EFFECTS OF SLEEP-DEPRIVATION ON LONG-TERM DEPRESSION OF EXCITATORY SYNAPTIC TRANSMISSION IN THE CA1 REGION OF THE HIPPOCAMPUS

by

Ramakrishna Tadavarty

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Abstract

In the central nervous system (CNS), synapses are considered to be the loci for memories. Activity dependent strengthening or weakening of synaptic strength via long-term potentiation (LTP) or depression (LTD), respectively, is widely postulated to underlie memory formation. Sleep, which is universally present across most species in the animal kingdom, aids learning and formation of memories. On the contrary, sleep-deprivation (short- or long-term), disrupts memory formation and task re-performance. Exactly how this happens is unclear. In the present study, we hypothesized that sleep-deprivation affects LTD, which may in-turn be responsible for cognitive deficits observed at the behavioral level. Following a 12 h period of sleep-deprivation, LTD of the population excitatory postsynaptic potentials (pEPSPs) induced using a 20 Hz, 30s tetanus to Schaffer collaterals in the CA1 region of the hippocampus, is enhanced in sleep-deprived (SD) rats. We investigated the role of metabotropic glutamate receptors (mGluRs), γ-Aminobutyric acid (GABA)-A receptors (GABA_A-Rs), GABA_B-Rs and N-methyl-D-aspartic acid receptors (NMDARs) in the LTD. The requirement of Ca^{2+} through L- and T-type voltage-gated calcium channels (VGCCs) and intracellular stores was also studied. Results indicate that mGluRs, a release of Ca^{2+} from intracellular stores and GABA_B-Rs are required for LTD. Studies with mGluR antagonists suggest that while mGlu1Rs are involved in both short-term depression and LTD, mGlu5Rs participate mostly in LTD. CGP-55845, a GABA_B-R antagonist, partially suppressed LTD in normally sleeping (NS) rats, while completely blocking in SD rats. Moreover, GS-39783, a positive allosteric modulator for GABA_B-Rs, suppressed the pEPSP in SD, but not NS, rats. Since both mGluRs and GABA_B-Rs seem to be involved in the LTD, especially in SD rats,
changes in receptor expression pattern and/or dimerization were examined using immunohistochemical, co-localization and co-immunoprecipitation (co-IP) techniques. Sleep-deprivation induced an increase in GABA_B-R1 and mGlu1αR expression in the CA1 region of the hippocampus. In addition, co-localization and heterodimerization between mGlu1αR/GABA_B-R1 and mGlu1αR/GABA_B-R2 is enhanced in SD rats. Taken together, our findings present a novel form of LTD sensitive to the activation of mGluRs and GABA_B-Rs, and reveal, for the first time, that sleep-deprivation induces alterations in the expression and dimerization of these receptors.
Preface

Publications that comprise the work in this thesis work.


   The data from chapters (3.2) are mainly included in this publication. I have contributed in the design, performance and analysis of all experiments (60%). Rajput P.S has helped me with immunohistochemical, co-localization and co-IP experiments (10%). Jennifer M. W helped with sleep-deprivation and in experiments using the agent GS-39783 (5%). Drs. Kumar and Sastry contributed significantly in the design of experiments and interpretation of results (25%).


   The data from chapters (3.1) are included in this publication. I have contributed in the design, performance and analysis of all experiments (70%). Kaan T. K helped with sleep-deprivation and performing few experiments on LTD (10%). Dr. Sastry contributed significantly in the design of experiments and interpretation of results (20%).

In this publication, as a co-author I have contributed in the design of experiments and interpretation of results (10%). Apart from this I have been involved in writing the manuscript and assisted in preparing figures (10%). Data from this paper has not been used in this thesis.


The animal experiment protocols used in this thesis were approved by the UBC Animal Care Committee (approval #s: A07-0536 and A10-0381)
Table of Contents

Abstract................................................................................................................................. ii
Preface................................................................................................................................. iv
Table of Contents................................................................................................................ vi
List of Tables ........................................................................................................................ xi
List of Figures ......................................................................................................................... xii
List of Abbreviations ............................................................................................................ xiv
Acknowledgements ............................................................................................................. xvii
Dedication ............................................................................................................................. xix

Chapter 1: Introduction ........................................................................................................ 1

1.1 Rat hippocampal formation............................................................................................. 1

1.1.1 Introduction.................................................................................................................. 1

1.1.2 Synapses...................................................................................................................... 3

1.1.3 Organization................................................................................................................ 3

1.1.3.1 Strata ....................................................................................................................... 3

1.1.3.2 Dentate gyrus ......................................................................................................... 5

1.1.3.3 CA3 ......................................................................................................................... 5

1.1.3.4 CA1 ......................................................................................................................... 7

1.1.3.5 Subiculum ............................................................................................................... 8

1.1.3.6 Interneurons ......................................................................................................... 8

1.1.4 Excitatory and inhibitory receptors........................................................................... 10

1.1.4.1 AMPA receptors .................................................................................................. 10
1.1.4.2 NMDA receptors ........................................................................................................ 11
1.1.4.3 Metabotropic glutamate receptors (mGluRs) .......................................................... 12
1.1.4.4 GABA_A receptors .................................................................................................. 12
1.1.4.5 GABA_B receptors ............................................................................................... 13
1.2 Memory .................................................................................................................................. 15
1.2.1 Hippocampus and memory .......................................................................................... 17
    1.2.1.1 Declarative memory ............................................................................................ 17
    1.2.1.2 Spatial memory .................................................................................................. 20
1.3 Memory consolidation and sleep ...................................................................................... 22
    1.3.1 Consolidation of memories ...................................................................................... 22
    1.3.2 Role of sleep .......................................................................................................... 25
1.4 LTP and LTD as cellular correlates for memory ................................................................. 29
    1.4.1 Synaptic plasticity and learning .............................................................................. 30
    1.4.2 Synaptic plasticity and sleep .................................................................................. 31

Chapter 2: Materials and Methods ......................................................................................... 33
2.1 Animals .............................................................................................................................. 33
2.2 Sleep-deprivation .............................................................................................................. 33
2.3 Brain-slice preparation .................................................................................................... 34
2.4 Recording chamber ........................................................................................................ 36
2.5 Media and drugs .............................................................................................................. 37
2.6 Recording and stimulation equipment ............................................................................. 40
    2.6.1 Patch pipettes ........................................................................................................ 40
    2.6.2 Data acquisition .................................................................................................... 40
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6.3 Stimulation and isolation units</td>
<td>41</td>
</tr>
<tr>
<td>2.6.4 Stimulating electrodes</td>
<td>41</td>
</tr>
<tr>
<td>2.7 Positioning of electrodes</td>
<td>42</td>
</tr>
<tr>
<td>2.8 Microscope and imaging</td>
<td>42</td>
</tr>
<tr>
<td>2.9 Patch-clamp and field recording setup</td>
<td>43</td>
</tr>
<tr>
<td>2.10 Electrophysiological recordings</td>
<td>44</td>
</tr>
<tr>
<td>2.10.1 Field recordings (for pEPSPs)</td>
<td>44</td>
</tr>
<tr>
<td>2.10.2 Whole-cell recordings (for EPSCs or IPSCs)</td>
<td>46</td>
</tr>
<tr>
<td>2.11 Immunohistochemistry</td>
<td>47</td>
</tr>
<tr>
<td>2.12 Indirect immunofluorescence</td>
<td>48</td>
</tr>
<tr>
<td>2.13 Western blot and co-IP</td>
<td>49</td>
</tr>
<tr>
<td>2.14 Corticosterone radioimmunoassay</td>
<td>51</td>
</tr>
<tr>
<td>2.14.1 Procedure</td>
<td>51</td>
</tr>
<tr>
<td>2.14.2 Calculations</td>
<td>52</td>
</tr>
<tr>
<td>2.15 Data analysis</td>
<td>52</td>
</tr>
<tr>
<td>2.15.1 Justification for analyzing slopes vs. amplitudes or area under the curve for pEPSPs</td>
<td>53</td>
</tr>
<tr>
<td>2.15.2 Slope and amplitude analysis of PSPs/Cs</td>
<td>54</td>
</tr>
<tr>
<td>2.15.3 LTD</td>
<td>55</td>
</tr>
<tr>
<td>2.15.4 Statistical analysis</td>
<td>55</td>
</tr>
<tr>
<td>Chapter 3: Experimental Protocols</td>
<td>57</td>
</tr>
<tr>
<td>3.1 Induction of LTD</td>
<td>57</td>
</tr>
<tr>
<td>3.2 Effects of sleep-deprivation on LTD</td>
<td>59</td>
</tr>
</tbody>
</table>
Chapter 3: Results

3.3 Effect of stress induced by sleep-deprivation ................................................. 60
3.4 Mechanisms involved in LTD ........................................................................ 61
3.5 Role of $[\text{Ca}^{2+}]$ in LTD ............................................................................ 63
3.6 GABAergic inhibition in LTD ......................................................................... 64
3.7 Expression of mGlu1Rs, GABA_B-R1s and GABA_B-R2s ......................... 67

Chapter 4: Results .................................................................................................. 71

4.1 LTD and effects of sleep-deprivation ................................................................. 71
  4.1.1 LTD (1 Hz, 15 min LFS) ............................................................................ 71
  4.1.2 LTD [20 Hz, 30 sec (1XSI)] ................................................................. 73
  4.1.3 LTD [20 Hz, 30 sec (2XSI)] ................................................................. 74
  4.1.4 Corticosterone radioimmunoassay ....................................................... 75
4.2 Mechanisms involved in LTD ........................................................................ 76
  4.2.1 Role of NMDARs in LTD ........................................................................ 76
  4.2.2 Role of mGluRs in LTD ......................................................................... 78
    4.2.2.1 Group I mGluR subtypes and LTD ................................................ 79
      4.2.2.1.1 Role of mGlu1Rs ................................................................. 79
      4.2.2.1.2 Role of mGlu5Rs ................................................................. 80
    4.2.3 Role of $\text{Ca}^{2+}$ in LTD ................................................................... 82
  4.2.4 GABAergic inhibition and LTD ............................................................... 84
    4.2.4.1 Role of $\text{GABA}_A$-Rs in LTD ....................................................... 84
    4.2.4.2 Effects of 20 Hz LFS on $\text{GABA}_A$-R mediated IPSCs .................. 85
    4.2.4.3 Masking of LTD by co-occurring LTP .......................................... 87
    4.2.4.4 Role of $\text{GABA}_B$-Rs in LTD ....................................................... 88
4.2.5 Immunohistochemistry, co-localization, western blots and co-IP ...................... 92

4.2.5.1 Expression of mGlu1αR, GABA_B-R1 and GABA_B-R2 in the CA1 hippocampal region of normally sleeping and SD rats ........................................ 92

4.2.5.2 Western blot analysis of mGlu1αR, GABA_B-R1 and GABA_B-R2 expression in the CA1 hippocampal region of normally sleeping and SD rats ......................... 95

4.2.5.3 Co-localization of mGlu1αR and GABA_B-R1/R2 in normally sleeping and SD rats ...................................................................................................................... 97

4.2.5.4 GABA_B-R1 and GABA_B-R2 receptors are expressed in mGlu1α receptor immunoprecipitate in normally sleeping and SD rats .............................. 100

Chapter 5: Discussion ................................................................................................. 103

5.1 LTD in the CA1 region of the hippocampus .................................................... 103

5.1.1 Induction ........................................................................................................ 103

5.2 Enhancement of LTD in SD rats ........................................................................ 104

5.3 Mechanisms involved in LTD ........................................................................... 105

5.3.1 Involvement of mGluRs and GABA_B-Rs .................................................... 105

Chapter 6: Conclusions .............................................................................................. 111

Chapter 7: Future Directions .................................................................................... 113

Bibliography ............................................................................................................. 116
List of Tables

Table 1. Theories on hippocampal function ................................................................. 19
Table 2. Media and drugs .............................................................................................. 39
Table 3. Effect of various pharmacological agent used on LTD. .................................. 91
List of Figures

Figure 1. Hippocampal anatomy ........................................................................................................... 2
Figure 2. Brain regions and memory types .......................................................................................... 16
Figure 3. Positioning of stimulating and recording electrodes .............................................................. 44
Figure 4. Whole-cell recording procedure ............................................................................................ 45
Figure 5. Calculation of slopes and amplitudes of PSP/Cs ................................................................. 54
Figure 6. LTD (1Hz, 15 min) ................................................................................................................ 72
Figure 7. LTD (20 Hz, 30sec- 1XSI) .................................................................................................. 74
Figure 8. LTD (20 Hz, 30 sec- 2XSI) .................................................................................................. 75
Figure 9. Role of NMDARs in LTD ...................................................................................................... 77
Figure 10. Role of mGluRs in LTD ...................................................................................................... 78
Figure 11. Role of mGlu1Rs in LTD .................................................................................................... 80
Figure 12. Role of mGlu5Rs in LTD .................................................................................................... 81
Figure 13. Role of Ca\(^{2+}\) in LTD ...................................................................................................... 83
Figure 14. Role of GABA\(_A\)-Rs in LTD ............................................................................................ 85
Figure 15. Effect of a 20 Hz LFS on GABAergic IPSCs ...................................................................... 86
Figure 16. Masking of LTD by LTP .................................................................................................... 88
Figure 17. Role of GABA\(_B\)-Rs in LTD ............................................................................................ 89
Figure 18. Effects of GS-39783 on pEPSPs ........................................................................................ 90
Figure 19. Distribution of GABA\(_B\)-R1/R2 and mGlu1\(\alpha\)Rs .............................................................. 94
Figure 20. Western blot analysis of GABA\(_B\)-R1, GABA\(_B\)-R2 and mGlu1\(\alpha\)Rs ............................... 96
Figure 21. Co-localization of GABA_B-R1/GABA_B-R2, GABA_B-R1/mGlu1αR, and GABA_B-R2/mGlu1αR....................................................................................................................... 100

Figure 22. Heterodimerization between GABA_B-R1/mGlu1αR, GABA_B-R2/mGlu1αR and GABA_B-R1/R2............................................................................................................... 102
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>CA</td>
<td>Cornus ammonis</td>
</tr>
<tr>
<td>LM</td>
<td>Lacunosum moleculare</td>
</tr>
<tr>
<td>IS</td>
<td>Interneuron selective</td>
</tr>
<tr>
<td>AMPARs</td>
<td>α-amino-3-hydroxy-5-methyl-isoxazole-propionic acid receptors</td>
</tr>
<tr>
<td>NMDARs</td>
<td>N-methyl-D-aspartate receptors</td>
</tr>
<tr>
<td>mGluRs</td>
<td>Metabotropic glutamate receptors</td>
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<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;-Rs</td>
<td>Γ-Aminobutyric acid- A receptors</td>
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<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt;-Rs</td>
<td>Γ-Aminobutyric acid- B receptors</td>
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<td>VGCCs</td>
<td>Voltage gated Ca&lt;sup&gt;2+&lt;/sup&gt; channels</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>NKCC1</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;-K&lt;sup&gt;+&lt;/sup&gt;-Cl&lt;sup&gt;-&lt;/sup&gt; co-transporter type 1</td>
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<tr>
<td>E&lt;sub&gt;GABA-PSC&lt;/sub&gt;</td>
<td>Equilibrium potential for GABAergic postsynaptic current</td>
</tr>
<tr>
<td>KCC2</td>
<td>K&lt;sup&gt;+&lt;/sup&gt;-Cl&lt;sup&gt;-&lt;/sup&gt; co-transporter type 2</td>
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<tr>
<td>GIRK channels</td>
<td>G-protein dependent inward-rectifying K&lt;sup&gt;+&lt;/sup&gt; channels</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>MTLS</td>
<td>Medial temporal lobe structures</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>S-R</td>
<td>Stimulus response</td>
</tr>
<tr>
<td>S-C</td>
<td>Stimulus conditioning</td>
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<tr>
<td>SPWs</td>
<td>Sharp waves</td>
</tr>
<tr>
<td>NREMS</td>
<td>Non-rapid eye movement sleep</td>
</tr>
<tr>
<td>REMS</td>
<td>Rapid eye movement sleep</td>
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<tr>
<td>SWS</td>
<td>Slow wave sleep</td>
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<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>PSWs</td>
<td>Paradoxical sleep windows</td>
</tr>
<tr>
<td>PGO waves</td>
<td>Ponto-geniculo-occipital waves</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>APV</td>
<td>DL-2-Amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>DNQX</td>
<td>6,7-Dinitroquinoxaline-2,3-dione</td>
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<tr>
<td>MCPG</td>
<td>RS-α-Methyl-4-carboxyphenylglycine</td>
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<tr>
<td>LY-367385</td>
<td>(S)-(+) α-Amino-4-carboxy-2-methylbenzeneacetic acid</td>
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<tr>
<td>MPEP</td>
<td>2-Methyl-6-(phenylethynyl)pyridine hydrochloride</td>
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<tr>
<td>CGP-55845</td>
<td>(2S)-3-[[1(S)-1-(3,4-Dichlorophenyl) ethyl] amino-2-hydroxypropyl] (phenyl methyl) phosphinic acid hydrochloride</td>
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<tr>
<td>GS-39783</td>
<td>N,N'-Dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>pEPSPs</td>
<td>Population excitatory postsynaptic potentials</td>
</tr>
<tr>
<td>EPSCs</td>
<td>Excitatory postsynaptic currents</td>
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<tr>
<td>IPSCs</td>
<td>Inhibitory postsynaptic currents</td>
</tr>
<tr>
<td>SD</td>
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</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
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<td>Diaminobenzidine tetrachloride</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>RIPA</td>
<td>Radio-immunoprecipitation assay</td>
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<tr>
<td>BCM</td>
<td>Bienenstock, Cooper and Munro</td>
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<td>HFS</td>
<td>High frequency stimulation</td>
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<tr>
<td>LFS</td>
<td>Low frequency stimulation</td>
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<tr>
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<td>Stimulus intensity</td>
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<td>NS</td>
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<td>Protein phosphatase</td>
</tr>
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<td>GPCRs</td>
<td>G-protein coupled receptors</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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</table>
Acknowledgements

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Dedication

I dedicate this thesis to my late grandfather.
Chapter 1: Introduction

1.1 Rat hippocampal formation

1.1.1 Introduction

The hippocampal formation (hippocampus proper and other regions) extends as a part of the cerebral cortex as a long, C-shaped structure, along the curvature of the lateral ventricles. The hippocampus proper (Cornus Ammonis or CA) has three subdivisions: CA3-CA2 (regio inferior) and CA1 (regio superior). The existence of the CA2 region is subject to much debate. Although it is comparatively less well-defined (for e.g. in rats), evidence indicates the presence of a narrow CA2 field, that can be distinguished clearly from other subfields (Amaral and Witter, 1989). Other regions include: dentate gyrus (fascia dentata), subiculum, presubiculum, parasubiculum (grouped as subicular complex) and entorhinal cortex (Blackstad, 1956; Lorente de Nó’, 1934; Ramón y Cajal, 1893).
**Figure 1. Hippocampal anatomy**

Schematic illustrating the various cell types and interconnections between entorhinal cortex (EC), dentate gyrus (DG), Cornu Ammonis layers 3 (CA3), CA1 and subiculum. This picture was originally drawn by Santiago Ramon y Cajal. It was published prior to Jan 1st 1923, and is out of copyright.

The hippocampus is highly organized into parallel segments or lamellae, capable of functioning as independent units (Andersen, et al., 1969; Anderson, et al., 1971). The exact orientation of these lamellae, however, appear to be species dependent (Blackstad, et al., 1970). Stimulation of the entorhinal afferents, sequentially **excite** principal neurons in the dentate gyrus (granule cells), CA3 and CA1 (pyramidal cells), along the trisynaptic loop [Entorhinal cortex → Dentate Gyrus (synapse 1); Dentate Gyrus → CA3 (synapse 2); CA3 → CA1 (synapse 3)].
1.1.2 Synapses

Synapses fall into two structurally distinct categories, type I and type II (Gray, 1959). Type I synapses are expressed mainly on dendritic spines (pl. see (Megias, et al., 2001) for type I synapses on dendritic shafts), possess an asymmetrical synaptic specialization (heavily stained presynaptic, compared to postsynaptic, density), and contain excitatory synapses (Andersen, et al., 1966). Type II synapses, on the contrary, are mostly seen on the dendritic shafts and around the neuronal somata, possess a symmetrical synaptic specialization (presynaptic and postsynaptic densities stain uniformly), and contain inhibitory synapses (Blackstad and Flood, 1963; Gray, 1959).

1.1.3 Organization

1.1.3.1 Strata

The entorhinal cortex projects axons carrying sensory information from various sources via the perforant path to, subiculum (from layer III), dentate gyrus and the CA3 region (from layer II). The dentate gyrus is, therefore, considered to be the first stage in the intrahippocampal trisynaptic loop. It is divisible into three distinct layers, of which the molecular layer (stratum moleculare, ~ 250 μm thick in rats) is closest to the hippocampal fissure. Afferents from the lateral and medial entorhinal cortex terminate into the outer one-third and the middle one-third of the molecular layer respectively; the granule cell layer
(stratum granulosum), comprising granule cells (8-12 µm in diameter, layer thickness- 4-8 somata); the polymorphic layer, enclosed within the characteristic V- or U- shaped structure formed by the molecular and granule cell layers. This layer is the deepest of all and is populated by a variety of neuronal types.

In the hippocampus, pyramidal cell layer (stratum pyramidale) forms the principal cell layer. While it is tightly packed with pyramidal cells (excitatory) in the CA1 region, neurons are relatively sparse or loosely packed in the CA2 and CA3 fields. Some interneurons (inhibitory), basket cells, bistratified cells, and radial trilaminar cells may also be present. Deep to the stratum pyramidale, layers, stratum oriens and alveus can be distinguished. Stratum oriens (referred as the infrapyramidal region) mainly consists: axons originating from CA3 and CA1 pyramidal neurons; cell bodies of interneurons, basket cell and horizontal trilaminar type; basal dendrites of pyramidal cells; septal and commissural inputs from the contralateral hippocampus. In the CA3 field, superficial to the stratum pyramidale, stratum lucidum contains the mossy fibers from granule cells. Stratum radiatum (referred as the suprapyramidal region) extends superficial to stratum lucidum in the CA3 region and above stratum pyramidale in CA2 and CA1. This stratum contains: Schaffer collateral projections from the CA3 region; septal and commissural fibers; interneurons such as, basket cells, bistratified cells and radial trilaminar cells. Stratum lacunosum-moleculare is the most superficial layer of the hippocampus consisting of afferents from the entorhinal cortex, nucleus reuniens of the thalamus, and a variety of interneurons.
1.1.3.2 Dentate gyrus

The dentate gyrus is considered to be the first stage in the intrahippocampal trisynaptic loop. Granule cells are the principal cells of dentate gyrus. They have a small soma, with two radially oriented, densely spiny dendrites with a cone-shaped arbor, reaching up to the hippocampal fissure. The entire dendritic arbor is confined to the stratum moleculare. Axons called mossy fibers collateralize extensively in the polymorphic layer and enter the CA3 field, forming synapses with the proximal dendrites of the pyramidal neurons (Blackstad, et al., 1970; Claiborne, et al., 1986; Gaarskjaer, 1978; Gaarskjaer, 1986). Mossy cells, present in the polymorphic layer, have dense spiny dendrites with thorny excrescences, located mainly in the hilar region. They project axons to a third of molecular layer, in ipsi- and contralateral hippocampus, with collaterals in the hilus (Amaral, 1978; Laurberg and Sorensen, 1981; Ribak, et al., 1985). Both granule and mossy cells excite interneurons through recurrent collaterals (Frotscher and Zimmer, 1983; Ribak, et al., 1986).

1.1.3.3 CA3

The CA3 field is the second stage of the trisynaptic loop. Pyramidal neurons (large somata) are the targets of granule cell axons, mossy fibers and projections from entorhinal cortex (Amaral and Witter, 1989). Loosely packed in a layer of ~ 60-120 µm thickness, they give rise to one or two spiny apical dendrites. These dendrites branch radially into the strata - radiatum and lacunosum-moleculare, and extend into the hippocampal fissure, the border of
the hilus (Ishizuka, et al., 1995; Turner, et al., 1995). They form synapses with large mossy terminals in the stratum lucidum (Blackstad and Kjaerheim, 1961; Lorente de No´, 1934; Ramón y Cajal, 1893). Basal dendrites, in contrast, are numerous, bear large complex spines and traverse obliquely along the stratum oriens, and into the alveus. Basal and/or apical dendrites, in addition, possess spines of a special type, called “thorny excrescences” at – first 20- 120 µm from the soma, depending on their specific location of the neuron within CA3 (Amaral and Dent, 1981; Blackstad and Kjaerheim, 1961; Chicurel and Harris, 1992). The CA3 region can be further subdivided into: area CA3c, penetrating the dentate hilus; area CA3a, region adjacent to CA2; and CA3b, the area in between. Basal and apical dendritic tree of the CA3c neurons are rather uniform, and relatively short, innervated extensively by mossy fibers. Mossy synapses are generally rare in regions CA3a and CA3b. Dendritic length and branching pattern is, therefore, heterogeneous, depending on the location, as well as, on the diameter of the cell body [8-10 mm, 16-18 mm for 20, 30 µm soma, respectively (Amaral, et al., 1990; Ishizuka, et al., 1995)]. Axons of the CA3 pyramidal neurons collateralize extensively within CA3 (creating associational connections and a highly excitable network), and also project to the CA1 field, as Schaffer collaterals (Schaffer, 1892) spanning across some two-thirds of the hippocampus (Li, et al., 1994; Sik, et al., 1993). Axons from CA3a neurons innervate both strata (-radiatum and –oriens); CA3b neurons terminate in stratum radiatum and to a lesser extent in oriens; CA3c neurons, mainly innervate stratum radiatum. In addition, CA3 neurons also send commissural projections to contralateral hippocampus and to the lateral septal nucleus (Swanson, et al., 1980).
1.1.3.4 CA1

The CA1 field represents the last stage of the trisynaptic loop. Pyramidal neurons (small somata) are arranged in a layer ~ 50-100 µm thick, and receive inputs from the CA3 pyramidal cell axons, the Schaffer collaterals (Schaffer, 1892) and entorhinal afferents (Pare and Llinas, 1995). A single, radially oriented apical dendrite arborizes in the stratum radiatum (proximal apical) and stratum lacunosum-moleculare (distal apical). The density of the tree is gradually reduced into a tuft of thin branches in the stratum lacunosum-moleculare, reaching up to the hippocampal fissure. Basal dendrites are numerous, branching extensively in the stratum oriens and reaching into the alveus (Ishizuka, et al., 1995). Both apical and basal dendrites are densely spiny, but unlike in the CA3 region, lack thorny excrescences. Axons emerge from the soma, close to the apical dendrites, and radiate deep into the alveus. They project mainly to the subiculum and entorhinal cortex (Meibach and Siegel, 1977) thereby completing the “cortex-hippocampus” feedback loop. Local collaterals and associational interactions between CA1 neurons are relatively sparse (Amaral and Witter, 1989; Ramón y Cajal, 1893), except in the developing hippocampus (Aniksztejn, et al., 2001). Extrinsic projections include those to the nucleus accumbens, lateral septum and the olfactory bulb (Swanson and Cowan, 1977).
1.1.3.5 Subiculum

Pyramidal cells in this region are categorized into regular-spiking or bursting types (Mason, 1993; Taube, 1993). They are diffusely packed, with a relatively less dense apical and basal dendritic arbor. Apical dendrites extend up to the molecular layer or in many instances into the hippocampal fissure (Greene and Totterdell, 1997; Staff, et al., 2000). As in other regions, dendrites are studded with spines, but their density or morphology is not well known. Inputs to subicular pyramidal neurons are mainly from the entorhinal cortex projections (Witter and Amaral, 1991), and axons of the CA1 neurons (Finch and Babb, 1980). In addition, they receive modulatory inputs from septal nucleus (cholinergic), locus coeruleus (noradrenergic), ventral tegmental area (dopaminergic), thalamus, and raphe nuclei (serotonergic). Axons collateralize extensively within the subicular region, and also project to presubiculum, parasubiculum, and several other cortical and sub-cortical areas (Paxinos, 1995).

1.1.3.6 Interneurons

At least six types of interneurons have been identified and they seem to spread across all layers of dentate gyrus and the hippocampus (Freund and Buzsaki, 1996). They target different domains on the soma and dendrites of the principal cells, and play a major role in shaping their output, synchronizing neuronal firing and generating behavior-related large scale network oscillations, such as, θ, γ and ripple activity (Klausberger, et al., 2004; Nimmrich, et al., 2005). Basket cell interneurons form a basket-like axonal plexus around the
soma and proximal dendrites of the pyramidal neurons. They are further divided based upon the expression of cholecystokinin or parvalbumin, axonal morphology and firing properties (Kawaguchi and Kondo, 2002). Their dendrites branch into strata oriens, radiatum and lacunosum-moleculare. They are excited by pyramidal neurons, in a feed-back and feed-forward type inhibition (receive ~2000 excitatory inputs from a large number of neurons), and form ~ 2-10 synapses with as many as 1000 pyramidal neurons (Andersen, et al., 1963; Freund and Buzsaki, 1996; Freund and Katona, 2007). Axo-axonic cells reside close to the pyramidal cell layer, and spread across the entire hippocampal strata. Typically, 4-10 axo-axonic cells target axon-initial segments of pyramidal cells, with each of them individually connecting to ~ 1200 pyramidal neurons (Somogyi, et al., 1982). O-LM cells have their cell bodies and dendrites in the stratum oriens and project axons into stratum lacunosum-moleculare, where they inhibit distal apical dendrites of pyramidal neurons, which in turn excite them through recurrent collaterals (Lacaille, et al., 1987). Bistratified cells are also located close to the pyramidal cells and extend their dendrites and axons into strata oriens and radiatum. However, unlike other interneurons, they do not reach the stratum lacunosum-moleculare (Halasy, et al., 1996). Other types of interneurons include, LM neurons located mainly in stratum lacunosum-moleculare, superficially in stratum radiatum (Lacaille and Schwartzkroin, 1988), and IS (interneuron-selective) neurons which make synaptic contacts only with other interneurons (Gulyas, et al., 1996). Like the principal neurons, interneurons express α-amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA)-, N-methyl-D-aspartate (NMDA)-, metabotropic glutamate (mGlu), Γ-Aminobutyric acid (GABA) _A_ - and GABA _B_ - receptors along with a repertoire of voltage-dependent Ca^{2+} channels (VGCCs).
However, they differ considerably in their subunit composition, which confers uniqueness to their firing properties, permeability to ions, such as, $\text{Ca}^{2+}$ (McBain and Fisahn, 2001).

### 1.1.4 Excitatory and inhibitory receptors

Like elsewhere in the CNS, glutamate is the main excitatory neurotransmitter in the hippocampus. It depolarizes target neurons by activating three major ionotropic receptors: AMPA, kainate, and NMDA and metabotropic mGlu receptors (mGluRs). GABA is the main inhibitory neurotransmitter. It hyperpolarizes target neurons by activating ionotropic GABA$_A$ and metabotropic GABA$_B$ receptors.

#### 1.1.4.1 AMPA receptors

AMPA receptors (AMPARs) are tetramers of subunits, GluR1, GluR2, GluR3 and GluR4, in different combinations (Keinanen, et al., 1990; Ozawa, et al., 1998). Based upon the subunit composition, AMPARs vary in channel conductance (Bowie and Mayer, 1995; Donevan and Rogawski, 1995), $\text{Ca}^{2+}$ permeability (Geiger, et al., 1995; Sommer, et al., 1991), and time course of desensitization (Mosbacher, et al., 1994). In pyramidal neurons, AMPARs are composed of GluR1-2 or GluR2-3 subunits (Wenthold, et al., 1996). A rapid pulse of glutamate (in excess of ~ 100 µM) elicits AMPAR-mediated excitatory postsynaptic potentials/currents (EPSP/Cs), with a rapid rise time (100-600 µs), suggesting very fast binding kinetics and high open probability ~ 0.6 (Jonas, et al., 1993). They deactivate
following clearance of excess synaptic glutamate or by entering into a desensitized state (Colquhoun, et al., 1992).

1.1.4.2 NMDA receptors

NMDA receptors (NMDARs) are heterotetramers of two distinct subtypes, NR1 and NR2A-D (McBain and Mayer, 1994). NR1 hosts the binding site for amino acids, glycine, D-serine; NR2A-D subunits, on the other hand, contain the glutamate-binding site (Laube, et al., 1997). Unlike AMPARs, NMDARs present on pyramidal neurons exhibit very slow kinetics (activation time constant- 7 ms) and deactivate slowly (~ 200 ms to 1-3 s) with a lower open probability (0.05-0.3). NMDARs however, have higher affinity to glutamate than the AMPARs (1-10 µM vs. 100-500 µM in steady-state conditions), which may explain their slower kinetics (Dingledine, et al., 1999; Lester, et al., 1990). Affinity to glutamate further depends upon the subunit composition (Cull-Candy, et al., 2001). In addition, NMDARs, a) possess an obligatory glycine-binding site (Kleckner and Dingledine, 1988), b) are highly permeable to Ca\(^{2+}\) ions (Ascher and Nowak, 1988), and c) subjected to a voltage-dependent block by extracellular Mg\(^{2+}\) ions (Mayer, et al., 1984; Nowak, et al., 1984). Therefore, for NMDARs to be activated, a release of glutamate must be accompanied by postsynaptic depolarization (to < ~ -50 mV). This need for near simultaneous pre- and post- synaptic activity makes NMDARs function as coincidence detectors, a highly valuable property in Hebbian forms of long-term synaptic plasticity (Hebb, 1949; Wigstrom and Gustafsson, 1986).
1.1.4.3 Metabotropic glutamate receptors (mGluRs)

mGluRs are classified into three types: Group I, II and III (Schoepp, et al., 1999). Based upon their physiological activity and receptor structure, they are further subdivided into eight subtypes, Group I (mGlu1R and mGlu5R), Group II (mGlu2R and mGlu3R) and Group III (mGlu4R, mGlu6R, mGlu7R and mGlu8R). Group I receptors are positively coupled to phospholipase C via Gq, and stimulate an increase in inositol triphosphate (IP3) and diacylglycerol (DAG) (Fagni, et al., 2000). They are mostly postsynaptic [perisynaptic (Lujan, et al., 1996)] and modulate neuronal excitability in the hippocampus by activating Ca2+, K+, other non-specific cationic conductances (Guerineau, et al., 1994; Heuss, et al., 1999; Swartz and Bean, 1992) or IP3-mediated release of Ca2+ from intracellular stores (Miller, et al., 1996). Group II and III receptors are negatively coupled to adenylyl cyclase via Gi/Go, and inhibit cyclic adenosine monophosphate (cAMP) production (Schoeppef and Johnson, 1993). They are located on presynaptic terminals, sometimes within the active zones (Shigemoto, et al., 1997), inhibit VGCCs and suppress neurotransmitter release (Cartmell and Schoepp, 2000).

1.1.4.4 GABA_A receptors

GABA_A receptors (GABA_A-Rs) are heteropentameric consisting at least seven different subunits along with various splice variants, α1-6, β1-3, γ1-3, δ, ε, π, and θ (Mehta and Ticku, 1999). These subunits recombine to form GABA_A-Rs with unique properties at different regions in the CNS. In the hippocampus, GABA_A-Rs are a combination of two α
subunits, two β subunits, and either a γ or δ subunit (Chang, et al., 1996). The presence of distinct α, β, and γ or δ subunits determines the affinity for GABA, sensitivity to various pharmacological agents, anchoring to synapses (Barnard, et al., 1998), rate and extent of desensitization, and tonic or phasic inhibition (Nusser and Mody, 2002). GABA, acting via GABA_A-Rs, can induce a depolarizing (excitatory)- [in neonatal neurons, (Ben-Ari, 2002; Cherubini, et al., 1991)] or a hyperpolarizing (inhibitory)- response [in adult neurons, (Zhang, et al., 1991)], depending upon the electrochemical gradient for Cl⁻ ions. Cl⁻ accumulation through Na⁺-K⁺-Cl⁻ co-transporter type 1 (NKCC1), switches the equilibrium potential for GABAergic postsynaptic currents (PSCs) - E_{GABA-PSC} in the positive (depolarizing) direction, making it excitatory (Plotkin, et al., 1997). On the contrary, Cl⁻ extrusion through K⁺-Cl⁻ co-transporter type 2 (KCC2) (Deisz and Lux, 1982; Jarolimek, et al., 1999; Misgeld, et al., 1986; Thompson, et al., 1988) makes GABA-ergic PSCs hyperpolarizing (Owens, et al., 1996; Rivera, et al., 1999) and inhibitory. GABA_A-Rs are also permeable to HCO₃⁻ ions, which may under certain conditions account for GABAₐ mediated depolarizing responses in pyramidal cell dendrites (Andersen, et al., 1980; Kaila, et al., 1997). Although GABA_A-R kinetics vary depending upon the subunit composition (Benkwitz, et al., 2004), they have a generally faster onset and a slower decay compared to AMPAR-EPSCs.

1.1.4.5 GABA_B receptors

Metabotropic GABA_B receptors (GABA_B-Rs) are obligate heterodimers of GBR1 and GBR2 subunits and structurally homologous to mGluRs (Jones, et al., 1998; Kaupmann, et
They are expressed on pre-, post-, and extra-synaptic regions of synapses (Kulik, et al., 2003). When present presynaptically they are negatively coupled to 
Ca\(^{2+}\) channels, Ca\(_{v}\)2.2 (N-type) and Ca\(_{v}\)2.1 (P and Q type) via G-proteins and regulate 
neurotransmitter release (Anwyl, 1991; Mintz and Bean, 1993). When located 
postsynaptically, they mediate slow inhibition (lasting several hundred milliseconds) by 
activating G-protein dependent inward-rectifying K\(^+\) (GIRK) channels (Misgeld, et al., 1995) 
or in some cases, Ca\(^{2+}\) channels (Jones, et al., 1998; Mehta and Ticku, 1999).
1.2 Memory

Across the animal kingdom animals adapt to the ever changing environment through multiple forms of learning. Over time, knowledge gained through this process is stored as memories in the central nervous system (CNS). The ability to store, retain and recall past events ensures superior behavioral and/or biological responses at future events. Formation of memories, however, is a multi-step process involving distinct time-bound stages such as encoding, storage and retrieval (Squire, 1986). Following initial acquisition, a process termed “consolidation” is required to stabilize (Alberini, et al., 2006), enhance (Walker, 2005) and integrate the newly encoded information into novel or pre-existing memories (semanticization). Therefore, until memories become more permanent, they tend to remain in a fragile state, vulnerable to external amnesic influences (Walker, 2005). Post recall, memories may disintegrate, and require “reconsolidation” to take place (Stickgold and Walker, 2005). These basic processes seem to be true irrespective of the type of memory being processed or formed.

Memories are broadly classified into either declarative or non-declarative (Tulving, 1985). Declarative memory (also referred as, explicit or relational memory) is the memory for conscious recollection of fact- or event- based information. It is further subdivided into episodic memory (specific time-place events) and semantic memory (facts and events gathered through experience). Non-declarative memory (also referred as, implicit memory), on the other hand, is the memory for non-conscious information, which is perfected through repetition and practice (procedural memory for skills and habits). While hippocampus and
other closely connected medial temporal lobe structures (MTLS) seem to be critical for processing declarative memories (Squire, 1992), the neuroanatomical structures involved in the formation of non-declarative memories are diverse, comprising several cortical, subcortical and cerebellar networks (Daum and Ackermann, 1997; Nagao and Kitazawa, 2008). The evidence for the existence of specific brain regions (Fig. 2) to encode distinct memory subtypes comes mainly from neuropsychological or behavioral studies involving, amnesic or epileptic patients (Scoville and Milner, 1957), lesions (Zola-Morgan, et al., 1986), electroconvulsive therapy (Squire and Slater, 1983; Squire, et al., 1979; Squire and Zouzounis, 1988) and/or other pathological conditions, such as, the Korsakoff’s syndrome (Shimamura and Squire, 1986).

**Figure 2. Brain regions and memory types**

Schematic illustrating the importance of various brain regions in processing distinct subtypes of memory (each region, and the respective form of memory processed is represented in separate colors).
1.2.1 Hippocampus and memory

1.2.1.1 Declarative memory

Hippocampus and associated MTLS are critical to the formation of “declarative” or “explicit” memories (Eichenbaum, 2001; Scoville and Milner, 1957; Squire, 1992; Tulving and Markowitsch, 1998; Walker and Stickgold, 2006). This perhaps was most convincingly demonstrated in patient H.M, who underwent a bilateral resection of medial portions of the temporal lobe in an effort to be relieved of pharmacologically intractable epilepsy (Scoville and Milner, 1957). A post-surgical neuropsychological assessment performed on H.M and nine other similar patients demonstrated anterograde amnesia, temporally graded retrograde amnesia and a profound inability to convert short-term memory to long lasting forms (Scoville and Milner, 1957). However, previously formed explicit memories remained intact with no noticeable impairment in, acquiring new motor/technical skills (Corkin, 1968; Shadmehr, et al., 1998), perceptual learning, repetition priming (Corkin, 2002) or perceptual aftereffects (Savoy and Gabrieli, 1991).

The above observations suggest the presence of distinct brain regions for processing memories and indicate a time-limited role for MTLS in the formation of declarative memories. A “systems-level” consolidation may, therefore, be required to make declarative memories independent of the hippocampus and MTLS (McClelland, et al., 1995). Whether the hippocampus remains engaged or disengaged post-formation during the lifetime of memories, is currently disputed. Nevertheless, in both cases, “hippocampal-neocortical”
interactions (Buzsaki, 1989; Lavenex and Amaral, 2000) seem to play a major role in memory formation. The independence of declarative memories from hippocampus can be achieved via bi-directional information transfer (cortex «→» hippocampus). Considering the sequential and unidirectional flow of information between the cortex and hippocampus, this seems to be an fairly efficient means to offload newly processed information back to neocortical areas, thereby making them permanent repositories for long-term memory (Buzsaki, 1996). Furthermore, this temporary outsourcing of memories to be processed in the hippocampus may prevent the otherwise “catastrophic interference”, as envisaged in parallel distributed processing networks (McClelland, et al., 1995). Several other theories, namely the multiple-trace theory (Nadel and Moscovitch, 1997), dual-process theory (Eichenbaum, et al., 2007; Norman and O'Reilly, 2003; Rugg and Yonelinas, 2003), relational theory (Cohen, et al., 1997) and cognitive-map theory (O'Keefe and Nadel, 1978), have also been proposed to speculate on the cognitive function of the hippocampus. While all theories support a role for hippocampus in processing declarative and/or spatial memories, they seem to differ mainly on the type and the temporal aspects of information processed (Table. 1).
Declarative theory | Declarative memories are initially processed in the hippocampus and MTLS, but consolidated to neo-cortical sites disengaging the hippocampus in the process.

Multiple-trace theory | Hippocampus and MTLS remain engaged during the lifetime of episodic memories, but not semantic memories.

Dual-process theory | Hippocampus and MTLS independently process aspects within episodic memories, such that contextual-based recollection of events requires the former while familiarity-based recollection requires the latter.

Cognitive-map theory | Hippocampus plays a vital role in creating representations of the environment, to aid in navigation. Thereby, it processes the spatio-temporal aspects of episodic memory.

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<th>Table 1. Theories on hippocampal function</th>
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<td>The above table lists various current theories on hippocampal function. It summarizes their main ideas.</td>
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1.2.1.2 Spatial memory

In addition to declarative memories, the hippocampus plays a crucial role in the formation of spatial memories. This has been demonstrated in rodents. Rats navigate strategically through mazes, in short-cuts or round-about routes, depending upon the goal and/or reward (Tolman, 1948). This behavior could not be readily explained by the then dominant “stimulus-response” (S-R) or the “stimulus-conditioning” (S-C) theories (Restle, 1957). Tolman and colleagues (Tolman, 1948) contended that cognitive maps, if present, could serve as internal neural representations of physical space providing details of the animal’s position relative to various environmental cues (distal, proximal and extramaze). The animal can then consult these points-of-reference and navigate intelligently in the open field. The discovery of “place-cells” in the hippocampus strongly supported Tolman’s interpretation and laid the foundations for the “cognitive-map theory” (Moser, et al., 2008; O'Keefe and Dostrovsky, 1971; O'Keefe and Nadel, 1978), which has been improved upon ever since (Eichenbaum, et al., 1999). Place-cells fire robustly (~ 20-100 Hz), in a location-specific manner when the animal enters a particular grid, called a “place-field”. These cells are otherwise quiescent, firing at a rate of ~ 1 Hz. In rats, the field and the pyramidal cell’s firing rate within persist (Thompson and Best, 1990) as long as the environmental variables remain intact despite deafening, blind-folding or even after darkening the room (O'Keefe and Speakman, 1987; Quirk, et al., 1990). Altering the size, such as simply expanding the circular open-field in which the rat previously traversed, only results in a “scale-up” of some place-fields, whereas changes in shape of the environment often leads to aberrant firing patterns and unpredictable behavior (Muller, et al., 1987). Similarly, modifications in cues, strategies,
head-direction or the speed of animal’s movement, result in a dramatic re-mapping or reorganization of place-fields (Breese, et al., 1989; Markus, et al., 1995; McNaughton, et al., 1983).

Although evidence from studies on “place-cells” clearly emphasize a role for the hippocampus in processing spatial memories, the concept of a cognitive map, is currently disputed. Opposing views have emerged mainly because adjacent cells have been found to have largely un-correlated firing patterns (Tanila, et al., 1997), and rats seem to rely overtly on relative distances from and between cues rather than respective positions of all the cues (O’Keefe and Burgess, 1996). Therefore, it appears that space in hippocampus is represented in a rather discontinuous manner, with place-fields of neighboring cells “overlapping or clustering” upon each other, instead of lining up in a continuous “Cartesian type” topographical representation, as originally proposed in the cognitive-map theory (Eichenbaum, et al., 1989; Hampson, et al., 1996). Clearly, more studies are required to understand exactly how time and space are represented in the hippocampus.

Evidence as to the role of hippocampus in spatial memories also emerges from studies assessing the structural changes in hippocampus across species in different situations. Hippocampal size is plastic, and varies according to species dependence on navigational skills. For instance, hippocampal volume relative to brain/body size is higher in small mammals, birds and in humans, especially taxi-drivers, who rely extensively on spatial navigation (Lee, et al., 1998; Maguire, et al., 2000). These alterations in size can also be seasonal, and changes seem to occur according to impending demand (Smulders, et al.,
1.3 Memory consolidation and sleep

1.3.1 Consolidation of memories

Memories, as mentioned elsewhere, are formed in distinct stages: acquisition (learning), consolidation and recall (Squire, 1986). Consolidation is an active process during which newly encoded information is strengthened via a repertoire of cellular, sub-cellular and systems-level processes, and gradually incorporated into neocortex and other brain regions for long-term storage. Since consolidation is a time-bound and continuous process, it takes place during both wakefulness and sleep. Not surprisingly, therefore, distractions in periods immediately post-learning, or episodes of post-training sleep-deprivation, significantly interrupt memory formation and impair task re-performance (McGaugh, 2000; Müller and Pilzecker, 1900). Although it seems to be reasonably clear as to why the consolidation process itself is slow (Frankland and Bontempi, 2005; Kandel, 2001;
McClelland, et al., 1995), exactly how it takes place and the mechanisms involved are presently unclear.

In rats, theta oscillations can be recorded from dentate gyrus during exploratory activity (Bland, 1986). On the other hand, awake immobility is dominated by irregular sharp waves (SPWs- 0.02-3 Hz, 40-120 ms duration) and high-frequency ripples (140-200 Hz) (Buzsaki, 1986). Granule cells of the dentate gyrus are especially active during the theta, compared to non-theta states. In contrast, pyramidal cells of the CA3 and CA1 regions, fire synchronous population bursts during SPWs and are silent (except “place-cells”) during theta (Buzsaki, 1986; Buzsaki, et al., 1983). The locus for theta and SPWs therefore, appears to be the granule and pyramidal cells in the hippocampus, respectively (Buzsaki, et al., 1983; Olmstead, et al., 1973; Suzuki and Smith, 1985). Based upon these observations, Buzsaki et al., proposed a “two-stage model” for the consolidation of memories (Buzsaki, 1989).

According to this model, during exploration subcortical influences (via the entorhinal cortex) prime granule cells of the dentate gyrus. These neurons in-turn induce a mild heterosynaptic potentiation in certain “initiator cells” of the CA3 region. During SPWs, “initiator cells” may recruit additional cells and subsequently associate to alter the synaptic efficacy of their target CA1 and subicular neurons (Buzsaki, 1989; Buzsaki, 1996). The CA3 region, with its extensive associational interconnectivity, equips the neural network with an intrinsic capability to strengthen (via recurrent activation?) the otherwise labile memory traces. Sequential alteration in the synaptic excitability of selected neurons in this fashion may further ensure that the same set of neurons are activated during subsequent reactivations.
or recall of the memory (Buzsaki, 1989). The non-reciprocal and unidirectional interconnections along the hippocampal trisynaptic loop, as opposed to the largely reciprocal connections between various regions in the neocortex (Bartesaghi, et al., 1988; Ramón y Cajal, 1893), also ensures the preservation of the fidelity of neuronal information before it is fed back into the cortical regions of origin. Overall, it therefore, seems that the hippocampus and the various sub-regions within it are self-sufficient enough to confer discrete computational capabilities to neural networks for processing multimodal information, and creating an integrated representation for newly acquired information, which can then be stored in higher brain centers as memories at a later stage (Nakazawa, et al., 2002). The two-stage model, however, lacks experimental evidence for many of its assumptions. Despite shortcomings, emerging evidence in literature is supportive of the existence of “initiator cells”, and the need for “hippocampal-neocortical dialogue” (Buzsaki, 1996; Buzsaki and Czeh, 1992; Molle and Born, 2009) in forming memories. Since not all forms of memory are hippocampus dependent (Lehmann, et al., 2007), it appears that consolidation processes differ with respect to the type of memory. These aspects need to be further investigated.

Sleep is a physiological imperative that has been observed in most animals. Although it has important roles in a variety of functions (Siegel, 2005), its ability to somehow actively facilitate consolidation of newly acquired information is especially interesting and attractive. The discovery of the heterogenous nature of sleep (Aserinsky and Kleitman, 1953) led to speculations that sleep could indeed serve as a platform for “offline-reprocessing” of memories. In fact, stage specific oscillations during sleep have been shown to drive and synchronize network activity between thalamus, cortex and hippocampus, an arrangement
which is highly desirable for systems-level consolidation of memories (Born, 2010; Siegel, 2001).

1.3.2 Role of sleep

Sleep is sub-divided into distinct stages. It encompasses recurring cycles of non-rapid eye movement sleep (NREMS; stages I to IV) and rapid eye movement sleep (REMS), which in humans, alternate every 90-min across the night (Aserinsky and Kleitman, 1953). While the amount of time spent in slow wave sleep (SWS) or deep sleep (NREM stages 3 and 4) is higher early in the sleep cycle, the proportion of REM or dream sleep gradually increases until natural awakening. Electroencephalograms (EEGs) during NREM stages, I, II, III & IV comprise, alpha (8-13 Hz) to theta waves (4-7 Hz); sleep spindles (11-16 Hz) and K-complexes; and delta waves (1-4 Hz), respectively. Stages III and IV, are together referred to as the SWS. EEG during REMS mainly consists of fast desynchronized activity and theta waves [4-7 Hz] (Steriade, 2006).

The evidence for the role of sleep in consolidation of memories comes from disparate studies. Numerous studies have confirmed the positive effects of sleep on declarative and procedural memories (Marshall and Born, 2007; Robertson, et al., 2004; Smith, 2001). Compared to equal periods of post-learning wakefulness, episodes of sleep, significantly improve retention of declarative information, and procedural skills (Plihal and Born, 1999; Walker, et al., 2003). There is, however, no consensus either on the duration or the latency of the onset of specific sleep stages, for performance gains. Studies have reported benefits of
paradoxical sleep (or REMS) windows (PSWs) for place learning, fine motor procedural tasks, and implicit learning (Karni, et al., 1994; Plihal and Born, 1999; Smith, 1995). Selective paradoxical sleep-deprivation during, but not outside PSWs impairs re-performance (Smith and Butler, 1982). The characteristics of PSWs, however, seem to vary according to the strain of the animal, type of the learning task, and the learning protocol employed (Smith, 1985). Similarly, SWS has been implicated in the consolidation of mainly declarative and explicit memories (Barrett and Ekstrand, 1972; Fowler, et al., 1973; Plihal and Born, 1999; Yaroush, et al., 1971). However, other data indicate the need for a memory trace to be processed first in SWS and then in REMS, in a sequential, and dual-step manner (Giuditta, et al., 1995). The specific influence of REMS and NREMS in the formation of individual forms of memory, therefore, seems to depend mostly upon the type of learning. Hence, it is reasonable to conclude that our current understanding of the respective role of REMS and NREMS will be greatly enhanced by designing behavioral studies with stage-specific sleep-deprivation.

Despite clear indications of a role for sleep in memory, exactly how it mediates this process is unclear. It is thought that sleep creates a brain state distinct from wakefulness, which is highly conducive to the formation of memories. SWS oscillations (slow, delta waves, spindles), are correlated to rhythmic spike bursts in the thalamus and cortex during the up- and down- states (Amzica, et al., 1997; Steriade, 1999). The temporal relationship, which exists in this synchrony, could in fact facilitate the “cortical-hippocampal dialogue” (Isomura, et al., 2006; Siapas and Wilson, 1998; Sirola, et al., 2003; Wierzynski, et al., 2009) and provide adequate conditions for synaptic plasticity, the purported cellular mechanism for
learning and memory, to occur (Buzsaki, 1989). Ponto-geniculo-occipital (PGO) waves, which are prominent in the EEG during or slightly before the REMS, have also been implicated in brain development and synaptic plasticity (Datta, 1999). Furthermore, high or low levels of acetylcholine in the brain during REMS and NREMS, respectively, may directly modulate the efficacy of hippocampal synaptic transmission, by suppressing or potentiating (facilitate synaptic plasticity) them via subcortical afferents (Hasselmo, 1999). Indeed, fornix-fimbria lesions result in large amplitude sharp-waves [SPWs] (Buzsaki, et al., 1988; Buzsaki, et al., 1989). The various oscillations during sleep, therefore, aid synaptic plasticity and the formation of memories via reactivation.

In the hippocampus during sleep, reactivation of the original neural networks that were active during encoding in preceding wakefulness has been observed. Place-cells in the hippocampus fire uniquely when the animal navigates through individual place-fields during a spatial exploratory behavior (O'Keefe and Dostrovsky, 1971; Pavlides and Winson, 1989; Skaggs and McNaughton, 1996; Wilson and McNaughton, 1994). Single and en masse network recordings from these cells reveal that the firing pattern as well as the temporal order is played back, although, on a faster time scale (Nadasdy, et al., 1999) during REMS and SWS (Poe, et al., 2000; Qin, et al., 1997). Reactivation of firing patterns have also been discovered in the thalamus, striatum and the neocortex (Ji and Wilson, 2007; Lansink, et al., 2008; Ribeiro, et al., 2004).

Consolidation of memories is likely to involve a cascade of molecular events. Several studies have probed for changes in brain protein synthesis, and gene transcription (Cirelli,
There seems to be a positive correlation between learning, SWS duration and cerebral protein synthesis in monkeys (Nakanishi, et al., 1997). Blocking protein synthesis using anisomycin during REM sleep impairs learning (Smith, et al., 1991). The expression of Zif-268, an immediate-early gene, is increased in sleep post-exposure, compared to wakefulness. Non-exposed rats, however, showed a decrease in Zif-268 levels both in REMS and NREMS, compared to wakefulness (Ribeiro, et al., 1999).

Taken together, the data on memory, sleep and hippocampal-dependent learning, reveal how these processes are interlinked in a manner that facilitates learning, storage of memories, and recall. However, a clear understanding of the consolidating of memories, and the role for sleep is complete only when a corresponding process is identified at a more local level, i.e., at the level of the synapse. It has been long held that activity dependent modification of synaptic strength (plasticity) may underlie various forms of learning and that synapse itself could be the locus for memory (Hebb, 1949). The discovery of long-term potentiation (LTP) and long-term depression (LTD), was an important breakthrough, following which several laboratories projected them as candidate mechanisms for learning and memory at the cellular level (Bliss and Collingridge, 1993; Bliss and Lomo, 1973; Lynch and Baudry, 1984; Lynch, et al., 1977). If this were true, cognitive impairments evident at the behavioral level must manifest as changes to LTP and/or LTD at the cellular level. In fact, strong correlations have been found between synaptic plasticity and memory. Studies have consistently reported improvements in learning when LTP occurs and impairment in learning and disruption in memory consolidation when LTP is blocked, in a variety of learning paradigms (Martin, et al., 2000). LTD, however, has traditionally been viewed as a
counterpart for LTP, as a mechanism to undo its effects (Bear and Malenka, 1994). It is only recently that investigators have identified a much broader role for LTD in formation of certain types of memories (Manahan-Vaughan and Braunewell, 1999), in forgetting and amnesia (Tsumoto, 1993), and in preventing saturation of neural networks (Dayan and Willshaw, 1991). Therefore, it appears that both LTP and LTD are equally important in the context of learning and in the maintenance of the normal functioning and order in the CNS.

1.4 LTP and LTD as cellular correlates for memory

Certain properties of LTP and LTD make them excellent candidates for memory at the cellular level; a) LTP and LTD occur both in vitro as well as in vivo (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973; Levy and Steward, 1979; Lynch, et al., 1977), and has been found almost universally in the CNS, b) LTP can be rapidly induced following tetanic stimulation of the inputs and persists for several hours or days, in other words, it outlasts the cause just like the formation of memory itself (Abraham, 2003), c) LTP is input-specific and co-operative (Bliss and Collingridge, 1993; McNaughton, et al., 1978; Sastry, et al., 1986). Stimulus protocols that mimic endogenous firing patterns successfully to induce LTP & LTD (Larson, et al., 1986; Staubli and Lynch, 1987), d) activation of the test input along with a concurrent tetanic stimulation of other excitatory afferents or a simultaneous depolarization of the postsynaptic neuron can associatively induce LTP (Barrionuevo and Brown, 1983; Sastry, et al., 1986; Wigstrom, et al., 1986), while hyperpolarizing the postsynaptic neuron elicits LTD (Debanne and Thompson, 1996; Stanton and Sejnowski, 1989). The associative induction of LTP and LTD is thought to play an important role in consolidation of
associative memory, e) sleep, which has been shown to promote synaptic plasticity (Benington and Frank, 2003), facilitates neural development, learning, as well as, memory. On the contrary, total/partial sleep-deprivation significantly impairs LTP in hippocampus and other areas of the brain (Campbell, et al., 2002; Davis, et al., 2006; Kopp, et al., 2006; McDermott, et al., 2003; Shaffery, et al., 2006; Tartar, et al., 2006).

1.4.1 Synaptic plasticity and learning

Retention deficits in spatial memory during senescence have been correlated to the degree/persistence of LTP (Barnes, 1979; Barnes and McNaughton, 1985). Similarly, over expression of amyloid precursor protein (APP) impairs performance in a spatial alteration task (Hsiao, et al., 1996). This decline in performance is correlated to a parallel decrease in LTP (Chapman, et al., 1999). Saturation of LTP or LTD by repeated stimulations or by pharmacologically blocking NMDARs or mGluRs, reversibly occlude spatial learning, certain types of olfactory learning, contextual fear conditioning, and other tasks (Danysz, et al., 1988; Danysz, et al., 1995; Moser, et al., 1998; Nielsen, et al., 1997). NMDAR1 or GluR1 mutants (O'Keefe, 1993; Tsien, et al., 1996) lack LTP, and exhibit decreased performance in a water maze owing to abnormal place fields and an overall reduction in correlated firing (McHugh, et al., 1996). On the contrary, agents that enhance hippocampal LTP improve learning (Lynch, 1998; Seabrook, et al., 1997; Staubli, et al., 1994). Although much less is known on how LTD influences learning, studies indicate that LTD is important in spatial learning (McNaughton, et al., 1986) and novelty acquisition of an object-place configuration (Manahan-Vaughan and Braunewell, 1999).
1.4.2 Synaptic plasticity and sleep

Learning paradigms that promote plasticity alter subsequent sleep (Smith and Lapp, 1991). Sleep enhances (Benington and Frank, 2003; Frank, et al., 2001), whereas, sleep-deprivation is detrimental to learning as well as LTP (Campbell, et al., 2002; Davis, et al., 2006; Kopp, et al., 2006; McDermott, et al., 2003; Tartar, et al., 2006). Sleep entails patterns of activity that may facilitate synaptic remodeling (Buzsaki, 1998). High percentage of REMS during development is critical for brain maturation and plasticity (Marks, et al., 1995). Selective sleep-deprivation during this critical period can adversely affect brain re-wiring (Fagiolini, et al., 1994; Frank, et al., 2001; Marks, et al., 1995). Although a majority of studies have focused on the adverse effects of sleep-deprivation on LTP, not much is known as to how it affects LTD. LTD is implicated in a variety of functional roles in the CNS. It protects neurons by dampening neuronal hyper-excitability, prevents a saturation of neuronal network activity (Dayan and Willshaw, 1991), and facilitates the formation of certain types of memories (Manahan-Vaughan and Braunewell, 1999). In adult/aged rats, however, an enhanced susceptibility to LTD has been found to not only interrupt the maintenance of LTP (Norris, et al., 1996), but also account for a slower pace of learning, poor retention and faster forgetting (Foster and Kumar, 2007). These observations support a vital role for LTD in the formation of memories, the modulation of LTP, and in normal functioning of the CNS.

In the present study, we therefore hypothesized that “a 12 h period of sleep-deprivation affects LTD in the CA1 region of the hippocampus”. This hypothesis was tested by
evaluating Schaffer collateral stimulation induced changes in the excitatory and inhibitory synaptic transmission. Specifically, the role of mGluRs, NMDARs, GABA_A-Rs and GABA_B-Rs were tested pharmacologically. In additional experiments, changes to receptor expression, co-localization and dimerization were examined using immunohistochemistry, immunofluorescence and co-IP techniques. All experiments were performed in normally sleeping and SD rats.
Chapter 2: Materials and Methods

This section provides a detailed description of the source of the animals, hippocampal slice preparation, incubating and recording chambers, superfusion media, intracellular pipette solutions, recording and stimulating equipment, field and patch-clamp recordings, LTD induction, data analysis and experimental protocols used.

2.1 Animals

Male Wistar rats (3-4 week old) were purchased from the Animal Care Centre at The University of British Columbia (UBC) or Charles River facility. Animals were delivered in groups, 2-4 rats, to the Department of Anesthesiology, Pharmacology and Therapeutics in the mornings (between 9:00 and 10:00 AM). They were housed in the Department’s animal holding facility, and given about 2-3 days to acclimatize. Prior to experiments, animals were brought to the laboratory in clean, ventilated cages, with ample food, water and environmental enrichment. All experiments were performed in accordance to the approved guidelines of the Canadian Council on Animal Care and the UBC animal care (Certificate no’s: A07-0536 and A10-0381)

2.2 Sleep-deprivation

Investigators routinely use “gentle-handling”, “forced locomotion” or the “inverted flowerpot” techniques to sleep-deprive animals (Campbell, et al., 2002; Davis, et al., 2003;
McDermott, et al., 2003). Among these well established procedures, the gentle-handling method is considered to be most humane. The other two procedures have been shown to simultaneously elevate stress (using serum corticosterone levels as an index) and physical exhaustion, which are unrelated to sleep-disturbance *per se*. In our studies, animals were sleep-deprived (for 12 hours) using the gentle-handling technique. During this time, animals were under the constant observation of well-trained personnel (2 people were present at any time). Observer teams switched alternatively every 4-6 hours. If animals entered into sleep or recumbence, they were gently awoken with tactile or visual stimuli, which involved, for instance, prodding with a brush or placement of novel “safe” objects in their vicinity, to provoke behavioral and mental activity. Animals were housed in a clean cage, in the temperature-controlled laboratory, with ample food and water at any given time.

2.3 Brain-slice preparation

Brain slices were prepared as described in previous studies (Xie, et al., 1995). The dissection area was set up close to a large sink. The following surgical instruments and materials were used for brain removal: guillotine, folded paper towels, a #11 scalpel blade, surgical scissors, bone rongeurs, a modified spatula, synthetic artists brush (number 0 or 00), modified Pasteur pipette fitted with a rubber bulb, 110 mm diameter Whatman Filter paper, ice and Petri dishes. Sucrose-based artificial cerebrospinal fluid (ACSF) was used, to block synaptic transmission and minimize Ca$^{2+}$, NMDAR dependent excitotoxicity during dissection (see Media and Drugs; Section #2.5). Solutions were kept ice-cold during the
entire procedure to slow down the metabolic rate and decrease the oxygen demand (Hagerdal, et al., 1975; Nishizaki, et al., 1988).

Animals were anesthetized with halothane and decapitated using a guillotine at the end of the 12 h light period. The head was immediately immersed in ice cold sucrose based ACSF, bubbled with carbogen (95% O₂/5% CO₂). Using the #11 scalpel, an incision was made in the middle of the scalp starting from near to the nasal bone and running caudally up to the occipital bone. This procedure exposed the sutures on the dorsal surface of the skull. With the rat’s head firmly held, the cutting edge of the surgical scissors were placed superior to the foramen magnum at the posterior portion of the skull. Keeping the lower sheer against the skull’s inner surface, the calvarium was then cut through the occipital plate first, then along the sagittal suture, and rostrally upwards to the frontal plates. Lateral cuts were made at the base and top of the saggital cut. The bone rongeurs was then slid under the left and right parietal plates, squeezed to hold the plates, and the wrist rotated upwards to retract the bones and expose the surface of the brain. After a quick inspection for the dura mater that may be still attached to the temporal plates, the modified spatula was moved side-to-side and forwards-backwards to sever any cranial nerves. The brain was then scooped out into ice cold ACSF, and allowed to chill. This procedure was completed in ~ 35-40 seconds.

The brain was then placed on a dampened (with ACSF) Whatman filter kept over the lid of an ice-cold Petri dish. The two hemispheres were separated, and the olfactory bulb and brainstem-cerebellum severed. One hemisphere was discarded and the other glued to an acrylic cutting block with cyanoacrylate (Lepage Accu-Flo No.8 or Crazy glue). The
mounted tissue was then placed in a bath chamber filled with ice-cold oxygenated sucrose-ACSF, and positioned carefully on a Vibroslice (World Precision Instruments Inc.). Transverse sections were cut to a thickness of 400 µm. A 350-400 µm thick hippocampal slice is a compromise between obtaining a useful core of the tissue, with the dendritic arbor intact, and the diffusion barriers posed by a thicker slice. CA3 region was cut off from the slice to diminish the influence of spontaneous activity from these neurons. Typically 4 slices could be obtained from a single hemisphere in this fashion/animal.

The cut slices were immediately transferred to a holding chamber containing oxygenated normal-ACSF (flow rate ~ 1.5 ml/min; temperature ~ 25-26 ºC) (see Media and Drugs; Section #2.5), and gently placed over a submerged nylon mesh. Care was taken so as to not agitate slices while bubbling carbogen. Slices were allowed to equilibrate for at least 1-1.5 h before being placed in the recording chamber. This incubation period is imperative because slices immediately post-dissection are physiologically non-responsive. They display no spontaneous activity for various reasons (Teyler, 1980). Slices regain their electrophysiological and biochemical activity post-incubation, and continue to give responses to experimental manipulations for at least 6 hours thereafter.

2.4 Recording chamber

An interface-type chamber was constructed from Plexiglas, such that it fit snugly in to a circular adaptor plate. This was then affixed on to the Burleigh Gibraltar platform. Slices rested on the central flat rectangular area over a cover slip base. They were held in position
using a custom-made slice anchor (U-shaped flat stainless steel wire with nylon threads). The media entered the chamber via a gravity-fed system (at a rate of ~ 1.5-2 ml/min). 3-4 barrels (60 ml capacity) mounted high above the chamber onto an aluminum frame acted as reservoirs for holding different solutions. By raising or lowering the aluminum frame, the desired flow rate could be achieved. Aeration in each barrel was controlled using a single gas line with adjustable valves. Individual flow lines were switched on and off using butterfly clips, and passed through a manifold before entering the chamber’s inlet. This setup ensured rapid exchange of different media, when required. A vacuum line attached to the chamber’s outlet removed the superfusing medium as it flowed. Care was taken to minimize dead space and all feed lines were made free of large air bubbles before the start of the experiment. Temperature in the chamber was maintained at 25-26 ºC.

2.5 Media and drugs

Fresh solutions were prepared each day prior to the experiment. For slicing: sucrose-based ACSF (in mM): 234 sucrose, 2.5 KCl, 28 NaHCO₃, 1.25 NaH₂PO₄, 3 pyruvic acid, 1 ascorbic acid, 7 MgCl₂, 0.5 CaCl₂ and 10 dextrose (saturated with 95% O₂/5% CO₂); pH was adjusted to 7.35-7.4 (when bubbled with carbogen). For incubation and perfusion: normal-ACSF (in mM): 120 NaCl, 3 KCl, 1.8 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, 2 MgCl₂ and 10 dextrose (saturated with 95% O₂/5% CO₂); pH was adjusted to 7.35-7.4 (when bubbled with carbogen). Following incubation, individual slices were transferred into a recording chamber superfused with ACSF at a rate of 1.5-2 ml/min.
For intracellular recordings - patch-pipette solution (in mM): 135 K-gluconate, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 KCl, 1 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA), 5 Mg-ATP, 0.1 CaCl$_2$, 10 Na$_2$-phosphocreatine, 0.4 Na$_3$-GTP and creatine phosphokinase 50 U/ml; pH was adjusted to 7.2-7.3 with KOH). Mg-ATP and Na$_2$-phosphocreatine ensured the required ATP regeneration, necessary to prevent run down of GABA$_A$-R-mediated responses (Chen, et al., 1990; Ohno-Shosaku, et al., 1989). All patch-clamp recording solutions were filtered through a 0.22 μm pore size syringe filter (Nalgene #176-0020) prior to use.
<table>
<thead>
<tr>
<th>Drugs</th>
<th>Concentration (µM) &amp; solubility</th>
<th>Source (catalog no.)</th>
<th>Main pharmacological action</th>
</tr>
</thead>
<tbody>
<tr>
<td>D, L-2-amino-5-phosphonopentanoic acid (APV)</td>
<td>50 (in water to 100 mM)</td>
<td>Tocris Bioscience (0106)</td>
<td>NMDAR antagonist</td>
</tr>
<tr>
<td>6,7-dinitroquinoxaline-2,3-dione (DNQX)</td>
<td>20 In DMSO to 100 mM</td>
<td>Tocris Bioscience (0189)</td>
<td>Non-NMDAR selective antagonist</td>
</tr>
<tr>
<td>(RS)-α-Methyl-4-carboxyphenylglycine disodium salt (MCPG)</td>
<td>500 (in water to 100 mM)</td>
<td>Ascent Scientific (Asc-252)</td>
<td>Non-selective group I/group II mGluR antagonist</td>
</tr>
<tr>
<td>(S)-(+)−α-Amino−4−carboxy−2−methylbenzeneacetic acid (LY-367385)</td>
<td>100 (in 1.1 eq. NaOH to 100 mM)</td>
<td>Ascent Scientific (Asc-067)</td>
<td>Selective mGlu1αR antagonist</td>
</tr>
<tr>
<td>2-Methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP)</td>
<td>40 (in water to 5 mM)</td>
<td>Ascent Scientific (Asc-008)</td>
<td>Non-competitive mGlu5R antagonist</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>5 (in DMSO to 50 mM)</td>
<td>Tocris Bioscience (0601)</td>
<td>L-type Ca&lt;sup&gt;2+&lt;/sup&gt; channel blocker</td>
</tr>
<tr>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>50 (in water)</td>
<td>Sigma Chemicals (339350)</td>
<td>T-type Ca&lt;sup&gt;2+&lt;/sup&gt; channel blocker</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>1 (in DMSO to 100 mM)</td>
<td>Ascent Scientific (Asc-286)</td>
<td>Sarcoplasmic Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase inhibitor</td>
</tr>
<tr>
<td>Bicuculline methiodide</td>
<td>10 (in water to 20 mM)</td>
<td>Tocris Bioscience (2503)</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;-R antagonist</td>
</tr>
<tr>
<td>(2S)-3-[(1S)-1-(3,4-Dichlorophenyl) ethyl] amino-2-hydroxypropyl] (phenyl methyl phosphinic acid hydrochloride (CGP-55845)</td>
<td>2 (in DMSO to 100 mM)</td>
<td>Tocris Bioscience (1248)</td>
<td>Selective GABA&lt;sub&gt;B&lt;/sub&gt;-R antagonist</td>
</tr>
<tr>
<td>N,N’-Dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine (GS-39783)</td>
<td>10 (in DMSO to 10 mM)</td>
<td>Tocris Bioscience (2001)</td>
<td>Positive allosteric modulator of GABA&lt;sub&gt;B&lt;/sub&gt;-R function</td>
</tr>
</tbody>
</table>

Table 2. Media and drugs
The above table is a list of various pharmacological agents, along with information on the concentrations, solubility, source and function, used in this project.

- Drugs were stored in concentrated stock solutions (-20 or -80 °C, as needed), and diluted directly using normal-ACSF to their final concentrations.
- When dissolved in DMSO, the final concentration was at 0.1 % in the recording media.

2.6 Recording and stimulation equipment

2.6.1 Patch pipettes

Patch-clamp recording electrodes were made from thin-walled borosilicate glass capillaries (inner diameter- 1.17 mm; outer diameter 1.5 mm), purchased from World Precision Instruments, Inc. Electrodes were fabricated on a Flaming/Brown programmable micropipette puller (model P-87; Sutter Instruments) using preset parameters. When filled with patch-pipette solutions, the resistance varied between 7-10 MΩ.

2.6.2 Data acquisition

Synaptic potentials (intra- and extra- cellular) were recorded using the Axopatch 200A (Axon Instruments) amplifier. The Axopatch 200A had a very low open circuit noise of 0.16 pA rms (at 10 KHz bandwidth). The headstage (CV 201; Axon Instruments), had a
precision resistor (gain, $\beta=1$) that set the pre-amplifier gain to 10. Capacitance neutralization capacity was up to 100 pF. Evoked responses were monitored on a dual beam storage oscilloscope (Tektronix 5113), digitized and stored for offline analysis with a Digidata 1220 board (Axon Instruments) and MS-DOS based Fetchex software. Low pass filtering was set at 5 kHz. Data were analyzed offline using the Mini Analysis program (Synaptosoft).

2.6.3 Stimulation and isolation units

Current was generated using a Grass S88 dual output stimulator. The stimulator output was connected to photoelectric constant isolation unit (PSIU6, Grass Instruments) for pulsatile stimulation procedures or rectangular current pulses. Control stimulation frequency was set at 0.05 Hz with square pulses (0.1-0.2 ms duration), and a stimulus intensity high enough to evoke a half-maximal pEPSPs, EPSCs or inhibitory postsynaptic currents (IPSCs). This was done to allow enough room for potentiation or depression of responses following a conditioning stimulation.

2.6.4 Stimulating electrodes

Bipolar concentric platinum electrodes (SNEX-100, David Kopf Instruments) had a shaft length of 50 mm. The tip of the electrode had a contact length and diameter of 0.75 mm and 0.1 mm, respectively. The resistance of this electrode was constantly monitored (was ~ 1 M$\Omega$), and replaced if it exceeded 7 M$\Omega$. 

41
2.7 Positioning of electrodes

The stimulating electrode was attached to a Narishige micromanipulator (model NMN-21), which permitted a movement range along the three axes, for coarse (X15mm, Y15mm, Z15mm; full knob rotation ~ 4mm) and for fine (X6mm, Y6mm, Z6mm; full knob rotation 250 µm with a minimum graduation of 1 µm) movement. Recording electrodes were attached to Narishige Water Hydraulic micromanipulator (model MHW-3), which permitted a movement range along the three axes for coarse (X30mm, Y30mm, Z30mm; full knob rotation ~ 4mm) and for fine (X2mm, Y2mm, Z2mm; full knob rotation ~ 50µm with a minimum graduation of 0.2µm) movement. Both NMN-21 and MHW-3 systems were mounted on a Burleigh Gibraltar platform. This setup ensured precision movement and stability.

2.8 Microscope and imaging

For electrophysiological experiments, the setup included a Zeiss Axioskop 2 FS plus upright microscope, which was mounted on the Burleigh Gibraltar platform, which provided the X-Y stage. The field of view was ~ 23 mm. The system was equipped with Nomarski interference optics, which provided excellent optical sections with an optimum resolution for thick slices. The condenser was setup for Kohler illumination. HAL 100 provided a 100 Watt halogen light source with variable intensity. The microscope was fitted with high numerical aperture objectives (Zeiss A-Plan 10x, 0.24 and Achroplan 40x, 0.80W). The system was equipped with an IR-1000 infrared CCD (1/2” interline) monochrome video camera (with a
separate control unit for gain and black levels) connected to a Hitachi B/W monitor (VM-1720U). The IR-1000 offered excellent relative spectral sensitivity (> 80%) in the near IR and the entire visible spectrum (380 nm to 1200 nm), producing crystal clear images of the slice when illuminated using the IR light.

For immunohistochemical and co-localization studies, slides were visualized and photographed using a Leica DMLB motorized microscope equipped with a QImaging Retiga 2000R Peltier cooled CCD camera (quantum efficiency ~ 55% at 500 nm; 7.4µm x 7.4µm pixel size; 12 bit). The system was equipped with a, a) metal halide arc lamp source, which produced distinct peaks at wavelengths 365, 405, 436, 546 and 579 nm, b) filter block sets for Alexa 488 and Alexa 594, and c) Leica PL FLUOTAR 2.5x, 0.07; HC PL APO CS 10x, 0.40; HCX PL APO 40x, 1.25-0.75 oil and HCX PL APO 63x, 1.40 oil objectives. For co-localization, images were acquired on two channels (green and red) and merged. In merged images, yellow fluorescence indicates co-localization of red- and green- fluorescence tagged proteins. Images were acquired using the QCapture Pro software and analyzed.

2.9 Patch-clamp and field recording setup

The Burleigh Gibraltar platform, which supported the microscope and micromanipulators, stood on a vibration-free isolation table (Nitrogen controlled). The entire setup was enclosed within a Faraday cage, which was grounded to a separate line in the building. This minimized electrical noise arising from other extraneous sources.
2.10 Electrophysiological recordings

Figure 3. Positioning of stimulating and recording electrodes

The figure depicts a hippocampal slice preparation on the left. On the right, the enlarged area shows the respective areas where the stimulation (STIM) and recording electrodes (REC 1 & 2) are placed. In our experiments, REC 2 was the position for pEPSPs and REC 1 for EPSCs or IPSCs.

2.10.1 Field recordings (for pEPSPs)

The stimulating electrode was positioned (using NMN-21) in the stratum radiatum of the CA1 region ~ 300-400 µm from the planned recording site, and ~ 200 µm from the stratum pyramidale (Fig. 3). The depth of the placement varied between 100-200 µm. The recording electrode (filled with normal-ACSF) was positioned in the dendritic region (apical) at a similar distance from the stratum pyramidale. pEPSPs were evoked at a rate of 0.05 Hz (square pulses 40-100 µs duration, 10-30 µA current). The recording electrode was then
gradually advanced into the planned site with MHW-3. Usually field responses increased to a certain depth and started to decrease. At this point, the lowering was stopped, and stable pre-tetanic controls (15-20 min) were obtained.

**Figure 4. Whole-cell recording procedure**

The relative position of the patch electrode and the cell is illustrated schematically on the left. To the right are changes to the test-pulse at different stages, as the resistance across the patch electrode tip goes up or down. A reduction in test-pulse current indicates a closer distance between the electrode tip and the cell. In (A), the recording electrode is just above the cell, not in direct contact, so the
resistance is low (~ 1-10 MΩ) and the test-pulse current is large. In (B), as the electrode touches the cell surface, the resistance goes up slightly and the test-pulse current becomes smaller. In (C), a gigaseal is established, which pulls a small patch of membrane up into the electrode tip, increasing the resistance to about > 2 GΩ. The electrode capacitive transients are also visible. In (D), the electrode capacitance transients are nulled and finally, in (E), rupture of the cell membrane is achieved by a strong suction, entering the whole-cell mode. Note the drop in resistance and large capacitance transients.

2.10.2 Whole-cell recordings (for EPSCs or IPSCs)

Pyramidal neurons were visually identified by their shape and location using the Zeiss Axioskop 2 FS plus microscope. The stimulating electrode was placed in a similar fashion, as described in the field recordings (but closer to stratum pyramidale for IPSCs; Figure 4). Before the recording electrode (connected to the headstage, which served as a preamplifier) was lowered into the recording chamber, slight positive pressure was applied via a 10 ml syringe connected to the electrode holder through a Teflon tubing (this procedure prevented the contamination of the tip of the electrode with cell debris). Once the recording electrode was lowered into the media, a 0.45 nA, 50 ms hyperpolarizing rectangular current pulse was delivered through the pipette every 2 s. The changes to the test-pulse were carefully monitored on an oscilloscope as the electrode approached the cell, as explained in Figure 4A, B, C, D, E) and whole-cell configuration achieved. If the cell could not be patched in first attempt, it was discarded and the same steps were repeated on a different cell. Fast capacitative transients were offset with a compensation circuit built into the amplifier. Series
resistance was calculated by measuring the peak amplitude of the capacitative transient and using the equation \( R_s = \frac{V_{\text{step}}}{I_{\text{cap}}} \). The values ranged between 10-30 MΩ. Series resistance compensation was at \( \sim 75\% \).

PSCs were evoked by constant current pulses (0.1-0.2 ms duration, 0.05 Hz). Cells were voltage-clamped at \( \sim -60 \text{ mV} \) and accepted for further study if PSCs were stable during the initial 15-20 min pre-tetanic control recording period. Recordings were considered stable if series resistance and capacitance could be compensated to 75% and if PSC’s holding current was stable. If series resistance changed by more than 15% during a recording, the cell was not used for analysis. In this configuration, the recording of whole-cell currents could last for more than \( \sim 1 \text{ h} \) without any significant changes in the response associated with cell dialysis \textit{per se}. While perforated patch-clamp recording was considered, the method was not used for the following reasons, a) it usually takes a long time for the antibiotics (nystatin or gramicidin) to punch holes on the attached cell membrane, b) increases the access resistance and noise in recordings significantly, c) prevents the usage of larger molecules in the intracellular solution (owing to smaller pore sizes) and d) over time, the patch of membrane tends to rupture, resulting in a whole-cell recording.

2.11 Immunohistochemistry

Immunohistochemical studies were performed on coronal sections of rat brains, as described previously (Rajput, et al., 2009). Briefly, normally sleeping or sleep-deprived (SD) rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital, and the brains
fixed by transcardial perfusion with 0.9% cold heparinized saline and 4% paraformaldehyde. Post-fixation, the brains were taken out and cryoprotected in 20% sucrose and 40% sucrose solution. Following a wash in cold water, 40 μm thick sections were prepared using the Leica 1200s vibratome. Free-floating hippocampal sections were incubated in 1% H₂O₂ (for 15 min) and 0.2% Triton X-100 (for 15 min), washed 3X in Tris-Buffered Saline (TBS, used at 20 mM; 1 L, 50 mM TBS contains, Tris-HCl 6.61g, Tris Base 0.97g, NaCl 8.77g, H₂O 800ml, with pH adjusted to 7.4) post-incubation between each treatment. The sections were then blocked in 5% normal goat serum (NGS; for 1 h at RT), and incubated with primary antibodies, specific to mGlu1αR (mouse anti-mGlu1αR, BD Pharmingen #556389), GABA₉-R1 (goat anti- GABA₉-R1, Santa Cruz Biotechnology #sc-7338) or GABA₉-R2 (goat anti-GABA₉-R2, Santa Cruz Biotechnology #sc-31457) at 1:300 dilution in 1% NGS, overnight at 4°C in a humid atmosphere. Following three subsequent washes in TBS, sections were incubated for 1 h with biotinylated secondary antibodies. The avidin-biotin complex method was used to detect the antigen (ABC kit, Vector laboratories, Burlingame, California) and 3, 3'-diaminobenzidine tetrachloride (DAB, 0.2 mg/ml) containing 0.001% H₂O₂ was used to visualize the reaction. Sections were then mounted on slides, viewed and photographed using a Leica DMLB microscope equipped with a Retiga 2000R camera.

2.12 Indirect immunofluorescence

Co-localization of mGlu1αR with GABA₉-R1/R2 and GABA₉-R1 with GABA₉-R2 was studied in normally sleeping and SD rats, as described previously (Rajput, et al., 2009). Briefly, brain sections passing through hippocampus were selected and incubated in 0.2%
Triton X-100 (for 15 min) and washed 3X with TBS for 10 min. The sections were then blocked in 5% NGS for 1 h at RT, and incubated overnight at 4°C in a humid atmosphere with primary antibodies (at 1:300 dilution in 1% NGS) in the following combination: mGlu1αR and either GABA_B-R1 or GABA_B-R2 (mouse anti-mGlu1αR, BD Pharmingen #556389; goat anti- GABA_B-R1, Santa Cruz Biotechnology #sc-7338; goat anti- GABA_B-R2, Santa Cruz Biotechnology #sc-31457); GABA_B-R1 (guinea pig anti-GABA_B-R1, Millipore #AB2256) and GABA_B-R2 (goat anti- GABA_B-R2, Santa Cruz Biotechnology #sc-31457). This was followed by incubation with mixtures of Alexa 594 (red) and Alexa 488 (green)-conjugated anti-mouse, anti-guinea pig or anti-goat secondary antibodies. Alexa Fluor dyes are exceptionally bright and photo-stable. The absorption spectra for Alexa 594 and Alexa 488 are well differentiated with no overlap. Finally, the sections were mounted on slides, viewed and photographed using the Leica DMLB microscope equipped with a Retiga 2000R camera.

2.13 Western blot and co-IP

Western blot and co-IP were performed on the tissue lysate prepared from the hippocampus of normally sleeping and SD rat brains, as described previously (Rajput, et al., 2009). Briefly, the hippocampal tissue lysate was solubilized in a homogenizing buffer containing (in mM): 62.5 Tris-HCl, 50 dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), and 10% glycerol. Protein concentration in the tissue was estimated using the Bradford protein assay. 20μg of protein was then solubilized in Laemmli buffer with 5% 2-mercaptoethanol and heated at 99°C for 5 min. Samples were then fractionated by
electrophoresis on a 7% SDS polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk for 1 h at room temperature and subsequently incubated overnight at 4°C with primary antibodies (at 1:500 dilutions in 5% bovine serum albumin) specific to mGlu1αR (mouse anti-mGlu1αR, BD Pharmingen #556389), GABA<sub>B</sub>-R1 (goat anti- GABA<sub>B</sub>-R1, Santa Cruz Biotechnology #sc-7338) and GABA<sub>B</sub>-R2 (goat anti- GABA<sub>B</sub>-R2, Santa Cruz Biotechnology #sc-31457). Membranes were then incubated with goat anti-mouse or anti-goat secondary antibodies for 1 h at room temperature. Bands were detected with a chemiluminescence reagent and images were taken using the Alpha Innotech Fluorchem 800 (Alpha Innotech Co., San Leandro, CA) gel box imager. β-actin was used as the house-keeping protein.

For co-IP experiments, tissue lysates prepared from normally sleeping and SD rats were centrifuged and the pellet was further solubilized in 1 ml of radio-immunoprecipitation assay (RIPA buffer; containing, 150 mM NaCl, 50 mM Tris–HCl, 1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate, pH 8.0), for 1 h at 4°C. Tissue lysates were then incubated with monoclonal mGlu1αR or polyclonal GABA<sub>B</sub>-R1 antibodies (at 1:500 dilutions) overnight at 4°C on a rocking shaker. 25 μl of protein A/G-agarose beads were added to each tube to immunoprecipitate antibody for 2 h at 4 °C. Beads were then washed three times in RIPA buffer and solubilized in Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol. The samples were heated at 99°C for 5 min before being fractionated by electrophoresis on a 7% SDS-polyacrylamide gel. The fractionated proteins were transferred to a 0.2 μM nitrocellulose membrane in transfer buffer. Membranes were blotted
with antibodies specific to GABA$_B$-R1 (Millipore Corporation, Billerica, MA) and GABA$_B$-R2, as described in the western blot analysis section.

2.14 Corticosterone radioimmunoassay

2.14.1 Procedure

Rats were decapitated at the end of the light period and trunk blood collected in culture tubes, serum separated and frozen. A radioimmunoassay (RIA) was performed in blood samples obtained from two groups (normally sleeping and SD rats) to estimate the serum corticosterone levels using standard procedures described in the protocol (\(^{125}\text{I}\) RIA kit, MP Biomedicals #07120102). Briefly, rat serum was diluted (1:200) with the steroid diluent by taking 10 µl of the sample to 2.0 ml. 0.3 ml of steroid diluent was added to tubes 1 and 2 (non-specific binding, NSB tubes). Supplied corticosterone calibrators (0.1 ml; 25 to 1000 ng/ml) were added to tubes 5 to 16. Reconstituted controls (0.1 ml) and diluted (1:200) rat serum were added to tubes 17 to end of assay. Corticosterone-\(^{125}\text{I}\) was added to all tubes, followed by anti-corticosterone (to tubes 3 to end of assay). All assay tubes were vortex mixed and incubated at room temperature for 2 h. After incubation, precipitant solution (0.5 ml) was added to all tubes and vortexed thoroughly. All assay tubes were then centrifuged at 2300-2500 rpm (1000 x g) for 15 min. The supernatant was aspirated and the precipitate was counted in a gamma counter.
2.14.2 Calculations

All duplicate tubes were averaged. The values of the NSB (blanks) were subtracted from the averages obtained, which yielded corrected values. The % bound was calculated by dividing the corrected values by the zero calibrator value,

\[
\frac{\%B}{Bo} = \frac{\text{CPM (sample)} - \text{CPM (NSB)}}{\text{CPM (0calibrator)}} - \text{CPM (NSB)} \times 100
\]

CPM = Average counts of duplicates  
Sample = Particular serum or calibrator being calculated  
NSB = Non-specific binding tube (blank)  
0calibrator = 0 tube (100% binding tube)

The values were plotted (% bound vs. concentration of corticosterone) for all calibrators (25-1000 ng/ml) to construct a calibrator curve. Sample values were then directly inferred from the curve. Corticosterone levels estimated by the RIA method are very similar and comparable to those obtained using other methods such as, high performance liquid chromatography combined with mass spectroscopy or fluorimetric methods (Rattner, et al., 1979; Shimizu, et al., 1983). The baseline range varies from ~ 50 to 300 or 400 ng/ml in mice and rats, depending on the time of the day (Shimizu, et al., 1983).

2.15 Data analysis

This section summarizes the methods used for the analysis of data.
2.15.1 Justification for analyzing slopes vs. amplitudes or area under the curve for pEPSPs

Changes to the synaptic excitation were quantitated by measuring the slopes of pEPSPs. Since inhibitory responses were not blocked in our studies (unless otherwise mentioned), evoked pEPSPs contained both excitatory and inhibitory events. The kinetics of AMPAR-mediated pEPSPs/EPSCs is, however, clearly distinct from both GABA\textsubscript{A} and GABA\textsubscript{B} -R mediated IPSCs, with the latter most likely affecting the peak of the response, while the former affecting the rising phase of the response. Therefore, slopes (10-90 % of the rising phase of the response) and not amplitudes of pEPSPs (a measure of distance from the baseline to the peak of the response) are truly representative of changes in synaptic excitation. Furthermore, although pEPSPs were evoked and recorded such that there was no contamination from population spikes (by placing the recording electrode far away from the cell body region, close to the dendrites), in some instances, spikes that were negligible in a pre-tetanic response, shifted in position relative to peak following tetanic stimulation (e.g. move closer to peak when the post-tetanic response is potentiated). Changes such as these, in the amplitude and/or position of the population spikes make it difficult or sometimes impossible to quantitate the amplitude of the pEPSP. Slopes, however, remain largely unaffected. For the same reasons, an area under the curve is also not a true estimate of changes in synaptic excitation. In addition, it is prone to potential errors and misinterpretation of results owing to inclusion of phases, such as the recovery. For responses which involve activation of any one receptor or receptor-mediated events such as, miniature EPSCs, quantitating area under the curves may be more accurate. Taking into account, our
Experimental conditions, pEPSPs were always quantitated by measuring slopes. Since, EPSCs or IPSCs that were recorded intracellularly are not prone to the above mentioned errors; amplitudes were analyzed for data from whole-cell experiments.

2.15.2 Slope and amplitude analysis of PSPs/Cs

![Figure 5. Calculation of slopes and amplitudes of PSP/Cs.](image)

A standard pEPSP record with (1) illustrating the analysis of slope and (2) illustrating the analysis of amplitude.

pEPSP slopes (mV/ms) were measured as the inclination between low % and high % (in our case, the 10 - 90%) of the peak amplitude. All waveform voltage points from slope beginning time point to end time point (sampling of 100 µs generated ~ 10 to 21 AD samples) were used to construct a linear regression line (using least squares fit). Slopes (1) were then ascertained automatically using the Mini Analysis program from Synaptosoft. Amplitudes for EPSCs or IPSCs, on the other hand, were measured as the distance of the vertical line (2) from the baseline of the recording to the peak of the response (Fig. 5).
In all cases, slope values of three consecutive records were averaged (one-min bin). Data were further normalized to their 10 min pre-tetanic controls and a time-course of pEPSP slopes constructed using the GraphPad Prism software.

2.15.3 LTD

A variety of stimulation paradigms (1 Hz, 15 min; 20 Hz, 30 s, 1XSI; 20 Hz, 30s, 2XSI, Section #4.1), were used to establish a reliable protocol for inducing LTD. LTD induction was considered successful when pEPSPs were depressed by 20-25 % at 20 min post-tetanus in normally-sleeping rats. While in all cases (in both normally sleeping and SD rats), post-tetanic pEPSPs depressed, only the 20 Hz, 30 s, 2XSI induced a depression which lasted for at-least 30 min post-tetanus. The depression induced in this fashion also exhibited a stable time-course, which plateaued at ~ 15-20 min post-tetanus, satisfying our criteria for LTD.

2.15.4 Statistical analysis

Two statistical tests were used to analyze the data in this study. In our experiments, LTD was quantitated at 20 min post-tetanus. The Student’s t-test was used to compare LTD when two different treatments were involved (normally-sleeping vs. SD rats; effects of different pharmacological agents on LTD, etc.). When experiments involved comparison of two factors, such as different treatments (post-tetanic depression at various time points) or conditions (normally-sleeping vs. SD rats), a two-way repeated measures ANOVA was used.
GraphPad Prism and SigmaStat softwares were used to perform Student’s t-test or ANOVA, respectively. In both statistical tests, the level of significance (P value) was arbitrarily chosen at 0.05. P<0.05 was considered significant. Corresponding graphs were constructed using GraphPad Prism software. Each point in the graph represents the mean ± SEM of responses at that particular time. n refers to number of slices studied; only one slice per animal was used. For immunohistochemical, co-localization and co-IP studies, the analysis was qualitative. Alteration in receptor expression was confirmed through Western Blot analysis. Respective bands were quantitated using densitometric analysis and changes in the protein expression were calculated as the ratio of band of interest with the density of β- actin.
Chapter 3: Experimental Protocols

The introductory chapters provided an overview on the, a) anatomy and physiology of the hippocampal formation, b) memory formation and the role of hippocampus, c) effects of sleep on memory, d) LTP and LTD as models for cellular learning and memory and e) effects of sleep on plasticity. In this section, LTD is discussed in more detail with relevant background literature, which forms a basis for the design of individual studies. The experimental protocol followed for each of these studies is further discussed.

3.1 Induction of LTD

The likelihood of LTP or LTD at a particular synapse seems to depend upon the prior synaptic activity (Coan, et al., 1989; Huang, et al., 1992). According to the Bienenstock, Cooper and Munro (BCM) model, if afferents are active during low levels of postsynaptic activity, they are more likely to depress (LTD), whereas, the opposite is true in vice versa (Bienenstock, et al., 1982). The BCM model also predicts the existence of a certain modification threshold ($\theta_M$), which serves as a “cross-over” point for LTP or LTD. For instance, high levels of postsynaptic activity swing $\theta_M$ towards the right, making LTP difficult and LTD easier to obtain (Abraham, 2008; Bienenstock, et al., 1982). This fine balance perhaps ensures that the neural networks are protected from excessive saturation and maintained within their dynamic functional range (Dayan and Willshaw, 1991). Not
surprisingly, therefore, high- or low- frequency stimulations (HFS or LFS), have been used to successfully induce LTP and LTD, respectively.

At naïve synapses, LTD in the CA1 region can be elicited using a variety of LFS paradigms. LFS [~ 1-20 Hz, (Bolshakov and Siegelbaum, 1994; Dudek and Bear, 1992; Sastry, et al., 1984; Tadavarty, et al., 2009)] readily elicits LTD in young/juvenile rats. In adult rats (~ 4-5 week old), however, certain LFS protocols, such as, 1 Hz, 600-900 pulses, seem to be ineffective (Dudek and Bear, 1993; Errington, et al., 1995). In these animals, LTD requires a stronger stimulation paradigm (Berretta and Cherubini, 1998; Kemp and Bashir, 1997; Tadavarty, et al., 2009). These age-related differences in LTD induction could be due to a parallel increase in GABA-ergic inhibitory strength (Kerr and Abraham, 1995; Wagner and Alger, 1995), changes in subunit composition of NMDARs, or some other modifications in the synaptic machinery (Kemp and Bashir, 2001).

A 20 Hz LFS to the Schaffer collateral afferents also reliably induces an LTD of EPSP in the CA1 region of the hippocampus, in both juvenile and adult rats, a protocol that has been routinely used in our laboratory (Sastry, et al., 1984). In the CNS, activity around 20 Hz seems to be crucial for synchronizing network activity (Boddeke, et al., 1997; Marshall, et al., 2002). The stimulation itself is powerful enough to unmask the otherwise latent excitatory connections in hippocampal neurons (Miles and Wong, 1987). Moreover, several neurons in the hippocampus and neocortex fire action-potentials (also as bursts) in this range during learning and behavior (O'Keefe and Dostrovsky, 1971). The temporal synchrony that exists between the SWS associated sleep spindles (~ 12-14 Hz), para-
hippocampal spindles and slow oscillations, is important in initiating the “hippocampal-neocortical” dialogue, a phenomenon considered crucial for offline re-processing of memories (Clemens, et al., 2007; Wolansky, et al., 2006).

The induction of LTD, therefore, seems to be affected by the age of the animal, receptor expression (inhibitory and excitatory) and the extent of prior postsynaptic activity in the synapse. Since our studies involved the use of adult rats (~ 3-4 weeks old), the first set of experiments were designed to establish a stimulation paradigm which ensures a reliable LTD, stable for at least 40 min post-tetanus. Since the early part, immediately post-stimulation is more likely to involve short-term depression, LTD was measured routinely at 10 min post-tetanus. Short-term depression differs significantly from LTD, in properties, as well as, in mechanisms. We tested the reliability of 1 Hz, 15 min, 20 Hz, 30s 1X stimulus intensity (SI), and 20 Hz, 30s, 2XSI low frequency tetanic stimulation protocols in inducing LTD.

3.2 Effects of sleep-deprivation on LTD

A 20 Hz, 30s, 2XSI stimulation protocol induced a stable and reversible LTD. Using this protocol, in these set of experiments, LTD was induced in sleep-deprived rats. Sleep-deprivation was done using the gentle-handling technique for a period of 12 h, as described in Section #2.2. The extent of LTD was compared between normally-sleeping and sleep-deprived rats.
3.3 Effect of stress induced by sleep-deprivation

Procedures used for sleep-deprivation, mainly the inverted flower-pot- or forced locomotion- techniques, have been previously shown to impart behavioral stress in the animal (Campbell, et al., 2002; McDermott, et al., 2003). In literature, the extent of acute/chronic behavioral stress is measured by quantitating the serum/plasma corticosterone levels. An elevated corticosterone level in the hippocampus can affect synaptic plasticity (Diamond, et al., 1992). To minimize the effect of behavioral stress on LTD, we took a two pronged approach. Firstly, we chose sleep-deprivation by gentle-handling in all our studies. It is well established that this method is significantly less stressful compared to the above mentioned classic methods. Secondly, while other investigators in the field have sleep-deprived rats for extended periods (up to 4 days), we employed 12 h. We believe shorter durations of sleep-deprivation are more realistic and directly applicable to our society and today’s 24 X 7 culture. Furthermore, choosing shorter durations had an additional benefit of inducing significantly lower behavioral stress in rats. Evidence in literature shows a correlative increase in the serum corticosterone levels proportional to the duration of sleep-deprivation.

In our studies, we quantitated serum corticosterone levels at the end of the light period in normally sleeping rats and SD rats (in this case, after the 12 h period of sleep-deprivation). Corticosterone levels in both conditions were estimated by performing a radioimmunoassay (refer to Section #2.14 for methods).
3.4 Mechanisms involved in LTD

A 20 Hz, 30, 2XSI protocol reliably induced LTD in the CA1 region of the hippocampus. Experiments were further designed to ascertain the mechanisms involved in LTD. In the CA1 region, NMDAR- and mGluR- dependent forms of LTD are well established. Both forms can be induced pharmacologically using receptor-specific agonists (Lee, et al., 1998; Palmer, et al., 1997), or through varying conditioning protocols (Bolshakov and Siegelbaum, 1994; Dudek and Bear, 1992). NMDAR- and mGluR- LTDs can also co-exist in the same synapse, without occluding each other, mainly due to their mutually exclusive mechanistic pathways. These alternating forms can be induced either by changing the frequency of induction protocol or by altering the Ca\(^{2+}\)/Mg\(^{2+}\) ratio (Kemp, et al., 2000; Oliet, et al., 1997). NMDARs are tetramers of various subunits (see Section #1.1.4.2). Genetic and pharmacological studies reveal that selective activation of specific NMDAR subtypes (NR2B type) may be necessary for NMDAR-LTD (Liu, et al., 2004), but only under special circumstances (Bartlett, et al., 2007; Brigman, et al., 2010; Duffy, et al., 2008). Other studies, however, have failed to reproduce this effect (Morishita, et al., 2007). For the mGluR-LTD, activation of group I mGluRs seem to be necessary. Application of MCPG, a group I mGluR selective antagonist, blocks both LFS and pharmacologically induced mGluR-LTD (Bolshakov and Siegelbaum, 1994; Stanton, et al., 1991). The involvement of other mGluR subtypes (see Section #1.1.4.3), however, is less clear. Interestingly, both mGluR and NMDARs may interact synergistically to induce LTD. Such an interaction has been observed in the perirhinal cortex (Cho, et al., 2000).
LTD is also age-dependent. Several studies have shown that while mGluR-LTD is readily induced in adult rats, NMDAR-LTD is not (Dudek and Bear, 1993; Kemp and Bashir, 2001; Staubli and Ji, 1996). Although the reasons behind this discrepancy are unclear, a variety of factors, including, stronger inhibitory tone (Steele and Mauk, 1999; Wagner and Alger, 1995), alterations in glutamate transport mechanisms (Collingridge, et al., 2010; Yang, et al., 2005), in adult rats, seem to play a role. For instance, application of GABAₐ- or adenosine-receptor antagonists, or blocking the L-type VGCCs, facilitate induction of LTD in these animals (Kemp and Bashir, 1997; Kerr and Abraham, 1995; Norris, et al., 1998; Wagner and Alger, 1995).

The involvement of both glutamatergic and GABA-ergic mechanisms in LTD is not surprising. As mentioned in detail in the introductory chapters (Section #1.1.3.6), interneurons (inhibitory) and pyramidal cells (excitatory) are arranged sequentially in a laminar format in the hippocampus. Conditioning stimulations, therefore, are likely to affect both these neuronal types, and thereby, induce and/or affect LTD. It is however, unclear as to what effect a 20 Hz, 30s, 2XSI stimulation, produces on these receptors. Therefore, in this set of experiments, we first investigated the role of NMDARs, mGluRs, specifically type I, mGlu1R and mGlu5Rs were tested using antagonists, APV (50 µM), MCPG (500 µM), LY-367385 (100 µM) and MPEP (40 µM), respectively (see Table. 2 for more information on drugs and concentrations used). In all cases, drugs were applied prior to the tetanic stimulation after analyzing the effects of the drugs used on a pre-tetanic control pEPSP.
mGlu1Rs and mGlu5Rs were specifically chosen considering their expression pattern in 3-4 week old rats, and data from published literature on their roles in LTP and LTD in the hippocampus. Although other mGluRs, 2, 3, 4, 7 & 8 subtypes are equally important, their functional significance in modulating synaptic plasticity in the hippocampus remains largely unknown. They seem to however, modulate neurotransmitter release at certain synapses, not surprising, owing to their mainly presynaptic origins (Shigemoto, et al., 1997).

### 3.5 Role of \([\text{Ca}^{2+}]_i\) in LTD

For both NMDAR- and mGluR LTDs, a postsynaptic elevation in \([\text{Ca}^{2+}]_i\) seems to be necessary. In the hippocampus, \([\text{Ca}^{2+}]_i\) rise can occur through a variety of sources, some of which include, VGCCs (Christie, et al., 1996), NMDARs (Cummings, et al., 1996), \(\text{Ca}^{2+}\)-induced \([\text{Ca}^{2+}]_i\) release (Alford, et al., 1993), or \([\text{Ca}^{2+}]_i\) release from intracellular stores (Reyes and Stanton, 1996). All the above sources have been implicated in NMDAR and mGluR LTDs (Bear and Abraham, 1996). Since the induction of LTP also entails a similar rise in \([\text{Ca}^{2+}]_i\), through the same sources, the specific role of \([\text{Ca}^{2+}]_i\) on LTD was widely investigated. Studies revealed that the direction of plasticity (LTP or LTD) is mainly governed by the magnitude, temporal and spatial characteristics of the \(\text{Ca}^{2+}\) signal (Yang, et al., 1999). Not surprisingly, therefore, LTD could be selectively induced by buffering postsynaptic \([\text{Ca}^{2+}]_i\), to levels (~ 500 nM), whereas, maintaining levels (at ~ XXX nM), resulted in LTP. Interestingly, the differences in \([\text{Ca}^{2+}]_i\) levels, seem to modulate the calcium-binding protein calmodulin (CaM) distinctly. Large increases preferentially activate CaM-dependent protein kinases, whereas, a modest rise activates phosphatases (Lisman, 1989).
Once activated, these enzymes seem to influence AMPAR function either by phosphorylating (Barria, et al., 1997; Mammen, et al., 1997) or dephosphorylating (Snyder, et al., 2003) specific sites on the receptor. While the former leads to LTP (Ca-CaM complex → CaMKII → AMPAR phosphorylation), the latter leads to LTD [Ca-CaM complex → protein phosphatase 2 B (PP2B or calcineurin) → inhibitor 1 → protein phosphatase 1 and/or 2 (PP1/2) → AMPAR dephosphorylation]). In agreement with this thinking, drugs that block protein kinases or phosphatases have been shown to inhibit LTP or LTD, respectively (Morishita, et al., 2001; Mulkey, et al., 1994; Mulkey, et al., 1993).

To understand if \([\text{Ca}^{2+}]\) played a similar role in the 20 Hz LTD, we studied the roles of L-type, T-type VGCCs and intracellular stores in LTD. L- and T-type VGCCs were blocked using nitrendipine (5 µM) or Ni\(^{2+}\) (50 µM), respectively. The requirement for \(\text{Ca}^{2+}\)-release from intracellular stores was tested using thapsigargin (1 µM). In all cases, drugs were applied prior to the tetanic stimulation after analyzing their effects on a pre-tetanic control pEPSP.

### 3.6 GABAergic inhibition in LTD

As mentioned previously, in addition to the influence of NMDARs and mGluRs on LTD, the strength of GABAergic inhibition is likely to influence LTD. GABA\(_A\)- and GABA\(_B\)-Rs are located strategically (see Chapter 1, Section #1.1.3.6) to regulate both “inputs-to” and “outputs-from” the pyramidal neurons, and impart a powerful regulatory influence on their net-excitability (Stelzer, 1992). Studies in literature have reported that,
while a decreased GABA$_A$-R activity facilitates induction of LTP of the pEPSP (Stelzer, 1992; Wigstrom and Gustafsson, 1983) and heterosynaptic LTD (Tomasulo, et al., 1993; Zhang and Levy, 1993), stimulation paradigms that would have otherwise induced LTP favored induction of LTD, in the presence of GABA$_A$-R agonists (Grover and Yan, 1999; Steele and Mauk, 1999). Moreover, an age-dependent increase in GABAergic inhibition has been shown to be responsible for the loss of NMDAR-LTD in adult rats (Wagner and Alger, 1995). Sleep-deprivation even for a few hours seems to alter the expression and function of several receptors in the brain (Longordo, et al., 2009). Alterations in the efficacy of the inhibitory transmission (through age- or sleep- dependent processes), can therefore, modulate LTP and/or LTD of the pEPSP.

Tetanic stimulations used to induce plasticity at excitatory synapses can lead to either short-term (Davies, et al., 1991; McCarren and Alger, 1985; Thompson and Gahwiler, 1989) or long-term changes (Shew, et al., 2000; Xie, et al., 1995) of the GABAergic IPSCs. Afferent activity also produces a shift in $E_{\text{GABA-PSC}}$ (Ouardouz and Sastry, 2000; Ouardouz and Sastry, 2005; Xu and Sastry, 2007). Changes to $E_{\text{GABA-PSC}}$, can shunt (depending on the direction of the shift) PSPs more effectively which can manifest as an enhanced LTD of the EPSP. Therefore, increases in the amplitude of IPSC via an tetanus-induced LTP or shift in $E_{\text{GABA-PSC}}$, will significantly affect the induction of LTD.

GABA$_B$-Rs, on the other hand, have been shown to modulate mainly NMDAR-LTD, via disinhibition. Application of CGP-35348, a GABA$_B$-R antagonist, significantly attenuated NMDAR-LTD. Blocking both GABA$_{A&B}$-Rs however failed to have any effect on
NMDAR-LTD, indicating that the effects of GABA$_B$-Rs on LTD was via a presynaptic control of GABA$_A$-R-mediated IPSCs on pyramidal neurons (Wagner and Alger, 1995).

Taking the above evidence into consideration, experiments were designed to understand the influence of GABA$_A$-Rs and GABA$_B$-Rs in LTD. The involvement of GABA$_A$-Rs was tested by stimulating the inputs using the 20 Hz protocol in the presence of bicuculline (10 µM). In earlier studies, Stelzer (1992), Wigmstrom and Gustafsson (1983), found that in a naïve state, the influence of a GABA-ergic IPSC is such that it can completely mask an underlying EPSC. In fact, they demonstrated that an EPSC is visible only on blocking the IPSC. Moreover, in additional experiments, it was demonstrated that the induction of LTP of the EPSC is facilitated when IPSCs are selectively blocked. One of the reasons being, the activation of NMDARs becomes easier when all the inhibition mediated by GABA$_A$-Rs is blocked. We therefore reasoned that, if application of bicuculline blocked LTD, it could be due to a development of LTP, which may mask LTD. So, in additional experiments, this hypothesis was tested by co-applying bicuculline (10 µM) with APV (50 µM) and thereby, simultaneously blocking GABA$_B$-Rs and NMDARs, respectively.

Since conditioning stimulations can affect or induce a short- or long-term plasticity of the IPSC, we designed experiments to observe changes to the amplitude of IPSCs following a 20 Hz, 30s, 2XSI stimulation. The rationale being, any changes to the strength of inhibition, will influence net excitability, and in fact, LTD of the pEPSP.
To elucidate the role of GABA<sub>B</sub>-Rs in LTD, LTD was induced in the presence of CGP-55845 (2 µM), a potent GABA<sub>B</sub>-R antagonist. In additional experiments, the effect of GS-38783 (10 µM), a positive allosteric modulator for GABA<sub>B</sub>-Rs, was tested on naïve pEPSPs. The use of a positive allosteric modulator has several advantages over conventional agonists, such as baclofen. Of these, reduced receptor desensitization is a significant advantage. Also, since the allosteric modulator does not activate the receptor but facilitates the effect of the endogenous ligand once the receptor is activated, the temporal and spatial efficacy of GABA is adequately maintained. We therefore hypothesized that, any change in the slopes of pEPSPs in the presence of GS-39783, is a clear indication of an alteration in either the function or the expression of GABA<sub>B</sub>-Rs.

### 3.7 Expression of mGlu1Rs, GABA<sub>B</sub>-R1s and GABA<sub>B</sub>-R2s

Results from electrophysiological studies indicated that LTD is enhanced in SD animals. GABA<sub>B</sub>- and mGlu- Rs, but not NMDARs were involved in 20 Hz-LTD. The antagonists to mGluRs and GABA<sub>B</sub>-Rs differentially affected the time-course of LTD in normally-sleeping vs. SD rats. Moreover, the presence of GS-39783 selectively suppressed evoked pEPSPs in SD rats. Taken together, these data indicated that sleep-deprivation induces changes in the function of mGluRs, GABA<sub>B</sub>-Rs or both. Alterations in receptor function can arise via modifications in the receptor number and/or their sub-cellular localization. If sleep-deprivation induces an alteration in the expression of either mGlu- or GABA<sub>B</sub>-Rs, it may explain the enhancement of LTD in SD rats. Indeed, the role of these
receptors in maintaining a balance between inhibitory and excitatory networks, and synaptic plasticity in the CNS is well established.

In the hippocampus, GABA\textsubscript{B}-Rs and mGlu1Rs are both localized in the perisynaptic regions of the dendritic spines/shafts (Kulik, et al., 2003; Lujan, et al., 1996). Depending upon their location (pyramidal cells or interneurons), they modulate neuronal excitability via actions on pre- and/or post-synaptic targets. Activation of mGluRs (mainly, group II & II) & GABA\textsubscript{B}-Rs expressed on glutamatergic- and GABA-ergic- axon terminals regulate neurotransmitter release by inhibiting presynaptic VGCCs and/or by interfering directly with the transmitter release machinery (Blackmer, et al., 2005; Swartz and Bean, 1992). Stimulation of mGluRs (mainly, group I), localized on postsynaptic neurons, depolarize them through actions on various Ca\textsuperscript{2+}, K\textsuperscript{+} and other non-specific cationic conductances (Guerineau, et al., 1994; Swartz and Bean, 1992). Conversely, postsynaptic GABA\textsubscript{B}-Rs, when activated, cause a G-protein-mediated alteration in K\textsuperscript{+}/Ca\textsuperscript{2+} conductance, leading to either a shunting or hyperpolarizing inhibition of the target neuron (Solis and Nicoll, 1992).

Although GABA\textsubscript{B} and mGlu-Rs may independently affect LTD enhancement, recent studies indicate that these receptors may “cross-talk” at a functional level, and co-operate to elicit effects. In the cerebellar parallel fiber-Purkinje cell synapses, activation of GABA\textsubscript{B}-Rs seem to enhance mGlu1R-mediated responses, mainly due to an interaction at the level of second messenger systems (Hirono, et al., 2001). Since GABA\textsubscript{B}-Rs and mGluRs are structurally homologous G-protein coupled receptors (GPCRs) and expressed in close proximity, a physical interaction between them is an interesting possibility (Kulik, et al.,
GPCRs were traditionally thought to exist and function in monomeric entities. However, accumulating biochemical and biophysical evidence indicates that most, if not all GPCRs assemble as homo- and/or heterodimers (Kumar and Grant, 2010; Milligan, 2009). For GABA\textsubscript{B}-Rs, heterodimerization seems to be obligatory. In a GABA\textsubscript{B}-R1-GABA\textsubscript{B}-R2 complex, the GABA\textsubscript{B}-R1 fraction imparts sensitivity to endogenous ligands, whereas the GABA\textsubscript{B}-R2 subtype enables coupling of the receptor to G-proteins. This association seems to be sufficient to overcome the endoplasmic reticulum (ER) retention signal for GABA\textsubscript{B}-R1 subtype, thereby allowing the receptor to traffic and express on the cell surface. The co-expression of both subtypes is therefore necessary for a functional GABA\textsubscript{B}-R (Jones, et al., 1998; Kaupmann, et al., 1998; White, et al., 1998). GABA\textsubscript{B}-Rs and mGlu1\alpha-Rs, in addition, have been shown to independently form stable heteromeric complexes with other receptors, such as, calcium sensing receptors (Gama, et al., 2001), a variety of cytoskeletal, scaffolding and signaling proteins like MUPP1, Homer and Shank (Fagni, et al., 2004; Kornau, 2006). A physical interaction between them is, however, currently disputed and not known to occur in the hippocampus. Receptor dimerization may change the basic function of either receptor, such that the activation of one receptor produces a synergistic/antagonistic effect on the function of the other, mainly through effects on receptor surface expression (Marshall, et al., 2002), sensitivity of receptors to endogenous ligands (Gomes, et al., 2000; Rocheville, et al., 2000), signal transduction (Kumar and Grant, 2010) and receptor internalization (Jordan, et al., 2001; Rocheville, et al., 2000). Since all the above mechanisms have been shown to influence LTD (Malinow and Malenka, 2002), modification in any of these aspects following sleep-deprivation could explain the enhancement of LTD in SD rats.
We therefore hypothesized that sleep-deprivation alters, a) the expression of mGlu1αR, GABA<sub>B</sub>-R1 and GABA<sub>B</sub>-R2, b) the degree of co-localization between mGlu1αR and GABA<sub>B</sub>-R1/- or GABA<sub>B</sub>-R2, and c) the extent of complex-formation between mGlu1αR and GABA<sub>B</sub>-R1/- or GABA<sub>B</sub>-R2. Accordingly, the expression pattern of mGlu1αR, GABA<sub>B</sub>-R1 and GABA<sub>B</sub>-R2 was studied by classical methods, immunohistochemical staining and western blot analysis. The interaction between receptors was studied using immunofluorescence and co-IP techniques (see Sections #2.12, #2.13 for methods).
Chapter 4: Results

4.1 LTD and effects of sleep-deprivation

In the current studies, pEPSPs or EPSCs were evoked by stimulating in the stratum radiatum at a rate of 0.05 Hz (see Fig. 3). At the start of each experiment, a full input-output curve was constructed. Stimulus intensity was set at a level sufficient to evoke a half-maximal pEPSP/Cs. Responses were allowed to stabilize over an initial 10-15 min waiting period. A 10 min pre-tetanic control was then recorded, and LTD induced using the 1 Hz, 15 min or 20 Hz, 30 sec LFS protocols. For the latter, two stimulus intensities (SI) were used (1XSI and 2XSI). This was done to standardize an appropriate protocol for obtaining a stable, and lasting LTD (for at least 30-40 min). These experiments were performed in slices from normally sleeping and SD rats.

4.1.1 LTD (1 Hz, 15 min LFS)

When the 1 Hz, 15 min stimulation protocol was used to induce LTD of the pEPSPs, in normally-sleeping rats no LTD was observed at 20 min post-tetanus (pEPSP slope as a % of pre-tetanic control; 20 min post-tetanus: 114.9 ± 9.1, n=11; Fig. 6). Similarly, no LTD was observed in SD rats (pEPSP slope as a % of pre-tetanic control; 20 min post-tetanus: 97.73 ± 4.3, n=11; Fig. 6). However, the slopes of pEPSPs were significantly lower in SD rats when
compared to normally-sleeping rats (p<0.05). This protocol was not used in further studies because it did not meet our criteria for LTD induction (Section #2.15.3).

**Figure 6. LTD (1Hz, 15 min)**

Arrow indicates the low frequency tetanic stimulation (1 Hz, 15 min). Graph depicts the time course of LTD in normally sleeping (open circles, n=11) and sleep-deprived rats (filled circles, n=11). Representative pEPSP traces were taken at the indicated time points. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM. At 20 min post-tetanus, pEPSP slopes were significantly suppressed (p<0.05) when compared to normally sleeping rats.
4.1.2 LTD [20 Hz, 30 sec (1XSI)]

The 20 Hz protocol (1XSI), induced a suppression of pEPSPs in normally sleeping rats, which recovered back to pre-tetanic control levels in about 10 min (pEPSP slope as a % of pre-tetanic control; 10 min post-tetanus: 94.2 ± 9.1, n=11; p>0.05; Fig. 7). However, similar to the results obtained using the 1 Hz, 15 min protocol, in SD rats, the suppression of pEPSPs was more pronounced and significant (pEPSP slope as a % of pre-tetanic control; 10 min post-tetanus: 78 ± 5.4, n=11; p<0.05; Fig. 7). Since the 20 Hz (1XSI) protocol did not induce LTD in normally sleeping rats, it was not used for further experiments.
Figure 7. LTD (20 Hz, 30 sec- 1XSI)

The 20 Hz, 30 s (1XSI) tetanic stimulation (indicated by an arrow) did not induce LTD in normally sleeping rats (open circles, n=11). The time course of pEPSPs in normally sleeping and sleep deprived conditions (filled circles, n=11) indicate a larger suppression of synaptic excitation in the latter case. This effect was significant (p<0.05). Representative pEPSP traces were taken at the indicated time points. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.

4.1.3 LTD [20 Hz, 30 sec (2XSI)]

The 20 Hz LFS (2XSI) reliably induced an LTD, which lasted at-least 30 minutes. LTD of the pEPSP in SD rats (pEPSP slope as a % of pre-tetanic control; 20 min post-tetanus: 53 ± 5.5, n=6; Fig. 8A) was significantly higher (p<0.05) when compared to normally sleeping rats (pEPSP slope as a % of pre-tetanic control; 20 min post-tetanus: 76.4 ± 10.7, n=6; Fig. 8A). Similarly, in whole cell recordings, SD rats showed a significantly elevated LTD (EPSC amplitude as a % of pre-tetanic control; 20 min post-tetanus: 64.8 ± 4.6, n=6; Fig. 8B), when compared to normally sleeping animals (EPSC amplitude as a % of pre-tetanic control; 10 min post-tetanus: 81.2 ± 3.2, n=6; Fig. 8B). A two-way RM-ANOVA showed a significant group [df(1,120); F = 6.42; P < 0.05] and time effect (P < 0.0001) in LTD of pEPSPs. This effect was also observed in whole-cell recordings, in factors, group [df(1,80); F = 7.50; P < 0.05] and time (P < 0.0001).
Figure 8. LTD (20 Hz, 30 sec- 2XSI)

A 20 Hz, 30 s (2 X SI) low frequency tetanic stimulation (arrow) was used to induce LTD. Sleep-deprivation potentiates LTD of pEPSP/-or EPSCs in A and B, respectively. The graph in A and B depicts the time course of LTD in normally sleeping rats (open circles: n = 6) and sleep-deprived rats (filled circles: n = 6). In each case, representative pEPSP/EPSC traces were taken at the indicated time points. The pEPSP slopes or EPSC amplitudes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.

4.1.4 Corticosterone radioimmunoassay

A RIA was performed to test the corticosterone levels in normally sleeping and SD conditions (Section #2.14). The corticosterone levels (ng/ml) in SD rats (154.4 ± 15, n=6) were not significantly different from normally sleeping animals (125.7 ± 14.3, n=6). Corticosterone levels estimated by the RIA method are very similar and comparable to those
obtained using other methods such as, high performance liquid chromatography combined with mass spectroscopy or fluorimetric methods (Rattner, et al., 1979; Shimizu, et al., 1983). The baseline range varies from ~ 50 to 300 or 400 ng/ml in mice and rats, depending on the time of the day (Shimizu, et al., 1983).

4.2 Mechanisms involved in LTD

Literature evidence cited in the Section #3.4 clearly indicates that two forms of LTD, NMDAR- and/or mGluR- type can be induced. The mechanisms involved are highly dependent upon the age of the animal and/or the stimulation parameters used in the induction of LTD. Since we used rats aged 3-4 weeks, and a 20 Hz, 15 min (2XSI) protocol to induce LTD, the role of NMDARs and mGluRs, was investigated in the following experiments. LTD was induced in the presence of antagonists specific to NMDARs or mGluRs, APV (50 µM) or MCPG (500 µM, a broad spectrum group I/II mGluR antagonist), respectively (Bolshakov and Siegelbaum, 1994). When used in these concentrations, neither APV nor MCPG had any significant effect on the control pEPSPs.

4.2.1 Role of NMDARs in LTD

LTD was not affected in APV (pEPSP slope represented as a % of the pre-tetanic control in normally sleeping rats- 20 min post-tetanus: 72.4 ± 2.9, n=6; p<0.05; Fig. 9), indicating that the LTD does not require the activation of NMDARs. For this reason, experiments were not performed in SD rats in the presence of APV. However, sleep-
deprivation itself may alter NMDARs and perhaps bring in a change such that LTD in SD rats may be sensitive to APV. This aspect needs to be further investigated in future studies.

Figure 9. Role of NMDARs in LTD

LTD was induced using a low-frequency tetanic stimulation (LFTS, 20 Hz for 30s, arrow). In slices from normally sleeping rats, the presence of APV (50 µM), a NMDA receptor antagonist did not block LTD. Graph depicts the time course of LTD in no-drug controls (open circles, n=6) and in APV (filled circles, n=6). Representative pEPSP traces in no-drug controls and treated conditions were taken at the indicated time points. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.
4.2.2 Role of mGluRs in LTD

The application of MCPG attenuated LTD in normally sleeping (pEPSP slope represented as a % of the pre-tetanic control; in normally sleeping rats- 20 min post-tetanus: 102.8 ± 5.5, n=6; p>0.05; Fig. 10A) and SD rats (pEPSP slope represented as a % of the pre-tetanic control; in SD rats- 20 min post-tetanus: 95.9 ± 11.1, n=5; p>0.05; Fig. 10B). Since MCPG blocks both group I/II mGluRs, it can be concluded that an activation of these receptors is required for the induction of LTD. In a separate set of experiments (Section #4.2.2.1), the roles of mGlu1Rs and mGlu5Rs in LTD were examined (please refer to Section #3.4 for justification).

Figure 10. Role of mGluRs in LTD

LTD was induced using a tetanic stimulation (20-Hz for 30s, arrow). In A and B, slices from normally-sleeping (NS) rats and sleep-deprived (SD) rats, respectively, LTD was significantly
attenuated in the presence of MCPG (500 µM), a group I/II mGluR antagonist. Graph depicts the time course of LTD in no-drug controls (A, B; open circles, n=6) and in MCPG (A, filled circles, n=6; B, filled triangles, n=6). Representative pEPSP traces in no-drug controls and treated conditions were taken at the indicated time points. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.

4.2.2.1  Group I mGluR subtypes and LTD

To investigate the role of group I mGluRs, LTD was induced in the presence of either LY-367385 (100 µM) or MPEP (40 µM), mGlu1R and mGlu5R receptor specific antagonists, respectively. At the above concentrations, LY-367385 or MPEP neither affect the slopes of control pEPSPs nor display any cross-reactivity with other mGluR subtypes (Pin and Duvoisin, 1995).

4.2.2.1.1  Role of mGlu1Rs

Application of LY-367385 (100 µM) significantly suppressed LTD in normally sleeping rats (pEPSP slope represented as a % of the pre-tetanic control, in no-drug controls- 20 min post-tetanus: 75.76 ± 7.97, n=6; in LY-367385- 20 min post-tetanus: 105.0 ± 12.56, n=6; p<0.05; Fig. 11A) and in SD rats (pEPSP slope represented as a % of the pre-tetanic control- in no-drug controls- 20 min post-tetanus: 54.54 ± 3.62, n=6, in LY-367385- 20 min post-tetanus: 80.69 ± 2.777, n=6; p<0.05; Fig. 11B).
Figure 11. Role of mGlu1Rs in LTD

LTD was induced using a tetanic stimulation (20 Hz for 30s, arrow). In A and B, slices from normally-sleeping (NS) rats and sleep-deprived (SD) rats, respectively, LTD was significantly attenuated in the presence of LY-367385 (100 µM), a mGlu1R antagonist. Graph depicts the time course of LTD in no-drug controls (A, B; open circles, n=6) and in LY-367385 (A, B; filled circles, n=6). Representative pEPSP traces in no-drug controls and treated conditions were taken at the indicated time points. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.

4.2.2.1.2 Role of mGlu5Rs

While the application of MPEP (40 µM) did not affect LTD in normally sleeping rats (pEPSP slope represented as a % of the pre-tetanic control, in no-drug controls- 20 min post-tetanus: 75.76 ± 7.97, n=6; in MPEP- 20 min post-tetanus: 86.57 ± 5.291, n=6; p>0.05; Fig. 12A), it was significantly suppressed in SD rats (pEPSP slope represented as a % of the pre-
tetanic control- in no-drug controls- 20 min post-tetanus: 54.54 ± 3.62, n=6, in MPEP- 89.06 ± 5.896, n=6; p<0.05; Fig. 12B).

Figure 12. Role of mGlu5Rs in LTD

LTD was induced using a tetanic stimulation (20 Hz for 30s, arrow). In A and B, slices from normally-sleeping (NS) rats and sleep-deprived (SD) rats, respectively, while LTD was unaffected in the presence of MPEP (40 µM), a mGlu5R antagonist in NS rats, it was significantly blocked in SD rats. Graph depicts the time course of LTD in no-drug controls (A, B; open circles, n=6) and in MPEP (A, B; filled circles, n=6). Representative pEPSP traces in no-drug controls and treated conditions were taken at the indicated time points. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.
4.2.3 Role of Ca$^{2+}$ in LTD

In the present study, the requirement of [Ca$^{2+}$]$_i$ for 20 Hz LTD was tested. LTD was induced in the presence of nitrendipine (5 µM), Ni$^{2+}$ (50 µM) or thapsigargin (1µM), drugs that block L- type, T- type VGCCs or Ca$^{2+}$ release from intracellular stores, respectively. These drugs did not affect control pEPSPs.

Bath application of thapsigargin (1 µM), which blocks Ca$^{2+}$-release from intracellular stores dampened LTD induction. This effect was significant at 20 min post-tetanus (pEPSP slope represented as a % of the pre-tetanic control in normally sleeping rats- 20 min post-tetanus: 106.1 ± 13.7, n=6; p>0.05; Fig. 13). The presence of nitrendipine (5 µM; pEPSP slope represented as a % of the pre-tetanic control in normally sleeping rats- 20 min post-tetanus: 79.9 ± 8.8, n=6; p<0.05; Fig. 13) or Ni$^{2+}$ (50 µM; pEPSP slope represented as a % of the pre-tetanic control in normally sleeping rats- 20 min post-tetanus: 79.9 ± 4.7, n=6; p<0.05; Fig. 13) did not affect LTD. These results indicate that Ca$^{2+}$ release from intracellular stores, but not L- or T- type voltage-gated Ca$^{2+}$ channels (VGCCs), are required for LTD induction.
Figure 13. Role of Ca\(^{2+}\) in LTD

LTD was induced using a low-frequency tetanic stimulation (LFTS, 20-Hz for 30s, arrow). In slices from normally sleeping rats, LTD was blocked in the presence of thapsigargin (1µM), which blocks Ca\(^{2+}\)-release from intracellular stores. Nitrendipine (5 µM), an L-type voltage-gated calcium channel (VGCC) antagonist or Ni\(^{2+}\) (50 µM), a T-type VGCC antagonist, however, did not affect LTD. Graph depicts the time course of LTD in no-drug controls (open circles, n=6), in thapsigargin (filled inverted triangles, n=6), in nitrendipine (filled circles, n=6) and in Ni\(^{2+}\) (filled triangles, n=6). Representative pEPSP traces in no-drug controls and treated conditions were taken at the indicated time points. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.
4.2.4 GABAergic inhibition and LTD

In the present study, the influence of GABA$_A$-Rs and GABA$_B$-Rs on LTD was tested. LTD was induced in the presence of bicuculline (10 µM) or CGP-55845 (2 µM), antagonists to GABA$_A$-Rs and GABA$_B$-Rs, respectively. In separate studies, the effects of the 20 Hz LFS on the amplitude of IPSCs were studied. This was done to examine if the alterations in LTD (enhancement in SD rats) occur at the level of GABAergic transmission. All experiments were performed on normally sleeping and SD rats (see Section #3.6 for justification).

4.2.4.1.1 Role of GABA$_A$-Rs in LTD

In the presence of bicuculline (10 µM), LTD was blocked in normally sleeping rats (pEPSP slope represented as a % of the pre-tetanic control- 10 min post-tetanus: 116.6 ± 25.3, n=6; p<0.05; Fig. 14A) and in SD rats (pEPSP slope represented as a % of the pre-tetanic control; 20 min post-tetanus: 138.4 ± 22.1, n=6; p<0.05; Fig. 14B). Interestingly, the blockade of GABAergic inhibition with bicuculline seemed to induce a potentiation of pEPSPs, following the 20 Hz protocol in both normally sleeping and SD rats. This potentiation was much higher in SD rats. Whether these effects were due to a stronger basal inhibition following sleep-deprivation was unclear. Therefore, in the next set of experiments, changes to GABA$_A$-R mediated IPSCs were directly examined.
Figure 14. Role of GABA$_A$-Rs in LTD

LTD was induced using a tetanic stimulation (20 Hz for 30s, arrow). In A and B, slices from normally-sleeping (NS) rats and sleep-deprived (SD) rats, respectively, LTD was significantly blocked in the presence of bicuculline (10 µM), a GABA$_A$-R antagonist. Graph depicts the time course of LTD in no-drug controls (A, B; open circles, n=6) and in bicuculline (A, B; filled circles, n=6). pEPSP slopes were averaged every min and normalized to respective pre-tetanic controls. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.

4.2.4.1.2 Effects of 20 Hz LFS on GABA$_A$-R mediated IPSCs

IPSCs were evoked by stimulating in the stratum radiatum (see Fig. 3, 4), in the presence of APV (50 µM) and DNQX (20 µM), drugs that block excitatory synaptic transmission by antagonizing NMDARs and AMPARs, respectively. Following the 20 Hz LFS, no significant difference was seen in IPSC amplitudes between normally sleeping rats (IPSC amplitude represented as a % of the pre-tetanic control; 20 min post-tetanus: 115 ±
12.4, n=6; p>0.05; Fig. 15) and SD rats (IPSC amplitude represented as a % of the pre-tetanic control; 20 min post-tetanus: 111 ± 11.9, n=6; p>0.05; Fig. 15), or compared to their respective pre-tetanic controls.

**Figure 15. Effect of a 20 Hz LFS on GABAergic IPSCs**

Effects of 20 Hz LFS on GABA\(_A\)-mediated IPSCs. Control IPSCs were stimulated using the 20 Hz, 30 sec low frequency stimulation (LFS). Graph depicts the time course of post-tetanic IPSC amplitudes in normally sleeping (NS; open circles, n=6) and sleep-deprived (SD) rats (filled circles, n=6). As can be seen, the 20 Hz LFS protocol do not significantly affect IPSCs. IPSC amplitudes were averaged every min and normalized to respective pre-tetanic controls. Values were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.
4.2.4.1.3 Masking of LTD by co-occurring LTP

The effect of bicuculline on LTD was not due to an altered GABAergic synaptic strength as predicted. Studies have shown that in the absence of GABAergic inhibition, stimulations that would otherwise induce LTD, elicit LTP. Therefore, the LTD could simply be masked by a co-occurring LTP in the absence of GABA$_A$-R mediated inhibition. To test if this is the case, in separate experiments, LTD was induced in the presence of bicuculline and APV. The presence of APV in the medium ensured the blockade of NMDAR-LTP.

LTD in the presence of bicuculline and APV (pEPSP slope represented as a % of the pre-tetanic control; 20 min post-tetanus: 68 ± 5.4, n=6; p>0.05; Fig. 16) was not significantly different compared to no drug controls (pEPSP slope represented as a % of the pre-tetanic control; 20 min post-tetanus: 75.8 ± 8, n=6; p>0.05; Fig. 16).
Figure 16. Masking of LTD by LTP

LTD was induced using a low-frequency tetanic stimulation (LFTS, 20 Hz for 30s, arrow). In slices from normally sleeping rats, the presence of APV (50 µM) and bicuculline (10 µM), a NMDA- and GABA$_A$-R antagonist, respectively, did not block LTD. Graph depicts the time course of LTD in no-drug controls (open circles, n=6) and in APV + bicuculline (filled circles, n=6). Representative pEPSP traces in no-drug controls and treated conditions were taken at the indicated time points. The pEPSP slopes were normalized to respective pre-tetanic controls in each case. Data are represented as mean ± SEM.

4.2.4.1.4 Role of GABA$_B$-Rs in LTD

In these experiments, the role of GABA$_B$Rs in LTD was investigated. Bath application of CGP-55845 (2 µM), a GABA$_B$-R antagonist, did not block LTD in normally sleeping rats (pEPSP slope represented as a % of the pre-tetanic control- 20 min post-tetanus: 89.1 ± 4.3, n=6; p<0.05; Fig. 17A). In SD rats, however, LTD was completely blocked in the presence of the drug (pEPSP slope represented as a % of the pre-tetanic control- 20 min post-tetanus: 121.8 ± 9.8, n=6; p>0.05; Fig. 17B).
Figure 17. Role of GABA_B-Rs in LTD

LTD was induced using a low-frequency tetanic stimulation (LFTS, 20 Hz for 30s, arrow). In A and B, slices from normally sleeping (NS) and sleep-deprived (SD) rats, respectively, while LTD was partially suppressed in the presence of CGP-55845 (2 µM), a GABA_B antagonist, in NS rats, it was completely blocked in SD rats. Graph depicts the time course of LTD in no-drug controls (A, B; open circles, n=6) and in CGP-55845 (A, B; filled circles, n=6). Representative pEPSP traces in no-drug controls and treated conditions were taken at the indicated time points. pEPSP slopes were averaged every min and normalized to respective pre-tetanic controls.

The application of GS-39783, a positive allosteric modulator for GABA_B-Rs (Urwyler, et al., 2003), significantly depressed evoked pEPSPs in SD rats (pEPSP slope represented as a % of the pre-tetanic control; 10 min post-application: 50.7 ± 4.3, n=5 p<0.05; Fig. 18). No change was, however, noted in normally sleeping rats (pEPSP slope represented as a % of the pre-tetanic control; 10 min post-application: 97.3 ± 8.5, n=5 p>0.05; Fig. 18). Since GS-39783 affects GABA_B-R mediated responses only when
activated, it appears that in normally sleeping rats these receptors are not fully active under a 20 Hz stimulation but get recruited in SD rats.

Figure 18. Effects of GS-39783 on pEPSPs

Bath application of GS-39783 (10 µM), selectively suppressed evoked pEPSPs in sleep-deprived (SD) rats, but not in normally sleeping (NS) rats. Graph depicts the time course of evoked pEPSPs in NS (open circles, n=5) and SD (filled circles, n=5) conditions. Representative pEPSP traces in NS and SD conditions were taken at the indicated time points. pEPSP slopes were averaged every min and normalized to respective pre-tetanic controls.
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<tr>
<th>Pharmacological agents (action)</th>
<th>LTD in normally sleeping rats</th>
<th>LTD in SD rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>APV (NMDAR)</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>MCPG (group I/II mGluRs)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LY-367685 (mGlu1Rs)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MPEP (mGlu5Rs)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Thapsigargin (Ca(^{2+}) release from intracellular stores)</td>
<td>Yes</td>
<td>N/A</td>
</tr>
<tr>
<td>Nitrendipine (L-type VGCCs)</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>Ni(^{2+}) (T-type VGCCs)</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>Bicuculline (GABA(_A)-Rs)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Bicuculline + APV (GABA(_A)-Rs and NMDARs)</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>CGP-55845 (GABA(_B)-Rs)</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 3. Effect of various pharmacological agent used on LTD.

“Yes” indicates a blockade of LTD and “No” indicates no significant effect on LTD.
4.2.5 Immunohistochemistry, co-localization, western blots and co-IP

In this section, results from studies on receptor expression, co-localization and dimerization are presented.

4.2.5.1 Expression of mGlu1αR, GABA_B-R1 and GABA_B-R2 in the CA1 hippocampal region of normally sleeping and SD rats

In normally sleeping rats, strong GABA_B-R1-like immunoreactivity was observed in the stratum pyramidale. Pyramidal and non-pyramidal cells in the CA1 region displayed GABA_B-R1-like immunoreactivity at the neuronal perikarya, as well as, in intracellular compartments. A weak immunoreactivity was observed in the stratum radiatum (Fig. 19a, b). These findings are largely consistent with previous studies (Kulik, et al., 2003). Following sleep-deprivation, however, the GABA_B-R1-like immunoreactivity was observed in stratum pyramidale and in the apical dendritic regions of the stratum radiatum (Fig. 19g, h). When examined visually, our results suggest that the intensity of staining and the number of neurons immunopositive to GABA_B-R1 may have been increased in SD rats when compared to that in normally sleeping rats. Similar results were obtained for GABA_B-R2 expression between normally sleeping and SD rats. Following sleep-deprivation, upon visual examination, the immunoreactivity in stratum pyramidale and radiatum appeared more pronounced in SD rats (Fig. 19i, j) in comparison to normally sleeping rats (Fig. 19c, d).
The mGlu1αR like immunoreactivity, in both normally sleeping and SD groups, was observed in isolated pyramidal and non-pyramidal neurons in stratum pyramidale. The expression was uniform at the neuronal perikarya and in the cytoplasmic compartment. However, in axonal processes or dendrites, the immunoreactivity was mostly punctated (Fig. 19e, f). Importantly, when compared visually, SD rats points toward an increased number of mGlu1αR positive neurons. In addition, the mGlu1αR- like immunoreactivity at the neuronal perikarya and intracellular compartments of both cell types appeared more intense in stratum pyramidale and radiatum in SD rats.
Figure 19. Distribution of GABA<sub>B</sub>-R1/R2 and mGlu1αRs

Representative photomicrographs illustrating the expression of GABA<sub>B</sub>-R1/R2 and mGlu1αR in the CA1 region of the hippocampus of normally sleeping (A) and sleep-deprived (B) rats. GABA<sub>B</sub>-R1-
like immunoreactivity was well-expressed at the neuronal perikarya and cytoplasmic protein (a, b). Following sleep-deprivation, the intensity of staining and the number of neurons immunopositive to GABA$_B$-R1 increased (g, h). An enhanced immunoreactivity was also evident in the stratum radiatum (sr; b, h). For the GABA$_B$-R2 subtype, a moderate to weak immunoreactivity was observed in stratum pyramidale (sp) and sr of NS rats (c, d), with a modest increase in immunoreactivity in SD rats (i, j). mGlu1$\alpha$R-like immunoreactivity was strong-to moderately-expressed in isolated pyramidal and non-pyramidal neurons in the sp, sr and stratum oriens (so; e) in NS rats. Following sleep-deprivation, the intensity of staining and the number of neurons immunopositive to mGlu1$\alpha$ increased (k, l compared to e, f). The immunoreactivity in dendrites and axons was also enhanced. While the staining in pyramidal/non-pyramidal neuron perikarya was uniform, it appeared punctuated in dendrites and axons in both NS and SD rats. Scale Bar= 160 µm for panels on left in A & B; 20 µm for panels on right in A & B; and 5 µm for inset.

4.2.5.2 Western blot analysis of mGlu1$\alpha$R, GABA$_B$-R1 and GABA$_B$-R2 expression in the CA1 hippocampal region of normally sleeping and SD rats

Data from immunohistochemical studies therefore suggest an increase in GABA$_B$-R1 and mGlu1$\alpha$R immunoreactivity, with a modest change in GABA$_B$-R2 expression. We quantified these results by studying protein levels of GABA$_B$-R1, GABA$_B$-R2 and mGlu1$\alpha$R. The hippocampal tissue lysate prepared from normally sleeping and SD rats was processed for western blot analyses, as described in material and methods. As shown in Fig. 20, GABA$_B$-R1 (A), GABA$_B$-R2 (B) and mGlu1$\alpha$R (C) immunoreactivity was expressed as a single band at the expected molecular mass of ~130 kDa, ~120 kDa & ~142 kDa, respectively. A significant increase in GABA$_B$-R1 and mGlu1$\alpha$R protein levels was detected
in SD rats with only a subtle change in GABA$_B$-R2 immunoreactivity. Histograms in representative panels describe the quantitative analysis for the receptor-immunoreactivity. These findings corroborate and further strengthen our observations from immunohistochemical studies.

**Figure 20. Western blot analysis of GABA$_B$-R1, GABA$_B$-R2 and mGlu1αRs**

Hippocampal tissue lysate from normally sleeping (NS) and sleep-deprived (SD) rats were processed for western blot analysis as described in Materials and Methods section. GABA$_B$-R1 (A), GABA$_B$-R2
(B) and mGlu1αR (C) immunoreactivity was expressed as a single band at the expected molecular mass of ~130 kDa, ~120 kDa & ~142 kDa, respectively. Histograms in representative panels describe the quantitative analysis for the receptor-immunoreactivity. A significant increase in GABA<sub>B</sub>-R1 and mGlu1αR protein levels was detected in SD rats with only a mild change in GABA<sub>B</sub>-R2 immunoreactivity. β-actin was used as the control for loading protein. * p < 0.05, ** p<0.01.

4.2.5.3 Co-localization of mGlu1αR and GABA<sub>B</sub>-R1/R2 in normally sleeping and SD rats

It has been previously reported that GABA<sub>B</sub>-R1 and mGlu1Rs co-localize in cerebellum (Tabata, et al., 2004). However, it is currently not known if these receptors co-localize in the CA1 hippocampal region and if sleep-deprivation causes a change in their co-expression. Therefore, in the current study, we examined if GABA<sub>B</sub>-R1, GABA<sub>B</sub>-R2 and mGlu1Rs co-localize in normally sleeping rats and visually compared the results with SD rats.

To investigate whether GABA<sub>B</sub>-R1/GABA<sub>B</sub>-R2 or GABA<sub>B</sub>-R1/mGlu1αR and GABA<sub>B</sub>-R2/mGlu1αR co-express in the CA1 region of the hippocampus, double-labeled immunofluorescence co-localization was performed. In agreement with previous studies (Kulik, et al., 2003), an overlap in distribution of GABA<sub>B</sub>-R1 & R2-like immunoreactivity was observed in the CA1 pyramidal neurons between normally sleeping (Fig. 21a-c) and SD rats (Fig. 21j-l). Much of the co-localization was confined to the neuronal perikarya. Importantly, as illustrated, GABA<sub>B</sub>-R1 expression seems to have changed in SD rats (Fig.
21k) in comparison to normally sleeping rats (Fig. 21b). Co-localization, however, seemed unaffected (Fig. 21l, c).

We further extended our study to determine the co-localization between GABA$_B$-R1/mGlu1αR in normally sleeping- (Fig. 21d-f) and SD- (Fig. 21m-o) rats. In normally sleeping rats, co-localization between GABA$_B$-R1 and mGlu1αR was mainly restricted to stratum pyramidale and confined at the pyramidal/non-pyramidal cell perikarya. In addition, discrete neuronal population displayed punctated co-localization in the dendrites. However, in SD rats, visual examination points toward an increase in the immunoreactivity and co-localization for both GABA$_B$-R1 and mGlu1αRs in stratum pyramidale. Co-localization, as in the normally sleeping rats, was, however, mainly restricted to the neuronal perikarya, although some neurons displaying changes in co-localization in the dendrites.

As illustrated in Fig. 21, the GABA$_B$-R2/mGlu1αR displayed a qualitatively stronger co-localization in hippocampal CA1 region in SD (Fig. 21p-r) rats when compared with normally sleeping (Fig. 21g-i) rats. In stratum pyramidale, the number of individual pyramidal/non-pyramidal neurons co-expressing GABA$_B$-R2 and mGlu1αR altered in SD- when compared to normally sleeping rats. The increase was not only restricted to the neuronal perikarya, but also evident in the dendrites.
Figure 21. Co-localization of GABA_B-R1/GABA_B-R2, GABA_B-R1/mGlu1αR, and GABA_B-R2/mGlu1αR

Representative immunofluorescence photomicrographs illustrating co-localization of GABA_B-R1/GABA_B-R2 (top panel), GABA_B-R1/mGlu1αR (middle panel) and GABA_B-R2/mGlu1Rα (bottom panel) in CA1 area of normally sleeping (NS) and sleep-deprived (SD) rats. Receptors co-localization was performed as described in Material and Methods. Brain sections passing through hippocampus were stained with specific antibodies for GABA_B-R1, GABA_B-R2 and mGlu1Rα. Red and green fluorescence indicate individual receptor types while yellow (merged) shows co-localization. Note the specific increase in receptor immunoreactivity and co-localization in SD rats in comparison to normally sleeping rats. Arrows in representative panels indicate co-localization, arrow-heads indicate neuronal population devoid of co-localization, whereas, asterisks indicate mild co-localization. Scale Bar= 10 µm

4.2.5.4 GABA_B-R1 and GABA_B-R2 receptors are expressed in mGlu1α receptor immunoprecipitate in normally sleeping and SD rats

Our immunocytochemical, western blot and co-localization analysis of GABA_B- and mGlu1α- Rs suggest that these receptors may function as heterodimers in hippocampus. Whether GABA_B- and mGlu1α- Rs form a complex and functionally interact with each other in cerebellum is currently disputed (Rives, et al., 2009) and not known in the hippocampus. Therefore, we performed co-IP experiments to study the complex formation between GABA_B-R1/mGlu1αR and GABA_B-R2/mGlu1αR, as well as, between GABA_B-R1/GABA_B-R2. As shown in Fig. 22A, GABA_B-R1 expression was detected at the expected size of ~272 kDa indicating a possible heteromeric complex in mGlu1αR immunoprecipitate. Similarly, a
complex formation was also observed between mGlu1αR and GABA\(_B\)-R2 at the expected molecular weight of \(\sim 260\) kDa (Fig. 22B). We further extended our study to determine whether GABA\(_B\)-R1 forms heterodimers with GABA\(_B\)-R2. As expected, in Fig. 22C, the immunoprecipitate of GABA\(_B\)-R2, when probed with GABA\(_B\)-R1 antibody, displayed a band at \(\sim 250\) kDa. The GABA\(_B\)-R1/R2 dimerization is consistent with several previous studies in different brain regions (Jones, et al., 1998; Kaupmann, et al., 1998; White, et al., 1998). In conclusion, these results indicate that GABA\(_B\)-R1/mGlu1αR and GABA\(_B\)-R2/mGlu1αR dimerize in hippocampus, the extent of which is clearly enhanced in SD rats (Fig. 22A, B). However, since there is a significant decrease in GABA\(_B\)-R1/R2 heterodimerization in SD rats (Fig. 22C), our results also suggest that a dissociation of GABA\(_B\)-R1/R2 heterodimerization may lead to the formation of a heteromeric complex between GABA\(_B\)-R1/mGlu1αR and GABA\(_B\)-R2/ mGlu1αR respectively.
Figure 22. Heterodimerization between GABA_B-R1/mGlu1αR, GABA_B-R2/mGlu1αR and GABA_B-R1/R2

Co-IP analysis illustrating the heterodimerization between, GABA_B-R1/mGlu1αR (A), GABA_B-R2/mGlu1αR (B) and GABA_B-R1/R2 (C) in hippocampal tissue lysate from normally sleeping (NS) and sleep-deprived (SD) rats. As described in the Materials and Methods section, the tissue lysate was immunoprecipitated with mGlu1αR or GABA_B-R2 specific antibodies. The mGlu1αR- or GABA_B-R2-immunoprecipitate was then fractionated on a 7% SDS gel and probed with anti-GABA_B-R1/R2 or GABA_B-R1 antibodies, respectively. Note the formation of GABA_B-R1/mGlu1αR (A), GABA_B-R2/mGlu1αR (B) and GABA_B-R1/R2 heterodimers at the expected sizes of ~272, ~260 and ~250 kDa in NS and SD rats. While the complex-formation between GABA_B-R1/mGlu1αR and GABA_B-R2/mGlu1αR is clearly enhanced in SD rats, GABA_B-R1/R2 heterodimerization is reduced.
Chapter 5: Discussion

5.1 LTD in the CA1 region of the hippocampus

5.1.1 Induction

The 20 Hz (2XSI) LFS reliably induced LTD in 3-4 week old rats. The LTD lasted for the 30-40 min post-tetanus when the experiment was terminated (see Section #4.1.3). Consistent with reports in literature, the 1 Hz LFS, however, did not induce LTD in these animals (Dudek and Bear, 1993; Kemp and Bashir, 2001; Wagner and Alger, 1995). The 20 Hz LTD requires activation of mGluRs and GABA$_B$-Rs (see Section #4.2). Since these receptors are localized mainly around the synapse (Kulik, et al., 2003; Lujan, et al., 1996), it may be possible that strong stimulation (such as the 20 Hz), recruits these receptors via a spill-over of the neurotransmitter into the extra or perisynaptic space (Isaacson, 2000; Nicoll, 2004). In fact, GABA$_B$-Rs and mGluRs possess a higher affinity for GABA or glutamate (Nicoll, 2004; Nishi, et al., 2000), respectively, making them well-suited for sensing low levels of the neurotransmitter in these conditions. While a paired-pulse (PP)-LFS (900 pulses at 1 Hz, 200 ms inter-stimulus interval) induces NMDAR-LTD, increasing the stimulation-strength using a PP-LFS (900 pulses at 1 Hz, 50 ms inter-stimulus interval), selectively induces mGluR-dependent LTD (Kemp, et al., 2000). A PP-LFS (25 ms inter-stimulus interval) designed to prime GABAergic feed-back inhibition, however, induces NMDAR-LTD (Thiels, et al., 2002). These findings indicate that depending on the type of receptors LFS recruits, LTD mechanisms may vary.
5.2 Enhancement of LTD in SD rats

Sleep-deprivation for 12-72 h has been previously shown to disrupt the induction and/or maintenance of LTP in the hippocampus (Campbell, et al., 2002; Davis, et al., 2003; McDermott, et al., 2003). We report for the first time that LTD is enhanced following sleep-deprivation (Tadavarty, et al., 2009). An elevated LTD may indeed be an intrinsic adaptive mechanism to prevent the saturation of neural networks associated with prolonged wakefulness and reset excitability to within their functional range (Longordo, et al., 2009). This would ensure that the learning and memory functions of the CNS are unaffected. In this context, the development of LTD may in fact be responsible for the disruption of LTP in SD rats. Computational modeling of neuronal plasticity, as well as experimental evidence, supports the existence of a certain “sliding threshold” for LTP or LTD (Dayan and Willshaw, 1991; Huang, et al., 1992; Stanton, 1996). When neuronal activity is high, in circumstances generally leading to LTP, compensatory changes could occur at specific synapses, which suppress subsequent LTP and favor the induction of LTD. This requires LTP and LTD to functionally interact and counterbalance each other at the cellular level. In fact, this can be achieved through various means, including the convergence in mechanisms that underlie LTP or LTD. Both LTP and LTD have been shown to require the activation of NMDARs and/or mGluRs (Collingridge, et al., 2010; Malenka and Bear, 2004; Riedel, et al., 2003). The magnitude and spatial and temporal characteristics of the postsynaptic Ca$^{2+}$ signal can, indeed, determine the direction of plasticity (Yang, et al., 1999). Moreover, variations in the inhibitory tone can tilt synaptic plasticity in favor of LTP or LTD (Steele and Mauk, 1999; Stelzer, et al., 1994). Taken together, it therefore seems plausible that the sleep-deprivation
induced enhancement of LTD underlies the impairment of LTP observed in other studies. These aspects need to be studied in further detail.

5.3 **Mechanisms involved in LTD**

5.3.1 **Involvement of mGluRs and GABA\(_B\)-Rs**

The 20 Hz LTD is dependent on the activation of mGluRs and GABA\(_B\)-Rs (see Chapter 3, Section #4.2). Activation of mGluRs may independently affect LTD via modulating the expression of synaptic AMPARs. While an increased expression of AMPARs is associated with LTP, a decrease in receptor number is suggested to be important for LTD (Malinow and Malenka, 2002). Lynch et al. originally proposed that LTP could entail an increase in the number of synaptic glutamate receptors (Lynch and Baudry, 1984). The discovery of “silent synapses” (Isaac, et al., 1995; Liao, et al., 1995) provided the first electrophysiological evidence for this hypothesis. Synapses are silent postsynaptically if they express only NMDARs but lack AMPARs. Since non-synaptic AMPARs outnumber synaptic AMPARs (Shi, et al., 1999), and given that the synaptic and non-synaptic regions are only a few microns apart, a rapid delivery/-internalization of AMPARs into the synapse is possible following conditioning stimulations (Nusser, et al., 1998; Petralia, et al., 1999; Takumi, et al., 1999). Interventions that disallow membrane fusion and/-or exocytosis machinery block LTP, providing additional evidence for receptor trafficking (Lledo, et al., 1998). On the contrary, a significant loss of synaptic AMPARs through a clathrin-dependent endocytotic process might underlie LTD (Man, et al., 2000). Also, prolonged increases in
network activity induced by blocking GABA-ergic IPSCs and giving brief applications of glutamate in spinal cord, cortical cultures or hippocampal cultures, can cause AMPAR internalization (Lissin, et al., 1998; O'Brien, et al., 1998; Turrigiano, et al., 1998). Conversely, application of AMPAR antagonists for hours to days increase the receptor surface expression (Liao, et al., 1999; O'Brien, et al., 1998). The delivery of AMPARs into the synapse can further be modulated by other receptors. Exactly how this happens is unclear, but both NMDARs and group I mGluRs have been shown to trigger an internalization of AMPARs (Luscher, et al., 1999; Snyder, et al., 2001), thereby causing LTD.

Activation of mGluRs may induce LTD by modulating AMPAR function. Altering the phosphorylation state at two sites, Ser-845 (Kameyama, et al., 1998; Lee, et al., 1998) and Ser-831 (Barria, et al., 1997; Lee, et al., 2000) on the AMPAR can influence its function by modulating the single channel conductance (Derkach, et al., 1999) and/or the channel open probability [P_{\text{open}}; (Banke, et al., 2000)]. While an increase in channel conductance is implicated in LTP, the opposite seems to be true in LTD. mGluR-LTD is associated with a decrease in the phosphorylation of AMPARs. Blocking protein tyrosine phosphatase activity inhibits mGluR-LTD (Moult, et al., 2006). Therefore, activation of mGluRs can induce LTD by modulating the expression and/or the function of AMPARs.

The role of GABA_B-Rs in LTD is, however, perplexing. GABA_B-R influences NMDAR-LTD through disinhibition (Wagner and Alger, 1995). Our results from studies on IPSC amplitude and selective blockade of GABA_A-Rs rules out disinhibition as a possible mechanism. One possibility is that the site of GABA_B action is indirect, on interneurons; this
needs further investigation. In the presence of GS-39783, a positive allosteric modulator for GABA<sub>B</sub>-Rs, pEPSPs were selectively suppressed in SD rats. It is possible that sleep-deprivation induces certain alterations in the function and/or expression of GABA<sub>B</sub>-Rs. In agreement with this thinking, we found changes in the expression of GABA<sub>B</sub>-R1 and GABA<sub>B</sub>-R2 following sleep-deprivation. Exactly how this translates into LTD is unclear. Since GABA<sub>B</sub>-Rs may functionally interact with mGluRs (Hirono, et al., 2001), we studied the expression pattern of these receptors using co-localization techniques, in normally sleeping and SD rats. Our data reveal an overlapping distribution for mGlu1αRs and GABA<sub>B</sub>-Rs in the CA1 region in pyramidal/non-pyramidal cells of hippocampus, a finding that is consistent with previous observations (Kulik, et al., 2003; Lujan, et al., 1996). Since the degree of co-localization is altered during sleep-deprivation, we further hypothesized that GABA<sub>B</sub>-Rs and mGluRs might physically interact with each other, and that the enhancement in LTD in SD rats could be related to an alteration in this interaction. In line with this thinking, co-IP results revealed a complex-formation between mGlu1Rs and GABA<sub>B</sub>-R1/R2s, which was enhanced in sleep-deprivation.

Exactly how GABA<sub>B</sub>-Rs and mGluRs co-operate to affect LTD and its enhancement is not clear. The 20-Hz repetitive stimulation employed in this study, is powerful enough to not only induce homo- and hetero- synaptic depression (Sastry, et al., 1984), but also unmask the otherwise latent excitatory synaptic connections in hippocampal neurons (Miles and Wong, 1987). Stimulating afferent fibers at this rate may, therefore, be strong enough to simultaneously activate both GABA<sub>B</sub>-Rs and mGlu1αRs and provoke an interaction in a manner that is sufficient to induce and modulate LTD. Alternatively, previous studies have
shown that, in a functionally active heteromeric complex, the activation of a single protomer is sufficient to modulate its counterpart to trigger a physiological response (Kumar and Grant, 2010). Hence, in a scenario where mGlu1αRs and GABA_B-Rs form complexes, activation of one receptor may not only further stimulate the formation of a heteromeric complex, but also enhance/decrease the effect of the other. In fact, a functional cross-talk/dimerization between mGlu1αR and GABA_B-R in cerebellar parallel fiber-Purkinje cell synapses seems to lead to a synergistic enhancement in the effect of mGlu1αR (Hirono, et al., 2001). Conversely, mGlu1R antagonists seem to enhance GABAergic neurotransmission, thereby significantly reducing post-ischemic neuronal damage and epileptiform activity (Cozzi, et al., 2002). Interestingly, recent studies have shown that GABA_B-Rs and group I mGluRs modulate the expression of Ca^{2+}-permeable (CP) AMPARs in the cerebellar stellate cell synapses. Blockade of either group I mGluRs or GABA_B-Rs results in an increase of CP-AMPARs, indicating that under normal physiological conditions, mGluRs and GABA_B-Rs may co-operatively alter the expression of AMPARs (Kelly, et al., 2009). In fact, this finding strengthens our thinking that a physical interaction between mGluRs and GABA_B-Rs occurs in normally sleeping and SD rats.

GPCRs exist as homo- and hetero- dimers and in higher order as oligomers with a variety of proteins. Interestingly, this assembly is not restricted to only closely related GPCRs such as, δ- & κ-opioid receptors (Jordan and Devi, 1999), but observed even in distantly related receptors like somatostatin-SSTR5 & dopamine-D_2 receptors (Rocheville, et al., 2000; Rocheville, et al., 2000). GABA_B and mGluRs have been shown to form stable heteromeric complexes with calcium sensing receptors (Gama, et al., 2001) and scaffolding
proteins, MUPP1, Homer, Shank (Fagni, et al., 2004; Kornau, 2006). Other studies in literature indicate that GPCRs also dimerize with ionotropic glutamate and GABA receptors (Serulle, et al., 2007). While in most cases, specific domains on the carboxyl-terminal tails of GPCRs seem to be important for an interaction to take place (Cvejic and Devi, 1997; Grant, et al., 2004; White, et al., 1998), the cross-talk between GABA<sub>B</sub>-R amplification of mGlu1 receptor responses is intact even when it is co-expressed with mGlu1β receptor, a short splice variant with a much smaller carboxyl-terminal tail (Rives, et al., 2009). These data indicate that GPCR dimerization is a complex process which is unique to each receptor pair. Therefore, further studies are necessary to elucidate the type of interaction between GABA<sub>B</sub> and mGlu1αRs, and any possible alterations following sleep-deprivation. Functionally, GPCR dimerization seems to influence receptor surface expression (Marshall, et al., 2002), sensitivity of receptors to endogenous ligands (Gomes, et al., 2000; Rocheville, et al., 2000), signal transduction (Grant, et al., 2008; Somvanshi, et al., 2009; Watt, et al., 2009) and receptor internalization (Jordan, et al., 2001; Rocheville, et al., 2000). Intriguingly, although each of these aspects is known to affect the induction of LTP and LTD, the role of receptor dimerization in synaptic plasticity remains largely unexplored. Therefore, our current findings underscore the need to re-examine the classical view that activation of individual receptors alone affects short- and long-term plasticity of EPSPs and IPSCs in the CNS.

Sleep-deprivation in one form or other has become increasingly common in today’s 24X7 lifestyle. Other factors, but not limited to, age, prolonged warfare and/or pathological conditions such as, Alzheimer’s disease, sleep apnea, etc., also account for the loss of sleep. Despite this, our knowledge on how sleep-deprivation affects basal synaptic transmission and
plasticity in the CNS remains very limited. One of the major findings of the present study is that acute sleep-deprivation, for as little as 12 h can significantly enhance LTD. GABA\(_B\)Rs and mGluRs seem to co-operate differentially to affect LTD in normally sleeping and SD rats. The expression, co-localization and dimerization of GABA\(_B\)Rs and mGluRs is altered following sleep-deprivation. Modulation of LTD via GPCR dimerization is a novel concept. In our view, such alterations in the receptor function through homo- and heterodimerization may be a much more efficient means to regulate plasticity in the CNS. It may ensure that short-term alterations induced by changes in behavior (sleep or sleep-deprivation), are quickly reversed to physiological levels following periods of recovery. The participation of both inhibitory and excitatory systems in modulating LTD also emphasizes the need to study the contribution of both GABAergic and glutamatergic in synaptic plasticity, as opposed to current focus on mainly glutamatergic pathways as mechanisms for LTP and LTD of pEPSPs/EPSCs. Lastly, considering the fact that a majority of currently available drugs act on GPCRs, we believe that a careful examination of our current findings will aid in the identification of novel targets and design of better drugs to combat problems like sleep-deprivation induced amnesia.
Chapter 6: Conclusions

Based on the experiments conducted in the present study, the major findings are summarized as follows,

1. LTD in the CA1 region of the hippocampus can be reliably induced using a 20 Hz, 30 sec LFS (2XSI).

2. LTD induced in this fashion is significantly enhanced in rats following a 12 h sleep-deprivation by gentle-handling.

3. Activation of group I mGluRs, mainly mGlu1Rs and mGlu5Rs are necessary for LTD. NMDARs are however, not involved.

4. Release of Ca\(^{2+}\) from intracellular stores, but not L- or T- type VGCCs is required for LTD.

5. Activation of GABA\(_B\)-Rs but not GABA\(_A\)-Rs affects LTD in normally sleeping and SD rats. GABA\(_A\)-R mediated- IPSCs are not affected following the 20 Hz LFS. Electrophysiological results also indicated a change in GABA\(_B\)-R function in SD rats.

6. The expression of GABA\(_B\)-Rs and mGlu1Rs are altered following sleep-deprivation.
7. The co-localization between mGlu1R/GABA\textsubscript{B}-R1, mGluR/GABA\textsubscript{B}-R2 is enhanced in SD rats. There seems to a decrease in the co-localization between GABA\textsubscript{B}-R1/R2 in SD rats.

8. While mGlu1R/GABA\textsubscript{B}-R1 and mGlu1R/GABA\textsubscript{B}-R2 complex formation is enhanced in SD rats, the dimerization of GABA\textsubscript{B}-R1/R2 is decreased in SD rats.
Chapter 7: Future Directions

Results of studies pursued in this thesis work have provided valuable information on the possible effects of sleep-deprivation on LTD and receptor expression. The findings offer several avenues for further research. Some of these directions are summarized below,

1. Sleep-deprivation induced alterations in the expression and interaction of mGlu1αRs and GABA_b-Rs is a novel finding. Results from immunohistochemical studies indicate that receptor expression is altered in both pyramidal cells and interneurons. Given the fact that these cell types interact to control the overall excitability of neuronal networks, it is important to further establish electrophysiologically, as to how changes in receptor expression affect the basic synaptic transmission in pyramidal cells and interneurons, and what influence these alterations have on the LTD of pyramidal cell EPSPs. This can be achieved by, for instance, combining field recordings from CA1 pyramidal neurons with whole-cell recordings from interneurons.

2. Data from co-IP studies suggest that mGlu1αRs dimerize with GABA_b-Rs. The interaction between the receptor subtypes must be additionally confirmed through fluorescence resonance energy transfer (FRET) experiments. Following this, the mechanism of interaction, and how it is altered during sleep-deprivation must be
studied. Knowledge from these studies will unravel potential targets for combating sleep-deprivation induced deficits.

3. In our studies, the focus was on the roles of mGluRs and GABA\textsubscript{B}-Rs in LTD, mainly because LTD in normally-sleeping rats required the activation of these receptors. However, sleep-deprivation itself may bring in changes in the function and/or expression of NMDARs and GABA\textsubscript{A}-Rs, which did not influence LTD in normally-sleeping rats \textit{per se}. This possibility must to be further investigated.

4. Studies in literature have well established the role of \([\text{Ca}^{2+}]_i\) in LTD. The 20 Hz- LTD is sensitive to the release of \(\text{Ca}^{2+}\) from intracellular stores. This is not surprising because both mGluRs and GABA\textsubscript{B}-Rs are GPCRs and \(\text{Ca}^{2+}\) acts as an important second messenger to both. Exactly how a rise in \([\text{Ca}^{2+}]_i\) leads to LTD remains unclear. In literature, evidence indicates a significant role for calcium-binding proteins, protein kinases and phosphatases in LTD. These mechanisms must be further investigated in both normally-sleeping and sleep-deprived rats.

5. We studied the effects of a 12 h sleep-deprivation on LTD. This we thought was a realistic representation of today’s lifestyle. However, in certain pathological conditions, such as sleep apnea or in soldiers during war time, longer duration of sleep-deprivation is the most likely scenario. After carefully selecting an appropriate
protocol, sleep-deprivation must be extended and its effect on synaptic transmission and LTD tested.

6. Our studies provide a remarkable overview of how even acute sleep-deprivation can influence synaptic transmission in the hippocampus. Whether these changes are reversible is currently unknown. This aspect can be tested by allowing a period of sleep recovery following sleep-deprivation, and subsequently examining its influence on LTD and receptor expression.
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