, voices, music, memories and perceptions (Hebb, 1977). A major breakthrough in this area came with the studies on the patient H. M. (Squire, 2009). In 1957, Brenda Milner described that removal of regions of the medial-temporal cortex from patient H. M. led to a severe loss in memory without altering intellectual or cognitive abilities (Scoville and Milner, 1957). Milner went on to describe that procedural memory was unaffected in monkeys when parts of the hippocampus were removed (Orbach et al., 1960). This indicated that different regions of the brain are responsible for the formation of particular types of memory. Further evidence against Lashley’s theory came from the classic study done by John O’Keefe in 1971 which demonstrated that the rat hippocampus registers spatial memory through the firing of place cells. These cells fire when the animal is in a particular spatial area and this form of memory is dependent on a combination of sensory inputs (O’Keefe and Dostrovsky, 1971).
However, the field of learning still lacked studies which could describe how the activity in the neurons of a circuit was modified during learning. The circuits and molecules involved in these learning tasks were first identified during the late 60’s and mid 70’s by studies on olfactory learning in *Drosophila*, gill withdrawal reflex in *Aplysia*, and behavioral modifications in Crayfish and honeybee (Brunelli et al., 1976; Menzel and Erber, 1978; Quinn et al., 1974; Zucker et al., 1971). This preliminary work, done in organisms much simpler than mammals, supported Cajal’s theory and indicated that alterations in the strength of synapses may be a mechanism by which memory is stored. These studies, and particularly those done on gill withdrawal reflex in *Aplysia* in Eric Kandel’s lab, showed that the application of serotonin (5-HT) to mimic tail shock leads to cAMP dependent changes in both short and long term facilitation (Brunelli et al., 1976; Castellucci et al., 1978). The long term form of synaptic facilitation was dependent on protein synthesis and the action of CREB protein.

Seymour Benzer’s lab first identified a gene responsible for defects in olfactory associative learning in *Drosophila* (Dudai et al., 1976). This gene coded for a protein involved in the cAMP signaling pathway. In *Drosophila* the cAMP-PKA pathway has also been implicated in the classical Pavlovian olfactory learning paradigm (Tully and Quinn, 1985). Moreover, mutations in genes involved in the cAMP-PKA pathway are known to result in impaired olfactory learning in *Drosophila* (Keene and Waddell, 2007). The most prominent amongst the learning mutants are *dunce* and *rutabaga*. *Dunce* flies have a mutation in the cAMP phosphodiesterase enzyme (PDE) which leads to increased cytosolic cAMP levels (Dudai et al., 1976). *Rutabaga* results from a mutation in the
calcium-calmodulin dependent adenylyl cyclase which leads to a decrease in intracellular cAMP levels (Livingstone et al., 1984). The mushroom body in Drosophila is the center for olfactory sensation and cAMP signaling in this structure has also been shown to be required for the short term memory component (Margulies et al., 2005). Furthermore, the long term memory profile in Drosophila is known to result from the activation of cAMP dependent transcription factor CREB in the nucleus (Yin et al., 1994). Several other genes involved in the cAMP signaling cascade have also been shown to be involved in Drosophila olfactory learning (Heisenberg, 2003; Keene and Waddell, 2007). Thus, the cAMP signaling pathway has been demonstrated to be one of the important, if not the most important, signaling pathway in invertebrate learning.

In 1971 Timothy Bliss and Terje Lomo discovered that the synapses in the prefrontal and Schaffer collateral pathway of the hippocampus of rats were highly plastic and could be potentiated (Bliss and Lomo, 1973). This potentiation resulted from pre-synaptic excitation followed closely by post-synaptic activity (LTP). This phenomenon showed that memory/information is stored at the level of synapses, and has since been used as a model of choice to explore synaptic plasticity in mammals (Bliss and Collingridge, 1993; Malenka and Bear, 2004; Malenka and Nicoll, 1999). The phenomenon of LTP provides direct evidence for Donald Hebb’s theory and suggests that changes in plasticity brought about by various signaling molecules at the synaptic level can account for behavioral learning at the whole organism level.
In summary, these studies support Cajal’s theory that postulates synapses are plastic and that memory or information can be stored in the brain by strengthening existing synapses and by growing new synaptic connections (Cajal, 1894).

1.2: Various Forms of Synaptic Plasticity Involving the cAMP-PKA Pathway

The interaction of individual components of signaling pathways with each other and their downstream targets promotes neuronal synaptic plasticity. The variable biochemical signaling profile of a synapse may be responsible for synaptic strengthening and may also result in behavioral learning and memory (Bhalla and Iyengar, 1999). A key molecule involved in this variable signaling at the synapse is the second messenger cAMP, which in turn activates the serine-threonine protein kinase, Protein kinase A (PKA).

Alteration in synaptic transmission is mediated by many signaling molecules, including the cAMP-PKA pathway, and can be classified into different subtypes. A burst of high frequency pre-synaptic activity leads to a short term or transient increase in synaptic transmission (facilitation, augmentation), whereas multiple repeated high frequency bursts or stimuli lead to a prolonged or long term increase in synaptic transmission (potentiation) as seen at the single excitatory synapse (Alberini et al., 1995). Similar forms of altered synaptic plasticity are seen in mammalian brain slices in vitro, and are known as long term potentiation (LTP) and long term depression (LTD). Both of these are believed to account for several behavioral forms of learning in vertebrates. LTP has been studied extensively in various regions of the brain including the hippocampus, cerebral
cortex, amygdala and the cerebellum; whereas, LTD has been seen only in the cerebellum and the hippocampus (Malenka and Bear, 2004). These two opposing forms of activity-dependent synaptic strengthening result from altered signaling at the level of synapses. Various modulator molecules are known to play an important role in the process of LTP (β-adrenergic receptors, metabotropic glutamate receptors, dopamine receptors, NO synthase) and LTD (NMDAR, mGluR, endocannabinoids) (Malenka and Bear, 2004). Interestingly, the cAMP-PKA signaling pathway remains an important component of both of these processes (Nguyen and Woo, 2003). For example, the late phase of LTP in CA1 hippocampal neurons in mice can be stimulated by the application of cAMP (Frey et al., 1993); and, its downstream target PKA is required for the late phase of hippocampal LTM formation (Abel et al., 1997). Cyclic AMP is also needed to initiate the early and late phases of mossy fiber LTP in the hippocampus of mice (Huang et al., 1994).

In invertebrates like *Aplysia*, the cAMP-PKA pathway is involved in the process of short term facilitation which leads to the formation of short term memory. This process involves serotonin-GPCR mediated activation of the cAMP-PKA signaling pathway; whereas the long term memory component is dependent on cAMP-dependent protein synthesis (Kandel, 2001). Studies on the *Drosophila* neuromuscular junction (NMJ) and cultured neurons have also shown that cAMP alters pre-synaptic release probability and facilitation at the excitatory synapses (Griffith et al., 1994; Lee and O'Dowd, 2000; Zhong and Wu, 1991) which is impaired in cAMP pathway mutants (Zhao and Wu, 1997).
1.3: Components of the cAMP-PKA Pathway: Molecules and Mechanisms

The cyclic Adenine Monophosphate/Protein Kinase A (cAMP-PKA) pathway plays a central role in neurotransmission in both vertebrates and invertebrates (Kandel, 2009). The two basic mechanisms by which cAMP is produced in a cell are diagrammatically represented in Figure 1.01. Adenylate cyclase is most commonly activated through G-protein coupled receptors (GCPRs). This pathway is known as the NMDA independent pathway (Fig. 1.01 A). The binding of a signal molecule (e.g. neurotransmitters like dopamine, serotonin, octapamine, epinephrine, nor-epinephrine, tyramine) to its respective GPCR (e.g. D1, D2, 5HT7, β-adrenergic receptors) leads to the dissociation of the G-α-s subunit from the G-β and G-γ subunits, and the subsequent activation of G-α-s. The activated G-α-s is bound to GTP which can then activate the enzyme adenylate cyclase (AC). AC then converts ATP to cAMP (Dorsam and Gutkind, 2007).

Cyclic AMP can also be produced by an influx of calcium ions into the cell through glutamate receptors (NMDAR) or through other ion channels (calcium channels). This pathway is commonly referred to as the NMDAR dependent pathway (Fig. 1.01 B). The activation of the NMDA receptor by Mg²⁺/depolarization leads to an influx of calcium ions into the cell which in turn results in the activation of the calcium-dependent calmodulin (CaM) enzyme. This enzyme then binds to Ca²⁺/CaM dependent adenylate cyclase, which activates the cAMP-PKA pathway. This mode of cAMP production is known to induce LTP in experiments using vertebrate brain slices (Malenka and Bear, 2004; Malenka and Nicoll, 1999).
Various components of the cAMP pathway have been shown to be essential for learning and memory as described below:

1.3.1 Neurotransmitters

Neurotransmitters such as 5-HT/serotonin in *Aplysia* (Byrne and Kandel, 1996), PACAP in *Drosophila* (Waddell et al., 2000) and octapamine in honeybees (Hildebrandt and Muller, 1995) have been shown to bind to their respective GPCRs. This binding results in the activation of the cAMP-PKA pathway. This in turn has been shown to be essential for learning and memory.

1.3.2 NMDA, AMPA, GABA and ACh Receptors

In invertebrates like *Drosophila*, the NMDA like receptors in the mushroom body have been implicated in short term memory formation (Xia et al., 2005), while those in the ellipsoid body are known to regulate memory consolidation (Wu et al., 2007). NMDA and AMPA receptors in vertebrates have been shown to be critical for LTP and LTD induction in particularly the Schaffer collateral-CA1 synapse in the hippocampus (Malenka and Bear, 2004). GABA<sub>A</sub> receptor endocytosis and exocytosis on to the post-synaptic membrane has been shown to be essential for the induction of LTP at inhibitory GABAergic synapses in the deep-cerebellar nucleus (Ouardouz and Sastry, 2000) and at hippocampal granule cells (Nusser et al., 1998). Similarly, GABA-RDL receptor expression in the *Drosophila* mushroom body has been shown to be essential for olfactory associative learning (Liu et al., 2007). The α7 nAChR and M1 muscarinic AChR mediated LTP and LTD induction has been seen at the Schaffer collateral-CA1 circuit synapses in the mammalian hippocampus (Buchanan et al., 2010; Gu and Yakel, 2011).
1.3.3 Enzymes in the cAMP-PKA Pathway

Many enzymes of the cAMP-PKA pathway such as cAMP phosphodiesterase, CaM dependent adenylate cyclase and the enzyme coding for the catalytic subunit of PKA, have been shown to be essential for Pavlovian olfactory learning in *Drosophila* (Heisenberg, 2003). It has also been shown that over-expression of the G-α-s subunit leads to severe impairment of olfactory associative learning in *Drosophila* (Connolly et al., 1996). The calcium calmodulin kinase II (CaMKII) is required for spatial memory (Silva et al., 1992) and consolidation in mice (Frankland et al., 2001). Inhibiting CaMKII in *Drosophila* is known to lead to defects in habituation (Jin et al., 1998). Thus, the cAMP-pathway is critical for various forms of memory and learning.

1.3.4 Protein Kinase A (PKA)

Once PKA is activated by cAMP, it can act on a wide range of pre and post-synaptic targets to modulate activity-dependent neuronal excitability. Although the details of all possible targets of the cAMP-PKA pathway are beyond the scope of this discussion (Nguyen and Woo, 2003), some of the well-known targets of this pathway are described below:

i) The best known target of PKA is the transcription factor CREB which upon phosphorylation by PKA leads to the transcription of genes required for long term memory in the nucleus of the post-synaptic neuron. CREB has been shown to be required for LTP (Yamamoto et al., 1988), long term memory in mice and *Drosophila* (Bourtchuladze et al., 1994; Yin et al., 1994) and long term facilitation in *Aplysia* (Alberini et al., 1994).
ii) The cAMP-PKA pathway is also known to activate certain adhesion molecules like N-cadherin which are required for potentiation during LTP in mice (Bozdagi et al., 2000). These are thought to act by increasing the adhesion strength of the synapse.

iii) One of the best known functions of PKA is the phosphorylation of the GluR1 subunit of AMPA receptors at serine 845, the dephosphorylation of which leads to LTD (Zamanillo et al., 1999); whereas the phosphorylation of serine 831 by CaMKII leads to LTP in CA1 pyramidal neurons (Barria et al., 1997).

iv) PKA is also known to increase depolarization by phosphorylating voltage-gated potassium channels like the Shaker channel (Yao and Wu, 2001). Such changes may lead to defects in normal potentiation at the synapses. In short term facilitation in Aplysia, PKA is known to activate the S-type K+ channel which result in action potential peak broadening and larger influx of calcium into the pre-synaptic neuron, thus causing enhanced glutamate release and facilitation (Shuster et al., 1985).

v) PKA also interacts with members of other signaling pathways such as MAP kinase to activate CREB, and it inhibits the action of protein phosphatase 1 (PP1), which leads to the induction of LTP (Nguyen and Woo, 2003).

1.4 Regulation of Synaptic Excitation and Inhibition by the cAMP-PKA Pathway: Implications in Learning
The cAMP-PKA pathway is known to regulate NMDA-independent form of LTP seen in the mossy fibers (granule cells in the dentate gyrus) and CA3 pyramidal cells in hippocampal slices. It has been shown that mice which lack enzymes crucial for vesicle
docking before exocytosis (Rab3A and RIM1α) have impaired mossy fiber LTP (Castillo et al., 1997; Castillo et al., 2002). Interestingly, PKA is also known to alter pre-synaptic release mechanisms by phosphorylating Rab3A and RIM1α (Sudhof, 2004). In the granule cell to Purkinje cell synapse, cAMP has been shown to increase the pre-synaptic vesicle release probability via miniature EPSC frequency which enhances the strength of this synapse (Chen and Regehr, 1997). In Aplysia, PKA is known to act on the S-type K+ channels and broadening the action potential peak. This leads to an influx of Ca\(^{2+}\) into the pre-synaptic neuron and facilitates vesicle fusion and glutamate release resulting in short term facilitation (Kandel, 2001). In Drosophila, FM1-43 based synaptic vesicle labeling has indicated that altered levels of cAMP affect the pre-synaptic release probability (Kuromi and Kidokoro, 2002). Studies in the Drosophila NMJ and cultured neurons have shown that cAMP alters pre-synaptic release probability and facilitation at the excitatory synapses (Griffith et al., 1994; Lee and O’Dowd, 2000; Zhong and Wu, 1991). In keeping with these observations, it has been shown that facilitation is impaired in cAMP pathway mutants (Zhao and Wu, 1997).

The various studies described above show that the cAMP-PKA pathway plays an important role in regulating synaptic plasticity and is also involved in learning and memory. Despite the fact that the cAMP pathway and its role in modulating excitatory synaptic transmission are well-studied, not much is known about how cAMP signaling affects inhibition at the synaptic level. Though excitation is believed to be the primary driver for learning and memory, inhibitory synaptic transmission has been shown to be important for learning and memory in both vertebrates (Gogolla et al., 2009; Isaacson
and Scanziani, 2011) and invertebrates like Drosophila (Liu and Davis, 2009). Recently, several studies in the cerebral cortex of mammals and in Drosophila brain have shown the presence of a large number of inhibitory GABAergic interneurons. These interneurons have been shown to be important for various forms of information processing and behaviors including learning (Isaacson and Scanziani, 2011; Olsen and Wilson, 2008a; Sohal et al., 2009). Further support for the significance of inhibitory synaptic plasticity comes from a host of recent studies showing that GABAergic synapses are plastic and cAMP signaling can induce both LTP and LTD at GABAergic synapses (reviewed in Castillo et al., 2011).

1.5: Biogenic Amines and their Receptors in Synaptic Plasticity

Biogenic amines are probably the most important group of neurotransmitters in both the mammalian and invertebrate brain. This group of amines is synthesized from amino acids and consists of the neurotransmitters dopamine, epinephrine, norepinephrine (collectively called catecholamines since they share the common catechol group), serotonin and histamine in vertebrates. In invertebrates, the biogenic amines consist of the neurotransmitters dopamine, serotonin, octapamine and tyramine. Most of these biogenic amines are coupled to their respective GPCRs which are linked to Gs or Gi subunits of G-proteins. These G proteins regulate intracellular cAMP levels in a positive and negative manner, respectively. Perhaps the two best studied biogenic amines are dopamine and serotonin. Dopamine (DA) has been implicated in a wide variety of behaviors like reward, cognition, addiction, aggression, motor skill control and balance (Sealfon and Olanow, 2000). Serotonin is associated with mood, arousal, aggression,
sleep patterns, circadian rhythms, learning and memory (Hoyer et al., 2002; Lucki, 1998). Both these neurotransmitters have a wide variety of GPCRs associated with them. These GPCRs can be broadly classified based on whether they activate cAMP or inhibit its production. The DA receptors are divided into two groups – the D1-like and D2-like family of receptors – which have distinct molecular and pharmacological profiles. The D1-like family consists of D1 and D5 receptors that stimulate cAMP production by activating GPCR coupled G-α-s. On the other hand, the D2-like receptor family comprising D2, D3 and D4 receptor subtypes have GPCRs coupled to the G-α-i subunit and thus inhibit cAMP production (Neve et al., 2004). The D1/D5 group of receptors has been implicated in altered plasticity in the hippocampus (Li et al., 2003) and is important for hippocampal learning in mice (Lemon and Manahan-Vaughan, 2006). The D1 receptor homologs in invertebrates have been shown to be involved in aversive learning in Drosophila (Schwaerzel et al., 2003).

Serotonin has seven known receptors in mammals called the 5-HT1 to 5-HT7 receptors (Nichols and Nichols, 2008). 5-HT1 and 5-HT5 negatively regulate cAMP, whereas 5-HT4, 5-HT6 and 5-HT7 positively regulate cAMP production. While 5-HT3 acts as a ligand gated ion channel, 5-HT2 is known to elevate intra-cellular calcium levels (Barnes and Sharp, 1999). Serotonin has been shown to regulate sleep, mood disorders, sexual function, appetite regulation and a wide range of cognitive tasks in mammals (Perez-Garcia and Meneses, 2008) and is crucial for learning and memory in Aplysia (Kandel, 2001). The 5-HT1, 5-HT4, 5-HT6 and the 5-HT7 receptors have been implicated in various forms of learning including spatial learning (King et al., 2008; Leopoldo et al.,
This indicates that the 5-HT group of receptors is crucial for a range of learning behaviors. Both dopamine and serotonin receptors are expressed in various regions of the brain, including those implicated in learning and memory, such as the pre-frontal cortex, hippocampus and amygdyla (Lucki, 1998; Neve et al., 2004).

1.6: *Drosophila* as a Model System for Studies on Synaptic Plasticity

*Drosophila melanogaster* has been used as a model to study learning and memory since the discovery of the *dunce* memory mutant in Semyour Benzer’s lab (Dudai et al., 1976; Quinn et al., 1974). The powerful genetic tools like the Gal4-UAS binary expression system, along with the ease of creating mutations have made this an ideal organism to study (Brand and Perrimon, 1993; Venken and Bellen, 2005). Moreover, the well known Pavlovian olfactory learning protocol can be easily adapted to train flies and assess learning (Quinn et al., 1974). This has allowed the identification of various genes in *Drosophila* that lead to a loss in olfactory associative learning. Interestingly, most known memory mutants have defects in the cAMP-PKA pathway (Heisenberg, 2003).

Although there is plenty of information on the genes involved in learning at the whole organism level, those regulating plasticity at the single synapse level have remained largely unknown. The peripheral glutamatergic neuromuscular junction (NMJ) is probably the best studied synapse in *Drosophila*. Synaptic plasticity and signaling pathways like cAMP-PKA have been examined in great detail at the *Drosophila* NMJ (Renger et al., 2000; Wu et al., 1999). This is probably because the NMJ is easily
accessible, large in size and easy to record from, in contrast to the central synapses which are small and hard to access for synaptic current recording. Mutations in genes associated with memory loss also show altered synaptic transmission effects at the NMJ (Broadie, 1999; Rohrbough and Broadie, 2002; Rohrbough et al., 2000). However, the NMJ is primarily an excitatory synapse with no known inhibitory neurotransmitters. This makes it difficult to extend observations with mutants in the NMJ to central synaptic transmission since these synapses have both excitatory and inhibitory components.

A few methods for measuring synaptic transmission at central synapses have been developed over the last decade, including the dissection of the ventral nerve cord in situ (Baines and Bate, 1998) and accessing motor neurons of larval CNS (Rohrbough and Broadie, 2002). In the last few years, some studies have recorded synaptic currents from central synapses in vivo and in ex situ preparations. These recording have been done with neurons of the olfactory system, the mushroom body and the circadian system (Gu and O'Dowd, 2006; Sheeba et al., 2008; Wilson, 2011). Though these studies are very informative, they are mostly restricted to the superficial neurons of the Drosophila brain and can thus not be used on all central synapses. Similarly, the use of reporter dyes for two photon imaging of the brain structures involved in learning lack the spatial resolution provided by electrophysiological recordings from individual neurons. Moreover, such studies are limited by the number of photo-activable dyes available for use (Gervasi et al., 2010; Tomchik and Davis, 2009).
A relatively simple and highly reproducible method is to allow embryonic neurons to form synaptic connections in culture systems (Lee and O'Dowd, 1999; Wu et al., 1983). The central synapses formed *in vitro* have been shown to have functional excitatory cholinergic synapses and inhibitory GABAergic synapses. These currents result from the activation of nicotinic acetylcholine receptors and the GABA-Rdl subunits respectively (Lee and O'Dowd, 1999; Lee et al., 2003). This system has been used to demonstrate that the learning mutant *dunce* has altered cholinergic transmission (Lee and O'Dowd, 2000); and, that the cAMP-PKA pathway is involved in regulating excitatory synaptic transmission by dopamine through the D1 like receptors in *Drosophila* (Yuan and Lee, 2007).

*Drosophila* is an attractive model system to study olfaction and olfactory associative learning since the circuits are simple. Despite this, the fly can sense a range of odors; there are unique and non-redundant neurotransmitter receptors; and, a wide range of behavioral tasks can be examined (Wilson, 2011). Moreover, the neurons of the olfactory system are reasonably accessible for *in vivo* and *ex vivo* electrophysiology recordings (Gu and O'Dowd, 2006; Tanaka et al., 2009; Wilson, 2011). It is thus not surprising to note that the *Drosophila* has been extensively used for Pavlovian associative learning assays for decades (Davis, 2011; Heisenberg, 2003; Keene and Waddell, 2007).

The following section describes how the olfactory system is functionally organized and how various components of this system are involved in memory acquisition, consolidation and retrieval.
1.7: The *Drosophila* Adult Olfactory System

The representation of accurate sensory information from the environment to a higher brain center poses a significant challenge for mammals. This is critical not only for humans and other mammals with a highly sophisticated nervous system, but also for simple invertebrates like the fruit fly (*Drosophila melanogaster*). *Drosophila* adults have to sort a combination of odors in the atmosphere to locate a food source, decide between preferred and non-preferred food sources, and to find mates using pheromone clues. Though these tasks seem rather simple, work on the *Drosophila* olfactory system has shown that their olfactory system is highly sensitive and has significant parallels with the complex rodent olfactory system (Vosshall, 2000).

The discovery of the olfactory receptor genes in rodents (Buck and Axel, 1991), *C. elegans* (Sengupta et al., 1996) and *Drosophila* (Vosshall et al., 1999) by Richard Axel’s lab led to the unraveling of the olfactory system architecture. The *Drosophila* olfactory system consists of around 1300 olfactory sensory neurons (OSNs) distributed in the third antennal segment and maxillary palp (Selcho et al., 2009). Every OSN expresses a particular type of olfactory receptor and sends its axonal projections to one of 43 glomeruli in the antennal lobe (olfactory bulb in mammals). The ORNs which express the same OR send their axons to one specific glomerulus. This branching pattern is highly stereotypic and similar to that seen in the mammalian system (Vosshall, 2000).
The odor information from the antennal lobe (AL) is transmitted to the calyx glomeruli (dendrites of Kenyon cells of the MB) and the lateral horns by about 150 projection neurons (PN) which form the secondary olfactory center (Martin, 2002; Ramaekers et al., 2005; Wong et al., 2002). The dendritic projections of the PN lie in the antennal lobe glomeruli and the axonal processes terminate on various calyx glomeruli. Some specific PN send axons that terminate on corresponding calyx glomeruli whereas other PN bypass the calyx glomeruli and head directly to the lateral horn. Since the PN from specific antennal lobes terminate on their specific calyx glomeruli, this information relay system is highly stereotypic with an anatomical pattern similar to the OSN to AL mapping (Selcho et al., 2009).

The calyx glomeruli are made up of the dendritic processes of the Kenyon cells. Kenyon cells are the third order olfactory neurons and their branching axons make up a structure called the mushroom body (MB). The mushroom body can be considered similar to the piriform cortex in mammals. It is estimated that around 2500 Kenyon cells send out dendrites to make up hundreds of calyx glomeruli (Heisenberg, 2003). These dendrites then converge to form a structure called the peduncle. The cell bodies of the Kenyon cells are located on the posterior dorsal part of the brain. The axons of the Kenyon cells branch anterior to the peduncle to form two vertical lobes (α and α’) and three medial lobes (β, β’ and γ). These lobes were designated by variable gene expression patterns of genes such as DCO, PKA, RUT, FASII which are known to be highly expressed in the MB (Crittenden et al., 1998). In summary, the structure defined as the mushroom body consists of the Kenyon cells, the peduncle and the three lobes
formed by the axons of the Kenyon cells (Fig. 1.02 A). Each Kenyon cell sends dendrites to numerous calyx glomeruli and the response of the Kenyon cell to a particular odor is highly sparse as compared to the response seen at the ORNs or the PNs (Olsen et al., 2007; Perez-Orive et al., 2002; Wilson et al., 2004). The sparse response of the Kenyon cells to a particular odor suggests a system where odor concentration can be discriminated from a range of odors.

Thus, though simpler than the mammalian olfactory system, the *Drosophila* olfactory system does display adequate complexity to discriminate between various odor types from a mix of odors and amongst various odor intensities. This complexity means that the adult olfactory system in *Drosophila* may not be the simplest possible system to study the basic architecture and nature of odor coding in the higher brain center. Thus over the years the relatively simpler *Drosophila* larvae have emerged as an attractive model for studying olfaction and related higher order functions like olfactory learning.

**1.8: The Drosophila Larval Olfactory System**

The *Drosophila* larval olfactory system is much simpler than that seen in the adult (Selcho et al., 2009). Larvae do not require the high spatial odor discrimination ability necessary in the adults who must fly around to detect food sources and find mates based on cues from a low concentration of pheromone. The larvae have 21 OSN neurons (Singh and Singh, 1984), each of which has its own OR with unique odor profile (Kreher et al., 2008). Each OSN then makes a one to one connection on to 21 antennal lobe (AL) glomeruli. The AL glomeruli are then connected to the calyx glomeruli by 21 projection neurons
(Ramaekers et al., 2005). In each glomeruli the axonal process of each projection neuron (PN) synapses on to the dendritic tree of several Kenyon cells. Thus, although much simpler than the adult, the larval olfactory system still retains a high amount of stereotypy like the adult olfactory system.

The third order neurons in the larvae are in the mushroom body (MB) which contains around 600 γ neurons by the early third instar stage (Lee et al., 1999). The MB forms a functional unit which is capable of both aversive and appetitive olfactory learning (Honjo and Furukubo-Tokunaga, 2005, 2009; Scherer et al., 2003). It has been shown that like the adult, the larvae are capable of pairing aversive and appetitive stimuli with visual stimuli (Aso et al., 2009). It has been suggested that the one-to-one ratio seen between the various components of the larval olfactory system reduces the high signal to noise ratio apparent in the Drosophila adult (Selcho et al., 2009). This in turn leads to a limited spatial odor discerning capability which may suffice for the substrate dwelling larvae (Selcho et al., 2009).

1.9: Structure, Development and Significance of the Larval Mushroom Body

The Drosophila larvae have a mushroom body remarkably similar to the adult counterpart described in section 1.7 above. There are about 200-300 Kenyon cells of embryonic origin, which expand to about 2500 Kenyon cells in the adult (Ito and Hotta, 1992; Technau and Heisenberg, 1982). The calyx and peduncle seen in the larval mushroom body are similar to that in the adult (Fig. 1.02 B). However, the larval MB has
two lobes (one medial and one dorsal) instead of the five in the adult. The other three lobes emerge late during larval and pupal development.

The availability of genetic tools like the Gal4-UAS expression system (Brand and Perrimon, 1993) and MARCM (Mosaic Analysis with a Repressible Cell Marker) (Lee et al., 1999) has allowed tracking the development of MB neurons from the neuroblast. Each half of the mushroom body develops from a set of four neuroblast cells in the early embryo (Noveen et al., 2000). These neuroblasts divide continuously through larval and pupal development to give rise to the various MB neurons (Ito et al., 1997; Ito and Hotta, 1992; Prokop and Technau, 1991, 1994; Truman and Bate, 1988). Clonal analysis has revealed that the Kenyon cells which project their axons to the γ lobe are born first (before the beginning of the third instar stage). The neuroblasts then switch to give rise to neurons which project to the α' and β' lobes 72 hours after larval hatching and stop producing these after puparium formation (108-120 hours after larval hatching). They then give rise to α/β MB neurons (Lee et al., 1999). During development, the newly born Kenyon cell axons move into the central core of the pedunculus and push the older born axons towards the edges, thus creating a layered arrangement (Kurusu et al., 2002). Overall, each MB neuroblast gives rise to 600 MB neurons during development (Lee et al., 1999).

The mushroom body neurons in the adult fly have been shown to be critical for a range of higher order sensory functions like learning and memory (Keene and Waddell, 2007), sleep (Joiner et al., 2006; Pitman et al., 2006), visual learning (Liu et al., 1999), courtship behavior (McBride et al., 1999), and locomotor activity (Martin et al., 1998). Different MB
neurons and even some MB extrinsic neurons are known to be involved in olfactory associative learning in the adult fly. Specifically, the γ neurons have been shown to be essential for short term memory (Zars et al., 2000); the α/β lobe neurons are needed for long term memory (Pascual and Preat, 2001; Yu et al., 2006) and, for memory retrieval (Dubnau et al., 2001; McGuire et al., 2001; Schwaezel et al., 2002). The output from the α'/β' lobes has also been shown to be essential for acquiring and stabilizing memory (Krashes et al., 2007). The output from other MB intrinsic neurons (non-Kenynon) like DPM (Dorsal Paired Medial) and APL (Anterior Paired Lateral), which have axons projecting to the MB lobes, has been shown to be critical for memory consolidation (Keene et al., 2006) and suppression of memory formation (Liu and Davis, 2009), respectively. These studies indicate that the many subsets of MB neurons are critical for various forms of memory formation and hence sensory information processing in the higher brain center. Thus, accurate development and organization of these MB neuronal subsets may be critical for various stages of memory formation.

Despite having just the γ subset of MB neurons, the third instar stage Drosophila larvae have been shown to be capable of forming both middle term appetitive memory (Hendel et al., 2005; Honjo and Furukubo-Tokunaga, 2005) and aversive olfactory associative memory (Aceves-Pina and Quinn, 1979; Honjo and Furukubo-Tokunaga, 2009). However, the larvae seem to forget both these memories much faster than the adult flies. This may be due to the absence of α/β neurons or because of incomplete development of the α'/β' MB neurons, which are known to be critical for long term memory formation and memory consolidation in adults (Krashes et al., 2007; Pascual and Preat, 2001; Yu et
al., 2006). It is to be noted that in both the *Drosophila* adult and larvae, the output of the MB neurons is essential for memory retrieval but not for memory acquisition and consolidation (Dubnau et al., 2001; Honjo and Furukubo-Tokunaga, 2005; McGuire et al., 2001). This indicates that olfactory memory may be stored in the MB neurons.

Another critical aspect of memory formation is the circuitry outside of the MB neurons. These neurons are known as the MB extrinsic neurons and contain various classes of neurotransmitter releasing neurons. The following section describes the functions of these subsets of neurons in both the *Drosophila* adult and larvae.

**1.10: The Role of Extrinsic MB Neurons in Olfactory Learning**

In addition to the MB intrinsic neurons described above, there are a large number of neurons outside of the MB which are not a part of the MB neuropil. The anatomy, projection patterns and the classification of about 50 of these MB extrinsic neurons has been described in great detail (Tanaka et al., 2008). One group of neurons within these 50 MB extrinsic neurons, the MB-V2 neurons, has recently been shown to be essential for aversive memory recall but not memory formation. These MB-V2 neurons are cholinergic and project on to the tip of the α’ lobe of the MB (Sejourne et al., 2011). Apart from this, numerous other MB extrinsic neurons also send their axonal projections onto the various MB lobes and other parts of the brain. The neurotransmitter profile for most of these MB extrinsic neurons is still not known.
Many biogenic amine producing neurons in the *Drosophila* larval and adult brain are a part of the MB extrinsic neurons and may also provide inputs to the MB lobes. One such example is the ventral unpaired median cluster of neurons (VUM), which in the adult brain is thought to contain the neurotransmitter octopamine. These neurons extensively innervate the sub-esophageal ganglion (SOG) and also send out projections to the mushroom body calyx, the γ lobe and the antennal lobes (Busch et al., 2009; Busch and Tanimoto, 2010; Honjo and Furukubo-Tokunaga, 2009; Tanaka et al., 2008). In the *Drosophila* larvae also the expression of octopamine/tyramine is restricted to the MB calyx and the antennal lobe, with hardly any expression along the vertical MB lobes (Honjo and Furukubo-Tokunaga, 2009). The appetitive (sucrose-based) olfactory learning has been shown to be dependent on the output of octopaminergic neurons in both the adult (Kim et al., 2007; Schwaerzel et al., 2003) and the larvae (Honjo and Furukubo-Tokunaga, 2009; Schroll et al., 2006). Since the octapamine (OA) neurons extensively innervate the SOG and MB, it is likely that the sucrose signal may be conveyed to the MB via direct and in-direct (AL-MB) connections.

Dopamine (DA), another major monoamine, is also present in several neurons in the *Drosophila* brain. In the adult brain, dopaminergic neurons are known to innervate almost all lobes of the MB and the peduncle. A low level of innervation has been seen in the MB calyx as well. Some interglomerular innervation of the antennal lobe has also been reported (Honjo and Furukubo-Tokunaga, 2009; Tanaka et al., 2008). In the *Drosophila* larvae around 95 dopaminergic neurons extensively innervate the vertical MB lobe (Selcho et al., 2009). However, no innervation to the AL and the calyx is seen (Honjo
and Furukubo-Tokunaga, 2009). Like the OA neurons, the output of the DA neurons is also known to be critical for the acquisition of aversive learning in both the adult (Schwaerzel et al., 2003) and larvae (Honjo and Furukubo-Tokunaga, 2009; Schroll et al., 2006; Selcho et al., 2009). The projection of DA neurons to just the MB and not the AL may account for the fact that aversive memory decays faster than appetitive memory in both the *Drosophila* larvae and adult (Honjo and Furukubo-Tokunaga, 2009).

Most monoamines expressed in the fly brain have G-protein coupled receptors (GPCRs) which can modulate cellular cAMP levels and other downstream signaling pathways. These GPCRs may be essential for conveying the unconditioned stimulus (sucrose or electric shock) upon activation of the OA and DA neurons. Both the OAMB receptor (for OA) and two DA GPCRs, dDA1 and DAMB are expressed in the MB neurons (Han et al., 1998; Han et al., 1996). The dDA1 receptor has not only been shown to be critical for aversive learning in the adult, but is also known to partially influence appetitive learning (Kim et al., 2007). This suggests that in addition to the branching pattern of various mono-aminergic neurons on to the MB lobes, the expression of the monoamine GPCRs and the signaling downstream to them may be essential for memory formation.

1.11: Biogenic Amines and their Receptors Involved in *Drosophila* Learning

In mammals and invertebrates like *Drosophila*, both the biogenic amines and their receptors are equally important in learning behavior. Dopamine receptors have been shown to regulate locomotor activity, sleep, arousal and olfactory associative learning
(Riemensperger et al., 2011). The six identified clusters of dopaminergic neurons innervate almost all major structures of the brain including the central complex and mushroom body (Davis, 2011). The Dopamine D1 like receptor has been shown to be essential for aversive olfactory learning (Kim et al., 2007).

The invertebrate biogenic amine octapamine (OA) is a homolog of the mammalian epinephrine. This biogenic amine has been implicated in egg laying behavior, flight or fight behavior, sleep, circadian rhythms and appetitive learning and memory (Evans and Maqueira, 2005). The OA neurons innervate the mushroom body and the antennal lobe and OA has been shown to be essential for appetitive olfactory learning in both the Drosophila larvae and adult (Honjo and Furukubo-Tokunaga, 2009; Tanaka et al., 2008). Octapamine has four known GPCRs (Balfanz et al., 2005) amongst which the OAMB receptor is known to be expressed in the mushroom body (Han et al., 1998). Though this receptor activates cAMP there are no reports linking it to olfactory associative learning.

Serotonin, the last of the biogenic amines, has been relatively poorly studied in Drosophila, though it has been shown to be involved in sleep and aggression (Certel et al., 2010). A study done more than two decades ago showed that the brains of both the Drosophila adult and third instar larvae have 22 serotonin (5-HT) positive neurons (Valles and White, 1988). Much later, serotonin was shown to be involved in spatial learning in Drosophila (Sitaraman et al., 2008). A recent study has shown that the Dorsal-Paired Median (DPM) neurons, known to be involved in olfactory learning, contain serotonin (Lee et al., 2011). These serotonergic DPM neurons innervate the mushroom
body and mediate anesthesia resistant form of learning through the d5-HT1A receptors, which are expressed in the MB (Lee et al., 2011).

However, the role of serotonergic neurons and the serotonin GPCR receptors on olfactory learning has not been studied in detail. In particular, the functional consequence of the expression of the three known *Drosophila* 5-HT GPCRs (Saudou et al., 1992) in the mushroom body on learning and memory has remained unexplored. The d5-HT1A/1B receptor in *Drosophila* has been shown to be expressed in the mushroom body and is implicated in sleep regulation (Yuan et al., 2005). The d5-HT2 receptor expression has also been seen in the *Drosophila* brain and has been associated with courtship behavior (Nichols, 2007). However, the expression of the third GPCR, d5-HT7 receptor, has only recently been reported in the adult and larval brain (Becnel et al., 2011). Other than this, there is no study which examines the expression of the d5-HT7 receptor in the fly brain or explores its role in the context of learning.

**1.12: Learning Assay in the *Drosophila* Larvae: Principle and Design**

*Drosophila* larvae are a good model to study since their brain architecture is relatively simple and consist of a reasonably small number of connections, interneurons and feedback circuits. Moreover, there are just two lobes in the MB instead of the five seen in the adult. This system can thus be easily used to delineate the function of a specific molecule or receptor in olfactory learning since the results are straightforward to interpret in the absence of multiple receptor homologs and complex circuits.
Various larval olfactory associative learning assays have been described in the literature, including the one by Furukubo-Tokunaga and colleagues (Honjo and Furukubo-Tokunaga, 2005). This assay is essentially a modification of a larval chemosensory assay described earlier (Heimbeck et al., 1999). The assay checks if larvae can associate a particular odor with a positive or a negative reinforcer (1M Sucrose or 0.1% Quinine Hemisulphate, respectively). Thus, both appetitive and aversive forms of association can be studied. The third instar larvae used for this assay are a good system to study since only the γ neurons of the mushroom body are fully functional at this stage (Lee et al., 1999). While this assay allows for the association between a single odor and the reinforcer, it differs from the one developed by Stocker and colleagues (Scherer et al., 2003) in one key respect. In Stocker’s larval learning assay, individual or small groups (20-30) of larvae were allowed to associate an odor with a reinforcer and then asked to discriminate between two odors on a testing plate. The Furukubo-Tokunaga assay on the other hand relies on en masse training and testing. Importantly, both of these larval learning assays have been shown to depend on the same signaling molecules and receptors as those identified in adult learning assays (Selcho et al., 2009).

1.13: Major Aims of this Thesis

In this thesis I assess if changes in synaptic strength can be directly correlated to changes in learning behavior, as suggested by Cajal. I also examine the signaling molecules and receptors involved in the regulation of synaptic transmission and assess whether these molecules are also involved in behavioral learning.
The first part of the thesis deals with the role of cAMP signaling in the regulation of inhibitory GABAergic transmission. An increasing body of evidence suggests that inhibitory GABAergic interneurons are involved in both olfactory information processing (Wilson, 2011; Wilson and Laurent, 2005; Wilson et al., 2004), and in olfactory learning and memory (Liu and Davis, 2009; Liu et al., 2007). However it is not known if cAMP, a molecule known to be critical for olfactory learning (Heisenberg, 2003), is involved in the regulation of inhibitory GABAergic transmission. Using both Drosophila embryonic and pupal neuronal cultures as a model for central synapses (Lee and O'Dowd, 1999; Su and O'Dowd, 2003), I examine how cAMP modulates inhibitory GABAergic transmission. I specifically examine the molecular mechanism through which this regulation is achieved. I also look at olfactory learning and memory mutants and examine their MB neurons to assess if alterations in cAMP mediated inhibitory GABAergic transmission can account for their observed defects in learning and memory.

In the second part of the thesis I examine how serotonin (5-HT), an important neuromodulator of a range of behaviors (Lucki, 1998), regulate both excitatory and inhibitory synaptic transmission at central synapses. Although serotonin is involved in various behaviors, little is known about 5-HT mediated synaptic plasticity at central synapses. I thus examine the mechanisms by which 5-HT alters synaptic plasticity and also attempt to identify the downstream GPCR/GPCRs which mediate the effect of 5-HT on synaptic transmission.
In the third and final part of the thesis I examine if alterations in synaptic plasticity mediated by 5-HT and 5-HT GPCR/GPCRs can be correlated with changes in behavioral learning. For this, I use the olfactory associative learning assay in *Drosophila* larvae (Honjo and Furukubo-Tokunaga, 2005). I primarily examine if over-expression or down-regulation of the 5-HT GPCR/GPCRs in the neurons of the olfactory learning center can lead to changes in learning behavior.
Figure 1.01: Model for cAMP Activation

A) Diagrammatic representation of the NMDA-independent mechanism of cAMP generation.

B) The NMDA-dependent mechanism of cAMP generation.

The signaling molecules can be Serotonin (5HT), Dopamine, Octapamine and/or Tyramine. The GPCRs may be either the 5HT receptors, D1/D2 receptors and/or Octapamine receptors in Drosophila.
Figure 1.02: The *Drosophila* Mushroom Body
A) Schematic representation of the various anatomical structures in the adult *Drosophila* brain.
B) Confocal image of the larval mushroom body.
2.1: *Drosophila* Stocks

Flies were maintained on standard fly food medium containing cornmeal, agar, yeast, sucrose and dextrose mixed in water. All flies were kept at 24°C in a 14/10 hour light dark cycle. Wild-type flies used in all experiments were the \( w^{1118} \) (a “Cantonized” white eye stock) and the Canton-S genotype (Wt). The Tph-Gal4 line was a kind gift from Dr. Jaeseob Kim at KIAST. The UAS-5HT7R line was gifted by Dr. Julian Dow at University of Glasgow (Kerr et al., 2004) and the UAS-Shi\(^{ts1} \) line was a kind gift from Dr. Toshihiro Kitamoto at Univ. of Iowa (Kitamoto, 2001). MB-Gal80 strain was a gift from Dr. William Joiner at UCSD. All the other lines used were from the Bloomington Stock Center, details of which are encapsulated in Table 2.01. All homozygous lines used in this study having both the Gal4 drivers and the UAS constructs in the same fly line were made by standard genetic crosses using the \( w^{1118}; \) CyO/Sco; TM2/TM6B double balancer line. For P-element mobilization of the UAS-d5-HT7 gene from the II to the III chromosome, standard genetic cross scheme was used with a strain carrying the \( \Delta 2-3 \) transposase enzyme in the \( w^{1118} \) background. Flies obtained from Bloomington Stock Center are described in Table 2.01.
2.2: *Drosophila* Primary Neuronal Cultures

For the egg laying procedure, flies were allowed to lay eggs for four hours on an agar plate containing yeast paste to stimulate egg laying. After four hours, the embryos were collected and immersed in a 50% bleach solution for four minutes to dechorionate and then rinsed with sterile water several times. The dechorionated embryos were then transferred to new petri dishes and moved into a laminar flow hood (ThermoForma, Forma Sci Inc, MA). A 50 μL Drummond Wiretrol capillary tube (Drummond Scientific Company, PA) was pulled on an electrode puller PP-830 (Narishige International USA Inc, NY) to produce a sharp micropipette. These sharp micropipettes were attached to a suction tube and used to harvest neuroblast cells from the midgastrula stage embryos (O'Dowd, 1995). Cells from two embryos (or only one embryo for neuronal cultures with deficiency lines carrying a balancer chromosome with GFP, eg. CyO-GFP) were plated on uncoated glass coverslips (Bellco Glass Inc, Vineland, NJ). Three such coverslips were placed in a 35 mm BD Falcon culture dish (BD Biosciences, Canada) and flooded with *Drosophila* culture medium DDM1 containing high glucose Ham’s F12/DME medium (Irvine Scientific, CA), L-glutamine (2.5 mM, Irvine Scientific, CA), HEPES (20 mM) and four supplements: 100 μM putrescine, 20 ng/ml progesterone, 100 μg/ml transferrin, and 50 μg/ml insulin (Calbiochem, CA). All supplements were purchased from Sigma (Sigma-Aldrich, MO) unless otherwise specified. The dishes were maintained in an incubator supplied with 5% carbon dioxide at 24°C to 25°C for up to 9 days as previously reported (Lee and O'Dowd, 1999). Half of the culture medium (1 mL) was replaced with fresh medium after five days *in vitro*. G-α-s homozygous mutant cultures were obtained from single embryonic cultures made from embryos of the G-α-s
[R60]/CyO-GFP line described above. The GFP negative coverslips were selected under an inverted Olympus IX71 microscope at 20X magnification fitted with a UV laser and a phase contrast ring (Darya et al., 2009).

2.3: Pupal Neuronal Cultures

The pupal culture protocol was modified from the one described earlier (Su and O’Dowd, 2003). In brief, P8-P9 stage Drosophila pupae, 170-192 hours after egg-laying (Bainbridge and Bownes, 1981) were removed from the puparium with a pair of 253/8 gauge needles attached to 1 mL syringes and decapitated. A pair of sharp forceps was used to remove the brain from the head under a standard dissecting microscope (Olympus SZ40) at 4X magnification. Care was taken to remove the exoskeleton carefully to ensure that the entire brain was left intact. The optic lobes were discarded.

The entire dissection procedure was carried out in dissecting saline containing the following (in mM): 126 NaCl, 5.4 KCl, 0.17 NaH₂PO₄, 0.22 KH₂PO₄, 33.3 glucose, 43.8 sucrose, and 9.9 HEPES, pH 7.4. Eight to ten brains were dissected and kept in cold dissecting saline on ice until the next step in the culturing procedure. The brains were then placed in a laminar flow hood (ThermoForma, Forma Sci. Inc, MA) where they were washed thoroughly with sterile dissociation solution and then placed in an enzyme solution containing 5 U/ml papain, L-cysteine (5.5 mM), EDTA (1.1 mM) and 2-Mercaptoethanol (0.067 mM), all dissolved in the dissection saline solution. The brains were left in the enzyme solution for ten minutes at room temperature on a rocker. Brains were immediately washed 3-4 times in sterile Drosophila-defined culture medium (DDM2) composed of Ham’s F-12 DMEM (Irvine Scientific, CA) containing 20 mM
HEPES, 100 µM putrescine, 20 ng/ml progesterone, 50 µg/ml of insulin, 100 µM transferrin and 1 µg/ml of 20-hydroxyecdysone as supplements. 5 µl of DDM2 was then placed on the center of a glass coverslip coated with 0.01% of Poly-L-Lysine. Individual brains were transferred to the DDM2 with a 20 µl pipette attached to a regular pipette-man. Two 25½ gauge sterile needles were attached to 1 mL disposable syringes and then used to mechanically dissociate each brain into 4-6 fragments, under a standard dissection microscope. Care was taken not to mince the brain into too many small fragments. Freshly pulled glass pipettes were used to mechanically disperse the fragments using gentle suction pressure. The cells were allowed to settle on the coverslip for 30 minutes before flooding them with DDM2 and then maintained in a 24.5 ºC, 5% CO₂ incubator. 24 hours after plating the cells 0.5 mL of DDM2 and Neurobasal (containing L-glutamine) in 3:1 ratio was added to the dishes. This was done once every three days in vitro after day one. Pupal cultures 3 to 5 days in vitro were used for electrophysiology experiments (Fig. 2.01).

2.4: Electrophysiology

Each coverslip containing Drosophila neuronal cultures was transferred into a recording chamber containing the following external solution (in mM): 140 NaCl, 1 CaCl₂, 4 MgCl₂, 3 KCl and 5 HEPES, pH 7.2. Pipettes for recordings were prepared by pulling borosilicate glass pipettes (Cat#53432-921, VWR International USA) with an electrode puller PP-830 (Narishige International USA Inc, NY). Postsynaptic currents (PSCs) were recorded with whole-cell pipettes with tip resistance of 6-8 MΩ for embryonic cultures and 8-10 MΩ for pupal neuronal cultures under a 40X objective on either a Nikon Eclipse
TE200 inverted microscope (Nikon Instruments Inc., USA) or a Motic AE31 inverted microscope (Motic Corporation, Canada). All recordings were carried out with pipettes filled with internal solution containing (in mM): 120 CsOH, 120 d-gluconic acid, 0.1 CaCl₂, 2 MgCl₂, 20 NaCl, 1.1 EGTA and 10 HEPES, pH 7.2. An Axopatch 200B amplifier (Axon Instruments Inc., CA) was used to measure PSCs. For recording cholinergic PSCs the voltage was held at -45 mV, the reversal potential of GABAergic currents, and at 0 mV for GABAergic PSCs (Lee et al., 2003). Tetrodotoxin (1 μM) was added to the external solution during the recording of miniature PSCs (mEPSCs/mIPSCs). Cholinergic mEPSCs were recorded at -45 mV and GABAergic mIPSCs were recorded with the cell held at 0 mV. For all spontaneous action potential (AP) recordings the cell was in the cell-attached mode and was thus extracellular in nature. For the perfusion experiments, the bath solution was replaced with external solution containing 20 μM or 2 μM forskolin (FSK), while the cell was still attached to the recording pipette using a perfusion system with vacuum/solution flow control valves FR50 (Warner Instruments Inc, CT). For the PKI (6-22) amide experiments, the drug was added to the internal solution in the recording pipette. This allowed for the effect of the drug to be restricted to the post-synaptic neuron. All drugs and neurotransmitters were focally delivered for 30 seconds to the patched neuron by using a Picospritzer III (Parker Hannifin Corp., NJ). For the perfusion experiments 100 μM GABA was focally applied for 2 seconds.

2.5: Pharmacology

Forskolin (FSK, Sigma-Aldrich, MO) was used as an activator of adenylate cyclase. db-cAMP (Calbiochem, USA) was used to mimic cAMP. H-89 (Sigma-Aldrich, MO) was
used as an inhibitor of PKA. PKI (6-22) amide (Biomol, PA) was used as a membrane impermeable PKA blocker. SB258719 (Tocris Biosciences, MO) was used as a selective 5-HT7 receptor antagonist. 5-HT (Sigma-Aldrich, MO) and GABA (Sigma-Aldrich, MO) were used for focal application. Poly-L-Lysine (Sigma-Aldrich, St. Louis MO) was used to coat cover slips for pupal neuronal cultures. Pentyl-Acetate (Sigma-Aldrich, MO), Propionic Acid (Fluka) and Quinine Hemsulphate (Sigma-Aldrich, MO) of the highest available purity were used for the larval learning assay. Acid Red 52 (ScienceLab Inc., TX) a common food dye was used to color the larval gut.

2.6: Immunohistochemistry

Embryonic neuronal cultures between at 3–9 DIV (days in vitro) were fixed in 4% paraformaldehyde (dissolved in 10 mM phosphate buffer saline) for 40 minutes on ice. The cover slips were then washed thoroughly with 10 mM PBS (phosphate buffer saline) at room temperature. The permeabilization was carried out in 10 mM PBS containing 0.1% Triton X-100 and 5% Normal Goat Serum (Sigma-Aldrich, MO) for 30 minutes on ice. The cover slips were washed once in 10 mM PBS for 10 min before being incubated with the primary antibody overnight at 4°C. On the second day of the procedure, the cover slips were washed thoroughly with PBS and then incubated with the respective secondary antibody to fluorescently label the target molecules. In all the staining experiments DAPI (1:2000, Molecular Probes) was used to label the nucleus of the neurons. The immunostained cultures were then mounted on glass slides and sealed with rectangular glass cover slips using nail polish.
For staining the *Drosophila* adult and larval brains, the brains were dissected out from
the head using sharp forceps in ice cold dissecting saline solution. The brains were then
fixed in 4% paraformaldehyde for 30 minutes on ice and then washed thoroughly with
PBS. The brains were then permeabilized in 10 mM PBSTX (PBS + 0.1% Triton X-100)
and 5% Normal Goat Serum (Sigma-Aldrich, MO) for one hour at room temperature on
a rocker. Primary antibody incubation was overnight at 4°C. The following day, after
three 30 minutes washes at room temperature in PBSTX, secondary antibody incubation
was carried out for 2 hours at room temperature (adult brains) and overnight at 4°C
(larval brains). The secondary antibody was diluted in the blocking solution. The brains
were then washed thoroughly before mounting and imaging.

### 2.7: Reverse Transcription PCR Analysis

Exon1-Exon2 boundary spanning primers were designed for the 5-HT7R gene homolog
in *Drosophila melanogaster*. Primers for the RP49 housekeeping gene were used as a
positive control. The *Drosophila* mRNA sequence was used in PrimerBlast (NCBI) to
design the primers. Primers for the 5-HT7R homolog gene (Forward Primer: 
TCGTTGACCCAGTTCCCGACGA; Reverse Primer: CCAGTGCTGACCTGCTGCCC)
and RP49 primers (Forward Primer: GAGAACGAGGCGGCCGTTG Reverse primer: TGACCATCCGCCAGCATAC) were obtained from Eurofins MWG
Operon Inc. USA. These sets of primers were designed to yield a product size of 334
base pairs (5-HT7R) and 390 base pairs (RP49) with the isolated mRNA. Exon-exon
spanning primers allowed elimination of products amplified from contaminating
genomic DNA, as this would give a larger sized product than that expected with only
mRNA. The mRNA was isolated using the RNeasy Mini Kit (Qiagen Inc., CA) by following the protocol for animal tissue samples described in the product manual. 16-18 3rd instar larvae were isolated for each genotype and crushed in 300 μL RLT buffer using a pestle in a 1.5 mL tube. The lysate was passed through the QiaShredder (Qiagen Inc., CA) for homogenization and then an isolation protocol was followed as described in the product manual. After RNA isolation, the RNA was quantified using Nanodrop 1000 (Thermo Scientific, DE). Any contaminating genomic DNA was removed using the DNA-free kit (Ambion Inc./Applied Biosystems, TX) as per the manufacturer’s protocol. After genomic DNA removal, the RNA was re-quantified and converted to cDNA using the Omniscript RT Kit (Qiagen Inc., CA) for 1 μg of RNA in a final reaction volume of 20 μL as per the manufacturer’s protocol.

PCR was performed using a PCR kit (Clontech Laboratories Inc., WI), for a final volume of 30 μL. 10 mM stock solution of each primer for both sets and 1 μL of mRNA were added to the reaction mixture in both cases. Annealing temperature for 5-HT7R and RP49 primers was 65°C and 60°C respectively. A standard 30 cycle PCR reaction was set up on a PTC 150 Mini Cycler (MJ Research/Bio-Rad Laboratories., CA) for both the primer sets.

For the mRNA isolation from FACS sorted GFP+ and GFP- cells (see section 2.08 below), a total of one million GFP+ cells were taken by pooling together cells obtained from 3 separate sorts. The mRNA was isolated using the protocol described for animal cells in the Qiagen RNeasy Micro Kit (Qiagen Inc., CA). The mRNA was converted to cDNA
using the AffinityScript qPCR cDNA synthesis Kit (Stratagene, CA) for a final volume of 40 μL as per the manufacturer’s protocol. PCR was performed using a PCR kit (Takara Bio Inc., Japan) for a final volume of 20 μL. A 10 mM stock solution of each primer was used for both genes. The annealing temperature for 5-HT7R and RP49 primers was 65°C and 60°C respectively. A 40 cycle PCR reaction was set up on a PTC 150 Mini Cycler (MJ Research/Bio-Rad Laboratories, Hercules CA) for both primer sets.

2.8: Fluorescence Activated Cell Sorting (FACS) of Mushroom Body Neurons

To obtain a single cell suspension for FACS sorting, 3rd instar larvae from the 201Y-Gal4; UAS-mCD8::GFP homozygous strain (84-87 hours after egg-laying) were collected. The brains were dissected and the ventral nerve cords removed for 100 such larvae for each sort experiment. About 25 w^{118} (wild-type) larval brains were dissected simultaneously and served as the negative control for the FACS sorting. The brains were dissected and placed in ice cold dissecting saline solution (described in section 2.3) before processing. The brains were washed with 0.1% PBS solution twice and spun down in 1.5 mL Eppendorf tubes. A pestle was used to homogenize the brains before treatment with 0.25% Trypsin-EDTA (Sigma-Aldrich, MO) for 4-5 minutes at 37°C. Fetal Bovine Serum (Invitrogen, USA) to a final concentration of 4% was added to stop the action of Trypsin-EDTA. The suspension was passed through a 70 micron filter (Catalog# 35 2350, BD Biosciences, CA) and re-suspended in 0.1% PBS before sorting. For the sorting procedure, a pre-sort was done on BD Aria FACS sorter with wild-type cells to set the gates for sorting GFP+ and GFP- cells. Following this, GFP+/GFP- cells were sorted
from the 201Y-Gal4; UAS-mCD8::GFP strain (see Figure 5.06). The sorted cells were flash frozen in liquid nitrogen and stored at -80°C until mRNA isolation.

2.9: Flourescence and Confocal Imaging

All images of the cultured embryonic neurons were obtained using an Olympus Inverted IX71 fluorescent microscope equipped with a SPOT CCD digital camera (Diagnostic Instruments, MI). All images in this study were acquired as 12-bit TIFF images. In order to minimize variability of signal intensity between experiments, the image-acquisition settings were kept constant.

The adult brain and the larval brain images were taken using a Zeiss Laser Scanning Microscope 510 (Carl Zeiss, Inc., USA). For imaging the mushroom body in the adult fly brains, 1.5 micron z-sections were taken at 25X magnification and then stacked. For the larval mushroom body images, 1.0 micron z-sections were taken at 25X magnification and then stacked. The individual images were stacked to create a 3D image using the Zeiss LSM Zen 2008 software.

2.10: Larval Learning Assay

The larval learning assay was adapted from the one described earlier (Honjo and Furukubo-Tokunaga, 2005). Briefly, 3rd instar larvae, 84-87 hours after egg laying on fly food were separated from the fly food by using 15% glucose solution. Parental flies not more than 15 days old were used for egg laying. Since the density of the larvae was less than that of the fly food, the larvae floated to the top and were then transferred onto a
sieve. The 500 micron sieve (Newark Wire Cloth Company) retained the larvae on top of the mesh whereas small bits of food and the glucose solution passed through. The larvae were washed several times with water to remove any traces of glucose.

For the training stage about 100 washed larvae were taken on a freshly made 2.5% agarose plate (8.5 cm in diameter) containing 1 mL of 1 M sucrose solution spread as a thin film on top. The inner surface of the lid of the plate had a filter paper disc (Whatmann filter) spotted with 10 μL of undiluted odor (Pentyl acetate or Propionic acid). The lid was then put on top of the agar plate. For control experiments, a similar agar plate was spread with a thin film of 1 mL of ddH\textsubscript{2}O instead of the sucrose solution. About 100 larvae were put on this control plate in the same order as described above for the training plate. The larvae were allowed to associate the odor with the sucrose and distilled water for 30 minutes at 24.5°C in a uniformly lit fume hood with a white table top background.

For the testing phase of the experiment, 50-100 trained larvae were lined along the center of a fresh Petri dish containing 2.5% agarose. This dish had two filter discs, each placed on the top of a 1.5 mL Eppendorf tube cap pressed down into the agar at a distance of 0.7 cm from the each edge of the plate. Each cap along with the filter disc formed the center of a 3 cm semi-circle on both sides of the center line (Fig. 2.02). 2.5 μL of the odor used for training was then spotted on one of the filter discs and the lid was closed. The larvae were allowed to move towards the odor and non-odor side and the response index (R.I) were calculated after 3 minutes as described below:
Response Index (R.I) =

(No. of larvae in the 3 cm ring with odor – no. of larvae in the 3 cm ring without odor)

Total number of larvae in both the rings

2.11: Data Analysis

Individual PSCs were analyzed using the Minianalysis detection software (Synaptosoft, Decatur, GA, USA) with threshold criteria for individual events of 7.5 pA amplitude and 7 pF charge transfer for cholinergic PSCs (Lee and O’Dowd, 1999) and at 5 pA and 5 pF for GABAergic PSCs (Lee et al., 2003). Only events with a fast rising and slowly decaying phase were used for analysis. For each acquired trace of data, all events were detected and the frequency was calculated every 5 seconds. If the PSC frequency declined continuously over 20 seconds before drug application, a rundown of PSCs was suspected and the data were not analyzed further. For the frequency analysis, only PSCs (before drug application) with a stable frequency longer than 20 seconds were used to calculate the control average frequency. This calculated control average frequency was then normalized as 100%. The frequency of the entire recording was compared with the control average frequency, and then the percent frequency was calculated and plotted before, during and after drug application. The Minianalysis detection software was used for examining biophysical properties of cholinergic and GABAergic PSCs. Like the frequency analysis, the amplitude of cholinergic and GABAergic PSCs in the drug application phase was averaged and compared with the control amplitude. The rise time and decay of PSCs were analyzed in the same way and plotted in Origin 7.0 (OriginLab, MA).
Table 2.01: Fly Strains Obtained from Bloomington Stock Centre

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Bloomington Stock No.</th>
<th>Complete Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT7R mutant</td>
<td>#24705</td>
<td>(w^{1118}; Mi{ET1}5-HT7^{MB04445}) (CG31008^{MB04445})</td>
</tr>
<tr>
<td>5-HT7R Deficiency</td>
<td>#24142</td>
<td>(w^{1118}; Df(3R)ED6346/TM6C, cu^{1} Sb^{1})</td>
</tr>
<tr>
<td>UAS-5-HT7R-RNAi</td>
<td>#27273</td>
<td>(y^{1} v^{1}; P{TRiP.JF02576}attP2)</td>
</tr>
<tr>
<td>UAS-GFP (III) T10</td>
<td>#1522</td>
<td>(w^{*}; P{UAS-GFP.S65T}T10)</td>
</tr>
<tr>
<td>(rut1)</td>
<td>#9404</td>
<td>(rutabaga^{1})</td>
</tr>
<tr>
<td>(dnc1)</td>
<td>#6020</td>
<td>(dunce^{1})</td>
</tr>
<tr>
<td>201Y-Gal4</td>
<td>#4440</td>
<td>(w^{1118}; P{GawB}Tab2^{201Y})</td>
</tr>
<tr>
<td>c309-Gal4</td>
<td>#6906</td>
<td>(w^{*}; P{GawB}c309)</td>
</tr>
<tr>
<td>1407-Gal4</td>
<td>#8751</td>
<td>(w^{*}; P{GawB}1407)</td>
</tr>
<tr>
<td>30Y-Gal4</td>
<td>#30818</td>
<td>(w^{*}; P{w^{+mW.hs}=GawB}30Y)</td>
</tr>
<tr>
<td>G-(\alpha)-s null mutant</td>
<td>#6340</td>
<td>(P{neoFRT}42D bw^{1}) (Gsa60A^{R60}/SM6b, P{eve-lacZ8.0}SB1)</td>
</tr>
<tr>
<td>tubP-Gal80(^{ls})</td>
<td>#7019</td>
<td>(w^{*}; P{w^{+mC}=tubP}- GAL80[ts])20; TM2/TM6B, Tb[1]</td>
</tr>
<tr>
<td>Delta 2-3 transposase</td>
<td>#3664</td>
<td>(y[1] w^{*}; ry[506] Sb[1]) (P{ry^{+t7.2}=Delta2-3}99B/TM6)</td>
</tr>
<tr>
<td>Putative 5HT7R-Gal4#1</td>
<td>#46627</td>
<td>(w[1118]; P{w^{+mC}=GMR70A04-GAL4}attP2)</td>
</tr>
<tr>
<td>Putative 5HT7R-Gal4#2</td>
<td>#46629</td>
<td>(w[1118]; P{w^{+mC}=GMR70A10-GAL4}attP2/TM3, Sb[1])</td>
</tr>
</tbody>
</table>
Figure 2.01: Pupal Cultures
A, B) Diagrammatic and pictorial representation of P8-P10 staged pupae inside the cuticle, respectively.
C) Represents an image of 9 pupal brains after they were removed from the cuticle and dissected out.
D) Bright-field image at 20X magnification of 3-DIV pupal neurons from the wild-type strain.
Figure 2.02: The Larval Learning Assay Set-Up
A) A schematic representation of the larval learning training plate.
B) A schematic representation of the larval learning testing plate.
C) An actual image of the larval learning training plate containing odor spotted on the inside of the filter paper attached to the lid.
D) An image of the larval learning testing plate before the beginning of testing (before the larvae were added).
CHAPTER THREE

Suppression of Inhibitory GABAergic Transmission by cAMP Signaling Pathway: Alterations in Learning and Memory Mutants

3.1 INTRODUCTION

A strengthening in the efficacy of excitatory transmission underlies enhanced synaptic transmission such as hippocampal long-term potentiation (LTP) and facilitation (LTF) in Aplysia (Milner et al., 1998). Such enhancement of synaptic transmission, LTP in particular, may be essential for neural information storage (Bliss and Collingridge, 1993). cAMP signaling is known to regulate hippocampal LTP in the Schaffer collateral pathway by activating its downstream target protein kinase A (PKA) (Frey et al., 1993). In Aplysia, blocking PKA mediated signaling in the sensory neurons which are presynaptic to the gill results in impaired synaptic facilitation (Castellucci et al., 1982). In Drosophila a number of short-term memory genes are essential molecular components of the cAMP signaling cascade. For example, dunce encodes a phosphodiesterase which breaks down cytoplasmic cAMP (Dudai et al., 1976) and rutabaga codes for the cAMP synthesizing enzyme adenylate cyclase (Livingstone et al., 1984). These genes are expressed preferentially in the mushroom body (MB), a brain region involved in various cognitive processes, including olfactory learning (Keene and Waddell, 2007). The over-expression of wild-type rutabaga in the MB neurons is known to be sufficient to overcome defects in olfactory learning (Zars et al., 2000). This suggests that this pathway plays a significant role in Drosophila learning.
The *Drosophila* MB, thought to be an equivalent of the mammalian hippocampus (Cayre et al., 2002), receives inputs from a large number MB extrinsic neurons (Tanaka et al., 2008) containing the neurotransmitters acetylcholine (Sejourne et al., 2011), γ-aminobutyric acid (GABA) (Liu and Davis, 2009), octapamine (Busch et al., 2009), serotonin (Lee et al., 2011) and dopamine (Tanaka et al., 2008). All these neurons have been shown to be involved in olfactory learning. Amongst these, GABA is the only inhibitory neurotransmitter (Liu and Davis, 2009) shown to be involved in learning and memory.

γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter (Bormann, 1988), plays an important role in neuronal communication (Paulsen and Moser, 1998). In mammals, GABAergic neurons innervate the memory center hippocampus (Freund and Buzsaki, 1996). GABA is known to be critical for higher brain functions such as learning/memory and coordinated behaviors (Fernandez et al., 2007; Floyer-Lea et al., 2006). Inhibitory synaptic plasticity has also been observed at GABAergic synapses in rodent hippocampus CA1 neurons (Chevaleyre and Castillo, 2003; Lu et al., 2000). However, unlike excitatory synaptic plasticity (e.g. LTP), inhibitory GABAergic plasticity is relatively unexplored in the mammalian hippocampus. This is probably because of the complexity of hippocampal GABAergic circuits (Castillo et al., 2011) and GABA\(_\lambda\) receptor subunit composition in the hippocampus (Nusser et al., 1999). Though a few studies indicate an important role for GABAergic transmission in learning and memory (Fernandez et al., 2007; Floyer-Lea et al., 2006) it is still unknown if cAMP-dependent plasticity at inhibitory GABAergic synapses can regulate these processes.
Similar to the hippocampus, the *Drosophila* MB is also extensively innervated by inhibitory GABAergic neurons (Yasuyama et al., 2002). Sparsening of odor representation through GABAergic inhibition in the mushroom body (MB) neurons is thought to be a possible mechanism for information storage in locusts (Perez-Orive et al., 2002). GABAergic local neurons are known to be involved in olfactory information processing in *Drosophila* (Olsen and Wilson, 2008b; Wilson and Laurent, 2005) indicating that GABAergic transmission plays a crucial role in shaping odor response. The GABAergic neurons projecting on to the MB have also been implicated in olfactory learning (Liu and Davis, 2009). Further, it has been demonstrated that a *Drosophila* GABA\(_A\) receptor RDL inhibits olfactory associative learning (Liu et al., 2007). These reports suggest that GABA in the MB may be important for learning in *Drosophila* similar to that seen in the mammalian system.

Although it is known that GABA and GABA receptor expression are involved in learning, it is not understood how these components alter synaptic plasticity at central synapses in *Drosophila*. Though some studies have used *in vivo* electrophysiology to record from MB intrinsic neurons (Gu and O'Dowd, 2006) and cells of the olfactory system (Wilson et al., 2004), an extensive use of this technique is limited by the fact that recordings can only be done from superficial neurons around the brain. Thus an *in vitro* neuromuscular junction (NMJ) model has traditionally been used to study excitatory synaptic transmission in *Drosophila* memory mutants (Gasque et al., 2006). These cAMP mutants show alterations in facilitation and/or post-tetanic potentiation at the NMJ
(Rohrbough and Broadie, 2002; Zhong and Wu, 1991). However, NMJ preparations in Drosophila lack inhibitory GABAergic synapses and are thus not suitable to study inhibitory synaptic plasticity.

Cultured primary Drosophila neurons offer an alternate in vitro model from which both excitatory and inhibitory synaptic transmission can be recorded (Lee and O'Dowd, 1999; Lee et al., 2003). This culture system has been used to demonstrate that the cAMP and learning mutant dunce\textsuperscript{1} shows altered excitatory synaptic plasticity (Lee and O'Dowd, 2000). Inhibitory GABAergic synaptic transmission in these central synapses has also been shown to be mediated through RDL containing GABA\textsubscript{A} receptors (Lee et al., 2003). Thus this in vitro system can be used to examine the role of cAMP on inhibitory GABAergic plasticity and to record from identified subsets of neurons.

In this chapter, I use whole-cell patch-clamp to examine physiological mechanisms underlying the regulation of cAMP mediated GABAergic plasticity in Drosophila primary neuronal cultures. I also assess how alterations in this form of plasticity in the Drosophila mushroom body may affect the learning mutants. The broad aims addressed in this chapter include:

a) Examining the effect of cAMP on inhibitory synaptic plasticity at central synapses and assessing whether this form of plasticity is altered in cAMP mutants

b) Understanding the mechanism (pre-synaptic vs. post-synaptic) through which cAMP modulates inhibitory synaptic plasticity
c) Examining if this form of plasticity is altered in anatomical structures of the *Drosophila* brain involved in learning and memory (mushroom body)

### 3.2 RESULTS

#### 3.2.1 Forskolin Inhibits GABAergic IPSCs in Embryonic Neuronal Cultures

The effect of cyclic AMP (cAMP) on GABAergic inhibitory post-synaptic currents (IPSCs) in the central synapses of *Drosophila* has not been explored. I have thus used whole cell patch clamp on *in vitro* embryonic neuronal cultures (Lee and O'Dowd, 1999) to examine the effect of cAMP on inhibitory synaptic plasticity. My results indicate that focal application of 20 µM Forskolin (FSK) causes a significant decrease in the frequency of the GABAergic IPSCs (Fig. 3.01 A). Forskolin is an activator of the enzyme adenylate cyclase, which synthesizes cAMP. cAMP is known to activate the downstream enzyme Protein Kinase A (PKA). I thus explored whether the effect of cAMP on IPSCs is mediated through PKA. I observed that the application of 20 µM FSK in the presence of 50 µM H-89 (a membrane permeable inhibitor of PKA) in the external recording solution, resulted in no change in the frequency of the GABAergic IPSCs (Fig. 3.01 B). There was also no change in the baseline frequency of GABAergic IPSCs before the application of FSK both in the presence and absence of H-89 in the external recording solution. This suggests that the PKA blocker H-89 by itself does not cause a decrease in the baseline frequency of GABAergic IPSCs. My results thus suggest that the inhibition of GABAergic IPSCs in wild-type (w<sup>1118</sup>) primary neuronal cultures is mediated by cAMP in a PKA dependent manner.
3.2.2 The Effect of Forskolin on GABAergic IPSCs can be Mimicked by a cAMP Analog

Some studies have shown that forskolin can act on targets other than adenylate cyclase, such as *Shaker* potassium channels, to alter cellular excitability (Harris-Warrick, 1989; Hoshi et al., 1988) in *Drosophila* neurons. I thus assessed whether the effect of forskolin on synaptic plasticity was specifically due to cAMP and not a result of its action on other non-specific targets. For this, I used a cAMP analog db-cAMP and tested its effect on the inhibitory GABAergic IPSCs. In my experiments, the focal application of 10 mM db-cAMP for thirty seconds was able to mimic the effect of 20 µM FSK on the GABAergic IPSCs (Fig. 3.02 A). By varying the concentrations of both FSK and db-cAMP, I ascertained that the effect of FSK and db-cAMP on the GABAergic IPSCs was dose dependent (Fig. 3.02 B). I noted that much higher concentrations of db-cAMP were needed to obtain results similar to those seen by much lower concentrations of FSK. This can be explained by the fact that FSK acts on the enzyme which constitutively produces large amounts of cAMP. To achieve similar levels of cAMP in the cell, large concentrations of the cAMP analog are necessary. I thus conclude that the effect of FSK on GABAergic IPSCs is specifically due to the activation of adenylate cyclase.

3.2.3 The Effect of Forskolin on GABAergic IPSCs in cAMP Signaling Mutants

My results so far suggest that the inhibitory GABAergic IPSCs are modulated by cAMP and its downstream target PKA. This raises the possibility that FSK can differentially modulate GABAergic IPSCs in cAMP signaling mutants. To address this, I used two well characterized cAMP signaling mutants *dunce1* (*dnc1*) and *rutabaga1* (*rut1*). While
dnc1 has a mutation in the gene responsible for the breakdown of cAMP leading to elevated cellular cAMP levels (Dudai et al., 1976), the rut1 strain has a mutation in the Ca\textsuperscript{2+}/CaM dependent adenylate cyclase gene resulting in lower cellular cAMP levels (Livingstone et al., 1984). I observed that focal application of 20 µM FSK on the neuronal cultures of both these strains caused a significantly smaller decrease in the frequency of GABAergic IPSCs as compared to the wild-type neurons (Fig. 3.03). My results thus indicate that cAMP signaling is essential for the inhibition of GABAergic IPSCs. Since both these mutants are known to have defective olfactory learning (Keene and Waddell, 2007), it is possible that inhibitory synaptic plasticity has a role in olfactory learning.

3.2.4 cAMP Modulates GABAergic IPSCs through a Post-Synaptic Mechanism

Inhibitory GABAergic IPSCs have been shown to be mediated by post-synaptic GABA-Rdl receptors in *Drosophila* primary neuronal cultures (Lee et al., 2003). I thus assessed whether FSK-mediated suppression of GABAergic IPSCs is pre-synaptic or post-synaptic in nature. I addressed this point using the membrane impermeable PKA inhibitor PKI 6-22 amide. When added to the internal solution in the recording pipette, this PKA blocker is expected to stay restricted to the post-synaptic neuron, once whole cell mode is achieved. Thus the effects of the PKA blocker would be restricted to the post-synaptic neuron. In my experiments, I observed that 200 µM of PKI 6-22 amide was able to overcome the suppression of GABAergic IPSCs induced by the application of 20 µM FSK (Fig. 3.04). Thus the presence of the PKA blocker in the post-synaptic neuron was able to rescue the effect of FSK on GABAergic IPSCs. This indicates a post-synaptic locus for the action of FSK on GABAergic IPSCs. Interestingly, I also noted that when
400 µM of PKI 6-22 amide was added to the recording pipette, the frequency of the GABAergic IPSCs increased even during the application of 20 µM FSK (Fig. 3.04). This increase in frequency in the presence of PKI 6-22 amide in the post-synaptic neuron suggests that forskolin may be acting on the pre-synaptic terminal to increase synaptic plasticity. Normally this effect is masked due to the presence of PKA action in the post-synaptic terminal. However when PKA signaling was blocked in the post-synaptic terminal, I saw an increase in frequency of GABAergic IPSCs.

3.2.5 Forskolin causes an Increase in the Frequency of Spontaneous Action Potentials in GABAergic Neurons

Spontaneously occurring action potentials (APs) in primary neuronal cultures have been reported as a good indicator of cellular excitability (Hodges et al., 2002). I recorded APs from an inhibitory GABAergic synapse. The GABAergic synapses were marked by the GAD1 (Glutamate Acid Decarboxylase1) enzyme tagged to RFP (red fluorescent protein). I noted an increase in the frequency of spontaneous APs in the presence of 20 µM forskolin in my recordings from these inhibitory GABA neurons (Fig. 3.05). This suggests that FSK causes an increase in cellular excitability probably through an increase in pre-synaptic vesicle release at the GABAergic terminal. However this does not explain how FSK results in a decrease in the frequency of the GABAergic IPSCs. To address this, I examined whether FSK acts on certain post-synaptic targets in addition to increasing pre-synaptic excitability.
3.2.6 cAMP Signaling alters the Response of GABA-Rdl Receptor to GABA

Post-synaptic GABA receptors are some of the best characterized targets of cAMP and PKA. The *Drosophila* GABA-Rdl is a GABA$_A$-like picrotoxin sensitive receptor that has been shown to mediate inhibitory synaptic currents in both embryonic (Lee et al., 2003) and pupal neuronal cultures (Su and O'Dowd, 2003). Since these receptors mediate GABAergic IPSCs, blocking them would lead to decreased frequency of the GABAergic events in whole cell patch clamp. If these receptors are located on a post-synaptic terminal, then a PKA blocker restricted to the post-synaptic neuron should result in overcoming the effects of FSK on GABAergic IPSCs. To test this, I applied GABA to the post-synaptic neurons in the presence and absence of 20 µM FSK in the external recording solution. I applied 100 µM GABA to the same neuron before and three minutes after the perfusion with 20 µM FSK added to external solution. My results showed that after perfusion with 20 µM FSK, the response of the GABA receptor to GABA was strongly reduced (Figs. 3.06 A, B). However both the cAMP mutant *dnc1* and *rut1* neurons showed a significantly lower decrease in their response to GABA after FSK perfusion. These results indicate that FSK leads to a decrease in the sensitivity of the GABA receptor to GABA. This decreased sensitivity is not seen in the cAMP signaling mutants (Figs. 3.06 C, D and E). These results also suggest that the effect of cAMP on GABAergic IPSCs is primarily mediated by the alteration of GABA receptor sensitivity at the post-synaptic terminal.
3.2.7 Forskolin Modulates GABA Receptor Sensitivity in the Mushroom Body Neurons

GABA-Rdl receptors are known to be expressed in the olfactory learning center (mushroom body) of *Drosophila* and have been implicated in olfactory learning (Liu et al., 2007). The output of the mushroom body (MB) extrinsic GABAergic anterior paired lateral (APL) neuron, which sends its projections on to the MB, has also been shown to be essential for olfactory learning (Liu and Davis, 2009). I thus asked if the effect of FSK on post-synaptic GABA receptor sensitivity is also seen in the mushroom body neurons. In addition, I examined whether the receptor sensitivity is altered in the MB neurons of cAMP mutants. For these experiments, I used the Gal4-UAS system to drive the expression of Green Fluorescent Protein (GFP) in the MB neurons using MB specific Gal4 drivers of *dnc*1, *rut*1 and wild-type w1118 flies. My results showed that the perfusion of FSK for three minutes leads to a significant decrease in GABA receptor response in wild-type mushroom body neurons. However this suppression of GABA receptor response was much lesser in the *dnc*1 and *rut*1 MB neurons. I noted that increasing concentrations of FSK in the external solution were directly proportional to the decrease in GABA receptor response to the application of GABA (Fig. 3.07A). Moreover, the application of both 20 µM and 2 µM FSK in the cAMP mutant MB neurons resulted in a significantly smaller decrease in GABA receptor sensitivity to GABA as compared to the wild-type MB neurons (Fig. 3.07B). These observations are similar to those seen in the normal non-MB neurons (Fig. 3.06).
Thus, my results indicate that cAMP signaling is required for the modulation of GABA receptor sensitivity to GABA. Alterations in cAMP signaling and loss of cAMP homeostasis lead to increased GABA receptor response in both the MB and non-MB neurons. The increased receptor response can account for the higher frequency of GABAergic IPSCs observed in the \textit{dnc1} and \textit{rut1} mutants (Fig. 3.03). Thus the altered GABA receptor sensitivity in the MB neurons can account for altered inhibitory synaptic plasticity and defects in behavioral learning seen in the cAMP mutants.

3.2.8 Forskolin Modulates GABA Receptor Sensitivity in the Late-stage Pupal Mushroom Body Neurons

My results from the previous section demonstrate that cAMP modulates the sensitivity of the GABA receptors in the MB neurons on the application of GABA. However, these effects were studied in embryonic MB neuronal cultures that may be very different from adult neurons. I thus examined these effects in late stage pupal neuron cultures (Su and O'Dowd, 2003). The pupal MB neurons from the wild-type, \textit{dnc1} and \textit{rut1} genetic backgrounds were tagged with GFP using MB specific Gal4 driver (c309-GAL4). I observed that the application of 100 µM GABA three minutes after perfusion of 2 µM FSK evoked no response from the GABA receptors. This effect was seen both in wild-type (Fig. 3.08 A) and cAMP mutant MB neurons.

Since FSK perfusion for 3 minutes completely abrogated GABA receptor response, I decreased the duration of FSK perfusion to 90 seconds. The application of 100 µM GABA under this condition evoked a quantifiable response from the GABA receptor in wild-
type (Fig. 3.08 B), dnc1 and rut1 MB neurons (Figs. 3.09 A & B). The perfusion of 2 μM FSK for 90 seconds resulted in a significantly larger decrease in the receptor response in wild-type as compared to cAMP mutants (Fig. 3.09 C). This alteration in GABA receptor response was comparable to that seen earlier in embryonic neuronal cultures (Fig. 3.06). The similarity between the results from embryonic and pupal MB neurons indicates that the modulation of GABA receptor sensitivity by cAMP is conserved through development. These results are of particular interest given the fact that MB neurons are responsible for processing olfactory information and have been shown to be critical for olfactory learning (Heisenberg, 2003).

In summary, the results described in this section indicate that cAMP mediated modulation of inhibitory synaptic plasticity is mediated predominantly through post-synaptic GABA_A-like (GABA-Rdl) receptors. cAMP modulates the sensitivity of the GABA receptor and this modulation is altered in cAMP signaling mutants. Two such cAMP mutants (known olfactory learning mutants) dnc1 and rut1 show altered synaptic plasticity at the inhibitory synapse due to altered receptor sensitivity at the post-synaptic terminal. This effect is seen not only in the normal neurons but also in the MB neurons which are involved in olfactory information processing. I hence propose that this form of inhibitory synaptic plasticity may be a critical cellular mechanism required for behavioral learning.
3.3 DISCUSSION

The output of monoamine neurons, such as the dopaminergic and octopaminergic neurons, is known to be essential for olfactory associative learning in *Drosophila* (Schwaerzel et al., 2003). These monoamines act through G-proteins to activate Ca2+/CaM dependent adenylate cyclase (AC) and thus produce cAMP. AC is known to function as a coincidence detector during learning in both *Drosophila* (Gervasi et al., 2010; Tomchik and Davis, 2009) and *Aplysia* (Abrams et al., 1991). Moreover, AC dependent cAMP activation can change pre-synaptic excitability at *Drosophila* excitatory synapses (Lee and O’Dowd, 2000; Zhong and Wu, 1991). However, the effects of cAMP at the inhibitory GABAergic synapses and its possible consequences on learning and memory have remained unexplored.

In this chapter I show that forskolin, an activator of cAMP, suppresses the frequency of inhibitory GABAergic IPSCs in *Drosophila* primary neuronal cultures (Fig. 3.01). I also demonstrate a concentration dependent effect of FSK and a cAMP analog, db-cAMP, on GABAergic IPSCs (Fig. 3.02). These results indicate that the effect of FSK on GABAergic IPSCs is through cAMP and is not a non-specific effect of FSK (Harris-Warrick, 1989; Hoshi et al., 1988). Recent imaging studies with intact fly brains have activated PKA using FSK and db-cAMP at concentrations similar to those in my experiments (Gervasi et al., 2010; Tomchik and Davis, 2009). This suggests that the concentrations of FSK used by us may be efficacious *in vivo*. I observe that inhibitory GABAergic IPSCs are altered in cAMP mutants *dunce1* and *rutabaga1* (Fig. 3.03). Both these mutants show a significantly higher frequency of GABAergic IPSCs in the presence of FSK as compared
to the wild-type strain (Figs. 3.03 C & D). Despite having physiologically different levels of cAMP, both these mutants showed similar defects in GABAergic IPSCs. This suggests that the altered inhibition may contribute to their commonly observed learning defects.

I next addressed the mechanism by which FSK modulates GABAergic IPSCs. I was particularly interested in examining if a common molecule/effector was altered in both the learning mutants described above. My results show that an inhibitor of PKA (H-89), a downstream target of cAMP, is able to rescue the effect of FSK on GABAergic IPSCs in wild-type neurons (Fig. 3.04). Interestingly, when a membrane impermeable PKA blocker was restricted to the post-synaptic neuron, an increase in the frequency of GABAergic IPSCs was noted during FSK application (at higher concentrations). In these experiments, FSK was also able to increase extra-cellular excitability at the inhibitory GABAergic synapses (Fig. 3.05). This suggests there might be a dual mode of regulation of GABAergic IPSCs by FSK.

GABAergic IPSCs have been shown to be mediated by post-synaptic GABA-Rdl receptors in previous studies (Lee et al., 2003; Su and O’Dowd, 2003). I observed that the GABA-Rdl receptor response was significantly decreased in the wild-type neurons in the presence of FSK (Figs. 3.06 A & B). Interestingly, both the learning mutants showed a similar decrease in GABA receptor sensitivity in the presence of FSK (Figs. 3.06 C, D & E), mimicking the effect of FSK on GABAergic IPSCs. This indicates that FSK acts on GABAergic IPSCs through the suppression of the GABA receptor response. This
regulation is similarly altered in both learning and memory mutants and results in a higher frequency of GABAergic IPSCs.

The results from this section also demonstrate a novel dual regulatory role of cAMP by showing that it increases overall presynaptic function on one hand; and, acts specifically on post-synaptic GABA_A receptors to decrease GABAergic plasticity on the other. Previous work from our laboratory has shown that the FSK can increase excitability at the cholinergic synapses (Yuan and Lee, 2007). This, along with the results described here, indicates that cAMP may result in a global increase in excitability (by increasing cholinergic EPSCs and decreasing GABAergic IPSCs). This alteration in the excitation/inhibition balance might be important for learning (Yizhar et al., 2011).

Finally, I examined whether inhibitory synaptic transmission is altered in the neurons of the MB, an anatomical structure involved in learning and memory. I observed that both embryonic (Fig. 3.07) and pupal (Figs. 3.08 & 3.09) MB wild-type neurons showed a similar decrease in GABA receptor response in the presence of FSK. In both the learning mutants (dunce and rutabaga) the embryonic and pupal MB neurons showed a decrease in GABA receptor sensitivity in the presence of FSK as observed earlier with the non-MB neurons (Fig. 3.06). It is interesting to note that both the mutants show a similar change in GABA receptor sensitivity in the presence of FSK despite having markedly different levels of cellular cAMP.
Recent imaging studies in the *rutabaga* mutant have shown that adenylate cyclase (AC) is required for coincidence detection in the MB neurons (Gervasi et al., 2010; Tomchik and Davis, 2009). FSK application also fails to increase PKA to wild-type levels in the MB neurons of *rutabaga* (Gervasi et al., 2010). Thus in my experiments, the changes in receptor response in *rutabaga* can be explained by a lack of increase in cAMP/PKA levels due to defects in FSK mediated AC activation. However the *dunce* mutants with high cellular levels of cAMP behave like the *rutabaga* mutants with a low level of cellular cAMP. It is possible that the GABA_A receptors in *dunce* MB neurons are less sensitive or they may alter their response to chronically high levels of cAMP. Nonetheless, my results from *dunce* mutants are consistent with those described in rat retinal neurons where chronic intracellular application of cAMP results in an increase in GABA_A receptor response (Feigenspan and Bormann, 1994). Moreover, several studies have shown that *dunce* and *rutabaga* have similar defects in growth cone motility, excitatory synaptic plasticity and more importantly, short-term memory (Gasque et al., 2006; Kim and Wu, 1996). My results on GABAergic plasticity in *dunce* and *rutabaga* neurons by cAMP are therefore consistent with these observations.

My results suggest that the GABA-Rdl subunit may be a common target in both the learning mutants. It is possible that defective phosphorylation of GABA-Rdl by PKA may result in similar defects in inhibitory GABAergic transmission in both of these mutants. Of the three known GABA_A receptor gene homologs in *Drosophila* – RDL, LCCH3 and GRD – only RDL is known to form homomultimers (Hosie et al., 1997). GABA_A RDL subunit is widely expressed in several regions of the *Drosophila* brain.
(Harrison et al., 1996) and its expression in the MB is inversely correlated with olfactory learning (Liu et al., 2007). Even though RDL and LCCH3 can co-assemble in *in vitro* expression systems, these co-assembled channels are picrotoxin insensitive unlike RDL homomultimers which are picrotoxin sensitive (Zhang et al., 1995). Since RDL and LCCH3 are known to be expressed during different stages of development (Aronstein et al., 1996), it is unlikely that their heterodimers will be present at any stage *in vivo*. The third subunit, GRD, is cation sensitive and is thus unlikely to play a role in inhibitory transmission (Harvey et al., 1994). My data does not provide direct evidence of receptor phosphorylation or the particular subunit involved in regulation of GABAergic IPSCs. However, based on the observation that the GABA<sub>α</sub> RDL subunit regulates GABAergic IPSCs in *Drosophila* primary neuronal cultures (Lee et al., 2003), I propose that the action of FSK on GABAergic IPSCs is through the GABA<sub>α</sub> RDL subunit. There is currently no information available on the receptor subunit distribution and/or phosphorylation pattern in the MB neurons of learning mutants. It would be thus interesting to examine if such receptor phosphorylation are indeed responsible for alterations in learning.

The experiments described in this chapter provide physiological data indicating that altered GABAergic transmission through the GABA<sub>α</sub> receptors may be critical in the MB of cAMP mutants *dunce* and *rutabaga*. The results suggest that the effect of cAMP on GABAergic inhibition may lead to some of their defects in short term learning.
Figure 3.01: Suppression of Inhibitory GABAergic Post-Synaptic Currents (IPSCs) by Forskolin (FSK)

A) After obtaining a stable GABAergic IPSC trace from a wild type neuron (3 days in vitro), FSK (20 µM) was focally applied. GABAergic IPSCs were almost completely suppressed by FSK. Sample IPSC traces are shown on an expanded time scale below the whole recording trace. Holding potential (VH) = 0 mV.

B) Graph shows the reduction in GABAergic IPSC frequency on application of 20 µM FSK in wild type neurons in the absence (n=9; circles) and presence (n=4; triangles) of 50 µM H-89 (a membrane-permeable PKA inhibitor). The reduction seen in IPSCs in the presence of H-89 is almost negligible as compared to the reduction seen in the absence of H-89. FSK was applied for 30 seconds (indicated by bar) at 62.5 seconds after the initiation of the IPSC recording. The IPSC frequency was calculated every 5 seconds and normalized to that of controls as described in the methods. (Bars indicate SEM).
Figure 3.02: Inhibitory Effects of cAMP on GABAergic Synaptic Transmission

A) The graph shows the reduction in GABAergic IPSCs on the application of 10 mM db-cAMP in wild type neurons (n=9). Almost complete suppression was seen during 30 seconds of db-cAMP application. In each experiment, db-cAMP was applied at 62.5 sec after initiation of IPSC recording. Bars indicate SEM.

B) The graph shows the effects of different concentrations of db-cAMP (100 µM-10 mM) and forskolin (2-100 µM) on GABAergic IPSCs. The suppression potency was determined by averaging percent frequencies for 20 sec from 70-90 sec. Bars indicate SEM.

(*** p<0.005 by ANOVA)
Figure 3.03: Modulatory Actions of Forskolin (FSK) on Inhibitory GABAergic Post-Synaptic currents (IPSCs) in Learning and Memory Mutants

A) Representative trace of the effect of FSK on GABAergic IPSCs in a dnc1 neuron. 20 µM FSK was focally applied for 30 seconds (bar) after establishing a stable IPSC trace from a 3 day old dnc1 neuron. An expanded time scale is shown below the whole recording trace. VH = 0 mV.

B) Effects of FSK on GABAergic IPSCs in a rut1 neuron (3 days old).

C) A comparison of the reduction in GABAergic IPSCs by 20 µM FSK in wild type (n=9), dnc1 (n=5) and rut1 (n=7).

D) Reduction in frequency of GABAergic IPSCs during the focal application of 20 µM FSK in wild type, dnc1 and rut1 neurons. Bars indicate SEM. (**p<0.005 by Student's T-test).
Figure 3.04: cAMP Modulates GABAergic IPSCs through a Post-Synaptic Mechanism

A) FSK action on GABAergic transmission was inhibited by a membrane-impermeable PKA inhibitor PKI (6-22) amide which was added into the whole-cell recording solution.

B) The graph shows inhibition of FSK modulatory action by PKI amide and H-89. In all cases, 20 µM FSK was applied for 30 seconds. Bars represent SEM. (*p<0.05, **p<0.01, ***p<0.005 by Student's t-test).
Figure 3.05: Effect of Forskolin on the Frequency of Spontaneous Action Potentials in GABAergic Neurons
A) 40X magnified image of a GABAergic neuron marked by the enzyme GAD1-RFP in primary embryonic neuronal cultures
B) A representative trace showing spontaneous action potentials (AP) recorded from a GABAergic neuron in cell attached mode. Focal application of 20 µM forskolin increases the frequency of these spontaneous APs. A magnified view of spontaneous APs before and after the application of forskolin is also shown
Figure 3.06: Effect of GABA Puffing in the Presence and Absence of Forskolin

A, B) Representative traces from a single neuron showing the effect of 100 µM GABA application on a wild-type (w1118) neuron for two seconds. In A, the control trace (grey) shows the effect of perfusing only the external solution on the same neuron; and, B shows the effect in the absence (black) and presence (grey) of 20 µM FSK.

C, D) Representative traces showing the effect of 100 µM GABA application for two seconds in the absence (black) and presence (grey) of 20 µM FSK on a single dnc1 (C) and rut1 (D) neuron.

E) Graph shows the percentage of original response to 100 µM GABA before and after perfusion with 20 µM FSK for 3 minutes. Response left in dnc1 and rut1 after FSK perfusion is significantly higher than w1118 neurons. Bars indicate SEM. (** indicates p<0.005 by Student's T-test)
Figure 3.07: Effect of GABA Puffing on Embryonic Mushroom Body Neurons

A) Cumulative response plot showing the percentage of original response to 100 µM GABA before and after perfusion for three minutes with varying concentrations of FSK. All responses were seen in wild-type (w^{1118}) embryonic mushroom body neurons. The mushroom body neurons were marked with GFP in w^{1118}; c309-Gal4; UAS-GFP

B) Cumulative bar graph showing percentage of original response to 100 µM GABA before and after 2 µM FSK for three minutes. Mushroom body neurons expressed GFP in the w^{1118}; c309-Gal4; UAS-GFP, the rut1; c309-Gal4; UAS-GFP and the dnc1; c309-Gal4; UAS-GFP genotypes. Response seen in the dnc1 and rut1 background embryonic MB neurons after FSK perfusion was significantly higher than w^{1118} neurons. Bars indicate SEM.

(*** p<0.005 by Student's T-test)
A. ++; c309-Gal4; UAS-GFP(III) pupal MB neuron

- 90 secs after 20µM FSK Perfusion
- Before FSK perfusion

20 pA
10 secs

100µM GABA for 2 secs

B. ++; c309-Gal4; UAS-GFP(III) pupal MB neuron

- 90 secs after 2µM FSK Perfusion
- Before FSK perfusion

50 pA
10 secs

100µM GABA for 2 secs

Figure 3.08: Effect of GABA Puffing on Late Stage Wild-type Pupal Mushroom Body Neurons
Representative traces showing the effect of GABA (100 µM) applied for 2 seconds before (grey) and after (black) perfusion with:
A) 20 µM FSK for 90 seconds on a single wild-type pupal MB neuron.
B) 2 µM FSK for 90 seconds on a single wild-type pupal MB neuron.
Figure 3.09: Effect of GABA Puffing on Late Stage Mutant Pupal Mushroom Body Neurons

Representative traces showing the effect of GABA (100 µM) applied for 2 seconds before (grey) and after (black) perfusion with 2 µM FSK for 90 seconds in:
A) A single *dnc1* pupal MB neuron and, B) A single *rut1* pupal MB neuron.
C) Graph shows percent original response to 100 µM GABA before and after perfusion with 2 µM FSK for 90 seconds. MB neurons were labeled with GFP in the *w*¹¹¹⁸, *rut1* and *dnc1* backgrounds using Gal4-UAS system. Bars indicate SEM. (***p<0.005).
CHAPTER FOUR

*Serotonin (5-HT7) Mediated Regulation of Central Synaptic Transmission through cAMP Signaling*

4.1 INTRODUCTION

The monoamine serotonin has been implicated in a range of functions such as sleep, circadian rhythms, appetite control, aggression, sexual behavior and learning in vertebrates (Lucki, 1998). Reward related learning in particular is regulated through the serotonergic system in vertebrates (Hayes and Greenshaw, 2011) and alterations of this system have been observed in patients with schizophrenia (Emsley, 2009). Most of these behaviors are modulated by the effect of 5-HT on its downstream receptors (Raymond et al., 2001) which are expressed in regions of the mammalian brain involved in learning and memory such as the pre-frontal cortex, hippocampus and amygdala (King et al., 2008). It is thus not surprising that many agonists of the 5-HT receptors are used to treat psychiatric and learning disorders (Millan, 2000). The clinical significance of this pathway has thus prompted studies that explore the mechanism of 5-HT action through its downstream GPCRs in promoting learning.

In invertebrates like *Drosophila* 5-HT is involved in a similar range of behaviors including aggression, sleep, locomotion, reproductive behavior and spatial learning (Blenau and Thamm, 2011). The 5-HT GPCRs homologs are known to be expressed in the *Drosophila* and honeybee mushroom body (Blenau and Thamm, 2011), a brain region involved in olfactory learning and memory (Keene and Waddell, 2007). 5-HT has also
been implicated in the regulation of spatial memory in *Drosophila* (Sitaraman et al., 2008), and shown to be necessary for the anesthesia-resistant form (ARM) of olfactory associative learning (Lee et al., 2011). It has further been shown that the output of 5-HT neurons projecting to the lobes of the mushroom body is required for olfactory learning. This action of 5-HT is dependent on the expression of the 5-HT1A-like receptor homolog in the mushroom body (Lee et al., 2011). Apart from this work, there is almost no other information on 5-HT circuits or the role of other 5-HT receptor homologs in the context of olfactory learning in *Drosophila*.

Most known 5-HT coupled GPCRs in vertebrates and invertebrates are predominantly coupled to the cAMP signaling pathway (Lee et al., 2009; Nichols and Nichols, 2008; Saudou et al., 1992). The cAMP signaling pathway has been shown to be essential for learning and memory in both vertebrates and invertebrates (Kandel, 2001, 2009). In vertebrates, the three known 5-HT coupled GPCRs (5-HT4, 5-HT6 and 5-HT7) which positively activate cAMP have been shown to be involved in various cognitive tasks and in learning and memory (King et al., 2008; Roberts and Hedlund, 2011). Interestingly, cAMP expression in the mushroom body has also been reported to be critical for learning in *Drosophila* (Keene and Waddell, 2007). This suggests that 5-HT mediated activation of the cAMP pathway may regulate learning in *Drosophila*.

Behavioral learning has been proposed to be the cumulative output of changes in synaptic plasticity at individual synapses in a circuit (Wilson, 2011). However it is difficult to integrate synaptic plasticity at the individual synapse with behavioral output
due to technical limitations of experimental systems. Though 5-HT is known to be involved in a range of behaviors, it is not known if 5-HT mediated changes in synaptic strength can shape behavioral responses at the circuit level. It is difficult to explore the role of a particular neurotransmitter at a circuit level because of the existence of complex connectivity and feedback circuits responding to a single neurotransmitter. Thus, in vitro assays with defined cell populations provide a tractable system that allows one to explore the effect of a particular neurotransmitter on neurons from a specific region of the brain. In vertebrates in vitro brain slices have been extensively used to study synaptic plasticity via mechanisms of long term potentiation (LTP) and depression (LTD) (Malenka and Bear, 2004). Experiments using such systems have shown that 5-HT can regulate both LTP and LTD in the vertebrate brain (Edagawa et al., 2001; Garraway and Hochman, 2001).

Mammalian systems have multiple 5-HT receptors activating a particular signaling pathway (Raymond et al., 2001) and are thus not the best model systems to clearly define the effect of 5-HT on synaptic transmission. This receptor redundancy complicates data interpretation even in studies with animals that lack receptor expression. Moreover the antagonists and agonists of various 5-HT receptors are not specific and are known to bind other 5-HT receptors and even other non-specific targets (Leopoldo et al., 2011). For example, knocking down the 5-HT7 receptor (5-HT7R) has been shown to affect only long-term learning while having no effect on short-term memory, spatial learning, novel object recognition and learning in a modified Morris water maze (Roberts and Hedlund, 2011). Since other mammalian 5-HT receptors (5-
HT4 and 5-HT6) also signal through the cAMP pathway, it is probable that these receptors compensate for the loss of 5-HT7 receptor in most behavioral forms of memory. It is thus difficult to conclusively prove that a single receptor is necessary and sufficient for learning and memory.

The invertebrate Aplysia has extensively been used to study 5-HT mediated short term facilitation and long term aversive learning in the gill withdrawal reflex (Kandel, 2009). A recent study in this system has indicated that 5-HT7 homolog mediated activation of cAMP signaling is important in synaptic facilitation in the gill-withdrawal reflex (Lee et al., 2009). 5-HT has also been shown to enhance neurotransmitter release in peripheral glutamatergic synapses of Drosophila and crayfish (Dasari and Cooper, 2004; Sparks and Cooper, 2004). However, these experimental set-ups examine only the modulation at peripheral synapses. Thus it is still not known if 5-HT can modulate synaptic plasticity at the central synapses in Drosophila.

To examine the effect of 5-HT, I chose the invertebrate model system Drosophila since it has only one known 5-HT GPCR that is positively coupled to cAMP signaling (Becnel et al., 2011; Saudou et al., 1992). Earlier experiments with Drosophila primary neuronal cultures have shown that cAMP mediated synaptic plasticity is involved in the regulation of excitatory cholinergic (Lee and O'Dowd, 1999, 2000) and inhibitory GABAergic transmission particularly in the MB neurons of Drosophila (Chapter 3). Thus the Drosophila primary neuronal cultures serve as a good model to study 5-HT mediated central synaptic transmission since neurons from specific anatomical regions of the brain
(particularly those involved in learning) can be identified and recorded from using the Gal4-UAS system (Brand and Perrimon, 1993). Moreover, the ease with which genetic manipulations can be done in *Drosophila* makes it an ideal system to study the effect of 5-HT in a receptor null backgrounds without the complications of redundancy.

In this chapter I use whole-cell patch-clamp to examine physiological mechanisms underlying the regulation of 5-HT mediated synaptic plasticity in *Drosophila* primary neuronal cultures. I primarily focus on identifying the 5-HT receptor involved in the regulation of synaptic plasticity and examine downstream signaling. I also examine whether the effect of serotonin on excitatory and inhibitory synaptic plasticity is mediated by pre- or post-synaptic mechanisms. The broad aims addressed in this chapter include:

a) Examining the effect of 5-HT on both excitatory and inhibitory synaptic plasticity at *Drosophila* central synapses

b) Identifying the 5-HT GPCR/GPCRs involved in the regulation of 5-HT mediated synaptic plasticity and the downstream signaling pathway

c) Exploring the mechanism (pre-synaptic vs. post-synaptic) through which 5-HT modulates excitatory and inhibitory synaptic plasticity
4.2 RESULTS

4.2.1 Effect of Serotonin (5-HT) on Cholinergic EPSCs in Wild-type (w^{1118}) Neurons

To examine the effect of 5-HT on central synaptic transmission, I used an *in vitro* primary neuronal culture assay developed in *Drosophila* (Lee and O'Dowd, 1999). In wild-type (w^{1118}) cultures, the focal application of 20 µM 5-HT for 30 seconds caused a significant increase in the frequency of excitatory cholinergic EPSCs (424.61±44.71%, n=9) as compared to the normalized baseline EPSC frequency (117.32±8.11%, n=9) before the application of 5-HT (Fig. 4.01). These results are consistent with those described earlier at the *Drosophila* and crayfish NMJ (Dasari and Cooper, 2004; Sparks and Cooper, 2004) where 5-HT application causes an increase in the frequency of evoked EPSCs. Thus my results suggest that 5-HT positively modulates excitatory cholinergic synaptic transmission in *Drosophila* primary neuronal cultures.

4.2.2 The Effect of 5-HT on the Frequency of Cholinergic EPSCs in the Presence of a PKA Blocker (H-89)

The effect of 5-HT on various behaviors, including those related to cognition, is through the 5-HT GPCR receptors which activate a wide range of signal transduction pathways (Nichols and Nichols, 2008; Raymond et al., 2001). Since certain 5-HT receptors in *Drosophila* are coupled to the cAMP signaling pathway (Saudou et al., 1992), I examined if the regulation of cholinergic EPSCs by serotonin is modulated through cAMP signaling. In order to address this question, I blocked signaling downstream of cAMP by blocking the action of its major target PKA by adding a chemical inhibitor (H-89) in the
external recording solution (bath solution). In the presence of 50 µM H-89 in the bath, the application of 20 µM 5-HT had no effect on the frequency of cholinergic EPSCs (Fig. 4.02). The frequency of cholinergic EPSCs during the application of 5-HT in the presence of H-89 (102.07±5.76%, n=5) was similar to that observed in wild-type (w^{1118}) neurons in the absence of H-89 (117.32±8.11, n=9). This indicated that a major part of the effect of 5-HT on cholinergic EPSCs, if not all of it, is mediated through cAMP signaling.

4.2.3 The Effect of 5-HT on Cholinergic EPSCs in a G-α-s Null Mutant Background

In the vertebrate system at least three major 5-HT GPCRs (5-HT4, 5-HT6 & 5-HT7) are positively coupled to cAMP (Barnes and Sharp, 1999). This makes it difficult to isolate a behavior brought about through the action of a single GPCR (Roberts and Hedlund, 2011). However, invertebrates such as *Drosophila* and *Aplysia* have only a single 5-HT7 receptor homolog positively coupled to cAMP (Lee et al., 2009; Witz et al., 1990). Since the *Drosophila* 5-HT7 receptor homolog (d5-HT7) is known to activate adenylate cyclase through the G-α-s protein and enhance cAMP levels, I examined if 5-HT mediated activation of the d5-HT7 receptor is involved in the regulation of cholinergic EPSCs. Taking advantage of the genetic tools available in *Drosophila*, I tested the effect of 5-HT on cholinergic EPSCs in a G-α-s null mutant background (Bloomington stock center, stock #6340). Homogenous G-α-s mutant cultures (GFP positive) were identified (Darya et al., 2009) and used for recording. In these cells the application of 20 µM 5-HT resulted in a significant decrease in the frequency of cholinergic EPSCs (36.57±3.20%, n=5) as compared to their frequency before 5-HT application (100.00±3.50%, n=5; p=0.002). This
was in sharp contrast to the effect of 5-HT on wild-type (w^{1118}) neurons (Fig. 4.03 A), where 5-HT caused an increase in the frequency of cholinergic EPSCs. This indicated that the G-α-s protein may significantly contribute to 5-HT mediated increase in the frequency of cholinergic EPSCs seen in the wild-type neurons.

If the effect of 5-HT is indeed through cAMP signaling, the activation of the enzyme adenylate cyclase (a target of the G-α-s subunit and an activator of cAMP) in the mutant background should be able to restore or partially rescue the effect of 5-HT on cholinergic EPSCs. To test this, I applied 100 μM forskolin (FSK) to the G-α-s null mutant neurons. I observed a significant increase in the frequency of cholinergic EPSCs (299.65±33.65%, n=9, p ≤ 0.001) as compared to their frequency in the wild-type (w^{1118}) strain before 5-HT application (117.32±8.11%, n=9; Fig. 4.03 B). These results indicate that the effect of 5-HT on cholinergic EPSCs is mediated through G-α-s directed activation of cAMP signaling.

Since the d5-HT7 receptor is the only known G-α-s coupled 5-HT GPCR my data suggests that this receptor may be involved in the regulation of cholinergic EPSCs via 5-HT. It should however be noted that the decrease seen in the frequency of cholinergic EPSCs during 5-HT application may also reflect an effect of 5-HT on other non-G-α-s coupled GPCRs.
4.2.4 Identification and Characterization of a d5-HT7 Mutant (#24705) in Drosophila

5HT7 receptors have been implicated in a range of behaviors including those related to higher cognitive functions like learning, sleep and aggression in vertebrates (Roberts and Hedlund, 2011). In Aplysia the 5-HT7 like receptor has also been implicated in synaptic facilitation during gill withdrawal reflex (Lee et al., 2009). However in Drosophila the function of the 5-HT7-like receptor is poorly understood. Although this receptor has been implicated in courtship and mating (Becnel et al., 2011) there are no reports on the role of this receptor in synaptic transmission. A recent study (Johnson et al., 2011) provides limited evidence for a possible function of this receptor in olfactory learning. However it is unclear if this receptor modulates learning through changes in synaptic plasticity. My results indicate that this receptor may regulate excitatory synaptic transmission. To conclusively prove a requirement for this receptor in synaptic transmission I searched for Drosophila strains mutant for this receptor. Upon examining the various publicly accessible Drosophila mutant strains with P-element insertions in the stock collection (Bloomigton stock center), I identified a strain (#24705) which has a Minos (P-element) insertion in the d5-HT7 gene (Metaxakis et al., 2005). To confirm that the insertion of this element into the d5-HT7 gene leads to a disruption of receptor expression, I designed exon spanning primers for the d5-HT7 gene (Fig. 4.04 A). I then isolated mRNA from the larvae of wild-type (w1118 and Canton-S) and the putative d5-HT7 mutant (#24705) strains, converted the mRNA to cDNA and conducted a PCR analysis with primers against the d5-HT7 gene and a control housekeeping gene (RP-49). I obtained products of expected molecular weight (334bp) in both the wild-type strains
but no amplification was seen in the d5-HT7 mutant (#24705) strain. All three strains gave the expected product with the RP-49 housekeeping gene (Fig. 4.04 B). Since the primers for the d5-HT7 were exon spanning and the gene has an intron >5kB between both the exons, the correct size product would be obtained only from mRNA and not from genomic DNA. Thus the primer design excludes the possibility of amplifying from any contaminating genomic DNA. Thus my results conclusively prove that the #24705 strain lacks the expression of a functional copy of the d5-HT7 gene. I will henceforth refer to this strain as a d5-HT7 null mutant.

4.2.5 The d5-HT7 Receptor Modulates the Effect of 5-HT on Excitatory Cholinergic EPSCs

Having identified and molecularly characterized a d5-HT7 receptor null mutant strain (#24705), I examined the effect of 5-HT on cholinergic EPSCs in this mutant background. The application of 20 µM 5-HT for 30 seconds on #24705 mutant neurons led to a significant difference in the frequency of cholinergic EPSCs (86.89±6.81%, n=7; p<0.005) as compared to their frequency in the wild-type (424.61±44.71%, n=9) during the application of 5-HT (Fig. 4.05 A & Fig. 4.05 B, light grey bar). In conjunction with the results from section 4.2.3 the results from this section strongly suggest that the d5-HT7 receptor mediates the effects of 5-HT on cholinergic EPSCs.

To further explore the involvement of the d5-HT7 receptor in the regulation of 5-HT mediated cholinergic EPSCs, I tested the effect of 5-HT in the presence of a well-defined 5-HT7 receptor (SB-258719) antagonist (Mahe et al., 2004) in the bath solution. My results
show that when 60 µM SB258719 was included in the bath solution, 20 µM 5-HT application had no effect on the frequency of cholinergic EPSCs (Fig. 4.05 B, dark grey bar, n=5) as compared to wild-type (w^{118}) neurons (Fig. 4.05 B, white bar). This result further supports the hypothesis that the d5-HT7 receptor modulates the effect of 5-HT on cholinergic EPSCs via the activation of cAMP signaling.

4.2.6 5-HT Modulates Cholinergic EPSCs Primarily through Pre-Synaptic Mechanisms

5-HT has been shown to modulate Shaker K+ channel activity in Drosophila photoreceptor neurons (Hevers and Hardie, 1995) and in decreasing delayed rectifier potassium currents (IKDR) in in vitro Drosophila neuronal cultures (Alshuaib et al., 2003). These studies indicate that 5-HT may alter pre-synaptic excitability and neurotransmitter release. Earlier studies with Drosophila primary neuronal cultures have also shown that the elevation of cAMP causes an increase in the frequency of cholinergic EPSCs at the pre-synaptic locus (Yuan and Lee, 2007). The results described earlier in this chapter indicate that 5-HT acts through the d5-HT7 receptor to modulate cholinergic EPSCs in a cAMP dependent manner. To identify the locus (pre vs. post-synaptic) of 5-HT action on cholinergic EPSCs, I recorded miniature EPSCs (mEPSCs) in the presence of 1 µM TTX which blocks all action potential-mediated neurotransmitter release. I observed that the application of 20 µM 5-HT resulted in a significant increase in the frequency of mEPSCs during (185.89%±6.95) and after (168.39±5.91) the application of 5-HT as compared to before (101.91±4.96%; n=3; p<0.005) the application of 5-HT (Fig. 4.06). However I observed no change in the amplitude of mEPSC before (21.54±4.77 pA), during
(19.20±1.837 pA, n=3, p=0.6638) or after (21.86±4.48 pA) the application of 20 µM 5-HT. These observations strongly indicate that 5-HT increases pre-synaptic neurotransmitter release in *Drosophila* primary neuronal cultures, which results in an increase in the frequency of cholinergic EPSCs. 5-HT may increase pre-synaptic excitability by either acting on potassium channels or on pre-synaptic calcium channels as reported in *Aplysia* (Laurienti and Blankenship, 1997).

### 4.2.7 5-HT Causes a Decrease in the Frequency of GABAergic IPSCs

In chapter 3, I demonstrated that cAMP causes a decrease in the frequency of GABAergic IPSCs through its action on post-synaptic GABA receptors. The balance between synaptic excitation and inhibition has been proposed to be critical for cognitive processes such as learning (Rubenstein and Merzenich, 2003). In *Drosophila* inhibitory GABAergic synaptic transmission has particularly been shown to be critical for olfactory information processing (Wilson et al., 2004). Given the involvement of 5-HT in various cognitive processes (Lucki, 1998) and the 5-HT7 receptor in cognitive processes such as learning (Roberts and Hedlund, 2011), I assessed if 5-HT regulates inhibitory GABAergic transmission at central synapses. I observed that the application of 20 µM 5-HT on inhibitory GABAergic IPSCs results in a significant decrease in the frequency of GABAergic IPSCs (Fig. 4.07). The frequency of GABAergic IPSCs during the application of 5-HT (27.13±4.21%, n=6; p<0.005) was significantly lower than their frequency before 5-HT application (96.15±3.90%, n=6). The results described in chapter 3 implicate cAMP signaling in the modulation of GABAergic IPSCs. Given the fact that only the d5-HT7 receptor in *Drosophila* is positively coupled to cAMP, it is likely that the effect of 5-HT on
GABAergic IPSCs may be through d5-HT7 receptor mediated activation of the cAMP signaling pathway.

4.2.8 The Effect of 5-HT on the Frequency of GABAergic IPSCs in the Presence of a PKA Blocker (H-89)

As described in the previous chapter (Fig. 3.04), the cAMP-PKA signaling pathway can modulate the frequency of GABAergic IPSCs. To examine if the effect of 5-HT on GABAergic IPSCs is mediated through the cAMP signaling pathway, I used an inhibitor for PKA (H-89), a downstream target of cAMP. I observed that the application of 20 µM 5-HT in the presence of 50 µM H-89 in the bath solution had no effect on the frequency of GABAergic IPSCs (Fig. 4.08). The frequency of GABAergic IPSCs during application of 5-HT (114.18±7.31%, n=4) was similar to that seen without H-89 in the bath solution (96.15±3.90%, n=6) in w1118 neurons. This indicates that 5-HT mediates the frequency of GABAergic IPSCs through the cAMP signaling pathway.

4.2.9 The d5-HT7 Receptor Mediates the Effect of 5-HT on GABAergic IPSCs

I next examined if the effect of 5-HT on GABAergic IPSCs is mediated through the d5-HT7 receptor. For this, I recorded the effect of 5-HT on GABAergic IPSCs in the d5-HT7 receptor null mutant background (Fig. 4.04). I observed that the application of 20 µM 5-HT on GABAergic IPSCs did not decrease their frequency (118.45±5.529%, n=7, Fig. 4.09A&B) as seen in the wild-type (w1118) neurons. In fact, there was a slight increase in the IPSC frequency which was not statistically significant. These results indicate that the d5-HT7 receptor may be involved in 5-HT mediated regulation of GABAergic IPSCs.
through the cAMP signaling pathway. To further confirm the role of the d5-HT7 receptor, a d5-HT7 receptor specific antagonist (SB258719) was added to the external recording solution. In the presence of this antagonist the application of 20 µM 5-HT caused no change in the frequency of GABAergic IPSCs (98.88±5.60%, n=4) in wild-type w¹¹¹⁸ neurons. This suggests that the d5-HT7 receptor is a key molecule involved in mediating the effect of 5-HT on GABAergic IPSCs.

4.2.10 Effect of 5-HT on Miniature IPSC (mIPSCs) in Wild-type (w¹¹¹⁸) Neurons
A significant part of spontaneous GABAergic IPSCs in Drosophila primary neuronal cultures are composed of action potential-independent miniature IPSCs (mIPSCs). These mIPSCs are brought about through the action of pre-synaptic voltage gated calcium channels in the absence of any external stimulus (Lee et al., 2003). Data from our lab suggests that cAMP signaling causes a decrease in the frequency of mIPSC in wild-type (w¹¹¹⁸) neurons (Ganguly and Lee, unpublished observations). Since there was a decrease in the frequency of spontaneous GABAergic IPSCs during 5-HT application in a cAMP dependent manner, I examined if 5-HT brought about this effect by reducing pre-synaptic neurotransmitter release. The application of 20 µM 5-HT in the presence of 1 µM TTX + 1 µM curare in the bath solution did indeed decrease the frequency of mIPSCs (Fig. 4.10) both during (35.42±3.39%) and after (37.22±4.39%) 5-HT application as compared to the frequency before 5-HT application (110.98±7.62%, n=3). However, there was no change in the amplitude of GABAergic mIPSCs before, during or after the application of 5-HT. According to the previous work at excitatory synapses (Lee and O’Dowd, 2000; Yuan and Lee, 2007), these results would indicate a pre-synaptic mode of
regulation. However at the inhibitory GABAergic synapses cAMP mediated increase in pre-synaptic GABA release along with a simultaneous decrease in post-synaptic GABA<sub>A</sub>-receptor response leads to an overall reduction in sIPSC (spontaneous) and mIPSCs (see chapter 3). This indicates that the effect of 5-HT seen here may be due to a decrease in post-synaptic GABA<sub>A</sub>-receptor response and not due to a decrease in pre-synaptic neurotransmitter release.

4.2.11 5-HT Regulates GABAergic IPSC (sIPSCs) by Decreasing Post-Synaptic GABA<sub>A</sub> Receptor Response

To examine if post-synaptic components are involved in 5-HT mediated regulation of GABAergic IPSCs, I first blocked all post-synaptic PKA. For this, 200 µM of a membrane impermeable PKA inhibitor (PKI 6-22 amide) was added to the internal recording pipette. In the presence of PKI the application of 20 µM 5-HT caused a significant increase in the frequency of GABAergic IPSCs (350.65±13.87%, n=5) in wild-type (w<sup>1118</sup>) neurons (Figs. 4.11 A & B). This was directly opposite to the effect of 5-HT on the frequency of both sIPSC (Fig. 4.07) and mIPSCs (Fig. 4.10). This suggests that in the absence of post-synaptic PKA 5-HT increases pre-synaptic GABA release. It is also possible that this overall decrease in the frequency of GABAergic IPSCs may be due to its effect on post-synaptic GABA<sub>A</sub> receptors (as seen in chapter 3).

To examine this possibility, I measured GABA<sub>A</sub> receptor response before and after the perfusion of 20 µM 5-HT. The application of 100 µM GABA for 2 seconds after 3 minutes of 5-HT perfusion caused a significant reduction in GABA<sub>A</sub> receptor response
(24.33±4.86%, n=6) as compared to the receptor response before (101.24±4.16%, n=6) 5-HT perfusion (Figs. 4.11 C & D). This clearly indicates that 5-HT perfusion leads to a reduction in post-synaptic GABA$_A$ receptor response. This response is reminiscent of the effect of forskolin on GABA$_A$ receptor response seen earlier (see chapter 3).

### 4.3 DISCUSSION

The results described in this chapter show that 5-HT modulates both excitatory cholinergic and inhibitory GABAergic PSCs in a cAMP-PKA dependent manner. Although previous work with *Drosophila* and crayfish Neuromuscular Junction (NMJ) has show that 5-HT modulates excitatory synaptic transmission at the peripheral glutamatergic synapses (Dasari and Cooper, 2004; Sparks and Cooper, 2004), there is no evidence indicating the modulation of synaptic transmission at central synapses in *Drosophila*. My results thus shed novel insights on the role of 5-HT mediated regulation of central synaptic transmission in *Drosophila*.

Though 5-HT has the highest affinity for the 5-HT GPCRs, other biogenic amines in insects (dopamine, octapamine etc.) also show a low affinity for most invertebrate 5-HT receptors (Blenau and Thamm, 2011). This raises the possibility that my results may be due to the binding of 5-HT to other biogenic amine receptors. Previous work from the lab has shown that the application of dopamine decreases the frequency of cholinergic EPSCs via the D1 (dDA1) receptor (Yuan and Lee, 2007) whereas it increases the frequency of GABAergic IPSCs through the dD2R receptor (Yuan and Lee, unpublished observations). Similarly, the application of high concentrations of octapamine (0.1 & 1
mM) is known to decrease the frequency of cholinergic EPSCs (Ganguly and Lee, unpublished observations). This is directly opposite to my observations with the application of 5-HT since I see an increase in cholinergic EPSCs and a decrease in GABAergic IPSCs (Figs. 4.01 & 4.07). This suggests that the effect of 5-HT on both the excitatory and inhibitory synaptic currents is not non-specific but is due to the specific binding of 5-HT to one of its GPCRs.

The effects of 5-HT are usually through its downstream GPCR coupled receptors (Raymond et al., 2001). Studies in vertebrates have shown that out of the seven major sub-classes of 5-HT GPCRs, the 5-HT(1A), 5-HT4 and 5-HT6 receptors are essential for behavioral learning and memory and in in vitro brain slice models of learning, such as long term-potentiation (LTP) at the glutamatergic synapses (King et al., 2008). Recently, the 5-HT7 receptor has also been implicated in the regulation of both LTP and behavioral learning assays (Roberts and Hedlund, 2011). The 5-HT(1A) receptor is negatively coupled to the cAMP signaling pathway whereas the 5-HT4, 5-HT6 and 5-HT7 receptors are positively coupled to the cAMP signaling pathway. This makes it difficult to identify the role of a particular receptor in regulating synaptic plasticity or in learning behavior even in mutants lacking the expression of a particular receptor. This situation is exacerbated by the fact that the antagonists and agonists for the various 5-HT receptors bind to various non-specific targets and to other 5-HT receptors (Leopoldo et al., 2011).
Since *Drosophila* has only one known 5-HT GPCR (d5-HT7) which activates cAMP signaling through the G-α-s protein (Witz et al., 1990), it makes a good model system to study the effects of 5-HT. I identified and molecularly characterized a null mutant for the d5-HT7 receptor (#24705) and showed that 5-HT fails to modulate both excitatory and inhibitory synaptic transmission in this receptor mutant background as compared to the wild-type neurons (Figs. 4.05 & 4.09). Using an antagonist for the 5-HT7 receptor (SB258719) I was also able to demonstrate that this compound can block the effect of 5-HT on both excitatory and inhibitory PSCs (Figs. 4.05 & 4.09). I further show that 5-HT dependent modulation of excitatory synaptic transmission is through receptor-mediated G-α-s activation of adenylate-cyclase and subsequent activation of the cAMP-PKA pathway (Fig. 4.03). These results suggest the cAMP-PKA activating 5-HT GPCRs may be essential for modulating central synaptic transmission in *Drosophila* similar to the observations in vertebrate model systems (Roberts and Hedlund, 2011). My results are consistent with those reported with other invertebrates like *Aplysia* where the 5-HT7 like receptor homolog has been shown to mediate the effect of 5-HT on synaptic facilitation at a sensory-motor synapse in a cAMP dependent manner (Lee et al., 2009). Previous work from our lab (Lee and O’Dowd, 2000; Yuan and Lee, 2007) and my results from Chapter Three (Fig. 3.04) suggest that cAMP signaling may be a key modulator of synaptic transmission at *Drosophila* central synapses. These observations, together with the results described in this chapter, suggest that 5-HT acting through the d5-HT7 receptor may be a key molecule in regulating synaptic transmission through cAMP signaling.
To determine if the effect of 5-HT on synaptic plasticity is pre or post-synaptic in nature, I recorded miniature PSCs in the presence of TTX in the bath solution. This compound blocks all AP-dependent pre-synaptic release but does not affect spontaneous AP-independent neurotransmitter release. My results show that the frequency of mEPSCs significantly increases in the presence of TTX (Fig. 4.06). 5-HT is a well known modulator of K+ channel function in *Drosophila* (Alshuaib et al., 2003; Hevers and Hardie, 1995). It is possible that the regulation of pre-synaptic K+ channels may result in prolonged depolarization and influx of Ca^{2+} through voltage sensitive calcium channels. Such influx of pre-synaptic calcium has been shown to be essential for the regulation of cholinergic mEPSCs in primary neuronal cultures (Lee and O'Dowd, 1999). It is thus probable that 5-HT acts on pre-synaptic ion channels to modulate the frequency of mEPSCs.

My results show that 5-HT application decreases the frequency of mIPSCs (Fig. 4.10). Earlier observations from our lab have suggested that activated cAMP signaling suppresses both sIPSC (Fig. 3.01) and mIPSCs in wild-type neurons (Ganguly and Lee, unpublished observations), while simultaneously increasing pre-synaptic GABA release (Fig. 3.05). Since cAMP is a key modulator of 5-HT’s effect on inhibitory synaptic transmission, it is possible that activation of cAMP via the binding of 5-HT to the d5-HT7 receptor regulates mIPSC frequency. 5-HT may directly modulate post-synaptic GABA receptor function to decrease receptor response, or it may alter pre-synaptic excitability (by decreasing neurotransmitter release) and thus decrease mIPSCs. My results show that when all post-synaptic PKA was blocked, 5-HT increased the
frequency of GABAergic IPSCs (Figs. 4.11 A & B). On the other hand the post-synaptic GABA$_A$ receptor response decreased significantly after 5-HT perfusion (Figs. 4.11 C & D). This indicates that d5-HT7 mediated activation of cAMP causes a decrease in GABA receptor function. Although 5-HT increases pre-synaptic GABA release in the absence of post-synaptic PKA, the decrease in the post-synaptic receptor response by 5-HT results in an overall decrease in the frequency of both mIPSCs and sIPSCs, irrespective of the pre-synaptic effects. This is remarkably similar to the effect of cAMP on sIPSCs and mIPSCs and suggests that 5-HT through d5-HT7 receptor mediated cAMP-PKA activation decreases post-synaptic GABA$_A$ receptor response.

Cyclic AMP signaling in the *Drosophila* MB has been shown to be critical for olfactory associative learning (Heisenberg, 2003). Although the 5-HT(1A) receptor homolog is known to be expressed in the *Drosophila* MB and modulate sleep (Yuan et al., 2006) and memory (Lee et al., 2011), no information is available on the role of the d5-HT7 receptor and its expression in the *Drosophila* MB. Since this is the only 5-HT receptor in *Drosophila* which activates cAMP, it is possible that it mediates synaptic plasticity in the MB neurons. Modulation of synaptic transmission may eventually lead to changes in cognitive behavior such as learning and memory. Thus in the next chapter I explore if the expression of d5-HT7 in the MB is required for olfactory learning and memory in the *Drosophila* larvae. This would allow us to assess if this receptor is essential for the regulation of synaptic transmission as well as behavioral learning in *Drosophila*. 
Figure 4.01: Effect of 5-HT on Cholinergic EPSCs in Wild-type (w^{1118}) Neurons
A) Representative trace from a single wild-type (w^{1118}) neuron showing that the application of 20 μM 5-HT (for 30 seconds) causes an increase in the frequency of cholinergic EPSCs.
B) A cumulative trace from multiple wild-type w^{1118} neurons (n=9) showing the effect of 5-HT on cholinergic EPSCs.
Figure 4.02: Effect of 5-HT on Cholinergic EPSCs in the Presence of a PKA Blocker (H89)

A) Representative trace from a single wild-type (w^{1118}) neuron showing that the application of 20 \( \mu \)M 5-HT (for 30 sec) in the presence of a PKA inhibitor (H-89) in the bath solution causes no change in the frequency of cholinergic EPSCs.

B) A cumulative trace showing the effect of 5-HT on wild-type (w^{1118}) cholinergic EPSCs without H-89 (circles, n=9) and with 50 \( \mu \)M H-89 in the bath solution (squares, n=5)
Figure 4.03: Effect of 5-HT on Cholinergic EPSCs in a Gαs Null Mutant Background
A) Cumulative trace showing the effect of 5-HT on cholinergic EPSCs in wild-type (w^{1118}, circles, n=9) and in a Gαs null mutant background (squares, n=5).
B) Representative trace from a single Gαs null mutant neuron showing that 100 μM forskolin (FSK), an activator of cAMP causes an increase in the frequency of cholinergic EPSCs, similar to that seen with 5-HT in the wild-type neurons.
Figure 4.04: Identification and Characterization of a d5-HT7 Mutant (#24705) in *Drosophila*

(A) Diagrammatic representation of the d5-HT7 gene showing the P element (Minos) insertion and the exon-exon spanning primers for the d5-HT7 gene (arrows).

(B) Agarose gel picture showing the reverse transcriptase PCR (RT-PCR) products obtained from mRNA isolated from the d5-HT7 mutant (#24705) and wild-type [w^{1118} & Canton-S (CS-Wt)] larvae. The image shows that the d5-HT7 null mutant strain lacks a functional copy of the gene while the wildtype strains express the d5-HT7 gene. RP49, a housekeeping gene was used as a control and both wild-type and #24705 mutant show normal expression of this gene.
Figure 4.05: Effect of 5-HT on Cholinergic EPSCs in a d5-HT7 Mutant and in Wild-type Neurons in the Presence of a d5-HT7 Receptor Antagonist

A) A representative trace from a single neuron of the d5-HT7 null mutant (#24705) strain showing that the application of 20 μM 5-HT has no effect on the frequency of cholinergic EPSCs.

B) Bar graph shows the % frequency of cholinergic EPSCs during 30 seconds of 20 μM 5-HT application. 5-HT application in the wild-type (w1118) neurons led to an increase in frequency of cholinergic EPSCs (white bar, n=9), which was blocked in the presence of 60 μM SB-258719 (a 5-HT7 antagonist) dark grey bar (n=5). The light grey bar shows the effect of 5-HT on the frequency of cholinergic EPSCs in the #24705 mutant neurons.
Figure 4.06: Effect of 5-HT on mEPSCs in Wild-type (w^{1118}) Neurons

A) Cumulative trace showing the % frequency of miniature EPSCs (mEPSCs) in wild-type (w^{1118}) neurons during the application of 20 μM 5-HT (n=3). The mEPSCs were recorded in the presence of 1 μM tetrodotoxin (TTX) in the bath solution.

B) Bar graph showing the average % frequency of mEPSCs from three wild-type (w^{1118}) neurons before (white bar), during (dark grey bar) and after (light grey bar) 5-HT application. (**p<0.005)
Figure 4.07: Effect of 5-HT on Wild-type (w^{1118}) GABAergic IPSCs
A) A representative trace from a single wild-type (w^{1118}) neuron showing that the application of 20 μM 5-HT (for 30 seconds) causes a decrease in the frequency of GABAergic IPSCs.
B) A cumulative trace from multiple wild-type (w^{1118}) neurons (n=6) showing the effect of 20 μM 5-HT on GABAergic IPSCs.
Figure 4.08: Effect of 5-HT on Wild-type (w^{1118}) GABAergic IPSCs in the Presence of a PKA Blocker (H89)

A) A representative trace from a single wild-type (w^{1118}) neuron showing that the application of 20 μM 5-HT (for 30 sec) in the presence of a PKA inhibitor (H-89) in the bath solution causes no change in the frequency of GABAergic IPSCs.

B) A cumulative trace showing the effect of 5-HT on wild-type (w^{1118}) GABAergic IPSCs without H-89 (circles, n=6) and with 50 μM H-89 in the bath solution (squares, n=4).
Figure 4.09: Effect of 5-HT on GABAergic IPSCs in the d5-HT7 Null Mutant (#24705) Neurons

A) Representative trace from a single d5-HT7 null mutant (#24705) neuron showing that 20 μM 5-HT (FSK) causes no change in the frequency of GABAergic IPSCs.

B) Graph shows the frequency of GABAergic IPSCs during the application of 20 μM 5-HT in wild-type (w1118) neurons in the absence (white bar, n=6) and presence of 60 μM 5-HT7 receptor antagonist (SB258719, gray bar, n=4). The cumulative frequency of GABAergic IPSCs during the application of 5-HT on the d5-HT7 receptor null mutant (#24705) is shown by the light gray bar (n=7).

(***p<0.005)
Figure 4.10: Effect of 5-HT on mIPSCs in Wild-type (w^{1118}) Neurons

A) A cumulative trace showing the % frequency of miniature IPSCs (mIPSCs) in wild-type (w^{1118}) neurons during the application of 20 μM 5-HT (n=3). The mIPSCs were recorded in the presence of 1 μM tetrodotoxin (TTX) and 1μM curare (to block acetylcholine receptors) in the bath solution.

B) Bar graph shows the average % frequency of mIPSCs from three wild-type (w^{1118}) neurons before (white bar), during (dark grey bar) and after (light grey bar) 5-HT application. (**p<0.005)
Figure 4.11: Serotonin Regulates GABAergic IPSCs by Decreasing Postsynaptic GABA<sub>A</sub> Receptor Response

A) Cumulative trace showing the effect of 20 μM 5-HT application on GABAergic IPSCs in the presence of 200 μM membrane impermeable PKA blocker (PKI 6-22 amide) in the internal recording pipette. In the presence of PKI, the frequency of GABAergic IPSCs increases significantly during the application 20 μM 5-HT (n=5).

B) Graph shows the effect of 20 μM 5-HT alone (n=6), in the presence of 50 μM H-89 in the bath solution (n=4) and, in the presence of 200 μM PKI amide in the internal recording pipette (n=5) on the frequency of GABAergic IPSCs in w<sup>1118</sup> neurons.

C) Trace from a single neuron showing the effect of 100 μM GABA application for two seconds before and after the application of 20 μM 5-HT.

D) Shows the cumulative amplitude of GABA receptor response left after 3 minutes of perfusion with external recording solution alone (n=5, light gray bar) and external recording solution with 20 μM 5-HT (n=6, dark grey bar). (** p<0.005)
CHAPTER FIVE

_Drosophila 5-HT7 Receptor (d5-HT7) in the Mushroom Body Regulates Larval Olfactory Learning_

5.1 INTRODUCTION

The representation of accurate sensory information from the environment to a higher brain center poses a significant challenge not only for mammals but also for simple invertebrates like the locusts, honeybees and the fruit fly. The adult insects have to sort from a combination of odors in the atmosphere to locate a food source, decide between preferred and non-preferred food sources and to find mates using pheromone cues. The primary center for olfactory information processing and integration in the insects is a neuropile structure called the mushroom body (Strausfeld et al., 1998). In the fruit fly _Drosophila melanogaster_ the mushroom body has been shown to be involved in learning and memory (Heisenberg, 2003) and a range of other critical behaviors including sleep, visual learning, temperature sensation, and odor perception (Tanaka et al., 2008). Olfactory associative learning, where the flies associate an odor (conditioned stimulus – CS) with a sugar/bitter reward or electric shock (unconditioned stimulus – US), has been shown to be critically dependent on the mushroom body intrinsic neurons, the Kenyon cells (Keene and Waddell, 2007). While the odor information is mostly processed through the olfactory system, the sensory information (electric shock, sweet/bitter food reward) is conveyed through a range of signaling pathways (monoamines in particular) which provide inputs to the mushroom body. _Drosophila_ has become the organism of choice for studying olfactory information processing and
integration (Heisenberg, 2003) because of the availability of a wide variety of genetic tools (Venken and Bellen, 2005), ease of manipulation of gene expression in neuronal circuits (Brand and Perrimon, 1993; Lee and O'Dowd, 1999), and a range of known behavioral assays.

The olfactory odor information is conveyed to the mushroom body (MB) via the olfactory receptor neurons (first order neurons) in the 3rd antennal segment to the antennal lobes (AL). The projection neurons (second order neurons) then carry the information from the AL to the MB neurons through the inner antennocerebral tract (iACT). The intrinsic neurons of the mushroom body (Kenyon cells) send dendritic projections to the calyx, which receives odor information from the projection neurons. There are about 2500 Kenyon cells in the MB which send out axonal processes and these collectively form the peduncle. The axons of the Kenyon cells branch out to give rise to the $\alpha$, $\alpha'$, $\beta$, $\beta'$ and $\gamma$ lobes of the MB (reviewed in (Heisenberg, 2003; Keene and Waddell, 2007; Selcho et al., 2009; Tanaka et al., 2008). Kenyon cells are subdivided into three major classes depending on the lobe to which they send their axonal projections. Thus, the $\gamma$ neurons project to the $\gamma$ lobe, the $\alpha/\beta$ neurons to the $\alpha$ and $\beta$ lobes, and the $\alpha'/\beta'$ neurons project to $\alpha'$ and $\beta'$ lobes.

The different subclasses of MB neurons, and even some MB extrinsic neurons, are known to be involved in olfactory associative learning in the adult fly. Specifically, the $\gamma$ neurons have been shown to be essential for short term memory (Zars et al., 2000); while the $\alpha/\beta$ lobe neurons are needed for long term memory (Pascual and Preat, 2001; Yu et
and memory retrieval (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). The output from the $\alpha'/\beta'$ lobes has also been shown to be essential for acquiring and stabilizing memory (Krashes et al., 2007).

Clonal analysis using MARCAM (Lee et al., 1999) has revealed that unlike the adult MB neurons, all larval MB neurons (till the mid 3rd instar) are of only one type, i.e. the $\gamma$ neurons. The axons of the $\gamma$ neurons split to form only two lobes (one medial and one dorsal) instead of the five seen in the adult (Crittenden et al., 1998). Thus the Drosophila larvae, with just one subset of neurons, make a good model to study learning and memory as the contribution of just this sub-type of neurons in learning can be clearly defined.

Even with just the $\gamma$ subset of MB neurons, the Drosophila third instar larvae have been shown to be capable of forming both middle-term appetitive memory (Hendel et al., 2005; Honjo and Furukubo-Tokunaga, 2005) and aversive olfactory associative memory (Aceves-Pina and Quinn, 1979; Honjo and Furukubo-Tokunaga, 2009). Recently, a study has shown that larvae are capable of pairing aversive and appetitive stimuli with visual stimulus (Aso et al., 2009). In both the larvae and the adult the output of the MB neurons has been shown to be essential for memory retrieval but not for memory acquisition and consolidation (Dubnau et al., 2001; Honjo and Furukubo-Tokunaga, 2005; McGuire et al., 2001). This indicates that olfactory memory might be stored in the MB neurons. Thus although simple, the larval olfactory system does retain significant stereotypic
connections like the adult olfactory system, has a low signal to noise ratio and is able to form associative memories (Gerber and Stocker, 2007).

The sensory stimuli from the environment to the mushroom body are conveyed by various groups of neurotransmitter releasing neurons. In experimental set-ups, these stimuli are usually in the form of sugar/bitter reward or electric/heat shock. These signals are primarily conveyed by three major monoaminergic neurons – the octapamine, dopamine and serotonergic neurons. Recently, GABA releasing APL (Liu and Davis, 2009) and acetylcholine releasing MB-V2 neurons (Sejourne et al., 2011) have also been shown to be involved in olfactory associative learning.

Octapamine (OA) positive neurons innervate the sub-esophageal ganglion (SOG) extensively and also send out projections to the mushroom body calyx and the γ lobe and the antennal lobes (Busch et al., 2009; Busch and Tanimoto, 2010; Honjo and Furukubo-Tokunaga, 2009; Tanaka et al., 2008). In the *Drosophila* larvae, the expression of octapamine/tyramine is restricted to the MB calyx and the antennal lobe, with little expression seen along the vertical MB lobes (Honjo and Furukubo-Tokunaga, 2009).

Appetitive (sucrose) olfactory learning has been shown to be dependent on the output of the octapaminergic neurons in the *Drosophila* adult (Kim et al., 2007; Schwaerzel et al., 2003) as well as the larvae (Honjo and Furukubo-Tokunaga, 2009; Schroll et al., 2006). Based on the extensive innervation patterns of OA neurons onto the SOG and MB, it has been proposed that the positive reward (sucrose signal) is conveyed to the MB via direct and in-direct (AL-MB) connections (Honjo and Furukubo-Tokunaga, 2009).
Dopamine (DA) is also expressed in several neurons in the *Drosophila* brain. In the adult brain, dopaminergic neurons innervate almost all lobes of the MB and the peduncle, with little innervation in the MB calyx. There is also some interglomerular innervation in the antennal lobe (Honjo and Furukubo-Tokunaga, 2009; Tanaka et al., 2008). In the *Drosophila* larvae about 95 dopaminergic neurons (Selcho et al., 2009) innervate the vertical MB lobe extensively. However, no innervation of the antennal lobe and the calyx has been seen (Honjo and Furukubo-Tokunaga, 2009). Similar to OA neurons, the output of the DA neurons is critical for the acquisition of aversive learning in adult (Schwaerzel et al., 2003) as well as the larvae (Honjo and Furukubo-Tokunaga, 2009; Schroll et al., 2006; Selcho et al., 2009).

Of all the monoaminergic systems, serotonergic neurons in the *Drosophila* brain and their innervation patterns on to the MBs have been least studied. In a recent study, 38 to 41 serotonergic neurons were identified in the adult *Drosophila* brain and were found to be required for place learning (Sitaraman et al., 2008). Most of these overlapped with those identified previously (Valles and White, 1988). Among these are the DPM neurons which are known to be required for olfactory learning (Keene and Waddell, 2007). These serotonin positive neurons innervate the α/β lobe of the *Drosophila* mushroom body and are essential for anesthesia resistant memory (ARM) in the *Drosophila* adults (Lee et al., 2011). In the 3rd instar larval brain 36-43 serotonin positive neurons have been recently identified (Giang et al., 2011). Thus the role of serotonergic neurons in olfactory
associative learning and higher cognitive function in both *Drosophila* adults and larvae has remained relatively unexplored.

Most monoamine receptors in the fly and larval brain are G-protein coupled receptors (GPCRs) which can modulate cellular cAMP levels through G-protein components. Both the OAMB receptor (for OA) and two DA GPCRs, dDA1 and DAMB are expressed in the MB neurons (Han et al., 1998; Han et al., 1996). The dDA1 receptor has not only been shown to be critical for aversive learning in the adult but it is also known to partially impair appetitive learning (Kim et al., 2007). In comparison, the expression and the possible contribution of the three reported *Drosophila* serotonin receptors (Saudou et al., 1992) on olfactory learning and other cognitive functions is relatively poorly understood.

Among the serotonin receptors found in *Drosophila*, the expression of d5-HT1A receptor has been shown in the mushroom body where it has been implicated in sleep (Yuan et al., 2005) and the ARM (Anesthesia-resistant memory) form of olfactory learning (Lee et al., 2011). The d5-HT2 receptor expression in the *Drosophila* brain has been shown to be necessary for aggression and courtship behaviors (Nichols, 2007). The d5-HT7 receptor expression has been shown in the ellipsoid body and is known to be required for courtship behavior (Becnel et al., 2011). A recent study has also implicated various d5-HT receptors in olfactory learning using a combination of antagonists and RNAi strains (Johnson et al., 2011).
The 5-HT7 receptor is expressed extensively in the mammalian brain including the cortex, hippocampus, thalamus and hypothalamus and has been implicated in regulation of circadian rhythms, learning and memory (Hedlund and Sutcliffe, 2004). In the mammalian system, three 5-HT receptors (5-HT5, 5-HT6 & 5-HT7) activate cAMP signaling (Barnes and Sharp, 1999) and this makes it extremely difficult to carefully identify the role of any individual 5-HT7 receptor in learning (Roberts and Hedlund, 2011). In *Drosophila* however only one 5-HT7 receptor like homolog (d5-HT7) is known to be positively coupled to $G_\alpha_s$ and to thus activate cAMP (Witz et al., 1990). This and the availability of powerful genetic manipulation tools in *Drosophila* led us to examine the role of d5-HT7 in olfactory associative learning.

In the previous chapter I have shown that 5-HT modulates both excitatory and inhibitory synaptic plasticity via the d5-HT7 receptor through cAMP signaling. Since cAMP signaling in the MB neurons of *Drosophila* is known to be essential for olfactory associative learning, in this chapter I examine the role of the d5-HT7 in olfactory associative learning. I ask whether the d5-HT7 receptor is expressed in the MB neurons and if this expression contributes to olfactory learning. To assess this I used combination of expression analysis and a simple larval learning assay to assess the role of the d5-HT7 in both the appetitive and aversive olfactory learning paradigms (Honjo and Furukubo-Tokunaga, 2005, 2009). In this chapter I address the following broad questions:

a) Is the d5-HT7 receptor required for appetitive olfactory learning?

b) Is the d5-HT7 receptor is expressed in the olfactory learning center (MB)?
c) Does this learning require the expression of the d5-HT7 receptor in mushroom body of the *Drosophila* larvae and can defects in learning be rescued by expressing the receptor in a mutant background?

d) Is this receptor also required for aversive learning and does this process involve the mushroom body?

5.2 RESULTS

5.2.1 Appetitive Associative Learning: Pentyl Acetate versus Propionic Acid

Using the appetitive olfactory learning assay (Honjo and Furukubo-Tokunaga, 2005) I show that *w*1118 larvae trained with propionic acid and sucrose showed a significantly higher Response Index (RI) than larvae trained with propionic acid and distilled water (Fig. 5.01). Thus, these larvae were able to associate the odor with the sucrose reward. On the other hand, two well-characterized olfactory learning mutants *dunce1* and *rutabaga1* (Keene and Waddell, 2007) did not show any significant change in RI when trained with odor paired with sucrose and distilled water. Thus these larvae were unable to associate the odor with the reward.

Although my results showed a significant increase in response when larvae were trained with propionic acid and sucrose or distilled water, the difference between the two observed values was relatively small (0.763±0.02 for sucrose and 0.637±0.04 for DW respectively). I therefore used another odor, pentyl acetate (banana odor), to train the larvae and to better discriminate between the two groups. Wild-type (*w*1118) larvae trained with pentyl acetate and sucrose gave an RI of 0.583±0.02 (as shown in Fig. 5.02)
while the larvae trained with odor and distilled water showed an RI of 0.294±0.02. This relatively large increase in the larval response index indicated a clear learning phenotype and was thus a more robust combination than propionic acid. I thus used this odor for all subsequent assays since it would allow discerning the response of genetic mutants that may have subtly different gene expression.

5.2.2 Appetitive Associative Learning in Wild-type and known Learning Mutants

I next examined the response index of wild-type and known olfactory learning mutants in my assay using the odor pentyl acetate. I also tested Canton-S (CS), a well known wild-type strain. This strain is genetically distinct from the w^{1118} strain used earlier but still gave RI scores were similar to those observed with the w^{1118} strain. In my experiments, the Canton-S strain larvae showed a significantly higher RI when trained with pentyl acetate (PA) and sucrose (0.586±0.02) as compared to PA and distilled water (0.294±0.04), shown in Fig. 5.03.

I then tested the olfactory learning mutants dnc1 (dunce1) and rut1 (rutabaga1) (Keene and Waddell, 2007). These mutants did not show a significant change in RI when trained with odor (pentyl acetate) and distilled water as compared to PA and sucrose. I thus conclude that my larval learning assay is sensitive enough to discriminate between strains with normal olfactory learning and the learning mutants.
5.2.3 The d5-HT7 Receptor is Required for Olfactory Associative Learning

In the previous chapter I described the characterization of a publicly available d5-HT7 mutant (#24705 Bloomington Stock Center). This strain showed no change in excitatory or inhibitory synaptic transmission on focal application of serotonin (5-HT) unlike the wild-type strains (Figs. 4.05 & 4.09). I next assessed whether d5-HT7 mutant larvae behaved like the olfactory learning mutants.

In addition to the #24705 (d5-HT7 receptor null, see Fig. 4.04) strain I also examined the #24142 strain which carries a large deletion in the third chromosome encompassing the d5-HT7 gene. This strain served as a negative control in my experiments. My results showed that the response index scores three minutes after the beginning of testing were similar for both distilled water and sucrose trained larvae (Fig. 5.04). The RI values were similar for both the strains which lacked a functional copy of the d5-HT7 gene. Thus both these strains were incapable of forming associations with odor and positive reward. These results are similar to those seen with dunce1 and the rutabaga1 mutants earlier (Fig. 5.03).

Using the Gal4-UAS binary expression system (Brand and Perrimon, 1993) I also expressed a d5-HT7-RNAi under a UAS sequence with a Gal4 driver (1407-Gal4) known to be expressed in all neurons of the central and peripheral nervous system (Luo et al., 1994). The progeny containing both the Gal4 and the UAS-d5-HT7-RNAi (1407-Gal4;UAS-d5-HT7-RNAi) are expected to have a reduced level of d5-HT7 receptor
expression. This strain did not show a significant increase in RI when trained with PA and sucrose versus PA and distilled water (Fig. 5.04).

I thus conclude that the d5-HT7 receptor is required for olfactory associative learning and mutations in this gene result in behavior similar to that of known learning and memory mutants.

5.2.4 d5-HT7 Expression in Drosophila Mushroom Body Neurons

It is important to note that the strains used in the learning assays described above lack d5-HT7 receptor in the larval nervous system and not just in a specific anatomical brain region. To identify the specific neuronal subsets responsible for the impaired olfactory associative learning, I first examined the MB neurons since they are known to be directly involved in larval olfactory learning (Honjo and Furukubo-Tokunaga, 2009). For this, I identified a Gal4 strain specific to the MB neurons since this would allow regulating d5-HT7 expression in just the MB neurons. The 201Y-Gal4 and 30Y-Gal4 are known to drive Gal4 specifically in the MB neurons of Drosophila larvae (Honjo and Furukubo-Tokunaga, 2005, 2009; Mochizuki et al., 2011; Pauls et al., 2010). To confirm this, UAS-mCD8::GFP was expressed under the control of 201Y-Gal4 and 30Y-Gal4 (Pauls et al., 2010) to assess expression patterns in the MB Kenyon cells of 3rd instar larvae. Confocal imaging of larval brains expressing GFP under the control of the two Gal4 drivers (Fig. 5.05) showed that both 201Y-Gal4 and 30Y-Gal4 are expressed specifically in the MB neurons of 3rd instar larvae. Counter-staining with the GFP antibody confirmed the expression pattern.
The results described in chapter 4 indicate that the d5-HT7 receptor is involved in regulating both excitatory and inhibitory synaptic transmission via the cAMP-PKA signaling pathway. In *Drosophila* the expression of cAMP pathway genes in the mushroom body (MB) is critical for higher order cognitive function including behavioral learning (Heisenberg, 2003). I thus explored whether the d5-HT7 receptor expression in the MB neurons could regulate cAMP levels and may thus be essential for learning. For this, the Gal4-UAS system was used to express GFP specifically in the MB neurons (201Y-Gal4; UAS-mCD8::GFP, Fig. 5.05) of 3rd instar *Drosophila* larvae. The GFP+tive MB neurons were sorted by FACS (Fluorescence activated cell sorting) from a single cell suspensions of the larval brains (Fig. 5.06 A). Wild-type larval brains (w^{1118}) that did not express GFP were used as negative controls to set the sort gates for GFP expression. GFP+tive neurons obtained from three such sorts were pooled, mRNA isolated and converted to cDNA. An RT-PCR for d5-HT7 receptor expression in the cDNA from GFP+tive and GFP-tive cells showed the presence of receptor transcripts in both the populations. The control housekeeping gene RP-49 also showed expression in both the sorted cell populations (Fig. 5.06 B). Our results thus convincingly demonstrate that the d5-HT7 receptor is expressed in the MB neurons of *Drosophila* larvae.

5.2.5 Appetitive Olfactory Learning Requires d5-HT7 Expression in the MB Neurons of *Drosophila* Larval Mushroom Body

My previous results have shown that the d5-HT7 receptor gene is expressed in the MB neurons of the 3rd instar larvae (Fig. 5.06). To specifically assess the effect of d5-HT7 in the larval olfactory learning, I next used the Gal4-UAS system to down-regulate d5-HT7
receptor expression exclusively in the olfactory learning center of the *Drosophila*, the mushroom body (MB). For this, I crossed UAS-d5-HT7-RNAi strain (#27273 Bloomington Stock Center) with the 201Y-Gal4 strain.

The response indices of homozygous 201Y-Gal4; UAS-d5-HT7-RNAi larvae trained to associate PA and sucrose (0.34±0.05; Fig. 5.07) were similar to those trained with PA and distilled water (0.297±0.05). However the homozygous larvae of the 201Y-Gal4 and UAS-d5-HT7-RNAi strains showed normal olfactory learning (Fig. 5.07). I thus conclude that the down-regulation of d5-HT7 receptor specifically in the MB neurons of the mushroom body impairs olfactory associative learning.

5.2.6 Over-Expression of the d5-HT7 Receptor in the MB neurons Enhances Appetitive Learning Response

In the previous section I have shown that the down-regulation of the d5-HT7 receptor in the MB neurons of the mushroom body impaired learning. I next examined whether the over-expression of this receptor in the MB neurons can enhance learning scores as compared to the wild-type larvae. I thus generated a 201Y-Gal4; UAS-d5-HT7 homozygous line. These larvae showed a significantly higher RI when trained with PA and sucrose (0.690±0.005) as compared to larvae trained with PA and distilled water (0.306±0.03; Fig. 5.08). The control 201Y-Gal4/+; UAS-d5-HT7/+ larvae gave RI scores similar to those seen earlier (Fig. 5.07). The RI of larvae trained with PA and sucrose in 201Y-Gal4; UAS-d5-HT7 larvae was significantly higher than w1118 larvae trained with PA and sucrose (0.690±0.005 vs. 0.568±0.02, Student’s t-test, p=0.055, n=7). Thus, the
over-expression of d5-HT7 receptor in the MB neurons of the larval mushroom body leads to enhanced olfactory learning.

5.2.7 Temperature Sensitive Down-Regulation of d5-HT7 Receptor Specifically in the MB Impairs Olfactory Associative Learning

In the previous sections I have shown that the down-regulation and over-expression of the d5-HT7 receptor in the mushroom body MB neurons are directly related to the larval learning scores. However, in both these cases the receptor was up-regulated or down-regulated in the MB throughout larval development. Thus the defects in olfactory learning may be due to a requirement of this receptor during larval development. To rule out this possibility I spatio-temporally restricted d5-HT7 receptor expression in the MB in a temperature sensitive manner using the Gal4/Gal80ts expression system. Gal80, a yeast protein, is an inhibitor of Gal4. The Gal80ts is a temperature sensitive form which gets denatured at 30°C and cannot inhibit Gal4 but acts normally at 19°C. To test this system, I first drove UAS-mCD8::GFP in the 201Y-Gal4 line and thus expressed GFP in the MB neurons. When I crossed the 201Y-Gal4;UAS-mCD8::GFP strain to the tub-P-Gal80ts1;TM6 strain, the corresponding F1 genotype (w1118; 201Y-Gal4/tub-P-Gal80ts1; UAS-mCD8::GFP/TM6) showed no expression of GFP in the MB neurons at 19°C (Fig. 5.09 C). These larvae had been allowed to develop to the 3rd instar stage at 19°C. These same larvae were then kept at 30°C for 16 hours before training to degrade Gal80ts. After induction at 30°C the larvae showed GFP expression specifically in the MB neurons (Fig. 5.09 D), confirming that my system worked.
I then repeated the above described experiments with the UAS-d5-HT7-RNAi line (w^{1118}; 201Y-Gal4/tub-P-Gal80ts1; UAS-d5-HT7-RNAi/TM6). Larvae which had developed at 19°C were able to associate PA with sucrose and learn as well as the wild-type w^{1118} larvae. However, when the same larvae were kept at 30°C for 16 hours, Gal80 was denatured and could not inhibit Gal4. This allowed for the expression of UAS-d5-HT7-RNAi in the MB neurons for 16 hours prior to training resulting in impaired olfactory learning in these larvae (Fig. 5.10).

These results clearly demonstrate that the defect in olfactory learning is not because of a developmental role for the receptor but due to impaired expression in specifically the MB. Interestingly, just 16 hours of expression of UAS-d5-HT7-RNAi in the MB neurons was sufficient to impair olfactory learning.

5.2.8 Expression of the d5-HT7 Receptor in Mutant Background Rescues the Impairment in Olfactory Associative Learning

I next assessed if the expression of the d5-HT7 receptor in a d5-HT7 mutant background can rescue the defects in learning. I approached this problem in two different ways. I first crossed the homozygous w^{1118}; 201Y-Gal4; UAS-d5-HT7-RNAi strain with the MB-Gal80 strain. This strain expresses Gal80 specifically in the mushroom body, and would thus inhibit Gal4 activity specifically in the MB. The w^{1118}; 201Y-Gal4; UAS-d5-HT7-RNAi larvae were unable to learn as shown earlier (see Fig. 5.07). When crossed to the MB-Gal80 strain the F1 progeny were able to associate PA with sucrose (0.60±0.04) significantly more than PA and distilled water alone (0.36±0.03). This indicates that
blocking Gal4 driven RNAi activity in the MB alone can rescue learning behavior in an otherwise mutant background (see Fig. 5.11).

I then confirmed these results by expressing a wild-type copy of the d5-HT7 gene (UAS-d5-HT7) specifically in the MB neurons of a d5-HT7 null mutant (#24705 strain). For this, I mobilized the P element in the #24705 strain from the 2nd to the 3rd chromosome since the wild-type d5-HT7 gene was also on the 3rd chromosome. I then generated a w\textsuperscript{1118}; 201Y-Gal4; P-24705 homozygous strain and crossed it to the w\textsuperscript{1118}; UAS-d5-HT7; P-24705 strain. The resulting progeny expressed the d5-HT7 gene specifically in the MB neurons in a mutant (#24705) background. The RI for w\textsuperscript{1118}; 201Y-Gal4/UAS-d5-HT7; P-24705/24705 larvae trained with 1M sucrose and pentyl acetate was significantly higher (0.568±0.03) as compared to distilled water and odor (0.256±0.02, n=4). My results indicate that the expression of a wild-type copy of the d5-HT7 receptor gene in the MB neurons alone can completely rescue the defects in olfactory learning seen in the d5-HT7 null mutant.

Thus the expression of a single functional copy of the d5-HT7 receptor gene, or blocking Gal4 activity specifically in the MB neurons in an otherwise mutant/null background can rescue the learning phenotype. The results described in this section strengthen my previous observations and strongly indicate that the expression of the d5-HT7 receptor in the MB neurons is critical for appetitive olfactory learning.
5.2.9 The d5-HT7 Receptor is Required for Aversive Olfactory Learning

I have so far described that the expression of d5-HT7 receptor in the mushroom body neurons is essential for appetitive olfactory learning. In literature, it has been suggested that appetitive and aversive learning pathways are independent of each other. While appetitive olfactory learning requires an output from octapamine neurons, aversive olfactory learning needs output from dopaminergic neurons in the *Drosophila* adult (Schwaerzel et al., 2003) and larvae (Honjo and Furukubo-Tokunaga, 2005, 2009). It has also been shown that some receptors like dDA1 that are expressed in the mushroom body are needed for both appetitive and aversive learning (Kim et al., 2007). I thus examined if d5-HT7 receptor is similarly required for both these types of learning in the *Drosophila* larvae.

To test for aversive learning I used a bitter compound quinine hemisulphate (Honjo and Furukubo-Tokunaga, 2009). Wild-type larvae (Canton-S strain) were trained to associate pentyl acetate with distilled water or 0.1% Quinine Hemisulphate (QH). The RI scores for odor and QH trained larvae were significantly lower than those from larvae trained with odor and distilled water. Thus wild-type larvae trained with odor and QH learnt to avoid the odor when tested (Fig. 5.12). Moreover the #24705 d5-HT7 mutant larvae were clearly unable to avoid the odor even after training with the bitter compound (Fig. 5.12). This indicates a defect in aversive olfactory learning. I thus conclude that the d5-HT7 receptor may also be involved in aversive learning.
5.2.10 Expression of the d5-HT7 Receptor in the MB Neurons of the Mushroom Body is Required for Aversive Olfactory Learning

I next examined the effect of down-regulating d5-HT7 receptor specifically in the MB neurons of the larval mushroom body to assess if receptor expression in these neurons is essential for aversive olfactory learning. Larvae of the homozygous w^{118}; 201Y-Gal4; UAS-d5-HT7-RNAi strain described earlier (section 5.2.5) were trained with pentyl acetate and distilled water or 0.1% QH. I noted that the RI scores of the larvae trained with either QH or distilled water were similar (Fig. 5.12). While the 201Y-Gal4/+ larvae were able to associate the bitter compound with the odor and avoid the odor during testing, the down-regulation of the d5-HT7 receptor in the MB neurons impaired the formation of this association (Fig. 5.12).

Thus my results indicate that d5-HT7 receptor expression in the MB neurons of the mushroom body is required for aversive olfactory learning in the *Drosophila* 3rd instar larvae.

5.2.11 Controls to Determine Larval Sensory Discrimination Ability

I finally assessed if the d5-HT7 receptor mutation alters the sensory system. If this is the case, then the changes in the response indices would not be due to a failure in associating odor with positive or negative reinforcement but due to altered sensory discrimination ability. Thus, the experiments described below were done as an essential control for all the experiments described so far.
I first examined the naïve odor response of each genotype to a particular odor. The 3rd instar larvae were tested for attraction to pentyl acetate without any training with distilled water or sucrose. In my experiments, all the genotypes gave a response index ranging from 0.32±0.02 to 0.35±0.04 for pentyl acetate (Fig. 5.13 A). These scores were not significantly different from those obtained from wild-type larvae trained with distilled water and pentyl acetate. I thus conclude that neither the d5-HT7 receptor mutants nor the various Gal4-UAS strains used in this study had any defects in naïve odor attraction to pentyl acetate. Therefore, the alteration in the d5-HT7 receptor expression does not alter the odor discrimination ability of the mutant strains.

I next examined if the larvae were able to taste the sucrose solution even when d5-HT7 receptor expression was altered. For testing this, a 0.5% agar solution was prepared in either water or sucrose solution. The two solutions were poured separately in a divided petri-dish. For testing, 3rd instar larvae were placed on the center divider of the plate and allowed to move freely. Five minutes later the number of larvae on each side was noted and a ratio of larvae on the side with sucrose versus the one with distilled water was estimated. My results indicated that both the d5-HT7 receptor mutant lines and the various Gal4-UAS lines always had more larvae on the side with sucrose. The values ranged from 0.450±0.03 to 0.575±0.03 (Table 5.01) and none of the genotypes showed a significant change from the wild-type strains. This suggests that alterations in d5-HT7 receptor expression does not change taste discriminatory abilities of the larvae.
I finally assessed if the larvae were actually ingesting the bitter compound QH during the aversive olfactory association training. If the larvae failed to ingest this compound, the purpose of the experiment would be defeated. To confirm this, I added a coloring agent Acid red-52 to the 0.1% QH solution and to distilled water during training with pentyl acetate. This dye stains the larval gut and fluoresces orange under UV light (Fig. 5.13 B). My experiments showed that about 90% larvae ingested distilled water and about 87% of the larvae ingested QH along with the dye. There thus appears to be no alteration in the ingestion/uptake capabilities of the larvae due to the absence/down-regulation of d5-HT7 receptor.

5.3 DISCUSSION

The experiments described in this chapter use the larval learning assay (Honjo and Furukubo-Tokunaga, 2005, 2009) to test olfactory learning behavior in wild-type and d5-HT7 receptor mutants. The reproducibility of the assay was assessed using two odors, propionic acid and pentyl acetate. Larvae were trained to associate these odors individually with positive (sucrose) and negative (bitter compound) cues and the attraction/repulsion of the larvae towards the odor larvae was tested. This gave a response index score (RI) which was indicative of the learning capacity of the larvae. My preliminary experiments were aimed to ascertain if the assay could be used to discriminate between wild-type (normal learning) and known learning and memory mutants (dunce1 and rutabaga1). I observed that wild-type larvae gave significantly higher R.I. scores when trained with sucrose as compared to distilled water alone (Figs. 5.01 and 5.03). Moreover, both the mutants were unable to learn when trained with
either of the two odors (Figs. 5.01 and 5.03). I thus established that my assay served as a good indicator of olfactory association index. Though my results demonstrated that both the learning mutants show no short-term learning, as described earlier (Aceves-Pina and Quinn, 1979; Honjo and Furukubo-Tokunaga, 2005), I observed that the dunce1 strain in particular shows a higher RI when trained with distilled water and odor as compared to the wild-type larvae. Similar observations have been made earlier (Honjo and Furukubo-Tokunaga, 2005). It thus appears that the dunce1 larvae may have increased attraction towards particular odors as compared to the wild-type when trained with only odor and distilled water. Despite the higher attraction to odor I saw no defects in their ability to taste sucrose (Table 5.01). These mutants were still unable to show any olfactory associative learning.

I next tested the associative learning ability of d5-HT7 receptor null mutant (#24705) and a deficiency line lacking the d5-HT7 receptor gene (#24142). In my experiments, both these strains are clearly incapable of associative learning when trained with odor (PA) and sucrose or distilled water (Fig. 5.04). These results indicated that the d5-HT7 receptor maybe essential for appetitive olfactory learning.

Cyclic AMP signaling in the Drosophila mushroom body has been shown to be critical for olfactory associative learning (Heisenberg, 2003). The mushroom body has been suggested to be the equivalent of the mammalian hippocampus (Cayre et al., 2002). Although the d5-HT1A receptor homolog is known to be expressed in the Drosophila MB and modulate sleep (Yuan et al., 2006) and memory (Lee et al., 2011), no information is
available about the role of the d5-HT7 receptor expression in the *Drosophila* MB. I demonstrate convincingly in this chapter that the d5-HT7 receptor is expressed in the MB neurons of 3rd instar larvae (Fig. 5.06).

In the 3rd instar *Drosophila* larvae only one class of mushroom body neurons – the γ neurons – are functionally expressed between 87-92 hours after egg laying (Lee et al., 1999). Combining this with the powerful genetic tools available in *Drosophila* gave me a simple yet powerful system to study associative learning in its most rudimentary form. I examined whether the expression of d5-HT7 receptor in these γ neurons of the MB was essential for olfactory learning. My results showed that both the down-regulation and up-regulation of d5-HT7 receptor in only the γ neurons of the MB affects olfactory associative learning (Figs. 5.07 and 5.08). Spatio-temporally restricting d5-HT7-RNAi expression in the MB neurons in a temperature dependent manner using the Gal4/Gal80 expression system led to a reversible form of learning (Fig. 5.10). I further showed that the expression Gal80 only in the MB neurons can restore olfactory learning in an otherwise learning mutant (Fig. 5.11). In addition, the expression of a functional copy of the wild-type d5-HT7 receptor gene in the γ neurons could rescue the learning phenotype in a d5-HT7 receptor null mutant background (Fig. 5.11). My control experiments indicate that impaired learning is not due to a defect in sensory system but is associated with improper expression in the brain (Fig. 5.13 & Table 5.01). My results thus establish that the expression of the d5-HT7 receptor specifically in the mushroom body is critical for appetitive olfactory learning.
Appetitive and aversive olfactory learning are known to depend on two distinct signaling pathways in both *Drosophila* larvae (Honjo and Furukubo-Tokunaga, 2005, 2009) and adult (Schwaerzel et al., 2003). I thus tested whether the d5-HT7 receptor is needed not only for appetitive but also for aversive learning. My preliminary experiments with d5-HT7 receptor null mutants indicated that this gene was essential for aversive learning (Fig. 5.12). I further showed that the expression of this receptor specifically in the MB neurons is required for aversive learning (Fig. 5.10). Thus my results indicate that the expression of d5-HT7 receptor in the mushroom body MB neurons is essential for both appetitive and aversive learning. This suggests that the receptor may be expressed in neurons responsible for the integration of both appetitive and aversive forms of learning.

The results described in the previous chapter showed that the d5-HT7 receptor is essential for regulation of 5-HT mediated changes in synaptic plasticity. Expression data from this chapter also showed that d5-HT7 mRNA is expressed in the MB neurons of 3\textsuperscript{rd} instar larvae. This novel receptor expression data in the MB neurons along with the results described in this chapter clearly suggest that d5-HT7 receptor expression in the MB is critical for olfactory associative learning. To the best of my knowledge, this is the first report in *Drosophila* conclusively indicating a functional role for d5-HT7 receptor in the MB.

At this time it is unknown if the serotonin neurons project on to the larval MB lobes and provide input signals during larval learning. A recent study in the adult *Drosophila* brain
has shown that serotonin neurons do project on to lobes of the MB and their input is responsible for olfactory learning (Lee et al., 2011). This study also implicates a role for an MB extrinsic serotonin expressing neuronal subset (DPM), that have earlier been shown to be essential for memory consolidation (Keene et al., 2006). It is thus possible that several such MB extrinsic neurons sending projections on to the MB (Tanaka et al., 2008) contain serotonin. Though this remains a subject for future studies, my results clearly show that d5-HT7 expression in the MB is required for olfactory learning.

Along with the electrophysiology data described in the previous chapter, the results described here indicate that d5-HT7 may be an example of a molecule that links changes in synaptic plasticity with behavioral learning.
Figure 5.01: Response Index (3 minute) for Larvae Trained with Propionic Acid and Distilled Water (DW) or 1M Sucrose (SUC)

The w^{1118} larvae show a significant increase in response index after training with sucrose (n=9), whereas dunce1 (n=8) and rutabaga1 (n=6), two well known learning mutants, fail to show any learning after being trained with 1M SUC and propionic acid. (** indicates p<0.005)
The w1118 larvae show a significant increase in response index after paired training with both odors propionic acid (n=9) and pentyl acetate (n=7). The difference in response index seen when trained with odor + DW versus odor + SUC was much higher with pentyl acetate as compared to propionic acid.

(***) indicates p<0.005

**Figure 5.02: Response Index (3 minute) for Larvae Trained with DW + Odor or 1M SUC + Odor**
Figure 5.03: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 1M SUC + Pentyl Acetate for Two Wild-type and Two Learning Mutant Strains

Both w^{1118} (n=7) and wild-type Canton-S (Wt CS, n=7) strain larvae show a significant increase in response index after training with sucrose and pentyl acetate. The two learning and memory mutants dunce1 (n=6), rutabaga1 (n=6) fail to show any learning. (** indicates p<0.005)
Figure 5.04: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 1M SUC + Pentyl Acetate for Wild-type and Two d5-HT7 Receptor Mutant Strains

The w^{1118} (n=7) strain larvae show a significant increase in response index after being trained with 1M sucrose and pentyl acetate. The d5-HT7 receptor mutant # 24705 (n=6) has a P-element insertion which disrupts expression of the receptor. The #24142 (n=5) deficiency mutant strain where the d5-HT7 gene is missing. Both these mutants fail to show any learning after training. The expression of UAS-d5-HT7 RNAi in a pan neuronal manner leads to an impairment of learning in larvae.

(*** indicates p<0.005)
Figure 5.05: Expression of GFP Specifically in MB neurons (Kenyon cells) using the Gal4-UAS Binary Expression System

A) Schematic representation of the Gal4-UAS system. The yeast Gal4 gene is placed downstream to a promoter of a protein expressed specifically in the *Drosophila* mushroom body. When these flies are crossed with those carrying the UAS (upstream-activating-sequence) gene promoter tagged to GFP gene sequence, GFP expression is seen in the mushroom body neurons alone.

B) Representative image from one such MB specific Gal4 driver (201Y-Gal4) showing that the expression of GFP is restricted to the mushroom body neurons of *Drosophila* 3rd instar larvae.

C) An image from another MB specific Gal4 driver (30Y-Gal4) showing the expression of GFP in the MB neurons in *Drosophila* 3rd instar larvae.
Figure 5.06: Detecting d5-HT7 Expression in FACS Sorted GFP+ive MB Neurons
A) FACS plot showing the forward scatter/ side scatter (left panel; representing the size and granularity) of neurons isolated from 201Y-GAL4; UASmCD8::GFP flies which express GFP only in the mushroom body. Cells under gate P1 were assessed for GFP expression (left panel) and the GFP+ and GFP- cells were sorted as shown (left panel). The average frequency of GFP+ cells in three separate sorts was ~2.0%.

B) The gel picture shows the RT-PCR product for RP49 (housekeeping gene) and d5-HT7R in mRNA isolated from GFP+ (~90,000 cells) and GFP- (~600,000) cells. The gel picture shows that the intensity of 5HT7R band is lower than that seen in the GFP- cells, indicating lesser expression.
The w^{1118} (n=7) larvae show a significant increase in learning after being trained with 1M sucrose and pentyl acetate. In the 201Y-Gal4; UAS-d5-HT7-RNAi strain, the expression of d5-HT7 is down-regulated specifically in the Kenyon cells of the larval mushroom body (olfactory learning center). Strains expressing only the 201Y-Gal4 driver (n=6) or the UAS-d5-HT7-RNAi (n=6) learn as well as the wild-type. However, larvae expressing both the Gal4 and UAS are unable to associate odor with 1M Sucrose (n=6).

(*** indicates p<0.005)
A) The w1118 larvae (n=7) show a significant increase in response index after training with 1M sucrose and pentyl acetate. The strains expressing only the 201YGal4 driver (n=6) or the UAS-d5-HT7 sequence (n=6) show normal learning. However, larvae in which d5-HT7 is over expressed in the MB Kenyon cells (201Y-Gal4; UAS-d5-HT7) show a learning score greater than that seen with wild-type larvae (n=7).

B) Shows the expanded view of the dotted-box in (A). Over-expression of the d5-HT7 gene in the mushroom body results in a significant increase over the wild-type in 3 minute score for larvae trained with pentyl acetate and 1M sucrose.

(*** indicates p<0.005, * indicates p<0.05)
Figure 5.09: Spatio-Temporally Restricted Expression of GFP in the Mushroom Body Neurons Using the Gal80\(^{ts1}\)/Gal4 Expression System

A) Schematic representation of the Gal80\(^{ts1}\)/Gal4 expression system. Gal80\(^{ts1}\) is a temperature sensitive mutant of Gal80, an inhibitor of Gal4. At 19\(^\circ\)C, Gal80\(^{ts1}\) inhibits Gal4 and does not allow the expression of GFP downstream of the UAS sequence. However, at 30\(^\circ\)C Gal80\(^{ts1}\) is denatured and thus GFP expression is turned on.

B) Representative image of larvae expressing the MB specific Gal4 driver (201Y-Gal4), the Gal80\(^{ts1}\) protein driven by tubulin promoter and the UAS-GFP sequence. Thus the genotype of the larvae was Tub-P-Gal80\(^{ts1}\)/201Y-Gal4; UAS-mCD8::GFP/TM6. The larvae were kept at 19\(^\circ\)C throughout development and show no GFP expression in the MB neurons.

C) The larvae shown in (B) were kept at 30\(^\circ\)C for 16 hours after developing at 19\(^\circ\)C for 6 days to inactivate Gal80\(^{ts1}\). Once Gal80\(^{ts1}\) is broken down, Gal4 drives GFP in the MB alone, as seen in the image.
Figure 5.10: Response Index (3 minute) for Larvae Expressing d5-HT7-RNAi in a Temperature Sensitive Manner Specifically in the Mushroom body

The temperature sensitive form of Gal80 (Gal80{ts1}) is denatured at high temperatures (30°C). At 19°C, the Gal80{ts1} protein inhibits Gal4, which is driven here specifically in the mushroom body by the 201Y-Gal4 driver. Thus at 19°C, Gal80{ts1} inhibits Gal4 and UAS-d5-HT7-RNAi is not expressed in the MB resulting in normal learning. However, when larvae develop normally at 19°C and are given a heat shock at 30°C for 16 hours before training Gal80{ts1} is denatured, leading to the expression of Gal4 and UAS-d5-HT7-RNAi specifically in the MB. This results in memory impairment. The left side bar graph shows the response index of w^{1118} ; 201Y-Gal4/ tub-P-Gal80{ts1} ; UAS-d5-HT7-RNAi/ TM6 or TM2 larvae developed at 19°C for the entire life span and then trained with distilled water and 1M sucrose. Larvae show significant increase in learning scores after being trained with SUC and odor compared to odor alone (n=7, p=0.0039). However when the same larvae were allowed to develop at 19°C and then shifted to 30°C for 16 hours before training (right hand side bars) with sucrose, they fail to associate odor with SUC and hence show no associative learning (n=7, p=0.7788). This experiment suggests that down-regulation of the d5-HT7 receptor in the MB alone for as little as 16 hours is enough to impair olfactory associative learning.

(*** indicates p<0.005)
Figure 5.11: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 1M SUC + Pentyl Acetate for Wild-type and Strains Expressing MB-Gal80 and d5-HT7 Genes Specifically in the MB Neurons of d5-HT7-RNAi and d5-HT7 Null Backgrounds, Respectively

The w^{1118} (n=7) larvae show a significant increase in response index after being trained with 1M sucrose and pentyl acetate. The expression of d5-HT7-RNAi in the mushroom body neurons alone (201Y-Gal4; UAS-d5-HT7-RNAi, n=6) leads to a suppression of olfactory learning. However, when Gal80, an inhibitor of the Gal4 protein, is expressed specifically in the mushroom body (MB-Gal80) in this RNAi background the larvae are able to learn normally, like the wild-type larvae (n=8). Similarly, when the d5-HT7 gene is driven in the mushroom body neurons in the d5-HT7 null background (w^{1118} ; 201Y-Gal4/UAS-d5-HT7; P-24705/P-24705) larvae can associate odor with sucrose reward as the wild-type strain (n=7), leading to a complete rescue of the mutant phenotype. (** indicates p<0.005)
Figure 5.12: Response Index (3 minute) for Larvae Trained with DW + Pentyl Acetate or 0.1% Quinine Hemisulphate (Bitter Compound) + Pentyl Acetate

Wild-type (Canton-S) larvae show a significant decrease in response index after training with quinine hemisulphate (n=6) indicating aversion to the compound. The d5-HT7 null mutant (#24705 strain) shows no aversion to pentyl-acetate when trained with quinine hemisulphate (n=6). Similarly down-regulating the expression of the d5-HT7 receptor in the mushroom body of the larvae results in lack of learning similar to the d5-HT7 null mutant (n=5).

(*** indicates p<0.005)
Figure 5.13: Olfactory and Gustatory Discrimination Controls

A) Graph shows the naïve odor response of larvae to the odor pentyl acetate. The larvae were washed and transferred directly on to the training plate without any training. The 3-minute response index of the larvae was calculated for the genotypes shown in the graph (n=5 for each genotype).

B) The w^{1118} and #24705 (d5-HT7 null mutant) larvae were trained with 1M SUC + 0.2 mg/ml of the dye Acid-Red 52 (Sulphorhodamine B) for 30 mins to check for gustatory uptake. 90% of the total larvae in both genotypes picked up the dye. Stained intestines of individual wild-type (i) and mutant (ii) larvae are shown.
Table 5.01: Gustatory Response Index of 3\textsuperscript{rd} Instar Larvae of Different Genotypes
Larvae were placed on a divided petridish containing 0.5% agar in dH\textsubscript{2}O) on one side and 0.5% agar in 1M sucrose solution on the other side. Larvae were placed in the center and the number of larvae in each half was counted after 5 minutes. Gustatory response index was calculated as:

\[
\text{(No. of larvae on 0.5\% Agar in 1M Sucrose)} - \text{(No. of larvae on 0.5\% Agar in 1M Sucrose)} \div \text{Total no. of larvae in both quadrants}
\]

<table>
<thead>
<tr>
<th>Genotype</th>
<th>5 minute gustatory response index</th>
</tr>
</thead>
<tbody>
<tr>
<td>(w^{1118})</td>
<td>0.450 (n=4)</td>
</tr>
<tr>
<td>Canton-S (CS-WT)</td>
<td>0.473 (n=5)</td>
</tr>
<tr>
<td>(dunce 1)</td>
<td>0.575 (n=4)</td>
</tr>
<tr>
<td>#24705 (d5-HT7 null)</td>
<td>0.512 (n=5)</td>
</tr>
<tr>
<td>1407-Gal4; UAS-d5-HT7-RNAi</td>
<td>0.527 (n=4)</td>
</tr>
</tbody>
</table>
CHAPTER SIX

Discussion

6.1 Summary of Results

In the previous chapters I have described how cAMP signaling plays a crucial role in regulating synaptic plasticity in *Drosophila* primary neurons. In particular, I have explored how cAMP mediated plasticity is crucial for both excitatory and inhibitory synaptic transmission, especially in the mushroom body neurons of *Drosophila*. In this study I also assess if these cAMP mediated changes in synaptic transmission can be correlated to changes in behavioral learning. The mushroom body (MB) in *Drosophila* is the only known anatomical structure critical for olfactory associative learning (Keene and Waddell, 2007). My results show that cAMP signaling in the MB neurons is essential for synaptic plasticity and defects in this signaling can be correlated with a loss in behavioral learning.

Earlier work from the lab has shown that cAMP signaling enhances excitatory synaptic transmission (Yuan and Lee, 2007) and this effect is altered in the *Drosophila* learning and memory mutant *dunce* (Lee and O'Dowd, 2000). However, these studies could not explain the mechanisms by which cAMP mediated plasticity alters inhibitory synaptic transmission. My work demonstrates that cAMP signaling via PKA causes a decrease in inhibitory GABAergic transmission. This is primarily the consequence of the action of cAMP on post-synaptic GABA receptors which results in a decrease in the response of post-synaptic GABA receptors (Fig. 6.01). This effect was also seen in both the
embryonic and late-stage mushroom body neurons. I also observed that this regulation is altered in the two well-characterized learning and memory mutants, *dunce* and *rutabaga*. Even though these mutants have physiologically different levels of cAMP, neurons from both show similar lack of GABA_A receptor modulation in the presence of cAMP. Interestingly, the MB neurons from both embryos and pupae of the mutants also showed similarly deregulated GABA_A receptor modulation. Since learning mutants have a known defect in cAMP signaling, it is possible that dysregulated cAMP-mediated inhibitory synaptic transmission in the MB neurons results in impaired learning.

In the second part of my thesis I demonstrate that the biogenic amine serotonin (5-HT) is a key regulator of synaptic plasticity, and functions by activating the cAMP-PKA signaling pathway. My studies indicate that 5-HT mediated changes in excitatory cholinergic synaptic transmission occur through an increase in pre-synaptic excitability and neurotransmitter release (Fig. 6.02). My data also suggests that like forskolin, 5-HT regulates inhibitory GABAergic transmission by suppressing post-synaptic GABA_A receptor (Fig. 6.03). The d5-HT7 GPCR is the only known 5-HT receptor positively coupled to cAMP signaling (Witz et al., 1990). This receptor is associated with the G-α-s protein, which activates cAMP signaling through the enzyme adenylate cyclase (Witz et al., 1990). Both the G-α-s and the adenylate cyclase enzyme have been shown to be essential for olfactory associative learning in behavioral assays (Connolly et al., 1996; Zars et al., 2000). I demonstrate that the d5-HT7 receptor, which is upstream of both these signaling molecules, is crucial for 5-HT mediated changes in synaptic plasticity. Null mutants for this receptor fail to show a response in both excitatory and inhibitory
synaptic currents on the application of 5-HT. It still remains to be examined if the expression of d5-HT7, or the lack of it, in just the MB neurons can alter learning behavior as a direct consequence of alterations in synaptic transmission.

To further elucidate the role of this receptor in behavioral learning, I first examined the expression of this receptor in the larval brain. FACS sorting and mRNA analysis showed that this receptor is expressed in the MB neurons of the 3rd instar Drosophila larvae. I then used olfactory associative learning assays along with genetic tools to both spatially and temporally down-regulate this receptor in the MB neurons. These assays demonstrated that the expression of this receptor in the MB neurons is essential for olfactory associative learning. I further showed that the learning impairment in the d5-HT7 receptor null mutants can be rescued by expressing a wild-type copy of the receptor (Fig. 6.04). My novel findings strongly indicate a vital role of d5-HT7 receptor in appetitive olfactory learning. My preliminary data also suggests that this receptor may also be essential for aversive learning. In conjunction with the electrophysiology data, these results suggest that serotonin mediated cAMP signaling may effect changes in behavioral learning by altering synaptic plasticity in the MB neurons.

6.2 Implications of this Study

My data indicates that cAMP mediated modulation of GABA receptor response is essential for regulating inhibitory GABAergic transmission. I demonstrate that this regulation is altered in two learning and memory mutants, dunce and rutabaga. These mutants show defective GABA<sub>A</sub> receptor response in the mushroom body neurons from
both the embryonic and pupal stages. These observations add to the recent studies which show that the projection of GABAergic neurons (Liu and Davis, 2009) and the GABA-Rdl subunit (Liu et al., 2007) is required for the formation of olfactory associative learning. In particular, it has been suggested that the over-expression of the GABA-Rdl subunit causes a decrease in olfactory associative learning specifically in the mushroom body neurons (Liu et al., 2007). This indicates that there may be an overall increase in GABAergic transmission due to the over-expression of this receptor subunit. Similarly, my work suggests that increased inhibition in the MB neurons of both the learning and memory mutants may account for some of their defects in behavioral learning.

Recent imaging studies in the MB neurons have shown altered cAMP signaling in the learning and memory dunce and rutabaga. Paired application of both forskolin and the neurotransmitter dopamine and octapamine showed defects in PKA signaling in the lobes of the MB neurons (Gervasi et al., 2010; Tomchik and Davis, 2009). However it was not clear if these alterations in PKA signaling had any functional effect on inhibitory synaptic transmission. My study demonstrates that this indeed occurs in an in vitro model for central synaptic transmission in Drosophila. Since there are a large number of inhibitory local neurons in both the olfactory and the optic system (Olsen and Wilson, 2008a), it would be interesting to examine how cAMP dependent inhibitory plasticity in the intact Drosophila MB (in vivo preparations) shapes behaviors such as olfactory associative learning.
My work demonstrates that the biogenic amine 5-HT mediates both excitatory and inhibitory synaptic transmission in a cAMP-PKA dependent manner. At the excitatory cholinergic synapses this is possibly through the regulation of 5-HT mediated increase in pre-synaptic neurotransmitter release. This is supported by evidence from other studies in *Drosophila* and other invertebrates which show that 5-HT regulates pre-synaptic excitability by acting on potassium channels (Alshuaib et al., 2003; Hevers and Hardie, 1995; Mercer et al., 1995). However at the inhibitory GABAergic synapses, 5-HT causes a decrease in post-synaptic GABA<sub>A</sub> receptor response possibly through PKA mediated phosphorylation, similar to that seen with cAMP alone. My work indicates that the effect of 5-HT on both excitatory and inhibitory synaptic currents may be through d5-HT7 receptor mediated activation of cAMP-PKA signaling. These results are consistent with reports in the mammalian systems where the 5-HT7 receptor has been shown to regulate synaptic plasticity (Roberts and Hedlund, 2011). The expression of this receptor in the hippocampus is also known to be essential for regulating many other types of learning behavior (Roberts and Hedlund, 2011).

It is not known if 5-HT7 receptor mediated regulation of synaptic plasticity in the hippocampus is essential during memory acquisition. This is difficult to test in the mammalian system since at least three 5-HT GPCRs positively coupled to cAMP are co-expressed in the mammalian hippocampus (Lucki, 1998; Nichols and Nichols, 2008). To specifically examine the role of a single receptor in memory formation, simple invertebrate models have been used since they express only one homolog of the 5-HT7 receptor (Saudou et al., 1992). For example in *Aplysia*, the 5-HT7 receptor homolog has
been shown to be important for synaptic facilitation through cAMP signaling during gill-withdrawal reflex (Lee et al., 2009). My work with *Drosophila* also demonstrates an important role of this receptor in regulating central synaptic transmission.

I then asked if the d5-HT7 receptor regulates cAMP mediated synaptic plasticity in the structures of the *Drosophila* brain involved in olfactory associative learning. Using a combination of genetic tools, FACS sorting and mRNA analysis, I show that this receptor is expressed in the mushroom body neurons of the third instar *Drosophila* larvae. My experiments show that the expression of the d5-HT7 receptor specifically in the MB neurons is important for acquisition of both appetitive and aversive larval learning. This is the first report linking any *Drosophila* 5-HT receptor with olfactory associative learning. Thus my study highlights a novel role for the d5-HT7 receptor in the regulation of 5-HT mediated synaptic plasticity and in the olfactory associative learning. Since this receptor is positively coupled to the cAMP signaling pathway and is expressed in the MB neurons, it is probable that 5-HT mediated synaptic transmission also directly contributes to learning and memory. It remains to be explored if *in vivo* changes in 5-HT mediated synaptic plasticity through d5-HT7 receptor are essential for behavioral learning.

The 5-HT neurons innervate the MB (Lee et al., 2011) and antennal lobes in *Drosophila*, and are essential for olfactory information processing (Dacks et al., 2009). There is no evidence in literature for a role of serotonergic (5-HT) neurons in olfactory learning. Other biogenic amines such as octapamine have been shown to be essential for olfactory
associative learning (Honjo and Furukubo-Tokunaga, 2009; Schwaerzel et al., 2003), while dopamine is required for aversive olfactory learning (Honjo and Furukubo-Tokunaga, 2009; Schroll et al., 2006; Schwaerzel et al., 2003; Selcho et al., 2009). My data suggests that the d5-HT7 receptor is involved in both appetitive and aversive olfactory learning. It is thus probable that the 5-HT neurons are downstream of both the octapamine and dopamine neurons which are also present in the Drosophila MB and antennal lobes (Honjo and Furukubo-Tokunaga, 2009). The input from these neurons on to the 5-HT neurons either in the antennal lobe or MB may thus be essential for olfactory learning. Thus down-regulating or blocking d5-HT7 receptor expression affects both forms of learning in the Drosophila larvae.

6.3 Future Directions

One of the most challenging and unanswered questions in neuroscience is establishing whether changes in synaptic transmission can directly result in behavioral learning. This is a difficult point to address since most electrophysiology studies which provide information on synaptic plasticity are done either with simple in vitro preparations or whole brain explants, but rarely in vivo (Lee and O'Dowd, 1999; Wilson, 2011; Wilson et al., 2004). Though useful, in vivo recordings have so far been done only on the superficially placed neuronal subsets in Drosophila (Gu and O'Dowd, 2006; Sheeba et al., 2008). In the recent years advances in imaging have led to a range of photoactivable dyes which can be monitored using two photon imaging in the intact organism (reviewed in Olsen and Wilson, 2008a). These studies are limited by the fact that only a few photoactivable dyes are known and they can give only limited information on the
various ion channels working at individual synapses to alter synaptic plasticity. In mice the recently developed optogenetic tools have been used to activate a pre-synaptic neuron in a particular circuit by light and whole cell \textit{in vivo} recordings are then performed on the downstream neurons (Deisseroth, 2011). These techniques are very useful in exploring the role of a particular circuit or region of the brain involved in a particular behavior. In contrast to study behaviors such as learning and memory, it would be essential to monitor how individual synapses and the activity of ion channels in them, change during memory formation, storage and retrieval \textit{in vivo}. Though technically challenging, a recent study has shown that this is possible.

In an excellent \textit{in vivo} study with locusts (Cassenaer and Laurent, 2012), it was shown that pre-synaptic input (or odor) application at the MB neurons led to a corresponding increase in post-synaptic β lobe neurons resulting in spike timing dependent plasticity. However, when olfactory learning like situation was mimicked by pairing specific odors with an injection of octapamine, a corresponding decrease in synaptic response in the β lobe neurons was noted. This decrease was seen only with odors paired with octapamine and not with those applied alone. This study suggests that alteration of synaptic plasticity can directly be induced by octapamine (unconditioned stimulus) when paired with odors. Although such studies remain challenging if not heroic in nature, it would be interesting to use these techniques to explore how cAMP mediated plasticity is altered through the d5-HT7 receptor during \textit{in vivo} olfactory learning in the MB neurons. This will help bridge the existing gap between studies of \textit{in vitro} synaptic plasticity and behavioral learning assays.
Figure 6.01: cAMP Modulates Inhibitory GABAergic Transmission through Post-Synaptic GABA_A-Like Receptors
The figure shows a diagrammatic representation of possible mode of action of forskolin on inhibitory GABAergic synaptic transmission.

Our data suggests that forskolin binds to the enzyme adenylate cyclase which leads to the production of cAMP. cAMP then activates the enzyme protein kinase A (PKA). PKA probably phosphorylates one of the subunits of the post-synaptic GABA_A-like receptor. This leads to a decrease in the response of post-synaptic GABA_A-like receptor to the GABA. This regulation is altered in learning and memory mutants dunce and rutabaga, which have altered levels of cellular cAMP. This deregulation of GABA receptor function is also seen in the MB neurons of both the learning mutants.
Figure 6.02: Serotonin (5-HT) Regulates Excitatory Cholinergic Transmission through an Increase in Pre-Synaptic Neurotransmitter Release

My study indicates that 5-HT causes an increase in pre-synaptic neurotransmitter release through its action on d5-HT7 receptor. d5-HT7 activates Gαs upon binding 5-HT by converting GDP to GTP. The GTP bound Gαs protein activates adenylate cyclase, which converts ATP to cAMP. cAMP then activates PKA which can increase pre-synaptic neurotransmitter release possibly by altering potassium channel function. This leads to an increase in ACh release and an increase in the frequency of excitatory cholinergic EPSCs.
Figure 6.03: Serotonin (5-HT) Regulates Inhibitory GABAergic Transmission by Decreasing Post-Synaptic GABA_A Receptor Response

My study indicates that 5-HT decreases GABAergic IPSCs by modulating post-synaptic GABA_A receptor response. The binding of 5-HT to the d5-HT7 receptor results in the activation of cAMP-PKA signaling, which causes a decrease in GABA_A receptor response. 5-HT alone can increase the frequency of GABAergic IPSCs when all post-synaptic PKA is blocked. However, under conditions when PKA is not blocked, 5-HT mediated activation of cAMP and the subsequent decrease in GABA_A receptor response results in an overall decrease in the frequency of both sIPSCs and mIPSCs.
Figure 6.04: d5-HT7 is Expressed in Larval Mushroom Body Neurons (Kenyon cells) where it Regulates Olfactory Learning

A) Diagram shows the location of mushroom body in the third instar Drosophila larvae. 
B) Shows a magnified view of the Drosophila larval mushroom body and, 
C) Shows individual Kenyon cell neurons expressing the d5-HT7 receptor.

My data indicates that the expression of this receptor in the MB neurons is important for associative learning in the Drosophila larvae.
REFERENCES


APPENDIX

List of Publications

- **Archan Ganguly** and Daewoo Lee. Suppression of inhibitory GABAergic transmission by cAMP signaling pathway: alterations in learning and memory mutants. *(Under review)*

- **Archan Ganguly** and Daewoo Lee. The role of *Drosophila* 5-HT7 receptor on the modulation of excitatory synaptic transmission and its implication in olfactory associative learning in *Drosophila* larvae. *(Manuscript in preparation)*
