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Effects of Chronic Sleep and Food Deprivation on In Vivo Levels of Prepro-Hypocretin (PPH)

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EFFECTS OF CHRONIC SLEEP AND FOOD DEPRIVATION ON *IN VIVO* LEVELS OF PREPRO-HYPOCRETIN (PPH)

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Abstract

The hypocretin peptides are two hypothalamic peptides known to be involved in both sleeping and feeding behavior, however their specific roles in these domains are not well understood. The present study sought to determine the effect of chronic (72-hour) sleep deprivation and (48-hour) food deprivation on preprohypocretin (PPH), which is the precursor for the hypocretin peptides. PPH levels were visualized and quantified via *in situ* hybridization. A three-factor ANOVA (group x dorsal/ventral x medial/lateral) revealed a significant effect of subregion, specifically dorsal/middle and ventral/medial exhibited elevated PPH levels, however there was no effect of group. A between group one-way ANOVA revealed no effect of group on PPH levels. It is theorized that four possible domains may be responsible for these results: presence of hypothalamic neuronal subpopulations, role of circadian rhythm, role of hypocretins in locomotive behavior and inextricably confounded variables. These are discussed at length.

Introduction

A silent epidemic of sleep deprivation has grown among American citizens. A study recently released by the National Sleep Foundation states that 51% of American adults report sleepiness on the job to be interfering with optimal job performance; these adults further estimate that the quality and quantity of their work is regularly diminished by about 30% (National Sleep Foundation Omnibus Poll, 2000). This phenomenon is not limited to adults; 33% of young adults also report chronic daytime sleepiness. Despite this probably the most staggering finding is that over 100,000 automobile accidents are annually attributed to sleep deprivation.

Many empirical investigations have demonstrated that sleep deprivation can result in significant cognitive impairments. For example, one night's loss of sleep resulted in impairments in an auditory task and a logical reasoning task (Blagrove, Alexander, & Horne, 1995), and 34-36 hours of sleep deprivation resulted in impairments on a word fluency test as well as an impairment in the ability to attend to relevant environmental stimuli or exhibit optimal response to stimuli (Harrison & Horne, 1997). Researchers also reported a tendency for subjects to lose interest in stimuli more rapidly following a 34-36 hour sleep deprivation period versus controls, in addition to generally exhibiting slower and more variable processing of the stimuli (McCarthy & Waters, 1997). Several investigators have suggested this decrement in function may result from an increased prefrontal cortex workload that arises in an attempt to combat sleep loss (Drummond & Brown, 2001a; Kim, Guilleminault, Hong, Kim, Kim, Go, et al., 2001; Drummond, Gillin & Brown, 2001b).

One prominent study put the effects of sleep loss into perspective by comparing the effects of total sleep deprivation and alcohol consumption on hand-eye coordination. Subjects were divided into two groups; the first group of subjects were kept awake for a period of up to 28 hours and the second group of subjects were asked to consume 10-15g of alcohol every 30 minutes until their blood alcohol level reached 0.10% (note that current federal regulations stipulate a blood alcohol level of 0.08% to constitute legal intoxication). These researchers found that 17 hours of sustained wakefulness diminished hand-eye coordination to a level comparable to a 0.05% blood alcohol level and that a 24-hour extended wakefulness period produced deficits comparable to a 0.10% blood alcohol level (Dawson & Reid, 1997).

Finally, a meta-analysis revealed that cognitive performance and mood were significantly impaired following short and long term sleep deprivation periods (from 1 to 45+ hours of sleep loss) and that persistent partial sleep deprivation (less then 5 hours of sleep in a 24 hour period) resulted in higher cognitive and mood impairments than either the short or long term sleep deprivation (Pilcher & Huffcutt, 1996). This latter finding is most alarming as it most closely mimics the sleep deprivation experienced by a large part of today's societal members (National Sleep Foundation Omnibus Poll, 2000).

The cognitive deficiencies found in humans are also prominent in animal models of sleep deprivation (for review, see Siegel, 2001). Work from our laboratory, for example, has found a 72-hour sleep deprivation period (accomplished via the platform over water method) to be sufficient for producing impairments in a hippocampally-mediated spatial memory version of the Morris Water Maze task in adult rats (Billiot, Gordon, Bazan, & LaHoste, G.J. 2002). Further evidence suggests that the hippocampus may be particularly vulnerable to the disruptive effects of sleep deprivation. In rats deprived of sleep for 72-hours hippocampally-mediated contextual memory (as measured in a fear conditioning task) was impaired, whereas amygdala-mediated cued memory remained intact in the same animals (McDermott, LaHoste, Chen, Musto, Bazan & Magee, 2003; Ruskin, Liu, Dunn, Bazan & LaHoste, 2004). Hippocampal dysfunction as the neuroanatomical substrate for these behavioral deficits is further supported by *in vitro* and *in vivo* electrophysiological studies. Hippocampal long-term potentiation (LTP) was found to be diminished in hippocampal slices from rats deprived of sleep for 12 (Cambell, Guinan & Horowitz, 2002) or 72 hours (McDermott et al., 2003). Identical findings were obtained using *in vivo* recordings from anesthetized rats deprived of sleep for 72 hours (Billiot et al., 2002). These findings indicate that sleep deprivation disrupts the neural physiological mechanisms thought to be involved in memory processes.

The aforementioned information indicates that diminished cognitive performance due to sleep deprivation is a prominent epidemic in our society. However little is known about the neuronal consequences of sleep deprivation. The successful modeling of sleep deprivation-induced cognitive deficits in rodents opens the door to studies aimed at elucidating these neuronal consequences, thereby leading to the potential development of performance-preserving strategies.

Classical Sleep Pathways

In order to successfully combat the detrimental effects of sleep loss, or abnormal sleep conditions, it is important first to understand the mechanisms behind sleep. To date it has become clear mammalian brains have two opposing processes, namely the induction of arousal and the induction of sleep. The neural pathways of these two processes are independent from each other. The arousal pathway (often referred to as the ascending reticular activating system) is comprised of both a dorsal and ventral pathway. Both of these pathways originate in the pontomesencephalic brain stem. While the dorsal pathway travels through the thalamus and eventually finalizes into diffuse cortical dispersions, the ventral pathway passes through the hippocampus and the basal forebrain prior to branching into cortical projections (Siegel, 2002, p. 49).

The pathways responsible for sleep are also divided into two types, one for rapid eye movement (REM) sleep (also called 'paradoxical sleep' because of the awake-like pattern of cortical EEG) and one for non-REM sleep. Non-REM sleep, better known as slow wave sleep, results from a negative feedback system between the reticular nucleus of the thalamus and the cortex, while the major brain area associated with REM sleep is located in the mid to caudal pons area (Siegel, 2002, p117).

Hypocretins

It was not until recently that a new mechanism contributing to the sleep-wake pattern was discovered. Nearly simultaneously two independent groups of researchers announced the discovery of a pair of novel neuropeptides highly concentrated in the hypothalamus of rats. Termed either hypocretin 1 and 2 (de Lecea, Kilduff, Peyron, Gao, Foye, Danielson, et al., 1998) or orexin A and B (Sakurai, Amemiya, Ishii, Matsuzaki, Chemelli, Tanaka, et al., 1998), these neuropeptides are synthesized from a 130-amino acid precursor peptide known as prepro-hypocretin (PPH) or prepro-orexin, respectively (Sakurai et al., 1998). (The nomenclature of de Lecea et al. will be followed for the remainder of this paper.)

Hypocretin 1 and 2 consist of 33- and 28-amino acid residues, respectively (Sakurai et al., 1998). They can be found in dense-core vesicles at presynaptic axon terminals (de Lecea et al., 1998), and their interaction with specific receptors initiates postsynaptic intracellular signaling cascades (Ca^{2+} mobilization; (Holmqvist, Akerman, & Kukkonen, 2002), suggesting

that the hypocretins act as releasable neuropeptide neurotransmitters. Since the discovery of hypocretins in rats these peptides have subsequently been detected in humans, mice, pigs, cows, amphibians and non-human primates (Smart & Jerman, 2002). The amino acid sequence of hypocretin 1 is identical in all species examined, whereas that of hypocretin 2, although highly conserved, differs somewhat across species.

The hypocretins bind to two different G-protein coupled receptors, identified as hypocretin receptor 1 (Hypocretin-R1) and hypocretin receptor 2 (Hypocretin-R2). Investigations have linked Hctr-1 with Gq proteins while Hypocretin-2 has been linked with both Gq and Gi proteins (Mieda & Yanagisawa, 2002; Shiba, Ozu, Yoshida, Mignot, & Nishino, 2002). Hypocretin 1 and 2 both bind to Hypocretin-R2 with equal affinity. However, hypocretin 1 binds to Hypocretin-R1 with 30-100 fold higher affinity than does hypocretin 2 and its affinity for this receptor is similarly greater than its affinity for Hypocretin-R2 (hypocretin 2 binds with similar affinity to Hypocretin-R1 and Hypocretin-R2) (Sakurai et al., 1998). This has prompted the belief that Hypocretin-R1 is selective for hypocretin 1 transmission (Holmqvist et al., 2002).

Neuronal cell bodies and axonal projections. Neuronal cell bodies containing hypocretins are distributed exclusively within the tuberal region of the dorsal hypothalamus, extending 1 mm rostrocaudally beginning posterior to the paraventricular nucleus, ~1.5 mm mediolaterally beginning at the third ventricle, and slightly less than 1 mm ventral from the dorsal peak of the third ventricle (Peyron, Tighe, van den Pol, de Lecea, Helle, Sutcliff, et al. 1998). The hypocretin-positive cells form a roughly continuous mediolateral cluster across the dorsomedial nucleus of the hypothalamus, the perifornical region, and the lateral hypothalamic area, the perifornical region itself containing approximately 50% of the hypocretin neurons. The observed distribution is identical regardless of whether the cell bodies are visualized using immunocytochemistry with antibodies against the hypocretin peptides or with *in situ* hybridization using RNA probes complementary to PPH (Peyron et al., 1998). As of yet, however, there are no quantitative data on the regional expression of PPH mRNA within the hypothalamus.

Despite the discrete localization of the cell bodies, hypocretin axons project diffusely throughout the brain. The widespread projection pathways terminate in many brain areas including the cerebral cortex, hippocampus, amygdala, thalamus, septum, basal ganglia, central

gray and several raphe nuclei (Peyron et al., 1998; Nambu, Sakurai, Mizukami, Hosoya, Yanagisawa, & Goto, 1999; Smart & Jerman, 2002; Fadel, & Deutch, 2002). The specific locations of these connections, as well as their fiber densities, are summarized in Table 1. These projections, illustrated schematically in Figure 1, do not differ between diurnal and nocturnal species (Novak & Albers, 2002).

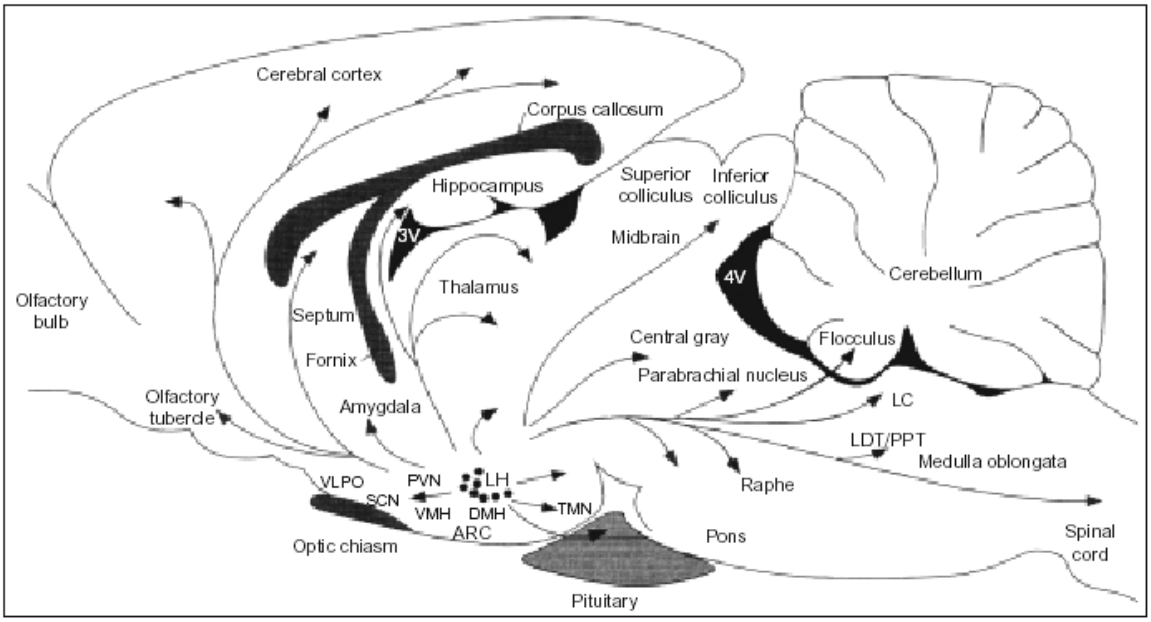

Figure 1. Schematic drawing of sagittal section through the rat brain to summarize the organization of the hypocretinergic neuronal systems. Dots indicate the location of hypocretin-like immunoreactive neurons, lines indicate some of the more prominent projection pathways, and arrows indicate terminal fields. [*Abbreviations*: 3V, third ventricle; 4V, fourth ventricle; ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; LC, locus coeruleus; LDT, lateral dorsal tegmental nucleus; PPT, posterior pretectal nucleus; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; TMN, tuberomamillary nucleus; VLPO, ventrolateral preoptic area; VMH, ventromedial hypothalamus] From Mieda and Yanagisawa, 2002.

Table 1- Hypocretin Projections: Anatomical Localization and Average Concentration	
Brain Region	**Fiber Density**
Cortex (Layers 1-6)	++
Hippocampus	+
Amygdala	
Central Nucleus and Anterior Amygdala Area	+++
Medial Nucleus	++
Basolateral, Basomedial, Cortical, Lateral and Intercalated Nuclei	+
Septum	
Lateral and Medial Nucleus	+++
Septofimbrial Nucleus	++
Diagonal band of Broca	
Horizontal Limb	+++
Vertical Limb	++
Bed nucleus of Stria Terminalis	
Ventral, Posterolateral nuclei	++++
Lateral, Medial, Posteromedial nuclei	+++
Basal Ganglia	
Accumbens nucleus, shell	++
Accumbens nucleus, core	+
Thalamus	
Central medial; Paraventricular; Subincertal; Subthalamic; Central Gray nuclei	++++
Rhomboid; Zona incerta, caudal; Parafasicular nuclei	++
Anterior Hypothalamus	
Substantia innominata	++++
Medial, Lateral preoptic area; Lateroanterior nucleus; Ventral Pallidum	+++
Medial preoptic nucleus; anterior hypothalamic area, anterior part	++
Posterior Hypothalamus	
Perifornical nucleus	+++++
Ventromedial; Dorsomedial; Arcuate; Submammillary; Tuberomammillary nuclei	++++
Dorsal, Lateral, Posterior hypothalamic areas; Tuberum cinereum	++++
Paraventricular nucleus	+++
Midbrain	
Substantia nigra, pars compacta; Central gray	++++
Substantia nigra, lateral part; Ventral Tegmental Area; Interpenduncular Nucleus	+++
Brain Stem	
Trigeminal, Facial, Hypoglossal and Vagus motor nuclei	+
Somatosensory Nuclei	
Nucleus of the Solitary Tract	++++
Lateral lemniscus; Parabrachial nucleus	+++
Superior, Inferior Colliculi; Cochlear nucleus, dorsal; Vestibular nuclei; Area postrema	++
Central Gray Nuclei	
Locus Coeruleus	+++++
Periaqueductal gray	++++
Barrington's nucleus; Laterodorsal tegmental nucleus	+++
Raphe Nuclei	
Raphe dorsalis; Raphe magnus	++++
Raphe median; B9	+++
Raphe pallidus; Raphe obscurus; Raphe linearis; Raphe Pontis	++
Rating Scale: +++++ - greatest fiber density, + - lowest fiber density, (Adapted from Peyron et al., 1999.)	

Table 2- Hypocretin Receptors: Anatomical Localization and Concentration		
Brain Region	**Hypocretin-R1**	**Hypocretin-R2**
Cerebral Cortex		
Neocortex, Layer VI	+	++
Neocortex, Layer V	+	-
Neocortex, Layer II	-	++
Cingulate cortex, Layer III	++	-
Piriform Area	-	++
Infralimbic Area	++	+
Prefrontal Cortex	++	-
Amygdala		
Bed nucleus of the stria terminalis	+++	++
Medial nucleus, anterolateral part	++	+
Cortical nucleus	+	++
Central nucleus	+	-
Substantia innominata	+	+
Hippocampus and Septum		
Taenia tecta	+++	+
Faciola cinerea	++	++
Lateral septal nucleus	-	+
Medial septal nucleus	-	+++
Nucleus of the diagonal band, vertical limb	+	++
Nucleus of the diagonal band, horizontal limb	++	+++
Dentate Gyrus	++	+
CA1	+	-
CA2	++	-
CA3	-	+++
Thalamus		
Centraolateral thalamus	+	++
Centromedial thalamus	+	++
Paraventricular nucleus thalamus	+++	++
Rhomboid nucleus	+	+++
Hypothalamic nuclei		
Anterior hypothalamic area, anterior	++	+
Anterior hypothalamic nucleus	+++	+
Arcuate nucleus hypothalamus	-	+++
Dorsal hypothalamic area	++	+
Lateral Hypothalamic area	+	+++
Posterior Hypothalamus	++	++
Midbrain		
Dorsal raphe nucleus	++	++
Locus coerulus	+++	+
Medial raphe nucleus	++	++
Periaqueductal gray matter	++	++
Substantia nigra, pars compacta	++	++
Ventral tegmental area	++	++
Rating Scale: +++: Highest receptor density, +: lowest receptor density, -: negligable receptor density. (Adapted from Marcus et al., 2001.)		

Receptors. Hypocretin-R1 and Hypocretin-R2 are expressed differentially throughout the brain. Although both Hypocretin-R1 and Hypocretin-R2 are expressed in brain regions such as the amygdala, the hippocampus, the thalamus, the hypothalamus and the midbrain area, the extent to which each receptor exists in any given nuclei is variable. The specific anatomical locations of Hypocretin-R1 and Hypocretin-R2 as well as their average density, are summarized in Table 2. (Marcus, Aschkensali, Lee, Chemelli, Saper, Yanagisawa, et al., 2001).

Role of endogenous hypocretins in sleep and arousal. The hypothalamic location of the hypocretinergic neurons suggests a role for Hypocretins in such in homeostatic mechanisms as sleeping and feeding behaviors. Indeed, these two behaviors were the first examined in the search for a functional role for the hypocretins. The role of hypocretins in sleeping behavior was recognized with the creation of hypocretin-knockout mice (Chemelli, Willie, Sinton, Elmquist, Richardson, Lee, et al., 1999). Disruption of the PPH gene prevented synthesis of either hypocretin 1 or hypocretin 2. Mice that were homozygous for the knockout allele (-/-) succumbed to numerous spontaneous periods of inactivity. Further investigation revealed these mice to be suffering from a form of narcolepsy. Around the same time it was discovered that narcolepsy in Doberman pinschers (a prominent dog model of human narcolepsy) resulted from a mutation in the Hypocretin-R2 gene (Lin, Faraco, Li, Kadotani, Rogers, Lin, et al., 1999). Human narcolepsy has since been associated with a substantial degeneration (approximately 85%-95%) of hypocretin-containing neurons, as determined *post mortem* (Thannickal, Moore, Nienhuis, Ramanathan, Gulyani, Aldrich, et al., 2000). (However, human narcolepsy is not familial and the cause of the hypocretinergic cell death is still unknown.) These serendipitous findings, implicating a role for the hypocretins in abnormal sleeping behaviors, prompted researchers to begin examining the role of hypocretins in normal sleeping behaviors.

Hypocretins have also been implicated in the circadian rhythm cycle. In laboratory rats**,** pontine hypocretin 1 immunoreactivity have been observed to fluctuate in a circadian manner. Hypocretin levels rise steadily just prior to the dark cycle and lower just prior to the onset of the light cycle (Taheri, Sunter, Dakin, Moyes, Seal, Gardiner, et al., 2000). Hypothalamic PPH mRNA also showed a steady decline towards lights out (Taheri et al., 2000). In order to rule out the chance that fluctuations in hypocretin levels are related to photic stimuli, Martinez and colleagues (2002) performed double immunocytochemistry staining for the immediate early gene

Fos and hypocretin in nocturnal (*Rattus norvegicus*, laboratory rats) and diurnal (*Arvicanthus niloticus*, Nile grass rats) rat species. Animals were sacrificed at six different time points throughout a 24 hour period. The proportion of hypocretin-positive neurons that were also Fos-positive (an indicator of neuronal activity) was reported to be most elevated at time points during which the animals were most active, regardless of light phase (Martinez, Smale, & Nunez, 2002). Another study that double-labeled for Fos and hypocretins also found the proportion of double-labeled cells to be elevated in nocturnal rats during the night (active phase); additionally, animals housed in 24-hour darkness also showed a rise in hypocretins levels during the subjective night phase of the circadian cycle (Estabrooke, McCarthy, Ko, Chou, Chemelli, Yanagisawa, et al., 2001).

A microdialysis study in cats revealed a positive correlation between wakefulness and hypocretin 1 levels in the hypothalamus (Kiyashchenko, Mileykovskiy, Maidment, Lam, Wu, John, et al., 2002). Interestingly, hypocretin 1 levels were increased in the basal forebrain and hypothalamus during REM sleep relative to slow-wave sleep. In the locus coeruleus, a major arousal area that receives the densest input of hypocretin fibers of any brain area (Peyron et al., 1998), baseline levels of hypocretin 1 in rats were observed to be significantly higher during the dark (waking) period than during the light (sleeping) period (Kodama & Kimura, 2002).

Effects of exogenous hypocretins in sleep and arousal. Intracerebroventricular (i.c.v.) administration of hypocretin 1 to rats resulted in a robust increase in wakefulness during the light (sleep) phase of the circadian cycle (time spent awake was increased to 100% from a control level of 39%). When administered during the dark (active) phase wakefulness was also increased to 100% (from control levels of 69%), although this active-phase effect was marginally nonsignificant (Espana, Plahn, & Berridge, 2002). In sleeping cats, microinjections of hypocretin 1 into the laterodorsal tegmental nucleus (an area that participates in the induction of sleep) produced a dose-dependent increase in the amount time spent awake (due to an increase in the number of waking episodes) and a significant decrease in the amount of time spent asleep (due to a decrease in the number of sleeping episodes) (Xi, Morales, & Chase, 2001).

Piper and colleagues (2000) reported that i.c.v. administration of hypocretin 1 to rats was accompanied by an almost instantaneous decrease in either slow-wave or REM sleep, and resulted in a dose-dependent increase in the amount of time spent awake as well as a marked

decrease in both REM and NREM sleep (Piper, Upton, Smith, & Hunter, 2000). Additionally, this effect could be reversed by prior i.c.v. infusion of the selective Hypocretin-R1 antagonist SB-334867-A (Smith, Piper, Duxon, & Upton, 2003). Radio telemetry analysis further indicated that i.c.v. infusion of hypocretin 1 in rats via a hydraulic minipump was able to reduce the quantity of slow wave sleep by up to 120 minutes as compared to controls. Even the less potent hypocretin 2 diminished slow wave sleep up to 30 minutes following post-infusion (Vogel, Sanchez, & Jennum, 2002).

Although the exact mechanisms responsible for the relationship between the hypocretins and sleep states are still unclear, evidence exists to support the theory that hypocretin 1 interrupts sleep stages via actions in the locus coeruleus. One study showed both unilateral and bilateral injections of hypocretin into the locus coeruleus sufficiently suppressed REM and slow wave sleep; the injections actually resulted in a 70% increase in wakefulness (Bourgin, Huitron-Resendiz, Spier, Fabre, Morte, Criado, et al., 2000). As mentioned above, baseline levels of hypocretin 1 are significantly higher in the locus coeruleus of nocturnal rats during the dark (waking) period than during the light (sleeping) period. Furthermore, intravenous administration of hypocretin 1 produced an increase in extracellular glutamate within the locus coeruleus (Kodama & Kimura, 2002).

Hypocretins and sleep deprivation. Although many studies have examined (and supported) a role for the hypocretins in sleep and arousal, few studies have investigated the consequences of sleep deprivation on the hypocretin system and those that have yielded inconsistent results. An *in situ* hybridization study in rats found no alteration in hypothalamic PPH mRNA levels as a result of short-term (2 to 4 hours) sleep deprivation (Terao, Peyron, Ding, Wurts, Edgar, Heller, et al., 2000). Dogs that were deprived of both food and sleep for 24 hours showed an increase in hypocretin 1 in cerebrospinal fluid; however, the authors of this study attributed this effect to an increase in general activity levels associated with the sleep deprivation paradigm (Wu, John, Maidment, Lam, & Siegel, 2002). In rats deprived of sleep for periods between 5 and 19 hours prior to sacrifice, the proportion of Fos/hypocretin double-labeled cells was increased compared to controls (Estabrooke et al., 2001). However, the use of Fos immunocytochemistry in this type of experiment is not ideal for two reasons. First, Fos protein remains in the cell for hours after a cell has become quiescent, resulting in poor temporal

resolution. This is important in studies on a time scale like the one used by Estabrooke et al. (2001). Second, and perhaps more importantly, Fos expression quickly desensitizes following repeated or continuous stimulation (Hope, Kosofsky, Hyman, & Nestler, 1992). Thus, during many hours of sleep deprivation, Fos expression may no longer be an accurate measure of neuronal activity.

Role of hypocretins in learning and memory. As described above, sleep is thought to play an important role in learning and memory (for review, see Siegel, 2001). Our studies, as well as those of others, have indicated that sleep deprivation results in cognitive deficits. Given these findings and the widespread distribution of hypocretin projections (including in areas known to be involved in learning and memory) (see Figure 1 and Table 1), it is reasonable to ask whether the hypocretins play a role in learning and memory *per se*. Recent electrophysiological findings suggest that the hypocretins may modulate activity within the neural substrates of learning and memory.

In mouse brainstem slices, application of either hypocretin 1 or hypocretin 2 had an excitatory effect on mesopontine cholinergic neurons within the laterodorsal tegmental nucleus (Burlet, Tyler, & Leonard, 2002). These cholinergic neurons provide a dense input to and have a strong excitatory effect on ventral tegmental A10 dopaminergic neurons (Oakman, Faris, Kerr, Cozzari, & Hartman, 1995; Oakman, Faris, Cozzari, & Hartman, 1999). Since release of dopamine by A10 terminals in the nucleus accumbens plays a central role in motivational processes (Wise & Rompre, 1989), excitation of this pathway by hypocretins has the potential to influence any of a number of higher cognitive processes. Further support for a role of hypocretins on the neural substrates of learning and memory comes from a study that employed discrete intracerebral injections in rats of the neurotoxin saporin conjugated to hypocretin 2 to produce a selective lesion of cells expressing hypocretin receptors (Gerashchenko, Salin-Pascual, & Shiromani, 2001). Injections of hypocretin/saporin were made in the area of the medial septum/diagonal band of Broca (MS/VDB), a site chosen because it is believed to be a source for the origination of hippocampal theta rhythms (Bland & Vanderwolf, 1972; Whishaw & Nikkel, 1975) and because it receives hypocretinergic input from the hypothalamus (Figure 1, Table 1). Theta rhythms are hypothesized to be involved with learning and memory and to assist with the induction of long-term potentiation (Bland & Vanderwolf, 1972; Leutgeb & Mizumori, 1999). Electroencephalographic (EEG) analysis showed that the loss of hypocretin-receptive neurons in

the MS/VDB produced no changes in either slow wave or REM sleep. However, hippocampal theta power was diminished by day-2 post-lesion and was completely abolished by day-12 post-lesion; theta activity did not return even after 1 month's time. These findings suggest that hypocretin neurons could facilitate hippocampal theta activity (Gerashchenko et al., 2001), and thus be involved directly with learning and memory. In contradiction to this hypothesis, however, Aou et al., (2003) found that i.c.v. hypocretin 1 inhibited LTP in CA1 of hippocampal slices *in vitro* (Aou, Li, Oomura, Shiraish, Sasaki, Imamura, et al., 2003).

Summary

In conclusion, investigations into the mechanisms of sleep deprivation-induced cognitive deficits have the potential to be advanced with the discovery of two newly discovered peptides. Termed the hypocretins, these peptides originate within the hypothalamus and send diffuse projections throughout the brain. Several lines of evidence suggest a role for the hypocretins in the neurobiology of sleep and waking, particularly in the initiation of arousal. There is additional evidence linking hypocretin function to learning and memory processes; whether these effects are specific for learning and memory or are the result of enhanced arousal remains to be determined. Limited sleep deprivation studies have provided mixed information. However, they do suggest that long-term, but not short-term, sleep deprivation may be correlated with an increase in the activity of hypocretinergic neurons.

Hypothesis

The aim of this study was to investigate the effect of sleep deprivation on neuronal hypocretinergic system(s) in rats. This was achieved by quantification of PPH mRNA levels within the hypothalamus using *in situ* hybridization. Unlike classical neurotransmitters, peptide transmitters are synthesized directly via gene transcription, the mRNA intermediaries of which are relatively short-lived. Therefore, quantification of changes in mRNA levels is a reliable indicator of peptidergic cell activity and neuropeptide release. The specific hypothesis to be tested were as follows.

Hypothesis: Prepro-hypocretin mRNA levels will be modified in response to a 72-hour total sleep deprivation period.

Modification of PPH mRNA levels may occur in one of two directions. If hypocretinergic neurons are capable of responding to behavioral conditions of forced wakefulness, one might expect increased activity, and therefore *increased* PPH mRNA in an attempt to maintain wakefulness in the presence of increased sleep drive. On the other hand, if the activity of the hypocretinergic neurons themselves is indicative of the state of sleep/wake drive, then one would expect PPH mRNA levels to *decrease* as sleep drive increases, i.e., during the course of sleep deprivation. Given the current state of knowledge about the role of hypocretins in sleep, it is impossible to predict which of these two outcomes is more likely to occur.

This 72-hour time point, which has not previously been studied, was chosen due to the extensive work in our laboratory that has shown a 72-hour sleep deprivation period is sufficient to produce pronounced inhibition of LTP and hippocampal, but not amygdala, mediated learning at this time point.

Methods

Animals

Twenty-four adult male Sprague-Dawley rats (approximately 300 grams at beginning of study) were singly housed upon arrival in hanging wire-mesh cages and randomly divided into three treatment groups: control, sleep deprived and food deprived (described fully below). Treatment groups, sample sizes, and time of sacrifice are summarized in Table 3. All animal procedures were approved by the University of New Orleans Institutional Animal Care and Use Committee and remained in compliance with standards set by the Public Health Service of the National Institutes of Health.

All animals were sacrificed within the circadian time (or Zeitgeber time (ZT)) interval of ZT6 and ZT7.

Animals were weighed daily between ZT6 and ZT7 in order to determine the amount of weight gained or lost as a result of experimental manipulations.

Table 3. Time Schedule of Sleep and Food Deprivation

	24-hours	48-hours	72-hours	Total
Sleep Deprived	-	-	8 rats	8
Food Deprived	-	8 rats	-	8
Home Cage Control	-	4 rats	4 rats	8
				24

Behavioral Procedures

Home Cage Controls. Home cage control animals were individually housed in wire-mesh hanging cages and had access to both food and water *ad libitum*. Due to the variation in optimal treatment durations for food (48 hours) and sleep (72 hours) deprivation, the control group was randomly divided into two equal subgroups, differing only in time of sacrifice. Forty-eight hours after the beginning of the experiment, four home cage control rats were sacrificed via rapid decapitation using a guillotine. The remaining four home cage control rats were sacrificed 72-hours after the beginning of the experiment.

Sleep Deprived Animals. Eight rats were deprived of all phases of sleep via the "platform over water" method (Pujol, Mouret, Jouvet, & Glowinski, 1968). Sleep deprivation chambers were constructed of Plexiglas® dimensionally measuring 38 x 46 x 24 cm in dimension (depth x width x height). The sleep deprivation platform was 5 cm in diameter and was fixed in a stationary manner in the middle of the chamber. The chamber was filled with water up to a point 1 cm below the surface of the platform. The nature of this paradigm is that, in order to avoid falling into the water, the rat will be forced to maintain wakefulness to remain standing on the platform. In the event the animal falls asleep it will lose its balance and fall into the water. Falling into the water should cause the animal to reawaken and regain its position upon the platform. Throughout sleep deprivation drinking water and food were supplied to the animal *ad libitum* via a grate that fits across the top of the chamber. Rats were removed from the chamber daily for the brief amount of time necessary to change the water inside the chamber. No animals were observed as sleeping during this brief period.

Our extensive work with this apparatus has proven it to be a very safe and successful method for maintaining wakefulness. Although excellent swimmers, rats find the water to be aversive and have no difficulty climbing back onto the platform even after 72 hours of sleep deprivation. This method of sleep deprivation has proven successful in our laboratory (McDermott et al., 2003) as well as others (Pujol et al., 1968; Murison, Urson, Coover, Lien, & Urson, 1982) for depriving animals of both REM and non-REM sleep. (Note that the small size of the platform effectively prevents all phases of sleep, including slow wave sleep. Other investigators have used larger platforms to induce selective REM sleep deprivation, which is not the case in the present study. Using a 5-cm platform and animals of the size used here, we have found that paradoxical sleep is almost completely abolished and slow wave sleep is reduced by >50%; McDermott et al., 2003.)

Animals were placed in the sleep deprivation chambers at the beginning of the experiment and remained in the chamber for a period of 72 hours. At the end of the 72-hour period the animals were removed and immediately sacrificed, as described above.

Food Deprived Animals. Food-deprived animals were included in this study to serve as positive controls. Previous studies have indicated that hypothalamic PPH mRNA levels increase

as a result of fasting (Sakurai et al., 1998; Yamamoto, Ueta, Hara, Serino, Nomura, Shibuya, et al., 2000; Nilaweera, Barrett, Mercer, & Morgan, 2003; Swart, Overon, & Houpt, 2001, Xin-Yun, Bagnol, Burke, Akil, & Watson, 2000, Kurose, Ueta, Yamamoto, Serino, Ozaki, Saito, et al., 2002). This manipulation was considered to be an important mechanism for the interpretation and clarification of results, especially in the event that PPH levels are unaffected by sleep deprivation.

At the beginning of the experiment, all rat chow was removed from the food hoppers of the eight rats in this treatment condition. Water was still accessible *ad libitum* throughout the experiment. Forty-eight hours after the food was removed all rats in this group were sacrificed, as described above. This time point was chosen to correspond with the positive results achieved by Sakurai et al. (1998) following a 48-hour time period.

Post-mortem Procedures

The dependent variable in this study is the amount of native PPH mRNA present in the hypothalamic region of the brains of the rats described above. Determination of this variable was achieved by quantification *post mortem* of PPH mRNA using *in situ* hybridization. Unlike classical neurotransmitters, peptide transmitters are synthesized directly via gene transcription and thus, have relatively short-lived mRNA intermediaries. Therefore, quantification of changes in mRNA levels is a reliable indicator of peptidergic cell activity and neuropeptide release.

In situ hybridization is a histochemical technique that permits the visualization of transcripts of a specific native mRNA within anatomically and morphologically preserved tissue sections (Feldman, Meyer, & Quenzer, (eds), 1997, pg 32). This is achieved by means of application to the *post mortem* tissue of a complementary nucleic acid 'probe.' The most sensitive type of probe is a radiolabeled cRNA antisense probe that is several hundred bases long. Using *in vitro* transcription, single-stranded RNA sequences are generated that have a radioactive [^{35}S] isotope attached to every uracil in the sequence. This 'antisense' probe binds with very high affinity to the native sense mRNA by Watson-Crick pairing. (The term 'hybridization' refers to the formation of a double-stranded RNA *hybrid* consisting of a native strand and a complementary artificial antisense strand.)

Brain tissue preparation. Upon being sacrificed the animals' brains were quickly removed and immediately frozen in liquid isopentane (-18°C). They were stored at -80°C until being sectioned on a cryostat in the coronal plane at a thickness of 20 μm. Sections through the tuberal region of the hypothalamus were saved and thaw-mounted onto Vectabond®-treated slides. Sections were then post-fixed in 4% paraformaldehyde in sterile 0.1 M phosphate buffer for 15 minutes, followed by a 5-minute wash in 0.05 M phosphate buffer and 1-minute wash in sterile deionized distilled water. Following post-fixing slides were stored at -80°C until hybridization.

Generation of antisense prepro-hypocretin probe. The antisense probe for PPH mRNA consisted of a single strand of 427 ribonucleic acid bases complementary to the region between bases 61 and 489 of the native 'sense' mRNA transcript. Double-stranded (ds) PPH DNA was amplified from rat brain cDNA via polymerase chain reaction (PCR) using the following primers:

Upstream: 5′-CGG ATT ACC TCT CCC TGA GCT-3′

Downstream: 5′-CAC CGG GTT CAG ACT CTG GAT C-3′

Non-underlined bases correspond to PPH sequences. The four 5'-most bases of the downstream primer (underlined) are part of a recognition sequence (5'-CCC TTC ACC-3') for the viral enzyme topoisomerase (see below) which therefore ensures cloning of the PPH-specific region into the plasmid in the correct orientation (in this case antisense) and at the correct locus (just downstream from the RNA polymerase promoter).

A sample of the resulting PCR product was then subjected to electrophoresis to determine the size, quality and approximate quantity of the product. Once the product was identified as being the correct size (Fig. 2) it was purified using Qiagen® spin columns. The PCR product was then inserted into the vector pcDNA3.1D/TOPO® by means of a 5-minute topoisomerase-mediated reaction at room temperature. The insert-containing plasmid was then used to transform bacteria (TOP10® competent *E. coli* cells) by heat-shock (20 min. on ice followed by 30 sec. at 42°C). After heat shock the cells were immediately placed back on ice. SOC medium was then added to the cells and the cells were incubated for one hour at 37ºC and swirled at 200 rpm. Subsequent to this incubation period the cells were spread thinly onto a bacteria culture plate containing Luria Broth with ampicillin (LBA). These cells were allowed to incubate on the

plates overnight at 37ºC thereby generating clonal colonies. The following day the number of bacterial colonies per plate was quantified and six independent bacterial colonies (those that had no contact with neighboring colonies) were selected from the plate. These colonies were placed into a culture tube containing 3ml of LBA to incubate overnight at 37ºC, 300 rpm. After the incubation period the test tube cultures were subjected to a miniprep procedure to purify the plasmid from the bacteria.

A sample of the purified plasmid minipreps was then digested with the restriction enzymes *Hind*III and *Xba*I. These restriction enzymes were chosen because their target sequences had been strategically engineered into the vector such that they were adjacent to the ends of the transfected PCR product and would therefore excise the target product. The digestion was performed at 37ºC for four hours and the end product (linear dsDNA corresponding to PPH) was visualized via gel electrophoresis. A digested sample was considered successful when the gel visualization revealed two distinctly different sized bands representing the insert (~427 bases long) and the vector (~5.5 kb).

A sample of the purified insert was subjected to full sequencing (Tulane DNA Sequencing Facility) to determine that the insert was of the correct sequence and had been transfected in the correct orientation. Correctly transfected miniprep samples were then linearized by digesting with the *Xba*I restriction enzyme for 4 hours at 37ºC. A 1µl sample of the digested product was visualized via gel electrophoresis to determine the accuracy of the digestion. The remaining product was purified using Qiagen® spin columns.

The final product (linearized dsDNA corresponding to PPH under the control of the T7 RNA polymerase promoter) was then used as a template for the transcription of single-stranded (ss) RNA in the presence of T7 polymerase, unlabeled ATP, GTP, CTP and radioactively labeled [^{35}S]UTP. Thus, every uracil in each probe (~100) was radioactive. Because of the directional insertion into the plasmid the resulting product was ssRNA that was perfectly complementary to native PPH mRNA—it was, thus, an *antisense* probe. For control purposes, ssRNA sense probe was generated in the same manner except that the CACC topoisomerase consensus sequence was added to the 5'-most base of the *up*stream PPH-specific primer sequence (see above) in the initial PCR reaction.

Antisense probes were expected to bind to native mRNA, thereby generating a detectable signal, whereas sense probes were expected not to bind to any nucleic acid sequence in the brain (due to the uniqueness of a 427-base sequence; $P = 1/4^{427}$), and thereby provided a negative control.

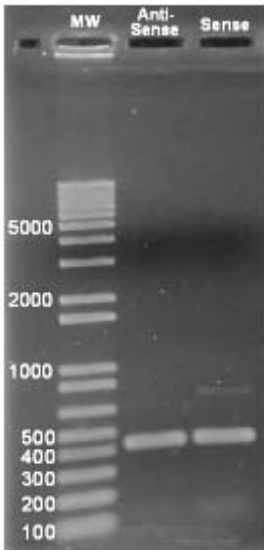

Figure 2: Gel electrophoresis visualization of PPH Antisense and Sense Riboprobe. Probes are approximately 430 nucleotides in length indicating successful PCR extractions of PPH Antisense and Sense strands.

Hybridization. At the time of hybridization the slides were removed from the freezer and allowed to warm to room temperature before being subjected to a series of acetylations and dehydrations. These washes served to facilitate the adherence of the probe to the mRNA as well as minimize non-specific probe binding. The slides were then saturated with a hybridization solution that contained either radiolabeled antisense or sense PPH probe. Slides were then coverslipped and incubated for a period of between 12 and 16 hours, after which time they were subjected to a series of high-stringency washes. These washes served to remove all non-specifically-bound probe, thereby generating a very high signal:noise ratio.

Autoradiography and quantification. Experimental slides and calibration slides (containing standards labeled with known quantities of ^{14}C) were exposed to photographic film (BioMax, MS-1; Kodak™) in the dark and enclosed within a light-tight film cassette. The film was exposed for approximately 20 days and was developed according to standard photographic procedures (5 minutes in developer, 30 seconds in a water rinse and 5 minutes in a fixative.)

For quantification, exposed film was placed on a light box. Close-up images were captured via a CCD video camera and transmitted to a computer-assisted image analyzer equipped with MCID™ software. A calibration curve was established for each piece of film by quantifying the relative optical densities of each ^{14}C standard and entering the known amount of radioactive emissions for each.

Results

Weight Loss

A repeated measures analysis revealed a significant between-group difference for weight loss ($F(2,14) = 14.186$, $p < .01$) (Fig. 3). Scheffé post hoc testing revealed that food-deprived animals lost significantly more weight then home cage animals ($p < .01$). Sleep deprived animals also suffered from significant weight loss compared to control animals ($p = .02$), however weight loss in sleep deprived animals was not as dramatic as that seen in food deprived animals ($p = .07$).

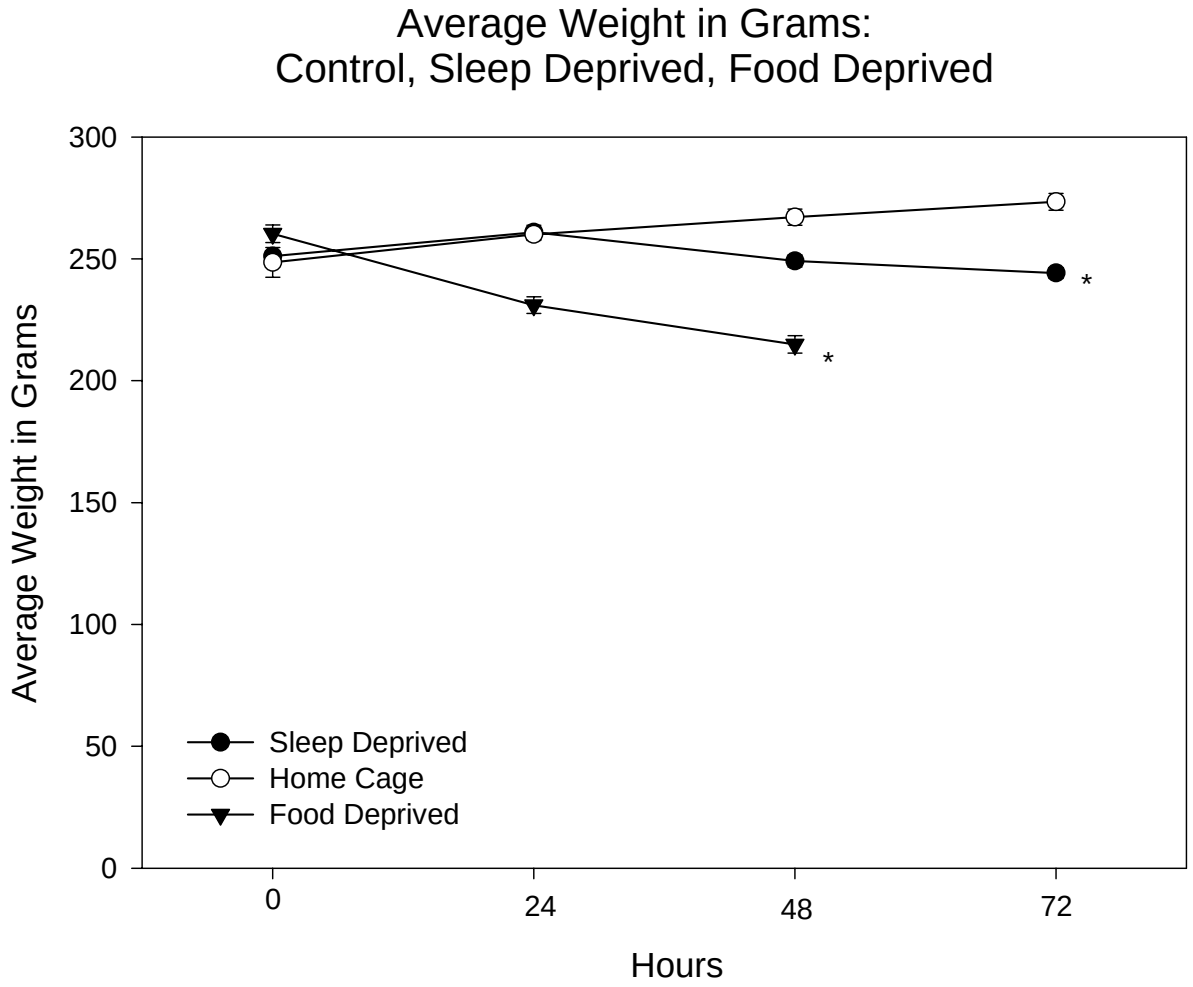

Figure 3: Average weight in grams. Significant between-group effect: Sleep and Food Deprived animals lost significantly more weight then control animals ($p < 0.05$, *). A Scheffe post-hoc test revealed total weight loss in Sleep Deprived animals was marginally non-significant when compared to Food deprived animals ($p = .07$).

Hypocretin Antisense Binding

The extremely restricted extent of Hypocretin-positive neurons combined with variations in anterior/posterior (A/P) levels of slides exposed to PPH antisense riboprobe precluded analysis of all twenty-three slides. Sixteen slides (consisting of five home cage controls, four sleep-deprived animals and 6 food-deprived animals) exhibited quantifiable signals. Sections on these slides corresponded to Plates 31-32 in the widely-used rat brain atlas of Paxinos and Watson (1998) (Fig. 4). For each brain section, relative optical densities were quantified for each of 6 subregions within a 2 x 3 matrix of 500 x 500-μm squares, as described in Methods. Subregions were classified as dorsal or ventral (2 within-subjects levels) and as medial, middle, or lateral (3 within-subjects level in the mediolateral dimension).

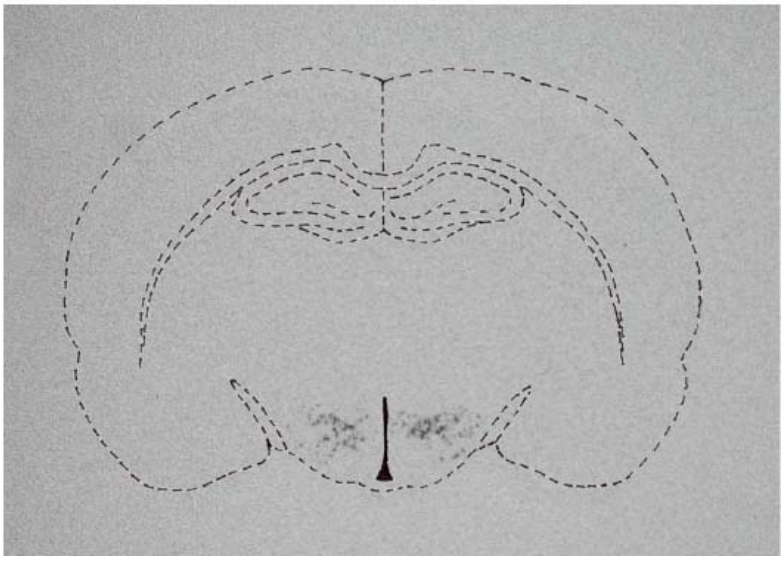

Figure 4: Illustration of preprohypocretin *in situ* hybridization superimposed over coronal brain section outline (Paxinos & Watson, 1988).

In situ hybridizations (and the molecular biology protocols leading up to them) were highly successful as judged by the pronounced appearance of signal and low background within the hypothalamic region where PPH-positive neurons have been shown to exist. A three-factor ANOVA (Group x Dorsoventral x Mediolateral) yielded highly significant subregional differences in the amount of PPH signal detected (Fig. 5). There was a significant mediolateral (ML) effect ($F(2,24)= 11.107$, $p<0.001$), a significant dorsoventral (DV) effect ($F(1,12)= 5.741$, $p=0.034$), and a significant interaction between the two (DV x ML: $F(2,24)= 9.779$, $p<0.001$).

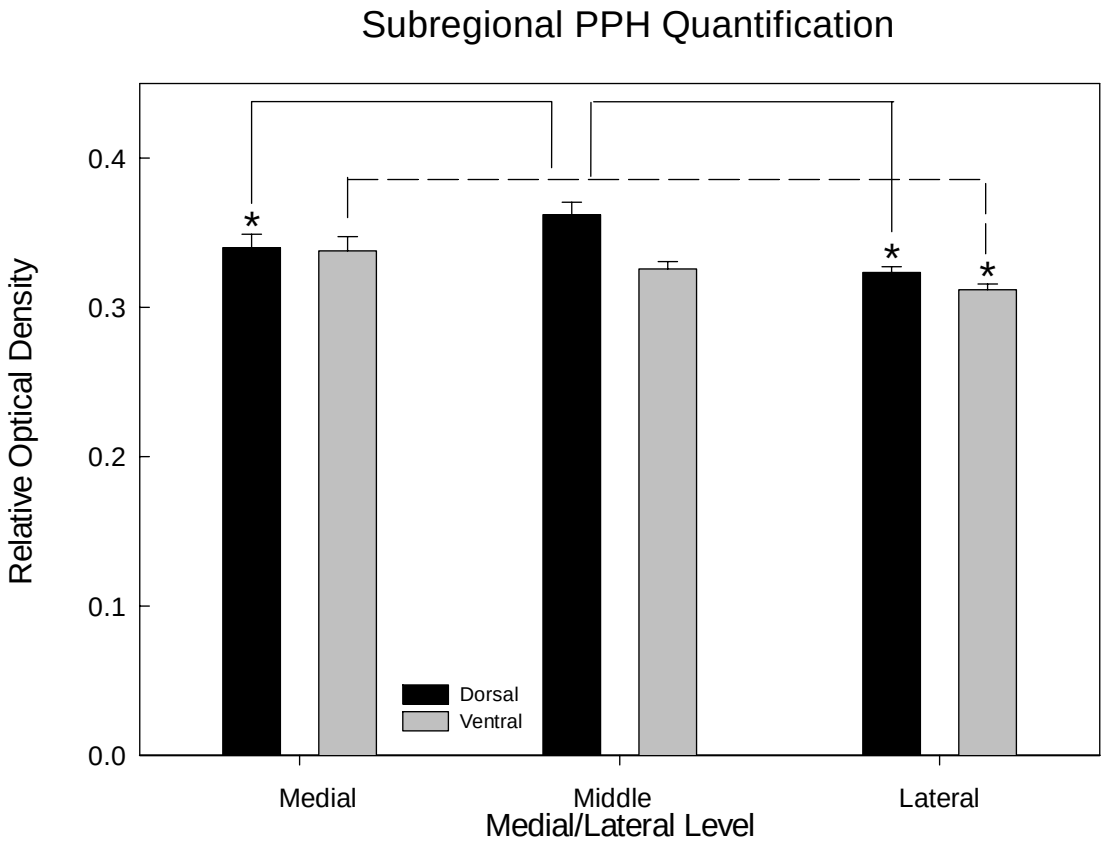

Figure 5: Dorsal (black) vs. Ventral (gray) bars illustrate subregional differences in relative optical density of PPH as measured via *in situ* hybridization. At the Dorsal level post hoc Scheffe tests revealed that the Middle subregion expressed significantly higher levels of PPH then either the Medial or Lateral subregions ($p < 0.05$, *). At the Ventral level the Medial subregion expressed a significantly higher level of PPH then the Lateral subregion ($p < 0.05$, *).

Subsequent Tests of Simple Effects showed that the ML effect was significant at both the dorsal ($p<0.002$) and ventral ($p<0.03$) levels. Post hoc Scheffé tests showed that at the dorsal level, PPH signal in the middle region was significantly higher ($p<0.05$) than either the medial or lateral regions, which did not differ significantly from each other. At the ventral level, PPH signal in the medial region was significantly higher ($p<0.05$) than that in the lateral region ($p<0.05$), whereas the middle region did not differ significantly from either the medial or the lateral. The DV effect was not significant at the middle ML level, was highly significant at the medial level ($p<0.001$; dorsal > ventral) and marginally nonsignificant at the lateral level ($p=0.053$).

The between-subjects factor Group (Home Cage vs. Sleep-Deprived vs. Food-Deprived) was not significant either alone or in interaction with any of the other factors ($F < 1$ in all cases)

(Fig. 6). Sections were also analyzed to determine whether the area of PPH mRNA signal differed significantly between groups. However, a oneway ANOVA revealed no effect of group ($F(2,11)= .12$, $p= .884$) on average mRNA area.

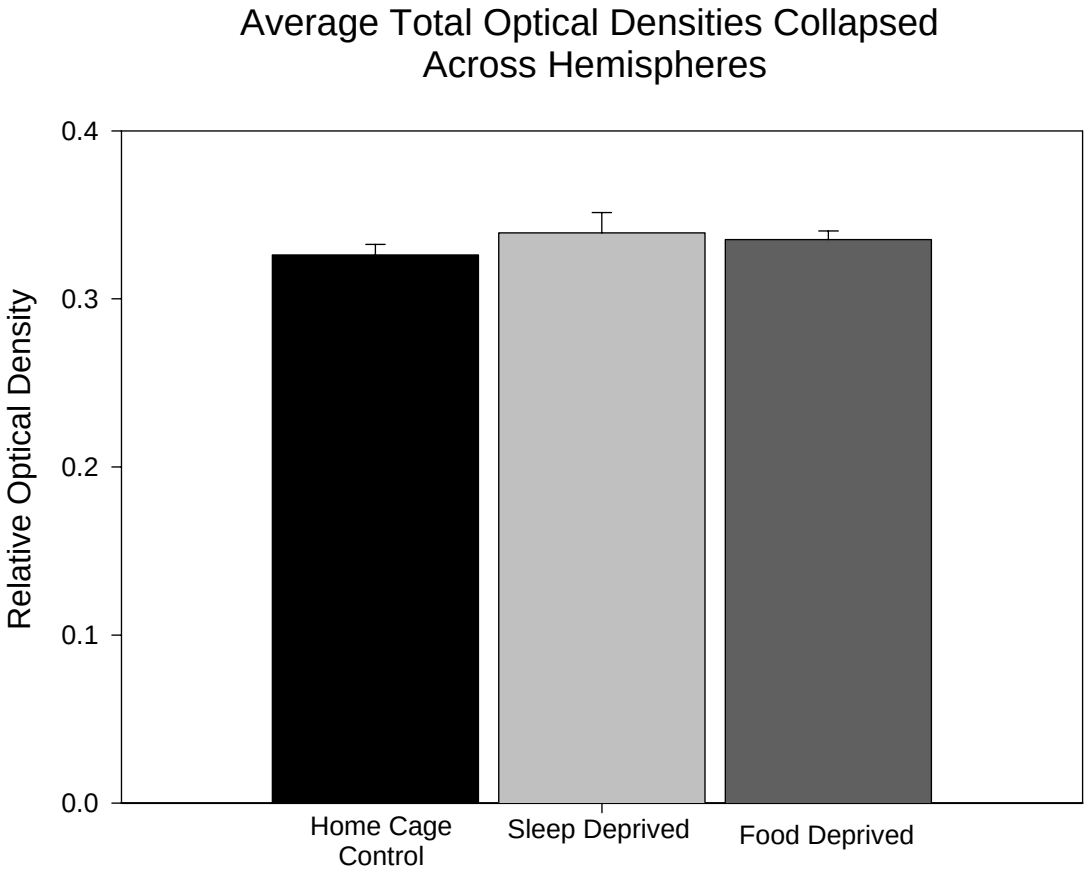

Figure 6: Average relative densities collapsed across hemispheres. Sections analyzed to determine whether PPH mRNA density differed significantly between groups. No significant between-group differences existed.

Hypocretin Sense Binding

When matched to antisense exposed slides for A/P level, sections exposed to the sense riboprobe exhibited no PPH mRNA binding ($p<.01$). This finding provides confidence that the antisense riboprobe was specific for PPH mRNA and not an artifact.

Discussion

The target region of the gene encoding PPH was successfully amplified from rat cDNA, cloned into a plasmid vector and transformed into bacteria for amplification and generation of sense and antisense riboprobes for use in *in situ* hybridization. The *in situ* hybridization with antisense probe was also successful, generating clear images of PPH signal against a low background of noise. Hybridization with a sense probe yielded only background images with no signal.

Quantitative analysis of PPH mRNA for the first time revealed anatomical differences in the amount of native PPH among different subregions of the tuberal hypothalamus, regardless of treatment group. In general there were decreasing medial-to-lateral and dorsal-ventral gradients indicating that discrete subnuclei within the tuberal hypothalamus probably play distinct roles in functions mediated by the hypocretin peptides. Future studies should take into account these regional differences.

With respect to treatment, no significant changes in the amount of PPH mRNA signal were detected, despite evidence indicating a role for hypothalamic PPH in both sleep and feeding. In the present study, hypothalamic PPH levels following a chronic (72-hour) sleep deprivation period were no different from those of home cage controls. Additionally, hypothalamic PPH mRNA levels were not elevated following a 48-hour total food deprivation period. This finding is in opposition to previous studies during which PPH mRNA levels were elevated following a 48-hour fasting period (Sakurai et al., 1998; Yamamoto et al., 2000; Nilaweera et al., 2003; Swart et al., 2001, Xin-Yun et al., 2000).

It was hypothesized that sleep deprivation might induce changes in hypothalamic PPH levels in one of two directions. Observed increases would lead to the conclusion that hypocretin levels reflected behavioral demands placed on an animal; thus, forced wakefulness would elevate the wakefulness-promoting Hypocretin peptides. Alternatively, if decreases in PPH levels had been observed, it may have been concluded that hypocretins serve as effector mechanisms of a putative sleep/wake 'homeostat,' responding to the level of sleep/wake drive. Assuming that sleep and feeding drive systems operate similarly, previous research on food deprivation had suggested the latter interpretation was not the case, suggesting indirect support for the first hypothesis. Further support for the former expectation was provided by the results of double

labeling of Fos and hypocretin 1 in sleep deprivation experiments (which may be problematic for reasons described in the Introduction). Nonetheless, no change in either direction was detected despite sufficient statistical power and despite the fact that significant regional differences were clearly detectable.

Failure of the food deprived group serving as a positive control to register a significant difference in PPH mRNA levels as compared to controls, or for a 72-hour sleep deprivation period to have an impact on hypothalamic PPH levels, is not necessarily indicative of poor experimental methodology, with regard to *in situ* hybridization. The unusually restrictive anatomical anterior/posterior extent of the hypothalamic neurons gave rise to an unexpected result; in each animal only a limited number of sections revealed PPH positive cells. Despite this, sections that did provide a signal did so with an appropriate signal-to-noise ratio. Furthermore, the sections revealing a signal were bordered immediately anterior and immediately posterior by sections completely void of any PPH signal (registering only a background signal). The specificity of this signal, namely the fact that no PPH positive cells were observed in sections very similar in anatomical level to PPH positive sections, supports the present experimental methods. In fact, the lack of signal observed in these anatomically surrounding sections was analogous to signal observed in anatomically matched sections hybridized with a sense (control) riboprobe. This strengthens the likelihood the obtained results are not due to methodological error.

It is therefore necessary to investigate the origin of the present findings. Potential explanations fall under four domains,

1) The inability to discriminate between hypothalamic neuronal subpopulations via *in situ* hybridization
2) Hypocretin fluctuations in accordance with the circadian cycle
3) The role of hypocretins in locomotive behavior
4) Inextricably confounded variables.

Inability to discriminate hypothalamic neuronal subpopulations via in situ hybridization

Our study revealed no difference between a sleep deprived group and a control group in hypothalamic PPH levels following a 72-hour sleep deprivation period at ZT6. As this study is the first to investigate PPH levels via *in situ* hybridization following chronic sleep and food

deprivation, the discrepancy between the present and previous results may signify that sleep or food deprivation-induced changes on the hypocretin system actually reveal a peptidergic effect of these manipulations and not a mRNA effect. In other words, it is possible that hypocretin release may be increased or decreased due to a change in homeostatic mechanisms (e.g. increases in proteolytic degradation of mature peptides or propeptides), yet PPH levels are so homeostatically entrained they are still produced at a constant level.

It is also notable that a variety of neuronal subpopulations exist within the hypothalamus (Swanson & Sawchenko, 1980). While an *in situ* hybridization technique is adequate for the visualization and quantification of neuronal cell bodies that are producing mRNA (in this case, PPH), this technique does not discriminate between the possible distinct subpopulations of PPH-positive neurons. For example, group differences may exist within the population of hypothalamic neurons that co-express PPH and norepinephrine, a transmitter commonly associated with sleeping behaviors (Espana & Scammell, 2004). Such an analysis is feasible via a double-labeled *in situ* hybridization technique; however this was beyond the scope of the initial investigation. Thus, it remains possible that chronic sleep and chronic food deprivation may have produced between-group differences in PPH levels within a subpopulation of PPH cells that were not distinguished in the present study.

Hypocretin fluctuations in accordance with the circadian cycle

Hypocretins have been observed to vary in a circadian manner. Taheri et al. (2000) reported that hypothalamic PPH levels rise steadily prior to the beginning of the dark cycle and fall just prior to the beginning of the light cycle (Taheri et al., 2000). However, Martinez et al. (2002) refuted the likelihood that hypocretins respond merely to photic stimuli. Estabrooke and colleagues (2001) observed an increase in Fos-positive hypocretin cells during the dark (but not the light) phase of the circadian cycle, and this elevation was also observed during the subjective night cycle of animals maintained on a 24-hour dark cycle schedule (Estabrooke et al., 2001).

In a study published after the present research was undertaken, Pederazzoli and colleagues measured hypocretin-1 cerebrospinal fluid levels at a 6-hour and a 96-hour sleep deprivation time point, as well as post-rebound sleep (Pederazzoli, Almeida, Martins, Machado, Ling, Nishino, et al., 2004). At each of these temporal points researchers sampled CSF levels from rats for two different circadian times ZT0 and ZT8. At the ZT0 measurement point no

significant group differences in CSF hypocretin-1 levels were observed between either the 6-hour sleep deprivation period, the 96-hour sleep deprivation period or the subsequent sleep rebound period (Pederazzoli et al., 2004). However, at ZT8 96-hours (but not 6 hours) of sleep deprivation led to a significant increase in CSF hypocretin-1 levels. Furthermore, hypocretin-1 levels were significantly reduced during rebound sleep (Pederazzoli et al., 2004). The failure of sleep deprivation to alter hypocretin CSF levels at ZT0 but create dramatic differences at ZT8 led Pederazzoli and colleagues to suggest the presence of a hypocretin "ceiling effect". It is possible that hypocretin levels at ZT0 may already be maximal and unable to increase further (Pederazzoli et al., 2004). More closely related to the present study is the finding that PPH mRNA levels were not reduced below the maximal level at ZT6 (Taheri et al., 2000), the time at which the present study was conducted. [It is important to report that evidence exists to indicate that CSF hypocretin levels are representative of hypothalamic hypocretin levels (Gerashchenko, Murillo-Rodriguez, Lin, Xu, Hallet, Nishino, et al., 2003)].

A similar finding regarding 24-hour food deprivation further substantiates this hypothesis. Fujiki and colleagues (2001) reported that hypocretin CSF levels were significantly increased following 24-hour total food deprivation at the ZT8 circadian time point, however no differences existed between the fasting group and the control group at ZT0 (Fujiki, Yoshida, Ripley, Honda, Mignot & Nishino, 2001). Hypocretin CSF levels failed to increase at ZT0 even after 72-hours of food deprivation, leading these researchers to hypothesize that hypocretin levels may reach a physiological maximum following extended stress and that this maximal level is attained independent of the circadian time point (Fujiki et al., 2001).

It remains possible that chronic sleep deprivation and chronic food deprivation did induce changes in PPH mRNA, yet differences were obscured in the present study by a ceiling effect resulting from the choice of ZT6 as the time of measurement

The role of hypocretins in locomotive behavior

Another behavior thought to be mediated by hypocretins, locomotion, may account for discrepancies between current and previous results. While hypocretins have been shown to have some involvement in such homeostatic mechanisms as sleeping and eating, evidence also exists to support a role for hypocretin in locomotive behavior. For example, Martins and colleagues (2004) found that hypocretin CSF levels measured at ZT0 were significantly decreased following

long-term immobilization, yet hypocretin CSF levels were increased following short-term forced swimming at ZT8. These results are striking in that they directly contrast with the consistent finding that hypocretin levels are elevated (and perhaps saturated) at ZT0 and decline by ZT8 (Pederazzoli et al., 2004, Fujiki et al., 2001). These researchers also reported that hypocretin CSF levels did not change in response a short-term immobilization period, in response to a total sleep deprivation period or in response to cold exposure, at either ZT0 or ZT8 (Martins, D'Almeida, Pedrazzoli, Lin, Mignot, & Tufik, 2004).

Further support for a role of hypocretin in locomotion comes from Kiwaki et al. (2004), who employed a cannula to inject 1 nmol of hypocretin 1 into the paraventricular nucleus of the hypothalamus in freely moving rats. These researchers reported that, irrespective of ZT time, 1nmol of hypocretin 1 resulted in a significant increase in spontaneous activity (as measured by infrared beam break count) that lasted up to two hours post injection. This response increased in a dose response fashion up to an injection level of 2nmol. This finding was corroborated by the fact that oxygen consumption (as measured by indirect calorimetry) increased at a rate comparable to spontaneous activity. Furthermore, injection of SB-334867-A (a hypocretin receptor 1 antagonist) attenuated this effect. Feeding behavior in response to hypocretin administration was also recorded and was only observed to increase when hypocretin 1 was administered early in the light phase (Kiwaki, Kotz, Wang, Lanningham-Foster, & Levine, 2003). These authors concluded that hypocretin 1 in the paraventricular nucleus could act to increase non-feeding associated physical activity (Kiwaki et al., 2003).

Another group of researchers utilized *in vivo* microdialysis in freely moving cats to measure hypothalamic hypocretin peptide concentration. Dialysate hypocretin peptide levels were elevated during both the active waking and REM-sleep periods as compared to levels recorded during slow wave sleep. These results led researchers to purport a role for hypocretins in the central programming of motor activity (Kiyashchenko et al., 2002). Additionally, Wu et al. (2002) reported that, in Doberman pinschers, hypocretin CSF levels remained unchanged following a 48-hour food deprivation period, whereas hypocretin levels were elevated up to 70% over baseline following a 24-hour sleep deprivation period. However, the magnitude of the hypocretin CSF level elevation during the sleep deprivation (which was accomplished by consistently walking the dogs) correlated positively with the level of motor activity the animal engaged in during the deprivation period. Moreover, a 2-hour period of exercise in the same test

subjects elevated hypocretin-1 CSF to a level of 57% over baseline, the extent of the increase being positively correlated with the level of motor activity (Wu et al., 2002).

The role of hypocretins in locomotive behavior is further demonstrated by the fact that microinjection of hypocretin-1 into the locus coeruleus (an area with dense hypocretinergic innervations and whose descending projections are partially involved with the facilitation of muscle tone regulation) of decerebate rats facilitated muscle tone in either the ipsi- or bilateral hindlimb muscle. Microinjection of either hypocretin 1 or 2 into the pontine inhibitory area, (another area with moderate hypocretinergic innervations that participates in the suppression of muscle tone during REM sleep), inhibited muscle facilitation. Despite this, direct application of hypocretin 1 or 2 to muscles did not result in any significant facilitation or inhibition of muscle tone. This led to the conclusion that hypocretin peptides influence muscle tone in a centrally mediated fashion (Kiyashchenko et al., 2001). Furthermore, microinjection of hypocretin-1 into medullary gigantocellular reticular alpha (GiA) and ventral (GiV) nuclei parts, both of which receive hypocretinergic innervations and participate in the induction of locomotion and muscle tone facilitation in decerebate animals, also resulted in an increase in muscle tone facilitation. This further strengthens the hypothesis that hypocretins actively mediate muscle facilitation and inhibition (Mileykovskiy, Kiyashchenko, & Siegel, 2002). Together this research provides strong evidence linking the hypocretins with locomotive behavior.

In the present study sleep deprivation greatly reduced the amount of locomotion that an animal could exhibit. Therefore, immobilization-induced decreases in PPH could have been offset by sleep deprivation-induced increases resulting in an apparent effect of zero.

The hypocretin influence and inextricably confounded variables

Previous efforts to investigate the role of the hypocretins in sleeping and feeding behavior have yielded inconsistent results. Experiments investigating the effect of sleep deprivation on hypocretin output have utilized multiple methodologies and reported either no effect on PPH levels via *in situ* hybridization (Terao et al., 2000), a nominal effect via hypocretin CSF levels in active Doberman pinschers (Wu et al., 2002) or a large increase inferred from Fos immunohistochemistry (Estabrooke et al., 2001). While experiments that involved hypocretin 1 i.c.v. microinjections (Piper et al., 2000; Espana et al., 2002, Vogel et al., 2002) all reported a significant increase in time spent awake and a significant decrease in time spent in REM or slow

wave sleep in response to the presence of hypocretin, none of these researchers failed to assess locomotive behavior as a potential factor affecting the outcome of the experiment. Due to the relationship between microinjections of hypocretin and muscle tone facilitation (Kiyashchenko et al., 2001, Mileykovskiy et al., 2002) as well as centrally mediated muscle facilitation or inhibition (Martins et al., 2004, Kiwaki et al., 2004, Kiyashchenko et al., 2002) it remains unclear whether the increase in time spent awake following i.c.v. injection of Hypocretin 1 is more attributable to an increase in locomotive behavior rather then more general increase in arousal.

The failure of experiments to continually yield consistent results regarding the role of the hypocretin peptides may be attributable to the failure of these studies to control for level of locomotion as a mediating factor. It may also be the case that these variables are not completely discernable. For example, while the sleep deprivation paradigm utilized by the present study successfully denies the animals of REM and nonREM sleep, it also constitutes a long-term immobilization paradigm. Long-term immobilization has been previously reported by Martins and colleagues to significantly reduce hypocretin CSF levels (Martins et al., 2004). Additionally, although evidence indicates a role for hypocretins in the circadian cycle, it is unknown the extent to which this cycle varies in accordance with locomotive manifestations. In fact evidence suggests that, with respect to stimulation of hypocretin levels, locomotion is perhaps the only variable that does not vary in respect to circadian rhythm (Martins et al., 2004).

It is therefore possible that the role hypocretin peptides play in such homeostatic drives as sleeping and feeding behavior will always result in an inherent measurement confound. For example, evidence exists that while rats are being deprived of food they may not achieve adequate amounts of sleep. EEG and EMG recordings of rats during an 80-hour total food deprivation period revealed that food deprivation led to a shortening of sleep episodes (specifically shortening the amount of time spent in REM and slow wave sleep stages), causing a change in the light-dark distribution of the sleep states (Borbēly, 1977). The present experiment revealed that animals sleep deprived for 72-hours lost a significant amount of weight as compared to control animals.

Based on the findings of the present study as well as previous experimental manipulations, it is hypothesized that the hypocretin peptides are so heavily ingrained within other homeostatic systems that it may not be possible to define a clearly established role for these

peptides. This is due to the inherent tendency of their constituent functions to create measurement confounds.

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Appendix

University of New Orleans

Institutional Animal Care and Use Committee (IACUC)

DATE: December 8, 2000
TO: Dr. Gerald J. LaHoste

FROM: Gerald J. LaHoste, Ph.D.
Chairman

RE: *IACUC Protocol No.* **024**
Entitled: **Sleep deprivation and LTP *in vivo***

Your revised application for the use of animals in research (referenced above) has been received and found to be acceptable. The approval date is December 8, 2000 and the expiration date is Decembers, 2003.

Vita

Originally from Upstate New York, Kelly Dunn received a Bachelor's of Arts in both Psychology (with honors) and Philosophy at Oswego State University in New York in 2002. During her junior and senior years at Oswego Kelly researched the effects of prenatal exposure to polychlorinated biphenyl's (PCB's) on learning and memory performance in rats with Dr. Paul Stewart. This research contributed to the Oswego Children's Study, an investigation into the cognitive functioning of children exposed prenatally to PCB's via Lake Ontario fish. Since arriving at the University of New Orleans Kelly has been working with Dr. Gerald LaHoste to investigate the effect of sleep deprivation on learning and memory in rats.