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CHANGES IN SLEEP ARCHITECTURE AND COGNITION WITH AGE AND PSYCHOSOCIAL STRESS: A STUDY IN FISCHER 344 RATS

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CHANGES IN SLEEP ARCHITECTURE AND COGNITION WITH AGE AND PSYCHOSOCIAL STRESS: A STUDY IN FISCHER 344 RATS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Heather Marie Buechel
Lexington, Kentucky
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2013

ABSTRACT OF DISSERTATION

CHANGES IN SLEEP ARCHITECTURE AND COGNITION WITH AGE AND PSYCHOSOCIAL STRESS: A STUDY IN FISCHER 344 RATS

Changes in both sleep architecture and cognition are common with age. Typically these changes have a negative connotation: sleep fragmentation, insomnia, and deep sleep loss as well as forgetfulness, lack of focus, and even dementia and Alzheimer's disease. Research has shown that psychosocial stressors, such as isolation from family and friends or loss of a loved one can also have significant negative effects on sleep architecture and cognitive capabilities. This leaves the elderly in a particularly vulnerable situation: suffering from cognitive decline and sleep dysregulation already, and more likely to respond negatively to psychosocial stressors. Taking all of these factors into account, it's surprising that little research has been done to elucidate the mechanisms behind aged subjects' enhanced vulnerability to new onset psychosocial stress.

Our lab embarked on a series of studies to test the effects of age and psychosocial stress on sleep architecture and cognition. Our first study measured sleep stages in young adult and aged F344 rats during their resting and active periods. Animals were behaviorally characterized on the Morris water maze and gene expression profiles of their parietal cortices were taken. We confirmed previous studies that found impaired cognition and decreased resting deep sleep with age. However, it was increased active deep sleep that correlated best with poor cognitive performance. In the second study rats were subjected to immobilization (restraint stress) immediately preceding their final water maze task. Hippocampi were prepared for synaptic electrophysiology and trunk blood was taken for corticosterone measurement after post-stress sleep architecture data was collected. Young subjects responded to acute stress with decreased cognition, elevated CORT levels and altered sleep architecture. In contrast, stressed aged subjects were statistically indistinguishable from control aged

subjects, suggesting that aged rats are less responsive to an acute psychosocial stress event. Together, these studies suggest that alleviating sleep dysregulation could therapeutically benefit cognition psychosocial stress resilience.

KEYWORDS: Aging, cognitive decline, Sleep dysregulation, Psychosocial stress, Fischer 344 rats

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CHANGES IN SLEEP ARCHITECTURE AND COGNITION WITH AGE AND PSYCHOSOCIAL STRESS: A STUDY IN FISCHER 344 RATS

By

Heather Marie Buechel

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12/4/13
Date

I would like to dedicate this work to my son, Maxwell. You light the dark.

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And finally, thank you to my mom and dad, for telling me, when I was a kid, that I can do anything that I want to do. I haven't forgotten, and of course, you were right.

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Study 1: Deep sleep and parietal cortex gene expression changes are related to cognitive deficits with age

Motivation

Aging is often accompanied by a decline in cognition as well as an increase in complaints of dysregulated sleep. However, very few studies have looked for a connection between the two events, even though sleep loss in young subjects is associated with aging- like metabolic and cognitive deficits (Spiegel et al., 2005, Banks and Dinges, 2007, Tasali and Ip, 2008). With this study, we hypothesized that there would be a connection between sleep architecture and cognitive performance in young and aged Fischer 344 rats.

Aging

Aging is a complex process that continues throughout a lifetime. It occurs so subtly that we typically only notice that we're aging when we begin to experience the ill effects that accompany the process. Those ill effects come in many varieties, both physical and cognitive in nature. What connects many of these physical and cognitive changes is the brain (Hinman and Abraham, 2007, Kumar et al., 2009, Lustig et al., 2009). Unhealthy brain aging (UBA) is involved in everything from the tremors of Parkinson's disease and the memory loss of Alzheimer's to the insulin dysregulation of metabolic syndrome and Diabetes. UBA is comprised of a variety of cellular and molecular changes including, calcium dysregulation, myelin sheath thinning, increased inflammation, changes in vasculature, and changes in insulin signaling, among many others (Thibault et al., 2001, Blalock et al., 2003, Norris et al., 2005, Latimer et al., 2011, Thibault et al., 2013).

The Hippocampus

The hippocampus, in humans, is a small 'seahorse' shaped brain structure that belongs to the limbic system, which also includes: amygdala, cingulate gyrus hypothalamus, thalamus, and basal ganglia (Sokolowski and Corbin, 2012). The limbic system controls emotional development and memory (Roxo et al., 2011). Structurally, the hippocampus is comprised of two interlocking c's, the Cornu Ammonis (or CA) region, and the Dentate Gyrus (El-Falougy and Benuska, 2006, Coward, 2010). The hippocampus is responsible for storing short term and spatial memory information, and it also plays a role in memory organization (Peinado-Manzano, 1990, Izquierdo and Medina, 1993, Schacter et al., 1996, Gilbert et al., 1998, Giovanello et al., 2004). The hippocampus is a central target of research in UBA because of its pivotal role in learning and memory (Winocur and Moscovitch, 1990, Fordyce and Wehner, 1993, Almaguer et al., 2002, Rosenzweig and Barnes, 2003, Kadish et al., 2009, VanGuilder et al., 2010). Subsequently, it has been extensively studied in mammalian aging (Landfield et al., 1978b, Fordyce and Wehner, 1993, McEwen, 1999, Almaguer et al., 2002, Bizon et al., 2004, Kadish et al., 2009)

Cognition and Aging

The bottom line of our aging research is to improve the quality of life for the aging population, and one of the main components of brain aging is declining cognition. Cognition is a broad term, defined by the Merriam – Webster dictionary as "conscious mental activities: the activities of thinking, understanding, learning, and remembering". More than ten thousand published studies have been done on 'cognition with age' and it's safe to say that, with age comes a decline in cognitive ability – across the mammalian class (Peters et al., 1996, Milgram, 2003, Bizon et al., 2004, Nagahara and Tuszynski, 2004). Aging research focuses on both improving cognition with manipulations such as exercise and drug intervention (Brach et al., 2004, De la Fuente et al., 2005, Seals et al., 2008, Allen and Morelli, 2011, Le Couteur et al., 2012, Simpson et al., 2012, Karolczak et al., 2013, Yu et al., 2013), as well as parsing out the molecular mechanisms that are associated with and may cause aging-related changes (Schouten et al., 2012, Lapak and Burd, 2013, Sibille, 2013).

An Animal Model of Cognition

The Morris water maze was established as a test of rodent cognition (Morris, 1984). Since then it has been used extensively with many variations to test everything from spatial memory to long term recall, with manipulations involving, for example, drugs, stress, and exercise (For a review of the versatility of the water maze as a cognitive task seeTerry, 2009). In our study, the water maze was used in two ways. First to assess the rats' ability to swim competently with a 'local visual' task, and second to measure spatial mapping and short term memory with a more traditional water maze paradigm (see methods chapter 2 & 3).

UBA and everyday life

UBA is does not occur in a vacuum. The events of our lives may have as much influence, if not more, than our genetic predispositions for how well we age. Relationships, education, self esteem, exercise, stress, and sleep quality are all play a role in the aging process, and these factors tend to fluctuate together over a lifetime. We build relationships, we exercise, we get educated, all of which build our self esteem, but when those relationships break, or we get injured or sick, it can cause stress which disrupts sleep which disrupts cognition, which all have been hypothesized to lead down the path of unhealthy brain aging over a lifetime. The potential change in stress response over age (McEwen and Seeman, 1999) is proposed to occur, at least in part, due to the consequences of life-long stress exposure (allostatic load) (McEwen et al., 1991, McEwen, 2003).

Sleep

Sleep is a vital brain function that we know surprisingly little about. Research is slowly teasing out the usefulness and the importance of a good night's sleep. We know that sleep architecture changes throughout life. While newborn babies can sleep up to 15 hours during a day, (Quillin, 1997) children tend to sleep around 10 consolidated hours in the night. Adults tend to sleep at least 7-8 hours a night and finally, aged adults tend to sleep seven hours a night or less (Krueger and Friedman, 2009). We also know that sleeping less than 7 hours a night is not healthy, and that age increases the chance of suffering from some type of sleep dysregulation such as insomnia, sleep fragmentation, or day time sleepiness (Avidan, 2003, Ancoli-Israel and Ayalon, 2006, Espiritu, 2008, Stamatakis and Punjabi, 2010) (Fig 1)

Figure 1. Sleep cycles throughout life.

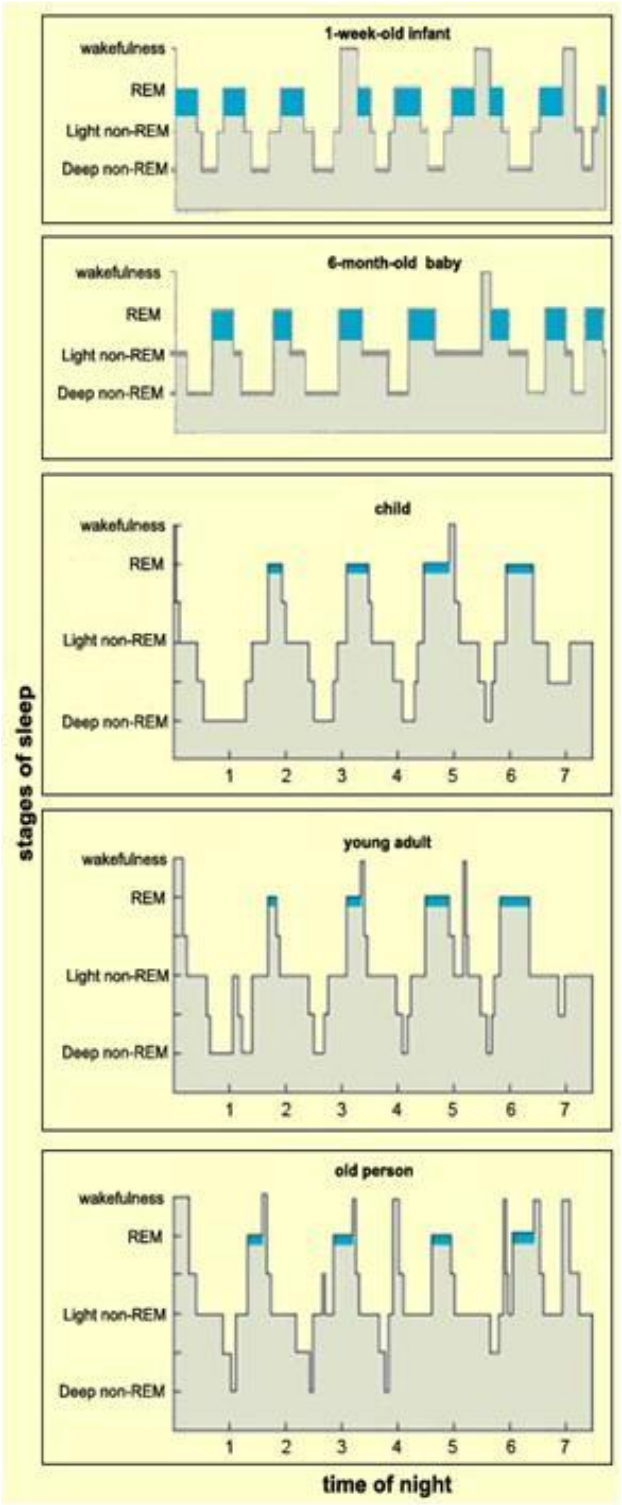


Figure 1. Sleep cycles throughout life From the top: Stages of sleep in 1 month old infants: Sleep is largely composed of REM and deep sleep. Stages of sleep in 6 month old babies: Duration of the sleep stages is increasing, REM sleep in consistent with a one month old, but deep sleep is becoming more consolidated. Stages of sleep in a child: Sleep cycles duration increases. Deep sleep is enriched early in the sleep period and REM sleep increases in duration through the sleep period. Stages of sleep in young adults: REM and deep sleep become more stratified through the sleep cycle. Stages of sleep in aged humans: Loss of deep sleep, frequent wakining REM becomes less stratified (Eric Kandel, 2012)

Classically, sleep was separated into 5 stages in humans. Recently it has been pared down to three stages of non REM (NREM) and one stage of REM. Stage 1 is the intermediate between wake and sleep. Stage 2 is a transitional stage between light and deep sleep that includes sleep spindles and k complexes (Kokkinos and Kostopoulos, 2011). Stage 3, is deep sleep composed of delta waves and largely synchronous brain activity thought to help process information gathered through the day (Dang-Vu et al., 2008, van Dongen et al., 2012). REM sleep can be considered a light sleep stage, but usually is categorized separately because of other parameters unique to REM such as muscle relaxation and dreaming, thought to improve and consolidate emotional memories (Maquet et al., 2005, Nishida et al., 2009). As we travel from child to adult, REM sleep decreases and deep sleep increases, steadying from the teen to middle aged years, and both of these sleep stages have been shown to decline with age (Van Cauter et al., 2000, Gaudreau et al., 2001, Javaheri and Redline, 2012).

We, and most other mammals, cycle through our stages of sleep. In humans, each cycle lasts around 90 minutes. Sleep starts with stage 1, going through stage 2, then 3, then backwards through the stages to complete the cycle with REM. After the initial cycle ends, humans typically jump straight from REM to stage 2 for the rest of the night's cycles. As the sleep period continues, deep sleep duration tapers off and REM sleep duration increases.

A Rat Model of Sleep

While rats follow a similar pattern of sleep cycling, there are a few fundamental differences between human and rat sleep. First, rats sleep during the day rather than at night, and their sleep is naturally more fragmented than ours. However, studies show that the total time in the various stages is consistent across subjects of the same species, allowing for observation of sleep disturbances (Datta and Hobson, 2000). Second, in our studies in rats, sleep is categorized as 'light', REM, or 'deep'. The electrodes to monitor EEG must be implanted on the dura of the rodent's brain to receive a strong enough signal to study. Rats were implanted after their 'local visual' water maze task was completed. They were given 2 weeks of recovery time and were continuously recorded for the duration of cognitive testing.

Since previous work shows that deep sleep is concentrated in the first few hours of the resting period, we chose to focus our analysis on the first four hours of each period (see methods, chapter 2 &3).

Transcriptional analysis

Transcriptional analysis through microarray is becoming increasingly popular as a method of observing a large number of genes present in a tissue of interest at a particular point in time – a transcriptional snapshot of a particular region of interest. The results can be used to compare the transcriptional snapshot of one region to another or one subject to another. In our case we compare our young and aged brains. For this study, we chose to analyze cortical tissue because sleep is known to occur in different regions of the brain at different times. The cortex is where our EEG data is recorded, so we reasoned that, although our animals were not being killed during sleep, the cortex transcriptional snapshot should more closely correlate with our sleep data than other regions.

Study 2: Aged Rat water maze, sleep architecture and blood glucocorticoid levels are hypo-responsive to acute restraint: implications for psychosocial stress in aging

Motivation

It is common to hear of cognitive decline with age, as well as an increased susceptibility to injury and disease, dysregulated sleep, and decreased mobility. However, what is often over looked is the impact of new onset psychosocial stress to an already over worked system. These experiments explore this new onset stress in the context of young vs. aged by testing Fischer 344 rats' water maze performance as well as sleep architecture, CORT levels, and hippocampal electrophysiology and gene expression, in the face of an acute restraint stress event.

Stress

There are many stressors, both physical and emotional that living creatures face. Vulnerability to those stressors also varies throughout life. A type of stress that humans are particularly vulnerable to is psychosocial stress. That is, stress that does not cause any physical pain, like becoming isolated from family or friends by a move, or preparing

to defend a dissertation (Umehara et al., 2007, Bartram et al., 2009, Buechel, personal communication). These stressors produce the same endocrine response as any other stressor, however, we habituate to them over time, When exposed to the same psychosocial stressor repeatedly, the endocrine response seems to decrease (Kant et al., 1985, Gerra et al., 2001). This may be particularly important when considering the stress response with age. It has been shown that many outside factors can impair the ability to habituate to stressors (McEwen and Seeman, 1999, Dubovicky and Jezova, 2004, Kudielka et al., 2006, Cyr and Romero, 2009), so the elderly, who are generally coping with other ailments, may be particularly vulnerable to the negative effects of new onset stress.

HPA Axis and Glucocorticoids

The stress response is initiated in the hypothalamus via central activation of the sympathetic and parasympathetic nervous system or peripherally via pain receptors on the body. Immediately after stress exposure, adrenaline and norepinephrine are released to regulate heartbeat and improve concentration (fight or flight response). At the same time, the hypothalamic- pituitary- adrenal (HPA) axis releases Corticotropin-releasing hormone (CRH) which quickly stimulates the release of adrenocorticotropic hormone (ACTH) to then stimulate corticosteroid (glucocorticoid [GC] and mineralocorticoid [MC]) production and release. (Fig.2) Glucocorticoids gradually increase, as necessary to respond to the stressor, for long term stress control. (For an excellent review of GC actions in stress, and of the stress response itself see Sapolsky et al., 2000).

The negative effects of a chronically elevated stress response are seen plainly in the hippocampus, a locus for spatial memory. The hippocampus is particularly vulnerable to stress because it is rich in glucocorticoid receptors (GR's) (Pruessner et al., 2005), which are activated during the stress response by glucocorticoids (cortisol in humans and corticosterone in rodents). While glucocorticoids are only part of the stress

Figure 2.Schematic overview of the typical endocrine stress response

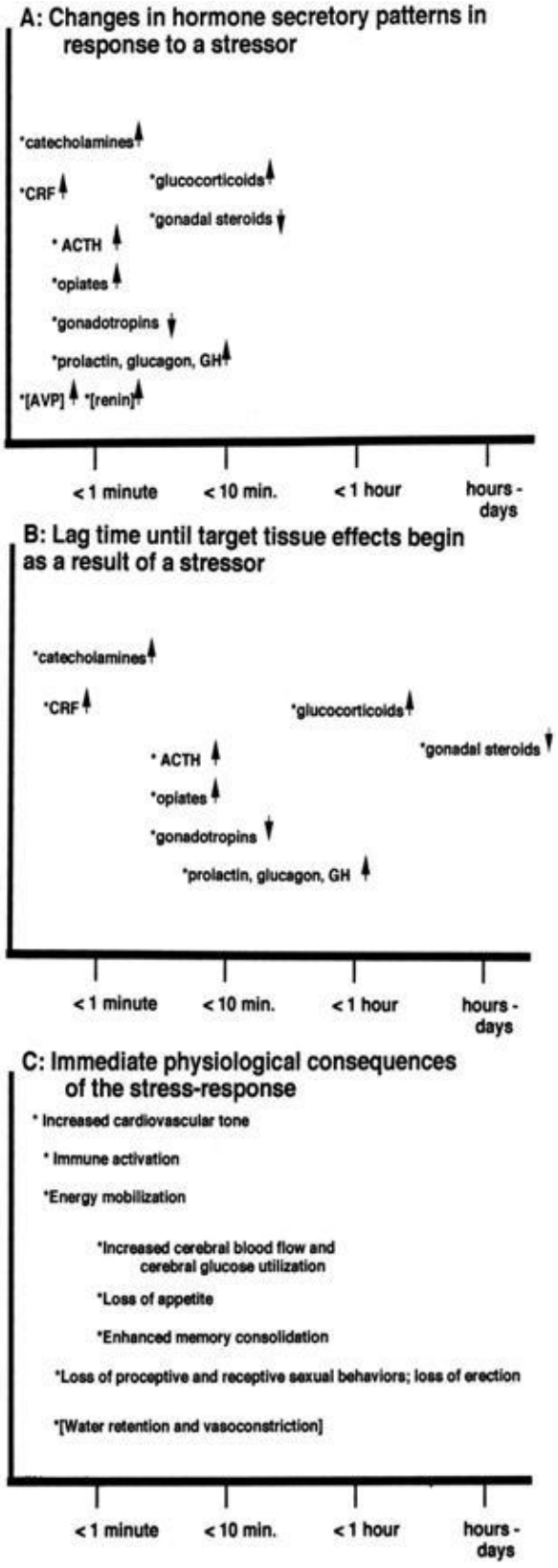


Figure 2.Schematic overview of the typical endocrine stress response. Schematic overview of the typical endocrine stress response. A, The time course of changes in hormone-secretory patterns in response to a stressor. B, The lag time until target tissue begin as a result of a stressor. C, Immediate physiological consequences of the stress response. *Asterisks* approximate where on the time line the particular hormone is first having its effect (A and B) or when on the time line the physiological consequence is initiated (C). There is no formal y axis—hormones or consequences are simply spaced vertically to facilitate reading. (Sapolsky et al., 2000)

response, they seem to be the most common and versatile molecule, preparing the brain and body for long term stress control. They perform many actions from suppressive to stimulatory, depending on their concentration and duration of exposure. However, in spite of their versatility in maintaining body homeostasis in the face of stress, elevated levels of glucocorticoids from HPA axis dysregulation over a life time have been implicated in unhealthy brain aging (Landfield et al., 1978a, Porter and Landfield, 1998). The glucocorticoid cascade hypothesis of aging postulates that elevated glucocorticoids cause hippocampal damage and atrophy, which is correlated with poor cognition Because the hippocampus also has descending inhibitory input to the HPA axis, having less hippocampal regulation results in glucocorticoids being over-secreted, further deteriorating the hippocampus (Sapolsky et al., 1986b) .

Stress over a lifetime

Many studies have shown that prenatal and newborn stressors can have lifelong negative effects on cognition. (Quillin, 1997, Graham et al., 1999, Van den Hove et al., 2006, Mesquita et al., 2007, Charil et al., 2010, Gomez-Gonzalez and Escobar, 2010, Morgan et al., 2011, Buss et al., 2012). However, as we leave infancy, and become more aware of our world, we become more resilient to stressors, recovering even from stress-induced structural plasticity changes such as dendritic retraction in the hippocampus (Conrad et al., 1999, Conrad, 2006) after stress has abated. When we reach old age, there is less certainty of the role new onset stress plays in our physiologic and cognitive health.

An Animal Model of Stress

Rats do not experience psychosocial stress in the same way that humans do, possibly because of the prefrontal cortex which is thought to control complex planning and reasoning, is a much less-developed structure in rodents (Koechlin et al., 1999). To use them in a model of psychosocial stress, we must look for pain free, stressful scenarios to place them in. Sound stress, strobe light stress and temperature stress all can fall into this category. However, we decided to use restraint stress as our model because of extensive previous research on the method in both the acute (our present study) and chronic (future direction) settings (Wood et al., 2003, Wood et al., 2004, McLaughlin et al., 2007, Colaianna et al., 2013, Lee, 2013). In this study, rats were restrained for 3 hours immediately prior to the water maze probe trial. We use plasma corticosterone,

water maze performance, body temperature, sleep architecture as well as the squeaks and struggles during restraint to assess perception and stress response in each group (see methods, chapter 3).

Sleep and Stress

Glucocorticoid aging research seems particularly focused on the hippocampus for reasons stated earlier, but GC's also play a role in sleep quality and quantity. Psychosocial stressors have been shown to alter sleep patterns (Akerstedt, 2006, Kim and Dimsdale, 2007, Bidulescu et al., 2010), and the HPA axis, along with mediating the stress response, is involved in sleep and circadian regulation (Buckley and Schatzberg, 2005, Wagner and Born, 2008, Postnova et al., 2013). CORT levels are lowest early in the night and increase to their maximum shortly after waking (Wagner and Born, 2008). (See Fig 3 on CORT levels throughout 24 hour period.)

Thesis Significance

This body of work was designed to test first, the changes in sleep architecture with age and second, the effects of restraint stress on Fischer 344 rats, both young and old. Sleep power and duration as well as performance in the water maze were our physical outcome measures in both studies. We used hippocampal microarray in the first study and a panel of hippocampal aging related genes in the second study as the molecular outcomes. The second study also used plasma corticosterone levels and hippocampal electrophysiology, to further gauge stress response in the young and aged populations.

These experiments were done in the hopes of beginning to understand the effects of acute psychosocial stress with age. This foundation will be used to perturb the stress response system, whether by drug intervention, environmental enrichment, or other method to improve stress response, sleep quality/ quantity and ultimately attempt to improve the cognitive decline so often seen with age.

Figure 3. Circadian rhythms in the hypothalamo–pituitary–adrenal (HPA) axis.

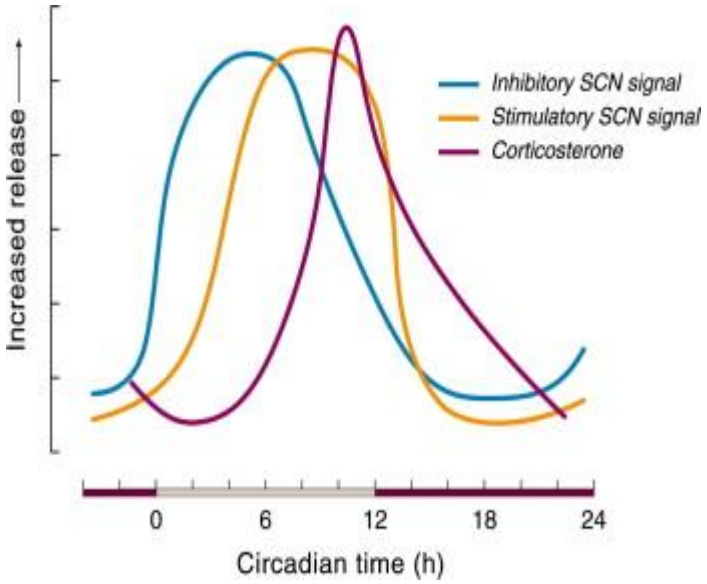


Figure 3. Circadian rhythms in the hypothalamo–pituitary–adrenal (HPA) axis
Schematic representation of the diurnal release pattern of SCN transmitters involved in the circadian control of corticosterone release. (Kalsbeek et al., 2011)

Chapter 2: Deep sleep and parietal cortex gene expression changes are related to cognitive deficits with age

Summary

Background: Age-related cognitive deficits negatively affect quality of life and can presage serious neurodegenerative disorders. Despite sleep disruption's well-recognized negative influence on cognition, and its prevalence with age, surprisingly few studies have tested sleep's relationship to cognitive aging.

Methodology: We measured sleep stages in young adult and aged F344 rats during inactive (enhanced sleep) and active (enhanced wake) periods. Animals were behaviorally characterized on the Morris water maze and gene expression profiles of their parietal cortices were taken.

Principal Findings: Water maze performance was impaired, and inactive period deep sleep was decreased with age. However, increased deep sleep during the active period was most strongly correlated to maze performance. Transcriptional profiles were strongly associated with behavior and age, and were validated against prior studies. Bioinformatic analysis revealed increased translation and decreased myelin/ neuronal pathways.

Conclusions: The F344 rat appears to serve as a reasonable model for some common sleep architecture and cognitive changes seen with age in humans, including the cognitively disrupting influence of active period deep sleep. Microarray analysis suggests that the processes engaged by this sleep are consistent with its function. Thus, active period deep sleep appears temporally misaligned but mechanistically intact, leading to the following: first, aged brain tissue appears capable of generating the slow waves necessary for deep sleep, albeit at a weaker intensity than in young. Second, this activity, presented during the active period, seems disruptive rather than beneficial to cognition. Third, this active period deep sleep may be a cognitively pathologic attempt to recover age-related loss of inactive period deep sleep. Finally, therapeutic strategies aimed at reducing active period deep sleep (e.g., by promoting active period

wakefulness and/or inactive period deep sleep) may be highly relevant to cognitive function in the aging community.

Introduction

Age-related cognitive deficits are a highly prevalent and important health risk in the human population (reviewed in (Bishop et al., 2010)), can presage development of age-related neurodegenerative disease (Chong and Sahadevan, 2005, Mariani et al., 2007, Petersen and Negash, 2008), and are a primary reason for elderly placement in assisted living facilities (Tornatore et al., 2003). Sleep dysregulation is also a common complaint among the elderly. During the night, the constellation of age-related sleep changes include circadian advance, sleep fragmentation, insomnia (Weinert, 2000, Avidan, 2003, Tractenberg et al., 2005, Ancoli-Israel and Ayalon, 2006, Espiritu, 2008, Stamatakis and Punjabi, 2010), and loss of deep, slow wave sleep (Kryger et al., 2004, Cajochen et al., 2006, Espiritu, 2008), while daytime symptoms include sleepiness, increased napping and breakthrough sleep. Further, healthy younger adults exposed to experimentally induced selective deprivation of night time (inactive period) deep sleep show some aging-like phenotypes, including daytime sleepiness (Banks and Dinges, 2007), blood chemistry changes similar to those seen in metabolic syndrome (a potential precursor to the development of type II diabetes) and cognitive deficits (Van Cauter et al., 1998, Spiegel et al., 2005, Tasali and Ip, 2008).

Although clearly vital to normal function, sleep is only grudgingly yielding to scientific inquiry regarding its role(s) in physiology. Recent studies suggest that deep, slow wave sleep during the inactive period promotes memory (Sejnowski and Destexhe, 2000, Born et al., 2006, Born, 2010, Diekelmann et al., 2011), possibly through localized synaptic (Huber et al., 2004, Tononi and Cirelli, 2006, Vyazovskiy et al., 2008) and macromolecular synthesis (Mackiewicz et al., 2008) effects. Thus, the dysregulated slow wave sleep seen with age might contribute to cognitive deficits seen with aging. Despite the seemingly similar effects of age and sleep dysregulation on cognition, and the high prevalence of sleep changes with age, relatively few studies have investigated possible mechanistic links between sleep architecture changes and age-related cognitive decline.

Here, we used the F344 rat model of aging to investigate this relationship. Young and aged rats were surgically implanted with wireless telemetry devices in order to measure sleep architecture. Each subject was evaluated for cognitive performance on the Morris

water maze. Further, microarray analysis assessed potential molecular relationships among aging, behavior, and sleep in brain tissue. Because sleep stages have been reported to be brain region specific, we selected parietal cortex for array analysis as it was closest to the recording electrodes (and therefore hypothetically most germane to correlations with sleep measures).

Materials and Methods

Subjects

Young adult (3 mo) and aged (21 mo) male Fischer 344 rats obtained from the NIA aging colony were individually housed with crinkled paper bedding and a cardboard tube. Animals were maintained on a 12:12 light/ dark cycle in the housing facility and were given access to food and water ad-libitum. All animals were evaluated for pathology (e.g., pituitary and mammary tumors, splenomegaly, and cataracts). Two aged subjects were excluded (one with a pituitary tumor, one with a mammary tumor) leaving n = 9 young and n = 9 aged for subsequent analyses. **Ethics statement:** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Kentucky Office of Research Integrity Institutional Animal Care and Use Committee. Surgical (isoflurane) and euthanatising (CO_2) anesthesia were used and all efforts were made to minimize suffering.

Surgery

All subjects were implanted with wireless EEG/EMG emitters according to standard procedures (Data Sciences International- TL11M2-F40-EET). Briefly, animals were anesthetized with isoflurane and placed in a stereotaxic frame. A two inch incision was made to expose the skull and spinotrapezius muscles. The emitter was placed under the skin between the left scapulae and the left ileum along the flank. The exposed dorsal region of skull was cleaned with 3% peroxide and the skull surface dried with sterile cotton swabs soaked in 70% ethanol. A 0.7mm hole was drilled 1mm from either side of the sagittal suture line and 1-2 mm anterior to the lambda suture line for the EEG leads. EEG leads were bent into a ‘u’ shape and the base of the ‘u’ inserted into the hole so that wire contacted the dura over the parietal cortex. They were then covered with dental cement and left to dry. EMG activity was recorded by surgically inserting two wire

electrodes perpendicular to trapezius muscle fiber. The free wire end was capped with insulation and both sides of the incision were tied off with surgical thread to prevent fluid infiltration into the insulation. The incision was then closed with 4-6 mattress stitches. Immediately after surgery, data was collected from the emitters implanted in the rats. This allowed us to monitor their surgery recovery and to evaluate when they had stable sleep/wake patterns. One young animals' emitter failed and his sleep architecture data is not included in the study.

Sleep Data Acquisition and Analysis

Animals were housed individually and cages were at least 18" apart to avoid interference during radiotelemetry data acquisition. For these nocturnal rodents, the first four hours of active (dark) and inactive (light) periods on the day prior to the water maze probe trial were analyzed. Two independent researchers scored each sleep segment using Neuroscore's analysis console. EEG, EMG, temperature and locomotor activity data were recorded continuously with DSI's Data Art acquisition software and binned in 10 second epochs. Epochs were scored in 30 second increments while being viewed in both 2 minute and 5 minute windows. EEG waves were stratified into 'low amplitude' ($\leq$ 50% of maximum) and 'high amplitude' (> 50% of maximum) tiers, and underwent fast Fourier transforms for each of 5 frequency ranges: Δ (0.5-4 Hz), Θ (4-8 Hz), A (8-12 hz), Σ (12-16 Hz) and B (16-24 Hz). EMG waves were stratified into 3 tiers: 'basal' $\leq$ 33% (seen during REM); 'intermediate' (between 33% and 66%); 'high' (> 66%). Stages were established as follows: Wake- intermediate or high EMG $\pm$ locomotor activity, EEG variable; Light sleep- low amplitude EEG, intermediate EMG, and no locomotion; REM (paradoxical) sleep- high frequency EEG, 'basal' EMG and no locomotor activity; deep sleep- high amplitude EEG activity enriched in delta band frequency, basal to light EMG activity, no locomotor activity. Prior assigned sleep stages informed subsequent assignments. Ambiguous epochs, those disagreed upon by independent scorers, as well as those containing artifacts, were not scored and accounted for < 5% of scored time.

Water Maze Testing

The water maze (black circular pool, 190 cm in diameter) was placed equidistant (~60 cm) to a continuous wall of black curtains hanging from the ceiling, making the environment relatively neutral. Three high contrast black and white cues (90 cm×90 cm, representing a circle, triangle and vertical lines), were placed on the curtains. Pool

temperature was maintained at 26 ± 2 °C. One quadrant contained a 15 cm diameter escape platform covered with black neoprene for improved traction. Illumination in the room was set such that the Videomex-V water maze monitoring system (Columbus Instrument, Columbus, OH) could reliably monitor animal movements with no artifacts. The Morris water maze (Morris, 1984) training and probe sessions took place between 10AM and 2PM as previously published (Rowe et al., 2007, Kadish et al., 2009, Blalock et al., 2010b). This 'standard operating procedure' for water maze testing in rats occurs during their inactive period. Thus, the duration of this procedure was kept consistent (4 hours) across all subjects on each day of behavioral acquisition to control for potentially sleep disruptive influences. Prior to surgery, animals were evaluated on a visual cue task (3 x 60s per day, 4 days). All animals were able to swim directly to the visual platform by the 12^{th} trial (data not shown). Animals were then implanted (see above) and allowed to recover for 2 weeks. Sleep architecture and behavior were then evaluated during the third week. For maze performance, a 5 day protocol was used (day 1: visual cue trial; days 2-4: 3 trials per day, hidden platform; day 5- probe trial with platform removed). On the cue and training days, each animal began in a different quadrant on each of three trials. They were given one minute to find the platform, one minute on the platform and a two minute inter-trial interval. On the probe day, the platform was removed and each rat was given one 60s trial. Path length and latency were measured to either the platform (trial) or a computer-superimposed silhouette of the platform (probe) (Videomex-V water maze monitoring system, Columbus Instrument).

Tissue Collection and Microarray Analysis

Immediately after the probe trial, rats were killed by CO_2 anesthesia and rapid decapitation. For a subset of animals selected by order of entry (the first 6 animals per age group) brains were removed and parietal cortex dissected out in chilled (0 ° C) artificial cerebrospinal fluid. Parietal cortex was selected for the present study to facilitate transcriptional profile correlation with slow wave activity measured during deep sleep as this was the cortical region beneath which the EEG recording electrodes were situated. Tissue was flash frozen and stored at -80° C for subsequent microarray analysis. RNeasy mini kit (Qiagen) was used to extract RNA and quality of starting RNA was measured with Agilent Bioanalyzer technology (RNA Integrity Number = 9.4 ± 0.1). Each sample (one per subject) underwent RNA extraction, purification, and cDNA labeling separately, as described previously (Blalock et al., 2003, Blalock et al., 2004, Kadish et

al., 2009, Blalock et al., 2010a, Blalock et al., 2010b) and according to standard Affymetrix procedures. Labeled cDNA from each subject was individually hybridized to an Affymetrix rat microarray (RAE230 2.0, 31099 probe sets). All arrays passed standard Affymetrix quality control (gene expression console v. 1.1): GAPDH 3′–5′ ratio 1.05 ± 0.03, RawQ 2.51 ± 0.16, Background noise 70.5 ± 4.1. Scaling factor, based on target intensity of 500, Young: 1.34 ± 0.25, Aged: 1.27 ± 0.20; as well as % Present-Young: 67.5 ± 1.0, Aged: 66.6 ± 1.9 were not significantly different across treatment groups (t-test, $p > 0.3$). Visual inspection of residual sign images of .cel files using Affy PLM (Bolstad et al., 2005) revealed no major geographic defects in microarray signal intensity.

The gcRMA probe level algorithm ('justgcRMA' command run in Bioconductor in the R operating environment) calculated signal intensities (Gentleman et al., 2004, Wu et al., 2004). Only unique probe sets/genes with 'A' grade annotations and at least 3 chips with signal intensities > 4.3 were retained for further analysis. Values were transferred to Excel (2007, Microsoft), Bioconductor (Gentleman et al., 2004), MultiExperiment Viewer [MEV, (Saeed et al., 2003)] and the DAVID suite of bioinformatic tools (Huang da et al., 2009a) for subsequent analysis. Specific statistical procedures are outlined in Results. The signal intensity (gcRMA) and images (.cel files) have been deposited to the MIAME compliant Gene Expression Omnibus (GEO) database [(Barrett et al., 2009) - accession #GSE24515].

Results

Water Maze

Animals were trained in the Morris water maze task 3 times per day for 3 days, and administered a probe trial on the 4th day (see Methods). Escape latencies across all 9 training trials were averaged for each animal and then treated as a single observation for that subject. As shown in Figure 4 (upper left) aged rats took significantly longer to find the hidden platform during training. Aged rats also took significantly longer than their

Figure 4. Aged rats show a deficit in performance on the Morris water maze.

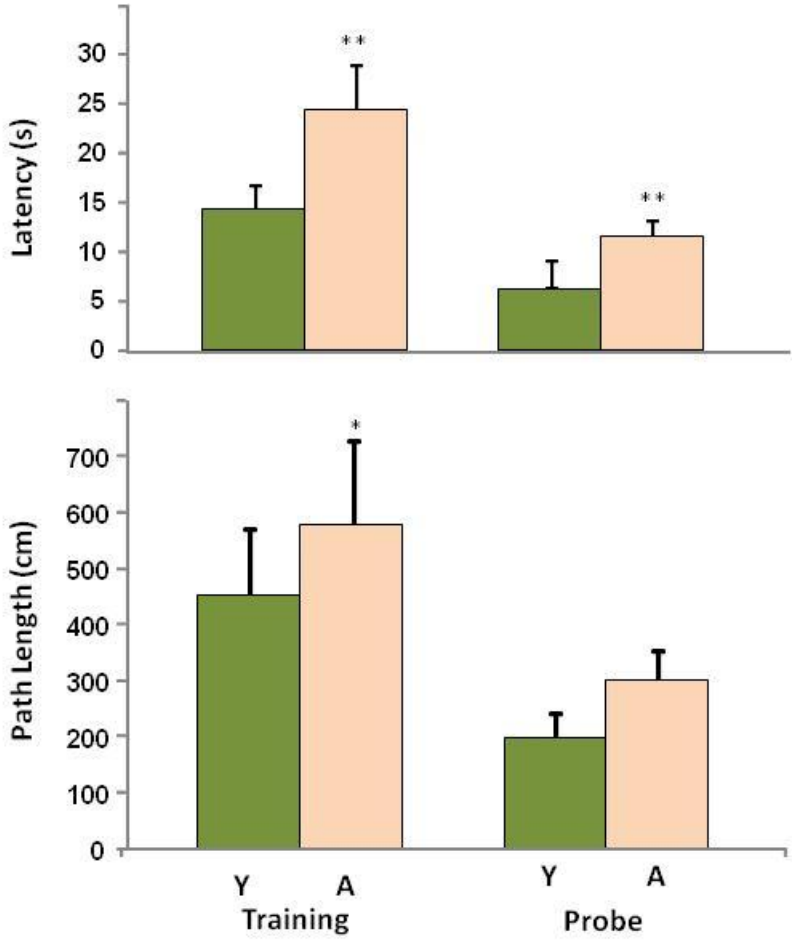


Figure 4. Aged rats show a deficit in performance on the Morris water maze.
Upper: Time to reach the platform (escape latency) averaged over training (left), or time to reach platform annulus during probe trial (right). **Lower:** Path length to reach platform during training (left) or platform annulus during probe trial (right). (* $p \leq 0.05$; ** $p \leq 0.01$; 2-ANOVA repeated measures, post-hoc pairwise Fisher's protected Least Significant Difference).

younger counterparts (latency; Fig. 4; upper right) to reach the goal annulus during the probe trial. Similar age-related deficits were revealed by escape path length measurements (Fig 4, lower panels, significant in training, trend to significant $p < 0.15$ in probe trial). These data confirm numerous previous studies in which aged animals do not perform as well as young (e.g.,(Gallagher et al., 1993, Norris and Foster, 1999, Blalock et al., 2003, Rowe et al., 2007)). There was no significant difference in swim velocity between young and aged subjects (probe trial: young 28.6 ± 2.3 cm/ sec; aged 28.4 ± 3.8 cm/ sec; $p = 0.96$; ttest), suggesting that an age-related change in swim speed did not account for increased latency.

Sleep Architecture

Each animal in the study was surgically implanted with a wireless EEG/ EMG/ temperature/ locomotion telemetry system (see Methods) at least 3 weeks prior to the water maze study. To evaluate potential changes in sleep architecture (duration and intensity of different stages of sleep) with age, we manually characterized sleep and wake behavior for the first four hours of a single active and inactive period (see Methods). As a positive control to validate our analysis system, we reasoned that animals would show relatively more sleep during the inactive period. Our results (Fig. 5a) clearly show a strong effect of period with both young and aged animals spending significantly more time sleeping in the inactive period, and no significant difference in total sleep time with age.

Prior studies have reported a loss of the deep slow wave sleep component with age in both rodents and humans (Naylor et al., 1998, Shiromani et al., 2000, Kirov and Moyanova, 2002, Kryger et al., 2004, Ancoli-Israel and Martin, 2006) although see reports (Rosenberg et al., 1979, Van Gool and Mirmiran, 1983). To evaluate age-related changes in deep sleep, we segregated the first four hours of the inactive period, a time frame reportedly enriched in deep slow wave sleep (Borbely et al., 1975, Roncagliolo and Vivaldi, 1991), into component wake, light, REM, or deep (slow wave) sleep (see Methods). The percentage of total time spent in each identified stage is plotted (Fig. 5B) and shows a significant age-related increase in light sleep at the expense of deep sleep.

We next performed a power analysis investigating the intensity of deep sleep during the inactive period. The first four bouts of inactive period deep sleep ('bout' defined as ≥ 2

Figure 5. Selective loss of deep slow wave sleep with age.

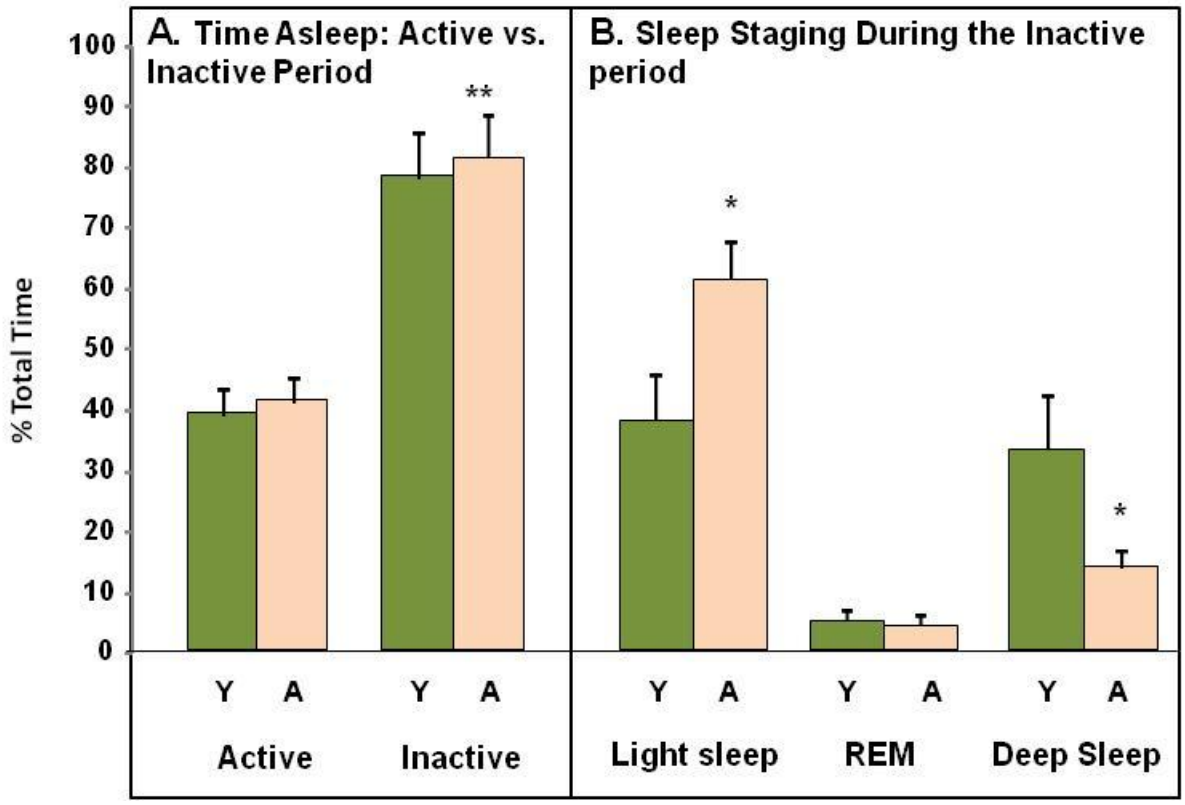


Figure 5. Selective loss of deep slow wave sleep with age. EEG, EMG, temperature, and locomotion radiotelemetry were recorded from young and aged subjects. **A**. % time asleep plotted as a function of age for 4 h blocks of the active vs. inactive periods. A significant increase in time asleep during the inactive period (** $p<0.01$, 2-ANOVA main effect of period), but no effect of age or interaction, was seen. **B**. Sleep staging analysis from first 4 hours of inactive period- staged as 'light' (low frequency, low amplitude EEG activity, basal – intermediate EMG activity), REM (rapid eye movement/paradoxical - high frequency, low amplitude EEG activity, basal EMG activity), and 'deep' sleep (low frequency, high amplitude EEG activity, basal – intermediate EMG activity- see Methods for complete description of staging analysis). Aged animals showed a significant increase in light sleep, no significant difference in REM sleep, and a significant decrease in deep slow wave sleep compared to their younger counterparts (repeated measures 2-ANOVA with a significant main effect of sleep stage [$p<0.001$] and interaction [$p<0.001$], with significant [* $p<0.05$] post-hoc Tukeys pairwise comparisons across age within deep sleep).

min. uninterrupted deep sleep) from each subject were analyzed. Fast Fourier EEG transforms were used to calculate power for each of 5 frequency ranges: Δ (0.5-4 Hz), Θ (4-8 Hz), A (8-12 hz), Σ (12-16 Hz) and B (16-24 Hz). Results were averaged within each frequency range across all four bouts for each animal and treated as a single observation. Results are plotted and analyzed as a function of age (Fig. 6- spectral analysis). Deep sleep's large Δ component was significantly and selectively decreased in aged subjects. Taken together, these results show that inactive period deep sleep duration and intensity (power) were reduced with age.

Relationship between deep sleep and water maze performance: Because a significant inactive period deep sleep loss paralleled age-related behavioral deficits in the Morris water maze, we hypothesized that as deep sleep was lost, maze performance would worsen in individual subjects. Both deep sleep duration and power were tested against water maze latency and path length. Contrary to our prediction, all results were non-significant (e.g., Fig. 7A $p > 0.9$, Pearson's correlation between inactive period deep sleep and training trial path length). Interestingly, there was a significant correlation between increasing maze training path length (worsening performance) and increased *active* period deep sleep (Fig. 7B- as active period deep sleep increased, maze performance worsened), suggesting that increased deep sleep during a normally wake-enriched period could be disruptive for maze performance. Further, if subdivided by age, the young subjects show no significant correlation between maze performance and active period deep sleep ($R = 0.4$; $p = 0.29$), while the aged subjects show a significant correlation ($R = 0.63$; $p = 0.03$), indicating that the overall correlation is primarily driven by the relationship between deep sleep and maze performance within aged subjects.

Microarray Transcriptional Profile of Parietal Cortex

Because EEG electrode placement was over parietal cortex, gene transcriptional profiles were taken from the same region to more accurately align results from these two different measurement systems. Gene transcription of the parietal cortex was analyzed for aging, behavior, and deep sleep associated transcriptional influences (Fig. 8A). Parietal cortex was removed from a subset of subjects (n = 6/ age group) and extracted RNA was hybridized to individual microarrays (Affymetrix RAE 230 v. 2). Probe sets that were not "A" grade annotated, unique, and present (i.e., had sufficient signal strength- Fig. 5C) were excluded from analysis. The remaining 8,080 genes were analyzed for

Figure 6. Loss of deep sleep delta power with age.

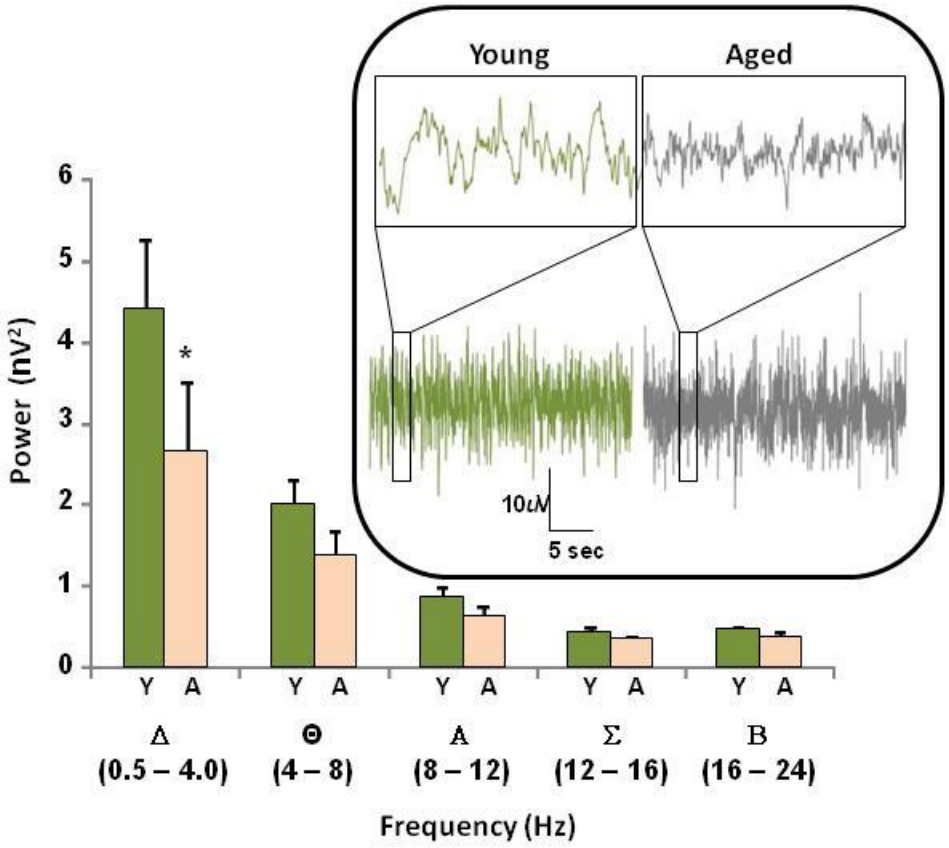


Figure 6. Loss of deep sleep delta power with age. Averaged power of delta, theta, alpha, sigma, and beta frequencies (x axis) are plotted as a function of age. Delta, the dominant hallmark frequency of deep sleep, is significantly reduced with age (2- ANOVA repeated measures; $p<0.001$ main effect of frequency; $p=0.08$ main effect of age; $p=0.03$ interaction term; * $p<0.05$ post-hoc Tukeys test). **Inset:** Representative EEG traces from young and aged subjects during deep sleep (upper detail- 1 s window) depicts reduced large amplitude, slow wave activity with age.

Figure 7. Maze performance is not correlated with duration of deep sleep during the inactive period (A. p>0.9), but worsens with increased active period deep sleep (B. p=0.023; Pearson's test).

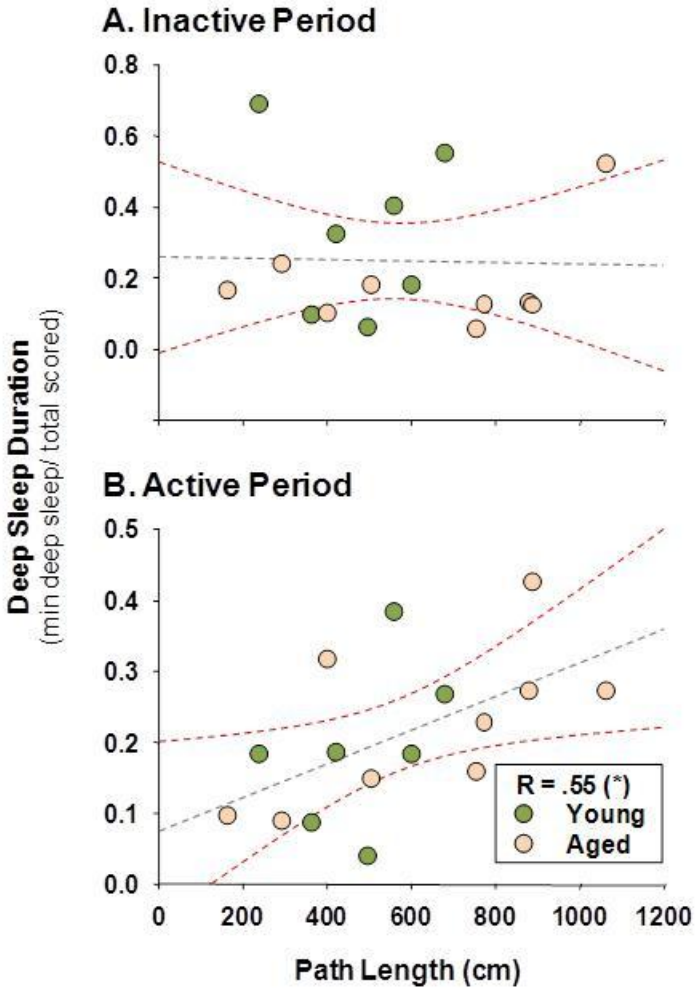


Figure 7. Maze performance is not correlated with duration of deep sleep during the inactive period (A. p>0.9), but worsens with increased active period deep sleep (B. p=0.023; Pearson's test). The proportion of deep sleep duration divided by total time scored (y axis) is plotted as a function of average training path length (x axis). Animals that experienced more deep sleep during the active period tended to have increased path lengths (worsened performance).

Figure 8. Microarray analysis overview.

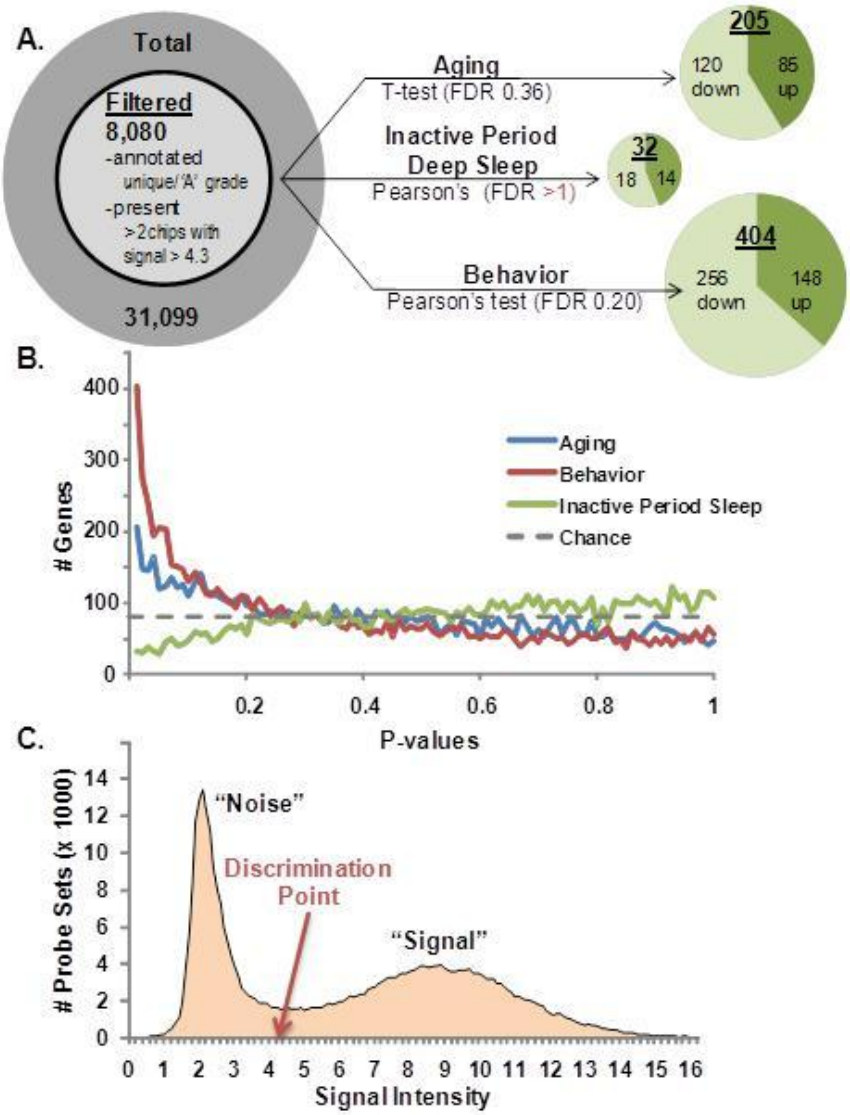


Figure 8. Microarray analysis overview **A.** *Left:* Microarray filtering strategy. Probe sets with low quality annotations were removed. To address redundancy, if two 'A' grade probe sets claimed to represent the same gene symbol, only the probe set with the highest average signal intensity was retained. Among these, probe sets for which more than 2 arrays reported a signal intensity >4.3 were retained for analysis. *Center:* 3 tests were performed on the data- Aging, Behavior (correlation with Probe Trial latency), and Sleep (duration of deep sleep during the inactive period). Type of test used (α=0.01 for all tests) and False Discovery Rate (FDR) are shown. *Right:* Significant results separated into up and downregulated for each test. Size of pie charts are roughly proportional to number genes found (note: sleep analysis finds fewer genes than expected by chance). **B**. P-value histograms for statistical results plots the number of genes found significant as a function of the p-value cutoff bin (0.01 increments) in which they were discovered. Both behavior (red) and aging (blue) show more genes than expected by chance (dashed gray line) at small p-values, while inactive period deep sleep (green) does not. **C**. Signal intensity frequency histogram depicts rationale for choosing 4.3 as a cutoff for presence calls. The number of probe sets (y axis) are plotted as a function of signal intensity (x axis). A narrow, low intensity 'noise' peak centered around 2.4, a broad high intensity 'signal' peak around 8.8, and a saddle region between the two from ~3.5–~5.5 are apparent. We selected the midpoint of that saddle region to discriminate signal-rich from noisy probe sets.

effects of aging (pairwise t-test comparison between young and aged subjects), behavior (correlation with water maze probe latency- Fig. 4) and deep sleep (correlation with duration during the inactive period- Fig. 5B right). Water maze performance, and to a lesser degree aging, appear to have strong, statistically reliable transcriptional signatures. Conversely, deep sleep during the inactive period correlates with fewer genes than would be expected by chance (false discovery rate > 1 for sleep in Fig. 8B). Because we observed a statistically significant correlation between maze performance and active period deep sleep, we also examined gene signatures associated with active period deep sleep (see 'Genes associated with active period deep sleep gain').

Aging Transcriptional Profile: 205 genes changed significantly with age (α= 0.01; Fig. 8A). Of these, 85 were upregulated and 120 downregulated. Pathway level investigations of the aging signature identified lysosomal and immune upregulation, as well as synaptic pathway downregulation groups (Table 1).

Upregulated aging-identified processes appeared similar to those found in other rodent brain aging microarray studies, particularly antigen presentation/ inflammatory and lysosome/ endosome related pathways (Prolla, 2002, Blalock et al., 2003, Moore et al., 2005, Burger et al., 2007, Rowe et al., 2007, Burger et al., 2008, Parachikova et al., 2008, Kadish et al., 2009, Di Stefano et al., 2010, Sarvari et al., 2010). Downregulated processes reflected a blunted stress response and cell signaling (purine nucleotide binding, ion transport, regulation of endopeptidase activity- possibly related to the stress response). However, there was a notable lack of downregulated aging processes related to neurons (with the exception of neuron projection morphogenesis) as compared to prior hippocampal aging studies (Blalock et al., 2003, Burger et al., 2007, Burger et al., 2008, Blalock et al., 2010b).

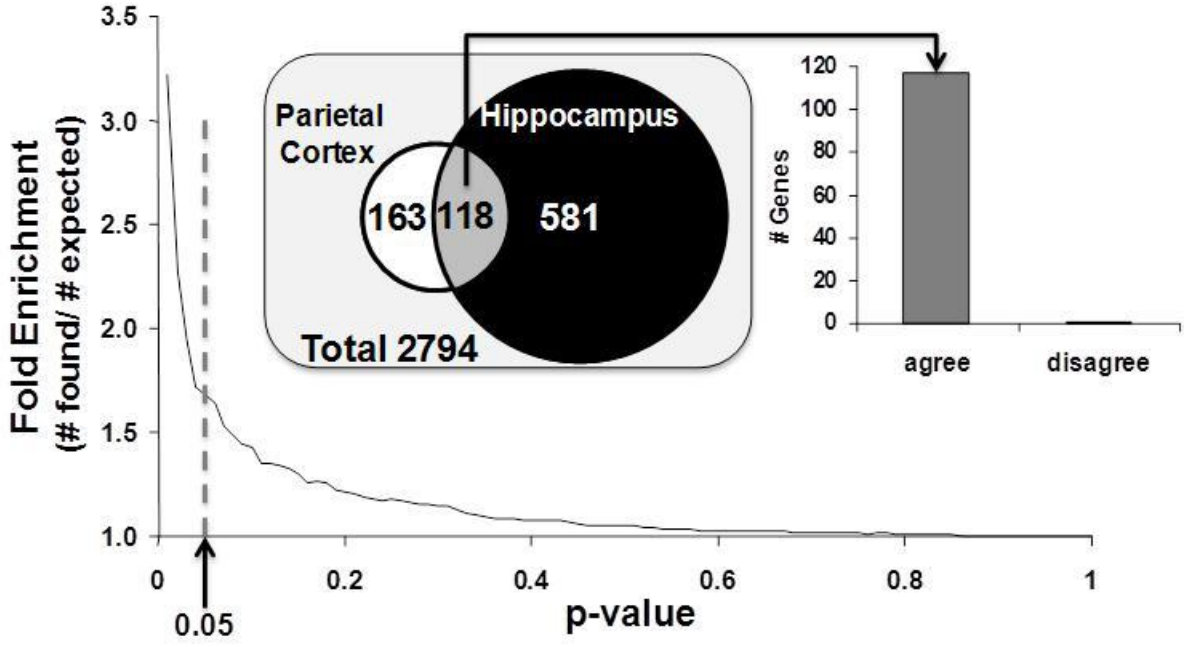
To formally test for similarity, we directly compared the aging transcriptional profiles of parietal cortex (present study) and hippocampus (Rowe et al., 2007, Kadish et al., 2009) at the gene, rather than the pathway, level. Only genes that were present and annotated in both studies (2794 genes) were evaluated. To determine a single Type I error cutoff (α level) for both studies, we constructed fold-enrichment graph (Fig. 9, upper) depicting the relative increase over chance discovery that real data comparisons show [as in (Blalock et al., 2010a)]. At a p-value cutoff of 0.05 (Fig. 9, dashed line), there

Table 1

Ontology	GO ID	Description	#	p-value
Upregulated with Age				
CC	GO:0005764	lysosome	13	1.88^{-09}
BP	GO:0019882	antigen processing and presentation	7	3.11^{-07}
CC	GO:0022627	cytosolic small ribosomal subunit	4	0.00776
CC	GO:0005770	late endosome	4	0.00899
BP	GO:0030595	leukocyte chemotaxis	3	0.01653
Downregulated with Age				
BP	GO:0006950	response to stress	21	0.00206
MF	GO:0017076	purine nucleotide binding	25	0.00212
BP	GO:0032412	regulation of ion transmembrane transporter activity	3	0.01071
BP	GO:0052548	regulation of endopeptidase activity	4	0.02458
BP	GO:0048812	neuron projection morphogenesis	6	0.04123
Increased as Animals Take Longer to Complete the Maze				
BP	GO:0012501	programmed cell death	18	0.00200
CC	GO:0005773	vacuole	12	0.00899
BP	GO:0015031	protein transport	23	0.01110
BP	GO:0007049	cell cycle	18	0.01216
MF	GO:0003743	translation initiation factor activity	6	0.01289
BP	GO:0016052	carbohydrate catabolic process	6	0.02935
BP	GO:0006412	translation	13	0.03957
Decreased as Animals Take Longer to Complete the Maze				
BP	GO:0006816	calcium ion transport	6	0.00493
BP	GO:0050804	regulation of synaptic transmission	7	0.01093
CC	GO:0014069	postsynaptic density	5	0.02272
Increased with Active Period Deep Sleep in Aged Animals				
BP	GO:0006414	translational elongation	11	0.00010
BP	GO:0006412	translation	15	0.00373
CC	GO:0005840	ribosome	12	0.00344
Decreased with Active Period Deep Sleep in Aged Animals				
BP	GO:0042552	myelination	9	0.00002
MF	GO:0046943	carboxylic acid transporter activity	8	0.00080
BP	GO:0006643	membrane lipid metabolic process	6	0.00806
CC	GO:0005856	cytoskeleton	30	0.01336
BP	GO:0008088	axon cargo transport	4	0.01388
CC	GO:0043005	neuron projection	19	0.04293

Table 1. Selected functional processes identified by the DAVID overrepresentation functional clustering algorithm (see Methods) are shown for aging, behavior, and sleep related genes. The filtered gene list (8080 genes) was used as background, and each of the six gene lists (Up or Downregulated with age; Increased or Decreased as a function of latency in the water maze; Increased or Decreased as a function of active period deep sleep duration) were analyzed separately in the Gene Ontology. A single process ($p \leq 0.05$) from each cluster was selected. *Abbreviations*: GO ID- Gene Ontology Accession ID; CC- cellular component; BP- biological process; MF- molecular function; #- number of genes significant in category; p-value- DAVID statistical test result.

Figure 9. Similarity in aging transcriptional profiles across brain regions.



Up regulated (81 genes)	#	P-value	Down regulated (36 genes)	#	P-value
lysosome	15	5.7^{-13}	glutamate signaling pathway	3	0.007
late endosome	6	1.5^{-5}			
antigen processing and presentation	6	3.6^{-6}			
small ribosomal subunit	7	3.7^{-7}			

Figure 9. Similarity in aging transcriptional profiles across brain regions The aging transcriptional signature for parietal cortex was contrasted with a prior study examining the transcriptional profile of aging in the hippocampus (Kadish et al., 2009). The proportion of genes found in the overlap divided by the number expected by chance in the overlap (y-axis) is plotted as a function of p-value cutoff (x-axis). We selected a p=0.05 cutoff (arrow- dashed line) for our overlap analysis. **Venn diagram:** Out of 2794 total genes present in both studies, far more were significantly changed with age in hippocampus. Of the genes changed with age in parietal cortex, nearly half were also identified in hippocampus. **Right:** 117/118 overlapping genes agreed in direction (up or downregulated in both studies). **Lower:** DAVID analysis of common age-regulated genes. Functional group name, number of genes (#) and probability that number of genes in that category would be identified by chance (p-value) are shown.

appears to be a sharp upturn in fold-enrichment, indicating that genes assigned a p-value ≤ 0.05 in both studies begin to show strong agreement with one another. Thus, the Type I error was set at $\alpha = 0.05$ for comparison across the two studies.

Parietal cortex yielded 281 significant genes and hippocampus 699 (Fig. 9, Venn diagram). 118 genes were common between the two studies. The probability of finding a certain number of genes in the overlap by chance can be estimated based on % significant genes in parietal cortex aging and % significant genes in hippocampal aging. The product of these two percentages gives the percentage of genes that should be found in the overlap (parietal cortex: 10% (281/2794) * hippocampus: 25% (699/2794) = OVERLAP: 2.5% (or 70 genes). Here, we expect 70 genes and find 118- the actual number of genes in the overlap exceeds that expected by chance and was highly significant ($p = 3.6^{-8}$, binomial test). Further, the majority of overlapping genes (117/ 118) agree in direction (Fig. 9, upper- inset) and, as reviewed above, reflect increased lysosomal and inflammatory processes with aging (Fig. 9, lower).

Behavioral transcriptional profile: 404 genes were significantly correlated to latency on probe trial (Pearson's test, $p \leq 0.01$). 148 significant genes with negative R values showed reduced expression, and 256 with positive R values showed increased expression, as maze performance worsened (Fig. 8A). Functional grouping analysis (Table 1) shows increased transcriptional activity related to apoptosis (programmed cell death, cell cycle), macromolecular synthesis (translation initiation factor, protein transport, translation), and energy (carbohydrate catabolism). Downregulated categories appeared to strongly represent the neuronal compartment. Although genes in these categories were negatively correlated with latency on the water maze (their mRNA levels decreased as performance on the maze worsened), they were not significantly altered with age. It is interesting to note that the majority of behavior-identified genes (395/ 404; 98%) showed the same direction of change with age (negatively correlated genes were decreased with age, positively correlated behavior genes were increased). Although most were non-significant with age, this 'directional agreement' appears highly unlikely to be a chance occurrence. Assigning a simple binomial test 50% probability of up or downregulation (that is- by chance assuming that any gene selected by behavioral correlation has a 50% chance of being up- or downregulated by age) yields $p < 1^{-12}$ likelihood such directional agreement could have occurred by chance.

Relationship between aging and behavioral profiles: Based on the observations above, we performed an overlap analysis of aging and behavior significant genes. Twenty one genes were significant ($p \leq 0.01$) in both tests, and all agreed in direction (e.g., genes positively correlated with latency on the Morris water maze were increased with aging). By chance, 10 genes would be expected in the overlap. This small but significant set of genes include-

> **Upregulated:** *Trem2* (Triggering receptor expressed on myeloid cells 2; may participate in activation of the immune response), *RT1-Aw2* (Class I histocompatibility antigen, Non-RT1.A alpha-1 chain; presentation of antigens to immune cells), *H2-M3 (*MHC class I antigen H-2M3), *Sult1a1* (**Sulfotransferase 1A1)**, and *Cngb1* (Cyclic nucleotide-gated cation channel beta-1).
>
> **Downregulated:** *Ccr5* (chemokine (C-C motif) receptor 5; receptor for inflammatory cytokines), *Dync2h1* (dynein cytoplasmic 2 heavy chain 1; involved in intracellular transport), *Dmgdh* (dimethylglycine dehydrogenase), *Neto2* (neuropilin [NRP] and tolloid [TLL]-like 2; receptor accessory subunit that increases kainate receptor activity), *Necab3* (N-terminal EF-hand calcium binding protein 3; a promoter of beta amyloid formation), *rCG_32844* (ubiquitin specific protease 43), *Emid1*(EMI domain containing 1), *Acss2* (acyl-CoA synthetase short-chain family member 2; activates acetate for lipid synthesis or energy production), *Serp1* (stress-associated endoplasmic reticulum protein 1; protects unfolded proteins in the ER from degradation), *Igf1* (insulin-like growth factor 1; similar to insulin with greater growth promoting activity), *Alcam* (activated leukocyte cell adhesion molecule; cell adhesion molecule involved in neurite extension), *Foxk2* (forkhead box K2; transcription factor that binds NFAT-like motifs in the interleukin 2 receptor coding region and can inhibit NFAT-mediated upregulation of cytokines), *Pdpk1* (3-phosphoinositide dependent protein kinase-1; phosphorylates and activates PKB/AKT, PKA, PKC-zeta, RPS6KA1 and RPS6KB1), *Nupl1* (nucleoporin like 1; Component of the nuclear pore complex, a complex required for the trafficking across the nuclear membrane), and *Ctsk* (cathepsin K; may play a role in extracellular matrix degradation).

By this analysis, 2.1x as many genes as would be expected by chance (21/ 10) were found in the overlap between behavior and aging. The same analysis in the Kadish data

set (isolated young and aged subjects only) revealed 231 genes common to both aging and behavior, while only 31 would be expected by chance (6.75x increase over chance). The relationship between aging and behavioral transcriptional profiles appears much stronger in hippocampus than parietal cortex. This is consistent with hippocampus' reportedly more direct role in water maze performance and enhanced vulnerability to aging (Morrison and Hof, 2007, Wang et al., 2009).

Genes associated with inactive period deep sleep loss: Although we clearly saw a loss in both the power and the duration of inactive period deep sleep (Figs. 5 and 6), correlation analyses with transcriptional profiles did not identify a statistically reliable transcriptional signature in parietal cortex (Fig. 8a and b- nor was there a reliable correlation signature among aged animals alone- data not shown). Data mining analyses against a prior 'extreme groups' rodent hippocampal aging microarray study (impaired vs. unimpaired cognitive aging n = 20 F344 per group; hippocampal microarray- similar to the overlap analysis in Fig. 9) (Rowe et al., 2007), did not reveal any significant similarity between sleep- and cognition- related genes in the two studies. However, significant genes from this analysis are included in Table 1 as this small subset could represent plausible candidates if replicated in future studies.

Genes associated with active period deep sleep gain: Because there was a significant, positive correlation between increased active period deep sleep and worsening maze performance (increased training path length), we also looked at transcriptional signatures correlated with this sleep behavior. The number of genes correlated with active period deep sleep did not exceed chance (~404 genes expected at α = 0.05, 431 genes found; FDR = .94- data not shown). If partitioned by age, young subjects showed a profile with an FDR > 1, while aged animals showed a statistically more reliable profile (~404 genes expected, 560 genes found, FDR = 0.72). Increasing the α stringency did not improve this relationship although relaxing it did worsen the FDR. As has been noted in prior work (Blalock et al., 2005), pathway-level information can be more reliable than gene-level information, at least in part because of a 'winnowing' effect (false positives are less likely than true positives to participate in similar pathways). Intriguingly, the functional overrepresentation analysis of this set of genes did identify upregulated translational and downregulated neuronal/ myelin pathways (Table 1, lower) that are consistent with current hypotheses regarding slow wave function in deep sleep.

Standardized tools (such as the DAVID suite of bioinformatic utilities used to help create Table 1 (Huang da et al., 2009b)) greatly facilitate identification of overrepresented functional pathways. However, assigning a probability to the likelihood that identified pathways support *a priori* hypotheses (like those related to slow wave sleep) requires a different approach. We re-examined DAVID output (note that the Functional Annotation Clustering option is used throughout DAVID analysis to reduce gene-level redundancy, an important consideration here). For positive correlations, there were 69 total gene pathways (clusters) identified by DAVID, 3 of which were significant. We then marked all 69 clusters as either supporting (6 clusters) or not supporting (63 clusters) macromolecular synthesis based on annotation content. 3/69 were significant (~4%), 6/69 were hypothesis-related (~9%), and the probability that a single pathway would be both significant and hypothesis-related is (9% * 4% = 0.4%). Overall, finding 3 significant and hypothesis-related pathways is highly unlikely ($p = 0.0002$; binomial test). Results for a similar analysis of downregulated pathways were: 89 total pathways (clusters): 6 (~7%) significant; 10 hypothesis-related (associated with neuronal or myelin-related function, ~11%); probability that 5 significant and hypothesis supporting pathways would be found by chance was highly unlikely ($p = 7^{-5}$, binomial test).

Discussion

Sleep and cognition

A deficit in water maze performance reported here confirms numerous prior studies supporting the rat as a model of human age-related cognitive decline (reviewed in (Driscoll and Sutherland, 2005)). The observed loss of inactive period deep sleep duration and power with age is also commonly seen in humans (Kryger et al., 2004, Ancoli-Israel and Martin, 2006). However, in rodent aging, researchers have variably reported no change in sleep (Imeri et al., 2004), reduced or fragmented sleep (Van Gool and Mirmiran, 1983), or changes in REM sleep (Rosenberg et al., 1979, Markowska et al., 1989, Stone et al., 1997). Our results lend support to prior rodent studies (van Gool and Mirmiran, 1986, Naylor et al., 1998, Shiromani et al., 2000, Kirov and Moyanova, 2002) showing deep, slow wave sleep loss during the inactive period with age. Discrepancies regarding sleep measures in prior studies may be related to different stress levels, a well understood disruptor of sleep (Pawlyk et al., 2008). Further, issues such as electrode placement and analysis technique could influence results. For

instance, our results show that deep sleep's loss is light sleep's gain during the inactive period. This "see-saw" effect may have been missed in studies combining light and deep sleep into a single 'non-REM' category (Naylor et al., 1998, Shiromani et al., 2000). Finally, the significant decrease in inactive period deep sleep was correlated with neither behavioral nor transcriptional profiles to an appreciable degree, contrary to our initial hypotheses.

Behavioral deficits did correlate significantly with *active* period deep sleep increases, particularly within the aged subjects. Prior work has shown that failed synaptic potentiation, and/or enhanced depotentiation corresponds to poor memory acquisition and is worsened with age (Barnes and McNaughton, 1985, Foster and Norris, 1997, Rosenzweig and Barnes, 2003, Foster and Kumar, 2007, Boric et al., 2008). Because two current hypotheses regarding the function of slow wave sleep (synaptic depotentiation (Tononi and Cirelli, 2006, Vyazovskiy et al., 2008); macromolecular synthesis (Mackiewicz et al., 2008)) could both conceivably contribute to this poor memory acquisition, we speculate that these deep sleep, slow wave driven processes, if engaged during the active rather than inactive period, may be disruptive to ordinary learning and memory processes. Regardless, understanding the mechanistic link between active period slow wave sleep and cognitive decline in aged rats may have important implications for understanding the role and consequences of breakthrough sleep, napping, and excessive daytime sleepiness on cognitive deficits in aging humans (Ancoli-Israel and Martin, 2006). Although generally not as well studied, it is interesting to note that there are parallels in the human population, where complaints of nighttime (inactive period) sleep disturbances are more common, but excessive daytime sleepiness more strongly predicts poor mental function (Reid et al., 2006) and has been associated with increased risk of age-related cognitive decline, dementia, and neurodegenerative disease (Foley et al., 2001, Abbott et al., 2005).

Microarrays

Transcriptional profiles of parietal cortex were interrogated for age, behavior, and sleep related gene expression. The aging profile was similar to that found in prior array studies. At the pathway level, upregulated inflammatory and endosomal processes appear consistent in rodent aging across multiple labs, array platforms, and brain regions (Prolla, 2002, Blalock et al., 2003, Moore et al., 2005, Burger et al., 2007, Rowe

et al., 2007, Burger et al., 2008, Parachikova et al., 2008, Kadish et al., 2009, Di Stefano et al., 2010, Ginsberg et al., 2010, Sarvari et al., 2010) (although see (Zahn et al., 2007)). Processes downregulated with aging have been more variable across brain regions, possibly because of regional differences in age-related selective neuronal vulnerability (Wang et al., 2009). Although we report downregulation of genes associated with stress response and cell signaling pathways in aging parietal cortex, there was a notable lack of downregulated processes related to neurons (with the exception of neuron projection morphogenesis) in contrast to consistent findings across prior hippocampal aging studies (Blalock et al., 2003, Burger et al., 2007, Rowe et al., 2007, Burger et al., 2008, Kadish et al., 2009, Blalock et al., 2010b). Further studies examining neuron (Ginsberg et al., 2006) and other brain cell-type specific array signatures (Lefebvre d'Hellencourt and Harry, 2005) may help to address these concerns. Nonetheless, our analyses juxtaposing aging parietal cortex to published hippocampal array data with the same rat strain, gender, age range, and microarray chip design yielded strong and significant validation (Fig. 9).

Behavior alone explained more of the variability in gene expression than either aging or sleep measures. As performance in the water maze worsened, the expression levels of genes related to synaptic structure and Ca^{2+} homeostasis declined. These findings support prior work attributing age-related cognitive deficits to reduced synapse number (decreased post-synaptic densities) (deToledo-Morrell et al., 1988, Rosenzweig and Barnes, 2003, Masliah et al., 2006, Morrison and Hof, 2007) and Ca^{2+} dyshomeostasis, a key and well-supported hypothesis of neuronal dysfunction with aging (Landfield and Pitler, 1984, Landfield, 1987, Khachaturian, 1989, Foster, 2007, Thibault et al., 2007, Toescu and Vreugdenhil, 2010). We also hypothesized that the relationship between behavior and age-related gene expression would be weaker in parietal cortex than hippocampus (a brain region known to be important for Morris water maze performance). We tested this by evaluating the number of genes whose expression levels were both significantly changed with aging as well as correlated with behavior. There was a 2-fold enrichment in parietal cortex, and a 6-fold enrichment in prior hippocampal studies. While both enrichments are significant, these data support the conclusion that aging gene signatures in the hippocampus appear more strongly related to water maze performance than those in the parietal cortex.

We tested the hypothesis that deep sleep and gene expression measures taken from parietal cortex would correlate. Parallel to behavioral associations, gene profiles were not related to inactive period deep sleep, but were tied to active period deep sleep, particularly among aged subjects. Unlike the behavior/ aging signature, no prior work exists with which to validate this signature. Therefore, results are provided separately and should be interpreted with care. Intriguingly, despite these cautions, it is interesting to note that, among this set of active period deep sleep correlated genes, increased expression profiles related to translation and decreased profiles related neuron/ myelin processes lend support to both the macromolecular synthesis (Mackiewicz et al., 2007) and synaptic depotentiating (Vyazovskiy et al., 2008) hypotheses of deep sleep function. As has been noted in prior work (Blalock et al., 2005), pathway-level information can be more reliable than gene-level information, at least in part because of a 'winnowing' effect (false positives are less likely than true positives to participate in similar pathways).

Caveats

This study likely represents a small piece of a much larger puzzle on sleep and cognition with aging involving other brain regions (e.g., hippocampus, hypothalamus), cell-types (e.g., astrocytes, neurons), and sleep stages (e.g., REM, light sleep- including temporal isolation of tissue during sleep stages (Cirelli and Tononi, 2000, Tononi and Cirelli, 2001, Mackiewicz et al., 2008, Mackiewicz et al., 2009)). Although there are several positive and new findings presented here, we also conclude that inactive period deep sleep, as measured from parietal cortex, does not correlate with maze performance or gene expression. Further studies increasing the number of subjects, or employing an alternative 'extreme groups' experimental design (for example see (Gage et al., 1988, Armstrong et al., 1993, Rowe et al., 2007)- separating aged subjects into impaired and unimpaired based on behavior) may reveal this to be a false negative. However, our analyses comparing results to prior 'extreme groups' data (see Results- genes associated with inactive period deep sleep loss) did not demonstrate any significant trends. Additionally, the present study was sufficiently powered to detect age-related maze deficits, deep sleep loss, transcriptional alterations, and even an intriguing correlation with active period deep sleep. Taken together, these observations suggest the more likely scenario that age-related maze performance deficits are more directly tied to active period deep sleep gain than to inactive period deep sleep loss.

Summary

The F344 rat models some common sleep architecture and cognitive changes seen with age in humans, including the cognitively disrupting influence of active period deep sleep. Microarray analysis suggests that the molecular processes, as far as they can be appreciated by mRNA measurement, engaged by active period deep sleep are consistent with the macromolecular and synaptic functions that have been ascribed to deep sleep. Thus, we propose that active period deep sleep is temporally misaligned but mechanistically intact in age. This leads us to the following observations/ conjectures. First, it appears that aged brain tissue is capable of generating the slow waves necessary for deep sleep, albeit at a weaker intensity than in young. Second, this activity, presented during the active period, appears disruptive rather than beneficial to cognition. Third, it is possible that this active period deep sleep is a cognitively pathologic attempt to recover age-related loss of inactive period deep sleep. Finally, therapeutic strategies aimed at reducing active period deep sleep (e.g., by promoting active period wakefulness and/or inactive period deep sleep) may be highly relevant to cognitive function in the aging community.

Chapter 3: Aged Rat water maze, sleep architecture and blood glucocorticoid levels are hypo-responsive to acute restraint: implications for psychosocial stress in aging

Summary

Cognitive processes associated with prefrontal cortex and hippocampus decline with age and are vulnerable to disruption by stress. The stress/ stress hormone hypotheses of brain aging suggests that brain aging, at least in part, is the manifestation of life-long stress exposure. In addition, as humans age, there is a profound increase in the incidence of new onset stressors, many of which are psychosocial (e.g., loss of job, death of spouse, social isolation), and aged humans are well-understood to be more vulnerable to the negative consequences of such new-onset psychosocial stress exposure. However, relatively little basic research has investigated the mechanistic underpinnings of this age-related shift in psychosocial stress response. Here, we separated young (3 mo.) and aged (21 mo.) male F344 rats into control and acute restraint (an animal model of psychosocial stress) groups (n = 9-12/ group) and assessed hippocampus-associated behavioral, electrophysiological, and transcriptional outcomes, as well as blood glucocorticoid and sleep architecture changes. Aged rats showed characteristic water maze, deep sleep, transcriptome, and synaptic sensitivity changes compared to young. Young and aged rats showed similar levels of distress (struggles and vocalizations) during the three hour restraint, as well as highly significant increases in blood glucocorticoid levels 12 hours after restraint. However, young, but not aged, animals responded to stress exposure with water maze deficits, loss of deep sleep, and molecular expression shifts. These results demonstrate that aged subjects are hypo-responsive to new-onset acute psychosocial stress. This suggests that age itself may act as a stressor occluding the influence of new onset stressors, and that such a lack of response may have negative long-term consequences for stress adaptation in aged subjects.

Introduction

Normal aging is a complex process resulting in functional decline and increased susceptibility to a variety of insults across multiple organ systems. The US Census Bureau predicts that the aging population will triple by 2050, dramatically increasing aging and age-related disease burden on health care infrastructure in the United States (Hebert et al., 2013). Brain tissue represents a critical point of failure, with-quality-of-life and autonomy worsening, and susceptibility to neurodegenerative disease increasing with age (Tornatore et al., 2003). Intense research over the past 40 years has forwarded several putative mechanisms of aging-related neurologic dysfunction including free radical damage (Finkel and Holbrook, 2000, Cutler et al., 2004, Poon et al., 2004), protein misfolding (Wickner et al., 1999, Soto, 2003), calcium ion dyshomeostasis (Landfield, 1987, Khachaturian, 1989, Landfield et al., 1992, Foster et al., 2001, Toescu and Verkhratsky, 2004, Thibault et al., 2007, Toescu and Vreugdenhil, 2010), and stress/stress hormone exposure (allostatic load/ glucocorticoid cascade) (Landfield, 1978, Landfield et al., 1978b, Sapolsky et al., 1986b, Kerr et al., 1989, Lupien et al., 1998, Porter and Landfield, 1998, McEwen, 1999, Landfield et al., 2007, Barrientos et al., 2012).

The hippocampus plays a critical role in cognitive function (Lupien et al., 1998, Yankner et al., 2008) and provides important feedback regulation over the hypothalamic-pituitary-adrenal (HPA) axis (McEwen et al., 1992, Rostene et al., 1995, Ziegler and Herman, 2002, Joels et al., 2013). HPA axis activity is associated not only with stress, but with sleep and circadian rhythm (Plihal and Born, 1999, Garcia-Borreguero et al., 2000, Van Cauter et al., 2000). Synergistic actions of stress and aging appear to be especially disruptive in the hippocampus, leading to memory impairments and weakened control over stress hormones (Sapolsky et al., 1984, Meaney et al., 1992, Stranahan et al., 2008). The convergence of age and stress at the hippocampus is highlighted by evidence of increased cognitive dysfunction in aged humans after exposure to new-onset stress, including jet lag, physical wounding, anesthesia, infection or psychosocial stressors (PS- stressors that do not involve nociceptive input) (Wofford et al., 1996, Bekker and Weeks, 2003, Lupien et al., 2005, VonDras et al., 2005, Barrientos et al., 2012).

Extensive work with animal models clearly shows that early life (neonatal, prenatal) stress exposure has life-long deleterious consequences (Meaney et al., 1988, McEwen

et al., 1999, Weaver et al., 2006, Harris and Seckl, 2011), and that glucocorticoids, stress exposure, or manipulations that promote stress signaling in young subjects recapitulates aspects of the aging phenotype (Wei et al., 2007), including impairment on spatial and working memory tasks (Wright et al., 2006, Ferrari and Magri, 2008, Barsegyan et al., 2010, Marin et al., 2011), enhancement or preservation of emotional memory (McGaugh and Roozendaal, 2002), neuronal functional deficit (Kerr et al., 1989, Kerr et al., 1991, Kerr et al., 1992, Krugers et al., 2012), transcriptional change (Porter et al., 2012, Chen et al., 2013), and disrupted sleep architecture (Pawlyk et al., 2008). Interestingly, prior stress exposure blunts response to a subsequent stressor (adaptation) in young (Paskitti et al., 2000, McEwen, 2001), but not aged (Spencer and McEwen, 1997) animals.

New onset PS is highly prevalent in aged humans and has negative sleep and cognition consequences (House et al., 1990, House et al., 1994) but basic research on the acute or chronic sleep and cognitive responses has lagged behind (Maines et al., 1998, Lupien et al., 2009, Porter et al., 2012). Here, we designed experiments to establish whether aged subjects responded differently than young to acute PS. Restraint, an animal model of PS (reviewed in Buynitsky and Mostofsky, 2009) was applied to young (3 mo) and aged (21 mo) male F344 rats. Sleep architecture, water maze performance, blood glucocorticoid level, circadian temperature oscillations, hippocampal electrophysiology and gene expression (NanoString evaluation of a panel of 200 previously defined aging sensitive hippocampal genes) was measured. Aged animals compared to young, as well as young animals responding to stress exposure, showed responses typical to those seen in prior work. However, aged animals showed either a weaker response, or a lack of response compared to their younger counter parts. This reduced response suggests, among other possibilities, that aging itself may act as a stressor, occluding the acute PS response. Our results raise the intriguing possibility that failure to respond to an acute stress may have long-term negative adaptive consequences for aged subjects.

Materials and Methods

Subjects

Young adult (3 mo.), n = 21 and aged (21 mo.) n = 19 male Fischer 344 rats obtained from the NIA aging colony were individually housed with enviro-dry paper bedding , a rat tunnel and a Nyla bone. Animals were maintained on a reverse 12:12 light/dark and were given access to food and water ad-libitum. Some subjects were excluded based on pathology (1 young, 4 aged), surgical complications (2 young, 5 aged), or failure to reach behavioral criteria (0 young, 2 aged). All experiments were performed in accordance with institutional and national guidelines and regulations, and conform to our approved protocol (University of Kentucky IACUC #2008-0347).

Surgery

All subjects were implanted according to standard procedures with wireless EEG/EMG emitters (Data Sciences International- TL11M2-F40-EET) as in prior work (Buechel et al., 2011). Prior to surgery, EEG wires were cut to length and a sterile 1/8" stainless steel screw was soldered to the end of each lead. To begin surgery, animals were anesthetized with isoflurane and placed in a stereotaxic frame. A two inch incision was made to expose the skull and spinotrapezius muscles. The emitter was placed under the skin between the left scapulae and the left ileum along the flank. The exposed dorsal region of skull was cleaned with 3% peroxide and the skull surface dried with sterile cotton swabs soaked in 70% ethanol. For EEG electrodes, a 0.7 mm hole was drilled 1 mm from either side of the sagittal suture line and 1–2 mm anterior to the lambda suture line. Screws were inserted into the holes and positioned so that the flat screw tip rested on the dura. Screw heads were covered with dental cement and left to dry. EMG electrodes were inserted through the trapezius muscle with a 21guage needle, perpendicular to the muscle fibers. The free wire end was capped with insulation and both sides of the incision were tied off with surgical thread to prevent fluid infiltration. The incision was then closed with 6 -8 mattress stitches.

Sleep data acquisition and analysis

Animals were housed individually and cages were positioned at least 18" apart to avoid interference during radiotelemetry data acquisition. EEG, EMG, temperature and locomotor activity data were recorded continuously with DSI's Data Art acquisition

software and binned in 10 second epochs. For these nocturnal rodents, the first four hours of their active period (dark) and the first four hours of their resting period (light) were evaluated for sleep architecture on the day prior to the start of water maze training (baseline), and following the stress/ probe trial paradigm. (Buechel et al., 2011). Architecture was scored using Neuroscore's analysis console in 30 second increments while being viewed in 2-5 minute windows. EEG waves were stratified into 'low amplitude' (≤50% of maximum) and 'high amplitude' (> 50% of maximum) tiers, and underwent fast Fourier transforms for each of 5 frequency ranges: Δ (0.5–4 Hz), Θ (4–8 Hz), A (8–12 hz), Σ (12–16 Hz) and B (16–24 Hz). EMG waves were stratified into 3 tiers: 'basal' ≤ 33% (seen during REM), 'intermediate' (between 33% and 66%), and 'high' (> 66%). Stages were established as follows: Wake- intermediate or high EMG ± locomotor activity, EEG variable; Light Sleep- low amplitude EEG, intermediate EMG, and no locomotion; REM (paradoxical) Sleep- high frequency EEG, 'basal' EMG and no locomotor activity; Deep Sleep- high amplitude EEG activity enriched in delta band frequency, basal to light EMG activity, no locomotor activity. Prior assigned sleep stages informed subsequent assignments. Ambiguous epochs, as well as those containing artifacts, were not scored and accounted for < 5% of scored time.

Water maze testing

The water maze task was performed as in previous studies (Buechel et al., 2011). A 190 cm diameter circular, black painted pool was centered (250 cm/ side) in a cubicle of floor to ceiling black curtains, making the environment relatively neutral. High contrast black and white cues (90 cm × 90 cm- circle, triangle and vertical lines), were placed, one to each of three curtains facing the maze, 60 cm above the maze rim. Maze temperature was maintained at 26 ± 2°C. One quadrant contained a 15 cm diameter escape platform covered with black neoprene for improved traction. Illumination in the room was set at 3.6 to 3.8 lux and a Videomex-V water maze monitoring system (Columbus Instrument, Columbus, OH) was used for analyses. All training and probe sessions took place between 12PM and 4PM (during the rats' active period).

Locally cued training (pre-surgery). The locally cued platform location task included an additional visual cue: a white Styrofoam cup suspended from the ceiling by black thread approximately 12 inches above the submerged platform. In the locally cued task, over 3 days, each animal was given three 60 second trials per day, with 60 seconds on

the platform and a 2 minute inter-trial interval. Criterion for performance was established as an ability to swim to the platform in under 30 seconds for 2/ 3 trials on the 3rd day.

Spatially cued training and probe (post-surgery). Two weeks after implantation surgery (to allow for recovery), the spatial water maze task was performed. Spatial cues outside the pool were used to triangulate on the submerged platform's location. A 4 day protocol was used (days 1–3: 3 trials per day, hidden platform; day 4-following restraint-probe trial with platform removed). On training days, each animal began in a different quadrant on each of three trials. They were given one minute to find the platform, one minute on the platform and a two minute inter-trial interval. On the probe day, the platform was removed and each rat was given one 60 s trial.

Restraint (psychosocial) stress

For 3 hours immediately prior to the water maze probe trial, half of the subjects were restrained with nylon coated canvas rat Snuggles® (Harvard apparatus). Animals were monitored continuously for vocalization and struggling throughout the 3 hour restraint, and tested on the probe trial immediately following restraint. Control animals stayed in their home cages in the housing facility until the probe trial began. After completing the task, animals were returned to their home cages in the housing facility and sleep architecture data was collected.

Tissue collection and analysis

On the following morning, animals were killed by CO_2 anesthesia and decapitation. Trunk blood was collected in lithium heparin vacutainers (BD biosciences), and centrifuged at 1200g for 10 minutes. Serum was removed for corticosterone measurement using a radio-immuno assay with a lower quantification limit of 20ng/ml (Antech GLP, Siemens Diagnostic, Los Angeles) and additional blood components measured (Abaxis Comprehensive Diagnostic Panel, VetScan2,University of Kentucky College of Medicine core facility). The hippocampus was dissected out of one hemisphere and prepared for electrophysiological testing. The anterior and posterior tips of those hippocampi were placed in dry ice and transferred to a -80°C freezer for Nanostring analysis at the end of the study. The second hemisphere was post-fixed in 4% formalin (24hr), cryoprotected in 15% sucrose solution and stored at -80°C for future use.

Electrophysiology

Hippocampi were prepared according to standard protocols (Searcy et al., 2012, Pancani et al., 2013). Briefly, hippocampi were removed and cut into transverse 400µm thick slices on a McIlwain chopper. They were placed in low calcium artificial cerebrospinal fluid (ACSF) chilled to 0°C, recipe as follows (in mM): 128 NaCl, 1.25 KH_2PO_4, 10 glucose, 26 $NaHCO_3$, 3 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$. Slices were transferred to an interface recording chamber kept at 32°C with 95% O_2, 5% CO_2 and normal-calcium ACSF (as above) with an additional 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Slices were allowed to incubate in the recording chamber for at least one hour before recording.

To begin testing, a 0.0055 inch, twisted bipolar Teflon coated stainless steel (A&M Systems) stimulating electrode, was placed in the perforant pathway. Glass recoding electrodes, pulled on a Sutter-Brown P-80 puller with tips broken back, were filled with ACSF and placed in the *stratum radiatum* of CA1 to record excitatory postsynaptic field potentials (EPSP's). We performed 3 experiments on each slice. First input/ output curves were constructed by varying input stimulator voltage and measuring the change in output EPSP. For the subsequent experiments, input voltage was set to generate 1/3 of maximum EPSP. Paired pulse facilitation was measured in triplicate with a 50, 100, 150 and 200 ms delays and a 30 second inter-trial interval. Frequency Facilitation (10 hz for 10 s) (Landfield et al., 1986, Thibault et al., 2001) was performed last in each slice as prior work suggests that FF can lead to permanent changes in synaptic physiology.

Gene expression analysis

Microarray data from three published microarray studies of hippocampal aging in the F344 rat (Blalock et al., 2003, Rowe et al., 2007, Kadish et al., 2009) (data available through the Gene Expression Omnibus GSE854, GSE5666, GSE9990) was compiled to establish lists of genes significantly upregulated (101 total) or downregulated (70 total) in at least 2/3 of these studies. This panel of 'male F344 rat hippocampal aging genes' was submitted to NanoString Technologies (Seattle, WA) who then constructed a custom nCounter multiplex code set (Geiss et al., 2008) for the detection of these mRNA species (and specifically, for the gene structures as assayed by the original Affymetrix microarray probe set designs). Hippocampal mRNA was extracted from fresh frozen hippocampal tissue as in as in prior work (Buechel et al., 2011, Porter et al., 2012). Briefly, the dorsal and ventral tips of hippocampi were placed in Ependorf tubes, frozen

on dry ice, and stored at -80°C. After all tissue was collected, RNA was extracted using the Trizol protocol and RNA quality was checked on Agilent Bioanalyzer (RNA Integrity Number-RIN: 7.27 ± 0.15). Although there was no significant difference with age or treatment, RIN values were lower than in prior work. Nanostring reports that the nCounter system is robust to lower quality RNA, but caveats should be appreciated in this data's interpretation. We suspect that the increased time spent preparing slices prior to freezing tissue may have resulted in this lessened quality. 171 pre-selected mRNA species were quantified with nCounter (12 animals- 3 per treatment group- were run on each nCounter plate, except the last, which measured 6 subjects- 4 young controls, 1 aged stress, and 1 young stress). Five subjects' nCounter data was excluded due to poor quality). Data from the remaining 37 subjects (n = 10 young control, 8 young stress, 10 aged control, 9 aged stress) were transferred to flat files for further analysis. Data were normalized according to standard nCounter procedures using Nanostring provided spike-in controls. Quality control measures were consistent across groups and within normal range (binding density: 0.6 ± 0.04; Fields of View Read: 599.7 ± 0.1; n.s. between treatment groups, two-way ANOVA). None of the probes selected failed to detect a signal.

Statistical analysis

Data was analyzed by conventional statistical analyses (e.g., two-way ANOVA, two-way ANOVA on repeated measures, Pearson's and Spearman's tests, and *post-hoc* pairwise contrasts as noted in Results) with $\alpha = 0.05$. Multiple testing error was estimated where appropriate using the False Discovery Rate procedure (Hochberg and Benjamini, 1990). Software used for statistical analysis included Excel (Microsoft, 2010), SigmaStat (v 3.5, Systat), and The Institute for Genomic Research's Multi-Experiment Viewer (Saeed et al., 2006).

Results

Water maze

Animals were initially trained to swim to a platform centered immediately under a visual cue ('locally' cued). The purpose of this task was to identify subjects that could not see or were too distressed to perform the task and remove them from the study. Over three successive days, latency to the platform was measured on three trials per day. Results are averaged and plotted (Fig. 10A). Both young and aged animals showed shorter latencies over time, but aged animals were significantly slower than young on all training days. Next, subjects were surgically implanted with sleep-monitoring equipment and given two weeks for recovery (Methods) and baseline sleep measurement.

After recovery, the local cue was removed from the water maze and animals were trained, 3 trials per day for 3 days, on the spatial Morris water maze. Here, distal cues are used to triangulate on the hidden platform's location, a hippocampus-dependent task. Young animals showed no change in performance over time while aged animals showed significantly shorter latencies over training days. Aged subjects were significantly slower on training days 1 and 2, but statistically indistinguishable from young by training day 3 (Fig. 10B). This allowed us to evaluate the effect of 3 hour restraint from a similar baseline in young and aged subjects. It should be noted that aged animals swam more slowly than young (young 33.7 ± 1.1 cm/s; aged 28.5 ± 0.9 cm/s; $p < 0.01$), and this reduced speed could in part explain longer latencies in aged subjects. However, with an average distance from all drop points to platform of 91.44 cm, young and aged subjects' minimum 'straight line' latency to platform, given their average swim speeds, would be 2.7s and 3.2s respectively. Therefore, swim speed alone accounts for about 0.5 seconds difference in latencies, a performance gap much smaller than observed on locally cued, and days 1 and 2 of spatially cued, training.

On the following day, half of the animals were restrained for 3 hours, and the hidden platform was removed from the maze for probe trial. During a 60s swim period, we counted the number of times each animal crossed the area originally containing the platform (Fig. 10C), as well as the animals' path length within the goal quadrant (Fig. 10C inset). As in prior work, aged animals dedicated less effort investigating the original platform location. However, while 3 hours of restraint significantly reduced young

Figure 10. Water Maze Pre and Post Stress.

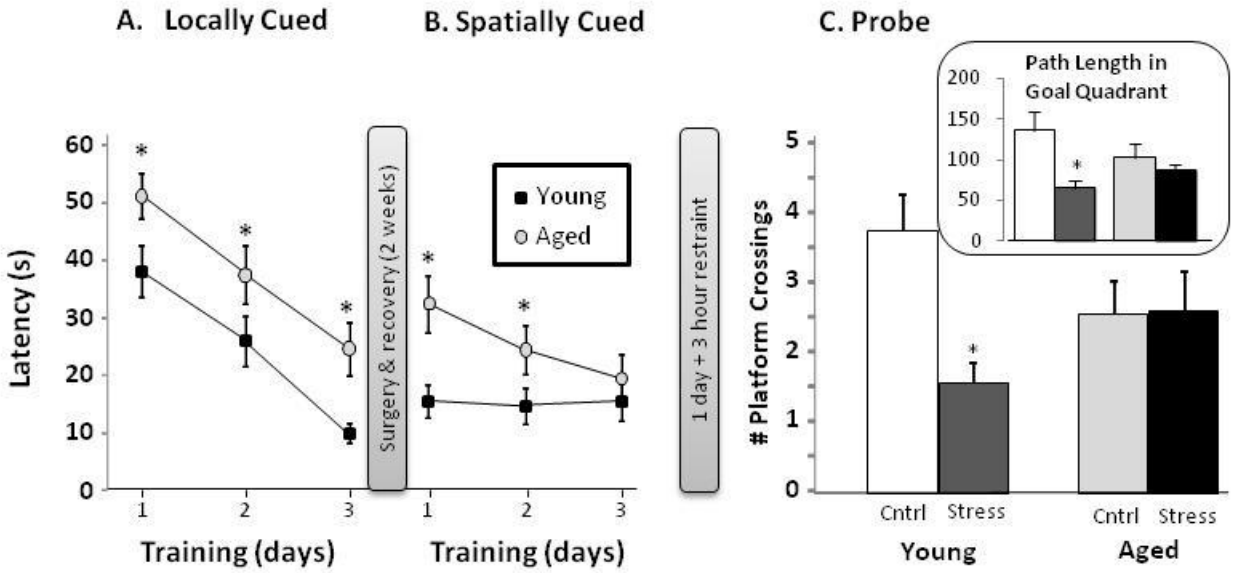


Figure 10. Water Maze Pre and Post Stress A. Locally cued water maze training. Latency to platform is plotted as a function of training day (two way ANOVA with repeated measures; significant main effects of training [$p < 0.001$] and age [$p < 0.001$], * $p \leq 0.05$ Fisher's LSD for all panels) **B.** Spatially cued Morris water maze training. Latency to platform is plotted as a function of training (as in A, two way ANOVA with repeated measures; significant main effects of training [$p < 0.01$] and age [$p < 0.001$] as well as significant interaction [$p < 0.01$]) **C.** Probe Trial. The number of times the original platform location was crossed (# platform crossings) is plotted for young and aged animals that were control (Cntrl) or 3 hour restraint treated (Stress). (two way ANOVA with significant interaction of age and stress [$p = 0.022$]) **Inset:** Path length (in cm) within the goal quadrant (two way ANOVA with significant interaction of age and stress [$p = 0.029$]).

animals' platform crossings and goal quadrant path lengths, an effect seen in multiple other restraint studies, aged subjects appeared relatively insensitive to restraint. Similar, albeit borderline significant patterns (p = 0.06 - 0.1) were seen with swim latency and path length to platform location (data not shown).

Restraint stress

For the 3 hours immediately preceding the spatial water maze probe trial on the fourth day, half of the young and aged animals received 3 hours of restraint. Animals were monitored throughout the restraint period for vocalizations and struggles. Qualitatively, both young and aged animals appeared distressed by the procedure. Quantitatively, we were unable to detect any age-related difference in perceived stress as measured by the number of struggles and vocalizations over 3 hours (Young 29.6 ± 7.9, Aged 29.6 ± 7.1; n.s., Student's t-test).

Sleep architecture

Polysomnographs were scored for duration of Wake, REM sleep, light sleep, and deep sleep. The first four hours of Resting and Active periods were evaluated before spatial water maze training, and again after restraint stress. As in prior work, control aged subjects showed less active period wake and less resting period deep sleep than young (Fig. 11A). Restrained young animals showed significantly more wakefulness, and less REM and Deep Sleep during the inactive period, while aged animals showed only elevated REM sleep (Fig. 2B upper). During the post-stress active period, young rats only showed a significant elevation in deep sleep (Fig. 11B lower). Note that previous work has shown, in both humans and rodents, that increased active period deep sleep is related to poorer cognition (Reid et al., 2006, Buechel et al., 2011)

Body temperature

We assessed potential age-related changes in diurnal rhythms by measuring body temperature (Adan et al., 2012, Menaker et al., 2013) . These data were acquired through the surgically implanted telemetry devices. Aged and young subjects are clearly in sync with light cues prior to spatial water maze training (Fig. 12A- the average of three 24 hour periods of days of baseline recording). Interestingly, studies have implicated stress in body temperature control (Oka et al., 2001). To investigate this, we measured

Figure 11. Sleep Architecture Pre and Post Water Maze.

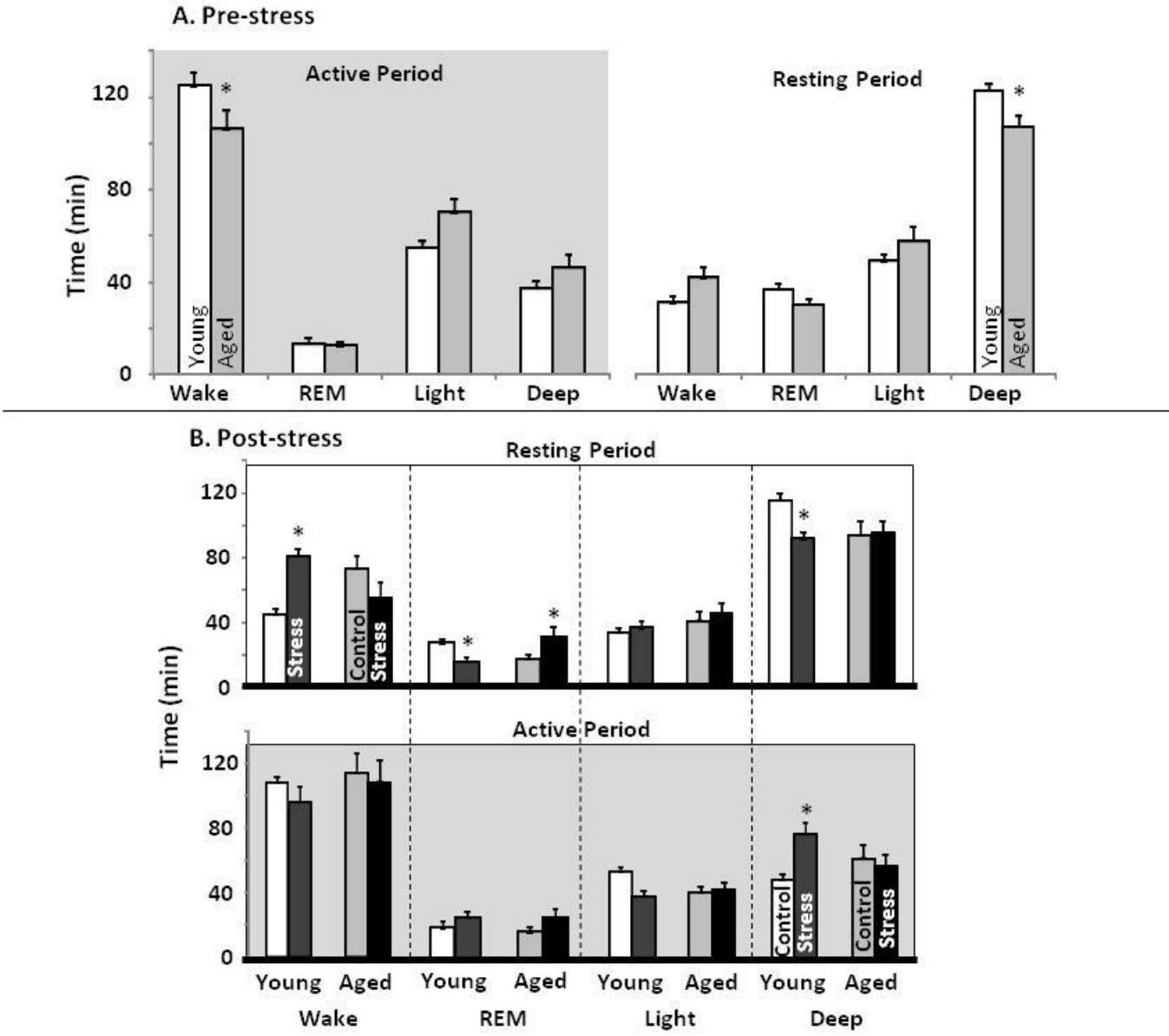


Figure 11. Sleep Architecture Pre and Post Water Maze A. Control young vs. aged sleep architecture (duration). **B.** Influence of stress exposure on young and aged sleep architecture: Resting period immediately following stress (resting period) and during subsequent active period (12 hours later). (Top: Repeated measures two way ANOVA with a significant interaction of age and stage [$p<0.040$], with significant [* $p<0.05$] post-hoc Tukeys pairwise comparisons of stress vs control within each age, Bottom: two way ANOVA with a significant interaction of age and stress [$p<0.01$], with significant [* $p<0.05$] post-hoc Tukeys pairwise comparisons of stress vs control within each age)

Figure 12. Body temperature in young and aged

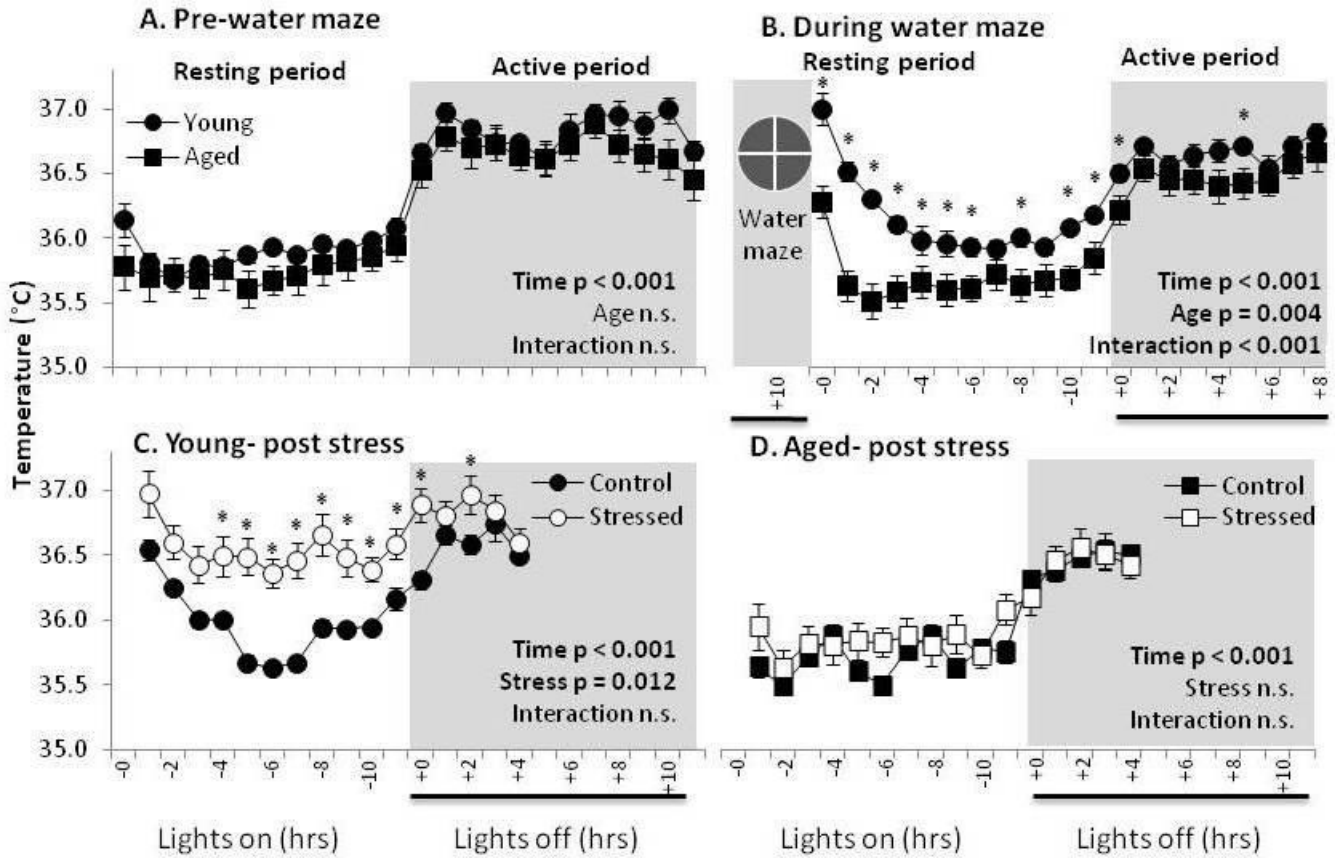


Figure 12. Body temperature in young and aged A. Pre-water maze baseline temperature measures show no difference between young and aged subjects B. During water maze training, young subjects show extended hyperthermia in the resting period after water maze exposure (x-axis rearranged to highlight post-water maze effect). C. Young subjects post-stress show elevated temperature compared to their control counterparts D. Aged subjects post- stress show no temperature differences from their control counterparts. (All tests are repeated measures two way ANOVA with post-hoc LSD).

body temperature during spatial water maze training as well as after restraint (Fig. 12B-D). Clearly, young animals show elevated resting period body temperatures during water maze training (Fig. 12C- average of 3 days training), although lights on/ lights off responses remain synced. Aged animals' body temperatures during water maze training are similar to their control levels. Finally, young animals show an exacerbated temperature elevation (Fig. 12C) following restraint, while aged animals' body temperature appears relatively unresponsive to restraint (Fig. 12D).

Hippocampal electrophysiology

Animals were killed the day following restraint and hippocampal slices were prepared for extracellular synaptic recordings. Input output (I/O) experiments assessed the relationship between synaptic response (output) and stimulus (input) (Fig. 13A). Aged control animals showed a significantly weakened input/output relationship, as has been seen in prior studies (Barnes and McNaughton, 1980, Landfield et al., 1986). This weakening appeared, at least in part, recovered in stressed aged subjects. We also tested for presynaptic calcium perturbations and synaptic plasticity changes using the paired pulse (PP) facilitation (Fig 13B) and Frequency Facilitation FF (Fig. 13C) protocols. As in previous studies (Landfield et al., 1978a, Wu and Saggau, 1994), no age-related changes were detected. Further, no change was seen in either of these measures with stress exposure.

Blood Corticosterone (CORT)

Corticosterone radio-immunoassays were performed on trunk blood that was collected at decapitation (during active period, 21 hours following restraint). It should be noted that 50-100 ng/ ml would be considered normal for young control animals. However, all animals had to be transported on the procedure day, adding additional stress exposure to all subjects. Thus, young control values are higher than expected. Despite this, young animals that had been restrained showed much higher blood CORT (Fig. 14), while aged subjects showed a blunted response. Future studies monitoring CORT levels throughout the stress exposure paradigm may help to discern whether this phenomenon might represent 'adrenal exhaustion' in the aged subjects. Additional measures taken from blood show that alkaline Phosphatase, amylase and potassium levels all changed significantly with aging and/or stress (two-way ANOVA).

Figure 13: Electrophysiology

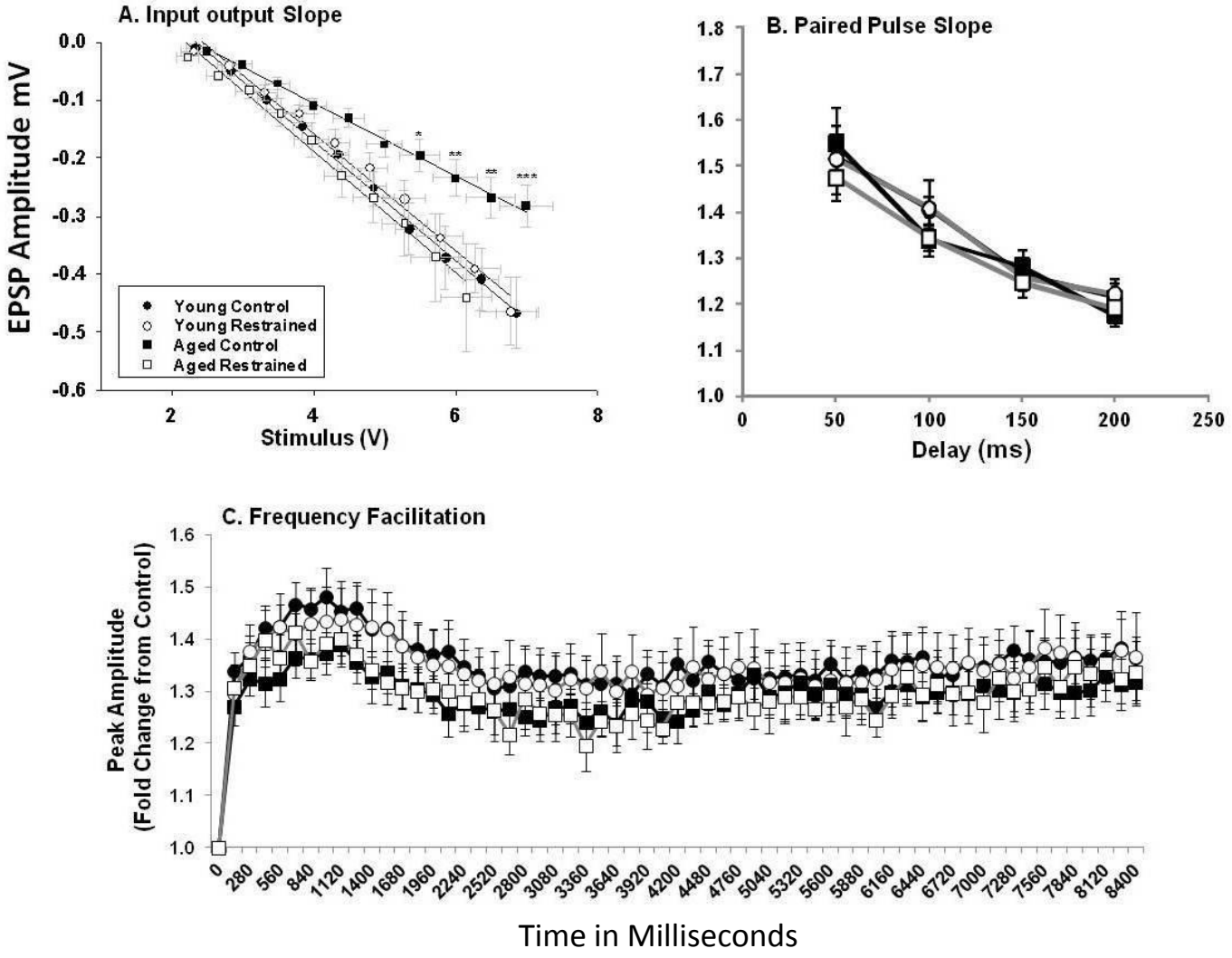


Figure 13: Electrophysiology A. EPSP amplitude plotted against stimulus voltage. There is a decreased response at higher voltages in the aged control animals. B. Paired pulse slope: no changes in Paired Pulse response from young to aged or stressed and control. C. Frequency Facilitation: No differences between young and aged, control and stressed.

Figure 14. Timeline and Corticosterone

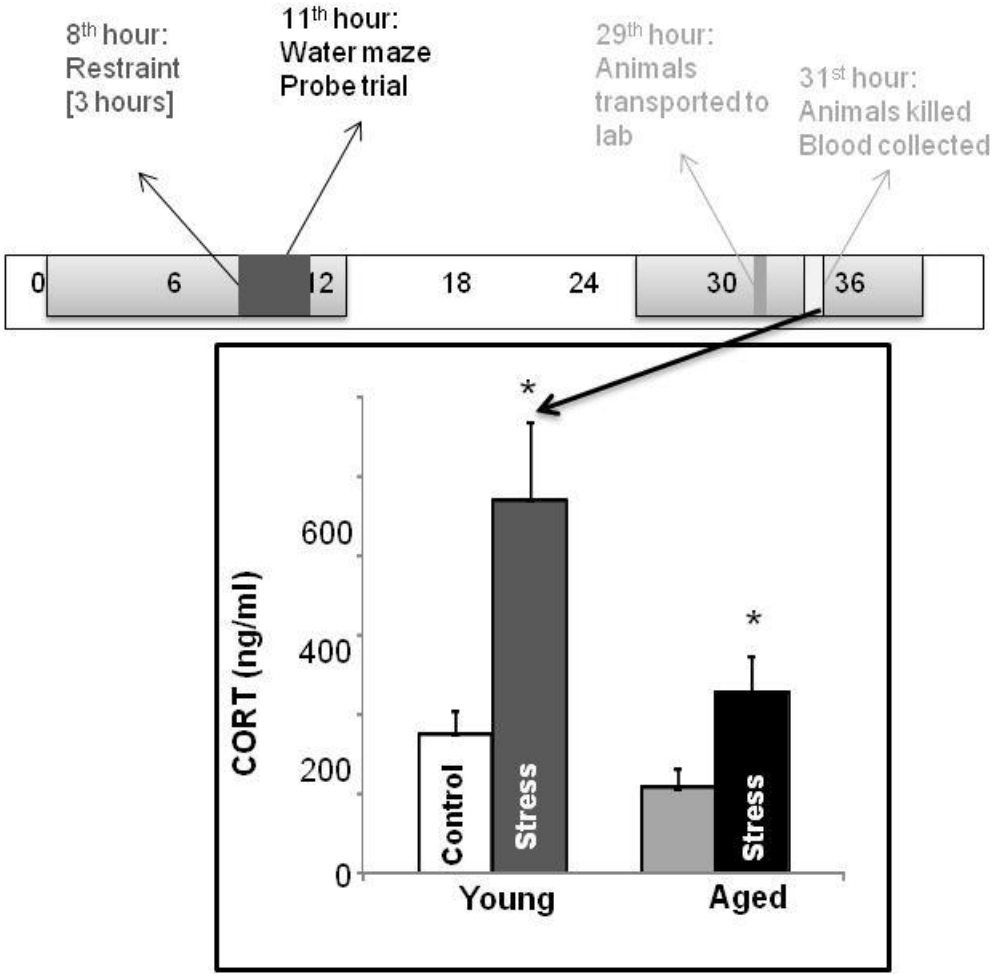


Figure 14. Timeline and Corticosterone A. Final 30 hours of our experiment: Restraint stress begins at the 8th hour. Water maze probe trial begins in the 11th hour. Animals are killed and trunk blood is taken for corticosterone measures in the 31st hour. B. CORT measurements: significant differences between young control and young stressed. Also significant differences between aged control and aged stressed, although they are on a different scale from the young subjects. Both stressed groups show an increase in CORT. (Repeated measures two way ANOVA with a significant main effect of Age[$p<0.011$] and Control vs Stress [$p<0.001$], with significant [* $p<0.05$] post-hoc Tukeys pairwise comparisons across age and across Control vs Stress).

Gene expression

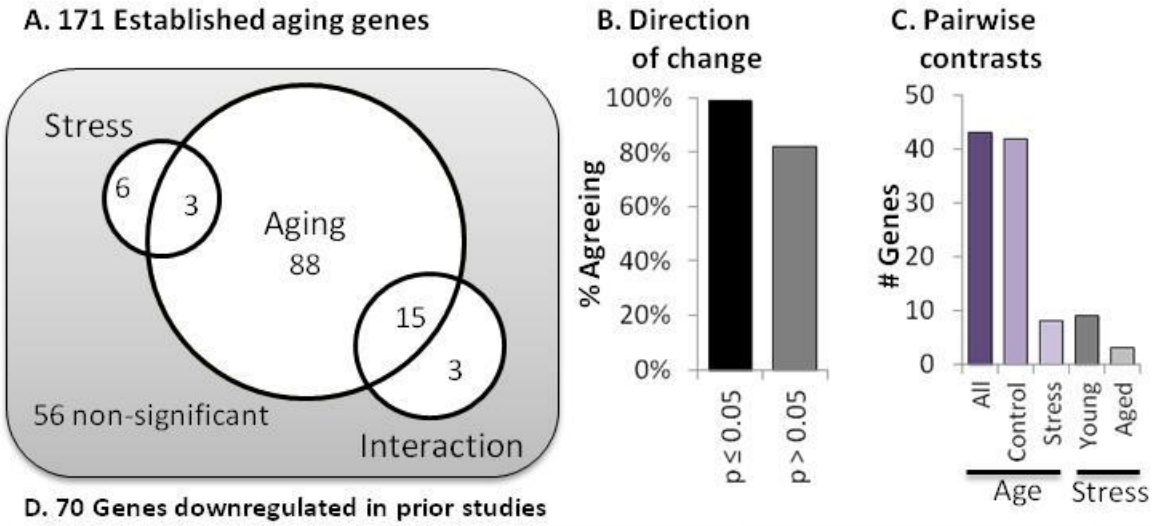
Hippocampal mRNA was tested for aging and stress changes against an 'aging' panel of 171 genes found to change significantly with age in at least two of three published hippocampal aging microarray studies in male F344 rats (see Methods). As predicted, far more genes are significant than could be reasonably explained by multiple testing error (115 found vs 9 expected with 171 tests at $\alpha = 0.05$) and are primarily centered on aging (Fig. 15A). Additionally, nearly all significant genes in the present study also agreed in direction of change with prior work (Fig. 15B). Even non-significant genes showed >80% agreement in direction of change with prior work ($p < 0.001$, binomial test). Genes altered with age were largely associated with results as reported in prior work, including downregulated neuronal and synaptic markers, and upregulated immune and inflammatory signaling. Specific genes in the downregulated and upregulated aging panel are shown in Fig. 15D and E, color-coded with their results in the present study.

Pairwise contrast analyses (Fig. 15C- 'All') clearly show a large cohort of genes significantly changed with age regardless of stress status. However, a second large group of genes changed with age in control, but not in stressed subjects. This suggests that young and aged subjects' transcriptional profiles become more similar under stress conditions. Of these 42 'stress-blunted' aging genes, 33 (79%) showed oppositional regulation in young vs. aged subjects. That is, if stress exposure increased expression in young, it decreased expression in aged (Fig. 15D and E, arrows).

Discussion

The purpose of this work was to determine whether aged subjects showed a differential response to acute psychosocial stress as modeled by restraint. Generally, our results suggest that aged animals show a blunted response to acute stress compared to young. Despite clinical data showing both an increased incidence of, and more deleterious response to, chronic psychosocial stress with age in humans, surprisingly little basic research has investigated psychosocial stress' negative influences on sleep and cognition. In our hands, aged and young animals showed equivalent discomfort during restraint (vocalizations and struggles- see Results, restraint stress). However, post-stress decline in water maze performance (Fig. 10C), deep sleep loss/ increased wake

Figure 15. Transcriptional Analysis



D. 70 Genes downregulated in prior studies

Ap2s1	Calb1	Chga	Cntn4	Col3a1	Cox7b	Crhbp$^{\downarrow\uparrow}$	Csrp2	Cyp51	Dcn
Dnaja1	Dpp3	E2f5	Egr1	Egr2	F2r	Fkbp1b$^{\downarrow\uparrow}$	Fyn	Gad1$^{\downarrow\uparrow}$	Gamt
Gap43	Ggh	Glra2	Kcnv1	Lpl	Map2k6$^{\downarrow\uparrow}$	Pcp4	Pcsk1	Ppid	Prkar2b
Prps1	Ptpro	Rab26	Rgc32	Sh3kbp1	Slc1a1	Sstr2	Suclg1	Trpc4$^{\downarrow\uparrow}$	Vgf
Agrn	Arc	Arg2	Car4	Cgref1	Cyp1b1	Fkbp4	Gnpat	Igfbp2	Lum
Lxn	Pacs1	Per2	Pfn1	Pou3f1	Ppib	Rab12	Rgs14	Sfpq	Slc15a4
Syp	Tceb1	Arf5	Chgb	Egr4	Eif2b4	Ide	Mal	Nudc	Serinc5

E. 101 Genes upregulated in prior studies

Aadat	Aif1	Aldh2	Anxa3	Apod	Apoe$^{\uparrow\downarrow}$	Arpc1b	B2m	Bin1$^{\uparrow\downarrow}$	C1qb
Cd37$^{\uparrow\downarrow}$	Cd53	Cd59	Cd63	Cd74	Cd81	Clu$^{\uparrow\downarrow}$	Cnn3$^{\uparrow\downarrow}$	Cryab	Csrp1$^{\uparrow\downarrow}$
Cst3$^{\uparrow\downarrow}$	Ctsb	Ctsd	Ctsh	Ctsl1$^{\uparrow\downarrow}$	Ctss	Decr1$^{\uparrow\downarrow}$	F3$^{\uparrow\downarrow}$	Fah$^{\uparrow\downarrow}$	Fcgr2a
Fn1	Fth1	Ftl$^{\uparrow\downarrow}$	Gatm$^{\uparrow\downarrow}$	Gfap	Gng8$^{\uparrow\downarrow}$	Grn	Il18	Itih3$^{\uparrow\downarrow}$	Klk6
Lamp1	Lamp2	Lcat$^{\uparrow\downarrow}$	Lgals1$^{\uparrow\downarrow}$	Lpar1$^{\uparrow\downarrow}$	Lyz2	Mag$^{\uparrow\downarrow}$	Maob	Mgst1	Nfe2l2
Padi2$^{\uparrow\downarrow}$	Pla2g16	Pla2g4a$^{\uparrow\downarrow}$	Prkch$^{\uparrow\downarrow}$	Psap$^{\uparrow\downarrow}$	Psme1	Qdpr	RT1-DMb	S100a4	S100b
Sepp1$^{\uparrow\downarrow}$	Serping1	Sgk1	Slc12a2$^{\uparrow\downarrow}$	Slc6a6$^{\uparrow\downarrow}$	Srebf1	Tcn2	Tgfa$^{\uparrow\downarrow}$	Vim$^{\uparrow\downarrow}$	Acadl
Bmp6	Csda	Ednrb	Fau	Gpx4	Grm3	Id1	Id3	Ifitm3	Litaf
Mt1a	Ncl	Pdk2	Phldb1	Pmp22	Ppap2b	Prdx6	Ptgds	Rpl10	Rpl19
Rps15	Rps17	Rps5	St18	Sts	Sult1a1	Trip10	Sult1d1	Hsd17b4	Sdc4
Timp2									

Blue= down, Red= up, dark background = age significant, **bold font** = stress significant, superscripted arrows-opposing regulation in young vs. aged when stressed)

Figure 15. Transcriptional Analysis A. Venn diagram. Of 171 established aging genes 115 are changed significantly in this study. **B.** Direction of Change. Nearly complete agreement with prior studies regarding direction of change for aging-sensitive genes. **C.** The largest group of genes changed expression with age, regardless of stress status. **D.** Genes downregulated in previous studies. **E.** Genes upregulated in previous studies. Color: Blue = down; Red = Up.

(Fig. 11B), greater CORT secretion (Fig. 14), and elevated resting period body temperature (Fig. 12C) all point to young subjects being more responsive than aged to stress. Young subjects even showed a temperature response to the water maze exposure during training (also considered a stressor, Fig. 12B) prior to restraint, while aged animals did not. Thus, our results point to decreased cognitive, sleep, thermal, and blood glucocorticoid secretion responses to acute restraint in aged subjects, although this glucocorticoid measurement comes with a caveat. Glucocorticoid in our study was measured 16 hours after stress exposure close to the predicted peak of diurnal glucocorticoid secretion. These data may serve as a baseline for the construction of an understanding of the more devastating influence of chronic psychosocial stress with age.

Restraint model of psychosocial stress

Psychosocial stressors have been implicated in a variety of disorders in humans, from diabetes to depression (For review see: Kajantie and Phillips, 2006). With age, psychosocial stressors tend to become more common, with deaths of loved ones, and distance from family and friends due to retirement or relocation to a care facility. Psychosocial stress is operationally defined as stress induced by non-nociceptive stimuli. In rats, one accepted model is restraint, but others include abrupt and unpredictable light/ sound/ cage mate changes. We chose restraint as our stressor based on extensive prior literature (Pawlyk et al., 2008) regarding its efficacy in inducing HPA axis activity. In our study young and aged subjects showed similar struggle/ vocalization responses to restraint although further analysis of blood CORT levels during the restraint period may help to reveal whether this restraint elicited a similar hormonal stress response during the stressor.

Water maze

Unlike our prior water maze studies, here we perform visual cue training first, and trained young and aged animals to the same level of performance prior to probe trial in order to compare the influence of restraint from the same performance baseline and this may have resulted in the young subjects being ‘over-trained’. Thus, the negative influence of stress on water maze performance in young animals may be underestimated. However, the aging deficits in water maze training seen in multiple studies (Gallagher et al., 1993, Tombaugh et al., 2002, Blalock et al., 2003, Rowe et al., 2007, Bizon et al., 2009, Mawhinney et al., 2011, Vanguilder et al., 2012, Speisman et al., 2013) were clearly

present in earlier training sessions. In support of our working hypothesis that age acts as a stressor occluding the influence of subsequent stressors, young, but not aged animals, suffered a deficit in water maze performance post stress (Fig. 10C). While this negative effect in young seems to point to an advantage of aging, we must consider that responding negatively to a stressor may be appropriate, and exert long term hormesis and/or adaptation benefits that are lost non-responding animals.

Sleep architecture

Consistent with prior studies, aged animals showed less deep sleep with age (Fig. 11A). However, young animals showed an aging-like shift in sleep architecture with stress exposure, increasing resting period wake, while losing REM and deep sleep. In human studies, increased wake/ decreased sleep in response to stress are associated with poor coping strategies(Cespuglio et al., 1995, Morin et al., 2003). Further, REM is reportedly associated with strengthening memory (Kim et al., 2005, Ellenbogen et al., 2006, Aleisa et al., 2011, Pace-Schott and Spencer, 2011, Zagaar et al., 2012) (Siegel, 2001, Genzel et al., 2009), while deep sleep is associated with de-potentiating hippocampal synapses for subsequent learning events (Vyazovskiy et al., 2008, Born, 2010). The loss of both with stress in young may be particularly disruptive to memory formation, while the increase in REM with age may help aged subjects' performance in the face of a stressful event and has been associated with a healthy response to stress (Cespuglio et al., 1995, Gonzalez et al., 1995).

Temperature and electrophysiological responses to stress

Temperature measurements were taken to evaluate both diurnal zeitgeiber response based on body temperatures circadian rhythm, as well as to assess stress-induced hyperthermia response (Oka et al., 2001). Aged and young animals both appear to show appropriate responses to light cues (Fig. 12), but the young, rather than the aged, showed a significant stress-hyperthermia after restraint (Fig. 12C and D). Surprisingly, this hyperthermic response was also present in young but not aged animals during water maze training prior to restraint stress (Fig. 12B), again highlighting that young subjects appeared more responsive to stressful events than aged subjects.

Stress' and stress hormones' influence on hippocampal electrophysiology have been characterized (Joels et al., 1997) although no work of which we are aware has investigated acute psychosocial stress' in aging hippocampus. Here (Fig. 13), aged

animals are no different from their control counterparts in both frequency facilitation and paired pulse measures (Fig. 13B and C), in agreement with prior work on stress hormone effects (Landfield et al., 1978a, Wu and Saggau, 1994). However, restraint significantly enhanced aged but not young input sensitivity (Fig. 13A). Like increased REM sleep, this may represent a beneficial response (Barnes et al., 1997, Thibault et al., 2001) that may provide compensation to aged subjects.

Corticosterone measures

Aged subjects show a significantly smaller CORT response to stress than young (Fig. 14). This seems to contradict the prevailing theory on CORT increases with age in both humans and rodents (Landfield et al., 1978a, Landfield et al., 1986, Van Cauter et al., 1996, Porter and Landfield, 1998). However, these results are not completely unprecedented (for review see, Segar et al., 2009). Measures of the Fischer 344 rat diurnal rhythm report blood CORT highest early in the active period 200-300 ng/ ml) and lowest early in the resting period (< 100 ng/ ml) and Fischers have have higher CORT levels both normally and in response to stress compared to other rat strains (Dhabhar et al., 1993, Stohr et al., 2000). Our samples were taken approximately 4 hours after peak CORT, putting our control results within an acceptable range. However, peripheral events are known to elicit an elevation in CORT (Covington and Miczek, 2005), and these animals were subjected to transport stress on the day of tissue preparation. Therefore, although all subjects received equal exposure to this stress, age and/or restraint treatment may have influenced response to transport.

While the aging hypothalamic-pituitary-adrenal axis is considered hyper-active (Meaney et al., 1992, Bizon et al., 2001, Herman et al., 2001, McEwen, 2008, Lupien et al., 2009), central components like excitatory amino acid level, corticotropin releasing hormone, glucocorticoid receptor level and/or brain region selective shifts in glucocorticoid response likely play a role (Lowy et al., 1995, Mabry et al., 1995, Herman et al., 2001, McEwen, 2001, Meyza et al., 2007). As opposed to exaggerated glucocorticoid responses to non-psychosocial stressors (Sapolsky et al., 1986a) aged animals appear to have either the same or a blunted corticosterone or nerve growth factor response to psychosocial stressors like restraint (Stewart et al., 1988, Scaccianoce et al., 2000, Herman et al., 2001, Shoji and Mizoguchi, 2010, Garrido et al., 2012). Regarding diurnal variation in corticosterone, an elevated diurnal trough and either no change or a blunted diurnal peak in glucocorticoid secretion is also seen with aging (Sonntag et al., 1987,

Goya et al., 1989, Carnes et al., 1994, Rowe et al., 1997, Gartside et al., 2003), suggesting a flattened diurnal rhythm.

Gene expression

To get a closer look at molecular changes that might be at play, we investigated gene expression for 171 'aging panel' genes (Fig. 15) with established age-related changes based on prior work (Blalock et al., 2003, Rowe et al., 2007, Kadish et al., 2009). The 101 upregulated genes are associated with immune and inflammatory signaling while the 70 downregulated genes have established roles in neuronal function. Here we discuss consequences of shifts in the total panel's expression rather than conjecture on individual genes and what changes in their transcript level might imply for functionality. Thus, the molecular panel was used to determine A) if the molecular profile of aging is identified (positive control), B) whether this aging panel is sensitive to stress, and C) if so, did stress modify the aging profile in young or aged subjects?

Clearly, we reliably detected the aging profile, with 93% of the genes showing directional agreement with prior studies. Many of those genes were significant with age regardless of stress status (Fig. 15C) and a second large cohort of 42 genes was significant with age in control, but not in stressed subjects. This latter observation suggests that stress's effect on aging-sensitive genes is to reduce the expression differences between young and aged. This could happen by moving young expression towards aging levels, by moving aging levels towards young, or, by moving both aged and young gene expression towards one another.

Because the following text discusses direction of change, rather than magnitude of change, we felt it prudent to label this section as conjecture and restrict it to the Discussion rather than the Results. With that caveat, we found it surprising that the latter appears to be the case. Fully 79% of these stress-blunted aging genes showed oppositional stress regulation with age- that is, if upregulated by age, then stress both reduced expression in aged subjects and increased expression in young subjects. Further, 91% (21/ 23) of these 'oppositional' genes that were also identified as 'oppositional' in a prior study identifying the age and glucocorticoid-sensitive transcriptional profile (Chen et al., 2013). This inversion of HPA-axis activity's action on gene expression may parallel age-related shifts in the action of estrogen (Sohrabji and Bake, 2006, Selvamani and Sohrabji, 2010, Rettberg et al., 2013, Singh et al., 2013).

Summary and conclusions

In this study, rats showed characteristic aging changes seen across multiple mammalian aging systems, including worsened cognition (Gallagher et al., 1993, Salthouse, 1996, Joseph et al., 1999, Bickford et al., 2000, Erickson and Barnes, 2003, Tombaugh et al., 2005, Foster and Kumar, 2007, Gunn-Moore et al., 2007, Cotman and Head, 2008), disrupted deep sleep (Stone, 1989, Van Cauter et al., 2000, Cajochen et al., 2006, Mackiewicz et al., 2006, Espiritu, 2008, Tasali et al., 2008, Ancoli-Israel, 2009, Monjan, 2010), blunted synaptic sensitivity (Barnes et al., 2000, Thibault et al., 2001), and shifted hippocampal gene expression (Cotman and Berchtold, 2002, Blalock et al., 2003, Toescu et al., 2004, Galvin and Ginsberg, 2005, Rowe et al., 2007, Kadish et al., 2009, Burger, 2010). Similarly, young animals exhibited typical psychosocial stress responses, including disrupted sleep architecture (Marinesco et al., 1999, Van Reeth et al., 2000), worsened cognition (Stillman et al., 1998, Sandi et al., 2005, Nicholas et al., 2006, Kim et al., 2007, Park et al., 2008), stress-induced hyperthermia (Oka et al., 2001), and increased blood glucocorticoid levels (Sapolsky et al., 2000, McEwen, 2007). Further, Morris water maze and sleep architecture changes in young stressed animals recapitulate, at least in part, changes seen in normal aging. In conjunction with extensive prior work (Kerr et al., 1989, Kerr et al., 1992, Bhatnagar et al., 1997, Oitzl et al., 2010) on exogenous glucocorticoid exposure in young animals, or long-term age-related consequences of pre- or perinatal stress exposure (Meaney et al., 1991, McEwen, 2002, Lupien et al., 2009, Eiland and McEwen, 2012), this work is consistent with one limb of the stress/allostatic load/ glucocorticoid hypotheses of brain aging, that young subjects exposed to stress exhibit aspects of an aging-like phenotype.

However, this begs the question- if stress/ glucocorticoid exposure leads to aging-like changes in young subjects' cognition and sleep, what happens to aged subjects exposed to the same stress? On the one hand, an acutely hyper-responsive HPA axis might result in an exaggerated stress response, but on the other, a chronically hyper-secreting HPA axis in aged may allow for blunted acute stress response if aging or aging-related changes are considered a chronic stressor. In this interpretation, age-related changes in central components like corticotropin releasing hormone (Segar et al., 2009) and localized glucocorticoid signaling (Chapman and Seckl, 2008), along with a flattened diurnal blood glucocorticoid oscillation (Sonntag et al., 1987, Goya et al., 1989, Carnes et al., 1994, Rowe et al., 1997, Gartside et al., 2003) may help to explain the

reduced response of aged subjects to acute psychosocial stress. Alternatively, the blunted response could be due to adrenal fatigue (Van Den Eede et al., 2007, Nater et al., 2008, Kumari et al., 2009). In humans adrenal fatigue is associated with chronic high stress environments like war (post-traumatic stress disorder) or constant pain (fibromyalgia, cancer), (Fries et al., 2005, Mease, 2005, Wu et al., 2012). However, here young subjects showed a hyper-thermic stress response prior to restraint while aged animals did not, CORT levels in controls were normal or even high, while low levels of cortisol are found in PTSD (Heim et al., 2000). Thus, hypo-responsiveness seems a more parsimonious explanation of the data.

Downstream consequences of this blunted acute response might be failed adaptation to repeated stress as seen in aged animals (Spencer and McEwen, 1997, McEwen, 1998) and/or reduced efficacy of hormesis-driven (Masoro, 2000, Mattson, 2008) anti-aging manipulations in late life such as environmental enrichment, exercise and caloric restriction (Masoro, 2005, Pang and Hannan, 2013, Voss et al., 2013) and possibly dietary manipulation (Gemma et al., 2010). Thus, aged animals may resist the cognition-worsening effects of acute psychosocial stress at the expense of their physiological capacity to adapt (McEwen, 2001, 2008). Despite over-activity of the HPA-axis having a bad reputation, and clear evidence of negative consequences, its hypo-response to acute psychosocial stress with age may also have long-term deleterious consequences.

Conclusion. For the present work, then, aged control animals showed characteristic changes in sleep architecture and cognition, and young animals that had been stressed showed aging-like changes in those same measures. Both young and aged showed elevated blood glucocorticoid levels with stress exposure, but young showed a larger magnitude increase. Young animals also show a greater stress-hyperthermia response, both after restraint, as well as simply in response to water maze exposure. Further, our assessment of age-related transcriptional response and hippocampal input/ output suggests that more than just being blunted by stress, aged subjects actually show a reversal, manifesting a more youthful profile with stress exposure. The most parsimonious conclusion is that aged animals are at least hypo-responsive to acute psychosocial stress. Because prior work demonstrates elevated HPA axis activity with age, we favor the interpretation that aging acts as a stressor whose presence occludes the influence of subsequent acute stressors. While this may be acutely beneficial for aged subjects, it may be deleterious in the long run, occluding stress adaptation and

reducing the efficacy of ‘beneficial’ stress (eustress) such as caloric restriction, exercise, and environmental enrichment.

Chapter 4 Final Thoughts

First study

In our first study looking at deep sleep and cortical gene expression, our aging rats modeled some common sleep architecture and cognitive changes seen with age in humans, including the cognitively disrupting influence of active period deep sleep. Aged animals showed a loss of inactive period deep sleep, as well as an increase in active period deep sleep which correlated with poor water maze performance. That led us to believe that for aged animals it may not be loss of deep sleep during the inactive period so much as the disorganization of deep sleep across both active as well as inactive periods that may contribute to cognitive disruption. However, we don't know if the sleep misalignment is a physiologic attempt to recover deep sleep duration lost in the resting period or a failure of sleep regulation throughout the sleep cycle. Regardless of the cause, methods for returning deep sleep to the resting period, via suppressing it in the active period, or enhancing it in the resting period may have cognitive benefit to the aging community. We also used microarray analysis on cortical tissue to look at gene expression with age, and sleep. Through transcriptional analysis, we confirmed the molecular aging effect that we have seen in previous work. However, the transcriptional profile did not correlate with sleep in either young or aged subjects. This may be partially due to the time of day when they were killed, and the fact that they were awake at the time of death. Previous research has shown that the molecular changes associated with each stage of sleep quickly change as the sleep state changes (Mackiewicz et al., 2009).

Second study

In our second study, we established that sleep changes reliably with age using our baseline sleep architecture data (aged animals showed decreased deep sleep in the resting period, and increased deep sleep in the active period). We also found that young animals exposed to stress showed aging like changes to their sleep architecture (decreased deep sleep in the resting period, and increased deep sleep in the active period). We also saw typical differences in water maze performance with age. However,

with stress young but not aged subjects showed a deficit in performance. Young and aged animals both show an increase in CORT 18 hours after stress, but the young subjects have a much larger elevation than aged subjects. With regard to electrophysiology, we only saw changes in input /output, and in this case, stress seemed to improve aged animals' synaptic throughput compared to their control aged counterparts. Young subjects showed a hyperthermic response to water maze exposure, as well as restraint, suggesting that, although they are outperforming their aged counterparts on water maze, they are having more trouble maintaining physiologic homeostasis after those stressful events. However, it is also possible that the intensely negative response seen after stress acts to preserve homeostasis in the long run. In which case the young animals, but not the aged may suffer a short term cognitive deficit in order to gain a long term benefit to stress exposure.

Finally, with our aging panel of genes, we found that these subjects' molecular profiles predominantly agree with previous aging microarray studies. By conventional statistical analysis, stress appeared to have little effect on this portion of the transcriptome. But interestingly, looking across the entire gene panel, stress showed a curious trend among age-related genes in that it appeared to move young genes towards aging levels and aged genes towards young levels, thus, creating a more youthful gene profile in aged subjects and a more aging like profile in the young.

All signs in this study point to acute restraint stress being more detrimental in young subjects than aged. However, when you take a step back from this study, and consider the body of literature on aging, there are other possibilities. There may be a hormesis effect, where aged animals have lived through life's stressors, so they've built an 'immunity' to stressful events. To me, the more likely possibility is that aged subjects, who at baseline, are underperforming compared to young, may already be responding to the long term stress of aging which may prevent them from mounting an adequate stress response during testing. This opens up two lines of possibility for future work: assessing the long term response to stressors in young subjects via cognitive and physiologic testing with the hopes of improving stress response to improve long term health with age And second, assessing aged animals' baseline data, as well as long term stress response to determine if they are unable to mount a proper stress response, or if restraint stress truly is a eustressor in an acute setting.

Pilot Study: Repeated Water Maze

In light of the interesting findings of our stress and aging study, I would also like to share some of the findings from a pilot study. Rather than devoting a whole chapter to this small study, I feel that it's more suited as a segue between the published data and our future directions. This pilot consisted of 10 aged (24 months) Sprague Dawley rats, 5 male and 5 female. Each was visually cued and implanted with EEG/EMG emitters, as with both of the published studies. After recovery, rats were exposed to a traditional water maze task (visual cue, 3 training days, probe trial), followed by 6 weeks of reversal training, where the platform was moved to a new location on the first day of each week and visually cued (as with the pre-surgery water maze), then the following day, the platform was removed and the 'platform crossings' of the new location were counted. A visual depiction of the procedure can be found in (Fig.16).

Water maze performance was consistent with prior work. However, deep sleep duration increased after each water maze exposure event. The deep sleep increase was short-lasting, and animals recovered to baseline before the beginning of the next week's water maze session. Further, this increase was exaggerated towards the end of the study (Fig. 17). Animals with the greatest initial deep sleep duration showed the greatest increase in deep sleep in response to water maze exposure. Further, early during maze testing, duration of deep sleep was significantly correlated with maze performance, and animals with more resting period deep sleep tended to do better on the water maze (Fig. 18). This correlation was lost with time, possibly because both water maze performance and sleep behaviors become much more consistent across all subjects at later time points (poorer performers started getting more deep sleep and began performing on par with their better performing counterparts). This led us to believe that the water maze may act as a stressor/ enricher- both of which have been shown to increase deep sleep, and that sleep plasticity may reflect stabilizing memory consolidation.

Figure 16. Experimental Setup: Repeated Water Maze.

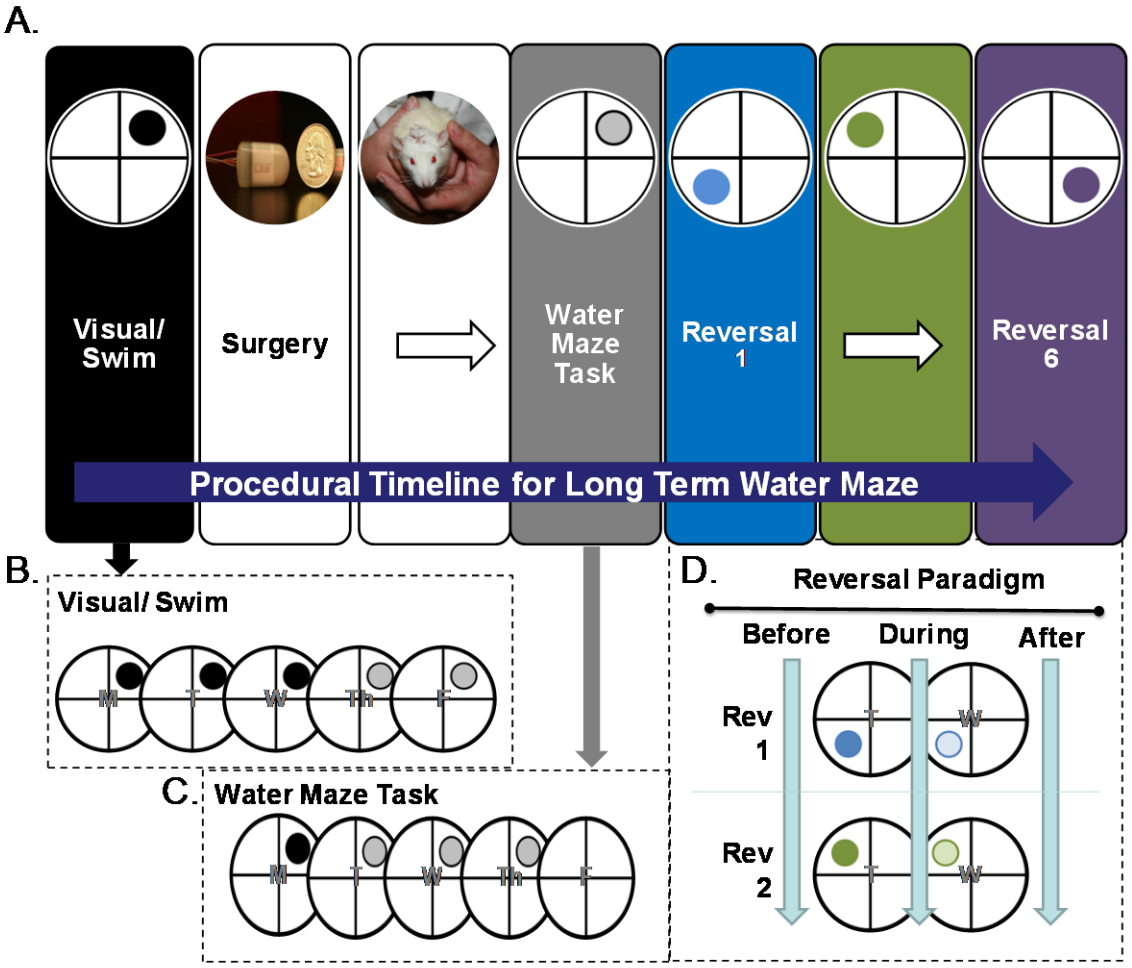


Figure 16. Experimental Setup: Repeated Water Maze A. Overview of study design – Week 1: visual cue Week 2: implantation, Week 3: Recovery, Week 4: Spatial water maze task, Week 5-11 Reversal training. Colored circles indicate platform location. B.Visual swim task**:** 3 days visible cue above platform (black circles), 2 days visual cue removed and only spatial cues were used for navigation (grey circles). **C.** Water maze task: Visible cue above platform on day 1, days 2-4 only spatial cues were used, day 5 probe trial with the platform removed. **D.** Reversals: (6 weeks) each week: day 1 visual cue above platform, day 2 only spatial cues; platform changes location each week. For all days except probe trial, subjects had 3, 1 min. swim sessions with a 2 min. intertrial interval. Sleep architecture measured in 4 hour blocks at reversal arrows.

Figure 17. Average Fold Change Deep Sleep.

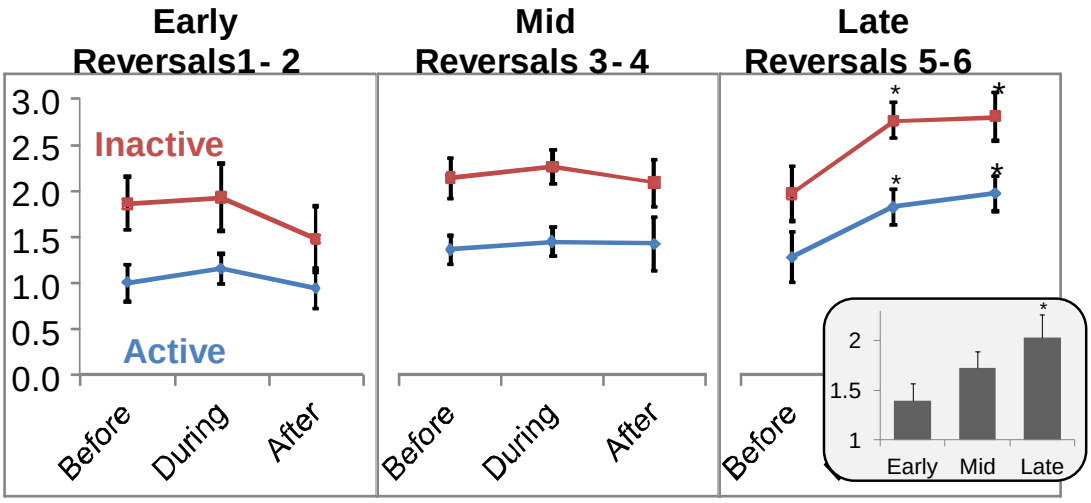


Figure 17. Average Fold Change Deep Sleep Subjects show more deep sleep during the inactive period. Deep sleep duration is unchanged in early reversals, but in Mid and Late trials, there is increased baseline deep sleep duration. Further, in the later trials, subjects showed an acute increase in deep sleep following water maze exposure (2-ANOVA RM; * Tukeys < 0.05). *Inset*: Averaged fold change in deep sleep for early, mid and late reversal trials (* $p < 0.05$; 1-ANOVA).

Figure 18. Deep Sleep Correlations.

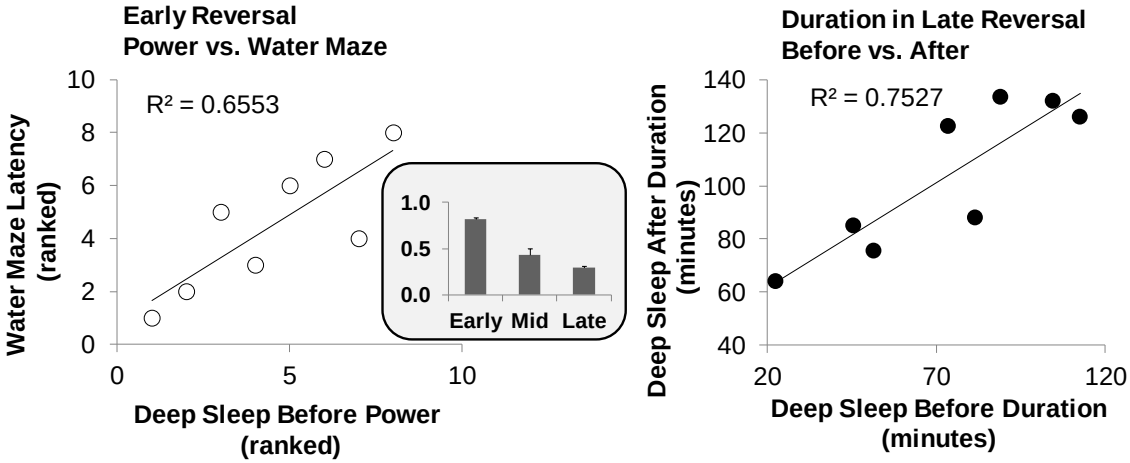


Figure 18. Deep Sleep Correlations Right: Deep sleep power (before, inactive) and water maze performance are strongly correlated in early reversals (Spearman correlation used because of dissimilar variables; $p = 0.015$). Inset: Average of deep sleep power to water maze correlation R values in early, mid and late reversals. **Left:** Deep sleep duration prior to and after water maze are strongly correlated in late reversal ($p < 0.001$).

This work is consistent with our published stress study, and gives us some insight on how our aged animals will react when faced with stresses that move from the acute arena into a more chronic scenario. Of course, everything is about dose, duration and timing. While the repeated water maze paradigm may have acted as a eustressor in that situation, it may not reflect the outcomes of a more intense chronic stress paradigm. This longitudinal water maze study does agree with other work that relatively little stress seemingly benefits both the sleep and cognition of aging rodents (Mirochnic et al., 2009, Arranz et al., 2010, Voss et al., 2011, Gomez-Pinilla and Tyagi, 2013, Hotting and Roder, 2013, Zinke et al., 2013), and further demonstrates that aged animals are capable of eliciting increased deep sleep given the appropriate manipulations.

Future Directions

Motivations

Studying an acute stress event is like taking a snapshot of stress' effects on our equilibrium. We see change, but we don't see the consequences of ongoing stress, or the biological responses to it, over time. To mimic a real life scenario, where stress doesn't occur in a vacuum, and doesn't always resolve in a few hours, or minutes, we need to study chronic stress. Psychosocial stressors can stay with us long after the event has passed. You don't recover from the loss of a loved one after a shower and a cold compress. Research has shown, that simply thinking of the stressful event can cause a rise in CORT levels in saliva (Wiemers et al., 2013) Interestingly, an exogenous application of CORT has also been associated with an increase in thinking about stressful events (Patten, 1999). It also has been shown that hippocampal damage, through over exposure to CORT causes a lack of response to stressful events (Buchanan et al., 2009). This supports our hypothesis that aged subjects show a lack of stress response, possibly due to an accumulation of hippocampal damage/ dysfunction/ vulnerability caused by elevated CORT levels over a lifetime. To parse out this hypothesis, we need to look at stress responses in our young and aged subjects over time. Do the aged animals continue to show a lack of response? Does the response get worse over time, or possibly better, as with our pilot study? How does the aged subject cognitively react to an intense stress over time? Is it different than the response to a mild

stress over time? How does sleep change after chronic stress exposure? Are the young animals experiencing any benefit from their more intense stress reaction, or are they setting themselves up for a future of cognitive deficit induced by elevated glucocorticoids? Do their bodies adjust to their allostatic load? What about after the stress has abated?

Cognitive testing

Our lab is also looking into other forms of cognitive tests for our stressed animals, as research has shown that different aspects of memory are affected differently by stressful events (Mello et al., 2008, Conrad, 2010). How will stress affect performance on other hippocampus-associated tasks like delayed match to sample or one way active avoidance? Can behavioral manipulations like environmental enrichment or exercise, or pharmacologic manipulations that increase slow wave sleep like gaboxadol or trazadone, help to alleviate the negative consequences of stress in aged subjects?

Stress Perfection

We are working on perfecting other, less invasive and less intensive methods of stressing our animals, namely, by using intense and unpredictable light as a stressor (Leonhardt et al., 2007). This will minimize physical manipulation of our psychosocial stress, as well as reduce the burden on the researchers who monitor the subjects. We first have to confirm that using a strobe light does elicit a detectable stress response in the rats then we can design experiments replacing the restraint stress with strobe light stress, which may be particularly useful for chronic stress studies.

CORT sampling

We are looking at methods for repeatedly sampling CORT levels throughout our study. With clear baseline data, we can better assess the changes in CORT seen in both young and aged animals with stress. Our biggest challenge is finding a method of CORT sampling that is compatible with our gold standard cognitive test, the water maze. In humans, salivary cortisol is often measured in stress studies (Nierop et al., 2006, Miller et al., 2013). However, very little literature is available on rodent salivary corticosterone. Cannulation of the jugular vein is a great option for minimally painful repeated plasma CORT measurement in rats (Ling and Jamali, 2003), but is less compatible with the water maze.

Sleep Manipulation

We showed reliable changes in sleep architecture in our two studies. We would like to determine if you can manipulate sleep in aged subjects in such a way that sleep architecture is realigned to emulate what is seen in young subjects. The simplest method for this would be to give a deep sleep enhancing drug such as gaboxadol in a time frame where it could act in the resting period, or the converse of that: giving a drug like caffeine so that it would act in the active period, suppressing sleep. We would answer the question of whether these drugs effectively manipulate sleep and if is there any cognitive benefit from their administration.

Environmental enrichment

Finally, we are looking into environmental enrichment, as it has been shown that animals living in an enriched environment show less negative, and more flexible responses when faced with stressors(Mitra and Sapolsky, 2009, Chen et al., 2010, Hutchinson et al., 2012). Interestingly, in humans and other animals, environmental enrichment is associated with higher baseline CORT levels, but a smaller change in CORT after a stressful event (de Jong et al., 2000, Moncek et al., 2004, Fiocco et al., 2007). Toys, voluntary exercise and friendly cage mates are typical environmental enrichments for animals. In humans, education is considered an environmental enrichment, but studies largely focus on the results of enrichment, such as high self esteem. Higher self esteem correlates with larger hippocampal volume and better scores on cognitive tests (Pruessner et al., 2004, Pruessner et al., 2005, Kubarych et al., 2012). Environmental enrichment is also associated with improved inactive period sleep in young animals (Gutwein and Fishbein, 1980, Mirmiran et al., 1982). It would be interesting to learn if the enrichment is still beneficial even if it's started later in life. Research suggests that even later in life, environmental enrichment has positive consequences (Harburger et al., 2007, Mirochnic et al., 2009).

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Achievements, Honors and Awards

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2012 Bluegrass Chapter of The Society for Neuroscience travel award nominee

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Seminar "Gray and White Matter with Laser capture and Microarray Analysis in Aging and Alzheimer's Disease" Dept. Pharmacology, UK

2011 Seminar "Changes in Sleep and Cognition with Age:
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2009-2011 Graduate student representative, Department of Pharmacology

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Seminar "Long-term water maze training increases deep sleep in aged rats" Dept. Pharmacology, UK

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2006 Cat Clinic participant, Dept. Biology, Duquesne

Peer-reviewed Publications

Blalock EM, **Buechel HM**, Popovic J, Geddes JW, Landfield PW (2011) Microarray analyses of laser-captured hippocampus reveal distinct gray and white matter signatures associated with incipient Alzheimer's disease. J Chem Neuroanat 42:118-126.

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Work Submitted/ in preparation/ in review

Buechel HM Thesis (in preparation) Changes in Sleep Architecture and Cognition with Age and Psychosocial Stress: A Study in Fischer 344 Rats

Buechel HM, Popovic J, Staggs K, Anderson K, Thibault O, Blalock EM (2013) Aged Rats are Hyporesponsive to Acute Restraint: Implications for Stress in aging

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Abstracts

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