THE EFFECTS OF SLEEP DEPRIVATION ON 5-HT1A AND 5-HT2A RECEPTOR EXPRESSION IN THE HIPPOCAMPUS

by

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Abstract

Sleep deprivation (SD) is becoming increasingly prevalent in our busy society. Teenagers, shift workers, and hard working professionals, among others, are all victims of this epidemic. The consequences of SD are dire. In the short term, they include cognitive deficits, impaired memory consolidation, experiencing negative mood states, and loss of homeostatic control. In a chronic context, SD is often associated with severe psychiatric disorders such as depression, psychosis and anxiety. Recent studies have found that the expression and heterodimerization of metabotropic glutamate receptors 1α (mGluR1α) and γ-aminobutyric acid B receptors (GABABR) were altered in the rat hippocampus following 12 hours of SD. Whether SD induces similar changes in other G protein-coupled receptors is unknown. 5-hydroxytryptamine (5-HT) regulates sleep, emotion, mood, and memory. Its receptors, particularly 5-HT1A and 5-HT2A, have been linked to multiple psychiatric disorders, as commonly used antidepressants, antipsychotics and anxiolytics theoretically exert their actions through these receptors. In this study, western blot and peroxidise immunohistochemistry revealed that 5-HT1A expression was increased in rat hippocampus following 12 hours of SD, specifically in the CA1 pyramidal layer. This up-regulation persisted despite 24 hours of recovery sleep, and recovered partially after 48 hours. 5-HT2A expression in whole hippocampal lysate was detected to be unchanged by western blot following 12 hours of SD, but immunohistochemistry revealed increased expression in the CA1 pyramidal layer, suggesting that 5-HT2A may be down-regulated in other regions of the hippocampus. Increased 5-HT2A expression in the CA1 pyramidal layer recovered back to control levels within 24 hours of recovery. The action of the agonist 5-HT on the field excitatory post-synaptic potentials (fEPSPs) of CA1 neurons was also investigated in hippocampal slices. 5-HT dose-dependently suppressed fEPSPs – an effect which was enhanced by 12 hours of SD, but the enhancement was reverted within 24 hours of sleep recovery. Heterodimerization between either 5-HT1A or 5-HT2A and either of mGluR1α, GABABR1 or GABABR2 was investigated by co-immunoprecipitation, but no evidence of heterodimers was found. These results suggest increased 5-HT transmission in the
hippocampus following SD, which could potentially lead to the development of psychiatric disorders and has consequences for drug dosing.
Preface

This thesis contains original, unpublished work by collaborating members of Dr. B. Sastry’s and Dr. U. Kumar’s labs. I conducted the western blot experiments of 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} in chapters 3.1 and 3.2, and all the co-immunoprecipitation experiments in chapters 3.3 and 3.4. A. Kwok conducted the peroxidase immunohistochemistry of 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} in chapters 3.1 and 3.2. In chapter 3.5, J. Hwang and Dr. H. Azizi conducted the electrophysiology measurements for control and sleep deprived animals; I conducted the electrophysiology measurements for 24-hour recovery and 48-hour recovery animals. Sleep deprivation of animals was a joint effort by me, A. Kwok, J. Hwang, Dr. H. Azizi, N. Kang, and A. Nguyen.

I contributed to the design and analysis of data for all of the experiments. Dr. B. Sastry and Dr. U. Kumar contributed significantly to the design of the experiments. Dr. R. Somvanshi contributed significantly to the design and analysis of the western blot and co-immunoprecipitation experiments.

The animal treatment protocols were approved by the UBC Animal Care Committee (#A10-0381 and #A15-0061).
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<th>Description</th>
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<tbody>
<tr>
<td>SD</td>
<td>Sleep deprivation</td>
</tr>
<tr>
<td>mGluR(1α)</td>
<td>Metabotropic glutamate receptor (1α)</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt;R(1,2)</td>
<td>γ-aminobutyric acid B receptor (1,2)</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>fEPSP</td>
<td>Field excitatory post-synaptic potential</td>
</tr>
<tr>
<td>CoIP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>NREM</td>
<td>Non-rapid eye movement</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movement</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalography</td>
</tr>
<tr>
<td>SWS</td>
<td>Slow-wave sleep</td>
</tr>
<tr>
<td>CA(1,3)</td>
<td>Cornus Ammonis (1,3)</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>Sub</td>
<td>Subiculum</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>ATN</td>
<td>Anterior thalamic nuclei</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-coupled inwardly-rectifying potassium channel</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>8-hydroxydipropyl-aminotetralin</td>
</tr>
<tr>
<td>LSD</td>
<td>Lysergic acid diethylamide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
</tr>
<tr>
<td>TBS(-T)</td>
<td>Tris-buffered saline (with Tween)</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitating</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal horse serum</td>
</tr>
<tr>
<td>SO</td>
<td>Stratum oriens</td>
</tr>
<tr>
<td>SP</td>
<td>Stratum pyramidale</td>
</tr>
<tr>
<td>SR</td>
<td>Stratum radiatum</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>25I-NBOMe</td>
<td>N-(2-methoxybenzyl)-2,5-dimethoxy-4-iodophenethylamine</td>
</tr>
</tbody>
</table>
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Chapter 1: Introduction

1.1 Sleep

Sleep is widely considered to be essential for the normal functioning of many animals, including humans. This intriguing state is characterized by immobility and severely reduced responsiveness to outside stimuli, but it is also readily reversible (Siegel 2005). During sleep, the brain displays distinct cycles of recurring electrical activity, represented as different stages.

1.1.1 Sleep Stages

Sleep is classically divided into the four stages of non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. Sleep begins with NREM stage 1 sleep which is considered the lightest sleep. During this stage the sleeper can be easily awakened and brain activity as measured by electroencephalography (EEG) is marked by a transition from alpha waves (8 to 12 Hz) to mixed frequency signals (Colten and Altevogt 2006; Rodenbeck et al. 2006). Progressing to NREM stage 2 sleep, the sleeper becomes less responsive to environmental stimuli, and sleep spindles (low amplitude activity bursts, 11 to 16 Hz) and K-complexes start to make an appearance on the EEG. Sleep spindle density was significantly increased in both rats and humans subjected to an extensive learning exercise prior to sleeping, suggesting that sleep spindles may play a role in memory consolidation (Eschenko et al. 2006; Gais et al. 2002). K complexes appear as relatively large waves marked by a slight negative voltage deflection, followed by a much larger positive deflection, and ending in an almost equally large negative deflection (Roth et al. 1956). They often occur when the sleeper is stimulated by the environment, for example, by light touching on the skin, and are thought to be involved in suppressing arousal (Cash et al. 2009; Roth et al. 1956).

NREM stage 3 and stage 4 sleep are often referred together as slow-wave sleep (SWS), during which the sleeper is even less responsive to environmental stimuli than the
previous two stages. The EEG consists of slow, delta wave activity (0.5 to 3 Hz) with sleep spindles also being observed (Offermanns and Rosenthal 2008). SWS is thought to be the most recuperative sleep and primarily responsible for alleviating the feelings of sleepiness and the urges to fall asleep during the day (Waterhouse et al. 2012). The final stage, REM sleep, is characterized by increases in heart rate and blood pressure, loss of muscle tone, theta (4 to 7 Hz) and alpha activity on the EEG, sexual arousal, and dreaming (Colten and Altevogt 2006). REM sleep is also referred to as paradoxical sleep because the EEG patterns are very similar to those observed during a state of wakefulness, but the individual is very difficult to rouse. Over the course of one night’s sleep, the average sleeper cycles through the NREM stages and REM sequentially 5 times, with each cycle lasting approximately 90 minutes, and the proportion of REM sleep in each cycle progressively increasing later into the night and towards awakening (Diekelmann and Born 2010).

1.1.2 Sleep Deprivation

In order to elucidate the functional role of sleep, many studies have investigated the effects of sleep deprivation on both animals and humans. Sleep deprivation leads to an array of undesirable outcomes in humans. These outcomes can be broadly categorized as cognitive deficits, negative mood states, or loss of homeostatic control. Cognitive deficits generally include loss of attention span, inefficient task performance, slow response time, inability to use divergent thinking to solve problems, and deficits in memory consolidation (Diekelmann and Born 2010; Goel et al. 2009; Stickgold and Walker 2005). Negative mood states include feelings of fatigue, unhappiness, lethargy, anxiety, and dissociation (Bianchi 2014). In terms of loss of homeostatic control, sleep deprivation has been found to decrease heart rate, lower body temperature, disrupt glucose metabolism, and increase appetite (Vaara et al. 2008; Knutson 2007). Similarly, in rats, extended sleep deprivation results in the inability to re-perform tasks (which is correlated to a deficit in memory consolidation), the inability to regulate body temperature, overeating, skin lesions, and ultimately death (McCoy and Strecker 2011; Siegel 2005).
One of the most studied consequences of sleep deprivation is its effect on memory consolidation. It is generally accepted that memories begin as labile traces which then must be consolidated into a more stable form for recall at a later time (McGaugh 2000). Any disruption in the consolidation process can cause the memory trace to be lost. For example, the learning of information can be easily disrupted by presenting the learner with additional, new, unrelated information to learn immediately after learning the original information, causing the original information to be forgotten (McGaugh 2000). The consolidation process is not well understood, but appears to depend upon the alteration of neuronal synapses and probably involves the participation of several brain regions, including the hippocampus, amygdala, and the neocortex, working in conjunction (McCoy and Strecker 2011). Observations of neuronal firing patterns support the idea of a consolidation process occurring during sleep. Place cells found in the hippocampus which fired together when a rat explored a certain location also fired concurrently during sleep afterward (Pavlides and Winson 1989). Furthermore, the temporal order of hippocampal pyramidal cell firing during wakefulness is also retained and replayed during sleep (Nadasdy et al. 1999; Skaggs and McNaughton 1996). Such reactivation patterns seem to be consistent with a consolidation process and occur for shorter periods of time in SWS sleep compared to REM sleep (McCoy and Strecker 2011; Siapas and Wilson 1998; Louie and Wilson 2001). Therefore, it has been suggested that the initial consolidation process begins during SWS while later stages of consolidation take place during REM sleep (McCoy and Strecker 2011; Giuditta et al. 1995).

Sleep deprivation is known to be a common comorbidity of psychiatric disorders (Colten and Altevogt 2006). A National Institute of Mental Health Epidemiologic Catchment Area study revealed that 40% of people suffering from insomnia also suffered from a psychiatric disorder, while normally sleeping individuals had a 16% incidence of being diagnosed (Ford 1989). Sleep deprivation leads to feelings of negative mood states, and if these mood states are exaggerated and extended for a long period of time, they may manifest themselves as psychological disorders. Some of the most common correlated psychiatric disorders include depression, general anxiety, and psychosis. This is not surprising given that the prominent structures of the limbic system responsible for
regulating mood and emotion, such as the hippocampus and the amygdala, are also key in modulating sleep. Whether sleep deprivation is the root of the problem or merely a symptom of another underlying disease is unknown, but this correlation points to shared neural architecture between sleep and proper cognitive functioning.

1.2 Rat Hippocampus

Located in the medial temporal lobe of the brain, the rat hippocampus lies below the neocortex and above the thalamus. It is a highly organized structure often described as two interlocking “C’s”. One of the “C’s” is the Cornus Ammonis (CA), also known as the hippocampus proper, which is divided primarily into the CA1 and CA3 regions. It has been argued that a region distinct from those two regions exists, called the CA2 region (Amaral and Witter 1989). The other C forms the dentate gyrus. Closely connected to the hippocampus and often regarded as part of the hippocampal formation are the subiculum and the entorhinal cortex (Witter et al. 2000).

Figure 1. Cross-section of a rat hippocampus. The illustration shows the different regions – dentate gyrus (DG), Cornus Ammonis 3 (CA3) and 1 (CA1), subiculum (Sub),
entorhinal cortex (EC) – and the neuronal connections between the different regions. It was originally drawn by Santiago Ramón y Cajal and is currently out of copyright.

### 1.2.1 Function

The major role of the hippocampus is considered to be the formation of memories. Two types of memory are recognized – declarative and non-declarative (Tulving 1985). Declarative memory describes memory which can be consciously recalled such as facts, knowledge, or events. Non-declarative memory, also known as procedural memory, is the ability to remember how to perform a skill. In 1957, Scoville and Milner observed that patients who had suffered bilateral damage to the hippocampus displayed anterograde amnesia (Scoville and Milner 1957). They were unable to form any new long-term memories, but it was clear that they retained previous declarative memory. Also, there was no difference observed in the ability to learn a motor skill. In fact, the types of learning classified as non-declarative such as associative learning, perceptual learning, and habits were all unaffected (Good 2002). It was, therefore, hypothesized that the hippocampus was involved in forming new declarative memory, but plays a limited role in forming non-declarative memory. More recent studies investigating hippocampus activation through neuroimaging have supported this hypothesis (Maguire 2001; Mayes and Montaldi 2001; Schacter and Wagner 1999).

The hippocampus also appears to be intimately involved in spatial memory. When an animal, such as a rat, explores a certain part of its environment, particular “place cells” in the hippocampus become active (O'Keefe and Dostrovsky 1971; Moser et al. 2008). These place cells fire every time the animal finds itself in the same location, given that the environmental cues are the same (Thompson and Best 1990), but are relatively silent outside of this location. Pyramidal cells in the Cornus Ammonis regions, granule cells in the dentate gyrus, and interneurons all participate in place cell firing (Moser et al. 2008). However, the proximity of neurons to each other in the hippocampus are uncorrelated to the place cells designated to adjacent locations in physical space (Tanila et al. 1997).
In addition to serving as a locus for memory formation, the hippocampus has been implicated in severe psychiatric disorders such as schizophrenia and depression. Namely, significant atrophy of the hippocampus has been observed (Kempton et al. 2011; Wright et al. 2000). Due to its extensive neuronal connections with other brain regions, the hippocampus is likely to play a role in regulating overall cognitive and emotional functioning as well.

1.2.2 Anatomical Organization

The dentate gyrus consists of three distinct layers. Adjacent to the hippocampal fissure lies the molecular layer, approximately 250μm thick, which houses the dendrites of granule cells, afferents from the entorhinal cortex, and a sparse array of interneurons. Deep to the molecular layer lies the granule layer. This layer is the principal cell layer of the dentate gyrus and consists of granule cells packed tightly together. Enclosed in the “U”-shaped granule layer is the polymorphic layer. The principal cell type residing in the polymorphic layer is the mossy cells (Amaral et al. 2007).

The hippocampus proper can also be separated into layers in a depth-wise manner. The deepest layer is the stratum alveus, followed by the stratum oriens just superficial to the alveus. Several types of inhibitory interneurons, including basket cells and horizontal trilaminar cells, are found in the stratum oriens. Also residing in the stratum oriens are the basal dendrites of pyramidal cells which synapse onto fibers from the contralateral hippocampus. Superficial to the stratum oriens lies the stratum pyramidale, which constitutes the main cell body layer. Densely packed excitatory pyramidal neurons enable this layer to be distinctly more visible than the surrounding layers. In the CA3 region, an additional stratum, stratum lucidum, exists superficial to the stratum pyramidale containing the mossy fibers projecting from the granule cells of the dentate gyrus. Otherwise, the next superficial layer is the stratum radiatum, which encompasses the axons projecting from the CA3 pyramidal neurons to CA1 (also referred to as Schaffer collaterals), as well as several types of interneurons and commissural nerve fibers. Lastly, the most superficial layers of the CA1-CA3 region are the stratum lacunosum and stratum
moleculare. Its chief residents are the afferent fibers originating from the entorhinal cortex.

1.2.3 Neuronal Connections

The trisynaptic loop in the hippocampus is a description of the mostly unidirectional flow of neuronal transmission from one hippocampal region to another. It begins in the entorhinal cortex which project and sends signals to excite granule cells in the dentate gyrus. The granule cells then relay these signals to the CA3 pyramidal cells via collaterals known as mossy fibers, named from their appearance (Blackstad et al. 1970). CA3 pyramidal neurons communicate with CA1 pyramidal neurons through the Schaffer collaterals. Then, from the axons of the CA1 neurons, signals are sent to both the subiculum and back to the entorhinal cortex (Meibach and Siegel 1977). The entorhinal cortex also forms direct connections to the dentate gyrus, CA3 and CA1 regions. Along each stage of the loop, various interneurons can modulate and synchronize excitatory transmission. Among the various types of interneurons are basket cells and chandelier cells. Basket cells appear to form a basket around the soma of principal cells with their highly branched axons. They communicate reciprocally with the target cell, often to help dampen excitatory responses (Freund and Katona 2007). Chandelier cells target the axon initial segments of pyramidal cells, and typically span across large areas of the hippocampus, forming synapses with over 1000 pyramidal neurons. Based on this observation, they are thought to be responsible for synchronizing the firing of large numbers of pyramidal cells (Li et al. 1992).

Outside of its own connections, the hippocampus is also very well connected to other brain regions. As part of Papez’s circuit for emotional processing, it is connected to the anterior thalamic nuclei (ATN), the mammillary bodies, the fornix, the cingulate cortex, the septal nuclei, and the amygdala (Bird and Burgess 2008). Through the subiculum and the entorhinal cortex, it is connected to the perirhinal cortex, which facilitates visual processing of the environment. Other connections to the hippocampus found throughout neocortical regions suggest that the hippocampus is capable of consolidating information.
from vastly different centres including the superior temporal gyrus (auditory processing), the insular cortex (interoceptive awareness, emotion, and consciousness), the orbitofrontal cortex (decision making), and the retrosplenial cortex (navigation) (Lavenex and Amaral 2000). Furthermore, innervations from regions known to control wakefulness and sleep such as the locus coeruleus and the dorsal raphe nucleus in the brainstem as well as the hypothalamus indicate a role for the hippocampus in modulating sleep (Born and Fehm 1998; Kayama and Koyama 1998).

1.3 5-hydroxytryptamine

5-hydroxytryptamine (5-HT, also referred to as serotonin) is one of the monoamine neurotransmitters in the central nervous system. It is synthesized in neurons using L-tryptophan as a precursor. When released from a nerve terminal, it acts on various 5-HT receptors and its actions are terminated via reuptake back into the presynaptic neuron. The serotonin transporter (SERT) is responsible for this reuptake process, and it is also the target of many existing drugs (Hyttel 1994), including the popular selective serotonin reuptake inhibitors (SSRI) citalopram (Celexa) and fluoxetine (Prozac).

1.3.1 5-HT Pathways

The raphe nuclei, located in the medial brainstem, constitutes the source of the majority of 5-HT released in the brain (Conrad et al. 1974). It projects serotonergic neurons to almost all pertinent brain regions, including the ventral tegmental area (reward system, motivation, and addiction), periaqueductal grey (pain and defensive behaviour), hypothalamus (sleep and circadian rhythm), cingulum bundle (emotion), thalamus (signal relay and sleep), the hippocampus, and the spinal cord (pain) (Conrad et al. 1974; Suzuki et al. 2004). In the hippocampus, 5-HT exerts direct effects on principal pyramidal cells as well as GABAergic interneurons in the dentate gyrus, CA1 and CA3 regions (Schmitz et al. 1998; Gulyás et al. 1999), but with a multitude of receptor targets, its precise functional role is not well understood. 5-HT is believed to participate in the generation of theta oscillations (Assaf and Miller 1978), which occur in rats during movement,
exploratory behaviour and REM sleep, leading to associations with motor behaviour, arousal, and memory (Hasselmo 2005). In particular, spatial memory appears to be affected (Richter-Levin et al. 1994; Richter-Levin et al. 1993). Moreover, total sleep deprivation was found to increase extracellular hippocampal 5-HT concentrations, suggesting the existence of a prominent relationship between 5-HT and sleep (Lopez-Rodriguez et al. 2003).

1.3.2 5-HT and Psychiatric Disorders

The classes of drugs known as antidepressants, anxiolytics, and antipsychotics currently available in the market today all attempt to target 5-HT transmission. In general, antidepressants bind to 5-HT reuptake proteins or monoamine oxidase enzymes to prevent the clearance of 5-HT from the synaptic cleft, thus it would appear that simply increasing 5-HT levels will alleviate symptoms of depression (Goodman et al. 2006). However, antidepressants typically have slow onset and are ineffective in a large number of patients, casting significant doubt on their mechanism of action and the contribution of 5-HT to depression (Artigas et al. 1996; Maes et al. 2008; Sanacora et al. 2012).

Anxiolytics are thought to function via a similar mechanism, as some antidepressants such as SSRIs are used for their anxiolytic effects. 5-HT1A receptor agonists, such as buspirone, are also used as anxiolytics, again pointing to the up-regulation of 5-HT transmission as a therapeutic pathway. Modern atypical antipsychotics have a high affinity to the 5-HT2A receptor and act as antagonists, suggesting that psychotic behaviour may be a result of hyper-activation of specific 5-HT receptors (Horacek et al. 2006).

1.4 G Protein-Coupled Receptors

G protein-coupled receptors (GPCR) are a family of functionally diverse membrane proteins responsible for signal transduction from the extracellular environment to the interior of the cell. They feature seven transmembrane segments, and are coupled to several different classes of heterotrimeric guanine nucleotide-binding protein (G protein) units inside cells. $G_{\alpha}$ activates adenylate cyclase, which converts adenosine triphosphate
(ATP) into cyclic adenosine monophosphate (cAMP). Generally considered to be excitatory, cAMP activates cAMP-dependent protein kinases which, in turn, modulate the opening of a channel elsewhere in the cell. $G_i$ inhibits adenylate cyclase, which prevents the conversion of ATP to cAMP. This decreases the activity of cAMP-dependent protein kinases, leading to overall inhibitory effects. $G_q$ initiates the activation of phospholipase C (PLC). PLC converts phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to diacyl glycerol (DAG), which activates protein kinase C (PKC), and inositol triphosphate (IP$_3$), which binds to specific IP$_3$ receptors to enable the opening of calcium channels, thus subsequently causing an increase in intracellular calcium concentration (Mikoshiba 2007).

The expression levels of GPCRs are often dynamically regulated in the physiological environment. They may be subjected to different forms of desensitization, leading to receptor endocytosis (Kelly et al. 2008; Krupnick and Benovic 1998). Certain disease states, such as multiple sclerosis, Parkinson’s disease, or obesity, and stress are known to permanently alter GPCR expression (Geurts 2003; Kaneda et al. 2005; Johnson and Kenny 2010; Harvey et al. 2003). In addition, recent work in our laboratory revealed that, after 12 hours of total sleep deprivation, the metabotropic glutamate receptor 1$\alpha$ (mGluR1$\alpha$) and the $\gamma$-aminobutyric acid B receptor (GABABR) subunit 1 were both significantly up-regulated in the rat hippocampus (Tadavarty et al. 2011).

While GPCRs are often referred to as single monomeric units, they are also known to participate extensively in homodimerization and heterodimerization (Milligan 2009; Terrillon and Bouvier 2004). For some receptors, such as the GABABRs, heterodimerization is necessary for the receptor to be transported to the cell surface (Margeta-Mitrovic et al. 2000). In other instances, dimerization appears to be a regulatory modification and seems to be able to modify agonist binding affinities (Rocheville et al. 2000; Jordan and Levi 1999). Nevertheless, physiological stressors are able to alter GPCR dimerization patterns. After 12 hours of sleep deprivation, mGluR1$\alpha$-GABAB$\alpha$R1 and mGluR1$\alpha$-GABAB$\alpha$R2 heterodimerization was increased in rat hippocampus, but GABAB$\beta$R1-GABAB$\beta$R2 heterodimerization was decreased (Tadavarty et al. 2011). Whether the agonist effects at these dimerized receptors also change has yet to be
determined, but given that protein structure often specifies function, potential changes are a likely possibility.

1.4.1 5-HT Receptors

At least 14 different subtypes of 5-HT receptors exist, all of which are classified as GPCRs, except 5-HT3, which is a cation-selective (Na+, K+) ligand-gated ion channel (Berumen et al. 2012). Almost every 5-HT receptor subtype can be found in the hippocampus. However, the most abundant and well-studied is the 5-HT1A receptor (Burnet et al. 1995). 5-HT1A receptors are located post-synaptically on pyramidal neurons in the CA1 and CA3 regions, as well as post-synaptically on granule cells in the dentate gyrus (Hannon and Hoyer 2008). They also demonstrate pre-synaptic occupancy to a lesser degree, presumably in a regulatory feedback role (Muchimapura et al. 2003). 5-HT1A receptors are coupled to the Gi protein and therefore exert an inhibitory effect on neuronal excitability. Inwardly-rectifying potassium (GIRK) channels are activated while calcium channels are inactivated, leading to hyperpolarization of the affected neuron (Polter and Li 2010). In terms of function, 5-HT1A receptors have been implicated in anxiety, depression, and memory. 5-HT1A knockout mice demonstrate more anxiety-related behaviour during exploratory tasks, suggesting activation of the receptor has an anxiolytic effect (Gardier 2009; Ramboz et al. 1998). Anxiolytic drugs such as buspirone which function as 5-HT1A agonists support this notion. The 5-HT1A agonists 8-hydroxydipropyl-aminotetralin (8-OH-DPAT) and buspirone are also effective in increasing activity in the forced swim test, a classic test to assess the degree of depression experienced by an animal (Kostowski et al. 1992). Greater activity is correlated to a less depressive state. The antidepressant effects of these agonists are blocked by 5-HT1A antagonists (Detke et al. 1995). Furthermore, brain imaging studies have revealed that 5-HT1A is reduced in the hippocampus, among other brain regions, of depressed patients, while mutations in genes causing alterations in 5-HT1A expression has been linked to both susceptibility to depression and responsiveness to antidepressant treatment (Savitz et al. 2009). Finally, 5-HT1A has been linked to fear-conditioned learning as well as spatial memory (Ogren et al. 2008). The administration of 5-HT1A agonists to animals appears to
impede learning while antagonists appear to be facilitative, likely via influence on cholinergic and glutamatergic neurons (Rowan et al. 1990; Madjid et al. 2006).

The 5-HT$_{2A}$ receptor is the primary excitatory 5-HT receptor in the hippocampus. 5-HT$_{2A}$ is coupled to G$_q$, which activates PLC, resulting in an influx of calcium ions to the cytosol from the endoplasmic reticulum. Similar to 5-HT$_{1A}$, 5-HT$_{2A}$ receptors are found post-synaptically on the pyramidal neurons of the CA1 and CA3 regions, and on the granule cells of the dentate gyrus (Berumen et al. 2012; Li et al. 2004). These receptors have also been linked to anxiety, depression, and memory, but the majority of studies identify 5-HT$_{2A}$ to be principally associated with psychosis. Psychedelic drugs such as lysergic acid diethylamide (LSD) exert their effect through 5-HT$_{2A}$ receptors (Moreno et al. 2011). Pimavanserin, an inverse agonist of 5-HT$_{2A}$, has been found to reverse psychotic behaviour in Alzheimer’s disease mice models (Price et al. 2012). The 5-HT$_{2A}$ antagonistic properties of the majority of current atypical antipsychotics also suggest that inhibition of this receptor can reduce psychotic symptoms (Horacek et al. 2006).

### 1.4.2 Glutamate and Metabotropic Glutamate Receptors (mGluRs)

Glutamate acts as a major excitatory neurotransmitter in the central nervous system, where in excess, it may cause excitotoxicity, leading to cell death and neurological disorders such as epilepsy (Gagliardi 2000, Meldrum et al. 1999). In the CA3-CA1 Schaffer collaterals of the hippocampus, glutamate is released from the pre-synaptic terminal of CA3 axons into the synaptic cleft, and exerts its effects on an array of glutamate receptors on the post-synaptic apical dendrites of CA1 pyramidal neurons. Rapid depolarization of the post-synaptic neuron is mediated chiefly by $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), which are ligand-gated ion channels specific for sodium and potassium (Platt 2007). They are responsible for giving rise to the excitatory post-synaptic potentials measured in the hippocampal CA1 region in electrophysiology studies. Another type of glutamate receptor, the N-methyl-D-aspartate receptor (NMDAR), is a voltage-dependent calcium-permeable cation channel important in mediating synaptic plasticity (Li and Tsien 2009).
Glutamate also acts on a class of GPCRs known as metabotropic glutamate receptors (mGluRs). Eight subtypes of mGluRs exist, which can be classified into three groups (Schoepp et al. 1999). Group I mGluRs are coupled to Gq, mediate primarily excitatory glutamate transmission, and are mostly located post-synaptically; Group II and III mGluRs are coupled to Gi, mediate primarily inhibitory glutamate transmission, and are represented both pre-synaptically and post-synaptically (Swanson et al. 2005). mGluR1 is part of Group I mGluRs, and thus play an excitatory role in glutamergic transmission by affecting Na+, K+, and Ca2+ conductance (Chu and Hablitz 2000; Miller et al. 1996). In the hippocampus, mGluR1 is abundant in great density in the granule layer of the dentate gyrus and the pyramidal cell layer of the CA3 region (Fotuhi et al. 1994). In the CA1 region, expression is less intense in the pyramidal cells (but nevertheless present), and can also be found scattered across the stratum oriens (Fotuhi et al. 1994). mGluR1 receptors appear to contribute substantially to the regulation of other glutamate receptors, namely the NMDARs for calcium entry into the cell, as well as synaptic plasticity (Skeberdis et al. 2001; Neyman and Manahan-Vaughan 2008).

1.4.3 GABA and GABAB receptors

GABA is the primary inhibitory neurotransmitter in the central nervous system. In the hippocampus, it is released by GABAergic interneurons to modulate transmission in the tri-synaptic loop (Freund and Gulyás 1997). GABA chiefly acts on receptor targets on pyramidal neurons, but some receptors are also found near the soma of interneurons located in the stratum radiatum (Pettit and Augustine 2000). Two types of GABA receptors exist – the GABA_A receptor, which is a ligand-gated Cl⁻ channel, and the GABA_B receptor (GABA_BR), which is a metabotropic GPCR.

As mentioned before, GABA_BRs actually exist as dimers of its two subunits, GABA_BR1 and GABA_BR2, on the cell surface. These receptors are ubiquitous in the hippocampus, existing at the post-synaptic membrane, the pre-synaptic membrane, as well as extra-synaptically, on both glutamatergic pyramidal neurons and GABAergic interneurons.
(Kulik et al. 2003). They mediate inhibition of the post-synaptic neuron by activating GIRK channels and by coupling to Gi to decrease calcium conductance (Misgeld et al. 1995; Scholz and Miller 1991). Strong GABA stimulation is required to activate GABABRs, presumably via GABA release from multiple interneurons and for GABA to spread to nearby areas, thus it has been hypothesized that GABABRs play a role in theta rhythm generation in the hippocampus (Scanziani 2000).

1.5 Study Objectives

Sleep deprivation (SD) is slowly becoming an epidemic in the modern society. Teenagers, shift workers, and busy professionals alike are suffering from a trend which has consequences we do not fully understand. It has been observed that, in the short term, SD can impair memory consolidation, induce cognitive deficits, cause negative mood states to develop, and disrupt homeostatic processes. Long term SD is associated with severe psychiatric disorders such as anxiety disorders, depression, and psychosis. The underlying cellular mechanisms responsible for these changes are largely unknown. Recently, mGluR1 and GABABR expression and heterodimerization have been shown to be altered following 12 hours of sleep deprivation, raising the possibility that other GPCRs may also change with SD.

In this study, we examined the effect of 12 hours of SD on the expression profiles of two relevant GPCRs in the rat hippocampus, the 5-HT1A and 5-HT2A receptors. 5-HT is an important neurotransmitter in the central nervous system that has been implicated in memory and severe psychiatric disorders. These particular receptors were chosen because they are the most widespread and well-studied out of all the 5-HT receptors in the hippocampus. 5-HT1A is expressed strongly in the pyramidal cells of the CA1 region and plays a role in memory, anxiety, and depression. 5-HT2A also displays significant expression in CA1 pyramidal cells and appears to have antipsychotic properties.

We also investigated whether 5-HT1A and 5-HT2A would form heterodimers with two of the more abundantly expressed GPCRs in the rat hippocampus, the mGluR1α and
GABAB receptor subunits, and whether heterodimerization patterns would change with SD. mGluR1α and GABABRs have previously been demonstrated to form heterodimers with each other (Tadavarty et al. 2011), and GABABRs naturally function as a heterodimer, suggesting an existing innate propensity to form heterodimers. In addition, the 5-HT receptors, mGluR1α, and GABABRs are all co-expressed in close proximity and in great abundance in the pyramidal neurons of the hippocampus, thus it is plausible that if an interaction is favourable, two receptors may interact to form a heterodimer. To a lesser degree, these receptors are all co-expressed in some interneurons as well (Pettit and Augustine 2000; Klempin et al. 2010).

Finally, because we anticipated changes in 5-HT receptor expression and/or dimerization following SD, we studied the effect of applying the endogenous agonist 5-HT on CA1 field excitatory post-synaptic potentials (fEPSPs) in hippocampal slices of normally sleeping and SD rats. Any changes to receptor expression and dimerization following SD can be compared and correlated to any changes in 5-HT agonist effects following SD. Alterations in 5-HT activity has implications for the development of psychiatric diseases affected by 5-HT transmission, such as depression, anxiety, and psychosis.
Chapter 2: Materials and Methods

2.1 Animals

All the animals used in this study were male Wistar rats, aged 21 to 28 days. They were ordered from Charles River through Animal Care Services at the University of British Columbia (UBC). Animals were delivered to the animal care facility at UBC hospital, where they were housed for at least one day up to a maximum of seven days before their use in an experiment. The housing of the animal for at least one day prior to treatment ensured that the effects we observed were not due to stress during transport. The animal care facility was responsible for all the general animal husbandry responsibilities, including caging, feeding, temperature control, and lighting control. To the best of our knowledge, all the animals had unlimited access to food and water. All experiments were conducted in accordance with the animal care protocols approved by UBC Animal Care Services (protocol no. A10-0381 and A15-0061).

2.2 Sleep Deprivation and Recovery

Sleep deprivation was performed in our laboratory. Rats were placed in a new cage and transported from the UBC animal care facility to our laboratory at the beginning of the sleep deprivation period. During any given weekday, the sleep deprivation period lasted from 7:00AM in the morning to 7:00PM in the evening, corresponding to 12 continuous hours through the light cycle. The animals were given unlimited access to food and water during this time. Sleep deprivation was achieved by experimenter observation, which was the method employed by Tadavarty et al. when they demonstrated GPCR changes after sleep deprivation. They have also shown that this method does not significantly increase corticosterone levels in sleep deprived rats (Tadavarty et al. 2009). As soon as the animal closed its eyes, or assumed a sleeping position, as determined by the experimenter, he or she would gently poke the animal with the blunt tip of a brush or gently rattle the cage to ensure the animal started moving from its position. At the end of the 12-hour period, animals were either sacrificed or left in the cage undisturbed for an additional 24 or 48
hours with ample food and water to study sleep recovery effects. After the additional 24 or 48 hours, recovery animals were sacrificed in the same manner as sleep deprived animals. Normally sleeping control animals were sacrificed at around the same time of day as sleep deprived and recovery animals.

2.3 Hippocampus Harvesting for Western Blot and Co-immunoprecipitation Experiments

To preserve the expression levels and dimerization patterns of proteins in the hippocampus after each treatment, the hippocampus must be harvested quickly and efficiently with minimal exposure to protein degradation opportunities. The goal of the following procedure was to extract the hippocampus as quickly as possible so that it can be frozen at -50°C having spent the minimal amount of time exposed to room temperature without blood supply. Controls or after treatment (sleep deprivation or recovery), animals were anesthetized with halothane. They were placed into a large glass chamber, approximately 3mL of liquid halothane was dropped into the chamber and the chamber was sealed. Animals were observed carefully during anesthetization to ensure they reached an appropriately sufficient anesthetized state. Specifically, the animal should still be taking slow, heavy breaths, it should be immobile, and the eyes should be open and non-blinking, indicating the loss of the eyelid reflex. The goal of choosing this particular anesthetic plane was to minimize cell death via hypoxia during anesthetic administration but also achieve sufficient anesthesia for the animal.

Once the animal had reached the target plane of anesthesia, it was moved from the chamber and placed on the lab bench where it was decapitated by guillotine immediately. Surgical scissors were used to make a sagittal cut in the skin of the head, revealing the skull beneath. By inserting one shear of the scissors into the posterior portion of the head where the spinal cord was disconnected, and keeping the other shear on the outside, two cuts parallel to the frontal plane were made on the lateral portions of the skull. The scissors were then positioned to make a sagittal cut to the roof of the skull, with one shear tucked against the inside of the skull, avoiding contact with the brain, and the other shear
on the roof of the skull outside the head. The sagittal cut was made carefully through the parietal and frontal bones, stopping near the eyes. Two transverse cuts were made on either side of the sagittal cut by inserting the tip of the scissors straight down into the head where the sagittal cut ended, then cutting laterally along the transverse plane. These series of cuts enabled easy access to the brain by simply pulling back the parietal bones. The parietal bones were pulled back with a bone rongeur, and the brain was scooped from the skull and placed on a small piece of aluminum foil.

The cerebellum was excised by razor and the brain was cut in half down the midline to reveal the subcortical structures. Working with the medial part of the brain, the hippocampus was visually identified and carefully isolated by pulling back the cortex and thalamus with metal forceps. Both halves were placed into an Eppendorf tube cooled in ice, and immediately frozen in a -50°C freezer until the tissue homogenization step.

2.4 Hippocampal Tissue Homogenization for Western Blot and Co-immunoprecipitation Experiments

Tissue homogenization was necessary to solubilize hippocampal proteins in solution so that it may be analyzed for its contents in western blot and co-immunoprecipitation experiments. Frozen hippocampal tissue was thawed and 200μL of tissue homogenizing buffer was added to each Eppendorf tube containing two hippocampal halves. A plastic tissue grinder was used to crush the hippocampus and shear the tissue against the wall of the tube. This was performed with the goal of physically breaking down the tissue into solubilized cells. Once the mixture appeared homogenous (i.e. no tissue bits could be seen in the mixture), the grinding step was considered complete and the tube containing the hippocampus in solution was set on ice until the sonication step. The purpose of the sonication step is to break down the lipid membrane of cells so that the intracellular proteins may be analyzed as well. Sonication was achieved by immersing the sonicator in the sample for 10 seconds, and then the sample was immediately placed in ice to cool. Samples were centrifuged at 7000rpm for 10 minutes to pelletize the insoluble contents of the tissue (for example, lipids), and the supernatant was extracted and placed into another
tube to use as the protein sample moving forward. If the size of the pellet was deemed to be too large, indicating that the physical breakdown of the tissue was inadequate, the grinding, sonication, and centrifugation processes were repeated to facilitate further breakdown of the tissue. After the supernatant was extracted, it was placed in a -60°C freezer until subsequent use.

2.5 Western Blot

In this study, western blot was used to assess the expression quantity of hippocampal 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} receptors in control, sleep deprived, and sleep recovery animals, with the ultimate goal of discerning whether expression changes would be altered following each treatment. Frozen hippocampal tissue was thawed and subsequently homogenized in a homogenization buffer by rigorous grinding of the tissue and sonication until a free-flowing liquid lysate was achieved (see section 2.4). A Bradford assay was used to estimate the protein concentration of each sample. 15\(\mu\)g of protein was taken from the sample and mixed with 5\% \(\beta\)-mercaptoethanol in Laemmli buffer (Bio-Rad) at a 1:1 volume ratio. The mixture was heated at 99°C for 5 minutes, and then loaded into a 10\% SDS-polyacrylamide gel for gel electrophoresis. The gel was always casted the day before the samples were loaded and allowed to set overnight.

Proteins were separated at 100 volts for the first 30 minutes, and then the setting was changed to 120 volts to run for an additional two hours, or until the blue dye escaped from the bottom of the gel. The bands were transferred onto a nitrocellulose membrane at 110 volts for 90 minutes, followed by blocking with 5\% non fat dry milk (Bio-Rad) for one hour, and incubation overnight with monoclonal mouse 5-HT\textsubscript{1A} primary antibody (EMD Millipore MAB11041, 1:1000 dilution in 5\% non fat dry milk) or polyclonal rabbit 5-HT\textsubscript{2A} primary antibody (Abcam ab16028, 1:1000 dilution in 5\% non fat dry milk) at 4°C. After washing three times with tris-buffered saline containing 0.2\% Tween 20 (TBS-T), the membranes were incubated in goat anti-mouse (1:1500 dilution in 5\% milk) or goat anti-rabbit (1:2000 dilution in 5\% milk) secondary antibodies for 2 hours at room temperature (RT). Following this, membranes were washed three more times with TBS-T.
and visualized immediately by chemiluminescence using the Alpha Innotech Fluorchem 800 imager (Alpha Innotech Co.).

Using β-actin as a loading control, relative bands intensities were determined by densitometric analysis in the imaging program. The intensities of the bands of interest were divided by the corresponding β-actin band intensities to generate a ratio. The ratios for each treatment condition were averaged from different replicates and plotted as a value +/- the standard error of the mean (SEM). Significance was determined by a two-tailed t-test. The two-tailed t-test is generally used for comparing two samples with equal variance when the direction of the change is unknown or thought to be possible in either way (increased or decreased).

### 2.6 Co-immunoprecipitation (CoIP)

CoIP was employed to determine whether heterodimers of 5-HT1A-mGluR1α, 5-HT1A-GABABR1, 5-HT1A-GABABR2, 5-HT2A-mGluR1α, 5-HT2A-GABABR1, or 5-HT2A-GABABR2 exist. This procedure involved using the antibody of one protein partner from a potential interaction to precipitate its associated proteins, then subsequent visualization of the co-precipitated proteins on a gel with the antibody of its proposed partner. If the presence of the partner protein is observed, this method would suggest an interaction exists.

Frozen hippocampal tissue was thawed and subsequently homogenized in a homogenization buffer by rigorous grinding of the tissue and sonication until a free-flowing liquid lysate was achieved (see section 2.4). A Bradford assay was used to estimate the protein concentration of each sample. 200μg of protein was diluted to a total volume of 1mL with phosphate buffered saline (PBS, from Life Technologies). The mixture was then incubated with the immunoprecipitating (IP) antibody of choice, either mouse polyclonal mGluR1α (BD Biosciences 556389), mouse polyclonal GABABR1 (Abcam ab55051), or mouse polyclonal GABABR2 (EMD Millipore MABN488), at 1:500 dilution overnight on a rotating tube rack at 4°C.
The next morning, protein A agarose beads were added to the samples, and the samples were spun on the rotating tube rack for 2 hours at RT to facilitate antibody binding to beads. The beads were centrifuged, washed three times with PBS to remove unbound proteins, re-suspended in 50 μL of 5% β-mercaptoethanol in Laemml buffer, and boiled at 99°C for 10 minutes to dissociate the remaining bound proteins from the beads. The supernatant was then loaded into a 7% SDS-polyacrylamide gel, which was always casted the day before running. The running of the gel, transfer to the nitrocellulose membrane, membrane blotting, and imaging steps were performed as described in the western blot section. The primary antibody used for blotting was either the polyclonal rabbit 5-HT1A antibody (Abcam ab44635, 1:500 dilution in 5% milk) or the polyclonal rabbit 5-HT2A antibody (Abcam ab16028, 1:1000 dilution in 5% milk). Secondary goat-anti rabbit antibody (1:2000 dilution in 5% milk) was used for chemiluminescent visualization.

2.7 Brain Harvesting for Immunohistochemistry

To preserve the hippocampus for protein analysis using immunohistochemistry, the hippocampus must be fixated shortly after removal from the blood supply. Fixation cross-links the proteins into a stable form and eliminates the activity of proteolytic enzymes, terminating the degradation process. Following fixation, the hippocampus must be equilibrated with 40% sucrose for preservation and to facilitate ease of sectioning.

Controls or after treatment (sleep deprivation or recovery), animals were anesthetized with halothane. They were placed into a large glass chamber, approximately 3mL of liquid halothane was dropped into the chamber and the chamber was sealed. Animals were observed carefully during anesthetization to ensure they reached an appropriately sufficient anesthetized state. Specifically, the animal should still be taking slow, heavy breaths, it should be immobile, and the eyes should be open and non-blinking, indicating the loss of the eyelid reflex. This particular anesthetic plane minimizes hypoxia-induced cell death while maintaining sufficient anesthesia for the animal.
Once the animal had reached the target plane of anesthesia, it was moved from the chamber and placed on the lab bench where it was decapitated by guillotine immediately. The head was quickly submerged in cold sucrose-rich artificial cerebrospinal fluid (ACSF) (see section 2.12 for contents of this solution). While maintaining the head in the solution, surgical scissors were used to make a sagittal cut in the skin of the head, revealing the skull beneath. By inserting one shear of the scissors into the posterior portion of the head where the spinal cord was disconnected, and keeping the other shear on the outside, two cuts parallel to the frontal plane were made on the lateral portions of the skull. The scissors were then positioned to make a sagittal cut to the roof of the skull, with one shear tucked against the inside of the skull, avoiding contact with the brain, and the other shear on the roof of the skull outside the head. The sagittal cut was made carefully through the parietal and frontal bones, stopping near the eyes. Two transverse cuts were made on either side of the sagittal cut by inserting the tip of the scissors straight down into the head where the sagittal cut ended, then cutting laterally along the transverse plane. These series of cuts enabled easy access to the brain by simply pulling back the parietal bones. The parietal bones were pulled back with a bone rongeur, and the brain was scooped from the head and quickly transferred into a fresh solution of cold sucrose-rich ACSF in another petri dish.

After five to ten seconds of bathing in fresh solution, the brain was transferred onto a piece of filter paper on a platform chilled with ice. The cerebellum and the anterior olfactory bulbs were excised, and then the whole brain was deposited into at least 45mL of 4% paraformaldehyde in a Falcon tube for fixation. The tube was placed at 4°C for 20 to 24 hours, after which the brain was removed from the paraformaldehyde and transferred into 20% sucrose in tris-buffered saline (TBS) for preservation. Once the brain sunk to the bottom of the 20% sucrose solution, indicating full equilibration, usually after 1 to 2 days, it was transferred into 40% sucrose in TBS. The brain was kept in 40% sucrose until the sectioning step.
2.8 Immunohistochemistry

Peroxidase immunohistochemistry was used to visualize both the localization and expression quantity of 5-HT$_{1A}$ and 5-HT$_{2A}$ in the hippocampal CA1 region after control and the various sleep deprivation and recovery treatments, with the ultimate goal of supporting the western blot data. Having ensured that the brain has fully equilibrated with the 40% sucrose solution (i.e. that it has sunk to the bottom of the tube), it was taken from the solution and washed under continuously running cold water for 15 minutes prior to sectioning. 40μm coronal sections were cut in PBS using the Leica VT1200S vibratome and sections containing a significant portion of the hippocampus were placed in individual wells of a 24-well plate in fresh PBS. Among animals treated under different conditions of sleep, we tried to choose sections in which the hippocampus had similar size, shape, and appearance. At least 4 sections from each animal were stained.

Once the sections to be stained had been chosen, they were incubated in 0.3% H$_2$O$_2$ for 15 minutes. After three 10-minute PBS washes, the sections were permeated with 0.2% Triton X-100 in PBS for 15 minutes. Sections were then washed three times in PBS again, and subsequently incubated in 5% normal horse serum (NHS) for one hour at RT for blocking. Following this, primary antibody was added – 5-HT$_{1A}$ (EMD Millipore ab15350, 1:400 dilution in 1% NHS) or 5-HT$_{2A}$ (Abcam ab16028, 1:300 dilution in 1% NHS). The entire plate was then incubated at 4°C overnight while slowly rotating on an orbital shaker. Afterward, sections were washed with PBS three times, and subsequently incubated with horse anti-rabbit biotinylated secondary antibodies (1:200 dilution in 1% NHS) for one hour at RT. The ABC Elite kit (Vector Laboratories) and peroxidase substrate kit (ImmPACT DAB, Vector Laboratories SK-4105) were used for biotin-avidin antigen detection and visualization, respectively. Sections were washed in a petri dish with PBS, carefully mounted onto slides and allowed to dry overnight before the imaging process. Imaging was performed with the Zeiss Axioplan 2 microscope.
2.9 Preparing Hippocampal Slices for Electrophysiology

The hippocampus preservation process for electrophysiology is even more rigorous than the preservation for western blot or immunohistochemistry, given that neurons must survive the slicing process in order to generate adequate recordings. Extraction and slicing of the hippocampus must be performed quickly and efficiently while immersed in cold, oxygenated artificial cerebral spinal fluid (ACSF) to prevent neuronal death induced by hypoxia.

Controls or after treatment (sleep deprivation or recovery), animals were anesthetized with halothane. They were placed into a large glass chamber, approximately 3mL of liquid halothane was dropped into the chamber and the chamber was sealed. Animals were observed carefully during anesthetization to ensure they reached an appropriately sufficient anesthetized state. Specifically, the animal should still be taking slow, heavy breaths, it should be immobile, and the eyes should be open and non-blinking, indicating the loss of the eyelid reflex.

Ensuring the animal had reached a sufficient plane of anesthesia, it was transferred to the lab bench and decapitated by guillotine. The head was quickly submerged in cold sucrose-rich ACSF (see section 2.12). While maintaining the head in the solution, surgical scissors were used to make a sagittal cut in the skin of the head, revealing the skull beneath. By inserting one shear of the scissors into the posterior portion of the head where the spinal cord was disconnected, and keeping the other shear on the outside, two cuts parallel to the frontal plane were made on the lateral portions of the skull. The scissors were then positioned to make a sagittal cut to the roof of the skull, with one shear tucked against the inside of the skull, avoiding contact with the brain, and the other shear on the roof of the skull outside the head. The sagittal cut was made carefully through the parietal and frontal bones, stopping near the eyes. Two transverse cuts were made on either side of the sagittal cut by inserting the tip of the scissors straight down into the head where the sagittal cut ended, then cutting laterally along the transverse plane. These series of cuts enabled easy access to the brain by simply pulling back the parietal bones.
The parietal bones were pulled back with a bone rongeur, and the brain was scooped from the head and quickly transferred into a fresh solution of cold sucrose-rich ACSF in another petri dish.

After five to ten seconds of bathing in fresh solution, the brain was transferred onto a piece of filter paper on a platform chilled with ice. The cerebellum and the anterior olfactory bulbs were excised, and the brain was cut in half by razor down the midline to reveal the medial portion of the hippocampus. Taking only one half of the brain, the most lateral 1mm was also excised to create a flat gluing surface. The brain was turned to rest on its lateral side and fixed via super glue (Lepage Accu-Flo No.8) to the vibratome stand. The Leica VT1200S vibratome was used to cut 400μm thick slices and the brain was kept submerged in cold sucrose-rich ACSF during cutting to minimize metabolic degradation. The slices containing significant portions of the hippocampus were retained, the CA3 region was excised from the slices by scalpel to prevent recording spontaneous activity from its neurons, and slices were then transferred to an incubating ACSF solution (see section 2.12) at room temperature to recover for at least one hour.

2.10 Electrophysiology

Field excitatory post-synaptic potentials (fEPSPs) were measured in the stratum radiatum of the CA1 region in the presence of various concentrations of 5-HT to determine whether responses to 5-HT change following sleep deprivation and sleep recovery. After incubation for at least one hour, slices were transferred in solution with a wide-mouth eye dropper to a recording chamber incubated with perfusion ACSF. The perfusion rate was adjusted to ~1.5mL/minute. A stimulating electrode was placed in the stratum radiatum in the CA1 region to excite axons projecting from CA3 pyramidal neurons (Schaffer collaterals). A recording electrode was placed in the stratum radiatum in the CA1 area, closer to the subiculum side than the stimulating electrode, to measure the fEPSPs generated upon stimulation. Stimulation was applied once every 20 seconds at a strength corresponding to half of the maximal fEPSP response observed. Half of the maximal strength was used to accommodate potential increases or decreases in fEPSPs during
recording. The fEPSP values for each slice was measured in perfusion ACSF for 20 minutes in the absence of 5-HT to establish a pre-drug baseline, then the recording chamber was flushed with perfusion ACSF solutions containing 1, 5, 10, 20, and 40μM of 5-HT (see section 2.12) sequentially for a duration of 5 minutes per drug concentration. After application of 5-HT, the chamber was re-infused with normal perfusion ACSF to allow the slices to recover for 30 minutes. The 10-90% fEPSP slopes, as measured by the MiniAnalysis program, were averaged for three consecutive measurements to generate a fEPSP value for each minute. The minute-averaged fEPSPs measured during the drug application and recovery periods were plotted as a percentage of the pre-drug baseline. Minute-averaged fEPSP values from different replicate recordings were averaged to generate a single curve for each treatment. Significance was determined by a two-tailed t-test. The two-tailed t-test is generally used for comparing two samples with equal variance when the direction of the change is unknown or thought to be possible in either way (increased or decreased).

### 2.11 Electrophysiology Setup and Equipment

The recording chamber was made from Plexiglass and set on a Burleigh Gibraltar platform. Hippocampal slices were held into position on the flat base of the chamber by a custom-made anchor, a stainless steel U-shaped apparatus with nylon fibers connecting the two arms of the “U”, which ensured that slices did not move during the recording. Polyethylene tubing carrying different ACSF solutions was connected to the inlet of the chamber; the solutions originated from a reservoir where they were saturated with oxygen. Because the reservoirs were placed above the recording chamber, gravity enabled the solutions to flow into the chamber, as long as resistance was minimized in the lines. To ensure this was the case, all lines were vacuum suctioned before each experiment to remove air bubbles. During the experiment, only one solution was allowed to enter the chamber at a time, the flow of the other solutions was prevented by clamping the lines with butterfly clips. The back of the recording chamber was connected to a vacuum line which was positioned to remove ACSF solution in the chamber at the same rate of infusion, ensuring a constant supply of fresh solution perfusing the slice.
A Grass S88 dual output square pulse stimulator connected to a photoelectric stimulus isolation unit (PSIU6, Grass Instruments) was used to supply constant current stimulation and to modulate the strength and rate of stimulation. To view the slice in the recording chamber, a Zeiss Axioskop 2 FS Plus microscope was positioned over the field of view and illuminated by a HAL 100 halogen lamp with adjustable brightness. fEPSPs were measured using the Axopatch 200A (Axon Instruments) amplifier, digitized with a Digidata 1220 interface (Axon Instruments), and monitored and saved with a MS-DOS Fetchex program. Traces could then be read and analyzed by the MiniAnalysis software.

Concentric bipolar electrodes (SNEX-100, David Kopf Instruments), 50mm long measured from the cementing mount, were used to stimulate the Schaffer collaterals in hippocampal slices. The contact length of the tip was 0.75mm and its diameter measured 0.1mm. The tip was rinsed in distilled water after each experiment and inspected for tissue debris prior to the start of each experiment in order to maintain constant resistance at approximately 1MΩ. The recording electrode was made from a silver wire treated with bleach to form a silver chloride coating around the wire. Borosilicate glass capillaries (1B100F-4, World Precision Instruments) pulled to a fine tip at one end using a micropipette puller (Flaming/Brown P-87, Sutter Instruments) were injected with ACSF solution and inserted with the silver/silver chloride recording electrode at the other end. The glass capillary was lowered onto the hippocampal slice to form a conductive bridge between the slice and the recording electrode.

2.12 Solutions

Tissues were homogenized in a tissue homogenization buffer: 31.25mM Tris-HCl, pH 6.8, 25mM dithiothreitol, 1% sodium dodecyl sulfate, 5% glycerol, with 1% protease inhibitor cocktail solution added freshly immediately before use.

For minimizing metabolic degradation during slicing, a cold sucrose-rich ACSF was used: 7mM MgCl₂, 0.5mM CaCl₂, 7mM dextrose, 234mM sucrose, 2.4mM KCl, 28mM
NaHCO₃, 1.5mM NaH₂PO₄, 3.75mM pyruvic acid, 1mM ascorbic acid, saturated with carbogen (95% O₂/5% CO₂).

For incubation of hippocampal slices after slicing, an incubating ACSF solution was used: 2mM MgCl₂, 2mM CaCl₂, 10mM dextrose, 125mM NaCl, 2.4mM KCl, 25mM NaHCO₃, 1.5mM NaH₂PO₄, 3.75mM pyruvic acid, 1mM ascorbic acid, saturated with carbogen.

For recording from hippocampal slices, a perfusion ACSF was used: 2mM MgCl₂, 2mM CaCl₂, 10mM dextrose, 120mM NaCl, 3mM KCl, 26mM NaHCO₃, 2mM NaH₂PO₄, saturated with carbogen.

5-HT drug solutions used to assess the fEPSP response of hippocampal slices to 5-HT: a 500μM stock solution was first made by dissolving 2.1mg of 5-HT (Sigma-Aldrich) into 20mL of fresh perfusion ACSF (see above). The 20mL of solution was separately aliquoted into 1mL portions and frozen at -20°C until subsequent use. Immediately prior to running an electrophysiology experiment, aliquots were thawed and diluted appropriately into fresh perfusion ACSF to make 25mL of 5-HT at the desired concentration.
Chapter 3: Results

3.1 The Effects of Sleep Deprivation on the Expression Profile of 5-HT$_{1A}$

Previous studies revealed that both the metabotropic glutamate receptor 1α (mGluR1α) and the γ-aminobutyric acid B receptor subunit 1 (GABAB$_R$1) were up-regulated in the rat hippocampus following 12 hours of sleep deprivation (SD). This increase in expression was detected by performing a western blot on the lysate of the whole rat hippocampus in both control and sleep deprived animals. In this present study, we investigated whether 5-HT$_{1A}$ undergo similar changes in response to SD. Increases in 5-HT$_{1A}$ expression could have possible implications for 5-HT transmission in the hippocampus, which, when altered, may facilitate the development of various psychological disorders such an anxiety or depression. The total expression of 5-HT$_{1A}$ in the rat hippocampus was measured by western blot in normally sleeping animals and animals subjected to 12 hours of sleep deprivation. We also wished to study whether potential changes could be reversed with 24 or 48 hours of recovery sleep, therefore western blot was also performed on 24-hour and 48-hour recovery animals.
Figure 2. Western blot of 5-HT$_{1A}$ expression in rat hippocampus following sleep deprivation. 5-HT$_{1A}$ expression in the rat hippocampus was measured by western blot in normally sleeping control animals, 12-hour sleep deprived animals (SD), and after 24 and 48 hours of sleep recovery (24hr and 48hr, respectively). The band at 46kDa represents 5-HT$_{1A}$. Darker bands indicate increased binding of antibody to protein and suggest that larger amounts of protein are present in a given lane. The loading control β-actin band is also shown for comparison. Graphical data displays the average results of densitometric analysis, with +/- error bars representing the SEM. (*) indicates a significant difference between SD and control animals, and between 24-hour recovery and control animals (p < 0.05). n = 4.

5-HT$_{1A}$ western blot data revealed that 5-HT$_{1A}$ expression was significantly increased in the hippocampus of SD animals (Figure 2). These findings, in conjunction with the previous reports of elevated mGluR1α and GABAB$_{R1}$ expression following SD, contribute further to the hypothesis that a variety of G protein-couple receptors (GPCRs) are dysregulated in a sleep deprived state.

After 24 hours of recovery, 5-HT$_{1A}$ expression remained elevated and even appeared to be slightly higher compared to immediately after SD, suggesting that one night of rest may not be sufficient to reverse some of the effects of SD. 5-HT$_{1A}$ receptor expression displayed partial recovery after 48 hours but did not revert completely back to control levels. The recovery profile of 5-HT$_{1A}$ emphasized the possible residual long-term effects of acute sleep deprivation for one night. The observation that GPCR changes have not recovered even after 48 hours raises some concerns about the amount of sleep we truly need to sleep to facilitate a full recovery. It is unclear whether mGluR1α and GABAB$_{R1}$ also require significant recovery times after increase with SD as previous experiments have not looked at the recovery profiles of these two GPCRs.

To corroborate the western blot findings, 5-HT$_{1A}$ expression was also investigated by peroxidase immunohistochemistry. The majority of 5-HT$_{1A}$ receptors in the hippocampus are located post-synaptically on pyramidal neurons. We focused specifically on the CA1
region so that we could correlate potential changes to changes in field excitatory postsynaptic potentials measured in this region. We anticipated intense 5-HT₁A staining in the CA1 pyramidal layer, and hypothesized that the intensity of staining would be different between control and sleep deprived animals, which would point to a change in receptor expression. Based on the western blot results, 12-hour sleep deprived animals should have more intensely stained CA1 regions than controls.

Figure 3. Immunohistochemistry of 5-HT₁A expression in rat hippocampus following sleep deprivation. A. Representative images of pyramidal neurons stained for 5-HT₁A in the CA1 region of the hippocampus in control, sleep deprived, 24-hour recovery, and 48-hour recovery rats. The top-left image is labelled to identify the stratum oriens (SO), the stratum pyramidale (SP), and the stratum radiatum (SR) layers in all the images. The dark band running horizontally across each image is the stained layer of pyramidal cells. The intensity of staining can be inferred from the darkness of the bands as the brightness and
contrast settings were kept constant throughout the imaging process. Several stained interneurons may also be found in the stratum oriens and the stratum radiatum. White scale bar = 100μm. n = 5.

B. Representative magnified samples of the images from part A showing the darkened stained outlines of CA1 pyramidal neurons more clearly. White scale bar = 25μm.

5-HT<sub>1A</sub> immunohistochemistry revealed that the CA1 pyramidal layer was more heavily stained following SD compared to controls, suggesting an up-regulation of receptors (Figure 3). This was consistent with the elevation of expression observed in 5-HT<sub>1A</sub> western blots. Based on these observations, the increase in total 5-HT<sub>1A</sub> expression in the hippocampus after SD can be attributed to the regional increase in 5-HT<sub>1A</sub> expression in the CA1 pyramidal neurons, at least partially if not fully.

High staining intensity persisted in the pyramidal neurons after 24 hours of recovery, and subsided slightly after 48 hours of recovery but did not fully revert back to control levels (Figure 3). Again, these results were consistent with the 5-HT<sub>1A</sub> western blot results. Both immunohistochemistry and western blot showed 5-HT<sub>1A</sub> increase after 12 hours of sleep deprivation, sustained increase after 24 hours of recovery, and partial recovery after 48 hours. Such a correlation suggests that the 5-HT<sub>1A</sub> expression increase specifically in the CA1 pyramidal layer is largely responsible for the increase in total hippocampal 5-HT<sub>1A</sub> expression.

5-HT<sub>1A</sub> staining was also observed in interneurons in both the stratum oriens and the stratum radiatum, confirming the existence of these receptors on interneurons. However, the number of interneurons stained positively for 5-HT<sub>1A</sub> was far less than the number of pyramidal neurons stained, which also confirmed that the majority of 5-HT<sub>1A</sub> receptors are located on pyramidal neurons (at least in the CA1 region). Interneuron staining intensity did not seem to change with SD. It was also difficult to determine whether the number of stained interneurons changed with SD due to the relatively few number of neuron candidates; nevertheless, no significant difference in the number of stained interneurons was discerned.
The western blot and immunohistochemistry data for 5-HT\textsubscript{1A} together provides evidence that 5-HT\textsubscript{1A} is indeed up-regulated in the hippocampus following sleep deprivation. This increase in expression may have consequences for the actions of 5-HT at 5-HT\textsubscript{1A} receptors, and overall 5-HT transmission in the hippocampus. 5-HT\textsubscript{1A} is also implicated in memory, anxiety, and depression. These results suggest that sleep deprivation may play a role in modulating certain aspects of memory and the progression of psychological disorders through changing 5-HT\textsubscript{1A} receptor expression.

3.2 The Effects of Sleep Deprivation on the Expression Profile of 5-HT\textsubscript{2A}

5-HT\textsubscript{2A} is another abundantly expressed GPCR targeted by 5-HT in the hippocampus. Similar to 5-HT\textsubscript{1A}, it is mostly located post-synaptically on the pyramidal neurons of the CA1 and CA3 regions. Unlike 5-HT\textsubscript{1A}, it is known for exerting excitatory effects. The actions of psychedelic drugs, such as lysergic acid diethylamide (LSD), are exerted through activation of 5-HT\textsubscript{2A}. Because of this observation, blocking 5-HT\textsubscript{2A} is thought to produce antipsychotic effects, while intense stimulation is thought to lead to psychotic behaviour. Similar to the expression levels of mGluR1\textsubscript{α}, GABA\textsubscript{B}R1, and 5-HT\textsubscript{1A} following sleep deprivation (SD), we anticipated changes to total hippocampal 5-HT\textsubscript{2A} expression. Thus, 5-HT\textsubscript{2A} expression levels were measured by western blot analysis of whole hippocampal tissue in normally sleeping and 12-hour sleep deprived animals. The 24- and 48-hour recovery profile of 5-HT\textsubscript{2A} was also investigated by western blot to determine if potential changes could be reversed within a short period of recovery.
Figure 4. Western blot of 5-HT$_{2A}$ expression in rat hippocampus following sleep deprivation. 5-HT$_{2A}$ expression in the rat hippocampus was measured by western blot in normally sleeping control animals, 12-hour sleep deprived animals (SD), and after 24 and 48 hours of sleep recovery (24hr and 48hr, respectively). The band at 53kDa represents 5-HT$_{2A}$. Darker bands indicate increased binding of antibody to protein and suggest that larger amounts of protein are present in a given lane. The loading control β-actin band is also shown for comparison. Graphical data displays the average results of densitometric analysis, with +/- error bars representing the SEM. n = 5.

In contrast to 5-HT$_{1A}$, the western blot of 5-HT$_{2A}$ revealed that the expression level of this receptor does not change following sleep deprivation (Figure 4). The intensities of the observed western blot bands were constant across each studied condition (control, SD, 24 hour recovery, and 48 hour recovery). It appeared that the response of 5-HT$_{2A}$ expression to sleep deprivation was quite different than the changes observed for other GPCRs, including mGluR1α, GABA$_B$R1, and 5-HT$_{1A}$. It is important to note, however, that GABA$_B$R2 was also found to be unchanged following SD, thus not all pertinent GPCRs are up-regulated following SD.
To corroborate these results, peroxidase immunohistochemistry was employed to visualize 5-HT$_{2A}$ expression in the hippocampus. Again, we focused on the CA1 region in an attempt to correlate our results with hippocampal field excitatory post-synaptic potential recordings in the CA1 region. Because 5-HT$_{2A}$ is known to be primarily expressed on the pyramidal neurons of the hippocampus, we expected intense staining in the CA1 pyramidal layer. Based on the western blot results, there should be no significant difference in the intensity of staining between control and sleep deprived animals.

**Figure 5. Immunohistochemistry of 5-HT$_{2A}$ expression in rat hippocampus following sleep deprivation.** A. Representative images of pyramidal neurons stained for 5-HT$_{1A}$ in the CA1 region of the hippocampus in control, sleep deprived, 24-hour recovery, and 48-hour recovery rats. The top-left image is labelled to identify the stratum oriens (SO), the stratum pyramidale (SP), and the stratum radiatum (SR) layers in all the images. The dark band running horizontally across each image is the stained layer of pyramidal cells. The intensity of staining can be inferred from the darkness of the bands as the brightness and
contrast settings were kept constant throughout the imaging process. Several stained interneurons may also be found in the stratum oriens and the stratum radiatum. White scale bar = 100μm. n = 5. B. Representative magnified samples of the images from part A showing the darkened stained outlines of CA1 pyramidal neurons more clearly. White scale bar = 25μm.

Several observations may be derived from the immunohistochemistry images of 5-HT2A. First, there was a distinct increase in the intensity of staining in the CA1 pyramidal layer in sleep deprived animals compared to controls, indicating an up-regulation of 5-HT2A receptors in this region (Figure 5). This starkly contrasted the western blot findings. The only theoretical difference between investigating protein expression with western blot and investigating protein expression with immunohistochemistry is that western blot measures the total 5-HT2A expression in the entire hippocampus, while immunohistochemistry focuses on a particular region, in this case the CA1 region. Our findings indicate that increased expression of 5-HT2A in the CA1 pyramidal layer following SD, as revealed by immunohistochemistry, is not driving a corresponding increase in the total hippocampal 5-HT2A expression, as revealed by western blot. This apparent conflict may perhaps be explained by down-regulation of 5-HT2A elsewhere in the hippocampus. When the hippocampus is extracted from the brain for western blot analysis, the CA3 region, the dentate gyrus, the subiculum, and the entorhinal cortex are all extracted along with the CA1 region. If 5-HT2A is decreased in any of the other regions, that may offset the increase in the CA1 region and appear as no difference in the western blot.

Secondly, immunohistochemistry revealed that, following 24 hours of sleep recovery, the increase in 5-HT2A observed after SD appeared to recover fully back to control levels (Figure 5). In contrast to the relatively long-lasting changes of 5-HT1A, increased expression levels of 5-HT2A appeared to recover relatively quickly, suggesting minimal long term effects from one night of sleep deprivation.
Finally, 5-HT$_2$A receptor expression in the pyramidal layer seemed to increase again after 48 hours of recovery (Figure 5). 5-HT$_2$A expression had already recovered within 24 hours, suggesting that a factor separate from sleep deprivation may be affecting these receptors. The identity of this factor is unclear, but it may be related to the experimental protocol. One explanation may be the social isolation of animals during the experiment. Animals which have been treated with 12 hours of SD followed by 48 hours of recovery have spent a total of 60 hours in isolation by the end of the treatment. Because rats are known to prefer social environments, it is not unreasonable that extended social isolation may initiate physiological changes.

A number of interneurons were also positively stained for 5-HT$_2$A in both the stratum oriens and the stratum radiatum, confirming the existence of 5-HT$_2$A receptors on interneurons in the hippocampal CA1 region. However, similar to 5-HT$_1$A, there seemed to be no relationship between the number of interneurons stained or their staining intensities and sleep deprivation.

### 3.3 5-HT$_1$A Heterodimerization

G protein-coupled receptors (GPCRs) are known to participate extensively in both homodimerization and heterodimerization. Recent studies have revealed that 12 hours of sleep deprivation changes the dimerization patterns of mGluR1α-GABAB$_1$R1, mGluR1α-GABAB$_2$R2, and GABAB$_1$R1-GABAB$_2$R2. GABAB$_1$R1-GABAB$_2$R2 has long been established as obligate heterodimers that only function when dimerized, while mGluR1α-GABAB$_1$R1 and mGluR1α-GABAB$_2$R2 heterodimers were only recently discovered, with no apparent indication of their function. It has been proposed that one subunit may regulate the activity of the other (Tadavarty et al. 2011). Given the propensity for mGluR1α, GABAB$_1$R1, and GABAB$_2$R2 to heterodimerize, we investigated whether 5-HT$_1$A would also form heterodimers with these receptors. Heterodimerization was probed using co-immunoprecipitation (CoIP). In this technique, an antibody specific for one potential dimer partner (the IP antibody) was used to precipitate all of its associated proteins. The associated proteins were separated on a gel and detected with an antibody
specific to the other protein partner (the blotting antibody). If the blotting antibody detected a specific band corresponding to the target dimer, it would indicate that the IP antibody was able to co-precipitate the protein partner via an interaction with the original IP protein. 5-HT\textsubscript{1A} heterodimerization was explored in control, sleep deprived, 24- and 48-hour recovery animals in order to observe potential changes in dimerization pattern caused by SD.

**Figure 6. CoIP of 5-HT\textsubscript{1A} and mGluR1\textalpha\;in rat hippocampus.** Possible dimerization bands in the hippocampus of control, sleep deprived (SD), 24-hour recovery (24R), and 48-hour recovery (48R) animals are shown. mGluR1\textalpha\;was used as the immunoprecipitating (IP) antibody and 5-HT\textsubscript{1A} was used as the blotting antibody for this gel. The kDa measurements indicate approximately where a particular sized protein is expected to appear. mGluR1\textalpha\;molecular weight: 133kDa; 5-HT\textsubscript{1A} molecular weight: 46kDa; the heterodimer is expected to appear at ~180kDa.

The CoIP between 5-HT\textsubscript{1A} and mGluR1\textalpha\;did not detect an interaction between these two receptors (Figure 6). The heterodimer was expected to have a molecular weight of approximately 180kDa. Therefore, if significant heterodimerization occurred in the hippocampus, a band should appear near the top of the gel above the 150kDa marker.
One prominent band appeared on the gel at ~130kDa, corresponding well to the mGluR1α monomer, indicating that perhaps the 5-HT₁A primary or secondary antibody was binding non-specifically to mGluR1α or residual mGluR1α antibodies. However, no other high molecular weight bands were detected.

**Figure 7. CoIP of 5-HT₁A and GABA_BR1 in rat hippocampus.** Possible dimerization bands in the hippocampus of control, sleep deprived (SD), 24-hour recovery (24R), and 48-hour recovery (48R) animals are shown. A control in which the immunoprecipitating (IP) antibody was omitted from the procedure was also employed (No IP Antibody Control). GABA_BR1 was used as the IP antibody and 5-HT₁A was used as the blotting antibody for this gel. The kDa measurements indicate approximately where a particular sized protein is expected to appear. GABA_BR1 molecular weight: 108kDa; 5-HT₁A molecular weight: 46kDa; the heterodimer is expected to appear at ~150kDa.

The CoIP between 5-HT₁A and GABA_BR1 did not reveal any bands near the anticipated ~150kDa target (Figure 7), suggesting that an interaction between these two receptors
does not exist. The highest molecular weight bands appeared at ~110kDa, which corresponds well with the IP antibody GABA\(_B\)R1. Again, it seemed that either the 5-HT\(_{1A}\) primary or secondary antibody was binding non-specifically to GABA\(_B\)R1 or its residual antibodies.

For this CoIP gel and all subsequent CoIP gels, a no IP antibody control was also run with the different experimental treatments. We employed this control because we felt that bands not specific to the co-precipitate of the IP antibody were being detected. Whether this was due to insufficient washing of the protein A agarose beads anchoring the co-precipitate or to unspecific secondary antibody binding is uncertain. However, non-specific bands created a real problem. In the 5-HT\(_{1A}\)-GABA\(_B\)R1 gel (Figure 7), the dark band slightly below the 75kDa marker can be detected in all five lanes, even in the lane where no IP antibody was used. Theoretically, no bands should be observed in that lane because nothing was co-precipitated, yet multiple bands were observed.

![CoIP gel](image)

**Figure 8. CoIP of 5-HT\(_{1A}\) and GABA\(_B\)R2 in rat hippocampus.** Possible dimerization bands in the hippocampus of control, sleep deprived (SD), 24-hour recovery (24R), and
48-hour recovery (48R) animals are shown. A control in which the immunoprecipitating (IP) antibody was omitted from the procedure was also employed (No IP Antibody Control). GABA_B2 was used as the IP antibody and 5-HT_1A was used as the blotting antibody for this gel. The kDa measurements indicate approximately where a particular sized protein is expected to appear. GABA_B2 molecular weight: 106kDa; 5-HT_1A molecular weight: 46kDa; the heterodimer is expected to appear at ~150kDa.

The CoIP between 5-HT_1A and GABA_B2 also did not indicate an interaction between these two receptors (Figure 8). The expected molecular weight of the dimer was approximately 150kDa, but no bands close to that particular size were found. In fact, the top half of the gel is considerably more barren than the two previous CoIP gels, and no bands greater than 100kDa were observed.

Taken together, the CoIP results of 5-HT_1A did not reveal any interactions with mGluR1_α, GABA_B1 or GABA_B2. The closest bands turned out to correspond to the size of the IP proteins. This is not unreasonable since the IP proteins would be present in large amounts in each sample, and could react with the blotting antibody if the antibody was not entirely specific. While we did not rule out that transient 5-HT_1A heterodimers may exist but was dissociated by the stringent conditions used in the CoIP protocol (with the use detergents and reducing agents), it is important to note that the mGluR1_α-GABA_B1, mGluR1_α-GABA_B2, and GABA_B1-GABA_B2 interactions were all detected using the same protocol. Perhaps this suggests that 5-HT_1A heterodimers are less prevalent than mGluR1_α and GABA_B_αR heterodimers in the hippocampus.

### 3.4 5-HT_2A Heterodimerization

Having found no significant 5-HT_1A heterodimers, we turned our attention to 5-HT_2A heterodimerization. Much of the same reasons for which 5-HT_1A was thought to possibly participate in heterodimerization may be applied to 5-HT_2A as well. 5-HT_2A is abundantly expressed in the hippocampus, and the bulk of its expression is on the post-synaptic terminals of pyramidal neurons. This puts 5-HT_2A in close vicinity to mGluR1_α,
GABABR1 and GABABR2 receptors, all of which are also abundantly expressed and have demonstrated the propensity to form heterodimers. Therefore, the possible heterodimerization between 5-HT2A and either mGluR1α, GABABR1 or GABABR2 was investigated by CoIP.

Figure 9. CoIP of 5-HT2A and mGluR1α in rat hippocampus. Possible dimerization bands in the hippocampus of control, sleep deprived (SD), 24-hour recovery (24R), and 48-hour recovery (48R) animals are shown. A control in which the immunoprecipitating (IP) antibody was omitted from the procedure was also employed (No IP Antibody Control). mGluR1α was used as the IP antibody and 5-HT2A was used as the blotting antibody for this gel. The kDa measurements indicate approximately where a particular sized protein is expected to appear. mGluR1α molecular weight: 133kDa; 5-HT2A molecular weight: 53kDa; the heterodimer is expected to appear at ~186kDa.

The CoIP between 5-HT2A and mGluR1α revealed no significant interaction between the two receptors (Figure 9). The portion of the gel where we anticipated observing a band of
the dimer, near 180kDa, was completely blank. In fact, all of the observed bands were less than 50kDa in size.

Figure 10. CoIP of 5-HT$_{2A}$ and GABA$_{B}$R1 in rat hippocampus. Possible dimerization bands in the hippocampus of control, sleep deprived (SD), 24-hour recovery (24R), and 48-hour recovery (48R) animals are shown. A control in which the immunoprecipitating (IP) antibody was omitted from the procedure was also employed (No IP Antibody Control). GABA$_{B}$R1 was used as the IP antibody and 5-HT$_{2A}$ was used as the blotting antibody for this gel. The kDa measurements indicate approximately where a particular sized protein is expected to appear. GABA$_{B}$R1 molecular weight: 108kDa; 5-HT$_{2A}$ molecular weight: 53kDa; the heterodimer is expected to appear at ~160kDa.

Similar to the other CoIP experiments, the CoIP of 5-HT$_{2A}$ and GABA$_{B}$R1 also revealed no significant intact heterodimers between the two receptors (Figure 10). The anticipated dimer band was not observed at ~160kDa. However, there was a band observed in the control and SD lanes at ~50kDa which could correspond to the 5-HT$_{2A}$ monomer. In the
no IP antibody control lane, this band appeared with significantly less intensity. This may suggest that GABA\textsubscript{B}R1 was able to co-immunoprecipitate 5-HT\textsubscript{2A}, but the two receptors dissociated during the preparatory heating step or during running of the gel. More work was required to determine whether a true interaction exists.

**Figure 11. CoIP of 5-HT\textsubscript{2A} and GABA\textsubscript{B}R2 in rat hippocampus.** Possible dimerization bands in the hippocampus of control, sleep deprived (SD), 24-hour recovery (24R), and 48-hour recovery (48R) animals are shown. A control in which the immunoprecipitating (IP) antibody was omitted from the procedure was also employed (No IP Antibody Control). GABA\textsubscript{B}R2 was used as the IP antibody and 5-HT\textsubscript{2A} was used as the blotting antibody for this gel. The kDa measurements indicate approximately where a particular sized protein is expected to appear. GABA\textsubscript{B}R2 molecular weight: 106kDa; 5-HT\textsubscript{2A} molecular weight: 53kDa; the heterodimer is expected to appear at ~160kDa.

The CoIP between 5-HT\textsubscript{2A} and GABA\textsubscript{B}R2 also did not support the existence of heterodimers (Figure 11). Once again, bands were absent from the top half of the gel,
which corresponds to a lack of high molecular weight proteins. A heterodimer would be expected to appear at ~160kDa, but all the observed bands were less than 75kDa. Interestingly, it seemed that the 50kDa band observed in the 5-HT$_{2A}$-GABA$_B$R1 gel (Figure 10) also appeared in this gel (Figure 11). The difference in this gel is that the band was very prominent in the no IP antibody control lane, suggesting that a non-specific interaction is giving rise to this band. If these were the same bands, which they appeared to be, then it was likely that the band in the 5-HT$_{2A}$-GABA$_B$R1 gel also represented a non-specific interaction and not an indication of heterodimerization.

Taken together, the CoIP results of 5-HT$_{2A}$ suggested that 5-HT$_{2A}$ does not form any significantly stable heterodimers with mGluR$_1 \alpha$, GABA$_B$R1 or GABA$_B$R2 receptors. Protein bands observed at ~50kDa, which corresponds to the size of the 5-HT$_{2A}$ monomer, were observed on two of the gels, perhaps indicating that 5-HT$_{2A}$ was able to be precipitated by GABA$_B$R1 or GABA$_B$R2, but dissociated during the preparatory heating step for the gel. However, significant amounts of this band were also detected in the no IP control lane of one of the gels, suggesting that this may be a non-specific interaction. While we did not rule out that transient 5-HT$_{2A}$ heterodimers may exist but was dissociated by the stringent conditions used in the CoIP protocol (with the use detergents and reducing agents), it is important to note that the mGluR$_1 \alpha$-GABA$_B$R1, mGluR$_1 \alpha$-GABA$_B$R2, and GABA$_B$R1-GABA$_B$R2 interactions were all detected using the same protocol.

3.5 The Effect of Sleep Deprivation on the Response of Hippocampal CA1 Neurons to 5-HT

The actions of 5-HT on the CA1 pyramidal neurons of the hippocampus are dependent on its activation of 5-HT receptors. Two of the more prevalent 5-HT receptor subtypes in the hippocampus are the 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. Despite the absence of 5-HT$_{1A}$ and 5-HT$_{2A}$ heterodimers, it was observed that the expression levels of these receptors were altered following 12 hours of sleep deprivation. We investigated whether such alterations would affect the field excitatory post-synaptic potential (fEPSP) response of CA1
pyramidal neurons to 5-HT in hippocampal slices. If the SD-induced changes observed in the receptors had any functional significance, we should be able to see corresponding changes to the effects of 5-HT by comparing the effects of 5-HT in control and sleep deprived animals. The 24-hour and 48-hour recovery time course of potential changes were also investigated to determine if these changes persist for a long time and if they match the recovery profile of 5-HT₁A or 5-HT₂A receptors specifically.

Figure 12. The Effect of Sleep Deprivation on the fEPSP response of CA1 neurons to 5-HT in rat hippocampal slices. The average fEPSP slope values across all replicates for control, sleep deprived, 24-hour recovery, and 48-hour recovery slices are plotted for each minute of time in the experiment. During the first 20 minutes, a pre-drug baseline was measured for each slice. All subsequent measurements are represented as a percentage of this baseline. Black vertical lines indicate the start of the application of a particular concentration of 5-HT (labelled in red at the top), beginning at 1μM and increasing steadily to 5, 10, 20, and 40μM. Each concentration was applied for a duration of 5 minutes. After application of 40mM 5-HT, slices were allowed to recover for 30 minutes in the absence of 5-HT. (*) indicates a significant difference at the end of the application of 10μM 5-HT (p < 0.05).
In hippocampal CA1 neurons, 5-HT depresses the fEPSP response in a dose-dependent fashion (Figure 12). This effect was observed in both control and sleep deprived animals. However, the dose-response curve appeared much steeper in SD animals, indicating that 5-HT has an enhanced depressant effect. The difference was most evident at the 10μM 5-HT concentration (Figure 13).

**Figure 13. The effect of sleep deprivation on the fEPSP response of hippocampal CA1 neurons to 10μM 5-HT.** A. Graphical representation of the average fEPSP values for control, sleep deprived, 24-hour recovery, and 48-hour recovery slices at 10μM 5-HT, with error bars showing +/- SEM. (*) indicates a significant difference between the control and sleep deprived conditions (p < 0.05). B. Representative traces of fEPSPs recorded during the pre-drug baseline period and during application of 10μM 5-HT.

The 24- and 48-hour recovery dose-response curves were more similar in steepness to the control curve than the SD curve, suggesting that the sleep deprivation effects recover within 24 hours. At 10μM 5-HT, it is clear that fEPSPs were less depressed in the recovery conditions compared to SD, and while they did not seem to recover fully back to
control levels, the difference between the control and recovery conditions was not significant.

The dose-response curves of 5-HT in control and sleep deprived animals revealed that the actions of applied 5-HT in the hippocampal CA1 region were altered following sleep deprivation. Thus, it appeared that changes in 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor expression following SD do lead to modification of the effects of their endogenous agonist. However, the changes in agonist effect cannot be correlated to 5-HT$_{1A}$ or 5-HT$_{2A}$ specifically as the recovery profiles do not match. They are likely modulated by both 5-HT$_{1A}$ and 5-HT$_{2A}$ expression changes, along with changes in other 5-HT receptors we have not yet studied. Nevertheless, greater susceptibility to 5-HT action induced by SD may be worrisome for patients already experiencing an imbalance of 5-HT, usually those suffering from psychological disorders such as anxiety, depression, and psychosis. Enhanced 5-HT transmission may further exacerbate their symptoms.
Chapter 4: Discussion

Sleep deprivation (SD) is slowly becoming a global epidemic. It affects individuals from many walks of life, from people who willingly sleep deprive themselves for enjoyment, such as teenagers and adolescents, to people who suffer SD based on employment circumstances, such as shift workers and busy professionals, to people who experience constant sleep disruption due to diseases, such as those suffering from insomnia, sleep apnea or narcolepsy. Some of the most severe consequences of SD include the inability to consolidate memory and association to psychological disorders such as anxiety, depression, and psychosis. The underlying cellular processes that mediate these consequences are largely unknown, but recent studies have implicated the involvement of G protein-coupled receptors (GPCRs). In particular, mGluR1α, and GABABR1 were found to be up-regulated in the rat hippocampus following 12 hours of sleep deprivation (Tadavarty et al. 2011). The dynamic expression nature of GPCRs makes them an intriguing target to study. One of the neurotransmitters in the central nervous system thought to be involved in memory, anxiety, depression, and psychosis is 5-HT. 5-HT receptors are abundantly expressed in the hippocampus, the majority of which are GPCRs. One of the objectives of this study was to investigate potential changes in the hippocampal expression of two prominent 5-HT receptors, 5-HT1A and 5-HT2A, induced by sleep deprivation, and to determine whether changes could be reversed by 24 and 48 hours of recovery sleep.

4.1 5-HT1A Increases Following Sleep Deprivation

5-HT1A expression changes were assessed by western blot and peroxidase immunohistochemistry. Western blot was performed using tissue from the entire rat hippocampus, while immunohistochemistry focused on the local expression of 5-HT1A in the CA1 region. From the western blot studies, we observed an increase in 5-HT1A receptor expression in the rat hippocampus following 12 hours of sleep deprivation, and immunohistochemistry found a similar increase in 5-HT1A expression in the pyramidal layer of the CA1 region. Immunohistochemistry corroborated western blot findings of the
recovery profile for 5-HT_{1A} as well. After 24 hours of recovery sleep, the expression of this receptor remained elevated in the hippocampus, and was arguably slightly higher than the levels observed immediately after SD. 5-HT_{1A} expression subsided partially after 48 hours of recovery, but it was still more highly expressed compared to control animals. This suggests that some effects of acute sleep deprivation may linger for greater than 48 hours after the SD episode.

The up-regulation of 5-HT_{1A} receptors has implications for memory, anxiety, and depression. 5-HT_{1A} has been linked to aversive learning and spatial memory (Ogren et al. 2008). In particular, excess 5-HT_{1A} activity appears to be detrimental to the learning process (Rowan et al. 1990; Madjid et al. 2006). 5-HT_{1A} up-regulation in the hippocampus following sleep deprivation likely increases 5-HT_{1A} activity, and thus has the potential to disrupt the learning process. This is consistent with the well-documented memory-impeding effects of sleep deprivation. Although the memory consolidation role of sleep is generally considered to be attributed to the reactivation of neuronal firing patterns during sleep (McCoy and Strecker 2011; Pavlides and Winson 1989), increased 5-HT_{1A} activity may exacerbate impairments.

Activation of 5-HT_{1A} receptors appears to play an anxiolytic role in the central nervous system (Gardier 2009; Ramboz et al. 1998). However, sleep deprivation is known to cause increased anxiety (Bianchi 2014), which would seem to contradict the increased 5-HT_{1A} expression findings. These findings can be reconciled by considering the functional roles of pre- and post-synaptic 5-HT_{1A} receptors. It has been suggested that applied 5-HT_{1A} agonists, including anxiolytic drugs such as buspirone, primarily targets pre-synaptic 5-HT_{1A} receptors, which acts to decrease overall 5-HT activity (Schreiber and De Vry 1993). This has been supported by studies demonstrating lower levels of 5-HT activity associated with reduced anxiety (Jennings et al. 2006) and the apparent anxiolytic effects of benzodiazepines, which have also been shown to reduce central 5-HT activity (Thiebot 1986). In the hippocampus, the majority of 5-HT_{1A} receptors are located post-synaptically on pyramidal neurons. Therefore, the increase of 5-HT_{1A} receptors in the hippocampus should enhance 5-HT transmission, which is consistent with the
observations that increased 5-HT activity and sleep deprivation both contribute to greater anxiety.

It has been observed that when depressed patients are sleep deprived for one night, they exhibit dramatic improvements to their depressive symptoms temporarily (Gerner et al. 1979). Given that 5-HT1A activation has been linked to antidepressant effects (Kostowski et al. 1992; Detke et al. 1995), the finding that 5-HT1A is increased following sleep deprivation would appear to support sleep deprivation as therapeutically beneficial for depression. In addition, other studies have found that extracellular 5-HT also increases in the hippocampus following sleep deprivation (Lopez-Rodriguez et al. 2003; Penalva et al. 2003). It is uncertain whether 5-HT1A increases as a response to increased 5-HT concentrations, or vice versa, or whether both are consequences of another underlying physiological modification, but nevertheless, 5-HT transmission would likely be increased dramatically. Increased neurotransmitter levels combined with increased receptor levels may help to explain why depression can be alleviated by sleep deprivation in such a profound manner, in contrast to taking SSRI antidepressants, which increases 5-HT levels in principle but has slow onset and is refractory in a large number of patients (Artigas et al. 1996).

4.2 5-HT2A Expression Following Sleep Deprivation

Similarly to 5-HT1A, 5-HT2A is also a highly expressed GPCR in the hippocampus, and given the findings which indicated the propensity of other GPCRs, such as mGluR1α, GABAbR1, and 5-HT1A, to change following sleep deprivation, it appeared likely that 5-HT2A may undergo SD-induced changes as well. This possibility was explored by analysis of hippocampal 5-HT2A expression using western blot and peroxidase immunohistochemistry in control, sleep deprived, and recovery animals.

Western blot of the entire hippocampus revealed that 5-HT2A receptor expression did not change following sleep deprivation, and its subsequent recovery profile after 24 and 48 hours was relatively constant as well. However, immunohistochemistry data showed
conflicting results. It clearly demonstrated that in the CA1 pyramidal layer of the hippocampus, sleep deprivation induced an increase in 5-HT$_{2A}$ expression. After 24 hours of sleep recovery, expression reverted back to control levels. These findings may point to 5-HT$_{2A}$ receptors being up-regulated in some areas of the hippocampus, but simultaneously being down-regulated in other areas of the hippocampus. Because we only focused on the 5-HT$_{2A}$ expression in the CA1 region of the hippocampus in immunohistochemistry, we are unable to make any conclusions on 5-HT$_{2A}$ expression patterns elsewhere. Future studies will be required to determine whether the CA3 region, the dentate gyrus, the subiculum or the entorhinal cortex experience decreased 5-HT$_{2A}$ expression after sleep deprivation.

An interesting effect was observed after 48 hours of recovery sleep in the immunohistochemistry studies. 5-HT$_{2A}$ expression in the CA1 pyramidal layer, after having already recovered within 24 hours, appeared to increase again. It seemed unlikely that this effect was related to sleep deprivation. Rather, we think perhaps the social environment of the animals played a role. After removing them from their litter mates at the UBC animal facility for experimental treatment, they are housed in isolation until the end of the treatment. Rats subjected to 12 hours of sleep deprivation and subsequent 48 hours of recovery have spent a total of 60 hours in isolation by the end of the treatment. Many studies have reported alterations in the behaviour of socially isolated rats, with some specifically implicating 5-HT neural circuitry changes and development of schizophrenia-like symptoms (Fone and Porkess 2008; Lukkes et al. 2009; McCool and Chappell 2009; Stevens et al. 1997). While the isolation treatment is of shorter duration in this experiment, it is possible that some changes are already starting to take place. As mentioned before, 5-HT$_{2A}$ activity has been associated with psychosis (Horacek et al. 2006), thus making the observation of schizophrenia-like symptoms more interesting. It is plausible that due to isolation for an extended period of time, 5-HT$_{2A}$ expression begins to increase, and this increase eventually leads to development of psychotic behaviour. Unfortunately, for the animals used in this study, we were not able to correlate psychotic behaviour to long periods of social isolation in 48-hour recovery animals. We were not looking specifically for psychotic behaviour, but we did not notice any significant
changes in the behaviour of 48-hour recovery animals compared to normally sleeping control animals. In the future, a control mimicking the isolation period, without the intervention of sleep deprivation, should be employed to determine whether social isolation of the animal alone for 60 hours can induce changes to 5-HT\textsubscript{2A} expression in the CA1 region.

Very few studies would agree that acute sleep deprivation for a short duration of time, for example one night, results in the development of psychotic symptoms. At worst, subjects would experience cognitive deficits and negative mood, but usually nothing that would be classified as psychotic. However, psychosis does eventually manifest after extended periods of continuous sleep deprivation, usually after five nights, as hallucinations, paranoia and profound confusion have all been observed (West et al. 1962). In this present study, we found conflicting changes in 5-HT\textsubscript{2A} following 12 hours of sleep deprivation. It is possible that this treatment was too short to elicit any significant changes in overall 5-HT\textsubscript{2A} receptor expression, but apparent increases detected in the CA1 region of the hippocampus may accumulate in the long-term to ultimately contribute to the development of psychotic behaviour.

4.3 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} Heterodimerization

GPCRs are known to form homodimers and heterodimers naturally (Milligan 2009; Terrillon and Bouvier 2004). For GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2, heterodimerization is necessary for proper targeting of the receptor to the cell surface (Margeta-Mitrovic et al. 2000). For other receptors, heterodimerization appears to play a regulatory role in altering receptor-agonist binding affinities (Rocheville et al. 2000; Jordan and Levi 1999). Tadavarty et al. reported recently that sleep deprivation induced changes in dimerization patterns of GPCRs in the hippocampus (Tadavarty et al. 2011). Specifically, mGluR\textsubscript{1α}-GABA\textsubscript{B}R1, mGluR\textsubscript{1α}-GABA\textsubscript{B}R2, and GABA\textsubscript{B}R1-GABA\textsubscript{B}R2 dimerization patterns were affected. In addition, it was the first reported discovery of mGluR\textsubscript{1α}-GABA\textsubscript{B}R1 and mGluR\textsubscript{1α}-GABA\textsubscript{B}R2 heterodimers. These findings raise the possibility that other novel GPCR dimers exist.
5-HT₁A and 5-HT₂A receptors are intriguing targets for heterodimerization. They are both located post-synaptically on the pyramidal neurons of the hippocampus, which puts them in close proximity to mGluR₁α, GABA₉₁, and GABA₂ receptors. mGluR₁α, GABA₂₁, and GABA₂₂ have all shown the ability to form heterodimers, thus it is not unreasonable that they also interact with 5-HT receptors. This study has already revealed that the expression patterns of 5-HT₁A and 5-HT₂A are altered by SD; could heterodimerization be another important regulatory element for these receptors?

Both 5-HT₁A and 5-HT₂A were individually probed for their possible heterodimerization with mGluR₁α, GABA₂₁, or GABA₂₂ via CoIP. However, CoIP revealed no evidence of any heterodimers – the regions of the gel where we anticipated seeing heterodimer bands were mostly devoid of bands.

In the case of 5-HT₂A and GABA₂₁, a band corresponding to the 5-HT₂A monomer was observed in the control and sleep deprived lanes, which may indicate that GABA₂₁ was able to co-precipitate 5-HT₂A, but their interaction was disrupted during heating of the sample. Two observations point to this band being a mere artefact rather than a true interaction. Firstly, the expression profile of this band defies rational explanation. The band appeared darker in the SD lane than the control lane, which suggests an increase following SD, but it was absent in the 24-hour recovery lane, which suggests subsequent abolishment, then reappeared faintly after 48 hours of recovery, which suggests the dimer reappears after initially disappearing at 24-hour recovery. Secondly, this band can be detected faintly in the no IP antibody control lane of this gel and the 5-HT₂A- GABA₂₂ CoIP gel, indicating that it is not specific to the precipitated proteins of GABA₂₁ (or GABA₂₂ for that matter).

In fact, very few examples of 5-HT receptor heterodimers exist in the literature. 5-HT₂A has been found to form dimers with dopamine D₂ receptors when co-expressed in human embryonic kidney 293 (HEK293) cells via the fluorescence resonance energy transfer (FRET) technique (Lukasiewicz et al. 2010). Because the conditions we used were relatively stringent (with the use of denaturing detergents and reducing agents), we do not rule out that transient heterodimers of 5-HT₁A and 5-HT₂A may exist. However, the
mGluR1α-GABAB1, mGluR1α-GABAB2, and GABAB1-GABAB2 heterodimers which were observed in previous studies used the same stringent conditions as the protocol employed in this study. Therefore, the mGluR1α and GABABR heterodimers appear to be more stable and resistant to dissociation than any potential 5-HT1A or 5-HT2A heterodimers.

4.4 Response of Hippocampal CA1 Neurons to 5-HT Following Sleep Deprivation

From a functional perspective, what do changes in 5-HT1A and 5-HT2A expression in the hippocampus following SD mean? Do they actually correlate to changes in hippocampal 5-HT transmission? This was investigated by measuring the effect of 5-HT on the field excitatory post-synaptic potentials (fEPSPs) of CA1 pyramidal neurons evoked by stimulation of the CA3-CA1 glutamatergic synapses in the rat hippocampus. These are well studied synapses which have been associated with memory formation (Bliss and Collingridge 1993). fEPSPs were measured in the absence and presence of different concentrations of 5-HT to generate a 5-HT concentration-fEPSP dose-response curve. The dose-response curve between control and sleep deprived animals were compared to determine if SD induces changes to the effects of 5-HT. The recovery of potential changes was also investigated by measuring fEPSPs in 24-hour and 48-hour recovery animals.

When 5-HT was applied to the hippocampus, fEPSPs measured in the stratum radiatum in the CA1 region were depressed. Response depression was observed from a range of 1μM 5-HT to 40μM 5-HT in a dose-dependent manner. Following sleep deprivation, the depressive actions of 5-HT was further enhanced – the same concentration of 5-HT produced a greater depression in the fEPSPs of SD hippocampal slices. This effect was most obvious at 10μM 5-HT, where the average fEPSP responses of SD slices were almost half the magnitude of control slices. No significant difference was observed at lower concentrations, which perhaps suggests that not enough 5-HT molecules were available at these concentrations to bind to the excess 5-HT1A and 5-HT2A receptors.
Once the 10μM concentration was reached, more 5-HT molecules were available to occupy and activate the excess 5-HT receptors. At higher concentrations, it appeared that substantial increases in 5-HT concentration were unable to produce proportionally large suppressions of fEPSPs, indicating the approach to a maximal effect. In fact, we observed that 40μM of 5-HT produced the maximum possible depression of fEPSPs. SD responses to 5-HT were also not significantly different from control responses at high concentrations. This is probably due to the depletion or occupation of intracellular secondary messengers responsible for the downstream effects of receptor activation. Such secondary messengers may include G proteins, adenylate cyclase, various protein kinases, or ions such as Ca$^{2+}$. Even with increased 5-HT receptor occupancy, the magnitude of downstream effects is mediated by the availability of secondary messengers to facilitate these effects. Generally, the number of receptors available far exceeds the number of occupied receptors required to elicit a full response (Lambert 2004).

The response to 5-HT demonstrated that alterations observed in 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor expression following sleep deprivation can be correlated to functional changes in agonist effects. However, the 5-HT effects did not correlate particularly well with either receptor specifically. For 5-HT$_{1A}$, which is known to exert inhibitory effects, increased expression after SD correlated well with enhanced 5-HT action after SD, but the actions of 5-HT recovered after 24 hours while increased 5-HT$_{1A}$ expression persisted despite 48 hours of recovery. For 5-HT$_{2A}$, increases in expression (at least in CA1 pyramidal neurons) and subsequent decrease after 24 hours of recovery seemed to correlate well with 5-HT actions, but no additional effects were observed for 5-HT actions after 48 hours of recovery while 5-HT$_{2A}$ expression appeared to increase again. Furthermore, due to its G protein coupling mechanism, 5-HT$_{2A}$ is commonly regarded as an excitatory receptor (Puig and Gulledge 2011), which, if increased, should produce an effect opposite to the observed 5-HT actions. Taken together, these results suggest that the overall 5-HT effect is not primarily mediated by one receptor subtype; rather, it is probably determined by the combined effects of 5-HT$_{1A}$ and 5-HT$_{2A}$ as well as possibly the other 5-HT receptor subtypes expressed in the hippocampus.
Nevertheless, enhanced 5-HT action following SD has implications for drugs targeting the serotonergic system. For drugs that theoretically increase 5-HT transmission, such as anxiolytics and antidepressants, SD patients may possibly experience a greater therapeutic effect, along with greater susceptibility for side effects. For drugs that theoretically decrease 5-HT transmission, such as atypical antipsychotics, SD patients may possibly experience less therapeutic effect compared to normally sleeping individuals.
Chapter 5: Conclusions

In this study, we investigated the effects of sleep deprivation on 5-HT activity in the rat hippocampus, with a particular focus on two of its receptors – 5-HT$_{1A}$ and 5-HT$_{2A}$. Western blot revealed that 5-HT$_{1A}$ expression was significantly increased in the hippocampus following 12 hours of sleep deprivation. Even after animals were allowed to sleep freely for the next 24 hours, 5-HT$_{1A}$ expression remained elevated. Expression decreased slightly after 48 hours of recovery but was still elevated compared to controls, although this difference was not statistically significant. Immunohistochemistry localized these changes to the CA1 pyramidal neurons of the hippocampus.

For 5-HT$_{2A}$ expression, western blot revealed no significant changes following 12 hours of sleep deprivation. However, immunohistochemistry showed that 5-HT$_{2A}$ expression was significantly increased in the CA1 pyramidal layer of the hippocampus, with subsequent recovery after 24 hours. Because the total hippocampal expression of 5-HT$_{2A}$ was unchanged, we think that 5-HT$_{2A}$ may be down-regulated in other areas of the hippocampus, such as the dentate gyrus, subiculum or entorhinal cortex, which were all included in the hippocampal tissue used for western blot analysis. Immunohistochemistry also revealed an unexpected increase in 5-HT$_{2A}$ expression after 48 hours of recovery. Because expression had already recovered fully within 24 hours after SD, we think that this effect may be caused by the isolation of animals during treatment rather than sleep deprivation.

The heterodimerization between 5-HT$_{1A}$ or 5-HT$_{2A}$ and either of mGluR1$\alpha$, GABABR1 or GABABR2 was investigated by CoIP. No evidence of heterodimerization was observed for any of the combinations, although we do not rule out that transient heterodimers exist due to the stringent conditions used in our protocol.

In rat hippocampal slices of control animals, 1$\mu$M to 40$\mu$M of bath-applied 5-HT dose-dependently suppressed the fEPSP response of CA1 pyramidal neurons. Following 12 hours of sleep deprivation, the dose-response curve was observed to be steeper, indicating
an enhancement in the suppressant action of 5-HT. This difference was most significant at a concentration of 10μM 5-HT, and recovered within 24 hours.
Chapter 6: Future Directions

This study revealed some important changes to 5-HT, 5-HT_{1A}, and 5-HT_{2A} in the hippocampus following sleep deprivation, but inconsistencies still exist. Namely, the actions of 5-HT do not correlate well with either 5-HT_{1A} or 5-HT_{2A} expression changes, indicating that other 5-HT receptors may play a crucial role as well. It is worthwhile to investigate whether other 5-HT receptor subtypes also change their expression after SD. Aside from 5-HT_{1A} and 5-HT_{2A}, some of the more commonly studied 5-HT receptors include 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2B}, and 5-HT_{2C}. Also, the specific contributions of 5-HT_{1A} and 5-HT_{2A} may be investigated using specific agonists and antagonists to these receptors in electrophysiology studies. Commonly used agonists include 8-Hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) for 5-HT_{1A} and N-(2-methoxybenzyl)-2,5-dimethoxy-4-iodophenethylamine (25I-NBOMe) for 5-HT_{2A}; commonly used antagonists include WAY100135 for 5-HT_{1A} and volinanserin for 5-HT_{2A}.

The recovery periods after sleep deprivation could be extended to resolve a couple of conflicts. Firstly, it appeared that 5-HT_{1A} increase had not fully recovered after 48 hours of recovery. Longer recovery periods, for example, up to one week, would enable us to determine when or if 5-HT_{1A} subsides to its original expression levels, or if these changes could be permanent. Secondly, the 5-HT_{2A} increase in the CA1 region observed in immunohistochemistry after 48 hours of recovery could be investigated to determine if the up-regulation persists beyond 48 hours, and whether it becomes worse. Alongside proper controls, which would involve isolating animals under normally sleeping conditions for 48 hours or more, we could determine whether social isolation is truly responsible for the observed changes in 5-HT_{2A} expression.

The FRET technique has revealed that 5-HT_{2A} appears to form heterodimers with dopamine D_{2} receptors. We did not observe any heterodimers with CoIP, but transient heterodimers may have been overlooked due to the stringent conditions we used in our procedure. FRET can be used as a better method of identifying such heterodimers by visualizing receptors in their natural state. In addition, potential associations may be
detected in vivo with this technique, ensuring that the observed interactions actually take place in a living cell.

5-HT is only one of the many neurotransmitters regulating hippocampal CA3-CA1 transmission. Glutamate, GABA, dopamine, and melatonin are all known to play a role. It would be interesting to study the agonist effects of these compounds on CA1 fEPSPs. Their targets comprise of many different GPCR subtypes, some of which may undergo changes following sleep deprivation. Possible candidates include the metabotropic glutamate receptor mGluR5, GABA receptors GABA_B1 and GABA_B2, dopamine receptors D_1 and D_2, and melatonin receptors MT_1A and MT_1B. Heterodimerization between these receptors are also a possibility. We could use the experimental design of this study and apply it to any of the mentioned neurotransmitters and receptors to develop a new worthwhile study.
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