Effect of Growth Hormone on Hippocampal Synaptic Function during Sleep Deprivation

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ABSTRACT

Long term potentiation (LTP) in the hippocampus is considered a cellular basis of learning memory. Sleep deprivation, especially rapid eye movement (REM) sleep deprivation, impairs learning and memory as well as LTP. Since most of the previous LTP studies were conducted in the in vitro condition, the full consequences of sleep deprivation (SD) in the living animal are yet to be found. Thus, I tested hippocampal LTP in living animals after 5 days of REM sleep deprivation to determine the effect of SD in vivo. SD also disrupts growth hormone (GH) release. Recent evidence indicates that GH regulates cognitive and hippocampal synaptic function. However, the relationship between GH and synaptic function during SD is not well established. Since the N-methyl-D-aspartate receptor (NMDAR) has an important role in inducing LTP, I hypothesized that loss of normal GH signals during SD would impair synaptic NMDAR expression and function, and treating SD animals with GH would restore normal NMDAR expression and function. To test my hypothesis, I treated animals with GH during 3 days of SD, and tested NMDAR dependent hippocampal synaptic

functions and measured synaptic expression of NMDAR subunits. In addition, I measured corticosterone concentration in control and sleep deprived animals to determine stress levels in each treatment. My results showed that LTP in vivo was impaired after 5 days of SD. NMDAR function was impaired and there was a selective loss of NR2B NMDAR subunits from synaptic membranes. These changes in NMDAR function and expression can explain the LTP impairment caused by SD. In agreement with my hypothesis, the LTP and NMDAR impairments were reversed by GH treatment during SD. Finally, there was no difference in corticosterone concentration between control and SD animals, demonstrating that differences in stress were not responsible for any of the changes I observed during SD.

DEDICATION

To my loving dearest husband, Han,
And my parents
For their endless love, support and patience

To my little friend, MJ,
For being there at my lowest point
And teaching me the joy of being a servant

To God
For giving me all of the above

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First and foremost, I thank God for giving me the strength to go through the course of this work.

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LIST OF ABBREVIATIONS/SYMBOLS

ACTH - adrenocorticotropic hormone

AMPA - α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

AP5 - 5-amino-phosphopentanoic acid or 5-amino-phosphovaleric acid (APV)

ACSF - artificial cerebrospinal fluid

BBB - blood-brain barrier

CA - Cornu Ammon of the hippocampus

CaMKII - calcium-calmodulin-dependent protein kinase II

CNS - central nervous system

CREB - cAMP-responsive element-binding protein

CRH - corticotrophin-releasing hormone

CSF - cerebrospinal fluid

EEG - electroencephalogram

EPSC - excitatory postsynaptic current

EPSP - Excitatory post-synaptic potential

ERK- extracellular signal-regulated kinase or mitogen-activated protein kinase (MAPK)

GABA - γ-aminobutyric acid

GH - growth hormone

GHR – growth hormone receptor

GHRH - growth hormone-releasing hormone

HPA - hypothalamic-pituitary-adrenal

IGF-1 - insulin-like growth factor 1

IRS-1 - insulin receptor substrate-1

JAK2 - Janus kinase 2

LTD - long term depression

LTP - long term potentiation

MAPK - mitogen activated protein kinase or extracellular signal-regulated kinase (ERK)

NMDAR - N-methyl-D-aspartate glutamate receptor

NO - Nitric oxide (NO)

PI3K - phosphatidylinositol 3-kinase (PI3K)

REM - rapid eye movement

rhGH - recombinant human GH

SD - sleep deprivation

STAT - signal transducers and activators of transcription

SWS - slow wave sleep

TBS - theta burst stimulation

Introduction

Hippocampus and Synaptic plasticity

The hippocampus has been recognized as playing a vital role in the formation of memory since the observations of Milner in 1957. Milner studied a patient, known as H.M., who had a bilateral hippocampal resection as a treatment for intractable epilepsy. As a result of his surgery, H.M. suffered dense, anterograde amnesia through the end of his life in 2008 (Scoville and Milner 1957). In 1982, Morris et al. used an animal model to confirm that the hippocampus is needed for spatial memory. Morris et al. surgically removed the hippocampus and then tested spatial memory in a water maze. Animals with hippocampal lesions had impaired spatial memory (Morris, Garrud et al. 1982).

The hippocampus is a cylindrical structure located inside the temporal lobe of the cerebral cortex. Humans and other mammals have two hippocampi, one in each side of the brain. The hippocampus proper can be divided into four regions, CA1 to CA4 (from the latin *cornu Ammon*, or Ammon's horn). The dentate gyrus, the subiculum and the entorhinal cortex are included in the more general term

hippocampal formation or hippocampal region. The main inputs to the hippocampus and dentate gyrus arise from the entorhinal cortex, the septal region, and the contralateral hippocampus. The output or principal neurons of the hippocampus are the pyramidal neurons found in CA3 to CA1 region. The pyramidal neurons in the CA1 region receive input mostly from CA3 then project heavily to neurons in the adjacent subiculum. The axons from the CA3 neurons which synapse onto CA1 pyramidal neurons are called Schaffer collateral. The Schaffer collateral/CA1 pyramidal neurons synapses have been studied most extensively for synaptic plasticity in the hippocampus (Shepherd, 1990).

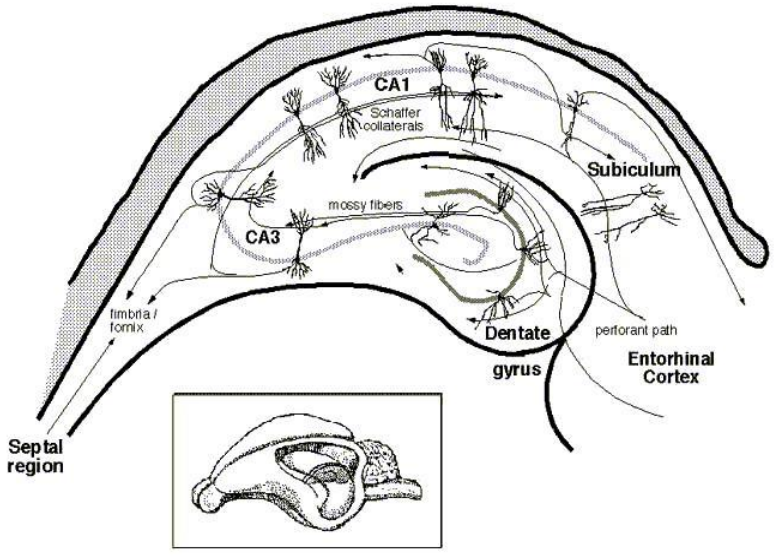


Figure 1. Cross-section of hippocampal formation

It is widely believed that a long-lasting change in synaptic function in the hippocampus is the cellular basis of learning and memory. The most thoroughly characterized example of such synaptic plasticity is long-term potentiation (LTP) (Malenka and Nicoll 1999). LTP was first demonstrated by Bliss and Lomo in 1973. They reported that brief high-frequency stimulation of excitatory pathways in the hippocampus caused an increase in synaptic strength that last for hours (Bliss and Lomo 1973). Induction of LTP requires the simultaneous activation of both pre- and postsynaptic neurons. Excitatory post-synaptic potentials (EPSPs) are measured as an indicator of postsynaptic activity. A single stimulation given to the presynaptic axons produces a relatively brief depolarization of the postsynaptic cell, whereas high-frequency (tetanic) stimulation (i.e., 100Hz, for 1s, which is commonly used to induce LTP) of the presynaptic axons produces a longer and larger compound, or summed, EPSP. Signaling events triggered during the postsynaptic response to high frequency presynaptic stimulation are essential for LTP induction. LTP may last for hours or days and this is the reason why it is called *long*-term potentiation (Craver 2003).

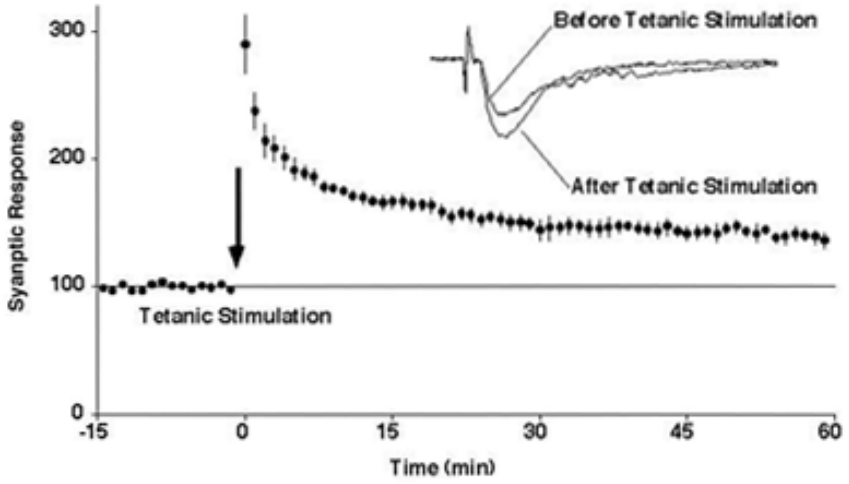


Figure 2. LTP in Hippocampal CA1 Region

Over the last couple of decades, knowledge of the mechanisms underlying LTP has increased rapidly. It is well established that increased calcium concentration in the postsynaptic cell during high frequency stimulation is required to induce LTP (Malenka and Nicoll 1999). The postsynaptic N-methyl-D-aspartate glutamate receptor (NMDAR) plays an important role as a calcium entry pathway during LTP induction. The neurotransmitter glutamate, which is released from

presynaptic terminals in the hippocampus, binds to two major classes of postsynaptic receptor: NMDA and AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate). The NMDA receptor is blocked by magnesium at resting membrane potentials and becomes activated only during coincident membrane depolarization and glutamate binding, which occurs during the high frequency stimulation used to induce LTP. Activated NMDA receptors are calcium permeable and therefore increase intracellular calcium levels in the postsynaptic cells during LTP induction. Increased calcium concentration in the postsynaptic cells after NMDA receptor activation leads to activation of several downstream signaling molecules. One of these molecules is calcium-calmodulin-dependent protein kinase II (CaMKII). It has been suggested that CaMKII becomes persistently activated through autophosphorylation during LTP induction, and this autophosphorylation helps to maintain LTP for several hours following induction (Lisman and Goldring 1988; Bliss and Collingridge 1993). In addition to phosphorylating itself, CaMKII phosphorylates AMPA receptors and thereby regulates their activity. AMPA receptors are permeable to sodium and potassium, and this permeability allows these receptors to rapidly change postsynaptic

membrane potential. Phosphorylation of AMPA receptors by CaMKII increases conductance which results in enhancement of AMPA receptor-mediated postsynaptic responses during LTP (Benke, Luthi et al. 1998). In addition, CaMKII controls insertion of new AMPA receptors into the postsynaptic membrane by activating rasGAP which stimulates mitogen activated protein kinase (MAPK, also known as extracellular signal-regulated kinase or ERK) and phosphatidylinositol 3-kinase (PI3K) (Malinow and Malenka 2002). Increased calcium concentration in the postsynaptic cell during LTP induction also induces presynaptic modification. Presynaptic modification is thought to occur through formation of a retrograde messenger in postsynaptic neurons which is released to alter presynaptic function. Nitric oxide (NO) is the leading candidate for this retrograde messenger. NO is released from cultured neurons following NMDA receptor activation (Garthwaite, Charles et al. 1988), and inhibitors of NO synthase block the induction of LTP (O'Dell, Hawkins et al. 1991; Haley, Wilcox et al. 1992). Although a complete description of the mechanisms of LTP induction does not yet exist, and some areas of controversy remain, the role of postsynaptic calcium in activating downstream signaling molecules is well established.

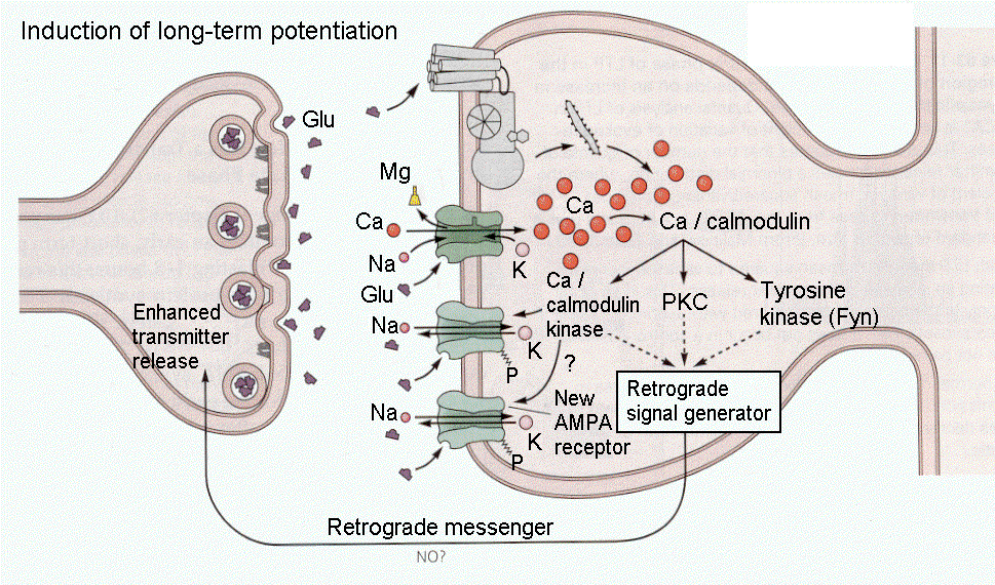


Figure 3. Mechanisms of LTP induction (Kandel, ER, JH Schwartz and TM Jessell (2000) *Principles of Neural Science*. New York: McGraw-Hill.)

Since the NMDA receptor has a critical role in inducing LTP, the relationship between LTP and learning and memory was tested by disrupting NMDA receptor function and looking for memory impairment. In 1986 Morris et al. showed that blocking NMDA receptors with the NMDA receptor antagonist, AP5 (5-amino-phosphopentanoic acid, or APV, 5-amino-phosphovaleric acid), inhibited both LTP and spatial learning (Morris, Anderson et al. 1986). Subsequently, Tsien et al. (1996) examined genetically manipulated mice in which the NMDA receptor was deleted in hippocampal area CA1 and reported that LTP as well as spatial memory were impaired (Tsien, Huerta et al. 1996). Manipulation of downstream targets of NMDAR receptor activation was also done to test the relationship between LTP and learning and memory. For example, it has shown that inhibition of ERK by a selective MAPK cascade inhibitor, such as PD098059, results in impaired induction of LTP (English and Sweatt 1997) as well as spatial memory (Blum, Moore et al. 1999). These studies have shown that disruption of components of the LTP signaling pathway impairs learning and memory. Because of these findings, LTP is considered a cellular basis of learning and memory.

Sleep, learning and memory, and LTP

The role of sleep in cognitive and brain function remains largely unknown even though there has been a rapid increase in understanding of the processes which generate and maintain sleep. A number of hypotheses have been proposed to link sleep and brain function involving, for example, energy conservation, brain thermoregulation, brain detoxification, and tissue restoration (Beatty 2000). Another hypothesis proposes that sleep periods are favorable for brain plasticity and for learning and memory (Blissitt 2001; Maquet 2001). A number of studies have shown that sleep has a beneficial effect and sleep deprivation (SD) has a negative effect on learning and memory. In 1977, Benson and Feinberg tested memory after 8 hr of sleep or SD. The subjects learned a paired-associate list of common nouns before sleep or SD treatment. After 8 hours of sleep or SD treatment, subjects had to recall each associated noun when the paired noun was presented. In this study the subjects who slept after learning recalled better than those who had been sleep deprived (Benson and Feinberg 1977). More recently, Harrison and Horne showed that SD impaired temporal

(recency discrimination) memory. The subjects learned two sets of 12 faces which were introduced as List A and List B (total 24 faces). During the test session, 24 faces (either from List A or List B) were randomly given to the subjects and the subjects were asked to specify whether the face was included in List A or List B. The results showed that the SD group scored significantly lower than control group indicating that temporal (recency) memory was impaired after 36 hr of SD (Harrison and Horne 2000).

Sleep consists of several distinct phases. Stages 1 to 4 are called non-rapid eye movement (REM) or slow wave sleep (SWS). Each stage is distinguished by distinct patterns of EEG (electroencephalogram) activity. Stage 1, the lightest sleep, is characterized by short periods of theta activity (4-7 Hz). Stage 2 is marked by the appearance of sleep spindles which are rhythmic bursts of 12 to 15 Hz EEG activity. Stages 3 and 4 show low frequency (1-4 Hz) delta waves. Stage 4 is the deepest stage of slow wave sleep, from which arousal is most difficult. The final stage is REM sleep in which the EEG is low amplitude and desynchronized, very similar to the waking EEG. However, unlike the waking or

non-REM sleep states, periodic suppression of muscle tone (except for ocular muscles), frequent bursts of rapid eye movement and vivid dreams occur during REM sleep. In humans, each of these stages occurs five to six times nightly with a complete cycle of stages lasting 60 to 90 minutes (Beatty 2000).

The role of each sleep stage in the learning and memory process is not clear. However, many experimental findings indicate that REM sleep has an important role in the learning and memory process (Siegel 2001). One line of evidence supporting a role for REM sleep in learning and memory is the increased duration or density of REM sleep which is observed after learning. Mandia et al. (1998) recorded EEG activities during the sleep periods of subjects who learned Morse Code before the onset of sleep. They found out that learning prior to sleep increased the number and duration of REM sleep episodes (Mandai, Guerrien et al. 1989). Smith and Lapp (1991) also recorded sleep in students after an intensive exam period. They found no change in REM sleep duration, but found an increase in the density of REM sleep eye movement (Smith and Lapp 1991). A second line of evidence indicating the importance of REM sleep in learning and

memory is the finding that REM sleep deprivation impairs spatial reference memory. Many of these studies have used animal models to test the effect of REM sleep deprivation on learning and memory. The 'inverted flower pot' or pedestal technique is perhaps the most widely used method for inducing REM sleep deprivation in animals. Animals, typically rats, are placed on a small platform (in the original studies, an inverted flower pot) surrounded by water. Upon entry into REM sleep, the rats lose muscle tone and fall into the water. Control animals are placed either on large platforms or are housed in normal cages (Horne and McGrath 1984). Animals also require special methods for testing learning and memory. The Morris water maze is widely used to investigate memory in animals. In the Morris water maze, the subject must find a platform hidden below the surface of water which has been made opaque by the addition of dye or nonfat powdered milk. Animals receive repeated trials with the hidden platform kept in the same location. The time required to find the hidden platform is used as a measure of spatial memory (Brandeis, Brandys et al. 1989). In 1997, Youngblood et al, used the 'inverted flower pot' method to induce REM sleep deprivation in rats and tested memory using the Morris water maze technique. They reported a

significant decrement in performance in the water maze task after 48hr of REM sleep deprivation (Youngblood, Zhou et al. 1997).

In addition to disrupting learning and memory, SD impairs synaptic plasticity in the hippocampus. In 2002, Campbell et al. showed that LTP was impaired in hippocampal slices from animals deprived of all sleep for 12hr (Campbell, Guinan et al. 2002). The effect of selective REM sleep deprivation on hippocampal synaptic plasticity and LTP has also been tested. McDermott et al. showed that 72hr of REM sleep deprivation impaired spatial memory in rats. In addition, they showed that CA1 neuron excitability and LTP were inhibited by SD (McDermott, LaHoste et al. 2003). In 2003, Davis et al. reported that maintenance of LTP was attenuated in hippocampal slices from 24, 48 and 72hr sleep deprived rats, and the induction of LTP was impaired by the two longer durations of REM sleep deprivation (48 and 72 hr) (Davis, Harding et al. 2003). These findings suggest that sleep, especially REM sleep, is required for learning and memory and synaptic plasticity in the hippocampus.

Growth hormone (GH) and Cognitive function

GH, a large 191 amino acid polypeptide (22 Kda), is secreted from somatotroph cells in the anterior pituitary. GH is essential for somatic growth and metabolism (Gotherstrom, Svensson et al. 2001). Synthesis and secretion of GH are regulated by two hypothalamic neurohormones: growth hormone-releasing hormone (GHRH), which simulates, and somatostatin, which inhibits GH. GHRH and somatostatin are secreted into the pituitary portal circulation at the median eminence, and they are carried into the anterior pituitary by the blood. GH secretion is also stimulated by exercise (Berg and Bang 2004), starvation, (Tanaka, Nakahara et al. 2004) and sleep (Obal and Krueger 2004; Steiger 2007), whereas aging decreases GH secretion (Corpas, Harman et al. 1993). GH has both direct and indirect actions on target tissues. Indirect effects of GH are mediated mainly by insulin-like growth factor I (IGF-1). IGF-1 is produced in response to GH stimulation in the liver and at other sites of GH action. IGF-1 in turn, feeds back on the hypothalamus and pituitary to inhibit GH release.

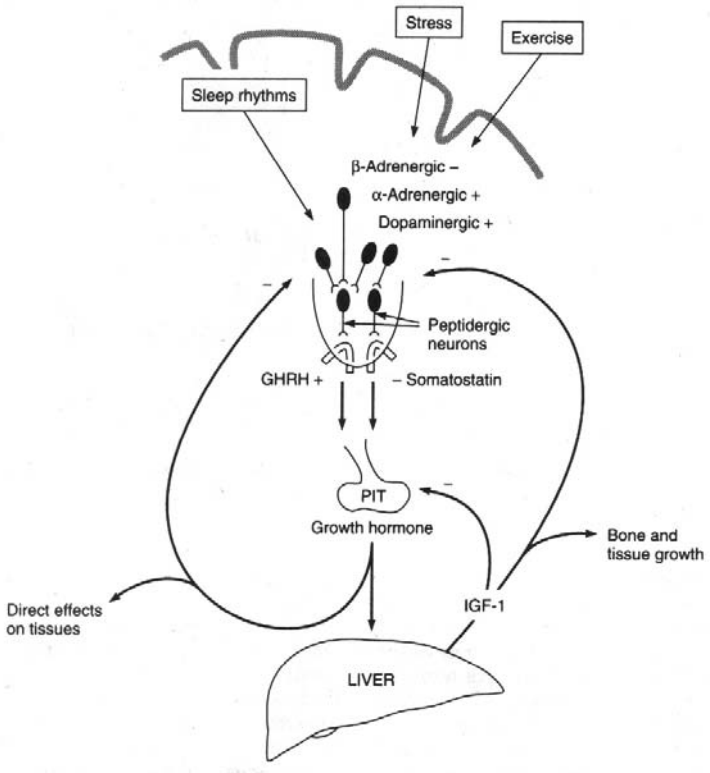


Figure 4. Schematic diagram of the control of growth hormone secretion

(modified from Reichlin S: Neuroendocrinology. In : Williams Textbook of Endocrinology, 7th ed. Wilson JD. Foster DV [editors]. Saunders, 1985)

The GH receptor (GHR) is expressed in several regions of the brain including the hippocampus. Immunohistochemistry and in situ hybridization in the neonatal rabbit brain revealed the strongest GHR signal in the cerebral cortex and the pyramidal cells of the hippocampus (Lobie, Garcia-Aragon et al. 1993). Previously, it was thought that the blood-brain barrier (BBB) was almost impermeable for most peptide hormones including GH. However, in recent years the ability of GH to cross the BBB has been recognized (Coculescu 1999; Aberg, Brywe et al. 2006). In 1995, Johnsson et al. showed increased GH concentration in the cerebrospinal fluid in GH-deficiency patient after recombinant human GH (rhGH) treatment (Johansson, Larson et al. 1995). In addition, GHRs are present in the choroid plexus at higher levels that in any other brain tissue, and these receptors may contribute to a transport system for moving GH across the blood-cerebrospinal fluid (CSF) barrier (Lai, Emtner et al. 1991). Others have provided direct evidence that GH enters the brain through a non-saturable process, suggesting that GH crosses the BBB by simple diffusion despite its large molecular weight (Pan, Yu et al. 2005). IGF-1 is also expressed widely in the brain (D'Ercole, Ye et al. 1996). In the CNS, IGF-1 promotes cell proliferation, cell migration, and

cell differentiation during brain development (Anlar, Sullivan et al. 1999). It is well established that IGF-1 reaches the CNS from the peripheral blood stream by a specific carrier in the BBB (Pan, Yu et al. 2005). However, it also has been reported that IGF-1 is locally produced in the brain (Sun, Al-Regaiey et al. 2005), suggesting that GH can regulate brain function directly through interaction with GHRs and indirectly through local production of IGF-1.

Recent evidence indicates that GH has effects on cognitive function (Nyberg 2000; Aberg, Brywe et al. 2006). Learning and memory impairment is a well known feature of GH deficiency in humans and a number of studies have shown that GH treatment improves learning and memory impairment in GH deficiency. Deijen et al. treated child-onset GH deficiency patients with GH and found that memory was improved after 1 year of treatment (Deijen, de Boer et al. 1998). With the same group of patients, Arwert et al. (2005) also reported that improved memory was maintained even after 10 years of GH treatment (Arwert, Deijen et al. 2005). In 2006, Le Greves, et at. treated hypophysectomized rats with recombinant human GH (rhGH) for 9 days. During treatment, they tested

memory using the Morris water maze and found that memory was improved with GH treatment in the hypophysectomized rats (Le Greves, Zhou et al. 2006). In addition, aging both inhibits GH release and impairs learning and memory. GH treatment improves spatial learning in aged rats (Ramsey, Weiner et al. 2004). Twenty-four month old rats were injected with porcine GH for 4 months and then spatial learning was tested. The GH treated aged rats showed improved spatial learning compared with saline treated aged rats. Moreover, there was no difference in learning between the GH treated 24 month old aged rats and saline injected 4 month old adult rats (Ramsey, Weiner et al. 2004).

GH and SD

GH is secreted in surges throughout the day but the largest surge of GH secretion occurs during sleep (Obal and Krueger 2004; Steiger 2007). Given the association between sleep and GH secretion, it is not surprising that sleep deprivation (SD) suppresses GH secretion. Kimura and Tsai (1984) investigated GH secretion in rats. They showed that GH secretion peaked during sleep onset and SD during this period prevented the normal high-level GH pulse (Kimura and Tsai 1984). In 2000, Brandenberger et al. showed a similar suppression of GH secretion during SD in human subjects (Brandenberger, Gronfier et al. 2000).

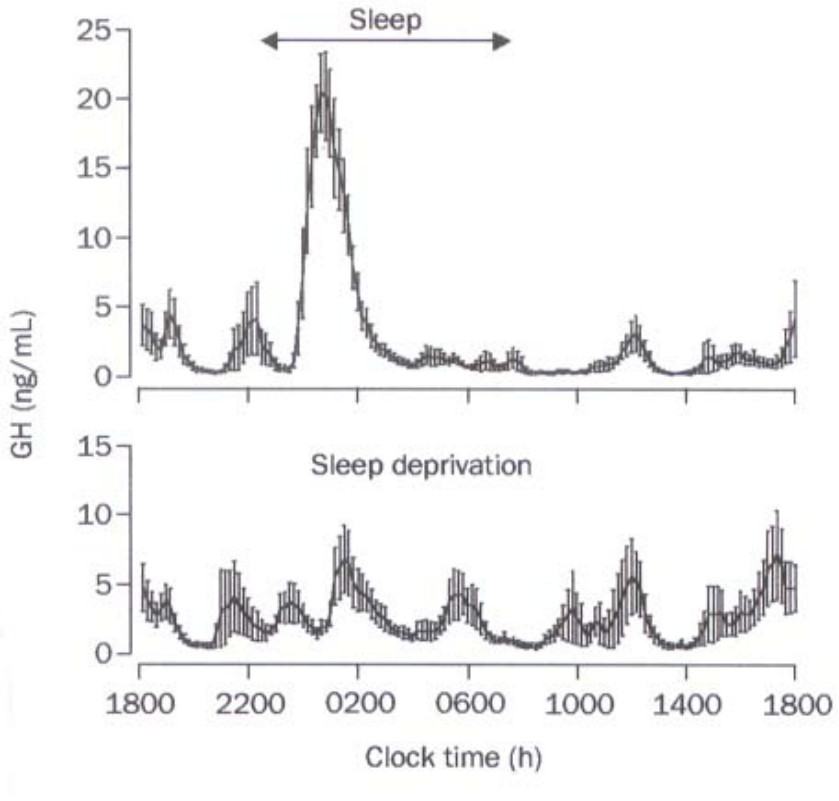


Figure 5. Effect of SD on 24 h GH secretion (Brandenberger et al. 2000)

Taken together, the previous studies have demonstrated that SD impairs NMDAR dependent hippocampal synaptic plasticity as well as GH release. Although both SD and GH have effects on cognitive and synaptic function, the effect of GH on synaptic function during SD has not been well investigated. To determine the effect of SD and GH on synaptic plasticity in the hippocampus, I addressed three related questions. First, does SD impair LTP in the hippocampus in vivo? To answer this question, I implanted electrodes directly into rat hippocampus and tested LTP after 5 days of REM sleep deprivation. Second, I hypothesized that if decreased GH release during SD is responsible for impairment of synaptic plasticity in the hippocampus, then GH treatment during SD should prevent this impairment. This hypothesis was tested by examining NMDAR function, which has an important role in inducing LTP, in hippocampal brain slices prepared from SD or control animals that had received GH or saline injections. To determine if GH treatment affected synaptic NMDAR expression, I used western blotting to measure NMDAR subunit levels in synaptosomal membrane fractions. And last, I asked whether the apparent effect of SD on synaptic plasticity might have resulted from stress which can be caused by SD procedures. To answer this

question, I compared the concentration of corticosterone, a stress related hormone, in serum from SD and control animals.

Chapter 1

Introduction

Long-term potentiation (LTP) in the hippocampus is widely used to study the cellular basis of learning and memory (Malenka and Nicoll 1999). LTP was first reported by Bliss and Lomo. They demonstrated that a brief high-frequency stimulation to excitatory pathways in the hippocampus caused an increase in synaptic strength that lasted for hours (Bliss and Lomo 1973). Activation of NMDA receptors and increased calcium concentration in the postsynaptic cell is required for induction of LTP (Malenka and Nicoll 1999). A number of studies have shown that blocking NMDA receptors impairs LTP as well as learning and memory (Morris, Anderson et al. 1986; Tsien, Huerta et al. 1996; English and Sweatt 1997; Blum, Moore et al. 1999). In 1986, Morris et al. showed that blocking NMDA receptors with the NMDA receptor antagonist, AP5, inhibited both LTP and spatial learning (Morris, Anderson et al. 1986). Subsequently, Tsien et al. (1996) examined genetically manipulated mice in which the NMDA receptor was deleted in hippocampal area CA1. They reported that LTP as well as spatial memory were impaired (Tsien, Huerta et al. 1996). These results indicate that the

learning and memory process and LTP induction share the same synaptic mechanisms.

Despite our increasing understanding of the processes generating and maintaining sleep, its function remains elusive. Among several hypotheses, it was suggested that sleep is involved in the brain plasticity which occurs during memory formation (Blissitt 2001; Maquet 2001). Sleep can be broadly considered as two states: rapid eye movement (REM) sleep and non REM sleep. REM sleep is characterized by periodic muscle suppression, frequent bursts of rapid eye movement and vivid dreaming. In addition, a low amplitude and desynchronized EEG, very similar to waking EEG activity, is prominent during REM sleep. By contrast, during non-REM sleep, the cortical EEG is slowed overall and progresses from intermittent spindling to a predominance of slow waves (Graves, Pack et al. 2001).

Several lines of evidences have suggested that REM sleep might play an important role in memory formation. It has been shown that REM sleep duration

and density is increased after learning (Mandai, Guerrien et al. 1989; Smith and Lapp 1991) and REM sleep deprivation impaired hippocampal dependent learning and memory (Youngblood, Zhou et al. 1997). REM sleep deprivation also impairs LTP in the hippocampus. Davis et al. (2003) reported that maintenance of LTP was attenuated in hippocampal slices from 24, 48 and 72hr sleep deprived rats and the longer durations of REM sleep deprivation (48 and 72 hr) impaired induction of LTP (Davis, Harding et al. 2003). In addition, McDermott et al. (2003) showed that neuronal excitability was reduced in CA1 neurons and induction of LTP was inhibited after 72 hr of REM sleep deprivation (McDermott, LaHoste et al. 2003).

Although previous investigations demonstrated disruption of LTP in isolated hippocampal slices following REM sleep deprivation, LTP in vivo may be affected differently due to REM sleep rebound and associated activity in endogenous neurotransmitter systems, influences which are lost during in vitro studies. REM sleep rebound was first proposed by Dement (1960) to describe the increase in frequency and duration of REM sleep after instrumental REM sleep deprivation (Dement 1960). It was suggested that REM sleep rebound occurs because the

REM sleep state has an important homeostatic role in the brain function and REM sleep loss has to be ‘repaid’ by a subsequent increase of REM sleep to maintain normal brain function (Jouvet 1994). In 2003, Wetzel tested the effect of REM sleep rebound on memory in rats. Rats were REM sleep deprived for 80 hr and then trained to learn a footshock-motivated brightness discrimination task in a Y-maze. After training, rats were allowed to freely sleep for 24 hr before a retention test. During post-training sleep, EEG activity was recorded to measure REM and non-REM sleep. There was a significant increase in number and duration of REM sleep episodes in the REM sleep deprived group compared to the control group. Also, the REM sleep deprived group scored significantly better on the retention test and there was a positive correlation between REM sleep rebound value and retention test scores (Wetzel, Wagner et al. 2003). These results show that REM sleep rebound after REM sleep deprivation has a positive effect on learning and memory.

REM sleep is regulated by three major neurotransmitters, norepinephrine,

serotonin, and acetylcholine. The noradrenergic (locus coeruleus) and the serotonergic (raphe nucleus) neurons in the brain stem are most active during waking and become progressively less active in the transition from non-REM (slow wave sleep) to REM sleep. On the other hand, the cholinergic neurons in the thalamus are active both during waking and REM sleep. During REM sleep deprivation, noradrenergic and serotonergic neurons continuously fire whereas cholinergic neurons decrease firing (Kalia 2006; Pal and Mallick 2007).

The hippocampus receives input from all three of these neurotransmitter systems. Serotonin receptors are found in the hippocampus and serotonin or serotonin receptor agonists have been shown to improve cognitive performance (Buhot 1997; Meneses 1999). Norepinephrine, briefly superfused during high-frequency stimulation in the rat hippocampal slice in vitro, produced a reversible increase in the magnitude, duration, and probability of induction of LTP in the CA3 subfield. Similar results were obtained with the β-adrenergic agonist, isoproterenol, whereas the β-adrenergic antagonist propranolol reversibly blocked long-term potentiation (Hopkins and Johnston 1984). Acetylcholine also has a

direct effect on hippocampal synaptic plasticity. Application of acetylcholine produced a gradually developing, long-lasting increase in the CA1 excitatory postsynaptic potential (Auerbach and Segal 1994). Collectively, these results indicate that REM sleep deprivation alters the function of these three neurotransmitter systems and that altered activity in these systems can in turn modulate hippocampal function, synaptic plasticity, and memory.

While long-lasting consequences of altered modulatory input to the hippocampus might be detectable in vitro, the full consequences may only be apparent when hippocampal function is assessed in vivo. I, therefore, investigated the consequences of REM sleep deprivation on hippocampal LTP in vivo, to compare with previous findings from in vitro studies.

Materials and Methods

Animals and REM sleep deprivation treatment

Male Sprague-Dawley rats (280-310 g) were subjected to REM sleep deprivation or control treatment. REM sleep deprived rats were placed individually on a small circular platform (10.5 cm diameter) in a water-filled tank (29 cm diameter). The platform was positioned 2.5 cm above water (6 cm depth). This procedure, the classic 'inverted flower pot' technique, selectively deprives rats of REM sleep (Horne and McGrath 1984): loss of muscle tone during REM sleep causes animals to contact the water and waken, but animals are not awakened out of non-REM sleep. Rats were treated for five consecutive days, except for a 1hr period each morning (10.00-11.00 a.m.), when rats were removed and placed in a home cage consisting of a clear circular tank with standard animal bedding. Animals were weighed and rectal temperatures were taken during this 1hr period. During the remainder of this 1hr period animals spent the majority of the time grooming, eating and drinking; animals rarely slept. Control rats were treated

identically to REM sleep-deprived rats, except the diameter of the platform was 28.0 cm, allowing them to obtain normal REM sleep. Food and clean water were freely available throughout treatment.

Figure 6. Control (left) and REM sleep deprivation (right) treatment using the 'inverted flower pot' technique. Control animals were housed on large platforms above water, which allowed REM sleep, whereas sleep deprived animals were kept on smaller platforms which prevented REM sleep. Food and fresh drinking water were continually available. Food was delivered through a tube attached to a wire cover, which has been removed to more clearly illustrate the relative sizes of the large and small platforms housing the animals.

Surgery

LTP was assessed in rats with chronic stimulating and recording electrodes implanted into the right hippocampus prior to treatment. Rats were anesthetized (halothane inhalation followed by chloral hydrate 300mg/kg i.p.), and mounted in a stereotaxic instrument (ASI Instruments). Two small holes were drilled for insertion of bipolar, teflon-insulated, stainless steel stimulating and recording electrodes into area CA1. Three additional holes were drilled for placement of stainless steel anchor screws. One of the anchor screws was used as an electrical ground point. Sterotaxic coordinates for the stimulating electrodes were 3.0 mm posterior to bregma, 2.0 mm lateral from the midline. Coordinates for the recording electrode, inserted at 10° from vertical, were 4.5 mm posterior to bregma, 4.0 mm lateral from the midline. Electrodes were initially lowered 3.0 mm, with final depths adjusted by monitoring evoked field potentials so that the largest negative field excitatory post-synaptic potentials (EPSPs) were obtained. After final positioning, electrode were secured with dental cement and placed into a plastic connector cap, which was fixed to the skull with dental cement.

Buprenophine (90 mg/kg, s.c.) was administered post-surgery for analgesia. Animals were allowed 7-10 days for recovery.

Recording

Animals received an initial recording session prior to treatment to allow habituation to the recording apparatus. All recordings were done in each animal's home cage. A flexible cable connected the plastic cap on the animal's head to a constant current, isolated stimulator (WPI A360) and an a.c. coupled amplifier (WPI DAM50). Field EPSPs were amplified (gain of 100-1000), bandpass filtered (0.1-2000 Hz), digitized and stored on a personal computer. Recording sessions began with a 30-60 min adaptation period. Next, an input-output curve was constructed by stimulation from subthreshold intensity to the intensity, which evoked a maximal response. The stimulus intensity, which evoked a response at 50% of maximum was determined and used for the remainder of the recording session (3 hr). Test stimuli were delivered every 15s.

After the initial recording session animals were assigned to either the REM sleep deprivation or control condition, and treatment began the next day. After 5 days of treatment, two additional recording sessions were made on consecutive recovery days. Recording on the first recovery day began approximately 1 hr following completion of treatment; the second recording session began 24 hr after the start of the first session. After 30-60 min of adaptation recording, an input-output curve was constructed, and the stimulus intensity was adjusted to evoke a response at 50% of maximum. After a 30min baseline-recording period at the 50% intensity, LTP induction was attempted, using ten 200 Hz, 100ms stimulus trains, delivered at a 30s inter-train interval. I delivered high-frequency stimulation while animals were awake because previous studies showed that the best LTP was obtained when animals were awake or in the REM sleep state (Bramham and Srebro 1989). Recording was continued for 3 hr following high-frequency stimulation.

On recovery day 2, animals were again connected for a second recording session. The initial stimulus intensity was set to the intensity used during the LTP recording on recovery day 1, so I could determine if any LTP remained. After 30-60 min, an input-output curve was constructed, and the stimulus intensity was readjusted to evoke responses at 50% of maximum. Following a 30 min baseline recording period at the 50% intensity, LTP induction was attempted with animals in a waking state. Recordings were continued for 3 hr following high-frequency stimulation.

Histology

Placement of stimulation and recording electrodes was verified by histological examination. After completing all experimental procedures, animals were deeply anesthetized (halothane inhalation followed by chloral hydrate 750 mg/kg, or Nembutal 100 mg) and perfused transcardially with formalin fixative. Brains were removed, sectioned, and thionin stained.

Statistical analysis

Field potentials were collected and EPSP slopes were determined using the WinWCP program (John Dempster, University of Strathclyde). Additional analysis used Excel (Microsoft) and Origin (OriginLab). All statistics are presented as mean ± one standard error of the mean. Statistical significance was assessed by paired and unpaired *t*-test, as appropriate, with $p<0.05$ considered significant.

Results

LTP was assessed in vivo on two consecutive recovery days following 5 days of REM sleep deprivation or control treatment. On recovery day 1, I found significant LTP for both the REM sleep-deprived and control conditions (see Fig. 7). Immediately after tetanization EPSPs were significantly increased in both REM sleep-deprived (169±27% of baseline, n=10, $p<0.01$) and control animals (181±39% of baseline, n=8, $p<0.05$). There was no difference between the two groups at this time point ($p>0.45$). At the end of the 3 hr post-tetanus recording period, hippocampal field EPSPs in REM sleep-deprived animals averaged 120±9% of the pre-tetanus baseline ($p<0.02$). For control animals, EPSPs were 153±29% of baseline ($p<0.05$). Although control animals showed numerically greater LTP, the difference between control and REM sleep deprived groups was not significant ($p>0.15$). Pre-tetanus recordings on recovery day 2 allowed us to determine if any LTP remained at the 24 hr post-tetanus time point. Although both REM sleep-deprived and control animals showed significant LTP at 3 h post-tetanus, by 24 hr post-EPSPs were no longer different from pre-tetanus ($p>0.03$,

0.10 for REM sleep-deprived and control, respectively).

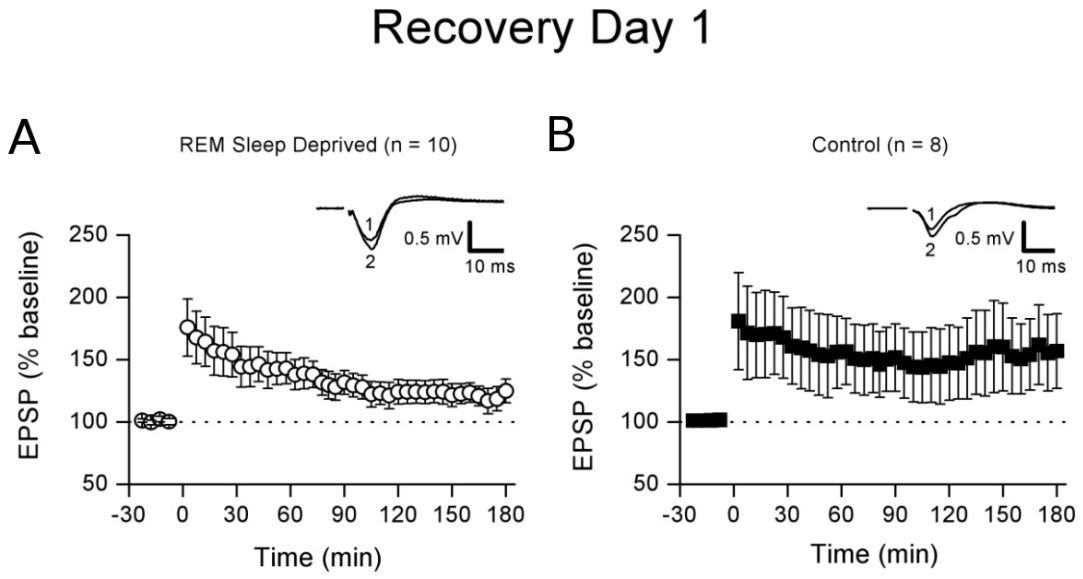


Figure 7. Significant LTP was obtained in both REM sleep-deprived and control group on the first recovery day following treatment. EPSPs were measured as the slope of the initial negative going portion of the response and were normalized by the mean of the pre-tetanus (baseline) response. EPSPs were averaged over 5 min periods and are plotted relative to time of tetaniztion (at 0 min). (A) REM sleep-deprived animals showed an immediated post-tetanic increase in field EPSP slope. Althought the potentiated EPSP declined substantially during the 3 hr post-tetanus recording, responses were significantly potentiated even at 3 hr post-tetanus (p<0.02) (B) control animals showed a similar increase in field EPSP immediately following tetaniztion, which remained up to 3 hr post-tetanus (p<0.05), and there was no significant difference between REM sleep-deprived and control animals at this time (p>0.15). Error bars show ± one standard error of the mean.

Although I found no differences between REM sleep-deprived and control animals on recovery day 1, differences emerged on recovery day 2 (see Fig, 8). The potentiation in REM sleep-deprived animals completely decayed over the 3 hr post-tetanus recording period (at 3 hr post-tetanus, EPSPs were 108±8% of baseline, $p>0.15$). In contrast, control animals on recovery day 2 continued to demonstrate significant LTP: at 3 hr post-tetanus, EPSPs were significantly enhanced compared to baseline (127±8%, $p<0.05$), and the difference between control and REM sleep deprived was significant ($p<0.05$). This difference in LTP occurred despite equivalent initial post-tetanic increase in field EPSP slope of 140±22% (REM sleep deprived) and 169±29% (control) of baseline ($p>0.25$)

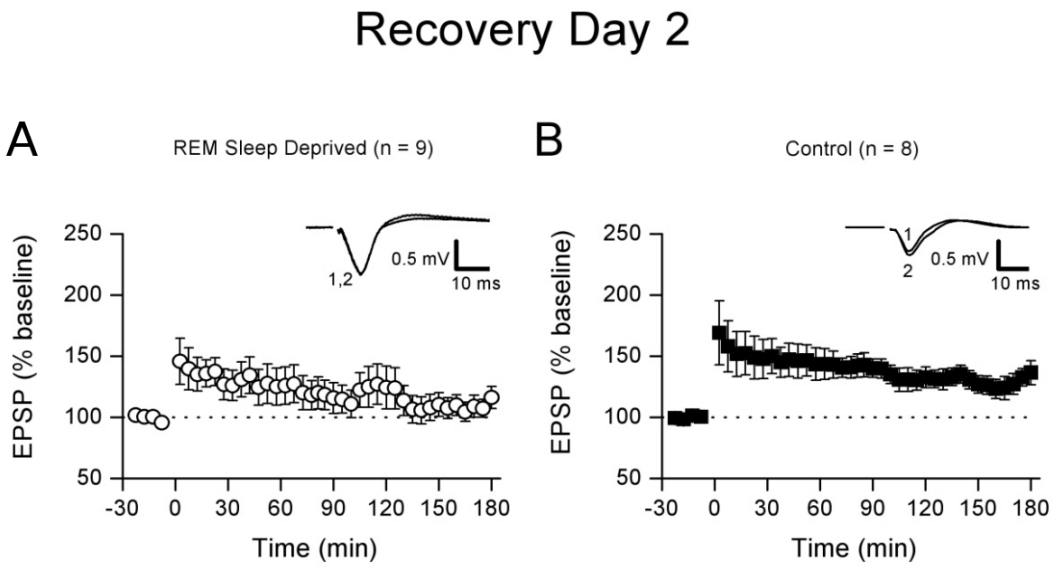


Figure 8. LTP was impaired in REM sleep-deprived animals on recovery day 2. (A) REM sleep deprived animals showed an immediate post-tetanic increase in field EPSP slope, but the potentiated EPSPs steadily declined during the 3 hr post-tetanus recording. After 3 hr, EPSP slopes were no longer different from baseline ($p>0.15$). (B) In contrast, control animals on recovery day 2 again showed LTP which was maintained throughout the post-tetanus recording at 3 hr post-tetanus, $p<0.05$), and which was significantly greater than in REM sleep-deprived animals ($p<0.05$). Error bars show ± one standard error of the mean.

Discussion

I found that 5 days of REM sleep deprivation significantly impaired LTP in hippocampal area CA1 in vivo, but only on the second recovery day following treatment. Three prior studies examined sleep deprivation for effects on hippocampal LTP (Campbell, Guinan et al. 2002; Davis, Harding et al. 2003; McDermott, LaHoste et al. 2003). In each of these studies, LTP was assessed in vitro using the brain slice preparation. In one study (Campbell, Guinan et al. 2002), 12 hr of total sleep deprivation was produced by forced locomotion. The remaining two studies (Davis, Harding et al. 2003; McDermott, LaHoste et al. 2003) used selective REM sleep deprivation procedures (using the 'inverted flower pot' method) for up to 72 hr. In this study, I induced 5 days (120 hr) of REM sleep deprivation using the 'inverted flower pot' method. Despite the variations in duration of SD, my in vivo study and previous in vitro studies are in general agreement: sleep deprivation inhibits LTP and selective REM sleep deprivation affects LTP persistence with little or no change in the maximal initial potentiation immediately following tetanization.

Although there is general agreement between my study and earlier in vitro studies, there are some differences. I found significantly impaired LTP only on the second recovery day following treatment, whereas prior in vitro studies found impaired LTP on recovery day 1. This difference might be explained by inputs to the hippocampus from serotonergic and noradrenergic nuclei. Neurons in these nuclei show altered activity during REM sleep deprivation and during the REM sleep rebound which follows REM sleep deprivation (Porkka-Heiskanen, Smith et al. 1995; Asikainen, Toppila et al. 1997). Asikainen et al. (1997) reported that serotonin metabolism in the rat brain including hippocampus was increased during SD (Asikainen, Toppila et al. 1997). In addition, Porkka-Heiskanen et al. (1995) measured norepinephrine concentration in the hippocampus after REM sleep deprivation. They found that norepinephrine concentrations were significantly increased after 72 hr of REM sleep deprivation (Porkka-Heiskanen, Smith et al. 1995). In addition, they showed an increase in tyrosine hydroxylase activity, a rate-limiting step in norepinephrine biosynthesis, and its mRNA levels after REM sleep deprivation (Porkka-Heiskanen, Smith et al. 1995). Since changes in serotonin and norepinephrine activities can modulate LTP (Hopkins and Johnston

1984; Auerbach and Segal 1994), and changes in the activity of these systems occur during sleep deprivation, the absence of this modulatory input when LTP is examined in vitro might account for my finding that LTP is intact on recovery day 1 in vivo, whereas previous in vitro studies found disrupted LTP at this time.

My finding of disrupted LTP on recovery day 2 can be explained by reduced REM sleep rebound on recovery day 2. In 2004, Machado, et al monitored sleep parameters during 96 hr of SD and during 4 days of recovery sleep. They showed that the 'inverted flower pot' method significantly reduced REM sleep during SD treatment, and caused a significant REM sleep rebound only during the first 24 hr of recovery sleep (Machado, Hipolide et al. 2004). Therefore, my finding of disrupted LTP on recovery day 2, when REM rebound is largely dissipated indicates an enduring disruption of LTP as a consequence of prior REM sleep deprivation. This disruption of LTP could reflect a loss or inhibition of any of the components of the signaling pathway which is activated during LTP induction, including the NMDA receptor. Recent findings indicate that NMDA receptor function and expression are, in fact, altered by sleep deprivation (Chen, Hardy et al.

2006; Kopp, Longordo et al. 2006; McDermott, Hardy et al. 2006). My next series of experiments examined a possible role for growth hormone in linking sleep to hippocampal NMDA receptor function and expression.

Chapter 2

Introduction

It is well known that sleep deprivation impairs cognitive function (Smith 1995; Youngblood, Zhou et al. 1997; Wilson 2002; McDermott, LaHoste et al. 2003). In addition, there are bidirectional interactions between sleep and the endocrine system. The plasma concentrations of many hormones display sleep-related variation suggesting that sleep influences hormone secretion. Growth hormone (GH) is the best documented hormone with a strong sleep-related secretory pattern. GH is produced by anterior pituitary somatotroph cells. Synthesis and secretion of GH are controlled by two hypothalamic neurohormones; growth hormone-releasing hormone (GHRH), which stimulates, and somatostatin, which inhibits GH (Gotherstrom, Svensson et al. 2001). Secretion of GH is strongly regulated by sleep. Although GH is secreted throughout the day, the major surge of GH secretion occurs during sleep (Obal and Krueger 2004; Steiger 2007). It has been shown that sleep deprivation (SD) suppresses GH secretion. Kimura and Tsai (1984) studied GH secretion in rats. These authors found that the peak of GH secretion appeared during the onset of the sleep cycle and SD during

this period prevented the high-level GH pulse (Kimura and Tsai 1984). A similar suppression of GH secretion is seen during SD in human subjects (Brandenberger, Gronfier et al. 2000).

In peripheral tissues, GH is essential for somatic growth and metabolism. GH has both direct and indirect actions on target tissues. Indirect effects of GH are mediated mainly by insulin-like growth factor 1 (IGF-1). IGF-1 is produced in response to GH stimulation in the liver and at other sites of GH action. IGF-1 in turn, feeds back on the hypothalamus and pituitary to inhibit GH release. In addition to peripheral tissues, GH also has effects on the central nervous system (CNS). As in peripheral tissues, the effects of GH on CNS can be either direct or indirect, mediated by IGF-1. In the CNS, IGF-1 promotes cell proliferation, cell migration, and cell differentiation during brain development (Anlar, Sullivan et al. 1999). Circulating IGF-1 reaches the CNS from the peripheral blood stream by a specific carrier in the BBB (Pan, Yu et al. 2005). However, IGF-1 is also produced locally in the brain suggesting that GH might stimulate brain tissues to produce IGF-1 within the CNS (Sun, Al-Regaiey et al. 2005). GH, like IGF-1, can cross the

BBB (Johansson, Larson et al. 1995; Coculescu 1999; Pan, Yu et al. 2005; Aberg, Brywe et al. 2006) and the GH receptor (GHR) is found in several brain regions including the hippocampus. GH effects on the brain can therefore be direct, indirect and mediated by circulating IGF-1, or indirect and mediated by IGF-1 produced within the CNS (Lai, Emtner et al. 1991; Lobie, Garcia-Aragon et al. 1993).

Recent studies have shown that GH affects cognitive function. Decreased GH release, with normal aging or with GH deficiency, is paralleled with cognitive impairment (Nyberg 2000; Aberg, Brywe et al. 2006). Moreover, GH therapy in GH deficiency, or GH treatment of aged animals leads to improved learning and memory (Nyberg 2000; Ramsey, Weiner et al. 2004; Aberg, Brywe et al. 2006). For example, Le Greves, et at. (2006) treated hypophysectomized rats with recombinant human GH (rhGH) for 9 days. GH-treated hypophysectomized animals showed improved performance in a behavioral test of memory using the Morris water maze (Le Greves, Zhou et al. 2006). In a similar study using aged rats, Ramsey et al (2004) found improved spatial learning in aged (24 month old)

rats which were treated with porcine GH for 4 months compared to control aged rats treated with saline vehicle for 4 months (Ramsey, Weiner et al. 2004). The positive effect of GH treatment on cognitive function could be a secondary effect of IGF-1. Administration of IGF-1 also improves age-related spatial memory deficits (Sonntag, Lynch et al. 2000). While GH and IGF-1 treatments both improve cognitive function, at least under conditions where endogenous hormone levels are decreased, the mechanisms underlying this improvement remain to be determined.

Although the exact mechanisms linking GH to cognitive function are still unclear, recent evidence suggests that GH can directly influence synaptic function and trigger synaptic plasticity in the hippocampus. In 2006, Mahmoud and Grover showed that acute application of GH onto hippocampal brain slices enhances excitatory synaptic transmission, indicating that GH can directly affect hippocampal synaptic function (Mahmoud and Grover 2006). In addition, the signal transduction pathways stimulated by the GHR and by hippocampal LTP induction share molecular components. The GHR is a member of the cytokine receptor superfamily. GHR dimerization upon binding to GH stimulates association with

and activation of Janus kinase 2 (JAK2) (Lobie, Zhu et al. 2000). Activated JAK2 in turn is responsible for activating numerous signaling cascades including the signal transducers and activators of transcription (STAT), Ras/Raf/MEK1/MAPK, and insulin receptor substrate-1(IRS-1)/PI3 kinase pathways (Liang, Jiang et al. 2000). Some of the kinases activated during GH signaling are also activated during LTP induction. For example, during LTP induction, NMDAR activation causes increased intracellular Ca^{2+} concentration which activates Ras and, in turn, activates MAPK. Activation of MAPK then stimulates activation of the cAMP-responsive element-binding protein (CREB), which is at least partially responsible for a late gene transcription and protein synthesis dependent phase of LTP (Lynch 2004). As in LTP, GH can also stimulate CREB activation (Lobie, Zhu et al. 2000) through the Ras-MAPK pathway (Winston and Hunter 1995). In addition, the PI3-kinase inhibitors, LY294002 and wortmannin, prevented LTP in rat hippocampal brain slices (Sanna, Cammalleri et al. 2002), suggesting that PI3-kinase which is activated in response to GH stimulation, is also involved in LTP.

In the hippocampus, N-methyl-D-aspartate receptors (NMDARs) play a critical role in LTP induction (Bliss and Collingridge 1993; Malenka and Nicoll 1999). The NMDAR is blocked by magnesium at resting membrane potentials, and only becomes activated during coincident membrane depolarization and glutamate binding. Activated NMDARs are calcium permeable, and their function in LTP induction is to increase intracellular calcium level in postsynaptic cells. Increased calcium concentration in postsynaptic cells leads to activation of CaMKII which, in turn, stimulates several downstream cellular signaling molecules that participate in LTP induction and maintenance.

The functional NMDAR is a heteromeric structure composed of two NR1 subunits and two NR2 (NR2A-D) subunits. The NR2 subunits are particularly important in shaping the functional properties of the receptor, affecting the kinetics of synaptic currents, interactions of the receptor with signal proteins, and the role of the receptor in LTP (Lynch 2004). NR2 subunit composition has an important role in regulating binding of the receptor to intracellular scaffolding proteins and kinases. One family of scaffolding proteins, the membrane-associated guanylate kinases

including postsynaptic density (PSD)-95, contains PDZ domains responsible for binding and stabilizing NMDARs in the plasma membrane. The C-terminal tail of the NR2A subunit binds to PSD-95, increasing its expression at the synapse while at the same time depressing synaptic expression of NR2B. During development, the overall expression of PSD-95 is increased, favoring an increase in NR2A and a decrease in NR2B at the synapse. As a consequence, NR2B subunits are present at higher levels in hippocampal synapses during development, but NR2A subunits predominate in mature synapses (Sans, Petralia et al. 2000).

In addition, NMDAR decay kinetics vary depending on the specific NR2 subunit contained in the receptor. These differences in decay (deactivation) kinetics have been investigated during rapid, brief glutamate application and also during synaptic release of endogenous glutamate. In general, NR2D containing receptors produce responses with the slowest decay time, whereas NR2B and NR2C containing receptors display more rapid deactivation time. NR2A containing receptors, like those that predominate in mature synapses, show the fastest decay time (Vicini, Wang et al. 1998; Cull-Candy and Leszkiewicz 2004).

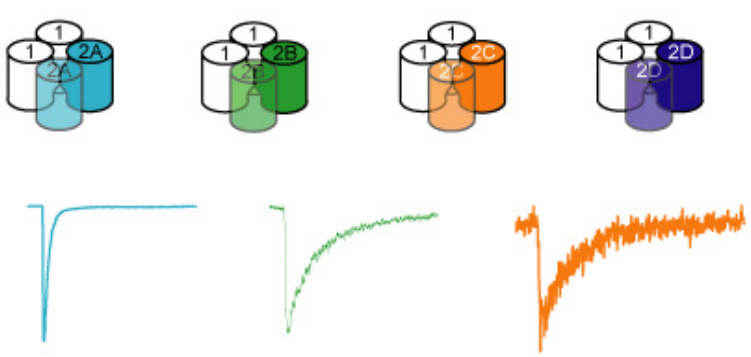


Figure 9. NMDARs form as tetramers composed of two copies of NR1 and two copies NR2 (top). Illustration of the different deactivation kinetics of the various NR2 subunits (bottom). The time constants of deactivation in response to a 10ms pulse of 1mM glutamate are roughly as follows; NR2A, 100 ms; NR2B and NR2C 250 ms; NR2D, 4 s. (Cull-Candy and Leszkiewicz 2004)

The role of specific NR2 subunits in LTP induction is controversial. For example, in hippocampal slices, selective pharmacological antagonism of NR2B subunits was reported to inhibit the induction of long term depression (LTD), a weakening of synaptic strength which can reverse LTP without affecting induction of LTP, whereas antagonism of NR2A subunits prevented the induction of LTP without affecting induction of LTD (Liu, Wong et al. 2004). In contrast, over

expression of NR2B in the forebrains of transgenic mice enhanced hippocampal LTP without affecting LTD (Tang, Shimizu et al. 1999). Although its role in LTP induction is equivocal, the NR2B subunit has an important role in targeting CaMKII to the postsynaptic membrane. CaMKII undergoes rapid autophosphorylation following NMDAR-mediated calcium influx at postsynapses. It has been shown that autophosphorylation of CaMKII induces high-affinity binding to the NR2B subunit in the postsynaptic membrane (Strack and Colbran 1998). Upon binding to the NR2B subunit, CaMKII controls intracelluar substrate phosphorylation and affects regulation of the kinase by protein phosphatases, which in turn contribute to enhancement of synaptic strength (Strack, Choi et al. 1997).

Since each “flavor” of NMADR may have a different role in synaptic function, previous studies have tested the effect of GH on NMDAR subunit expression. GH treatment in chronic GH deficiency or aging alters the mRNA abundance of specific NMDAR subunit in the hippocampus. In 2002, Le Greves et al. showed that 10 days of GH treatment increased the mRNA levels of the GHR and the NR2B subunit in adult rats, but NR1 and NR2A subunit mRNAs were increased in aged

rats (Le Greves, Steensland et al. 2002). On the other hand, mRNA for NR1, NR2A, and PSD-95 was increased after 9 days of GH treatment in hypophysectomized rats (Le Greves, Zhou et al. 2006). These findings indicate that GH availability can regulate hippocampus synaptic function by changing the pattern of mRNA expression for specific NMDAR subunits

Like GH, SD also affects hippocampal synaptic plasticity and hippocampal NMDAR subunit composition and function. In 2005, McDermott et al. reported that NMDA R currents in hippocampal neurons were decreased following 72 h of SD, and NR1 and NR2A subunits were reduced in hippocampal synapses. Similar results were reported by Kopp et al. (2006), who showed that the ratio of NR2A to NR2B subunits was increased after SD, and this change in NR2A/NR2B ratio was reversed after recovery sleep. Finally, Chen et al. (2006) showed that NMDAR1 subunit expression was reduced after 24h of SD. These SD-induced changes in NMDAR function and expression may underlie the LTP deficit and learning and memory impairment following SD.

GH is normally released during sleep and SD greatly reduces circulating GH. Because both GH and sleep regulate NMDAR expression and function in the hippocampus, I hypothesized that loss of normal GH secretion during SD might alter hippocampal NMDAR expression and function, and impair NMDAR-dependent synaptic plasticity. In addition, because GH treatment in GH deficiency improved cognitive function, I also hypothesized that GH treatment during SD would reverse the impairment of synaptic function caused by SD.

Materials and Methods

Animals and treatment

270-350g male Sprague-Dawley rats were divided into two groups for sleep deprivation (SD) or control treatment. Rats were placed on small or large (10.5 or 28cm diameter) platforms over water for 3 days SD or control treatment (Kim, Mahmoud et al. 2005). Half of the rats in each group were injected with rhGH (1 mg/kg/day; SD-GH, Cont-GH), and the other half received equivalent volume saline injections (SD-Sal, Cont-Sal). rhGH or saline injections were given every morning (between 9 – 11am) during 3 days of SD or control treatment.

Slice preparation

Rats were sedated by CO_2/air inhalation, and decapitated. The brain was removed and placed into chilled artificial cerebrospinal fluid (ACSF) composed of 124mM NaCl, 26mM $NaHCO_3$, 3.4mM KCl, 1.2mM NaH_2PO_4, 2.0mM $CaCl_2$, 2.0mM $MgSO_4$, 10mM glucose, pH7.35, equilibrated with 95% O_2, 5% CO_2. The

brain was cut in half along the longitudinal fissure. The hippocampus was removed from one of the hemispheres and immediately frozen on dry ice. The other hemisphere was trimmed and glued to the stage of a vibrating microtome (Campden Instruments), immersed in chilled ACSF, and sectioned into 400μm thick coronal slices. Transverse hippocampal slices were dissected free from surrounding structures, and stored at room temperature (20-22^{o}C) in an interface holding chamber. Individual slices were transferred as needed to a small volume (approximately 200μL) interface recording chamber with oxygenated ACSF (35°C, perfusion rate 1-1.5 mL/min).

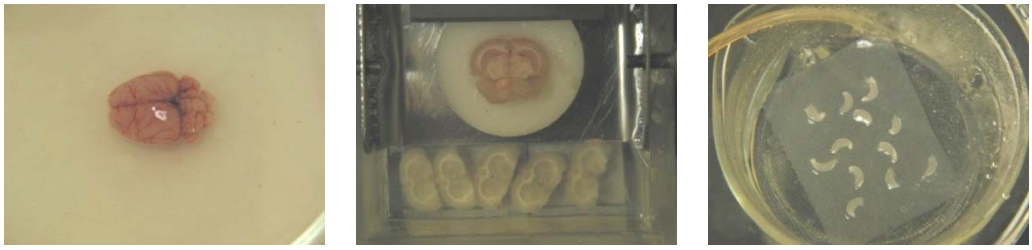


Figure 10. Preparation of rat hippocampal slices. Rat brains were collected and glued to the stage of a microtome to cut in sections. Hippocampal slices were isolated then incubated in an oxygenated chamber.

Electrophysiology

LTP was assessed by field potential recording. Extracellular potentials were recorded through low impedance (3-4MΩ) glass micropipettes filled with ACSF and placed into the stratum radiatum of area CA1. Signals were amplified (gain 100) and filtered (0.05-3,000 Hz, or 0.1-10,000 Hz), then digitized (10-100kHz; National Instruments) and stored on a personal computer using WinWCP software (Strathclyde Electrophysiology Software, John Dempster, University of Strathclyde). Baseline responses were recorded for 15 min, and then theta burst stimulation (20 bursts, each burst consisting of 4 stimuli at 100Hz, with bursts repeated at 200msec intervals) was given to induce LTP. Recordings were continued for 60min following LTP induction. For each slice, EPSP slopes were measured and normalized relative to the mean slope during the pre-tetanus baseline, and then expressed as percentage change from the baseline. For statistical analysis, the percent change in EPSP slope was averaged over the 25-30 min and 55-60 min post-tetanus periods.

Figure 11. The recording chamber (left) and placement of recording and stimulating electrodes (right).

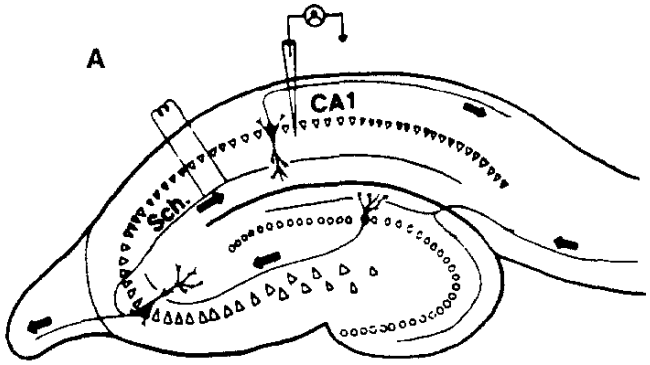


Figure 12. Diagram of a transverse section through the hippocampus illustrating the positioning of the electrodes for field potential recording. Stimulating and recording electrodes were positioned in area CA1 stratum radiatum, which contains the Schaffer collateral branches of CA3 pyramidal neurons (Sch.).

Somatic whole cell patch clamp recordings obtained from CA1 pyramidal neurons by the method of Blanton et al. (1989) were used to record spontaneous excitatory postsynaptic currents (EPSCs). Patch electrodes (3-4MΩ) were filled with 140 mM cesium gluconate, 10 mM sodium HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), and 3 mM $MgCl_2$, adjusted to 285-290 mOsm, pH 7.2. Recordings were done in the continuous voltage clamp mode of an Axoclamp 2B (Axon Instruments). Signals were amplified (gain 10) and low pass filtered (3 kHz), then digitized (10-100 kHz; National Instruments) and stored on a personal computer using WinWCP or WinEDR (Strathclyde Electrophysiology Software, John Dempster, University of Strathclyde) programs. Membrane potentials were corrected for a calculated liquid junction potential of 10 mV. EPSCs were recorded in low Mg^{2+} (50 μM) ACSF. EPSCs were recorded for a 5 to 10min baseline period, and then D-AP5 (50 μM) was added to block NMDAR-mediated currents. Spontaneous EPSCs were detected and analyzed using the Mini Analysis program (Synaptosoft). Detection threshold was set to 4 times the RMS baseline noise level. EPSCs from the baseline recording and after addition of D-AP5 were aligned and averaged separately. The amplitude of the

NMDAR-mediated synaptic current was determined by comparing the mean EPSC half-width from the baseline period with the mean EPSC half-width after addition of D-AP5

Synaptic membrane preparation and western blotting

Synaptic membranes were obtained as described earlier (Grosshans, Clayton et al. 2002; Goebel, Alvestad et al. 2005). Dounce homogenates were prepared from hippocampal tissues in sucrose buffer (SB) containing 10 mM Tris-HCl (pH 7.4), 320 mM sucrose, and phosphatase and protease inhibitor cocktails (Sigma, Roche). Homogenates were centrifuged at 1,000g for 10 min to remove nuclei and large debris (P1). The resulting supernatant (S1) was centrifuged at 10,000g for 15min to obtain a crude synaptosomal membrane fraction (P2). The P2 was lysed hypo-osmotically in water containing phosphatase and protease inhibitors for 30 min and then centrifuged at 25,000g for 20 min to yield the synaptosomal membrane fraction (LP1). Pellets were rinsed with cold SB after each centrifugation. The final pellets were resuspended in RIPA buffer and frozen

at -80^{o}C until further analysis.

For western blotting, samples were diluted to load 30µg of protein onto 8% SDS-PAGE gels. Proteins were separated by electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked at room temperature with 3% ECL Advance^{TM} blocking reagent (Amersham/GE Healthcare) in TTS (0.5% Tween20, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2 mM EDTA). Membranes were then incubated with primary antibody (to NR1, NR2A or NR2B) diluted in TTS either at room temperature for 1hour or overnight at 4^{o}C. After washing in TTS, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in TTS for 1 hour at room temperature or overnight at 4^{o}C. The blot was washed and proteins were detected on X-ray film using the ECL Advance^{TM} system (Amersham/GE Healthcare). Films were scanned and analyzed using Image J software (Wayne Rasband, NIMH). Blots were stripped (Pierce Restore^{TM} stripping buffer) and reprobed for other NMDAR subunits and for PSD-95.

Measurement of serum IGF-1

Trunk blood was collected at the time of sacrifice of animals. Blood was kept on ice for 30 min and then was centrifuged at 1000g for 15 min to separate serum. Serum IGF-1 was measured by ELISA, following the manufacturer's instructions.

Results

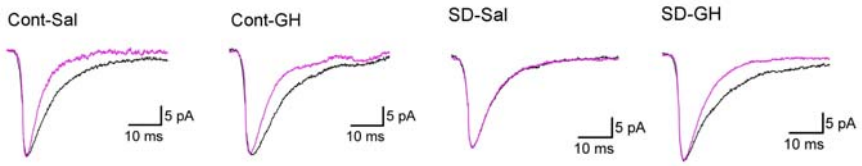
Previous investigations have established that GH is released during sleep, and prevention of sleep in turn suppresses circulating GH. If GH is a mediator of sleep effects on hippocampal synaptic function, then experimental restoration of GH to sleep deprived animals should reverse the effects of SD. I used three experimental approaches to test this hypothesized role for GH. First, I examined NMDAR-mediated synaptic currents using whole cell patch clamp recordings from hippocampal neurons. Second, I measured NMDAR-dependent LTP in hippocampal brain slices. Third, I measured NR subunit expression in hippocampal synaptosomal membranes.

NMDAR synaptic currents were impaired by SD but rescued by GH treatment (Fig. 13)

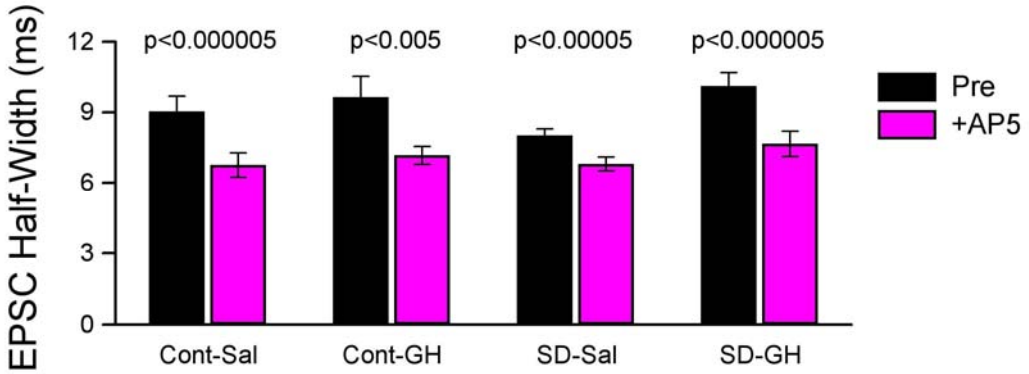
I recorded spontaneous EPSCs (sEPSCs) in CA1 pyramidal neurons in hippocampal slices prepared from animals after 3 days of SD or control (cont) treatment. These animals received either daily injections of saline vehicle (Sal) or

daily injections of GH. NMDAR function was assessed by whole cell patch clamp recording of sEPSCs in low Mg^{2+} (50μM) ACSF containing γ–aminobutyric acid (GABA) receptor antagonists. NMDAR function was assessed by comparing sEPSC half-width before and after D-AP5 (50μM) application. In Cont-GH and Cont-Sal cells, sEPSC half-width was reduced significantly by D-AP5 (Cont-GH, -23,1±4.0%, n=11; Cont-Sal, -25.0±2.8%, n=13). In SD-Sal cells, sEPSC half-width changed by only -15.1±2.4% (n=13, significantly different from Cont-Sal, $p<0.05$). In SD-GH cells, sEPSC half-width was reduced by -24.3±2.7% (n=15, not significantly different from Cont-GH or Cont-Sal, but significantly different from SD-Sal, $p<0.05$).

A


Cont-Sal
Cont-GH
SD-Sal
SD-GH
5 pA
10 ms

B


EPSC Half-Width (ms)
12
9
6
3
0
p<0.000005
p<0.005
p<0.00005
p<0.000005
Pre
+AP5
Cont-Sal
Cont-GH
SD-Sal
SD-GH

C

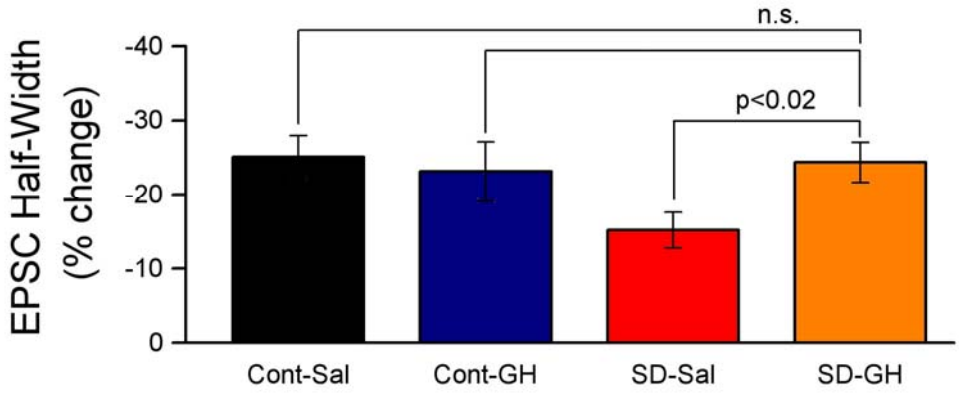

EPSC Half-Width
(% change)
-40
-30
-20
-10
0
n.s.
p<0.02
Cont-Sal
Cont-GH
SD-Sal
SD-GH

Figure 13. NMDAR synaptic currents were impaired by SD but rescued by GH treatment. A. Averaged sEPSCs recorded from individual CA1 pyramidal neurons in each of the four experimental groups, before addition of D-AP5 (black) and after addition of D-AP5 (magenta). The decrease in EPSC duration after AP5 application indicates the magnitude of NMDAR-mediated synaptic current. D-AP5 reduced EPSC duration in all cells except for the cell from the SD-Sal group. B. Mean EPSC half-width before (Pre) and after addition of the NMDAR blocker (+AP5). Although EPSC half-width was significantly reduced by D-AP5 in all four groups, the effect was substantially smaller in cells from SD-Sal animals. C. Percentage change in EPSC half-width. Cells from SD-Sal animals showed significantly reduced change, indicating impaired NMDAR function. GH injection had no effect on cells from control animals, but GH completely restored NMDAR function in cells from sleep deprived animals. N's = 13 (Cont-Sal), 11 (Cont-GH), 13 (SD-Sal), 15 (SD-GH).

LTP was impaired by SD but rescued by GH treatment (Fig. 14)

I recorded evoked field excitatory postsynaptic potentials (EPSPs) in stratum radiatum of area CA1 to assess LTP in response to theta burst stimulation (TBS) of Schaffer collateral/commissural afferents. Following a stable 15 min baseline recording period, each slice received TBS (20 bursts, 4 stimuli at 100Hz, with bursts repeated at 5Hz), which caused an immediate increase in EPSP slope. In Cont-Sal (n=10) and Cont-GH (n=8) slices, the EPSP slope remained potentiated throughout the post-tetanus period. In Cont-Sal and Cont-GH slices, at 25-30 min post-tetanus, EPSP slopes were increased by 39±5.0% and 44±8.4% compared to baseline, and at 55-60 min post-tetanus, EPSP slopes were increased by 24±5.4% and 35.6±8.1%.

In SD-Sal slices (n=7), LTP was greatly impaired: EPSP slopes were increased by only 16.9±2.8% at 25-30 min post-tetanus ($p<0.05$ compared to Cont-Sal), and no potentiation was present at 55-60 min post-tetanus (-1.2±5.0% change in EPSP slope; $p<0.05$ compared to Cont-Sal). However, in SD-GH slices

(n=10), LTP at both post-tetanus times was significantly greater than in SD-Sal slices ($p<0.05$). In addition, there was no difference between SD-GH and control slices: at 25-30 min post-tetanus, EPSP slopes were increased by 29.9±4.6%, and at 55-60 min post-tetanus, EPSP slopes were increased by 25.8±8.5%.

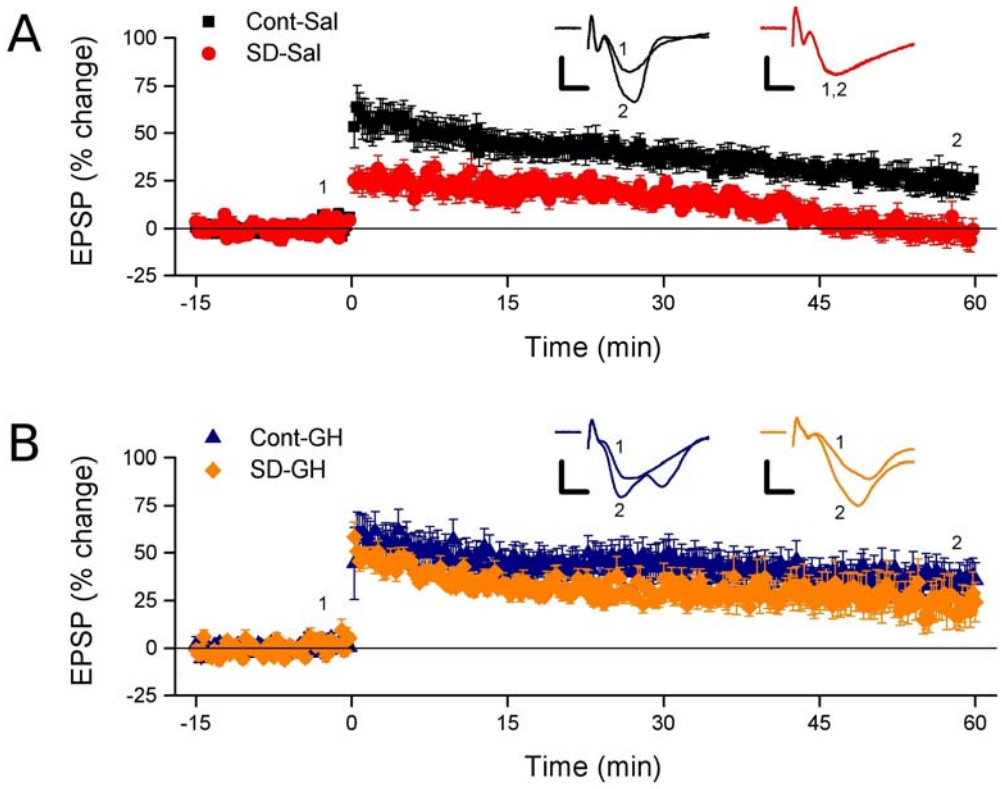
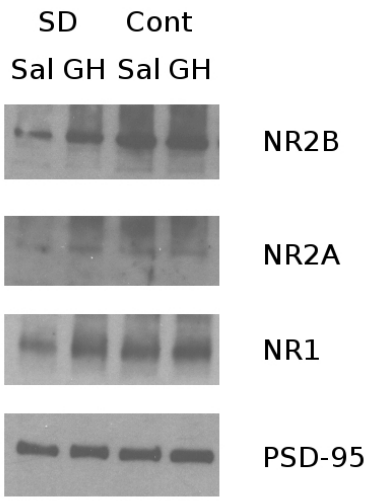


Figure 14. LTP was significantly impaired after sleep deprivation, but was rescued by GH injection. A. LTP was significantly reduced in slices (n = 8) from the SD-Sal group compared to slices (n = 10) from the Cont-Sal group ($p<0.01$ at both 30 min and 60 min time points). Insets (top) show EPSPs from representative slices in Cont-Sal (black) and SD-Sal (red) groups. EPSPs were averaged over (1) the final 5 min of the baseline period and (2) the final 5 min of the recording. Calibration bars show 1 mV, 2 ms. B. In contrast, there were no significant differences in LTP between the SD-GH (n = 10) and Cont-GH groups (n = 11; $p>0.10$ at 30 min, $p>0.25$ at 60 min). In addition, although daily GH injections had a pronounced effect on LTP in slices from sleep deprived animals, there was no effect on LTP in slices from control animals (Cont-Sal *vs* Cont-GH, $p>0.55$ at 30 min, $p>0.25$ at 60 min). Insets show EPSPs from representative slices in Cont-GH (blue) and SD-GH (orange) groups. EPSPs were averaged over 5 min periods at (1) the end of the baseline period and (2) the end of the recording. Calibration bars show 1 mV, 2 ms.

Synaptic NMDAR 2B subunit protein level was decreased by SD but restored by GH treatment (Fig. 15)

To determine the subunit composition of synaptic NMDARs in the hippocampal synapse, I prepared synaptosomal membranes from whole hippocampus, and quantified subunit protein levels using immunoblotting. Protein expression was quantified by film densitometry and normalized within each blot to the mean density of the Cont-Sal group. Normalized values were averaged across blots. There were no differences among the four conditions (all n's=5) for NR1 or NR2A subunit levels, nor were there differences in PSD-95 levels. NR2B subunits were significantly decreased after SD ($p<0.05$ for SD-Sal compared to Cont-Sal). Critically, GH treatment of SD animals restored NR2B subunit levels to normal (no difference between Cont-Sal and SD-GH, $p>0.05$).

A


SD
Cont
Sal GH Sal GH
NR2B
NR2A
NR1
PSD-95

B

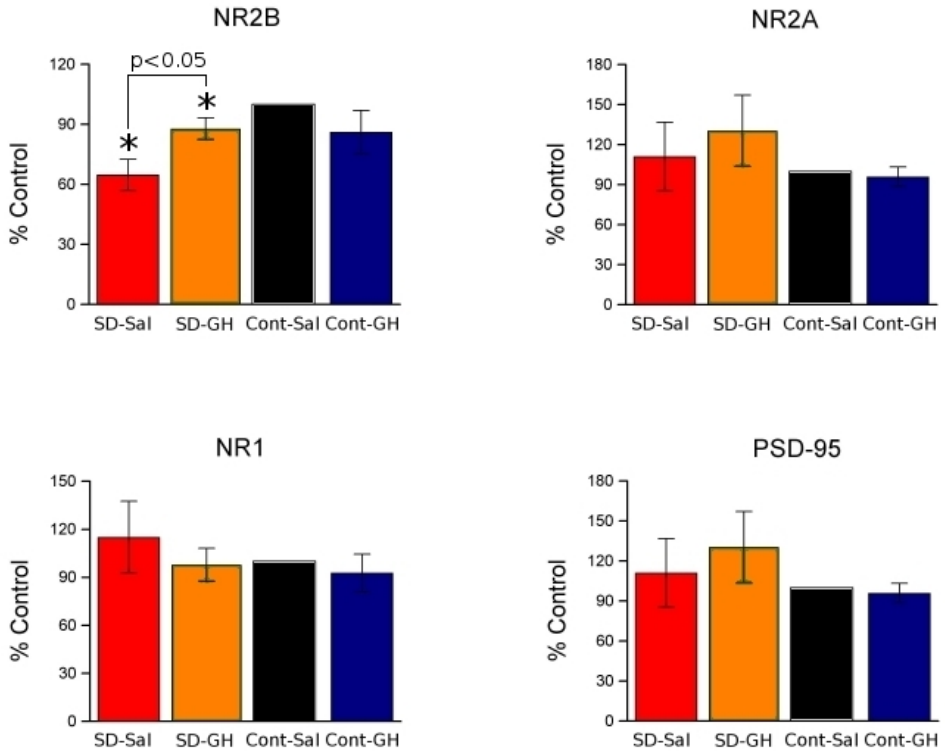

NR2B
p<0.05
% Control
SD-Sal
SD-GH
Cont-Sal
Cont-GH
NR2A
NR1
PSD-95

Figure 15. Hippocampal NR2B subunit expression was decreased after sleep deprivation, but restored by GH injection. (A) Results from a representative experiment. Four animals were examined. NR2B expression (top row) was substantially reduced by sleep deprivation in saline injected animals, but was restored by GH injection, whereas NR2A and NR1 subunits (middle rows) were less affected. Subunit expression did not differ between control animals (GH or Sal injected), and also did not differ between control and SD-GH. There were no differences in PSD-95 levels. (B) NR2B subunit was significantly reduced ($p<0.05$) in the SD-Sal group compared to Cont-Sal, and was significantly restored by GH injection ($p<0.05$, SD-Sal *vs* SD-GH).

IGF-1 level was not normalized by GH treatment (Fig. 16)

IGF-1 is produced by the liver and other target tissues in response to GH stimulation and IGF-1 in turn can alter brain and cognitive functions (Sonntag, Lynch et al. 2000). To verify that GH/IGF-1 signals were reduced in my SD animals, and to investigate whether GH treatment during SD restores IGF-1 production, I used ELISA to measure serum IGF-1 level in animals from each of my four experimental conditions. As expected, IGF-1 was significantly reduced in SD-Sal animals (1659.0±228.4 ng/ml, n=10) compared to Cont-Sal (3171.7±293.0 ng/ml, n=11, $p<0001$). Surprisingly, GH treatment did not restore IGF-1 in SD animals, with IGF-1 remaining significantly reduced in SD-GH animals (1909.5±259.3 ng/ml, n=8) compared to Cont-GH animals (3000.5±284.03 ng/ml, n=7, $p<0.02$).

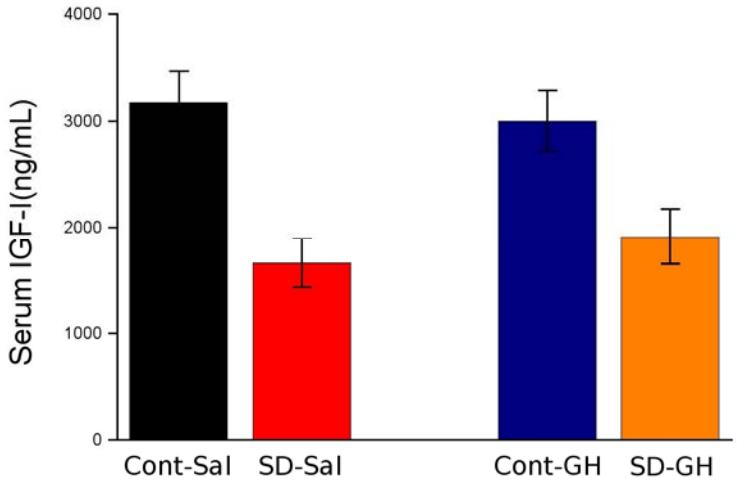


Figure 16. Serum IGF-1 was significantly reduced in SD animals regardless of whether saline or GH injections were given. Two way ANOVA demonstrated significant main effects for treatment (SD vs Cont, $p<0.001$), but not for injection (GH vs Sal, $p>0.80$) ; there was no interaction between treatment and injection ($p>0.45$); n's = 7 - 11).

Discussion

GH is released in a major surge during sleep (Kimura and Tsai 1984; Obal and Krueger 2004; Steiger 2007), and SD substantially reduces circulating GH (Kimura and Tsai 1984; Brandenberger, Gronfier et al. 2000). I hypothesized that loss of GH as a consequence of SD was responsible for the altered synaptic function which has been reported in the hippocampus after SD (Davis, Harding et al. 2003; McDermott, LaHoste et al. 2003; Chen, Hardy et al. 2006; Kopp, Longordo et al. 2006; McDermott, Hardy et al. 2006). My findings confirm a critical role for GH in maintaining hippocampal synaptic function. Hippocampal CA1 neurons had reduced NMDAR-mediated synaptic currents, reduced NMDAR-dependent LTP, and reduced synaptic NR2B subunit expression. Most importantly, restoring GH to SD animals caused recovery of NMDAR function and subunit expression. NMDAR function and expression were not affected by GH injection in control animals, which were not subjected to the SD procedure, demonstrating that the effects seen in SD animals represent a specific reversal of the consequences of sleep loss, and not a general enhancement of NMDA R function and expression

by GH.

The decrease in NR2B subunits in hippocampal synaptosomal membranes from SD animals may explain my observation of reduced NMDAR contribution to EPSC half-width. The decay kinetics of NMDAR synaptic currents depend on NR2 subunit identity: receptors composed of NR1 and NR2A subunits result in synaptic currents with faster decay times than receptors composed of NR1 and NR2B subunits (Vicini, Wang et al. 1998; Cull-Candy and Leszkiewicz 2004). The decreased NR2B subunit abundance I saw after SD would have left more hippocampal synapses in SD animals with faster NMDAR synaptic current decay time constants and therefore shorter half-widths. Restoration of normal NR2B subunit expression with GH treatment can also explain the recovery of EPSC half-width that I observed in pyramidal neurons from GH injection SD animals. The loss of NR2B subunits after SD, and the recovery of NR2B levels with GH treatment may also explain the changes in LTP that I report here. In my study, LTP was deficient in hippocampal slices from SD animals, which also had a lower NR2B subunit level. GH restoration to SD animals rescued LTP and NR2B

subunit expression in parallel. Previous studies suggest a mechanism for this association between NR2B subunits and LTP. NR2B is critical for targeting activated CaMKII – a downstream target for Ca^{2+} during LTP induction (Malinow, Schulman et al. 1989; Silva, Stevens et al. 1992) and loss or specific inhibition of NR2B containing NMDARs impairs LTP (Clayton and Browning 2001; Clayton, Mesches et al. 2002).

My results showing that hippocampal synaptic impairment caused by SD was restored by GH treatment add to a growing literature on the role of GH in cognitive and memory functions. In both humans and animal models, GH deficiency leads to a variety of cognitive impairments, including deficient memory function (Nyberg 2000; Aberg, Brywe et al. 2006). GH restoration to GH deficient humans and animals in turn normalize cognitive and memory function (Deijen, de Boer et al. 1998; Arwert, Deijen et al. 2005; Le Greves, Zhou et al. 2006). Changes seen during normal aging further emphasize the critical role of GH/IGF-1 in maintaining memory function. Circulating GH and IGF-1 are decreased during aging (Corpas, Harman et al. 1993), and GH or IGF-1 treatment improves memory-

dependent performance (Markowska, Mooney et al. 1998; Ramsey, Weiner et al. 2004). Aged rats show impaired hippocampal LTP (Geinisman, Detoledo-Morrell et al. 1995; Bergado, Fernandez et al. 1997), and hippocampal-dependent learning and memory (Ward, Oler et al. 1999; Ward, Stoelzel et al. 1999) that have been linked to decrease NR2B expression (Clayton and Browning 2001; Clayton, Mesches et al. 2002). In 2002, Le Greves et al. showed that chronic GH treatment increased NR2B mRNA in adult rats, and increased NR1 and NR2A mRNAs in aged rats (Le Greves, Steensland et al. 2002). These authors suggested that GH facilitates hippocampal function and enhances LTP in young adult rats by up-regulating NR2B gene transcription; however, they showed no change in NR2B subunit with GH treatment in aged rats. In addition, mRNA levels of NR1, NR2A, and PSD-95 were increased by GH treatment in hypophysectomized rats, but there was no change in NR2B subunit expression (Le Greves, Zhou et al. 2006). Although these results indicate that GH may regulate NMDAR composition differently under different circumstances, GH regulation of hippocampal memory-related functions through altered NMDAR expression is of general and considerable significance.

Many GH effects are mediated by IGF-1 produced by the liver and target tissues. Decreased GH release leads to reduced plasma IGF-1. Effects of GH on hippocampal synaptic function or NMDAR expression could be mediated by IGF-1. In support of this possibility, Le Greves et al (2005) showed that repeated injections of IGF-1 increased NR2B mRNA levels in young (11 weeks) adult rats. However, I found no change in serum IGF-1 concentration in GH treated animals, indicating that the GH effects I report here were not mediated through increased circulating IGF-1 (Le Greves, Le Greves et al. 2005). A direct role for GH in altering hippocampal synaptic function is supported by our laboratory's previous finding (Mahmoud and Grover 2006) that direct application of GH to *in vitro* hippocampal slices can enhance NMDAR mediated EPSP/Cs. My finding that GH treatment failed to stimulate circulating IGF-1 does not rule out a potential role for local, hippocampal IGF-1 production (D'Ercole, Ye et al. 1996).

GH regulation of IGF-1 production by the liver is well documented (Schwander, Hauri et al. 1983). It was surprising, therefore, that GH treatment of

SD animals did not restore circulating IGF-1 levels. Several factors could explain this lack of effect. First, GH is not the only circulating hormone whose levels are altered by SD: circulating thyroid hormone, insulin, corticosterone, leptin and ghrelin are all altered by SD (Steiger 2007). Second, although IGF-1 expression is regulated by GH, circulating IGF-1 concentration is dependent on the presence of IGF binding proteins (IGF-BPs). SD has diverse effects on metabolic function and hormone levels (Everson 1995; Copinschi 2005; Knutson, Spiegel et al. 2007), which in turn may alter production of IGF-1 and IGF-BPs, or decouple IGF-1 production from GH (Miell, Taylor et al. 1993; Thissen, Ketelslegers et al. 1994; Schmid, Brandle et al. 2004). Restoration of GH alone may not have been sufficient to restore control IGF-1 levels.

Taken together, my data indicate that GH regulates hippocampal synaptic function. When normal GH signals are lost during SD, NMDAR-mediated synaptic currents and NMDAR-dependent LTP are impaired, and this deficit in NMDAR function may reflect a specific loss of NR2B subunits from synaptic membranes.

Most critically, restoration of GH to SD animals rescued hippocampal NMDAR function and expression. While sleep has long been recognized as an essential regulator of biological function, including brain function, the signals linking sleep to brain function have not been known. In this study, I demonstrated a direct role for GH as a mediator between sleep and hippocampal function, establishing for the first time that GH is an essential link between sleep and brain function.

Chapter 3

Introduction

Sleep deprivation (SD) impairs learning and memory as well as synaptic plasticity in the hippocampus. In addition, SD causes stress and stress itself can interfere with learning, memory, and LTP in the hippocampus. Foy et al. (1987) tested the effect of stress on in vitro hippocampal LTP by subjecting animals to stress prior to the preparation of brain slices. The animals were placed in a restraining tube, and then received tail shocks every minute for 30 min. Hippocampal slices were prepared immediately after stress. LTP was significantly impaired in the animals which received uncontrollable stress (restraint + shock) (Foy, Stanton et al. 1987). Similar results have been reported when animals were exposed to more natural stressors. LTP in the CA1 area of hippocampus was suppressed when animals were food deprived (Diamond and Rose 1994) or exposed to a predator (Mesches, Fleshner et al. 1999). In addition, acute stress caused by exposure to a novel environment enhances long term depression (LTD), a weakening of synaptic strength which can reverse LTP, and that last hours or days (Xu, Anwyl et al. 1997). These studies showed that stress has negative

effects on hippocampal synaptic plasticity. Therefore, when assessing effects of sleep deprivation on cognitive and brain function, it is important to distinguish between the effect of sleep loss itself and stress which may occur secondarily to the loss of sleep.

One of the main neuroendocrine systems involved in the response to stressors is the hypothalamic-pituitary-adrenal (HPA) axis. A stressful stimulus perceived by the senses and evaluated in the brain ultimately induces the release of corticotrophin-releasing hormone (CRH) from the hypothalamus. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary and ACTH subsequently stimulates the release of glucocorticoids (cortisol in humans or corticosterone in rats) from adrenal cortex. Glucocorticoids produced in the adrenal cortex then negatively feedback to inhibit both the hypothalamus and the pituitary gland (Steiger 2007).

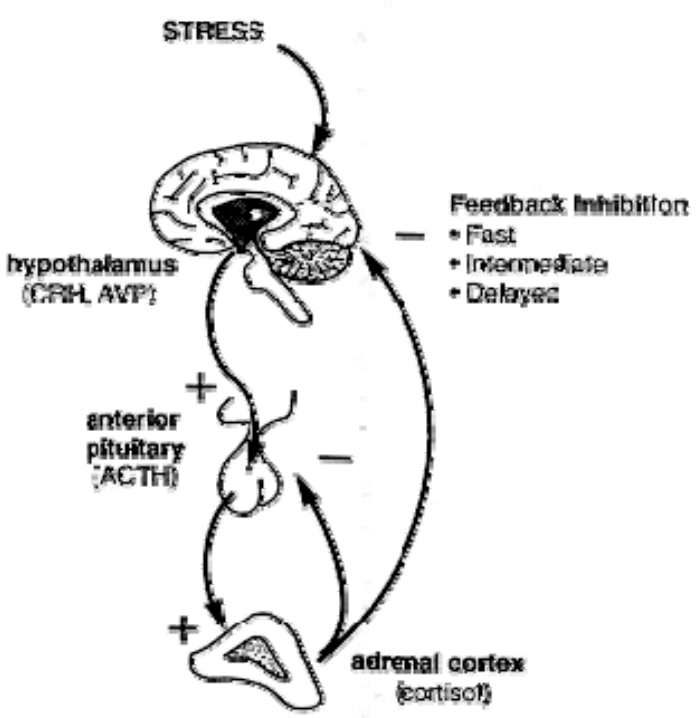


Figure 17. Hypothalamic-pituitary-adrenal (HPA) axis

Hippocampal function is strongly influenced by corticosterone. The hippocampus contains two types of corticosterone receptors, mineralocorticoid receptors and glucocorticoid receptors. Mineralocorticoid receptors have 10-fold higher affinity for corticosterone than glucocorticoid receptors, and they are saturated under basal conditions. At high corticosterone levels, such as during periods of stress, there is additional binding to the glucocorticoid receptors. Activation of glucocorticoid receptors has been shown, in several studies, to

suppress LTP. Pavlides, et al. (1993) administrated corticosterone over 21 days and showed LTP was impaired compared to vehicle controls. They also showed that acute injection of corticosterone impaired LTP demonstrating that corticosterone has both chronic and acute effects on hippocampal synaptic function (Pavlides, Watanabe et al. 1993). In 2003, Yamada, et al. used adrenalectomized animals to test the effect of stress induced elevations of corticosterone on hippocampal LTP. LTP was tested in vivo shortly after acute restraint stress. Adrenalectomized rats did not show the normal stress-induced suppression of LTP (Yamada, McEwen et al. 2003).

Sleep deprivation induced by the small pedestal ('inverted flower pot') procedure is generally acknowledged to be stressful because of the restricted mobility, periodic immersion in water and loss of sleep. Hipolide et al. (2006) measured corticosterone level after 4 days of SD induced by small pedestal procedure. Corticosterone levels were increased in sleep-deprived rats compared to control rats and remained elevated even after a 4 day recovery period (Hipolide, Suchecki et al. 2006). Meerlo et al. (2002) also found increased corticosterone

levels after a shorter, 2 day, period of sleep deprivation. In this study, SD was induced by confining the rats on a slowly rotating wheel, and blood samples were taken from the tail during the SD procedure. Corticosterone levels were significantly increased after only 6 h of sleep deprivation and remained elevated until the end of the 48 h sleep deprivation (Meerlo, Koehl et al. 2002). These results indicate that instrumental SD induction is stressful and causes increased corticosterone levels. However, these studies also showed that corticosterone level was significantly lower in control animals that were placed either in a home cage or on non-rotating wheels, indicating that control treatment in these studies were less stressful. Since corticosterone can affect synaptic function in the hippocampus, using an appropriate control group, one which shows comparable levels of corticosterone, allows isolation of the effect of sleep deprivation.

An appropriate control group for studying effects of SD on hippocampal synaptic plasticity would be treated as similarly as possible to the SD group and be subject to equivalent stress, but have normal, or closer to normal, sleep. A large pedestal procedure had been suggested as a control treatment for SD studies

(Plumer, Matthews et al. 1974). Pulmer et al. tested memory in rats that were placed on different sizes of the pedestal (7, 11.5, and 15 cm diameter) for 5 days. They found that animals that were placed on 15 cm diameter pedestal had better memory then the animals placed on smaller pedestals. They suggested that larger pedestal can be used as a control group because the lager pedestal is still surrounded by water, reproducing the environment of the small pedestal. But the large pedestal is less disruptive of sleep because the pedestal is big enough for animals to sleep without falling into water. In further support of the large pedestal control, there was no difference in adrenal gland weight between the SD and large pedestal control animals, indicating equivalent levels of stress during both procedures (Vogel 1975). Therefore, I used the large pedestal procedure as a control treatment in my previous studies to assess unique effects of SD. In this experiment, I measured corticosterone level in control and sleep deprived animals to verify that stress levels were similar in both treatments.

Materials and Methods

Measurement of serum corticosterone

Male Sprague-Dawley rats (280 – 310g) were sleep deprived for 1, 3, or 5 days using the inverted flower pot technique (10.5 cm diameter). Control animals were placed on a larger pedestal (28.0 cm diameter) which allowed them to obtain sleep for 1, 3, or 5 days. After completing SD or control treatment, rats were anesthetized by halothane inhalation, than decapitated. Trunk blood was collected at the time of sacrifice. Trunk blood from rats that were treated with either GH or saline during 3 days of SD was also collected at the time of sacrifice (for detailed methods see Chapter 2). After collection, blood was kept on ice for 30 min and then was centrifuged at 1000g for 15 min to separate serum. Serum corticosterone was measured using OCTEIA Corticosterone EIA kits (American Laboratory Products Company) according to the manufacturer's instructions. Samples and standards were loaded in duplicate on a 96-well plate coated with a polyclonal corticosterone antibody, along with HRP-labeled corticosterone. The

plate was incubated overnight (4°C), washed and developed with a chromogenic substrate (3,3',5,5'-tetramethylbenzidie). Absorbance was determined on a microplate reader at 450 nm (reference 650 nm). Corticosterone concentration in each sample was determined by comparison with the absorbance of known standard concentrations.

Results

No difference in corticosterone level between SD and control treatment (Fig. 18)

To determine if the differences between SD and control animals reported in Chapter 1 and 2 might have been caused by differences in stress level between SD and control treatments, I measured serum corticosterone levels, using ELISA, after 1, 3, or 5 days of treatment. In SD treatment, corticosterone level was significantly increased from day 1 (64.4±17.8 ng/ml) to day 3 (266.3±55.4, $p<0.05$) and day 3 to day 5 (517.3±59.4, $p<0.05$). However, the large platform control group also showed increased corticosterone level over 5 days of control treatment (67.6±23.9 ng/ml on day1; 178.3±41.9ng/ml on day3 and 436.9±31.0ng/ml on day5). For control and SD animals, the difference across treatment days was significant ($p<0.05$ for day 1 vs. day 3, day 1 vs. day 5, and day 3 vs day 5; n=6 on days 1 and 3, and n=7 on day 5). Finally, there was no difference between SD and control treatment on any treatment day (all $p>0.05$) indicating that my SD and

control treatments produced equivalent levels of stress, and any difference between these groups therefore reflects effects of sleep deprivation.

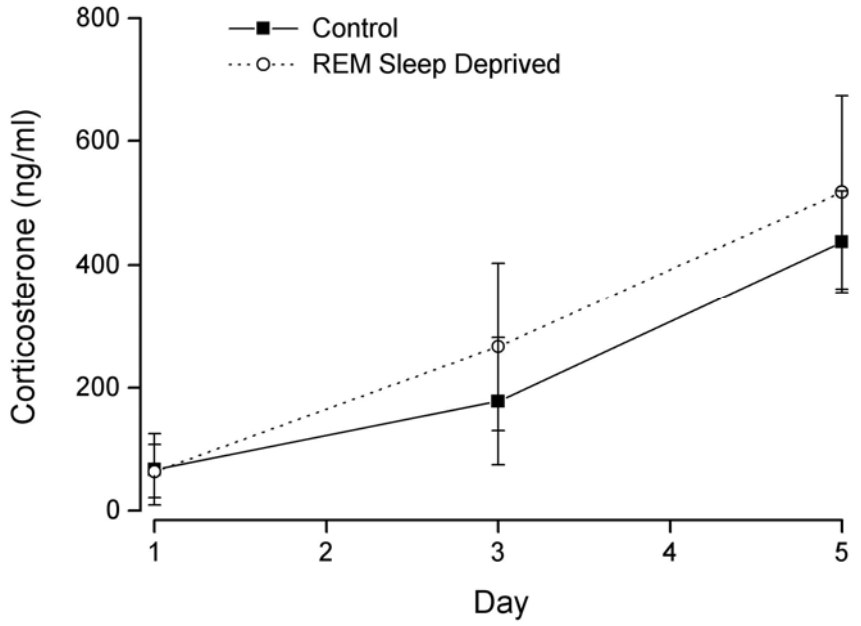


Figure 18. Circulating corticosterone concentration did not differ between SD and control animals after 1, 3, or 5 days of treatment. Although corticosterone concentrations increased across treatment days ($p<0.05$ for day 1 vs. day 3, and day 1 vs. day 5), concentrations were not different between SD and control animals on any day (all $p>0.05$). On day 1 and 3, n=6 for both SD and control, and on day 5, n=7 for both SD and control.

No differences in cotricosterone levels in GH and saline injected SD and control animals (Fig. 19)

I also measured serum corticosterone, using ELISA, after 3 days of SD or control treatment, with daily GH or saline injections. There were no significant differences in corticosterone concentration among these four conditions ($p>0.15$ for SD vs Cont, $p>0.25$ for GH vs Sal; two-way ANOVA). For Cont-Sal animals, corticosterone concentration averaged 31.7±9.0 ng/ml (n=10), for SD-Sal animals, corticosterone concentration was 65.7±20.3 ng/ml (n=14), for Cont-GH corticosterone concentration was 26.4±10.2 ng/ml (n=6), and for SD-GH animals corticosterone averaged 33.8±13.6 ng/ml (n=8). These data demonstrate that serum corticosterone was not affected by either treatment (SD or control) or injection (either GH or saline). Although there were no significant differences, corticosterone concentration appeared to be highest in SD-Sal animals at 65.7±20.3 ng/ml compared to concentrations between 26 and 34 ng/ml in the other three conditions. This apparent difference, however, reflected the presence of two outliers within this group, and removing these outliers left a corticosterone

concentration of 37.5±7.4 ng/ml (n=12), similar to the means of the other groups.

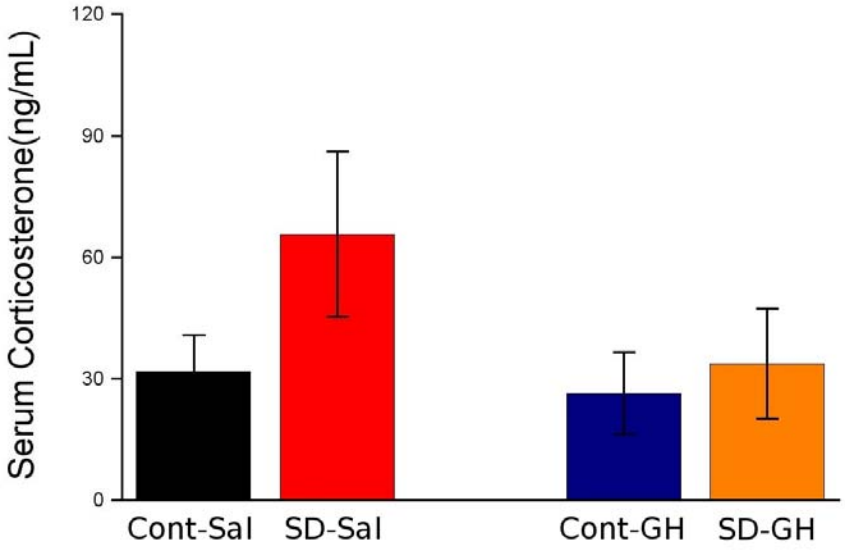


Figure 19. There were no differences in serum cotricosterone concentration between SD and Cont treatments, and between GH and saline injection groups. A two-way ANOVA revealed no main effect for treatment (SD vs Cont, $p>0.15$) and no main effect for injection (GH vs Sal, $p>0.25$).

Discussion

SD may be confounded with stress, and stress disrupts LTP in the hippocampus (Foy, Stanton et al. 1987; Pavlides, Nivon et al. 2002). Corticosterone is released in response to stress from the adrenal cortex (Axelrod and Reisine 1984). To determine if the effect of SD on synaptic plasticity was due to difference in stress level between SD and control, I measured serum corticosterone levels, using ELISA, after 1, 3, or 5 days of SD or control treatment. Corticosterone concentration was increased over 5 days of SD using the inverted flower pot technique. However, I also observed equivalent elevations in control animals which were placed on a larger pedestal indicating that there was no difference in stress between the two groups. In addition, there were no significant differences in corticosterone levels among the GH and saline injected animals that received either SD or control treatment. These results demonstrate that differences in stress cannot explain the synaptic changes as I reported in Chapter 1 and Chapter 2.

Although substantial evidence indicates the negative effect of stress-induced corticosterone elevation on synaptic plasticity (Pavlides, Watanabe et al. 1993; Yamada, McEwen et al. 2003), other data suggest that the relationship between corticosterone and synaptic plasticity is more complex. For example, animals that can terminate experimentally administered shocks show elevated corticosterone levels similar to animals which cannot terminate the shock. However, LTP was not impaired in brain slices from the animals that were able to escape from the shock, whereas brain slices from animals that received uncontrollable shock showed impaired LTP (Shors, Seib et al. 1989). Thus, although elevated levels of corticosterone are a critical determinant of the effect of stress on synaptic plasticity, an increase in glucocorticoid hormones alone does not impair LTP, and an interaction with other factors may be required to cause impaired synaptic plasticity. The present study showed corticosterone was increased in both SD and control animals, but there was no impairment of synaptic plasticity in the control group (as shown in Chapter 1 and Chapter 2) demonstrating that corticosterone was not responsible for the synaptic impairment I observed following SD.

Conclusion

Chronic sleep deprivation (SD) is a common feature of several pathologies, including those directly related to sleep such as insomnia and obstructive sleep apneas. In addition, SD can result from our modern around-the-clock lifestyle, increased work pressure, shift work and psychosocial stress. Since it has been proposed that sleep may play some essential role in the processes of learning and memory, it is important to know if brain functions involved in learning and memory are affected by SD. Previous studies have shown that sleep deprivation impairs cognitive function and synaptic plasticity in the hippocampus. SD also alters sleep related neuroendocrine systems, like the GH/IGF-1 axis. However, the relationship between these neuroendocrine systems and synaptic function during SD is not well established. The purpose of this study was to determine the effect of SD on synaptic plasticity and the role of GH in regulating synaptic plasticity during SD. My studies were divided into three stages. First, to compare with previous findings from in vitro studies, I investigated the consequences of 5 days of REM sleep deprivation on hippocampal LTP in freely behaving animals. Second, to determine if decreased GH release during SD is responsible for synaptic function impairment, I treated animals with GH during 3 days of SD and tested

hippocampal synaptic functions after completion of treatment. Third, since SD causes stress and stress in turn can cause synaptic function impairment, I compared stress levels by measuring corticosterone concentration in each treatment group of animals.

My results showed that hippocampal LTP was impaired after 5 days of SD. However, unlike the in vitro studies, the significant impairment was only seen on the second recovery day suggesting a possible compensation mechanism, such as REM sleep rebound, might be present in the in vivo condition. I also showed that GH treatment during sleep deprivation prevented the impairment of synaptic function which normally follows SD. My finding that NMDAR function was restored by GH treatment during SD is consistent with emerging evidence for a general role of GH in regulation of synaptic function. I found no differences in serum corticosterone concentration between control and SD animals, indicating that the effects I observed after SD were not the result of stress or stress-induced changes in corticosterone. Additional studies will be needed to fully elucidate the cellular mechanisms underlying the effects of GH on synaptic plasticity. These

future studies will need to address, for example, the signaling pathways through which GH regulates NMDAR expression and function.

In summary, I have shown, for the first time, that GH acts as a signal linking sleep to maintenance of normal brain function. The loss of normal GH signals impairs hippocampal synaptic functions involved in memory, and experimental restoration of GH rescues these functions even during continued sleep deprivation.

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