COMPARING THE EFFECT OF ANESTHESIA, SLEEP, AND WAKE ON NOVEL OBJECT RECOGNITION MEMORY

by

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ABSTRACT

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4

Recent research has supported the idea that sleep incurs a benefit for learning and memory. However, there is still debate about whether studies on sleep and its role in the facilitation of memory imply a function of sleep, or whether the benefit of sleep on memory results from a lack of interference. In this study, general anesthesia will be used on rats to induce a state similar to, but distinct from sleep. Research has demonstrated that volatile anesthetics, particularly isoflurane, have functional similarities to sleep but do not replace natural sleep, as measured by sleep debt and sleep rebound. The novel objection recognition (NOR) task was used to measure memory in this study. No significant differences were found in the recognition index between rats that were allowed to sleep (n = 14, m = 0.562, SEM = ±0.040), sleep deprived (n = 14, m = 0.524, SEM = ±0.027), received interference (n = 14, m = 0.527, SEM = ±0.029), or received anesthesia (n = 14, m = 0.567, SEM = ±0.044). These results demonstrate that NOR memory may not be sleep dependent as no difference was found between rats that received sleep and those that did not receive sleep.

TABLE OF CONTENTS

LIST OF FIGURES	. vi
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CHAPTER

Page

I. INTRODUCTION

Purpose of Sleep	1
Sleep in Mammals	2
Sleep Deprivation	3
Memory Consolidation	4
Interference	9
Anesthesia, Sleep and Memory	13
Novel Object Recognition	17
Summary and Hypotheses	19

II. METHODOLOGY

Animals and equipment	21
Design and procedure	18
Measurements	21
Figures	22
	źź

III. RESULTS

Recognition Index	
Novel Object Exploration	
Total Exploration	
Activity	
IV. DISCUSSION	

REFERENCES	. 35
APPENDIX	. 44

LIST OF FIGURES

FIGURE	Page
1. General experimental timeline	22
2. Experimental timeline for anesthesia group	. 24
3. Recognition Index score across treatments	26
4. Novel object exploration time across treatments	27
5. Total exploration time across treatments	28
6. Activity of cage control rats	29
•	

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CHAPTER I

INTRODUCTION

Sleep is a nearly ubiquitous behavior from flies to humans, yet despite decades of research across species, the purpose of sleep is still unclear. Many theories attempt to explain its function and purpose, from neuronal and somatic recovery to protection from predation. The necessity of sleep at the most fundamental level is noted in the seminal work by Rechtschaffen and Bergmann (2002), who showed that rats sleep deprived for two weeks died of unknown or yet to be proven causes. The authors went on to conclude that "sleep and paradoxical sleep are biological necessities" supporting the theory that a (or the) function of sleep is restorative, and necessary for vital functions (Rechstaffen & Bergmann, 2002, p. 27).

Earlier theories focused on sleep as serving a sole purpose of recovery, as individuals are typically immobile during sleep, but it only offers a 5-10% reduction in calories consumed compared to a waking state and it is unlikely that sleep's sole purpose is for conservation of energy (Frank 2006). One unique view on the purpose of sleep from an evolutionary perspective is that sleep reduces activity during times when an animal's behavior may not be most efficient and may be viewed as a period of adaptive inactivity (Siegel 2009). Siegel (2009) states that adaptive inactivity is not only limited to mammals, as other organisms undergo dormant states akin to sleep such as estivation in snails or seasonal dormancy in deciduous trees.

It is likely sleep serves numerous vital functions including survival and recovery in

addition to cognitive benefits. Frank (2006), in a review on current theories and perspectives of sleep function, concluded that sleep promotes neural recovery more than it promotes somatic recovery. This claim is supported by noting the number of contradictory studies on whether sleep benefits somatic recovery, specifically in epidemiological studies of sleep amount and effect on morbidity and mortality, as well as sleep's effect on endocrine and immune system function (Frank 2006). Frank and Bennigton (2006) note that until the mid 1990's, studies showing a positive effect of sleep and those that show no or a negative effect on memory were approximately equal. In the last 20 years however, positive findings have dominated the field (Stickgold & Walker, 2005). In explaining this discrepancy, Frank and Bennington (2006) suggest that sleep researchers have either corrected methodological mistakes committed earlier on in the study of sleep and memory or are focusing on methods or procedures that produce positive results.

Sleep in Mammals

Mammalian sleep is divided into two main sub-types: non-rapid eye movement (NREM) and rapid-eye movement (REM) sleep. Humans cycle between NREM and REM sleep at intervals of approximately 90 minutes while rats, the organism of interest in this study have sleep cycles of approximately 12 minutes (McCarley 2007). This cyclical nature also changes over the course of the night as the first half of the night tends to have a greater proportion of NREM sleep whereas the second half of the seep bout has a greater amount of REM sleep.

NREM sleep is further divided into three stages N1, N2 and N3, with these stages

differentiated by their electroencepholography (EEG) readings. Stage N1 is characterized by the presence of alpha waves. N2 is characterized by the appearance of sleep spindles (brief waves in the 12 - 14 Hz range) and K-complexes, in addition to the increase in theta activity and concomitant decrease in alpha activity. Humans spend approximately 50% of their sleep in stage N2. Stage N3 is the stage during which slow wave sleep (SWS) takes place and is characterized by the presence of slow, high amplitude EEG oscillations, or delta waves. REM or paradoxical sleep is characterized by muscle atonia below the neck, rapid eye movements, ponto-geniculo-occipital waves and is the stage of sleep when dreaming takes place. NREM and REM sleep are purported to incur a differential benefit on memory, though this is debated (Rasch & Born, 2013).

Sleep Deprivation

One method to study the function of sleep is to observe the effects of sleep deprivation and compare sleep deprived subjects to those that received normal sleep. Sleep is homeostatically regulated and the body tries to retain a certain amount of sleep over time. Deficits in sleep result in an accumulation of sleep debt, which represents the difference between the amount of sleep an organism should have received and the amount of sleep accumulated. When an organism accumulates sleep debt, during subsequent sleep bouts that are unrestricted, sleep rebound occurs, total sleep is increased and sleep debt is reduced. Selective sleep deprivation of either NREM or REM sleep results in sleep debt for the particular stage that was deprived and results in a sleep rebound for that same stage in later sleep bouts (Rechstchaffen, Bergmann, Gilliland, & Bauer, 1999).

Sleep deprivation can be accomplished through total sleep deprivation, selective

sleep deprivation, or sleep fragmentation. Total sleep deprivation attempts to deprive the organism of any sleep through requiring persistent mental or physical exertion. This is generally done by gently handling the rat for the desired time of sleep deprivation, or requiring constant or periodic movement through use of an automated device. Selective sleep deprivation attempts to deprive the organism of a specific type of sleep, typically REM sleep. This is done in animals either pharmacologically or by taking advantage of the muscle atonia that occurs during REM sleep by using the flower pot method (Colavito, Fabene, Grassi-Zuconi et al., 2013). Some researchers have argued that the method of sleep deprivation can have varying effects on behavioral tests following sleep deprivation, although Rechtschaffen (1998) counters that although one could argue different methods of sleep deprivation could lead to varied effects dependent on the method used. But in general sleep has been shown to be necessary both physically and mentally and without it there is cognitive decline, physical exhaustion and eventually sleep deprivation will lead to death.

Memory Consolidation

Sleep research has demonstrated that sleep deprivation causes impairments in cognitive functioning, including spatial reference memory, declarative and non-declarative memory, as well as executive function (McCoy & Strecker, 2011). Stickgold and Walker (2007), when discussing sleep and memory, use the terms memory consolidation and reconsolidation, to describe unconscious processes in which memory is stabilized and enhanced (as measured in tests of recall) during wake and especially during sleep. This process can take place over a span of years, as new memories are

integrated with old memories and can result in changes to the initial memory. Each time a memory is recalled, the memory temporarily enters a labile state during which it is subject to interference, degradation or reconsolidation. Although still unclear, degradation may result in a loss of the memory trace or the ability to recall the memory (Stickgold & Walker, 2007). Degradation and consolidation are especially relevant to sleep as memories are though to be "pruned" during sleep as consolidation and reconsolidation of memories occurs (Saletin, Goldstein & Walker, 2011).

Consolidation is also believed to take place at two levels: at the synapse and on a systems level. At the synaptic level, synapses are remodeled as they begin to form a representation of a memory that can potentially be further consolidated, whereas at the systems level these new memory representations are integrated with previous representations (Rasch & Born, 2013).

As new memories are integrated, total storage space is technically reduced which challenges whether to keep or erase old memories at the expense of new memories or efficiently. This is known as the stability-plasticity dilemma. One solution to how the brain likely overcomes this obstacle is by having a theoretical fast memory storage in the hippocampus and a longer term storage in the neocortex. Memories are first encoded in the hippocampus and later integrated into the larger neocortex. Evidence for this statement can be found in studies of individuals with hippocampal lesions. A hippocampal lesion results in the inability to gain new memories while older and more remote memories are left intact (Wixted 2004). The time period from which a memory is consolidated and becomes independent from the hippocampus is highly variable and dependent on both the individual and the information to be stored (Rasch & Born, 2013). Currently, there are considered to be two main categories of memory: declarative and non-declarative which are then further subdivided into more specific categories such as episodic or procedural memories. Declarative and non-declarative memories are differentiated by the involvement of medial temporal structures. NREM sleep is believed to preferentially benefit the consolidation of declarative memories, whereas non-declarative memories are believed to be consolidation during REM sleep. This idea is supported by research using night-half paradigm. The night-half paradigm is based on the fact that during the first half of the night, NREM sleep predominates but in the latter half there is a higher proportion of REM sleep (Rasch & Born, 2013).

Sleep Dependent Memory Consolidation

There are multiple theories that attempt to explain how sleep may benefit memory consolidation, one of which is the dual process hypothesis. This hypothesis assumes that NREM and REM sleep benefit declarative and nondeclarative memory, respectively (Rasch & Born, 2013). In a study that compared the effect of NREM sleep and REM sleep on memory consolidation, it was found that sleep mainly consisting of NREM sleep produces better performance on a test of declarative memory recall than sleep consisting of REM sleep. Participants were split into three groups, one that was given a list and tested after 4 hours of sleep, one that was provided a list following 4 hours of sleep and then tested after the second 4 hours of sleep, and one that was awake and tested for recall 4 hours later. The NREM group, the group that was given the list and tested 4 hours after sleep, a period consisting mainly of NREM sleep, performed better than both the REM sleep group and the group that was kept awake, which both performed similarly (Yaroush, Sullivan & Ekstrand, 1971). A similar paradigm was used to study REM sleep's benefit on nondeclarative memory, except focused on the latter half of the night. The study found that memory for implicit and procedural memory was improved during the periods in which REM sleep predominates (Plihal & Born, 1997). These two studies support one hypothesis of sleep dependent memory consolidation, the dual process hypothesis, in which the different stages of sleep play different roles in consolidation of memory.

Another hypothesis of how sleep can benefit memory, the sequential hypothesis, purports that the cyclical nature of REM sleep and NREM sleep aids in memory consolidation. Evidence for this hypothesis comes from studies in which the number of instances in which SWS was followed by REM sleep predicted performance on a declarative memory task (Giuditta, Ambrosini, Montagnese, Mandile, Cotugno, Grassi Zucconi & Vescia, 1995).

The sequential hypothesis states that during SWS, memories are selectively weakened or strengthened depending on whether the memories are adaptive, and then those memories that were strengthened were later integrated into the neocortex for long term storage (Giuditta. et al., 1995). More specifically the slow wave activity during SWS causes depotentiation of synapses which are later strengthened by the high frequency activity observed during REM sleep.

The active systems consolidation hypothesis combines the dual process hypothesis and the sequential hypothesis into a unifying theory, as it proposes that SWS reactivates memories, which then primes for storage during REM sleep. The purpose of the reactivation process is to transfer memory from a short term storage site to a storage site for integration into long-term memory (Rasch & Born, 2013). This hypothesis also presents sleep's impact on memory as an active instead of a passive process.

While memory can be measured at the most superficial level through use of behavioral tests, some researchers focus on memory at the molecular level, specifically with long-term potentiation and depotentiation. The function of long-term potentiation (LTP) is the strengthening of synapses due to a series of events originating from Schaffer collaterals within the hippocampus. This process is believed to be the molecular analog of learning and memory (Reasor & Poe, 2008). For LTP that occurs in the hippocampus, glutamate binds to a subunit on the AMPA receptor on the synaptic terminal which opens a Na⁺ channel, causing an influx of Na⁺ resulting in a depolarization event. This depolarization causes the NMDA receptor on the post synaptic neuron to release Mg²⁺ which allows influx of Na⁺ and Ca²⁺ through the NMDA receptor channel. The subsequent rise in calcium levels causes Camk II activation, which then initiates protein synthesis through promotion of transcription factors which then cause the strengthening of the synapse.

Long term depression or depotentiation (LTD) can be though of as the antagonistic equivalent of LTP, at least on the molecular level. Whereas LTP strengthens synapses, LTD weakens synapses and is involved in the process of forgetting. Although in some instances, such as in directed forgetting, LTD is considered part of the learning process (Frank 2012). In the hippocampus, LTD occurs in a similar manner except through a lower level of Ca2+. It is thought that the level of calcium determines whether a synapse is strengthened or weakened, as higher levels result in CaMKII phosphorylation of proteins and thus potentiation, whereas lower levels result in the depotentiation of synapses (Purves, et al., 2001).

Interference

One cannot mention learning and memory without the understanding of what it means to forget. Early research into forgetting focused on decay, or the passing of time, as the main cause of forgetting. This idea was quickly refuted in a study by Jenkins and Dallenbach (1924) that suggested interference, not decay, was the cause of forgetting. In this study, subjects memorized nonsense syllables and then either slept overnight or were kept awake. If decay were the cause of forgetting both groups should have performed similarly. Those who slept performed significantly better than those were awake despite the same passage of time. These results suggest that sleep either enhances memory or prevents it from being subjected to processes during wake that could lead to a diminished recall ability.

Of particular importance to this study is retroactive interference, when newly learned material interferes with previously learned material. During periods of wake, each bit of new information can potentially interfere with previous memories, with more recent memories more subject to this interference compared to more remote memories (Diekelmann 2014).

While asleep or in an anesthetized state, compared to a waking state, new information cannot be learned without arousal (Wyatt, Bootzin & Allen, 1994). Though an individual may appear lucid during sleep or anesthesia through having conversation or otherwise normal behavior, this is indicative of working memory, which can be left intact for a brief time during sleep and is useful during surgery (Reasor & Poe, 2008). The main reason why no information cannot be learned is thought to be due to inhibition of LTP. Thus, while asleep, the brain is not subject to the effects of retroactive interference.

In one study that tried to elucidate the interference mitigating effects of sleep, participants learned a list of associated words (A-B), and then were split into either a wake group (awake for the next 12 hours), a sleep group (with an extended period of sleep within 12 hours). and then further subdivided into an interference or no interference group. Testing took place 12 hours after the initial learning session, except the interference group learned a new list A-C, 12 minutes prior to testing. Participants were measured by their ability to recall items from the target list B, when given a cue, A. The study found that that sleep resulted in significantly better performance on this declarative memory task and sleep seemed to protect memories from interference significantly more than the wake group. Researchers also had a 24 hr group that learned A-B. went to sleep then had interference introduced immediately prior to the testing phase at 24 hr. The 24 hour group that received interference performed similarly to the 12 hour sleep group that received interference and significantly better than the wake group that received interference (Ellenbogen, Hulbert, Stickgold, Dinges & Thompson-Schill, 2006).

While the study by Ellenbogen, et al. (2006) seems to demonstrate sleep plays an active role in memory consolidation the authors do not take into account the fact that sleep occurs immediately after learning in both the 12 and 24 hour sleep conditions, which may suggest that the temporal relationship between the time of learning and when sleep occurs may also factor into sleep's protective ability from interference. Sleep has been shown to be more beneficial for learning if it occurs 3, rather than 10 hours after learning (Gais, Lucas & Born, 2006). This may suggest a temporal relationship between sleep and learning, suggesting a function of sleep – to reduce or prevent retroactive

interference. In addition to protecting memories from interference it also suggested that some memories may have "paradoxical sleep windows"—periods of time following learning that are required to occur for memory consolidation to occur during sleep, specifically REM sleep. This window is thought to be associated with REM-dependent memory such as motor and procedural memory tasks (Smith 1982; Smith, Aubrey & Peters, 2004).

A similar "window" effect has also been demonstrated with alcohol and benzodiazepines. When participants are presented a list of words then immediately administered alcohol or a benzodiazepine they perform better on a test of recall than if they were to learn the words while under the influence of benzodiazepines or alcohol (Mueller, Lisman & Spear, 1983; Fillmore, Kelly, Rush & Hays, 2001). The reason for this is thought to be two-fold, as the drugs are not only inhibiting LTP, but also inhibiting parts of the brain required for information processing, thus reducing the information that is input into the brain, and subsequently reducing the opportunity for new information to be consolidated into memory. Specifically, this process is referred to as retrograde facilitation and has been observed with alcohol and benzodiazepine administration, as well as sleep. During NREM sleep, induction of hippocampal LTP is blocked without affecting LTP that was previously induced, thereby reducing new memories from being formed during NREM sleep (Wixted 2004).

One study, in support of retrograde facilitation is one in which used a finger-tapping motor sequence task. Participants that slept after learning the task performed better than those who stayed awake, but when a new motor task was introduced shortly after the first task and before sleep, the sleep dependent enhancement in performance is nullified (Walker, Brakefield, Morgan, Hobson & Stickgold, 2002).

Recently, numerous studies have demonstrated that sleep plays a role in learning and memory, but debate still continues as to whether or not sleep positively affects learning and memory (re)consolidation or whether sleep merely protects memories from interference (Vertes & Siegel, 2005; Stickgold & Walker, 2005). Previous research has relied upon sleep deprivation to answer this question, but no research has attempted to induce a state that resembles sleep, i.e. a period during which an animal or human is not conscious.

Research has demonstrated that sleep deprivation can negatively impact learning and memory, but are these results seen because sleep serves a positive role in the facilitation of memory, or does the lack of sleep merely provide more opportunities for interference to occur? And would it also be possible to quantify the difference between the memory consolidation benefit of sleep versus that of a waking state during which there is minimal to zero interference? As previously mentioned, LTP is inhibited during NREM sleep and therefore theoretically no new memories can be formed during that time period. If there was a state of consciousness or unconsciousness that could be experimentally manipulated to protect newly learned material from interference, sleep's impact on learning and memory could be further elucidated. An anesthetized state is one such state that fits these characteristics.

Anesthesia, Sleep and Memory

Anesthesia was first used in surgery in the mid 19th century by Crawford Long. During the time long was in school, ether was frequently inhaled as a recreational drug, and occasionally individuals would run into or cause injury to themselves with knowing it until after the effect of the ether had worn off. During the same time period Charles Jackson found that ether could produce another effect - loss of consciousness (Walker & Mashour, 2008). This loss of consciousness is what causes most to equate sleep with an anesthetic state, as even anesthesiologists may often say they are putting a patient to sleep (Brown, Lydic & Schiff, 2010).

Behaviorally, sleep appears similar to a comatose or anesthetized state but is distinguished from a comatose or an anesthetized state by its rapid reversibility (Dinges, Rogers & Baynard, 2005). Anesthesia has multiple phases much like sleep, except that these stages are dose dependent and do not have a cyclical nature. EEG recordings of Phase 2 anesthesia resemble stage N3 or slow-wave sleep, a stage that is believed to play an essential role in sleep dependent memory consolidation (Brown, Lydic & Schiff, 2010).

One characteristic of sleep is that it reduces sleep debt, and a lack of sleep (or sleep deprivation) causes sleep rebound. A recent study in rats demonstrated that NREM sleep debt, but not REM sleep debt decreases in rodents after isoflurane administration, demonstrating a similarity between NREM sleep and anesthesia, at least in terms of affecting sleep rebound and debt (Pick, Chen, Moore, Sun, Wyner, Friedman, & Kelz, 2011). Sleep deprivation also enhances the effect of isoflurane, which further demonstrates the similarity between NREM sleep and isoflurane induced anesthesia, at least in the mechanism of the induction of sleep and general anesthesia (Tung, Szafran, Bluhm, and Mendelson, 2002).

Isoflurane is a Group 3 halogenated anesthetic which also includes sevoflurane, halothane, enthane and desflurane with the purpose of these anesthetics to induce hypnosis, immobility and amnesia (Forman & Chin, 2008). Isoflurane is the anesthetic of choice for this study, because its primary mechanism of action is through affecting the GABA receptor, a mechanism that resembles the natural induction of sleep (Dildy-Mayfield, Eger & Harris, 1996). This is not surprising as brain imaging research has demonstrated similar patterns between sleep-wake circuitry and those parts of the brain involved while in an anesthetized state (Alkire, Haier & James, 1998). Recent research has even demonstrated that emergence from anesthesia and sleep rely on similar neurons, specifically orexin neurons (Allada 2008).

One concern with administration of anesthetics is that they may contribute to a phenomenon known as post-operative cognitive dysfunction (POCD) that has received much attention of late as an aging population undergo an increasing number of surgeries and following these surgeries cognitive deficits have been noted (Guay 2011). In young and aged rats that were administered isoflurane in nitrous oxide, and tested 48 h later, performance on a spatial learning task was impaired compared to rats that did not receive anesthesia (Culley, Baxter, Yukhananov, & Crosby, 2004). Though a more recent study had mixed results with the use of only isoflurane, as the effect of isoflurane was dependent upon the age of rats as old but not young rats were negatively affected by isoflurane administration on performance in the Morris water maze task (Callaway,

Jones, & Royse, 2012).

The mechanism behind POCD is poorly understood, although rats administered isoflurane may show increased inflammation in the brain, specifically in the hippocampus, which could help to explain the impaired performance on spatial tasks (Lin & Zuo, 2011). Lin and Zuo (2011), tested the hypothesis that inflammation is the cause of cognitive impairment by co-administering lidocaine, a local anesthetic that serves as an anti-inflammatory agent with isoflurane. A spatial learning task was used to assess learning and memory deficits, and the study demonstrated that the co-administration of lidocaine and isoflurane in rats attenuated the cognitive deficits associated with POCD, compared to rats that received only isoflurane (Lin, Cao, Wang, Li, Washington & Zuo, 2012). Thus, administration of isoflurane by itself may interfere with learning and memory tasks, but the co-administration of lidocaine and isoflurane reduces the effects of POCD, a possible confound when attempting to induce a state similar to sleep.

In a study on contextual fear, a type of declarative memory, mice were placed in a fear conditioning chamber and received a shock, then tested either 12 or 24 hours later. The mice were also split into two groups that were both on a 12 hour light: 12 hour dark cycle, but one group was awake for the first 12 hours, while the other was asleep for the 12 hours. Only one significant difference was noted, specifically that the mice that spent the first 12 hours awake and were tested 12 hours after fear conditioning performed significantly worse on the contextual fear task (as measured by percent of time displaying a freeze response, a response that demonstrates fear) compared to the group that was awake first and then tested 24 hours later (Cai, Shuman, Gorman, Sage, & Anagnostaras, 2009). In the groups that slept in the first 12 hours after fear conditioning, no significant

difference was observed (Cai, et al., 2009). These results further support the idea that sleep, particularly within the first 12 hours after training, serves as a functional role either by protecting memories from retroactive interference, or as a unique function of sleep that aids in memory consolidation.

A study that did not use a contextual fear conditioning paradigm, but with a similar design, utilized an object-place recognition task to measure the effects of sleep on declarative memory consolidation. In the experiment rats were familiarized to two objects in two different corners of a square box, and were then tested 2 hours later. For the test, one of the objects was moved, and success on the task was measured by the amount of time exploring the object that moved. Rats that were allowed to sleep in the first 12 hours following the first exposure to the items, performed significantly better than those that were awake in the first 12 hours (Binder, Baier, Molle, Inostroza, Born, & Marshall 2012). Both of these studies, despite testing different forms of memory suggest, at least for rats, that behavioral testing should be done following a bout of sleep, at the start of the dark period.

In tasks that require rats to demonstrate learning of declarative memory, the rats that perform best are those who receive sleep within the first 12 hours following the task. From this idea, the current study will use similar experimental methods that will compare sleep and anesthesia, particularly within the first 12 hours following a learning trial. With regards to anesthesia, general anesthesia leaves long term memories intact, with a lesser effect on more remote memories, but its impact on interference has not been studied (Reasor & Poe, 2008).

Novel Object Recognition

The current study will use the novel object recognition (NOR) task to compare the effects of anesthesia and sleep on declarative memory. This task requires only one trial to test and does not rely on food deprivation or fear and instead relies on a rat's natural preference for novelty. Some argue that it tests episodic memory or episodic like memory, while others have argued that the test is one of declarative memory (Clark, Zola & Squire, 2000; Dere, Silva & Houston, 2004)

The NOR task was created by Ennaceur and Delacour (1988) who noted its value because of its lack of reinforcing stimuli and similarity to primate tasks of recognition. These characteristics make the test similar to those used in humans (such as the visual paired comparisons task) and thus it has a high level of applicability. The original version of the test consists of a period of habituation to the NOR chamber, followed by a sample trial, in which the animal is familiarized to an object (either a single object or two identical objects) and a test trial in which the animal was presented with that same object in addition to a new object. Exploration of the two objects are measured and then compared for a preference (or which object was explored more as measured through orientation towards the object within approximately 2 cm). The test has been demonstrated to be useful in measuring short, intermediate and long term memory, and it was determined in these early studies the preference for the novel object (and thus recognition of the familiar object) decreased as the time between the two trials was increased. Specifically, rats were shown to prefer the novel object when the familiarization and test trials were separated by 1 min or 1 hour, but not 24 hours later

(Ennaceur & Delacour, 1988).

The main brain structures implicated in the NOR task are the hippocampus and parrahippocampal regions of the temporal lobe (Antunes & Biala, 2012). The hippocampus has been demonstrated to be involved in time periods of less than 24 h, as measured by LTP in Schaffer collaterals, but may not be involved in the long term maintenance of NOR memory (Clarke, Cammarota, Gruart, Izquierdo, & Delgado-Garcia, 2010). The perirhinal cortex (a part of the parrahipocampal region) if lesioned has been shown to impair object recognition memory at periods greater than 10 minutes (Norman & Eacot, 2005). The perirhinal cortex has been implicated in all facets of object recognition memory from encoding to consolidation and maintenance (Dere, Huston & De Souza Silva, 2007).

In an evaluation of post-surgical behavioral deficits on the novel object recognition (NOR) task, two groups of rats were used, one which received a microdialysis probe, and another which only received anesthesia were tested before and after surgery to compare their effects (Frumberg, Fernando, Lee, Biegon & Shiffer, 2007). Pre-surgical NOR results showed no significant difference in performance between groups as both learned the task as they were able to discriminate between the novel and familiar object. However there was a significant effect of surgery as rats that only received anesthesia preferred the novel object but those that received the microdialysis probes did not prefer the novel object at 3, 7, 14 and 56 days following the procedure (Frumberg, et al., 2007).

Summary and Hypotheses

Previous research has demonstrated that rats perform better on declarative memory tasks at the beginning of the dark phase, after a bout of sleep (Cai, et al., 2012). Sleep prevents new memories from interference and has been demonstrated to aid in consolidation, possibly through retroactive facilitation. This has also been demonstrated with alcohol and benzodiazepines, which may indicate that the period of time between learning and recall is an important period for consolidation. Preventing interference is difficult while an animal is awake, and during anesthesia and sleep, no new memories can be formed. Anesthesia has similar properties to sleep, may function through similar circuits, and also reduces NREM sleep debt. Despite these similarities to sleep, anesthesia has been implicated in POCD, a term which describes cognitive decline following surgery. While it is not clear whether POCD results from anesthetic administration, the surgical process, or through an interaction of both, an anti-inflammatory drug lidocaine can negate the effects of POCD. Declarative memory, the type of memory involved in the NOR task has been demonstrated to benefit from sleep and is also maintained following administration of anesthesia.

It is expected that the group that receives interference will perform the worst based on data that show when new conflicting information is introduced in a waking period between learning and recall, performance is impaired (Wixted 2004). It is expected that the group that receives anesthesia will perform better than the interference group. And because lidocaine has been demonstrated to attenuate the deficit associated with POCD the group receiving isoflurane should perform as well if not better than the wake group (Lin, et al., 2012). If the group that receives anesthesia performs as well as the cage control rats (rats that are allowed to sleep), it will support the hypothesis that during a time in which there is no interference, memory consolidation occurs and that process may not be sleep dependent. If the rats that receive anesthesia perform worse than the sleep group, then it will support the idea that sleep serves an active role in memory consolidation.

CHAPTER II

METHODOLOGY

Animals

Adult male Sprague-Dawley rats were used for this study. Rats were housed together under a controlled 12 hour light/dark cycle (10:00 lights off, 22:00 lights on), and given *ad libitum* access to both food and water. The animals were housed for 5 days prior to any experimental manipulation.

Equipment

Open Field

The open field in this experiment is a square, opaque, plastic enclosure with interior dimensions of 70 x 70 x 30 cm, a size appropriate for adult rats (Antunes & Biala, 2012). The open field was housed in a room that is dimly lit, the activity room. During all stages of the experiment a red light was used over the open field to aid in video recording. Because the rats used are albino rats the red light should not be perceptible.

Activity Chamber

The activity chamber used for this experiment was a Columbus Instruments Opto-Varimex-Mini. The chamber monitored activity in 5 minute intervals and recorded the number of times a rat crossed an infrared beam.

Objects

A total of 9 sets of objects will be used in this study (objects A through I). These

objects did not resemble environmentally relevant stimuli to the rats. The objects were placed in symmetrical corners, 25 cm from each edge on top of a sticker that marks the center of this point. Objects were cleaned between trials with an ethanol solution.

Design and Procedure

The rats were randomly assigned to one of four conditions: cage control, sleep deprivation, anesthesia, and interference. Each rat underwent each condition with 6 days between condition. The object-recognition task took place in the open field. The procedure consisted of three phases: habituation, familiarization and test trial. The habituation phase allowed the rats to become acquainted with the open field prior to placement of any objects. In the familiarization phase, rats were placed in the open field with two identical objects placed 25 cm from the edges of the open field on opposite corners, and allowed to explore for 5 minutes. Two hours after the sample phase, the rats were subjected to the test trial and given 5 minutes to explore.



Figure 1. General experimental timeline for the novel object recognition test. Note: groups only underwent habituation for the first week of trials.

Cage control

Rats in the cage control condition were placed in an activity chamber following the habituation phase and monitored for 2 hours in the activity chamber before being moved to the activity room for the test phase.

lsoflurane

Rats in the isoflurane group were then placed in an induction chamber and received isoflurane in 100% O₂ until the rat lost its righting reflex. The rat was then removed from the chamber and received a tail vein injection of lidocaine, 1.5 mg/kg in an 8 mg/mL saline solution (Lin, et. al, 2012). Following the injection, rats received a continuous flow of 1.2% isoflurane in 100% O₂ through a nose cone for 1 hour. Rats were then recovered in a single cage. Two hours after the end of the familiarization trial, the rats were then brought to the activity room for the test phase.

Sleep deprivation

Following the familiarization phase, rats in the sleep deprivation group were placed in an automated sleep deprivation chamber (Pinnacle Technology 9000-K6-S) in which a bar alternatively rotated clockwise or counter clockwise for 4 seconds, every 16 seconds, for 2 hours. Following sleep deprivation, rats were then brought back to the activity room for the test phase.

Interference

Rats in the interference group also underwent sleep deprivation. After 1 hour the rats were then brought back into the activity room for the interference phase. Rats were then placed into the open field with two novel objects (/ and /) and allowed to explore for 5



minutes, removed and placed back in the sleep deprivation chamber.

Figure 2. Experimental timeline for interference group in the novel object recognition test. Note: Not shown is habituation phase, which took place during the first week of trials.

Open Field Habituation

Rats were placed in the open field, with no objects, and allowed to explore for 10 minutes, and then removed. This was done each day for 5 days prior to testing.

Familiarization Trial

Rats were placed into one corner of the open field facing away from the objects (A and A') located in adjacent corners to the rat. Rats were allotted 5 minutes for exploration. Following the familiarization trial, rats underwent experimental manipulation, dependent on the group and and began the test trial 2 hours following the cessation of the familiarization trial.

Test Trial

The test trial took place 2 hours after the sample trial. In this trial, object A was replaced by a novel object, B through H, (see Figure 1). Rats were placed facing away from the objects and then observed for 5 minutes for interaction and exploration with the

"familiar" and novel stimuli. The location of the novel object was alternated between trials to control for spatial or contextual bias.

Measurements

Each trial was recorded using a digital video camera mounted 4 ft above the covering of the open field. The transparent plastic covering of the open field contained two red squares that formed a 4 cm wide perimeter above the center of the objects to aid in assessing exploration. For measurement of exploration during the familiarization, interference, and test trials, exploration was defined when the rat oriented its head within a 45 degree angle, within 4 cm of the object (Antunes & Bialla, 2011). Exploration was scored on recorded video. When the qualifications for exploration were met a stopwatch was started to record the amount of time spent exploring an object and stopped whenever the rat no longer met the qualification for exploration. Time was recorded separately for both objects. If the rat reared up or sat on the object this was not counted and/or terminated the running exploration time for that object.

Recognition Index

To quantify the value of novel exploration *Recognition Index* (RI) was used, which describes preference for the familiar or known object versus the novel object on a scale of 0 to 1. Absolute preference for the familiar or known object would receive a score of 0 while absolute preference for the novel object would receive a score of 1 and preference for neither would receive a score of 0.5. The formula used for this index is $RI = (T_N) / (T_N + T_F)$, where $T_N =$ the amount of time spent exploring the novel object and $T_F =$ time spent exploring the familiar object. If the rat has learned the task, the rat will spend more time exploring the novel object because the rat is already familiar with the other object (Antunes & Biala, 2012).

CHAPTER III

RESULTS

Recognition Index

A one-way repeated measures ANOVA was calculated comparing the recognition index (RI) of CC, ISO, INT and DEP groups. No significant effect of treatment was found (F(3, 36) = .464, p = .709) between Cage Control (m = 0.562, SEM = ±0.040), *Isoflurane* (m = 0.567, SEM = ±0.044), *Interference* (m = 0.527, SEM = ±0.029), or Sleep Deprived (m = 0.524, SEM = ±0.027).



Figure 3. Recognition Index score across treatments. No significant differences were found between groups. Error bars represent the standard error of the mean.

Novel Object Exploration

A one-way repeated measures ANOVA was calculated comparing the time (in seconds) spent exploring the novel object. A significant effect of treatment was found (F(3,36) = 10.201, p < 0.001) between exploration time of *Cage Control* (m = 30.415 s, SEM = ±4.312), *Isoflurane* (m = 12.800 s, SEM = ±3.402), *Interference* (m = 21.285 s, SEM = ±2.056), and *Sleep Deprived* groups (m = 25.8 s, SEM = ±2.386). Bonferroni *post hoc* analysis indicated that *Isoflurane* was significantly less than *Cage Control* (p = .001) and *Interference* (p < .001), but not *Sleep Deprivation* (p = .306). No other between group differences were noted.



Figure 4. Novel object exploration time (in seconds) across treatments. A significant difference was found between groups (p < 0.05). Error bars represent standard error of the mean.

Total Exploration

A one-way repeated measures ANOVA was calculated comparing the total exploration time (in seconds) of each group. A significant effect of group was found (F(3,36) = 12.019, p < 0.01) between total exploration time of *Cage Control* (m = 52.354s, *SEM* = ±5.516), Isoflurane (m = 20.769 s, *SEM* = ±4.065), Inteference (m = 41.185 s, *SEM* = ±4.196), and Sleep Deprived groups (m = 47.515 s, *SEM* = ±5.653). Bonferroni *post hoc* analysis indicated that *Isoflurane* was significantly less than *Cage Control* (p< .001), *Interference* (p < .001), and *Sleep Deprivation* (p = .019). No other between group differences were noted.



Figure 5. Total exploration time across treatments. A significant difference was found between groups (p < 0.01). Error bars represent the standard error of the mean.

Activity

Activity level was measured for all rats during their cage control treatment. Activity level was measured by the number of times the rat crossed an infrared beam in the activity chamber (n = 13, m = 2802.15, SEM = ±250.152).



Figure 6. Activity of cage control rats. Intervals represent 5 minute intervals for a total of 2 hours. Error bars represent the standard error of the mean. Cage control rat activity greatly decreased during the first part of the light cycle.

CHAPTER IV

DISCUSSION

Despite the rats learning the NOR task and preferring the novel object, no significant effect of group was found to influence performance on the NOR task as measured by RI. The result of no effect for this study could be due to a number of factors, one of which is regarding the objects used in the study. The objects used in this study were selected by the researcher to represent different materials (plastic, porcelain, metal, glass), different patterns (striped, solid, transparent), and different shapes (oblong, short, square) and chosen as to not represent environmental stimuli. While every effort was used to try to minimize the similarity of the objects, it is possible that there could have been interfernce between the 9 total object the rats were exposed to over 4 trials and 24 days.

Previous studies have also utilized specific time requirements during the familiarization phase, such as requiring the rat to spend 30 seconds with each object before being removed whereas in this study rats were provided 5 minutes of exploration regardless of time spent with each object (Antunes & Biala, 2012). Due to technical constraints this was not possible for this study but future studies have begun to used automation as a technique to monitor exploration time and can be used to ensure rats are fully familiarized with objects after spending a set amount of time with each (Silvers, Harrod, Mactutus, & Booze, 2007). Previous research has also demonstrated that rats can visually inspect objects without being near (less than 2 cm away) which this study could also not measure (Winters & Reid, 2010).

In addition, albino rats have poor visual acuity and also the potential for visual problems in general compared to their hooded counterparts (Whishaw & Kolb, 2004, p. 55-57). The Sprague-Dawley rats used in this study likely relied more on olfactory cues compared to visual cues to discriminate between the novel and familiar objects. To combat the rats' poor visual acuity, the objects used in each trial were selected because of their distinct differences (width, height, and material) but it is not certain if the rats were able to see or process this distinction. Despite a visual disadvantage compared to other rat strains, object recognition memory has been demonstrated to persist for up to 4 hours in Sprague-Dawley rats (Bertaina-Anglade, Enjuanes, Morillon, & Drieu la Rochelle, 2006).

Another potential issue with this study is that rats were only tested at the beginning of their wake cycle and not at any other time. This does not take into account any issues related to circadian rhythms or time of day effects from only testing during the first 2 hours of the light period. Although this may be an issue previous research has demonstrated that at least for tasks of declarative memory, rats perform best at the beginning of the dark cycle, following a bout of sleep (Binder, et al., 2012).

While administration of the volatile anesthetic isoflurane has been shown to cause impairment on tests of cognition, isoflurane with lidocaine has been demonstrated to prevent the cognitive decline potentially associated with POCD. This result was supported by this study, as the group that received isoflurane and lidocaine performed similarly on the test of NOR. The isoflurane and lidocaine group did however have a lower mean exploration time, which may indicate a motor impairment due to the lingering presence of isoflurane in the body. Isoflurane is not eliminated from muscle, at least in humans, for 4 to 6 hours after cessation of administration (Schmidt 1987). Another issue is that rats can also explore items visually, even if they are not < 2 cm from the object (as was required for an exploration event in this study). Because isoflurane was still present isoflurane in muscle tissue, a visual strategy may have been more efficient than one that required olfaction or physical touch (Winters & Reid, 2010). Although, this could have had an effect on total exploration time it should not have had an effect on preference for either object (Dere, Huston, & De Souza Silva, 2007). Regardless, this study does demonstrate that either isoflurane does not cause cognitive decline associated with POCD or that those effects can be mitigated by an anti-inflammatory drug such as lidocaine on the NOR task.

It was anticipated that rats receiving interference should have performed worse on the NOR test compared to rats that slept, were anesthetized or received no interference, but this was not demonstrated. This study used a procedure that had not previously been used. Previous tasks have only used two objects when testing recognition memory and its possible the object did not even interfere with the familiar and novel object. Future research could focus on an effective way to create interference possibly by adding a spatial component instead of another object between the familiar and test phases.

Another concern is the use of the NOR task for studying sleep dependent memory consolidation. A recent study demonstrated that when the object recognition task is performed without a spatial or temporal component it may not be sensitive to the effects of sleep deprivation (Inostroza, Binder, & Born, 2013). Rats were tested for recognition memory during the first 80 minutes of their wake period. No significant differences were found for object recognition memory between the rats that were allowed to sleep and

those that were not allowed to sleep. In the study the researchers demonstrated that the rats received about 20 minutes of sleep and this started about 40 minutes after presented with the objects for the first time. However, the study did find that rats that slept performed significantly better on the object recognition tasks when either a spatial or temporal element was added, such as moving an object to a new location on the recognition trial, or introducing an object between the familiarization and test phases. In these tests, researchers demonstrated that if a rat did not sleep in the first 80 minutes after the familiarization phase they did not learn it. The researchers argued that spatial or temporal context made the memory an episodic one, and without either of these elements the memory was merely semantic in nature and did not rely on the hippocampus. Sleep has been shown to be essential for hippocampal dependent memories, but the evidence for non-hippocampal memories is sparse.

Broadbent, et al. (2004) found that if damage to dorsal hippocampus and ventral hippocampus were of a significant amount (greater than 75% and 50% respectively) then impairments of object recognition were observed. However, in one study that tested object recognition memory in rats that had cytotoxic lesions of the hippocampus, it was found that these lesions had no effect on object recognition from intervals of 1 to 120 minutes, even after meeting criterion set forth by the aforementioned study (Albasser, et al. 2012). What has been consistent in tests of object recognition memory is the need for an intact perirhinal cortex. The perirhinal cortex is involved in all facets of object recognition memory, from acquisition to retrieval (Dere, Huston, & De Souza Silva, 2007).

Because it is not clear if the one trial NOR task is hippocampal dependent, future

research on comparing consolidation of memory during sleep and wake could use an object-place recognition task (which is hippocampal dependent) to better understand the degree to which sleep actively consolidates memory. The study would also use materially uniform objects that could be more easily distinguished by a hooded-rat species as opposed to an albino species such as the Sprague-Dawley used in this experiment. This type of basic research could help to further understand to what extent sleep consolidates memory and could provide evidence for (or against) improving memory consolidation through decreasing interference from other daily activities. It could also help further the case for the importance of sleep in improving cognitive function, as college students stay up late to cram for exams, and employees work longer hours to meet more demanding criteria set forth by their employers. With evidence mounting in support of the benefits of sleep on cognitive function, one can only hope that sleep is eventually viewed not as something done by those who are lazy but by those who strive to be successful.

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APPENDIX A

University of Houston-Clear Lake Institutional Animal Care and Use Protocol

Federal animal welfare regulations require that the Institutional Animal Care and Use Committee (IACUC) must review and approve all activities involving the use of vertebrate animals prior to initiation of such use. Once approved by the IACUC, any change(s) to the following protocol must be submitted in a written amendment for review and approval of the IACUC prior to implementation of the change(s).

1. Title of Project

Anesthesia and sleep in learning

2. Principal Investigator:

Name:	Chris Ward		
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Secondary Contact Person involved in the study:

Name:	Ryan Kieltyka		
Email:	Kieltyka@gmail.com	Emergency Phone:	281-435-2206

3. Project Type

New Protoc	ol Renewal	Addendum Previous Protocol #	
If renewal:	Annual Renewa	al Protocol Renewal see Appendix E	
If addendum:	Personnel Chan	nge	
	Minor Revision	Major Revision see Appendix E	
Number of year continue:	s project is expecting	g to 1 year 2 years 3 years	
This protocol	Teaching	Research Breeding	
is for:			
If teaching:			
Course name	and number:		
Frequency co	ourse is offered:		
If research:			
How will this	s project be funded:		
If grant, this	project is:	Pending Funded – Federal	
		Funded – Other	
Grant title and/or contract number (if available):			
Has this proje	ect already received a	an independent scientific Yes	
peer-review?		No	
If yes, by wh	om?		

4. Location

Where will animals be housed?	UHCL Animal Research Facility 🗌 Field
	Study
Other:*	
Where will animal use take place?	UHCL ARF
Will animals be kept for over 12 hou	rs outside of housing 🚺 Yes* 🛛 No
area?	
If yes, give location and reason:	

*A standard operating procedure (SOP) to ensure proper welfare and housing of animals must be attached to this protocol in Appendix F. This does not apply to animals housed at other AAALAC accredited animal facilities (e.g., UH or NASA).

5. Lay Summary:

Describe the goals and intended benefits of the project in terms that can be understood by a non-scientist. Include the <u>species</u> and the <u>number</u> of animals to be used. This description should be no more than 250 words. Avoid the use of technical jargon and abbreviations.

Evidence suggests that sleep plays an important role in the consolidation of memories. However, a specific mechanism that explains this consolidation has not been identified. An alternate explanation is that sleep removes the interfering effects of a normal day that hinders memory consolidation. In the present experiment, Sprague Dawley rats (n=56) will be tested in a task that memory recall has been show previously to be enhanced following sleep. Rats will explore an open field with the same two common objects (figurine, bowl, etc.). Two hours later, one object will be changed and the memory of the rat for the previous object will be tested. During the two hours, rats will either be allowed to sleep, be given anesthesia, or kept awake. The recall of rats following these manipulations will help us to understand if sleep is actually vital to the consolidation of memory.

6. Animal Use:

Provide the specifications for all of the animals requested for use in this protocol. List each strain separately.

	_	Number Requested			ested	
Species	Breed/Strain	Vender/Source	Year	Year	Year	Total
(common name)			1	2	3	
Rat	Sprague-Dawley	Harlan	56			56

Not Applicable (this is a population field study)

If a population field study, check all vertebrate animals that are planned to be studied:

Fish Amphibians Reptiles Birds Mammals / Cetaceans

7. Personnel:

List all personnel having contact with animals, the species proposed and the years of experience the individual has with the species. List the specific roles the individual will have in the project and the date of last training received.

Name, Degree, Title	Species and Years of Experience	Specific Role in Project*	Train ing Date
Chris Ward, PhD, Assoc. Prof.	Rat – 15 yr	Supervision, anesthesia, surgery, monitoring, post-procedural care, euthanasia	
Ryan Kieltyka, RA	Rat – 2 уг	care/handling, anesthesia, behavioral testing, euthanasia	
Jennifer Pido, RA	Rat – 2 yr	care/handling, anesthesia, behavioral testing, euthanasia	

* Examples include: supervision, care/handling, anesthesia, surgery, monitoring, post-procedural care, euthanasia in the stated species

Student visitors will/may participate in this protocol and will be supervised by: Chris Ward

8a. Literature Search

Using at least two different databases, perform literature searches to determine alternatives to procedures that may cause more than a momentary or slight pain or distress to the animals, and unnecessary duplication of research.

Search Database (e.g., Agricola)	Date of Search MM/DD/YYYY	Years Covered (e.g., 1980 – 2010)	Keywords or Search Strategy Used in Search
PubMed	9/20/2013	1980-2013	Sleep, memory consolidation, anesthesia, object recognition
Google Scholar	9/20/2013	1980-2013	Sleep, memory consolidation, anesthesia, object recognition

8b. Rationale and purpose of animal use

State the overall rationale, purpose, and significance of this project.

Sleep research in both humans and rodents has demonstrated that sleep deprivation causes impairments in cognitive functioning, including spatial reference memory, declarative and non-declarative memory, and executive function. Thus, research has demonstrated that sleep deprivation can negatively impact learning and memory, but are these deficits observed because sleep serves a positive role in the facilitation of memory consolidation, or does the lack of sleep merely provide a greater number of opportunities for interference to occur? If there were a state of consciousness or unconsciousness that could be experimentally manipulated to protect newly learned material from interference, sleep's impact on learning and memory could be further elucidated.

No mechanism has been identified as to why sleep plays a role in memory consolidation. Previous research has relied upon comparisons of sleep deprivation and normal sleep to answer questions about the relationship between sleep and memory, but no research has yet attempted to induce a state that resembles sleep, i.e. a period during which an animal or human is not in an alert or awake state.

Behaviorally, sleep appears similar to a comatose or anesthetized state. Further supporting this similarity is that electroencephalography (EEG) recordings of anesthetized humans, demonstrate that Phase 2 anesthesia resembles Stage 3 NREM sleep. Although these two states appear similar, sleep is distinguished from coma or anesthesia by its rapid reversibility. Another main distinction between sleep, anesthesia, and a comatose state, is that sleep reduces sleep debt, and lack of sleep (or sleep deprivation) causes "sleep rebound." But a recent study in rats demonstrated that rapid eye movement (REM) sleep debt, but not non-rapid eye movement (NREM) sleep debt, increases in rodents after isoflurane administration, demonstrating a similarity between NREM sleep and anesthesia, at least in terms of affecting sleep rebound and sleep debt.

Isoflurane, a volatile anesthetic, is the anesthetic of choice for this study because its primary mechanism of action is through affecting the GABA receptor, a mechanism that resembles the natural induction of sleep. One concern with use of anesthesia is a deficit in cognitive function that can last for weeks following administration, an effect known as post-operative cognitive dysfunction (POCD). Although volatile anesthetics such as isoflurane have been demonstrated to cause POCD, co-administration of lidocaine with isoflurane has been shown to reduce the effect of POCD on both short and long term memory in rats compared to rats only receiving isoflurane (Lin et al., 2011 & Cao et al., 2012).

This study will use the novel object recognition task, a hippocampal dependent declarative memory task, to compare the effects of anesthesia and sleep on declarative memory. Novel object recognition task performance has been demonstrated to be enhanced by sleep compared to sleep deprived rats, specifically when the task was given at the end of the sleep/ beginning of the awake cycle. If memory enhancement is due to

specific mechanism of sleep, the free sleep will be the only group to show increased object recognition. However, if removing cognitive interference during a period of unconsciousness is the beneficial aspect of sleep, the anesthesia group should also so improvement on the object recognition task. Conversely, the group that receives extra interference should be low performing compared to sleep deprived control rats.

References

Lin D, Cao L, Wang Z, Li J, Washington JM, Zuo Z. Lidocaine attenuates cognitive impairment after isoflurane anesthesia in old rats. Behav Brain Res. 2012 Mar 17;228(2):319-27.

Cao L, Li L, Lin D, Zuo Z. Isoflurane induces learning impairment that is mediated by interleukin 1 β in rodents. PLoS One. 2012;7(12):e51431.

8c. Justification for animal use.

Explain why non-animal models such as isolated organ preparation, cell or tissue culture, or computer simulation cannot be used.

This study involves sleep; therefore, a mammalian species must be utilized. Though some invertebrates show circadian or sleep like behaviors, these behaviors are poorly understood which create difficulties in the generalization of results. The same is true for the sleep like behaviors of reptiles and some fish. To date, the only species that have a sufficient literature base in sleep research other than humans are dogs, cats, rats, and mice. Additionally, the behavioral task utilized has been previously validated in rats.

To date, the knowledge in the literature of the interaction between memory systems and sleep is poorly understood, especially all of the underlying mechanisms. Therefore, computer simulation is not possible in the proposed experiments.

8d. Justification for using this particular species.

Explain why the species and/or strain(s) requested is/are the most appropriate for this research. Statements that the planned species is traditionally used for the proposed research are not sufficient.

The rat is extensively used in studies of neurophysiology, sleep, and learning and memory. A great deal of literature exists describing the underlying physiology and behavior of sleep in rodents. Additionally, learning and memory tasks that utilize the hippocampus are well established in the rat. The techniques described in the current protocol are commonly used and accepted in the rat model. Additionally, the sleep characteristics in non-mammalian species is fundamentally different from that of human sleep, therefore, results are difficult if not impossible to generalize.

8e. Alternatives to Potentially Distressful Procedures

Describe considerations of alternatives to procedures that may cause more than a momentary or slight pain or distress to the animals, and determination that alternatives were not available.

Not Applicable (animals listed are <u>only</u> in USDA Category B or C)

Rats will be exposed to anesthesia and sleep deprivation. Isoflurane was chosen because it has a lower risk for adverse effects than other types of anesthesia. Sleep deprivation will be achieved through a commercially available sleep deprivation chamber that mimics gentle handling sleep deprivation. While this procedure does not produce a large stress effect, and rats in our lab have been sleep deprived for 24 hours without any problems, exposure will be limited to two hours in this experiment.

8f. Assurance of Non-Duplication

This experiment does not unnecessarily duplicate previous experiments. Otherwise, provide justification of the necessity of experiments proposed.

9. Justification of animal numbers.

Provide a detailed justification for the numbers of animals requested. Include number of animals per group and total number of animals. If power analysis was utilized, give appropriate details. If the determination was based on prior experience, please cite reference. If a population study in the field give justification of sampling method.

Based prior literature, power analysis was conducted with the G*Power 3.0 software package. Given a large effect size of f = .4, using an $\alpha = .05$, and power = .7, it was determined that n = 14 per group should provide enough statistical power to detect a significant difference if there is an actual difference. Statistical analysis of data will be conducted with an appropriate ANOVA model followed by post-hoc tests.

The following summarizes the experimental groups:

Isoflurane + Lidocaine	14
Free Sleep	14
Sleep Deprived	14
Sleep Deprived + Interference	<u>14</u>
Total	56

10. Pain, Discomfort, and Distress.

a. USDA Pain/Distress Classification

Check the category that indicates the highest level of pain/distress the animals will experience during the course of these studies. (Refer to the Instructions, Section 10 for help.)

Category B	Category C	Category D	Category E
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b. If Category E is selected, provide scientific justification why pain and/or distress could not be relieved. State methods or means used to determine that pain and/or distress relief would interfere with test results.

11. What will happen with animals at the end of their roles in the project.
a. Check all that apply and provide explanation if necessary.
Placed for adoption
Released into wild (field study)
Euthanasia:
Rodents:
CO ₂ - followed by secondary method (e.g. bilateral thoracotomy, cervical
dislocation)
State secondary method:
Injectable agent (Specify Agent, Route, Dose):
Inhalant agent (Specify Agent, Dose): isoflurane, overdose
Cervical Dislocation (rodents < 200 gm) w/ anesthesia- (Specify Agent. Route.
Dose):
Decapitation/Guillotine w/ anesthesia- (Specify Agent, Route, Dose):
Exsanguination w/ anesthesia (Specify Agent, Route, Dose):
Anesthetic + Perfusion (Specify Agent, Route, Dose):
Type of perfusion:
Amphibians, Fish, Reptiles:
Injectable agent (Specify Agent, Route, Dose):
External or topical agent (Specify Agent, Route, Dose):
Inhalant agent (Specify Agent, Dose):
Decapitation and pithing
Stunning and decapitation/pithing
X Other: Transferred to other protocols

b. Explanation / Justification

12. Additional Forms Attached

Check all that apply and attach appropriate forms. If a form is not needed, delete the page from the protocol.

Appendix A: Laboratory Research or Classroom

Appendix B: Surgical Procedures

Appendix C: Wild Animal and/or Field Research

Appendix D: Safety

- Appendix E: Renewal / Addendum
- Appendix F: Additional Information / Standard Operating Procedures

13. Check the following:

I certify that the use of all animals involved in this project will be carried out within the provisions of the Animal Welfare Act, the Guide for Care and Use of Laboratory Animals, the PHS Policy on Humane Care and Use of Animals, the University of Houston Policy on Care and Use of Animals and related animal welfare rules and regulations as issued by state and/or federal agencies.

I am aware that the Institutional Animal Care and Use Committee (IACUC) may make periodic inspections of all labs in which animals are used. I will permit unannounced inspections and observations of my animals and surgical techniques by a UH veterinarian or other representative of the IACUC.

I am aware that the IACUC is empowered to stop any objectionable procedure or project. Where procedures have caused severe distress to an animal which cannot be alleviated, UH staff veterinarians are authorized to humanely euthanize that animal. I understand that every attempt will be made to contact me before any action is taken. I understand that I cannot start this project until I have received approval from the IACUC.

I understand that I will make written notification to the IACUC of any proposed changes to the project. I understand that I will not be able to implement such changes until approval is received from the IACUC.

I certify that the above statements are true and that I will make written notification to the IACUC of any changes in the proposed project prior to proceeding with any animal experiment.

<u>9/20/1013</u>

Date

Signature of Principal Investigator or Instructor

Submitted Electronically: Instead of signature, protocol is emailed from the PI's UHCL email address

Appendix A: Laboratory Research or Classroom

Delete page if not needed.

1. Special Husbandry Requirements:

a. Will any of the following exceptions to routine animal care be required? Selections must be justified.

	Wire bottom cage	S	🛛 Individual housin	g 🔲 Special Diet
	Special Bedding		Special Water	Food/Water Restriction
X	Other (explain):	Live in	sleep deprivation char	nber for 3 days.

b. Justification:

Rats will be placed in solitary housing during testing.

For sleep interruption protocols, single rats will live in a special chamber (660 cm² floor area – recommendation for rats <400g is 258 cm²). The chamber contains a rotating arm that automates the sleep deprivation process. Rats will live in the chamber with free access to food and water for up to 3 days to allow for habituation. Active sleep deprivation will not exceed 2 hours. Control rats will be individually housed during the same time period as a control.

Rats would be individually housed only for the length of the study and then would be placed back into social housing.

2. Description of Experimental Design and Animal Procedures.

Provide a clear and concise sequential description of the proposed use of animals. This description should allow the IACUC to understand the course of an animal from its arrival through the experiment to the endpoint of the study, and final disposition. A flowchart or timeline of experimental activity is encouraged. Refer to the Instructions, Section A2 for information that should be included. Details of surgical procedures must be provided in Appendix B.

Rats will be identified by the use of numbered ear tags. The ear tags will be cleaned in alcohol prior to being placed on the animal. The rat will be held by one person as another uses an ear tag applier. The ear tag will be placed on the lateral base of the ear, approximately 3 mm from the edge of the ear pinna.

Open Field Test

The open field is a square, opaque plastic enclosure (70x70x30 cm). Common object will be placed in the open field. Objects will be made of glass or plastic and will not have rough, sharp edges, or features that would encourage chewing. Examples would include a container with a lid filled with marbles or a figurine. Object will not resemble environmentally relevant stimuli to the rats.

Rats will first be habituated to the open field the day before testing by being allowed 10 minutes to explore the open field. On the day of testing, identical objects will be placed in opposite but symmetrical corners and secured with hook and loop tape. The rat will be allowed 5 minutes to explore the open field. Two hours later, a test will take place where one of the two objects will be replaced with a novel object. The rat will be allowed to explore the open field for 5 minutes. All sessions will be recorded with a video camera and rat exploration will be scored at a later time. The open field and objects will be cleaned between trials with alcohol.

During the 2 hour period between exposure and testing, rats will be randomly assigned to one of four experimental conditions.

Isoflurane + Lidocaine Exposure

Rats will be placed in a chamber exposed to 4-5% isoflurane in oxygen until righting reflex is absent. Rats will be removed from the chamber and a nosecone will be used to deliver 1-3% isoflurane in oxygen. Attempts will be made to use the lowest possible dose of isoflurane to keep the rat sedated since a surgical plane of anesthesia is not needed. Rats will be kept on a warming pad during anesthesia. As in previous studies (Cao et al., 2012), lidocaine will be administered twice (1.5 mg/kg, IV at anesthesia onset and 2 mg/kg, IV during anesthesia). Injection volume will be less than 0.1ml. Prior to inject, the tail will be cleaned with gauze and alcohol. A 22-25 gauge needle will be used. After injection, the needle will be removed and proper hemostasis will be ensured. Following one hour of isoflurane administration, the rat will be allowed to fully recover to consciousness before being tested again.

Free Sleep

Rat will be allowed undistributed sleep in their home cage during the first 2 hours of the lights on period. Rats will be individually housed to control for the individual housing in the sleep deprivation chamber. Rats will be habituated to individual housing for no more than 3 days prior to testing.

Sleep Deprived and Sleep Deprived with Interference

Rats will be sleep deprived using an automated device designed by Pinnacle Technology (a product description and picture has been provided in Appendix F). A rotating bar (5-15 RPM) will nudge the rat for 4 sec and then turn off of 12 sec, repeating every 16 sec for the duration of sleep deprivation, 2 hours. Prior to sleep deprivation, rats will live in the sleep deprivation chamber for no more than 3 days to habituate to the chamber.

Half of the rats will be removed after 1 hour in the sleep deprivation device and placed in the open field for 5 minutes with 2 novel objects. Following this, they will be returned to the sleep deprivation chamber for the final 1 hour. Following the test of recall in the open field, all rats will be returned to social housing.

3a. Animal Well-Being and Minimizing Pain and Distress

What is the impact of the proposed experiments on the animals' well-being? Describe the procedures designed to assure that discomfort and pain to animals will be limited to that which is unavoidable for the conduct of this project.

Not Applicable (animals listed are only in USDA Category B or C)

As with any anesthesia, there is the possibility that a rat will respond poorly. An experimenter will be present with a rat the entire time it is under anesthesia. Anesthesia exposure will be for approximately one hour. In our lab, we routinely expose rats to Isoflurane for the same length of time for surgeries. Anesthesia will be removed if a rat is having trouble breathing.

Sleep deprivation will be achieved through a commercially available sleep deprivation chamber that mimics gentle handling sleep deprivation. While this procedure does not produce a large stress effect, and rats in our lab have been sleep deprived for 24 hours without any problems, exposure will be limited to two hours in this experiment.

3b. Criteria for Endpoint

The following list gives standard criteria for indications of pain or stress in animals. If study requires a change to these criteria, please provide a justification below.

Non-specific signs of illness to monitor for (more than one or two criteria could require experimental termination after consult with a veterinarian) Mammals

Weight loss (10%)	Lethargy	Kyphosis (hunched posture)
Unkempt appearance	Isolation from cage mates	Slow/lame/limping
Dermatitis	Uterine or rectal prolapsed	
<u>Aquatic</u>		
Decreased/abnormal activity	Change in ventilation rate	

Serious conditions requiring	more immediate experimental (termination
Mammals	-	
Dyspena (Difficulty	Non-responsiveness	Severe Fight
Breathing)	-	-
Weight loss (>15%)	Dehydration	Infection
Ataxia/ inability to access	Ulcerated tumors	Tumors >1.5

Dermatitis >10% body area Abdominal distention

Wounds

Tumors >1.5cm(mou se) or 2.5cm(rat)

Justification:

food/water

Appendix F: Additional Information / Standard Operating Procedures



9000-K6: Automated Sleep Deprivation Kit for Rats

Pinnacle offers a unique solution for sleep deprivation of rats without associated exercise effects. An adjustable metal bar, positioned above the bottom of the cage, rotates during periods of sleep or programmed sleep deprivation. This disturbs the subject such that the animal must wake up to move around the bar as it rotates. Rotation of the bar ceases once the animal has been awake for a predetermined period of time, limiting exercise and stress effects due to stimulus application. Preliminary studies have shown this to be an effective method for long-term sleep fragmentation and/or deprivation. Sleep onset parameters can be automatically determined by the software or controlled directly by the user. In the case of software control, EEG and EMG signals fed into the PAL 8200 software control suite are analyzed for signs of sleep (high EEG delta activity, low EMG amplitude, etc...) and the rotating bar stimulus is triggered when any of these parameters are met. Parameters may also be modified by the experimenter, when necessary. A second mode allows the rotating bar to be programmed independently to follow a controlled deprivation paradigm as defined by the experimenter.

The Sleep Deprivation System includes:

- 12"d circular cage for rats
- sleep deprivation actuator
- water bottle with holder
- sleep deprivation software module
- Touch screen controls
- 9009-RSD Adjustable Stand (~36" h)

To use in automated mode with rats the 8200-K2-SL is required, or the unit can be operated in stand-alone mode with no computer required.