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Deep Sleep, Cognition, Body Weight, Body Temperature, and Behavioral Distress Responses to New Onset Psychosocial Stressors are Blunted with Age in Male F344 Rats

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DEEP SLEEP, COGNITION, BODY WEIGHT, BODY TEMPERATURE, AND BEHAVIORAL DISTRESS RESPONSES TO NEW ONSET PSYCHOSOCIAL STRESSORS ARE BLUNTED WITH AGE IN MALE F344 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
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Lexington, Kentucky

Director: Dr. Eric Blalock, Associate Professor of Pharmacology and Nutritional Sciences
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2016

ABSTRACT OF DISSERTATION

DEEP SLEEP, COGNITION, BODY WEIGHT, BODY TEMPERATURE, AND BEHAVIORAL DISTRESS RESPONSES TO NEW ONSET PSYCHOSOCIAL STRESSORS ARE BLUNTED WITH AGE IN MALE F344 RATS

Complaints associated with aging, including cognitive deficits and sleep loss, are highly prevalent and negatively impact quality of life. Further, with increased age, humans are also more likely to experience new-onset psychosocial stressors, such as divorce, loss of a spouse, and social isolation. Stress has detrimental consequences that in many ways parallel the effects of aging on sleep and cognition. The long-standing stress/ glucocorticoid hypotheses of brain aging posit that stress exposure exacerbates aging symptoms, and extensive prior studies have shown that early life stress exposure does worsen phenotypic aging symptoms. However, despite its prevalence in aged humans, little basic research has investigated the response of aged subjects to new-onset psychosocial stress. Prior work in our lab showed aged rodents to be hyporesponsive to a new-onset acute psychosocial stress. Here, we assess the age-course of this acute response, as well as evaluate the consequences of chronic psychosocial stress exposure in aged animals. Our lab tested two hypotheses. First, we hypothesized that mid-aged animals will have an intermediate response between young and aged to acute psychosocial stress. Second, we hypothesized that aged animals' will continue to be hyporesponsive during a chronic psychosocial stress.

We focused on mid-aged animals for our first study because this age-point serves as the transition period from young to aged and could hold some key information about the transition from healthy to unhealthy brain aging. We used restraints to induce stress, the Morris water maze to test cognitive function, and telemetry devices to characterize sleep architecture and body temperature. We showed that, among age-related acute stress hyposensitive findings (deep sleep loss, hyperthermia, and cognitive deficit), mid-aged animals were hyporesponsive to sleep, but not body temperature or maze performance. This suggests that the failure to manifest a sleep response to stress precedes cognitive and body temperature related stress insensitivity.

In our second study, we investigated the influence of new-onset chronic psychosocial stress (three hours per day, four days per week for one month) in young and aged rodents. Aged animals were hyporesponsive to multiple common indicators of stress including distress during the restraint, weight loss, and cognitive deficits, all of which were easily detectable in young animals. These results suggest that the age-related blunting of the stress response is sustained from acute to chronic exposures. While the hyporesponsiveness may seem advantageous in the aged, a failed response could also be maladaptive, reducing a subject's ability to compensate for a changing environment. Together, this work supports prior observations that stress exposure makes young animals more aged like. Aged animals also showed a more limited response to stress, suggesting that age itself may act as an occluding stressor. Finally, this work points to deep sleep promoting interventions as potential therapeutic strategies for managing age-related changes in stress response.

KEYWORDS: Aging, Psychosocial Stress, Cognition, Sleep Architecture

Kendra Staggs
Student's signature

09/12/2016
Date

DEEP SLEEP, COGNITION, BODY WEIGHT, BODY TEMPERATURE, AND BEHAVIORAL DISTRESS RESPONSES TO NEW ONSET PSYCHOSOCIAL STRESSORS ARE BLUNTED WITH AGE IN MALE F344 RATS

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12/1/16

I dedicate this body of work to my son, Michael. You have brightened my entire world.

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Last, but certainly not least, thank you to my parents. Thank you for letting me grow and be my own person. Thank you for letting me carve out my own path in life. Most importantly, thank you for your support during graduate school. You

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Chapter 1 Thesis Overview and Significance

1.1 Aging

Aging is a chronic condition that involves multiple systems and impacts most species. Common ailments associated with normal aging include cognitive decline (Barnes, 1988; Barrientos et al., 2010; Klempin and Kempermann, 2007; Miller and O'Callaghan, 2005; West, 1993; Wimmer et al., 2012), altered sleep architecture (Bixler et al., 1984; Buechel et al., 2011; Ehlers and Kupfer, 1989; Espiritu, 2008; Foley et al., 1995; Hayashi and Endo, 1982; Kirov and Moyanova, 2002; Zepelin et al., 1972), and increased neuroinflammation (Godbout et al., 2005; Ownby, 2010). In humans, prevalence of neurodegenerative disorders, such as Alzheimer's disease (AD) is increased, leading to a poor quality of life and, often, eventually forcing them into assisted living facilities.

However, not everyone is susceptible to the negative consequences associated with aging and instead experience "successful aging." There are three factors that describe "successful aging": low probability of developing disease, high cognitive and physical function, and active engagement with society (Rowe and Kahn, 1997). It has been argued that a healthy diet (Fontana et al., 2010), exercise (Cotman and Berchtold, 2002; Hillman et al., 2008; Pedersen and Hoffman-Goetz, 2000; Penedo and Dahn, 2005), and cognitive stimulation (Nithianantharajah and Hannan, 2009; Whalley et al., 2004) contribute to successful aging.

1.2 Brain Aging

The brain plays a key role in regulating several functions in the body, such as cognition and memory, the physiological stress response, and sleep (McEwen, 2007; Walker and Stickgold, 2006). Because this organ is crucial to everyday function, it has been the center of a multitude of studies, especially relating to aging, trauma, and neurodegenerative diseases. Like other systems in the body, the brain is affected by aging and this can cause the aged human population to have a poorer quality of life including the need to move into an assisted living facility. Consequently, there have been many hypotheses that have explored the effects of aging on the brain, such as the calcium dysregulation, allostatic load, and glucocorticoid hypotheses.

1.2.1 Calcium Dysregulation Hypothesis

There are several theories to explain brain aging and in particular, unhealthy brain aging. One of the earlier lines of thinking proposed calcium dysregulation as a proponent for brain aging (Disterhoft et al., 1994; Gibson and Peterson, 1987; Khachaturian, 1989; Landfield and Pitler, 1984). One thing researchers observed was increased calcium influx in neurons via voltage-gated calcium channels (Moyer and Disterhoft, 1994; Pitler and Landfield, 1990; Thibault and Landfield, 1996), causing a surge in intracellular calcium concentrations and leading to prolonged afterhyperpolarizations (Landfield and Eldridge, 1994a; Landfield and Pitler, 1984) and increased long term depression (LTD) (Foster and Norris, 1997; Landfield, 1987b; Landfield and Pitler, 1984; Norris et al., 1998;

Norris et al., 1996). Later, it was noted that ryanodine receptors in the brain appeared to work in conjunction with voltage-gated calcium channels, and both contributed to aging-related calcium dysregulation (Thibault et al., 2007). Normally, ryanodine receptors activate calcium-induced calcium release from the endoplasmic reticulum via physical interaction with voltage-gated calcium channels (Blaustein, 1988; Giannini et al., 1995; Kostyuk and Verkhratsky, 1994). It appears that aging results in enhanced calcium-induced calcium release from ryanodine receptors and together with the voltage-gated calcium channels cause calcium dysregulation (Thibault et al., 2007).

In the hippocampus, long-term potentiation (LTP) and long-term depression (LTD) are involved in synaptic plasticity (Bliss and Collingridge, 1993). The concept of synaptic plasticity was first introduced by D. O. Hebb in 1949 when he hypothesized a mechanism for the formation of memories. In his hypothesis he states, “When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased (Viana Di Prisco, 1984).” LTP was first observed in the hippocampus (Bliss and Lomo, 1973) and was discovered to contribute to synaptic plasticity via the pairing of pre- and post-synaptic activity and increased synaptic excitability (Bear and Malenka, 1994; Martin et al., 2000; Thompson, 1986). Mechanistically, LTP occurs by when glutamate is released from

presynaptic terminals and to N-methyl-D-aspartate receptors (NMDA-R), causing depolarization of the postsynaptic membrane and the consequent influx of calcium via the postsynaptic membrane (Bear, 1996). Complementing LTP is LTD, which has been implicated in the storage of information via NMDA-R activation and an increase in intracellular calcium concentration (Bear, 1996).

Both of these are also altered in aging and neurodegenerative diseases. It has been shown that there is an increase in LTP decay and increased LTD induction in the aged hippocampus (Foster and Norris, 1997; Norris et al., 1996). Among other things, calcium influx is required for LTD induction (Mulkey and Malenka, 1992), but too much can lead to excitotoxicity. Specifically, the increased NMDA-R activation from prolonged glutamate exposure and consequently, increased concentration of intracellular calcium from NMDA-Rs and voltage-gated calcium channels can result in neuronal death (Norris et al., 2006).

The rise in calcium seen in aging could be explained by an increase in the density of the voltage-gated calcium channels (Thibault and Landfield, 1996). These channels have been shown to be positively correlated with deficits in cognition in aged animals (Thibault and Landfield, 1996). Also, the introduction of an antagonist specific to these channels improved cognition in impaired aged animals (Deyo et al., 1989; Disterhoft et al., 2004; Wu et al., 2002), supporting their role in age-related cognitive changes.

1.2.2 Allostatic Load/ Glucocorticoid Hypothesis

Two other closely related hypotheses are the allostatic load and glucocorticoid hypotheses. These describe the accumulation of repeated activation of the hypothalamic-pituitary-adrenal (HPA) axis as a result of the body trying to maintain allostasis (McEwen, 1998b; McEwen, 2001; McEwen and Stellar, 1993; Sapolsky et al., 1986b) in a stressful environment. Acute stress can transiently elevate glucocorticoids (Kirschbaum et al., 1996; McEwen and Sapolsky, 1995), but long-lasting problems, such as obesity (Epel et al., 2001), cardiovascular disease (Bjorntorp, 1990; Brindley and Rolland, 1989; Lupien et al., 1998; Sapolsky et al., 1986b; Troxler et al., 1977), and type 2 diabetes (Bjorntorp, 1990; Brindley and Rolland, 1989) are associated with chronic and/ or repeated elevation of glucocorticoids. Eventually, hippocampal damage (Lupien et al., 1998; McEwen et al., 1999; Sapolsky et al., 1990), cognitive impairment (Dellu et al., 1994; Martignoni et al., 1992), and brain aging (Landfield, 1987c; Landfield et al., 1978; Sapolsky et al., 1985) can accelerate brain aging from sustained glucocorticoid levels.

The glucocorticoid hypothesis of brain aging mechanistically describes the allostatic load hypothesis and posits that chronic exposure to glucocorticoids promotes brain aging (Landfield, 1978; Porter and Landfield, 1998). This was developed based on early studies and observations of glucocorticoid actions in peripheral tissues that mimicked aging (Finch, 1972; Wexler and McMurtry,

1983). It was also shown that glucocorticoid receptors are prominently expressed in the hippocampus (de Kloet et al., 1990; Joels and de Kloet, 1992; McEwen et al., 1968) and participate in the regulation of the stress response (Sapolsky et al., 1986a; Tsigos and Chrousos, 2002; Wolf, 2003). Over time, the hypothesis was modified: aging could increase or decrease glucocorticoid efficacy depending on the cell type (Landfield et al., 2007). For my studies, I suspected that aging decreased glucocorticoid efficacy because the aged animals in a prior acute stress study (Buechel et al., 2014) were hyporesponsive to acute stress. This blunted response suggested, to me, that the aged animals had difficulty utilizing glucocorticoids to mount a response similar to a classic stress response (Section 1.5) that is more commonly seen when faced with an acute stressor.

1.3 Rodent Cognition Model

There are several models used to test cognition, including delayed match to sample, active avoidance, and the Morris water maze. In a delayed match to sample task, the subject (animal or human) is trained to either press a lever or match pictures that were previously displayed. Correct responses are rewarded and incorrect responses result in a “time out” and no reward (Dunnett, 1985; Schon et al., 2004; Tagamets and Horwitz, 1998). Typically, the percentage of successful trials declines to chance as the time delay between first presentation and second match presentation increase. Correctly responding after longer delays is associated with improved hippocampal function. This has been shown with fMRI imaging and implicates the parahippocampal gyrus in participating in

the maintenance of the memory trace during the delay phase. The activity of this region during the delay phase positively correlates with long-term memory. (Schon et al., 2004).

Active avoidance involves placing the animal in a brightly lit “holding” chamber. At time “0” a door opens to a darker room in the chamber and when the animals escape to that room, they receive a foot shock. To test cognition, the animals repeat the task the next day, however there is no foot shock. Shorter latencies to escape the darker room (avoidance) are considered form of memory (Freeman and Young, 2000; Li et al., 2004; Nabeshima et al., 1990; Zarrindast et al., 2002).

The Morris water maze involves training the animal to find a submerged platform by using external cues (Morris, 1984) and has been extensively used in aging studies (Carter et al., 2009; Frick et al., 2003; Latimer et al., 2014; Ma et al., 2014; van Praag et al., 2005; Yau et al., 2002; Zyzak et al., 1995). This task allows for manipulations, such as drugs (Forcelli et al., 2012; Ishida et al., 2007; Niyuhire et al., 2007; Pedraza et al., 2009; Yamazaki et al., 1995), stress (Buechel et al., 2014; Markham et al., 2010; McKim et al., 2016; Woodson et al., 2003), exercise (Ben et al., 2010; Fordyce and Wehner, 1993; Lee et al., 2012; van Praag et al., 2005), etc. Using distal cues to spatially map the location of the hidden platform is considered a hippocampus-dependent task. While other cognitive assessments such as delayed match to sample and active avoidance

are valid and used quite often, we chose a method that was consistent with previous studies in our lab. Also, a task similar to delayed match to sample requires a long period of time to train the animals as well as food deprivation to motivate the animals' performance. Food deprivation can be considered as an additional stressor and we therefore chose the water maze. For our purposes, we use a locally cued task to assess the ability of the animals to learn and swim as well as a spatially cued task to assess the effects of stress on young, mid-aged, and aged animals' cognition.

1.4 Role of the Hippocampus

The hippocampus is part of the limbic system and is described, in humans, as a curved structure resembling a seahorse (Andersen, 2007; O'keefe and Nadel, 1978). In addition to the hippocampus, the limbic system includes the amygdala, hypothalamus, septal nuclei, epithalamus, and anterior thalamic nuclei (Mega et al., 1997). The limbic system is involved in processing emotion (Mega et al., 1997) and memory (O'keefe and Nadel, 1978).

One of the roles of the hippocampus is the consolidation of memory, in particular, short-term and spatial memory (Bird and Burgess, 2008). The hippocampus has been widely researched, but one of the more famous studies involved a patient referred to as "Patient H.M." He was thoroughly studied after he underwent a bilateral medial temporal-lobe resection in an attempt to cure epilepsy (Scoville and Milner, 1957). As a result, he suffered from severe memory defects and was

unable to form new, episodic memory and could not remember anything immediately prior to the surgery, despite maintaining the ability to recall childhood memories. Around the same time period, other patients diagnosed with psychiatric disorders unable to be ameliorated with conservative treatments had similar operations with comparable memory defects varying in the degree of severity, depending on the length of the resection. These were compared to other cases where the temporal neocortex was unilaterally lesioned to treat temporal epilepsy and resulted in no memory defects (Milner, 1954). This served as early evidence for the role the hippocampus played in memory consolidation (Scoville and Milner, 1957)**.** Together with extensive aging studies later on in human and animal models, the hippocampus was discovered to not only be important in memory, but was also a target for age-related memory deficits, as previously discussed. Furthermore, it also appeared to play a central role in regulating hypothalamic-pituitary-adrenal axis activity during a physiological stress response.

1.5 Stress and Hypothalamic-Pituitary-Adrenal Axis

The idea of stress dates back to the era of the great philosophers, such as Hippocrates, who expanded on Empedocles' theory (Chrousos et al., 2013) of all matter existing in a state of harmonious balance (now termed "homeostasis"). Hippocrates described the harmonious balance as a state of health, while disharmony was considered as a state of disease (Chrousos et al., 2013). Walter Bradford Cannon was the first person to describe the term homeostasis and coin

the phrase “fight or flight” which described an animal’s ability to prepare to fight or flee because of the activation of the sympathetic nervous system in response to an exogenous stressor (Fink, 2009).

Hans Selye was the first person to provide a clear definition of stress and developed the term “heterostasis” to describe how the body adapts to stress; this is thought to be an early idea of the concept of allostasis (Fink, 2009; Selye, 2013). Selye brought stress to the forefront of research and decades later, we have a clearer idea of the underlying mechanisms involved in a stress response. A couple of things occur when a stressor is introduced: the first response is the activation of the autonomic nervous system to release epinephrine and norepinephrine within seconds (Sapolsky et al., 2000; Wolf, 2003). The second response is slower and involves the production of glucocorticoids (Wolf, 2003). Both responses are driven by the hypothalamic-pituitary-adrenal (HPA) axis, however glucocorticoid action is the focus for our lab because a sustained increase in glucocorticoids in the hippocampus have been implicated in aging-related cognitive deficits. When this axis is activated (Fig. 1), corticotrophin releasing factor (CRH) is released from the paraventricular nucleus in the hypothalamus and binds to CRH receptors in the pituitary gland. From there, ACTH is released and travels to the adrenal gland, binds, and releases glucocorticoids (Sapolsky et al., 1986a; Tsigos and Chrousos, 2002; Wolf, 2003) in systemic circulation. Glucocorticoids are lipophilic and therefore have the

Figure 1. Stress Response

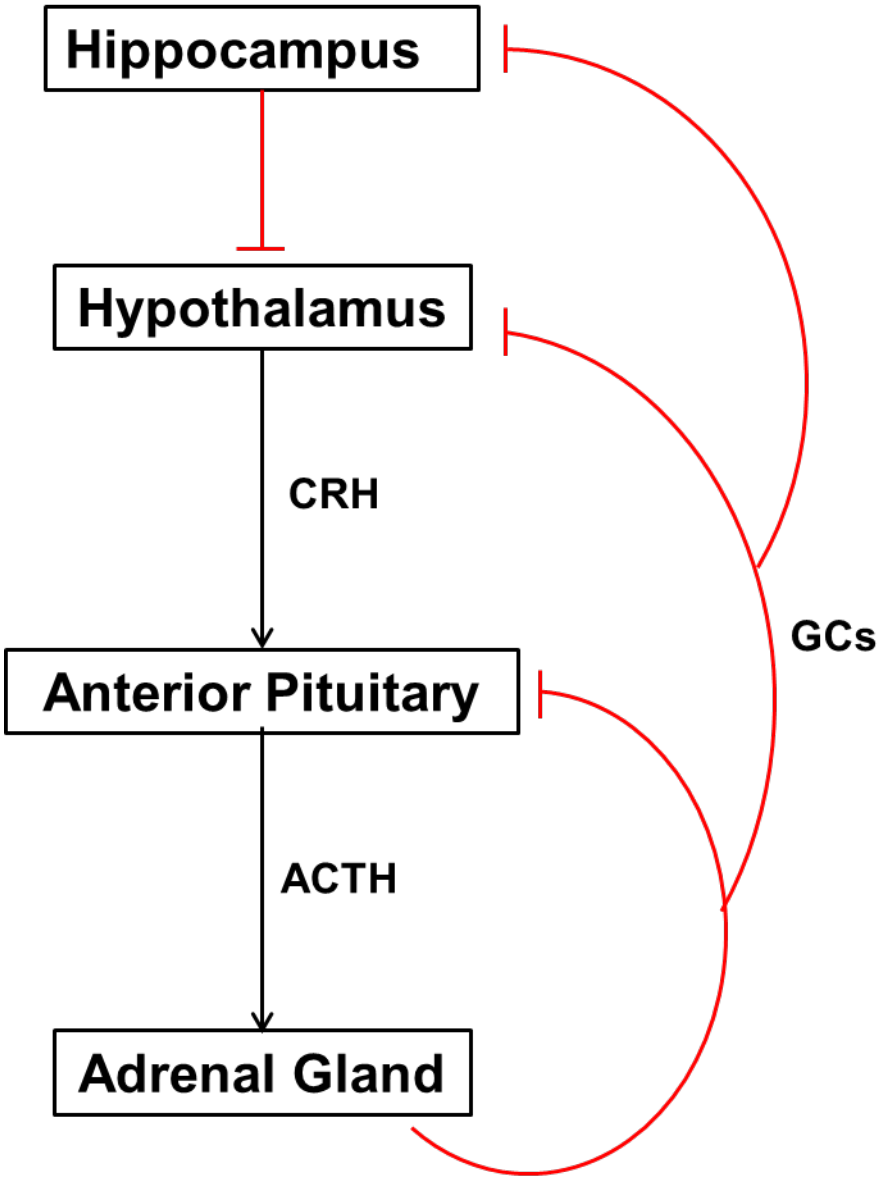

Figure 1. Stress response When a stressor is encountered, the hypothalamus releases corticotropin releasing factor where it binds in the anterior pituitary to release ACTH. The ACTH travels to the adrenal cortex, which releases glucocorticoids into the circulating bloodstream. Some of those glucocorticoids travel back to the hippocampus where they bind to mineralocorticoid and glucocorticoid receptors to trigger a negative feedback loop to shut down the stress response.

ability to cross the blood-brain-barrier in order to bind to receptors in the brain, predominantly in the hippocampus (Joels and de Kloet, 1992; McEwen, 1999; McEwen et al., 1992; Wolf, 2003). There are two receptors that bind glucocorticoids: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). These are nuclear receptors with a 94% sequence homology in the DNA binding domain (de Kloet et al., 1990). MRs have a 6- 10 fold greater binding affinity for glucocorticoids compared to GRs and typically bind glucocorticoids at basal levels (de Kloet et al., 1990; De Kloet et al., 1998; Reul and de Kloet, 1985; Reul et al., 1987; Srivareerat et al., 2009). During a response to a stressful stimulus, the elevation of glucocorticoids will result in the binding to the lower affinity glucocorticoid receptors (Windle et al., 1998) once the mineralocorticoid receptors have been occupied. One of the roles of glucocorticoids is to convert fatty acids into energy (Sapolsky et al., 2000) and prime the body to either fight the cause of the stress or retreat. In a normal stress response, glucocorticoids released from the adrenal cortex travel and bind to glucocorticoid receptors in the hippocampus to trigger negative feedback, shutting down the HPA axis (Wolf, 2003), hence signaling the resolution of the stress.

1.6 Psychosocial Stress

Because stress is such a large field of study and almost any stimulus can be considered a stressor, it is helpful to narrow the scope and define the specific type of stress our lab studies. Our lab studies psychosocial stress or stress caused by a non-noxious stimulus (Fink, 2009). In humans, examples of this

would include divorce, loss of a loved one, or social isolation. The onset of psychosocial stress can occur at any point in an individual's life, but often occurs later in life, and more often presents as a chronic stressor. Failure of the physiological response to resolve the stress (McEwen, 1998a) can also cause a normally acute stressor to become chronic and can have significant negative effects on the body. Combined with the existing consequences of aging, it is reasonable to deduce chronic stress to have the potential to lead to worsened health outcomes in the aging population.

The literature shows acute psychosocial stress can be both beneficial (McGaugh and Roozendaal, 2002; Roozendaal and McGaugh, 1996; Sandi and Rose, 1997) and detrimental (de Quervain et al., 1998; de Quervain et al., 2000; Diamond et al., 1999; Kirschbaum et al., 1996; Newcomer et al., 1994; Newcomer et al., 1999) to young subjects' cognition, depending on the time the stress occurred in reference to learning or recall. Some studies show stress can facilitate learning if the stress is encountered immediately following the training (Wolf, 2003). On the other hand, other studies have shown worsened spatial memory when the stress occurred immediately prior to a memory recall test (Buechel et al., 2014; de Quervain et al., 1998; de Quervain et al., 2000).

While HPA axis activation during acute stress can be beneficial and is necessary for survival (Tsigos and Chrousos, 2002), activation from chronic stress can be

harmful to an individual. The increased HPA response associated with chronic stress is a risk factor for diabetes (Brindley and Rolland, 1989), increased adiposity (Tsigos and Chrousos, 2002), and impaired cognition (Wolf, 2003). In the brain, chronic stress has also been shown to decrease neurogenesis (Gould et al., 1997; Pham et al., 2003) and hippocampal volume (McEwen, 2000b) as well as to decrease the survival of newly proliferated neurons (Heine et al., 2004; Malberg and Duman, 2003; Vollmayr et al., 2003).

1.7 Rodent Stress Model

Humans and nonhuman primates have a highly developed prefrontal cortex, giving them the ability to "learn across time," or anticipate particular outcomes based on previous experiences (Asaad et al., 1998). Evolutionarily, this can be advantageous because humans and nonhuman primates can quickly adapt new strategies to handle stressors based on previous outcomes with the same or similar stress (Modirrousta and Fellows, 2008). While rodents have been shown to have a prefrontal cortex, it is not as evolved (Ongur and Price, 2000; Uylings et al., 2003) and researchers must find alternative means to simulate a chronic psychosocial stressor. There are several methods to stress rodents, but for our purposes, we require a stressor that would be considered a non-noxious stressor. Our lab chose restraint stress, a widely used method of psychosocial stress (Buynitsky and Mostofsky, 2009; Luine et al., 1994; McEwen, 1999). Although there are other ways to model psychosocial stress, such as strobe light (Kapoor et al., 2009; Leonhardt et al., 2007) or water avoidance (Bradesi et al.,

2005; Santos et al., 2000), we have previously used restraint stress (Buechel et al., 2014) and chose to be consistent with those prior studies.

1.8 Sleep

1.8.1 Definition and Sleep Architecture

Sleep can broadly be defined as a decrease in the response to stimuli and voluntary motor activity (Fuller et al., 2006). It also has several different roles including memory processing (Kushida, 2012; Marshall and Born, 2007; Sejnowski and Destexhe, 2000; Tononi and Cirelli, 2006, 2012; Walker, 2009), energy conservation (Berger and Phillips, 1995; Walker and Berger, 1980), metabolism (Leproult and Van Cauter, 2010; Spiegel et al., 1999) and physical restoration (Adam and Oswald, 1977; Kushida, 2012). In humans, there are two broad categories of sleep: non-rapid eye movement (NREM) and rapid eye movement (REM). NREM can be further separated into light sleep (NREM stages 1-2) and deep sleep (NREM stages 3-4). During a typical night, a person will oscillate between light, deep, and REM sleep with the duration of deep sleep decreasing as the night progresses while the inverse is true of REM (Kryger et al., 2004; Kushida, 2012; Tononi and Cirelli, 2003).

Polysomnographic recordings of sleep indicate light sleep, in humans, consists of the transition of beta and gamma waves to the lower frequency theta waves as well as decreased muscle tone and slow eye movement (Fuller et al., 2006; Mendelson, 2012; Stickgold and Walker, 2010). In NREM 1, a person can be

easily awakened (Kushida, 2012). NREM 2 is characterized by the appearance of sleep spindles and K-complexes (Fuller et al., 2006; Kushida, 2012) and the loss of awareness (Fuller et al., 2006). It is thought that sleep spindles (sudden increase in wave frequency) and K complexes (sudden increase in wave amplitude) play a role in information processing and memory consolidation via thalamic and cortical neuron interaction (Fogel and Smith, 2011).

Deep sleep is dominated by delta waves (Kushida, 2012; Stickgold and Walker, 2010) and a person is not easily aroused from this stage (Kushida, 2012). During this stage, muscle activity, eye movement, respiratory and heart rates, and blood pressure are all decreased (Kushida, 2012; Stickgold and Walker, 2010). The appearance of spindles and K complexes are significantly less in this stage compared to NREM 2 (De Gennaro and Ferrara, 2003; Fogel and Smith, 2011).

REM is characterized by rapid eye movement, muscle atonia, and is enriched in theta waves, often described as ‘desynchronized’ sleep (Fuller et al., 2006; Kushida, 2012; Stickgold and Walker, 2010). In humans, REM oscillates every 90-110 minutes and is prominent in the last one-third of sleep (Kushida, 2012). Memory consolidation does occur during REM, especially for procedural memory (Marshall and Born, 2007; Siegel, 2001).

1.8.2 Deep Sleep and Significance

On an EEG recording, deep sleep can be detected by the prevalence of delta waves (Kushida, 2012; Stickgold and Walker, 2010) and is thought to occur when there are synchronized oscillations between the thalamus and the neocortex (Steriade, 2003b). Deep sleep plays an important role in macromolecular biosynthesis (Mackiewicz et al., 2007; Mackiewicz et al., 2009), the release of growth hormones (Steiger, 2006; Suchecki and Tufik, 2006), and learning and memory consolidation (Marshall et al., 2006; Marshall and Born, 2007; Stickgold, 2005; Tononi and Cirelli, 2006). It has also been shown that people with a reduced amount of deep sleep have increased insulin resistance and elevated risk of type 2 diabetes (Kawakami et al., 2004; Tasali et al., 2008).

In regards to learning and memory, there are three main processes in the formation of a memory: encoding, consolidation, and retrieving. During the encoding phase, information is taken in from the animals' interaction with their environment during the wakeful periods (Tononi and Cirelli, 2003), creating a memory trace. Mechanistically, learning has been shown to increase gene expression associated with LTP, resulting in the strengthening of synaptic neurons and hence, the synaptic weight (Knott et al., 2002; Silva, 2003; Sjostrom et al., 2001; Tononi and Cirelli, 2003). Lending more credence to this idea is the evidence that LTP-related gene expression is decreased during sleep (Cirelli and Tononi, 2000b; Tononi and Cirelli, 2003). It is thought that this increase in

synaptic weight increases the propensity for sleep and is in line with an established fact that deep sleep duration is proportional to the duration of wake from the previous day (Borbely, 2001; Tononi and Cirelli, 2003).

Consolidation of a memory trace involves the stabilization and storage of the trace. Deep sleep, or slow wave activity, is described as a slow oscillation consisting of a de-polarized up-phase where the neurons are rapidly firing and a hyperpolarized down-phase where the neurons are quiescent (Steriade, 2003a). This stage of sleep is thought to relieve the synaptic weight of all synapses by transferring the information to the neocortex for processing and storage (Mander et al., 2011; Maquet et al., 2003; Tononi and Cirelli, 2003; Van Der Werf et al., 2009) and there is a lot of evidence to support this idea. Comparative studies of the upregulation/downregulation of genes during wakefulness and deep sleep show a selective downregulation of LTP-related genes during NREM as well as a selective upregulation in molecules involved in depotentiation during the same period (Cirelli et al., 2004; Cirelli and Tononi, 2000a).

Finally, retrieval is the act of recalling the stored information (Rasch and Born, 2013). Researchers have shown that the hippocampus is involved in retrieval, though the signaling may be different than in acquisition and consolidation. For the latter and former, the NMDA receptor, PKA, and PKC have played role (Abel and Lattal, 2001; Goosens et al., 2000; Steele and Morris, 1999). Unfortunately,

the exact mechanism is still being investigated, however, it has been demonstrated that glucocorticoid exposure can impair retrieval depending on the timing of exposure in relation to recall (Buechel et al., 2014; Roozendaal, 2002).

1.8.3 Sleep with Age

Just like several other systems in the body, age influences sleep. With increasing age, the amount of total sleep time (Siegel, 2003; Weitzman et al., 1982), REM (Ehlers and Kupfer, 1989; Weitzman et al., 1982), and deep sleep (Foley et al., 1995; Siegel, 2003; Wolkove et al., 2007) decrease (Fig. 2). In exchange, there is an increase in wake and light sleep duration, as well as a circadian advance (Bliwise, 1993; Espiritu, 2008; Siegel, 2003). Other common sleep complaints include sleep fragmentation and increased daytime napping (Bliwise, 1993; Espiritu, 2008; Foley et al., 1995; Wolkove et al., 2007).

1.8.4 Sleep and Neurodegenerative Diseases

While augmented sleep architecture is a normal observation in elderly individuals, it is also common in neurodegenerative disorders, such as Alzheimer's disease (AD), which frequently appear in older populations (Corder et al., 1993). Pathophysiologically, AD typically presents with Aβ plaques and neurofibrillary tangles (Dubois et al., 2010; Glenner and Wong, 1984; Hardy and Higgins, 1992; Katzman and Saitoh, 1991). Clinical symptoms of AD include memory and executive function impairments as well as apraxia and

Figure 2. Sleep Architecture of Young and Aged Human

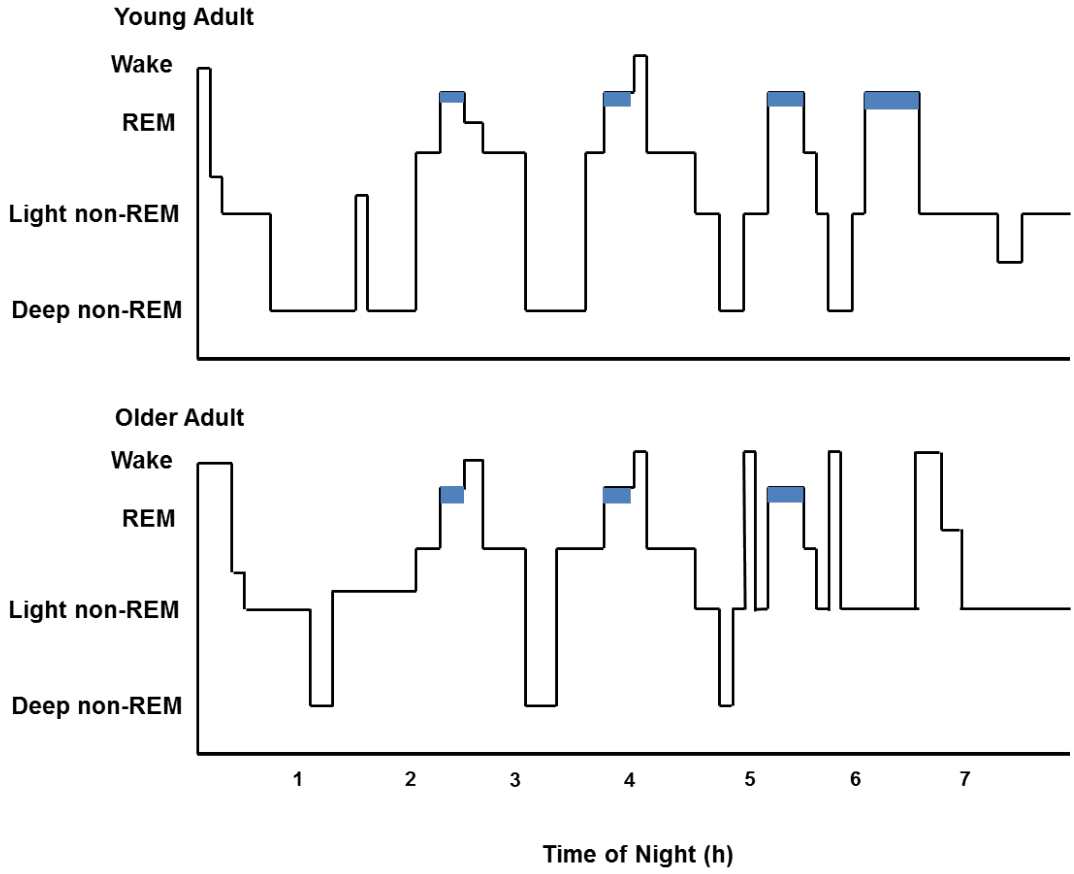

Figure 2. Sleep architecture of young and aged human. Top: Stages of sleep of young adult. Deep sleep dominates the first third of the night, while REM is increased during the last third of sleep. Bottom: Stages of sleep of elderly adult. The amount of deep sleep and REM is decreased and sleep fragmentation is present.

neuropsychiatric problems (irritability, apathy, etc) (Cummings, 2004; Dubois et al., 2010; Price et al., 1993).

Interestingly, sleep disturbances are associated with both of these diseases, as well as other neurodegenerative diseases. Most of the disturbances (circadian advance, reduced total sleep time, sleep fragmentation) seen in normal aging also present in these diseases (Bombois et al., 2010; Culebras, 2007), but it appears the disease state plays a role in worsening sleep. For example, sleep-wake disturbances are common in AD (Lavie et al., 2002; Malkani and Attarian, 2015; Zhong et al., 2011). Melatonin has been documented to be an important regulator of circadian rhythms and the sleep-wake cycle (Arendt and Skene, 2005; Srinivasan et al., 2005; Srinivasan et al., 2006). However, melatonin levels decline in normal aging (Bombois et al., 2010), but further decline with AD (Malkani and Attarian, 2015; Pandi-Perumal et al., 2005). This decrease can worsen the sleep disturbances and ultimately cognition in AD patients (Pandi-Perumal et al., 2005).

1.8.5 Rodent Sleep Model

To examine sleep in our studies, we use F344 rats that are surgically implanted with wireless telemetry devices (DSI International). These devices detect and record EEG and EMG signals that aide us in the determination of the stage of sleep the rats were in during the recording period. With this information, we can analyze the impact stress has on the duration of each stage of sleep compared

to a baseline recording taken at the very end of the recovery period immediately prior to the commencement of the restraint protocol, as well as to control animals.

Neuroscore software enables us to further analyze deep sleep power (quality) using a fast fourier transform that graphically displays the magnitude of frequencies within the EEG signal, hence allowing us to measure the change in magnitude stress has on the quality of deep sleep in young, mid-aged, and aged rodents.

1.9 Thesis Significance

Aging and stress have both been extensively studied as two separate entities and the negative consequences are well-documented. Cognitive decline, sleep architecture, and increased neuroinflammation have all been associated with aging. On the other hand, repeated or chronic exposure to stress can cause impaired cognition, decreased neurogenesis and eventually lead to obesity, metabolic syndrome, cardiovascular disease, and increased risk of infection or other long-term maladies. Increased prevalence of chronic psychosocial stress is also associated with aging, yet the influence of these stressors on an aged system has not been thoroughly investigated. This work was designed to tackle this very issue. The ability for the aging population to resolve, or at least cope with, these stressors could restore or preserve cognition and therefore delay the transition to assisted living facilities.

Secondly, this work studies the impact psychosocial stress has on deep sleep. Deep sleep plays a crucial role in learning and memory, but it is also thought to be a mechanism which helps restore the body to homeostasis after a stress period. Because it is nearly impossible to control the existence of exogenous stressors, this restoration likely is important in preventing permanent damage.

To simulate stress, we restrained F344 male rats and documented signs of distress to create a distress index. Performance in the Morris water maze was used to assess the impact stress had on cognition. Wireless telemetry devices provided insights on body temperature and sleep architecture. Finally, corticosterone was analyzed to biochemically evaluate stress.

This work was designed to bring together two naturally occurring and highly comorbid phenomena to hopefully better understand how they influence each other. Both are unavoidable and have the potential to have negative consequences. By understanding stress's influence on aging and vice versa, we can hopefully improve the health and cognitive abilities of the aging population, as well as improve quality of life.

Chapter 2 Acute psychosocial stress in mid-aged male rats: restraint causes cognitive decline and hyperthermia, but does not alter sleep architecture (in review at Neurobiology of Aging)

Authors: Kendra Hargis, Heather Buechel, Jelena Popovic, Eric Blalock

Summary

Aging is associated with altered sleep architecture and worsened hippocampus-dependent cognition, highly prevalent clinical conditions detract from quality of life for the elderly. Interestingly, exposure to psychosocial stress causes similar responses in young subjects, suggesting that age itself may act as a stressor. In prior work we demonstrated that young animals show loss of deep sleep, deficits in cognition, and elevated body temperature after acute stress exposure, while aged animals are hyporesponsive on these three measures. However, it is unclear if these age-altered stress responses occur in parallel over the course of aging. To address this, here we repeated the experiment in mid-aged animals. We hypothesized that mid-aged stress responses would be intermediate between those of young and aged subjects. Sixteen mid-aged (12 mo) male F344 rats were implanted with EEG/EMG emitters to monitor sleep architecture and body temperature, and were trained on the Morris water maze for three days. On the fourth day, half of the subjects were restrained for three hours immediately prior to the water maze probe trial. Sleep architecture and body temperature were measured during the ensuing inactive period, and on the following day, end point measures were taken. Restrained mid-aged animals showed an aging-like resistance to deep sleep loss, but demonstrated young-like

stress-induced water maze probe trial performance deficits as well as post-restraint hyperthermia. These data suggest that age-related loss of sleep-architecture stress sensitivity may precede both cognitive and body temperature related stress insensitivity.

2.1 Introduction

There are several consequences of normal aging including cognitive decline (Barnes, 1988; Driscoll et al., 2006; Gallagher and Pelleymounter, 1988; Klempin and Kempermann, 2007; Wimmer et al., 2012), reduced deep sleep (Buechel et al., 2011; Espiritu, 2008; Kirov and Moyanova, 2002; Zepelin et al., 1972) altered circadian rhythm (Dijk et al., 2000; Monk, 2005; Pace-Schott and Spencer, 2011), and increased neuroinflammation (Gemma and Bickford, 2007; Nikodemova et al., 2007). Additionally, hypothalamic-pituitary-adrenal (HPA) activity has been documented to be increased with aging (Paul et al., 2015). Loss of deep sleep and deficits in hippocampal function are also observed with stress exposure (Prenderville et al., 2015). The allostatic load hypothesis of aging (McEwen and Stellar, 1993) posits that stress exposure has a cumulative and exacerbating influence on age-related processes. Several studies have shown that stress in young rodents and humans has long-lasting consequences throughout the lifespan (Lupien et al., 2009). Further, the chances of experiencing a new onset stressor, particularly a psychosocial (non-painful stressor such as losing a job, death of a spouse, or becoming socially isolated, increases with age (House et al., 1994) and the negative consequences of a stress exposure can be more

severe in the aged population (Azuma et al., 2015; Machado et al., 2014; Prenderville et al., 2015; Stein-Behrens and Sapolsky, 1992). However, the lack of basic research on the age-related responses to new onset stressors represents an important area of investigation and has recently been referred to as part of the ‘stress-aging gap’ (Epel and Lithgow, 2014).

To address this, in prior work (Buechel et al., 2014), we used restraint to model of acute psychosocial stress in young and aged male F344 rats. This manipulation induces sleep loss, cognitive deficit, and body temperature elevation in young animals. However, the same treatment failed to elicit these canonical responses in aged animals, despite the aged animals showing clear signs of distress during the restraint. Whether these sleep, cognitive, and body temperature changes in stress response occur in parallel across aging is not known, and mid-age could represent a crucial transition period in the aging stress-response phenomenon.

In this study, mid-aged male (12 mo) F344 rats were implanted with EEG/EMG telemetry devices to measure sleep architecture and body temperature. Effects on cognition were evaluated with the Morris water maze. Three hour restraint was used to model psychosocial stress in half of the animals. Middle-aged animals showed post-stress cognitive deficits and hyperthermia, but did not show stress-associated deep sleep loss. These results indicate that age-related stress

hypo-responsiveness begins to develop earlier for sleep response than for cognitive and body temperature responses, suggesting sleep alteration may be a critical upstream target for therapeutic manipulation in protecting against age-related cognitive decline.

2.2 Materials and Methods

2.2.1 Subjects

Sixteen middle-aged (12 mos) male Fischer 344 rats were obtained from the NIA aging colony. Animals were individually housed with enviro-dry bedding, rat tunnel and Nyla bone. They had access to food and water *ad libitum* and were acclimated to a 12 hour reverse light/dark cycle (4:30 AM lights off, 4:30 PM lights on). Two additional animals were excluded due to surgical complications. Rats were randomly assigned to control and stress groups. 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).

2.2.2 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; Buechel et al., 2014). 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 21 gauge 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.

2.2.3 Sleep Data Acquisition and Analysis

Sleep data was acquired according to established protocols in prior work (Buechel et al., 2014). Animals were housed individually and cages were positioned at least 18” apart to avoid interference during radiotelemetry data acquisition. EEG, EMG, and temperature data were recorded continuously with DSI's Data Art acquisition software and binned in 10 s epochs. For these nocturnal rodents, the first 4 h of their active period (dark) and the first 4 h 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. Architecture was scored using Neuroscore's (v. 2.1.0 Data Sciences International) analysis console in 30 s increments while being viewed in 2–5 min

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 based on EEG/EMG signaling 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.

2.2.4 Water Maze

The water maze task was performed as in previous studies (Buechel et al., 2011; Buechel et al., 2014). 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 × 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 (v. 4.64 Columbus Instrument) was used for analyses. All training and probe sessions took place between 12PM and 4PM (during the rats' active period).

2.2.4.1 Visual Cue Training (pre-surgery)

The visual cue task took place over 3 days. A Styrofoam cup served as an additional cue and was hung by a black thread 12 inches above the submerged platform. Rats were given 3-60s trials and 60s on the platform with 2 minute inter-trial intervals. On the third day, all rats could reach the platform in under 30s for 2/3 trials (criterion for this study). The spatial cues for the spatially cued task were already present during this task.

2.2.4.2 Spatial Cue Training and Probe (post-surgery)

After rats recovered from surgery (2 weeks), they performed a spatially cued task as in prior work (Buechel et al., 2014). Briefly, spatial cues were provided and the Styrofoam cup used in the visual cue was removed. As in the visual cue task, rats had 3-60s trials/day for three days with one minute on the submerged platform and 2 minute inter-trial intervals. On the fourth day, the platform was removed and rats performed one 60s probe trial. Path length, latency, platform crossings and path length in goal quadrant were recorded. Placement of the platform remained unchanged through the duration of the study; however, the starting quadrant changed for each trial. Animals started in the quadrant opposite the quadrant containing the platform for the probe trial.

2.2.5 Restraint Stress

Stressed group rats were restrained in their home cages using Rat Snuggles (Harvard Apparatus) for 3 h immediately preceding the probe trial. They were continuously monitored for the duration of the restraint and their vocalizations and struggles were noted and analyzed to construct an index of apparent distress (the number of times each rat squawked or struggled to get out of the restraint was tallied for each hour of the stress period). Control animals remained in the housing facility during the stress period and joined the stressed group at the beginning of the probe trial. Animals were returned to the housing facility at the conclusion of the probe trial for sleep data collection during the ensuing 12 hour inactive period.

2.2.6 Blood and Tissue Collection

On the following morning, animals were anesthetized with isoflurane and decapitated. Trunk blood was collected in K_2EDTA vacutainers (BD biosciences) and immediately centrifuged at 1200 g for 10 min. The serum was collected for corticosterone and adrenocorticotropic hormone analysis (AniLytics Inc., Gaithersburg, MD). Brain tissue was rapidly removed as in prior work (Buechel et al., 2011; Buechel et al., 2014). Hippocampal tissue from one hemisphere was snap frozen while the other hemisphere was post-fixed in 4% formalin, cryoprotected in a 15% sucrose mixture and stored at −80°C for future use.

2.3 Results

Statistical tests and results are detailed in figure captions.

2.3.1 Water Maze

The visual cue task (Fig. 3 A1 inset) was used to determine if rats could perform non-spatial aspects of the water maze (all subjects performed to criterion- see Methods). Rats showed a significant improvement over training days. After this task, the animals underwent surgery and two weeks of recovery. Rats were then trained on the spatially cued Morris water maze task for three days and showed significantly shorter latencies and path lengths with training (Fig. 3 A1 and B1). On the fourth day, half of the animals (the 'Stress' group) were restrained and 32.1 ± 5.0 vocalizations/ struggles were noted over the 3 hour restraint period. Immediately following restraint, all animals were presented with the water maze probe trial. For the probe trial, the submerged platform was removed and the animals were placed in the tank and allowed to swim for 60s. During the probe trial, software tracked time and distance within four platform-sized and symmetrically spaced rings, with ring 3 (R3) positioned over the original trained platform location. The total amount of time (Fig. 3 A2) and distance (Fig. 3 B2) the animals spent in each of the rings was measured, and greater time/ distance in R3 interpreted as remembering the platform location. Control animals showed the expected preference for R3, while Stress animals spent significantly less time and distance in R3.

Figure 3. Water Maze Before and After Acute Stress

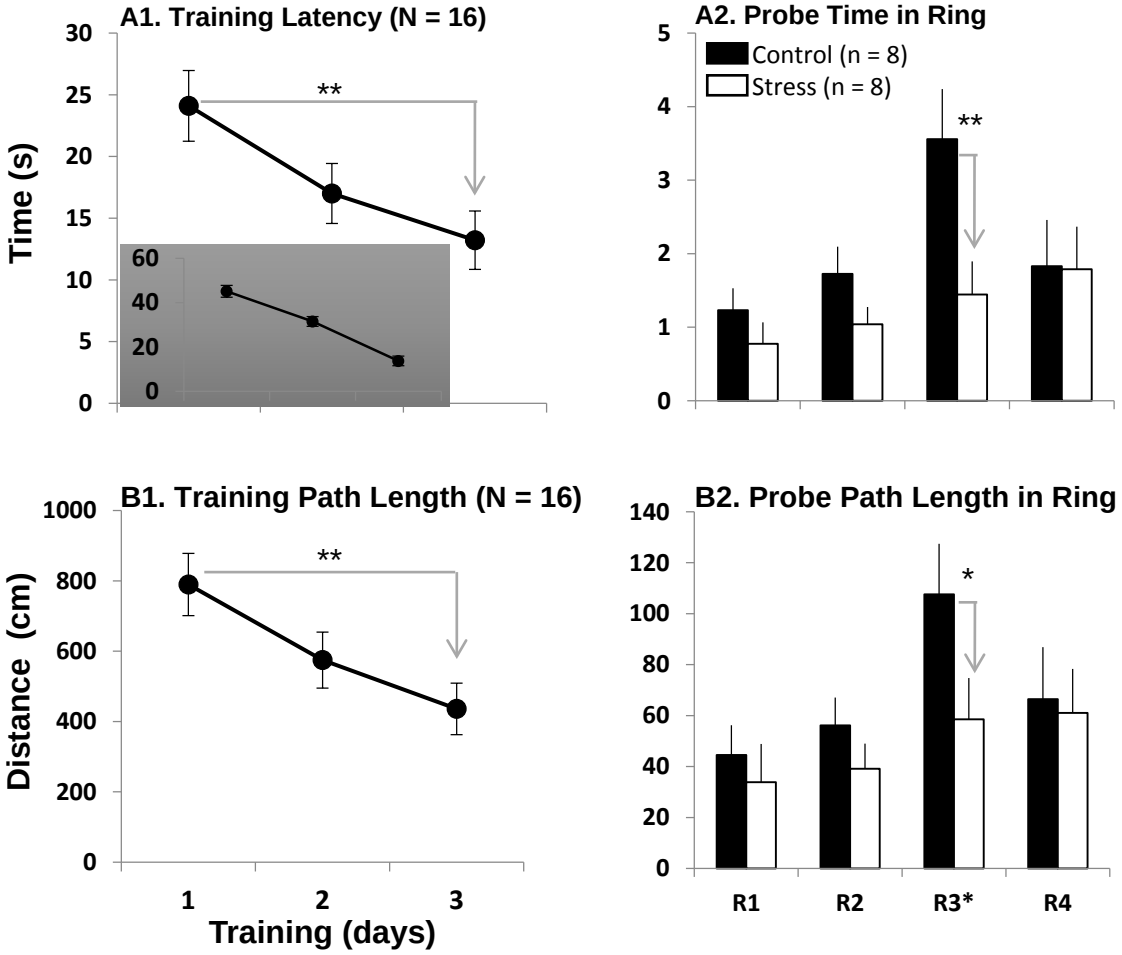

Figure 3. Water Maze Before and After Acute Stress. Training latency plotted as a function of training day. By the third day, the latency to the platform was significantly reduced ($p = 0.004$, repeated measures one-way ANOVA, ** $p =< 0.01$ Tukeys post-hoc day 1 vs day 3). Inset: Pre-surgery locally cued water maze training. Subjects' latency was reduced over the 3 day period ($p = 1.68E^{-10}$ Repeated measures one-way ANOVA, *** $p < 0.001$ Tukeys post-hoc all pairwise comparisons). A2. Time spent inside goal-sized circular areas (Rings- R) within each quadrant (1-4) during probe trial. R3*= goal ring. Control but not stressed rats spent significantly more time in the correct goal ring ($p < 0.05$ for main effects of Stress and Ring, ** $p = 0.002$ post hoc Tukeys pairwise control vs stress in R3*). B1. Spatially cued path length is plotted as a function of time. Path length was significantly reduced by the third day ($p = 0.005$ repeated measures one-way ANOVA, ** $p = 0.004$ Tukeys post hoc day 1 vs day 3). B2. Path spent inside goal-sized circular area (ring- R) within each quadrant during probe trial. R3*- goal area. Control but not stressed rats had significantly longer path lengths in the correct goal ring ($p = 0.03$ for main effect of Ring, * $p = 0.03$ post hoc Tukeys pairwise control vs stress in R3).

2.3.2 Sleep Architecture

Four-hour blocks of time from the start of the active and inactive periods on both the day before the spatial water maze task (baseline) and following the probe trial (post-stress) were analyzed. As expected, the baseline recording (Fig. 4A) shows characteristic active to inactive period sleep changes such as significant decreases in wake duration, and significant increases in all sleep stages. Following the water maze probe trial (Fig. 4B), the stress group's sleep stage duration architecture remained unchanged in both the active and inactive periods. However, there was a significant and selective increase in delta power during deep sleep during the inactive period (Fig. 4 Inset) in the stress group.

2.3.3 Body Temperature and Hormone Analysis

Psychosocial stress is well documented to induce relatively long-lasting hyperthermia in multiple mammalian species including rats and humans (Adriaan Bouwknecht et al., 2007; Vinkers et al., 2008). In prior work, young, but not aged, animals exhibited this response. Here, body temperature was monitored using surgically implanted telemetry devices.

The baseline temperature (Fig. 5A) follows a circadian rhythm that is not altered by water maze exposure, but post-stress (Fig. 5B) body temperatures are significantly elevated in the inactive period. The day after the restraint and probe trial, rats were euthanized and their trunk blood was collected for hormone analysis (see Methods). Neither CORT (ng/ ml: control 425 ± 124, stress 345 ± 97; n.s., Student's t-test) nor ACTH (control 361 ± 113, stress 394 ± 76; n.s.,

Figure 4. Sleep Architecture Before and After Stress

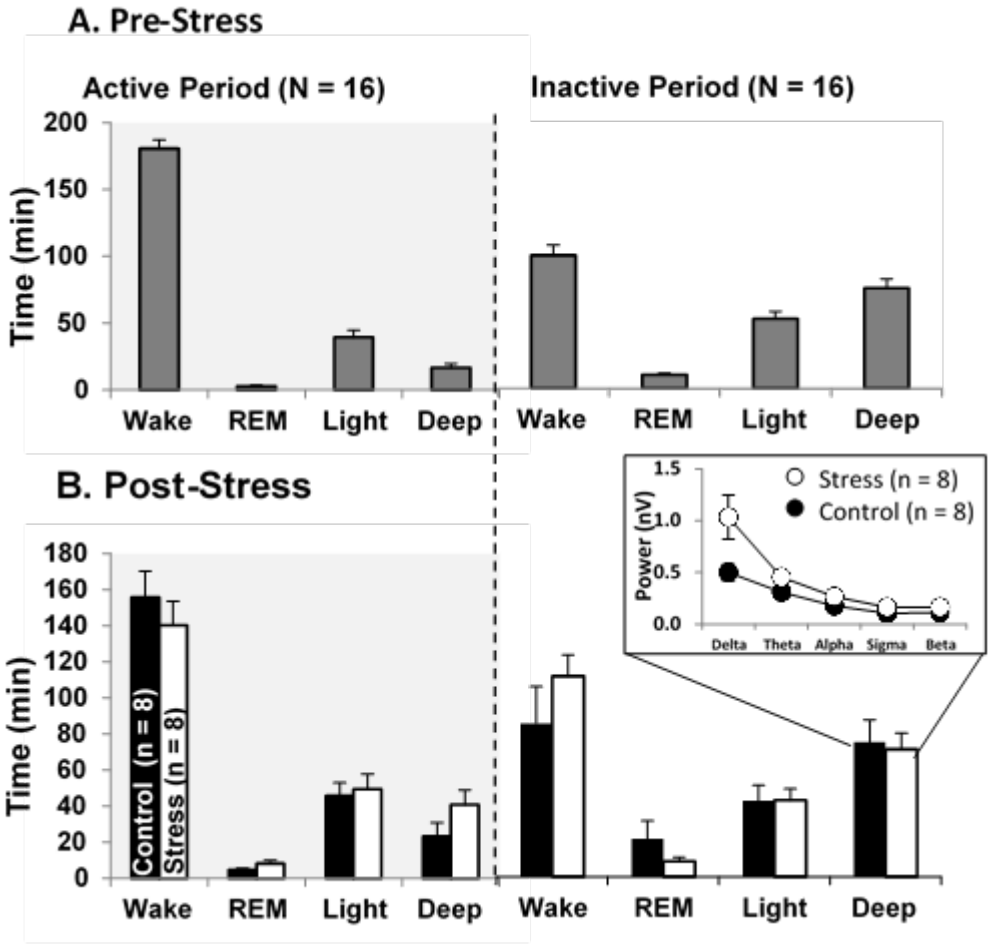

Figure 4. Sleep Architecture Before and After Stress. (A) The first four hours of the active and inactive periods prior to the first day of the spatially cued water maze task were scored. The duration was plotted for each stage of sleep. Subjects showed characteristic sleep architecture during these two periods prior to the restraint stress and the stages of sleep between the active and inactive periods are significantly different (Wake, REM, and Deep: paired t-test, $p < 0.01$; Light: paired t-test, $p \leq 0.04$). **(B)** Immediately following the restraint, the first four hours of the inactive and active periods were scored. The stressed subjects' sleep duration in each stage was unchanged in both the active (Wake, Mann-Whitney Rank Sum, $p = 0.281$; REM, t-test, $p = 0.135$; Light, t-test, $p = 0.753$; Deep, t-test, $p = 0.145$) and inactive periods (Wake, t-test, $p = 0.274$; REM, Mann-Whitney Rank Sum, $p = 0.281$; Light, t-test, $p = 0.989$; Deep, t-test, $p = 0.958$). **Inset.** The inactive period following the restraint stress showed significantly more power than control (2-way ANOVA RM, main effects of treatment and frequency, $p \leq 0.05$).

Figure 5. Baseline and Post-stress Body Temperature

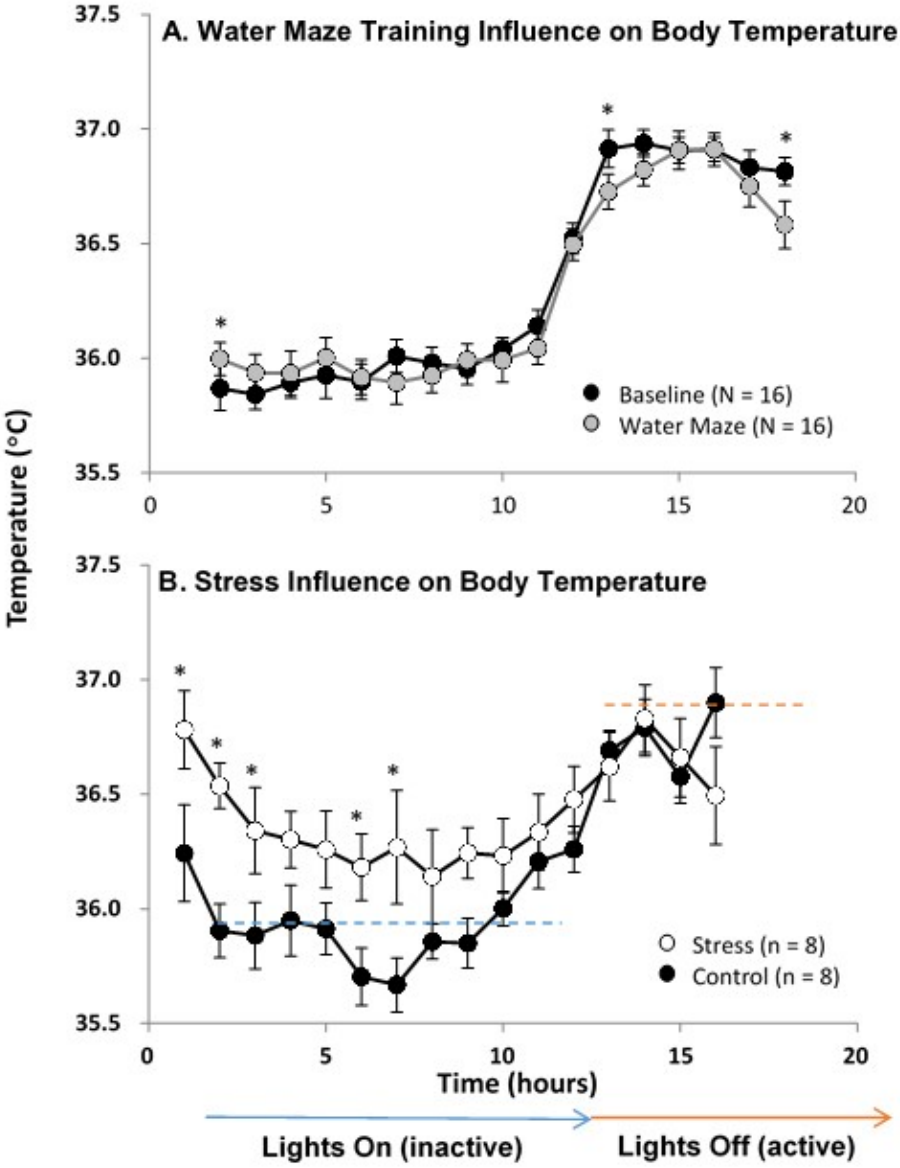

Figure 5. Baseline and Post-stress Body Temperature. A. Baseline and post-water maze exposure body temperatures are plotted as a function of time (0- lights on, 12- lights off). Temperature showed significant effects of time ($p < 0.001$) and interaction ($p = 0.002$) (repeated measures two-way ANOVA, * $p < 0.05$, Tukeys pairwise comparisons of baseline vs. water maze at indicated time). **B.** Stress-associated hyperthermia. Animals had significantly elevated inactive period body temperature after stress exposure ($p < 0.001$ for both time and interaction, repeated measures two-way ANOVA, * $p < 0.05$, Tukeys pairwise comparisons of control vs stress at specified time). Reference lines for baseline low (dashed blue) and baseline high (dashed red) body temperatures provided for reference.

Student's t-test) were significantly altered with treatment, although the reported values are elevated compared to prior work (Fig. 6A). Hormone levels were elevated, even in control, beyond normal physiological ranges previously reported (Sonntag et al., 1987), varied as a function of the time after transport/ kill order (Fig. 6B) and correlated strongly (Fig. 6C) with ACTH levels. This suggests that transport (~20 minutes in a covered cart) had a detectable stress effect that endured for approximately 2 hours after transport was completed. Although, subjects were balanced across treatment groups for kill order, these transport- related stress effects may have obfuscated the restraint paradigm's potential effects on blood stress hormone levels.

2.4 Discussion

Acute stress in young subjects causes spatial memory deficits (de Quervain et al., 1998; de Quervain et al., 2000; Stillman et al., 1998), elevated body temperature (Adriaan Bouwknecht et al., 2007; Vinkers et al., 2008) and loss of deep sleep (Kecklund and Akerstedt, 2004; Lesku et al., 2008; Vandekerckhove et al., 2011). In prior work (Buechel et al., 2014) we validated these effects in young animals, and further demonstrated that aged animals were hyporesponsive to the same stress exposure for these three measurements. However, prior work also clearly shows aged animals and humans are the more vulnerable chronic psychosocial stress population

Figure 6. Blood Hormone Analysis Following Stress

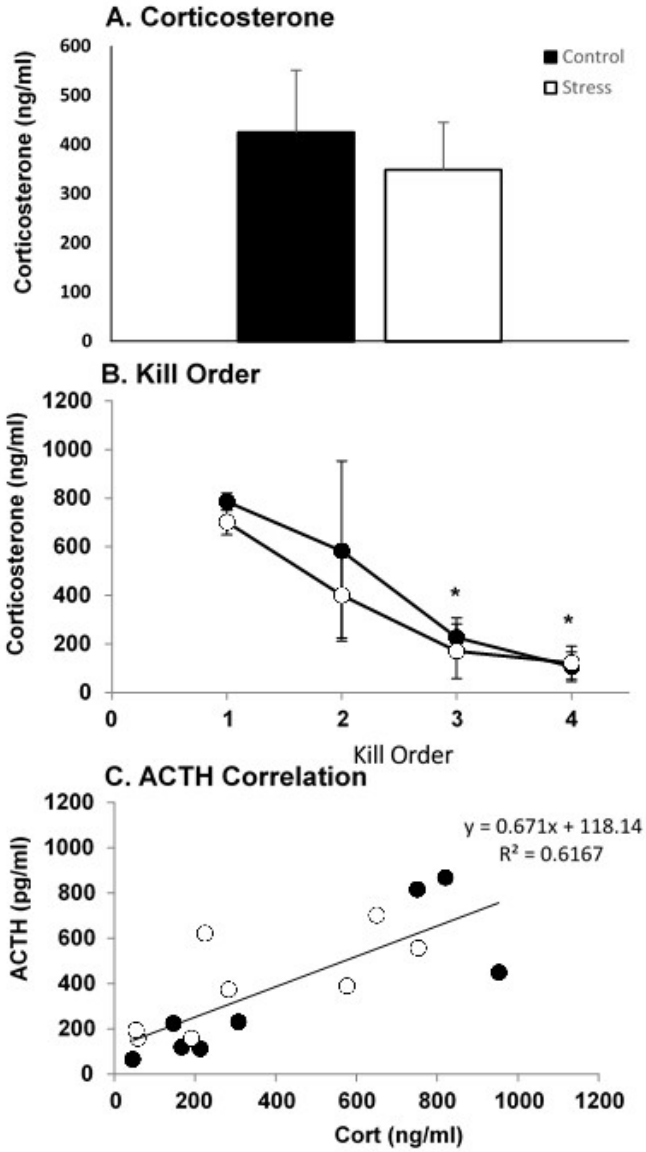

Figure 6. Blood Hormone Analysis Following Stress. Trunk blood was collected following decapitation for the analysis of CORT levels 18-20 hrs following restraint. (A) There was no significant difference between the groups (One Way ANOVA on Ranks $p = 0.798$). (B) The CORT levels were analyzed according to the order of kill to determine if transport stress was the reason behind elevated CORT levels compared to normal physiological ranges regardless of grouping. There was a significant effect of the kill order (Two Way ANOVA Kill order $p = 0.015$, Stress $p = 0.513$, Interaction $p = 0.936$ post hoc Tukey's pairwise comparison $^*p \leq 0.05$) (C) ACTH was plotted as a function of CORT and there was a strong correlation between the two measures.

(Azuma et al., 2015; Machado et al., 2014; Prenderville et al., 2015; Stein-Behrens and Sapolsky, 1992), that glucocorticoids themselves can be protective against stress (Rao et al., 2012), and that the aged hippocampus transcriptional profile in response to glucocorticoid exposure is fundamentally shifted from that of the young. Taken together, these data suggest that the lack of a response to stress can be maladaptive.

Very little work has investigated acute stress response in mid-aged animals, despite the importance of the mid-age time point in the trajectory of age-related changes. Here, we tested this response in the context of behavior, sleep architecture and body temperature. Water maze probe trial performance was significantly worsened by restraint stress (Fig. 3, 7A). While it's been shown that encountering stress before learning a task has minimal effects (Wolf, 2003), or can even promote memory (Roozendaal et al., 1997; Stamatakis et al., 2008; Zheng et al., 2007), stress immediately prior to memory retrieval is well-understood to have detrimental effects (de Quervain et al., 1998), possibly via neuroendocrine signaling directly in the hippocampus (Thomas, 2015) or via the innervation of stress-sensitive circuitry from the forebrain (Paul et al., 2015). Prior work (Chen et al., 2013) also shows that the glucocorticoid-responsive molecular machinery in the hippocampus is altered with age, suggesting that the transcriptome may play a role in the mechanism for the loss of stress-related recall disruption with age. Work here demonstrates that the

Figure 7. Summary Figure

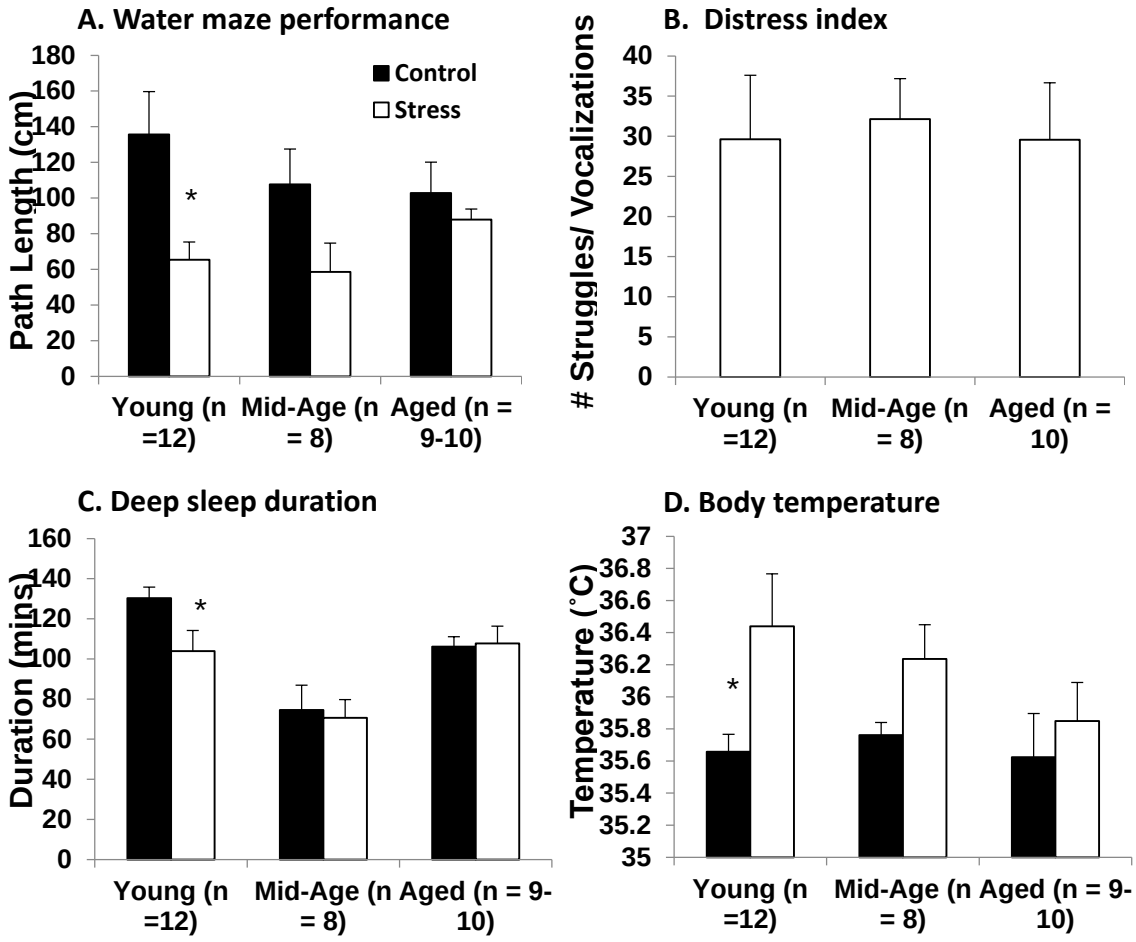

Figure 7. Summary Figure. Summarized mid-aged from the present study are compared to prior work (Buechel et al., 2014) on young and aged subjects (* indicates that significance was assigned in prior study) A. Path in goal ring during probe trial (Fig. 1) is re-plotted with prior young vs. aged study. B. Perceived distress was calculated as average # vocalizations and struggles per hour during the three hour restraint. C. Amount of time spent in deep sleep during the inactive period after restraint is plotted. D. Body Temperature response is the average of the three hour middle of the post-stress inactive period (9-11 PM).

recall-disrupting effects of stress exposure remain intact in mid-aged animals and may play a role in age-related cognitive decline detectable by mid-age (Blalock et al., 2003; Guidi et al., 2015; Pancani et al., 2013). Both REM and deep sleep are significantly decreased with aging (Behan and Brownfield, 1999; Ehlers and Kupfer, 1989; Espiritu, 2008; Foley et al., 1995; Pace-Schott and Spencer, 2011) and this loss is strongly associated with poor health and cognitive deficits (Ancoli-Israel, 2009). Previously (Buechel et al., 2014), we showed that young, but not aged, subjects had decreased deep sleep duration in the inactive period following a stress. Here, mid-aged animals showed an aging-like hyposensitivity to stress-induced deep sleep loss (Fig. 4, 7C), but did show young-like cognitive and body temperature stress responses, suggesting that age-related sleep changes may be early critical changes in the hyposensitive stress phenotype of age. Interestingly, mid-aged animals did show a significant increase in delta power during deep sleep (Fig. 2B, inset) after stress, an effect not seen in either young or aged animals exposed to stress in prior work (Buechel et al., 2014). Increasing deep sleep power is thought to improve stress resilience (Brand et al., 2014) and therefore may represent a unique mid-age stress-management phenomenon.

Mid-aged body temperature following stress was hyperthermic (Fig. 5B, 7D), characteristic of the stress-induced hyperthermia in young adult subjects. However, mid-aged animals did not show hyperthermia in response to water

maze exposure (Fig. 5A), a response that is statistically significant, albeit of lower magnitude, in young adult subjects, and absent in aged subjects. Thus, the mid-aged stress-induced hyperthermic response may be somewhat intermediate between that seen in young and aged subjects.

In our previous study (Buechel et al., 2014), stress-induced hyperthermia might have been associated with the increased wakefulness in the young during their inactive period, as body temperature is generally higher during wake. However, the middle-aged animals here did not show a significant increase in the time spent awake during their inactive period following the probe trial, but still showed an elevated body temperature. Alternatively, stress-induced hyperthermia could result from PGE_2 signaling in the preoptic area of the hypothalamus due to stress-induced norepinephrine release (Glavin, 1985; Nakane et al., 1994; Oka et al., 2001; Vellucci and Parrott, 1995) or corticotropin-releasing factor (CRF) stimulating brown adipose tissue (Morimoto et al., 1993; Oka et al., 2001). This latter observation may also help to explain why stress-induced hyperthermia (Buechel et al., 2014) declines with age, as brown adipose tissue content is decreased in aged animals (Cannon and Nedergaard, 2004; Pfannenberg et al., 2010).

The influence of transport on stress hormone signaling observed here (Fig. 6) has also been reported in other work (Dallmann et al., 2006). This is an important

observation for researchers who are considering studies where transport can have a significant influence on endpoint measurements associated with stress, and, based on these studies, we would advise allowing at least a two hour ‘cool down’ period between transport and measurement.

2.5 Conclusion

Despite the relative importance of characterizing the psychosocial stress response phenotype in mid-aged animals, relatively little work has investigated this time-frame. To facilitate comparison with young and aged subjects, results from the present study are juxtaposed with similar results from prior work (Buechel et al., 2014) (Fig. 7- no statistics are applied across studies, only the statistical results from within each study are noted). Like the young animals, the mid-aged showed a stress-associated decrease in path length in the goal ring during the water maze probe trial as well as hyperthermia during the inactive period following restraint (Fig. 7A, D). Based on vocalizations/ struggles, all age groups undergoing the stress procedure perceived the restraint to be stressful while it was occurring (Fig. 7B). Mid-aged animals showed young-like body temperature and cognitive responses to stress, but aged-like sleep architecture hyposensitivity to stress (Fig. 7C). This suggests that, by mid-age, subjects have already established an aging-like hyporesponsive phenotype for sleep stress-response that may presage the age-related development of cognitive and body temperature stress hyposensitivity. Interestingly, mid-aged animals also showed a significant elevation in delta power during deep sleep. As it is often associated

with improved sleep ‘quality’, increased post-stress delta power may represents a unique, mid-aged mechanism for compensating for psychosocial stress exposure, although further work will be needed to test this.

Chapter 3 Aged male F344 rats are hyporesponsive to chronic restraint stress (submitted to Behavioral Brain Research)

Authors: Kendra Hargis, Sara Qutubbin, Jelena Popovic, Eric Blalock

Summary

The allostatic load hypothesis of aging posits that exposure to repeated and/or unresolved stressors throughout life results in accumulated stress-associated changes that manifest as, or accelerate, brain aging. Indeed, a large body of literature supports the allostatic load hypothesis by demonstrating profound worsening of the aging phenotype in animals exposed to severe stress perinatally, or by showing that acutely stressing young adult animals results in acute age-like cognitive symptoms. However, little basic research has examined the response of phenotypically aged animals to new-onset stress. In humans, the incidence of new-onset psychosocial stress (PS- non-painful stimuli that evoke a stress response; e.g. social isolation, death of a loved one) increase with age and are associated with reduced quality of life and worsened health outcomes. Previously, we showed aged animals were hyporesponsive to acute PS. Here, we hypothesized aged animals would be hyporesponsive to chronic PS. Young (3mos) and aged (19mos) male Fischer344 rats were assigned to control or PS (restraint, 3 h/day, 4 days/week, 4 weeks; n = 7-10/ group) treatments. Morris water maze, sleep architecture, behavioral distress, body weight, body temperature, and corticosterone levels were measured. Chronic PS had no detectable effect on deep sleep, body temperature, or blood corticosterone levels at either age. However, aged animals were hyporesponsive on typical stress parameters including response to behavioral distress, weight loss, and cognitive

deficit. Taken together, the aged animals appear cognitively and behaviorally hyporesponsive to chronic PS. Restoring the blunted PS response in aged subjects represents a novel, age-selective intervention strategy.

3.1. Introduction

Aging is a strong positive risk factor for several conditions that dramatically reduce quality of life, including cardiovascular disease, type II diabetes, cancer, epilepsy, and Alzheimer's disease (Prince et al., 2015; Riedel et al., 2016; Stephen et al., 2006; Zhao et al., 2016). The chronic nature of these conditions, coupled with the projected increase in the worldwide aging population (He et al., 2016) is projected to overwhelm healthcare systems if the *status quo* between age and disease is maintained (Comlossy and Walden, 2013). The burgeoning field of 'normal aging' basic research focuses on age-related changes to understand the causes and reduce the risks for multiple age-related conditions. In the absence of disease, normal age-related changes include disruptions in: cognition (Craik and Salthouse, 2008; Gemma and Bickford, 2007; Harada et al., 2013; Salthouse, 2010), sleep architecture (Bonnet, 1985; Espiritu, 2008; O'Donnell et al., 2009; Wolkove et al., 2007; Zepelin et al., 1972), circadian rhythm (Kondratova and Kondratov, 2012; Van Someren, 2000), thermoregulation (Blatteis, 2012; Guergova and Dufour, 2011), and inflammation (Franceschi et al., 2000; Franceschi and Campisi, 2014).

Interestingly, many of these normal age-related changes are mimicked or exaggerated by stress and stress hormone exposure (Chen et al., 2013;

Goosens and Sapolsky, 2007; Holt-Lunstad et al., 2010; Kerr et al., 1992; Landfield and Eldridge, 1994b; Snyder-Mackler et al., 2014). The long-standing allostatic load hypothesis posits that stress responses to repeated and/or unresolved stressors results in accumulated changes that manifest as, or at least accelerate, brain aging and vulnerability to aging-related pathologies (McEwen, 2002). In the central nervous system, the hippocampus is thought to play a prominent role in this interplay between stress and aging (Hauger et al., 1994a; Landfield et al., 1978; Lupien et al., 1998; Meaney et al., 1995; Stein-Behrens et al., 1994).

Chronic psychosocial stress (PS) is a non-noxious stressor associated with major life changes such as divorce, loss of a loved one, reduced social status, or isolation. Much like physiological stressors that cause pain and injury, a highly conserved stress response is evoked by PS stimuli, activating the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos et al., 2013; Fink, 2009; Lazarus, 1966). The activated HPA axis facilitates changes in physiology and behavior that are thought to help a subject re-acquire homeostasis with the environment. However, in chronic PS, the HPA response, by definition, does not resolve the stressor. The resulting sustained stress response damages the hippocampus, causes cognitive, cardiovascular and metabolic deficits, thereby further reducing the subject's fitness (Heraclides et al., 2009; Krajnak, 2014; Meaney, 2015; Williams et al., 2013; Wood, 2014). PS is among the most common of environmental stressors in the human population, and some authors have suggested that

humans, based on our capacity for memory of the past and anticipation of the future, may be particularly vulnerable to its negative influences. PS elevates stress hormone levels (Leonard, 2005; Sapolsky, 1999), disrupts sleep (Akerstedt, 2006; Kim and Dimsdale, 2007) and impairs cognition (Conrad et al., 1996; Gouirand and Matuszewich, 2005; Luine et al., 1994).

In humans, the incidence of new-onset PS increases with age and is associated with reduced quality of life and worsened health outcomes (Kremen et al., 2012). However, little basic research has examined the response of phenotypically aged animals to new-onset chronic PS. Determining aged subjects' response to chronic PS may be critical to understanding the influence of an existing allostatic load for a new onset PS event, as both hyper- and hypo-responsiveness are considered maladaptive. Based on prior work (Buechel et al., 2014) demonstrating that aged animals were hyporesponsive to acute PS, we tested the hypothesis that aged animals would be hyporesponsive to new-onset chronic PS. Young (three month old) and aged (nineteen month old) male Fischer 344 rats were assigned to control or PS (restraint, 3 h/day, 4 days/week, 4 weeks; n = 7-10/ group) treatments. Morris water maze, sleep architecture, behavioral distress during restraint, body weight, body temperature, and corticosterone levels were measured. Neither young nor aged animals demonstrated significant effects on stress response measures associated with body temperature or deep sleep. Corticosterone levels in blood eighteen hours after the last stress exposure were borderline elevated in both ages. However, aged animals were

hyporesponsive on stress parameters to which young animals showed significant responses, including behavioral distress, weight loss, and cognitive deficit. Thus, this work supports the hypothesis that aging subjects are hypo-responsive to new-onset chronic PS and suggests therapeutic strategies focusing on restoring the stress response in aged subjects.

3.2. Materials and Methods

3.2.1 Subjects

Seventeen young (3 mos) and 17 aged (19 mos) male Fischer 344 rats were obtained from the NIA aging colony. Animals were individually housed with enviro-dry bedding. They had access to food and water *ad libitum* and were acclimated to a 12 hour reverse light/dark cycle (4:30 AM lights off, 4:30 PM lights on) and experimenter handling for two weeks prior to initiating study. Rats were assigned to control (7 aged, 8 young) and stress (10 aged, 9 young) groups and were weighed weekly. 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). Twelve additional animals (10 aged, 2 young) were removed from the study. Of these, two aged and one young were removed due to failure to reach cut-off on visual cue testing. Eight aged were euthanatized during the course of the study due to pale appearance, lethargy and weight loss. Gross pathology demonstrated enlarged spleens in all cases suggesting large granular lymphocytic leukemia

(Thomas et al., 2007) and one young subject due to developing ataxia (a pituitary tumor was found in gross pathology).

3.2.2 Surgery

All subjects were implanted according to standard procedures with wireless EEG/EMG emitters (Data Sciences International- TL11M2-F40-EET) as in previous work (Buechel et al., 2011; Buechel et al., 2014). 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 21 gauge 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.

3.2.3 Sleep Data Acquisition and Analysis

Sleep data was acquired according to established protocols (Buechel et al., 2014). Animals were housed individually and cages were positioned at least 18” apart to avoid interference during radiotelemetry data acquisition. EEG, EMG, and temperature data were recorded continuously with DSI's Data Art acquisition software and binned in 10 s epochs. For these nocturnal rodents, the first 4 h of their active period (dark) and the first 4 h of their resting period (light) were evaluated for sleep architecture during the 24 hour period following the final stress/probe trial paradigm. Architecture was scored using Neuroscore's (v. 2.1.0 Data Sciences International) analysis console in 30 s increments while being viewed in 2–5 min 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 based on EEG/EMG signaling 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 < 10% of scored time.

3.2.4 Water Maze

The water maze task was performed as in previous studies (Buechel et al., 2011; Buechel et al., 2014). A 190 cm diameter circular, black pool was centered (250 cm/side) in a cubicle of floor to ceiling black curtains. Maze water temperature was maintained at 26 ± 2°C. Except for probe trials, one quadrant always contained a 15 cm diameter escape platform covered with black neoprene. Illumination in the room was set at 3.6 to 3.8 lux and a Videomex-V water maze monitoring system (v. 4.64 Columbus Instrument) was used for analyses. All training and probe sessions took place between 12 PM and 4 PM (during the rats' active period).

The visual cue task took place over 3 days on the week before surgery to assess non-hippocampus-dependent function (Guidi and Foster, 2012; Guidi et al., 2014). A Styrofoam cup served as a visual cue and was hung by a black thread 12 inches above the submerged platform. Rats were given three trials (60s each) and the location of the platform, as well as the drop location, was changed for each trial. Animals that did not reach the platform within 60s were gently guided to the platform. All animals were allowed to stay on the platform for 60s after each trial and were then towel-dried and placed in a dry cage with excelsior bedding under a warming lamp for 2 min (intertrial interval). Reaching the

platform in under 30s for 2/3 trials on the third day was criterion for this study. The spatial cues were not present during the visual cue task.

In surgically implanted animals during the last week of the chronic restraint paradigm, rats performed a spatially cued Morris water maze task as in prior work (Buechel et al., 2014) immediately following restraint. Briefly, spatial cues (high contrast black and white 90 × 90 cm- circle, triangle and vertical lines, one to each of the three curtains facing the maze, 60 cm above the maze rim) were provided and the Styrofoam cup used in the visual cue was removed. As in the visual cue task, rats had 3 trials (60s each)/ day for three days with one minute on the submerged platform and 2 minute inter-trial intervals. On the fourth day, the platform was removed and rats performed one 60s probe trial. Path length and latency to platform, platform crossings and path length in goal quadrant were recorded. Placement of the platform remained unchanged throughout spatial cue training. However, the starting quadrant changed for each trial. Animals started in the quadrant opposite the goal quadrant for the probe trial.

3.2.5 Restraint Stress

Rats in the psychosocial stress (PS) group were moved from the vivarium to a procedure room, and placed in restraint (Rat Snuggle, Harvard Apparatus) while under brief (< 30s) isoflurane anesthesia. Restraint was applied 3h/ day, 4 days/ week for a total of 4 weeks. Animals were continuously monitored during restraint and vocalizations and struggles were quantified to construct an index of

observable distress. Animals were returned to the housing facility at the conclusion of stress. Control animals were maintained in the vivarium.

3.2.6 Blood and Tissue Collection

On the morning following the Morris water maze probe trial, animals were anesthetized with isoflurane and decapitated. Trunk blood was collected in K_2EDTA vacutainers (BD biosciences) and immediately centrifuged at 1200 g for 10 min. The plasma was collected for corticosterone quantification by radioimmunoassay (Antech, Irvine, CA). Brain tissue was rapidly removed as in prior work (Buechel et al., 2011; Buechel et al., 2014). Hippocampal tissue from one hemisphere was snap frozen while the other hemisphere was post-fixed in 4% formalin, cryoprotected in a 15% sucrose mixture and stored at −80°C for future use.

3.3. Results

3.3.1 Morris Water Maze

Following acclimation, and prior to surgery, all animals were evaluated on the visual cue task to determine if animals could swim were able to orient towards a visible, localized cue. Three animals (2 aged, 1 young) were removed from the study because they did not meet performance criteria by the last day of visual cue (see Methods). Remaining animals showed significantly reduced latencies (Fig. 8A) and path lengths (Fig. 8B) over training. In addition, aged animals were

Figure 8. Visual Cue (pre- chronic stress)

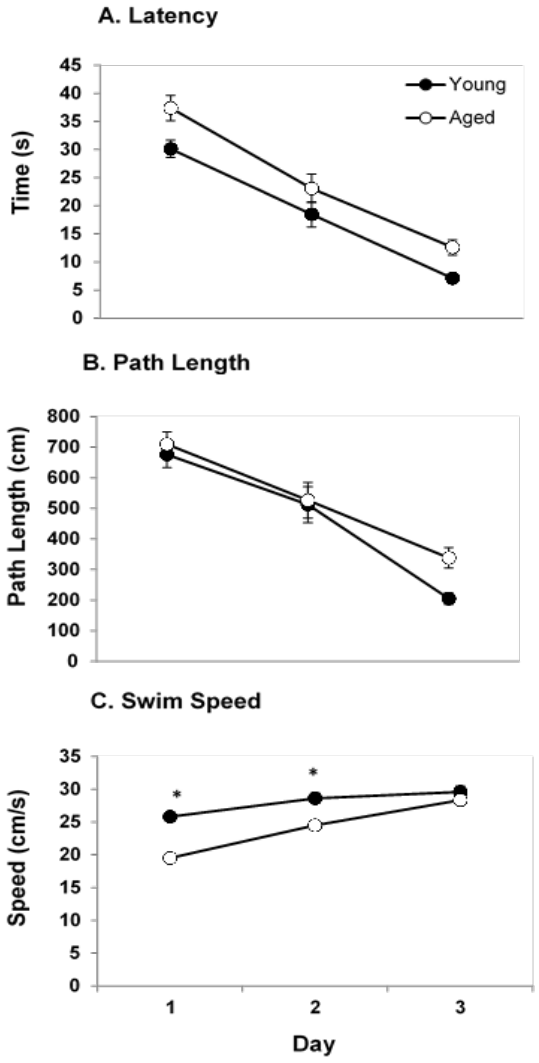

Figure 8. Visual Cue (pre-chronic stress). (A) Visual cue latency was plotted as a function of day. Animals in both age groups significantly improved their performance each day and the young animals were significantly faster finding the platform on day 1 than the aged animals. (Two-Way RM ANOVA Age $p = 0.006$, Day $p < 0.001$, Interaction $p = 0.698$ post hoc Tukey's pairwise comparison $*p \leq 0.05$) **(B)** Path length was plotted as a function of day. Both age groups significantly decreased their path length each day during visual cue. (Two-Way RM ANOVA: Age $p = 0.177$, Day $p < 0.001$, Interaction $p = 0.375$) **(C)** Swim speed was plotted as a function of day. Both age groups significantly improved their swim speed by day 3 and the aged animals were swimming on par with the young animals by the last day. (Two-Way RM ANOVA Age $p < 0.001$, Day $p < 0.001$, Interaction $p < 0.001$ post hoc Tukey's pairwise comparison $*p \leq 0.05$).

significantly slower swimmers initially (Fig. 8C), but their swim speeds were similar to those of the young by the third day of training.

During the final week of stress (week 4), all animals were trained on the spatial cue task immediately following the restraint period. There was a significant effect of training, but not stress, for both young (Fig. 9A1) and aged (Fig. 9A2) groups. Further, in young but not aged subjects, there was a significant interaction between training and stress, and post-hoc pairwise analysis indicated a significant performance deficit in stressed young animals on day 2. Immediately after the final restraint period, probe trial testing was done. As in prior work, aged animals took significantly longer paths than young to the goal area. Further, restraint stress was associated with a significant path length increase in young animals, while aged animals showed no significant effect of stress (Fig. 9B). These results suggest that aged animals, although showing performance deficits compared to young, are hyporesponsive to chronic stress-induced cognitive deficits.

3.3.2 Sleep Architecture

Following visual cue and prior to the start of restraint stress, all animals were surgically implanted with wireless telemetry emitters that recorded EEG, EMG, and temperature data. The first four hours of the final inactive and final active periods after the water maze probe trial were recorded and analyzed to determine the effect of chronic psychosocial stress in both age groups.

Figure 9. Spatial Cue and Probe Trial

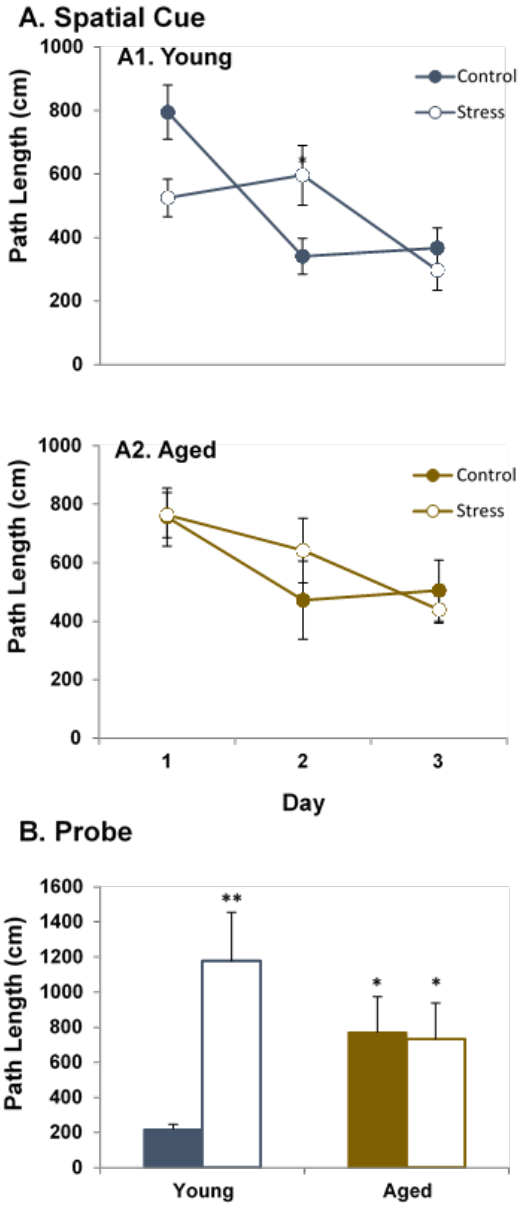

Figure 9. Spatial Cue and Probe Trial. Spatial cue training was conducted immediately following the 3 hr restraint period during the final week of stress. **(A)** Path length was plotted as a function of training day. A1. All of the young animals significantly improved their path length by the third day of training compared to the first day (Two-Way RM ANOVA Stress $p = 0.882$, Day $p < 0.001$, Interaction $p = 0.015$ *post hoc Tukey's pairwise comparison* $*p \leq 0.05$) A2. By the last day of training, only the aged control animals had significantly shorter path lengths compared to the first day of training (Two-Way RM ANOVA Stress $p = 0.632$, Day $p = 0.005$, Interaction $p = 0.423$ *post hoc Tukey's pairwise comparison*) **(B)** Probe trial was performed immediately following the final day of stress. Path length to the original platform location was plotted for each group. The young control group had a significantly shorter path length to the platform location compared to the age-matched stress group (Two-Way ANOVA Age $p = 0.816$, Stress $p = 0.05$ Interaction $p = 0.036$ *post hoc Tukey's pairwise comparison vs Young Control:* $*p \leq 0.05$; $** p \leq 0.01$)

Interestingly, there were no differences in deep sleep duration with stress or aging. However, there was a significant decrease in wake in the aged stressed animals during the active period (Table 1), similar to the decreased active period wake observed in prior work after acute restraint (Buechel et al., 2014).

3.3.3 Distress Index and Body Weight

The animals in the stress groups were restrained for 3 hrs/ day, 4 days/ week, 4 weeks. During each restraint period, the animals were continuously monitored and each struggle and/or vocalization was counted. A tally of the total struggles and vocalizations over the 3 hour period for each animals was defined as their behavioral stress index. Averaged per week by age (Fig. 10), behavioral stress indices were consistently higher in young than aged animals. Body weights were measured throughout the course of the study. Young adult control animals (Fig. 11) showed steadily increasing weight over time. Young stressed subjects significantly deflected from that trend by the 3rd and 4th weeks of treatment. Aged control animals were heavier than young adults and showed a plateaued, or slightly decreasing weight over time. Stress had no statistically detectable effect on aged animal body weight.

3.3.4 Body Temperature and Corticosterone Levels

In a previous study (Buechel et al., 2014), young, but not aged animals showed a delayed, long-lasting acute restraint stress-induced hyperthermia. Three hours after removal from restraint, animals showed a 9 hours hyperthermic response.

Table 1. Sleep Duration

						2-Way ANOVA		
Period	Sleep Stage	YC	YS	AC	AS	Age	Treatment	Interaction
Active	Wake	122.8 ± 12.2	139.9 ± 14.6	165.3 ± 12.6	74.6 ± 18.6	0.62	0.04	0.01
Active	PS	8.2 ± 1.4	6.1 ± 1.6	3.0 ± 0.9	9.9 ± 2.1	0.54	0.08	0.07
Active	SWS1	82.0 ± 9.6	59.3 ± 11.3	58.6 ± 6.0	83.2 ± 5.3	0.65	0.56	0.04
Active	SWS2	24.8 ± 5.4	18.9 ± 5.8	17.8 ± 2.4	22.0 ± 1.4	0.68	0.86	0.29
Inactive	Wake	59.0 ± 11.5	77.6 ± 2.7	76.8 ± 10.6	82.3 ± 5.8	0.62	0.04	0.01
Inactive	PS	38.7 ± 1.8	23.9 ± 1.1	30.9 ± 4.0	33.9 ± 5.2	0.54	0.08	0.07
Inactive	SWS1	111.5 ± 13.1	92.7 ± 4.9	93.2 ± 10.6	106.6 ± 7.3	0.65	0.56	0.04
Inactive	SWS2	93.2 ± 10.9	82.1 ± 11.1	96.5 ± 6.4	96.7 ± 7.1	0.68	0.86	0.29

Table 1. Sleep duration. The first four hours of the final (following the last restraint and probe trial) active and inactive periods were analyzed. There was a significant decrease in the aged stressed animals' wake and increase in SWS1 in the final active period but no changes in the inactive period. There were no significant changes in the duration of deep sleep for any group of animal in either the active or inactive periods.

Figure 10. Chronic Stress Distress Index

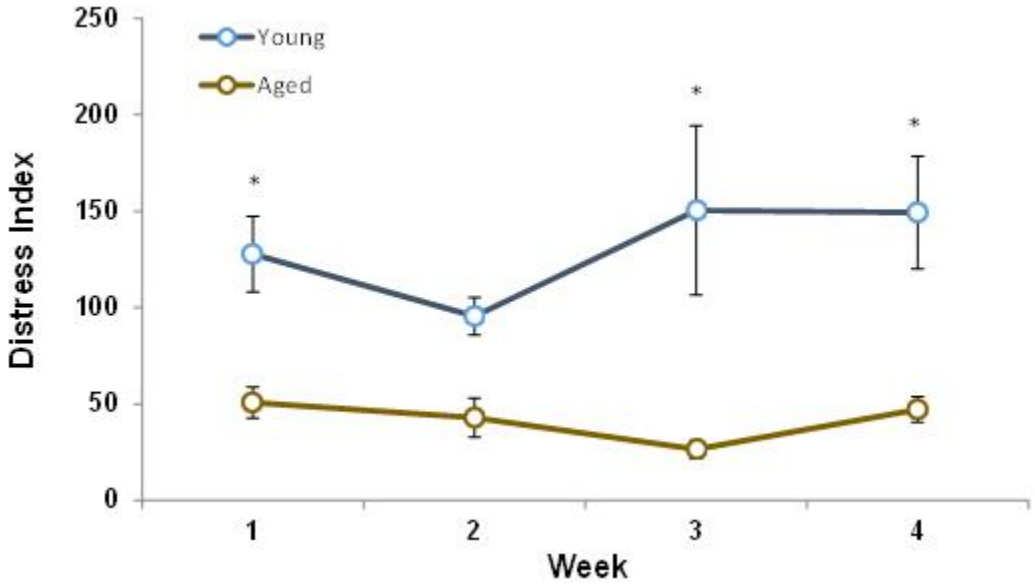

Figure 10. Chronic Stress Distress Index. The average number of struggles and vocalizations per 3 hour restraint period (distress index) is plotted by week for young and aged animals. The young had significantly more struggles than aged animals (Two-Way RM ANOVA Age $p < 0.001$ Week $p = 0.220$ Interaction $p = 0.106$ post hoc Tukey's pairwise comparison $*p \leq 0.05$).

Figure 11. Body Weight During Chronic Stress

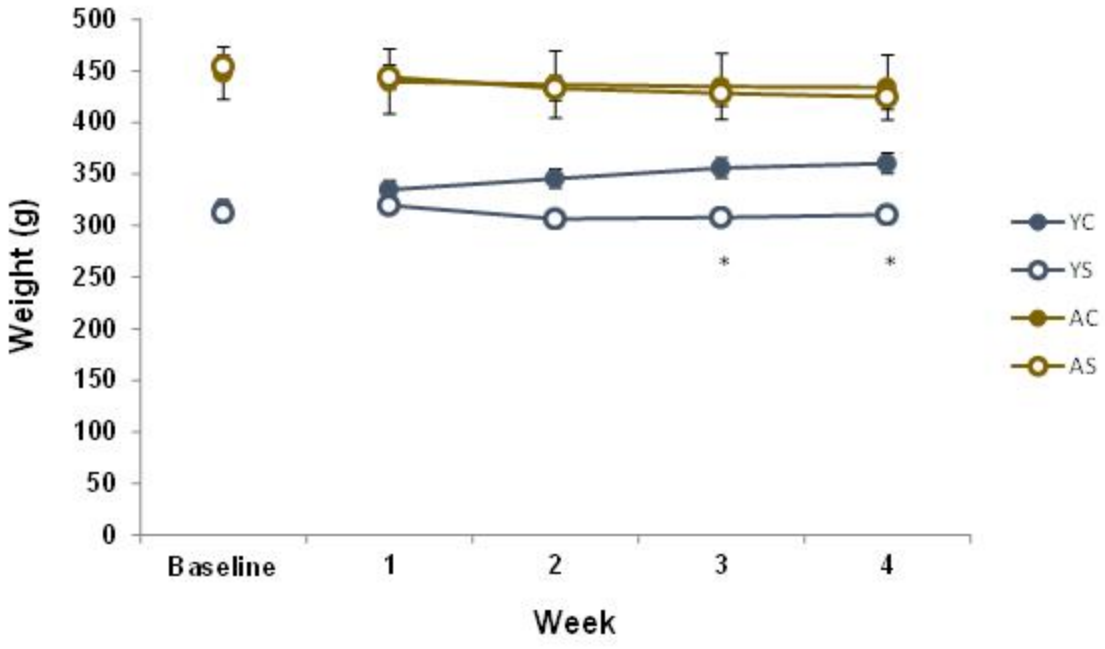

Figure 11. Body Weight During Chronic Stress. Animals were weighed throughout the entire study before the beginning of the restraint stress. The young stressed animals showed significant changes in weight compared to all other groups (Two-way RM ANOVA: Group $p \leq 0.001$, Week $p = 0.18$, Interaction $p \leq 0.001$ post hoc Tukey's pairwise comparison $^*p \leq 0.05$)

To determine if there was a hyperthermic response in the chronic restraint paradigm, we looked at the same post-stress time span. All groups showed a significant circadian effect of time on body temperature, with a consistent elevation associated with onset of the active period (dashed gray vertical line; Fig. 12A, B). Young subjects showed no significant influence of stress on body temperature or any significant interaction between time and stress. Aged animals showed a significant interaction between body temperature and time, with elevated stress-associated body temperatures early in the inactive period. However, this effect, though statistically significant, only occurred at one time point and did not endure through the inactive period. Trunk blood was collected at the end of the study (19 h following the end of the last restraint period) and analyzed for levels of corticosterone, a biomarker for stress. Chronic restraint showed a trend ($p = 0.1$) towards increasing cort levels (Fig. 13) and no effect of aging.

3.4. Discussion

We tested the hypothesis that aged animals would be hyporesponsive to new-onset chronic psychosocial stress (PS). The key findings were that aged animals were hyporesponsive on classic stress-sensitive measures, including cognitive impairment, weight loss, and behavioral distress. In addition, neither young nor aged animals showed deep sleep loss or stress-induced hyperthermia with chronic PS. In acute stress, glucocorticoid levels are elevated for hours to days

Figure 12. Temperature Following Chronic Stress

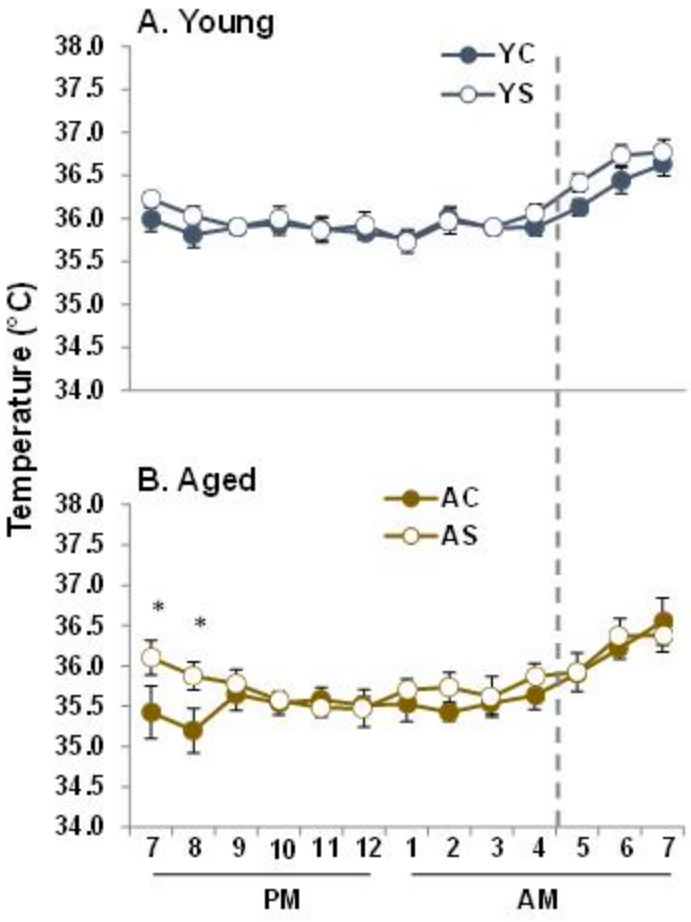

Figure 12. Temperature Following Chronic Stress. (A) Body temperatures of young animals following the final stress period and probe trial. Body temperature is plotted as a function of time over 12 hours starting at 7 PM and ending at 7AM with lights off marked (4:30 AM- beginning of the active period, dashed gray vertical line). (Two-Way RM ANOVA Treatment $p = 0.495$ Time $p < 0.001$ Interaction $p = 0.898$) **(B)** Body temperatures of aged animals following the final stress period and probe trial. (Two-Way RM ANOVA Treatment $p = 0.494$ Time $p < 0.001$ Interaction $p = 0.02$; *post hoc Tukeys pairwise* $*p \leq 0.05$)

Figure 13. Stress Hormone Analysis Following Chronic Stress

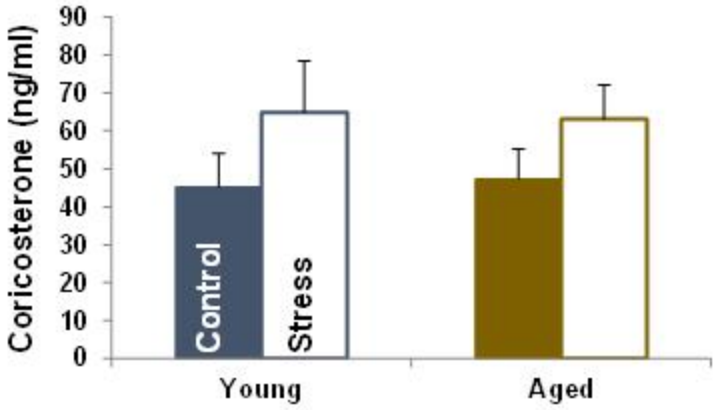

Figure 13. Stress Hormone Analysis Following Chronic Stress. Immediately following decapitation, trunk blood was collected to be analyzed for plasma corticosterone levels (Two-Way ANOVA Age p = 0.987, Stress p = 0.111, Interaction p = 0.855)

after stress (Buechel et al., 2014; Dunn et al., 1972; Ottenweller et al., 1992). However, in chronic PS, young and aged animals only showed borderline elevations in corticosterone blood levels eighteen hours after PS.

In the human population, both the incidence of new-onset chronic psychosocial stress (e.g., chronic illness/ death of a loved one, and socioeconomic upheaval) and the severity of health consequences after such exposure, including cancer, cardiovascular disease, metabolic disorders, and neurodegenerative disease, are worsened in aged subjects (Kremen et al., 2012). These clinical observations are consistent with the long-standing stress/glucocorticoid hypotheses of aging, and suggest an age-related decrease in the ability to adapt to a changing environment. However, little basic research has focused on the potential age-related differences in downstream stress responses.

3.4.1 Effects of Chronic PS in Young Subjects.

In this study, young subjects' cognition, body weight, and behavioral distress responses were consistent with prior work. Cognitive effects in young animals may be associated with stress/glucocorticoid-mediated dendritic atrophy (Conrad et al., 1996; Luine et al., 1994; McLaughlin et al., 2007; Tynan et al., 2010) (Conrad et al., 1999; Cook and Wellman, 2004; Lupien et al., 2009) and glutamate receptor downregulation (Yuen et al., 2012). Similarly, the stress-induced weight loss reported here is a well-established response to PS (Bielajew et al., 2002; McLaughlin et al., 2007; Tynan et al., 2010) and is thought to

proceed through central modulation of food intake (Retana-Marquez et al., 2003) (Scherer et al., 2011) and peripheral metabolic changes (Lemche et al., 2016).

Numerous studies report the importance of sleep and stress in relation to memory (Borbely, 2001; Marshall et al., 2006; Roozendaal, 2002; Stickgold, 2005; Wolf, 2003). However, in this study there were no significant differences in inactive period deep or REM sleep, the two stages most commonly associated with memory. Thus, the chronic PS-induced cognitive disruption seen in young animals either did not proceed through a sleep-related mechanism, or sleep disruption may have been an acute response to which animals adapted over time. Because promoting deep sleep has been associated with stress resiliency (Brand et al., 2014; Meerlo et al., 2008; Sadeghi Bahmani et al., 2016), deep-sleep enhancing agents may help preserve cognition in chronic PS.

Acute PS in young animals results in significant elevations in blood corticosterone levels for hours or days after the stressor is removed (Akerstedt, 2006; Buechel et al., 2014; Kuhlmann et al., 2005; Lupien et al., 2009). The borderline significant glucocorticoid blood levels reported here, coupled with the absence of stress-induced hyperthermia (Barnum et al., 2007; Tynan et al., 2010) suggest that using this chronic stress paradigm (3h/ day, 4d/ week for 4 weeks) results in stress adaptation or adrenal insufficiency/ exhaustion in which some effects (body weight, cognition, behavioral distress) are more sensitive to ongoing stress.

3.4.2 Chronic PS Hyposensitivity in Aged Animals.

Just as in our prior studies in aged animals exposed to acute stress (Buechel et al., 2014), the aged animals in this study maintained a hyporesponsive phenotype. Aged-matched control animals showed characteristic deficits in water maze performance compared to young (Gallagher and Pelleymounter, 1988), but further deficits were not observed with chronic PS. A similar pattern was observed for both weight loss and behavioral signs of distress. These results suggest that HPA axis signaling, and/or the downstream response to it, is dampened in aged animals. Because glucocorticoid levels were similar in young and aged, it seems reasonable that localized glucocorticoid signaling (Yau and Seckl, 2012), glucocorticoid receptor expression (Oitzl et al., 2010), or mediators of glucocorticoid action, could play a role in stress-hyposensitivity with age. Glucocorticoid receptors in the hippocampus play key roles in regulating the stress response (Jacobson and Sapolsky, 1991; Sapolsky et al., 2000) and stress (McEwen, 1998a; Sapolsky, 1996) and aging (Lupien et al., 1998; Porter and Landfield, 1998; Roth, 1974) both impair hippocampus-mediated feedback suppression of the HPA axis.

3.4.3 Conclusion

The allostatic load hypothesis posits the accumulation of stress responses over time can accelerate brain aging and eventually lead to negative health outcomes (Seeman et al., 2001; Upchurch et al., 2015). Chronic and/or repetitive stress

increases the allostatic load, resulting in either hyper- or hypo-response and could ultimately wear out the HPA axis (McEwen, 1998a) and accelerate symptoms of brain aging (Lupien et al., 1998; McEwen et al., 1999). Taken together, these results support a hypo-responsive interpretation of the allostatic load hypothesis (McEwen, 1998a; Seeman et al., 1997) in which aging acts as a stressor that mechanistically interferes with responses to new onset stress. A blunted stress response is maladaptive (Heim et al., 2000). Indeed, many conditions associated with increased allostatic load, including depression, post-traumatic stress disorder, and care giver stress, can result in blunted (Burke et al., 2005; McEwen, 1998a) (but see Chida and Hamer, 2008) responses. In the aged population, disruption of the HPA axis could explain worsened health outcomes, such as increased vulnerability to infection and disease (Glaser, 2005; Kiecolt-Glaser et al., 2003). Restoring stress-sensitivity in aged subjects could represent a novel target for the treatment of stress-related conditions in aged subjects. Finally, this work highlights the rationale and feasibility of modeling stress responses in age-appropriate systems in order to more accurately target interventions.

Chapter 4 Supplemental Data

Both studies either brought up more questions or had an aspect that deserved a closer look. I decided to further investigate and performed a few smaller studies to address these. While these smaller studies are not the main focus, they do deserve mention. Therefore, I have included this smaller section.

4.1 Corticosterone Levels During Restraint

Before I started both of my studies, I helped Dr. Heather Buechel with a small study measuring the corticosterone levels in young and aged animals during the restraint period. My lab as well as other studies (Buechel et al., 2014; Kuhlmann et al., 2005; Lupien et al., 2009), had already demonstrated a sustained corticosterone response several hours after the termination of stress, but we were interested in measuring corticosterone levels during the restraint in young and aged animals.

We used eight young (3 mos) and six aged (19 mos) male F344 rodents to accomplish this task. Blood samples were collected via tail prick beginning at the beginning of the restraint and then every hour for three hours (the length of our restraint period). Samples were immediately centrifuged and the plasma was collected and stored on dry ice until the end of the restraint period.

Consistent with other studies (Barlow et al., 1975; Marin et al., 2007), corticosterone was elevated during the entire restraint period in the young and aged animals (Fig. 14). The data from this small experiments served multiple purposes. First, it provided me with the confidence that the restraints sufficiently stressed both age groups. Second, I determined that it was not feasible for my lab to efficiently collect blood samples from control and stressed animals, accurately record observations from the stressed animals to create a distress index, and properly prepare blood samples for analysis (centrifuge and storage). For this experiment, the numbers were small and we focused specifically on stressed animals. We also had several people helping out. Unfortunately, I knew I would not have the manpower to accomplish this for my studies. Therefore, I decided to only collect trunk blood samples from my animals for all of my studies.

4.2 Transport Stress

When analyzing corticosterone data from the mid-aged acute stress study, I noticed the corticosterone levels were outside the normal physiological ranges for that time of day. Corticosterone levels follow a circadian rhythm and based on findings in the literature (Dhabhar et al., 1993; Hauger et al., 1994b; Morano et al., 1994; Sonntag et al., 1987), I was able to create an abstract representation of corticosterone levels during the day (Fig. 15) and predict the normal physiological range of corticosterone based off the time of day I sacrificed the animals. This prompted me to take a closer look at the data and I discovered there was a

Figure 14. Corticosterone During Restraint

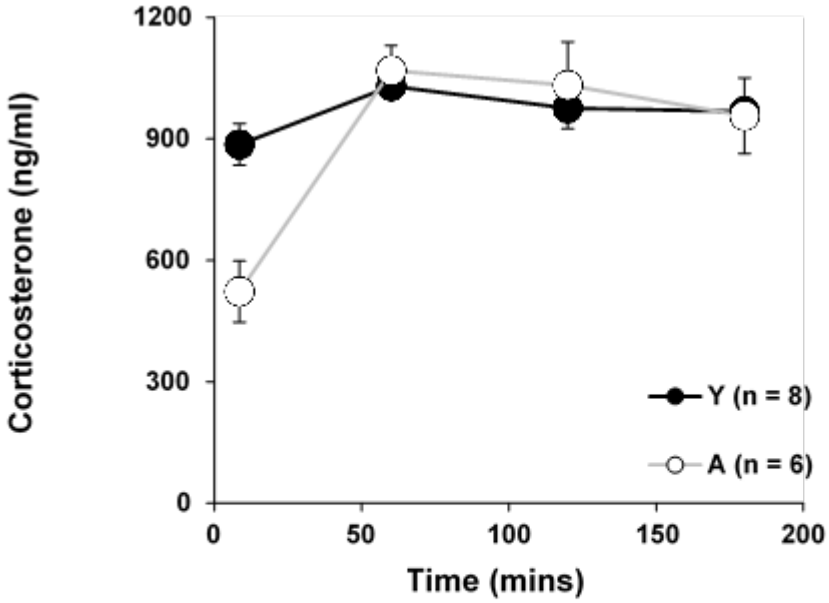

Figure 14. Corticosterone During Restraint. Blood samples were collected from young and aged animals during restraint stress and analyzed for corticosterone. Corticosterone was significantly elevated during restraint and remained elevated throughout the entire restraint period (Two-Way ANOVA RM Time $p < 0.001$ Age $p = 0.35$ Interaction $p = 0.001$)

Figure 15. Physiological Corticosterone Levels in Rodents

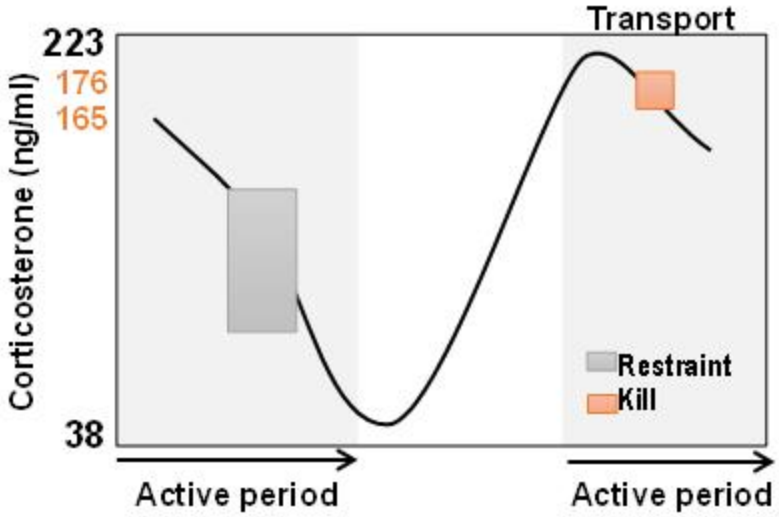

Figure 15. Physiological Corticosterone Levels in Rodents. Based on literature, I was able to create a graph showing the typical zenith and nadir of the corticosterone levels in rodents. The gray box represents the time of restraint and the orange box represents the time of transport and trunk blood collection. Using this data, I predicted the expected physiological range (165-176 ng/ ml) the corticosterone should fall in for my animals based on the time the animal was killed. This helped me determine how transport affected the animals' corticosterone levels and what adjustments could be made in the future.

pattern in the order the animals were killed (Fig. 16)—the animals (regardless of treatment group) killed immediately following transport had significantly higher corticosterone levels compared to the animals killed last (two hours after transport). Interestingly, the same observations have been documented in rats (Dallmann et al., 2006) and cattle (Palme et al., 2000; Trunkfield and Broom, 1990). While I could not comment on the effect acute stress had on corticosterone levels in the study, this raised an important point regarding transport stress and the need for a ‘cooling off’ period before sampling if stress hormones are being measured.

4.3 Pharmacologically Promoting Deep Sleep

Spontaneous deep sleep loss occurs during aging (Bixler et al., 1984; Bliwise, 1993; Espiritu, 2008) and deep sleep is thought to play a role in stress management (Brand et al., 2014; Sadeghi Bahmani et al., 2016). While I demonstrated that aged animals were hyporesponsive to chronic PS, a blunted response is still maladaptive (McEwen, 1998a). Additionally, young animals demonstrated cognitive deficits in chronic PS without any effect on deep sleep. Although neither age groups’ deep sleep was altered during chronic PS, promoting deep sleep could: 1. at least partially restore the stress response in aged animals and help them become resilient to PS and 2. in young animals, it could remedy some of the cognitive deficits observed after acute PS (Buechel et al., 2014).

In this study, 32 young (3 mos) and 40 aged (19 mos) male F344 rats were used

Figure 16. Kill Order After Transport

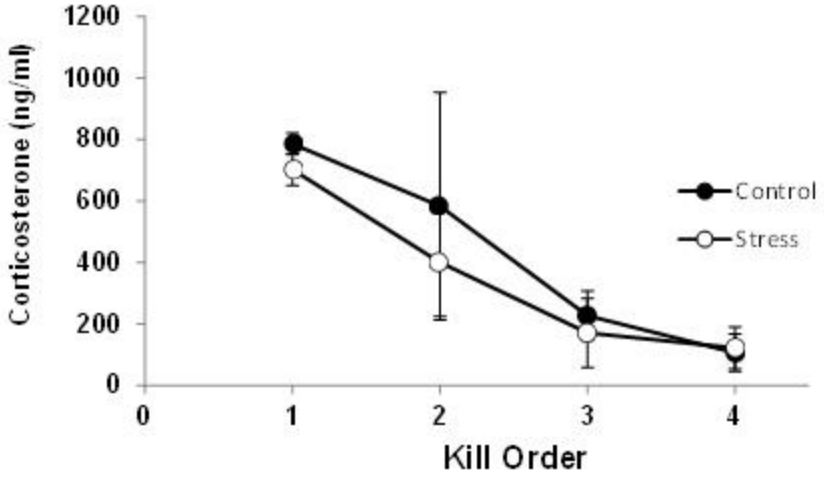

Figure 16 Kill Order After Transport. The CORT levels were analyzed according to the order of kill to determine if transport stress was the reason behind elevated CORT levels compared to normal physiological ranges regardless of grouping. Approximately 1- 1.5h after transport (4), the corticosterone levels have returned to normal physiological levels and are significantly lower than the animals killed immediately after transport. There was a significant effect of the kill order (Two Way ANOVA Kill order $p = 0.015$, Stress $p = 0.513$, Interaction $p = 0.936$)

to study the influence of administering Gaboxadol during acute PS. Gaboxadol is a selective $GABA_A$ receptors (Mathias et al., 2001a; Wafford and Ebert, 2006). This drug differs from others, such as benzodiazepines that bind to the BZD domain of $GABA_A$ receptors, in that it is selective to the delta subunit of extrasynaptic $GABA_A$ receptors (Wafford and Ebert, 2006). Unlike current sleep aids on the market (e.g. zolpidem and zopiclone) that do not actively enhance deep sleep, Gaboxadol lengthens episodes of deep sleep without altering sleep latency and REM sleep (Lancel, 1999). Animals were divided into four treatment groups (control + drug, control + vehicle, stress + drug, stress + vehicle). I, with the help of Sara Qutubuddin and Jelena Popovic, measured cognition, activity, behavioral distress, and corticosterone to determine gaboxadol's effect. The drug was administered daily 30 minutes prior to the onset of the inactive period. Restraint stress (3h/ day, 4 days) was used to model acute psychosocial stress. All animals were trained on the spatial cue task of the Morris water maze for three days immediately following restraint stress. The probe trial was conducted on the final restraint day after the termination of stress. Activity was monitored using Home Cage – Locomotor Activity (Accuscan Instruments) and the first five hours of the inactive and active periods were monitored daily.

Interestingly, the Gaboxadol appears to selectively work in young stressed animals. The drug had no effect on the cognition of the young control animals, but cognitive ability appears to have improved in the young stressed animals that

were administered the drug. While all young stressed animals experienced increased activity during the inactive period compared to control animals, gaboxadol was associated with a significant decreased activity during this period.

Consistent with prior data (Buechel et al., 2014), aged animals were hyporesponsive to stress in terms of cognition, but the drug did not appear to do anything to improve performance during the probe trial (Fig. 17A) as the aged animals' path length to the original platform location was unaffected. During acute and chronic PS, young stressed rodents have demonstrated cognitive deficits during the probe trial (Buechel et al., 2014). Interestingly, the young stressed animals that received Gaboxadol as an intervention improved their path length to the original platform location (Fig. 17B), suggesting that the drug was able to help restore stress-associated cognitive deficits. While the analysis is ongoing, it does appear that administering Gaboxadol at the onset of a new stress does help maintain stress resiliency in young animals and prevents stress-associated cognitive deficits. Unfortunately, this intervention did not seem beneficial to the aged animals. Similar to prior work, this highlights the differences in the stress responses in young and aged animals, and further suggests the need for age-specific interventions.

Figure 17. Probe Trial Following Acute Stress

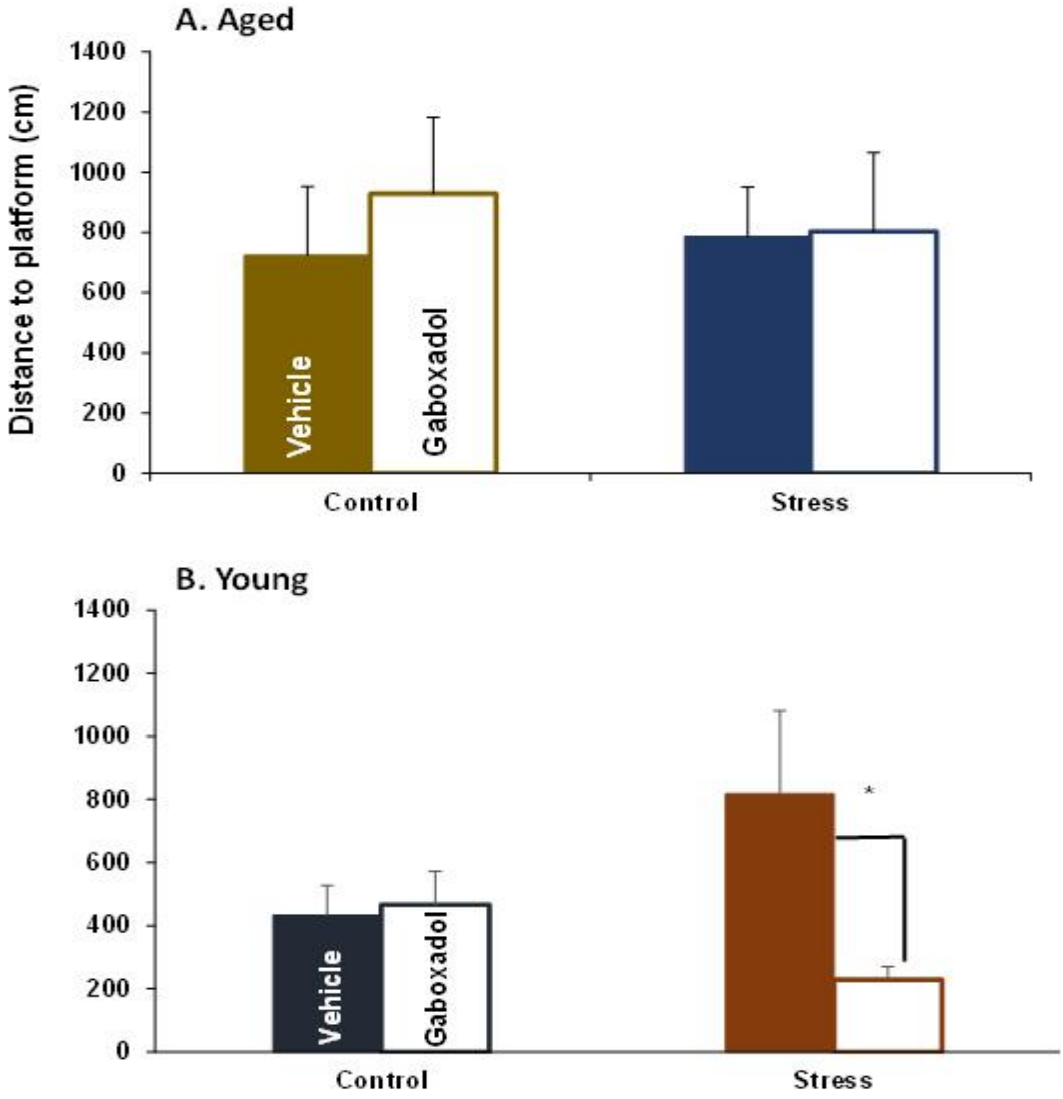

Figure 17 Probe Trial Following Acute Stress. The path length to the original platform location was recorded. A. The aged animals were hyporesponsive to the stress and drug intervention (Two-Way ANOVA Stress p = 0.962, Drug p = 0.778, Interaction p = 0.766). B. There was a trend towards a significant decrease in the path length of the young stressed animals receiving the drug intervention (Two-Way ANOVA: Stress p = 0.619, Drug p = 0.100, Interaction p = 0.056 (*Tukey's post hoc p = 0.015).

Chapter 5 Discussion

5.1 Summary of Introduction

Current research is quick to dedicate many resources to investigate the cause, and attempt to find a cure, for aging-related diseases, such as Alzheimer's disease. While that is important, especially in attempting to improve the quality of life for individuals suffering from those diseases, we should take a step back and look at the definition of aging. To summarize Hayflick's characterization of aging (2000), it is a phenomena that occurs in virtually all species after reaching reproductive maturity and is the result of the reduced capacity for cells to maintain their integrity. He goes on to eloquently argue that aging causes molecular disorder that increases the vulnerability to diseases and can ultimately lead to death in the aged population. To get to the bottom of this issue, researchers need to focus on the "molecular disorder" in the aging population in the absence of age-related disease—then we will begin to understand why the human population is living as long as it is (Hayflick, 2000).

The average human lifespan has remained 125 years (Hayflick, 2000), however, life expectancy in the United States was reported to be 79 years (Xu et al., 2016). The statement 'humans are living longer' is true, but what percentage of the population experiences good quality of life during the later portion of life? Additionally, how do humans increase the average life expectancy and is that something to even consider? Some interventions, such as caloric restriction

(Colman et al., 2009; Cox and Mattison, 2009; Mehta and Roth, 2009), resveratrol (Csiszar, 2011; de la Lastra and Villegas, 2005), and rapamycin (Blagosklonny, 2013; Harrison et al., 2009; Richardson, 2013) have been shown to increase maximal lifespan in animal models, but so far no one has unlocked a magical cure to aging.

Trying to increase human lifespan raises a very important argument between utopian and dystopian aging. The former describes improvements on health that essentially delay morbidity and disability until the end of one's life and ultimately improve one's quality of life (Schaie et al., 2013). The latter describes increasing life expectancy, but with a steadily worsening quality of life. The additional years added onto one's life are filled with chronic disease and the individual becomes increasingly less independent (Schaie et al., 2013). Advances in medical research and technology have vastly improved the lifespan and healthcare in developed countries. However, as the population lives longer, there is also an increased burden on society to care for these individuals because most are unable to care for themselves, suggesting dystopian aging. Some of the increased burden could be explained by the consequences of aging, such as neurodegeneration (Bombois et al., 2010; Hindle, 2010) preventing the elderly from maintaining their independence. However, not all the aging population experiences these consequences; in fact, some people experience what has

been termed “successful aging” and these people maintain the ability to care for themselves.

It is widely known that the aging population already experiences cognitive deficits (Erickson and Barnes, 2003; Klempin and Kempermann, 2007; Nithianantharajah and Hannan, 2009; Rosenzweig and Barnes, 2003; Whalley et al., 2004) and changes in sleep architecture (Ancoli-Israel and Alessi, 2005; Jaussent et al., 2013; Kryger et al., 2004; Naylor et al., 1998; Zepelin et al., 1972). There are also several potential factors that may increase the probability of a person to experience “unsuccessful aging,” such as genetics (Aviv et al., 2003; Franceschi et al., 2000), socioeconomic status (Adler et al., 1994; House et al., 1990), or social support (Rowe and Kahn, 1997). Another possibility could be exposure to stress and the resulting activation of the HPA axis in the attempt to physiologically re-establish homeostasis.

The HPA axis is a conserved physiological response to an exogenous stressor and is present in species, such as fish (Wendelaar Bonga, 1997), birds (Siegel, 1980) and mammals (Tsigos and Chrousos, 2002). This is also called the “fight-or-flight” response and functions to promote survival and re-establish homeostasis (Chrousos and Gold, 1992). Briefly, the hypothalamic-pituitary-adrenal (HPA) is activated to release glucocorticoids, epinephrine, and norepinephrine (Tsigos et al., 2000). These hormones participate in actions, such

as diverting energy away from non-essential muscles and regulating the stress response (Tsigos and Chrousos, 2002), that allow for the maximal chance of survival. Several researchers have argued that stress can be described as hormetic (Calabrese et al., 2012; Gems and Partridge, 2008; Rattan, 2001); it can be beneficial, but prolonged exposure or increased intensity can be harmful.

Not only does exposure to new onset stress have its own consequences, but the probability of experiencing new onset stress increases with age and may exacerbate the existing consequences of aging (Epel et al., 2004; Sapolsky, 1999). The allostatic load hypothesis posits that the accumulation of stress responses over time can accelerate brain aging and lead to a hyper- or hypo-response of the HPA axis (McEwen, 1998a). McEwen and colleagues describe the consequences of increased allostatic load and include suppressed neurogenesis and synaptic and dendritic remodeling (McEwen, 2000a) which lead to cognitive deficits. Ongoing allostatic load can lead to prolonged exposure to the stress hormones epinephrine, norepinephrine, and cortisol (Juster et al., 2010; McEwen, 2006). More specifically, the increased secretion of cortisol can not only lead to symptoms resembling Cushing ’s disease (McEwen, 2008), but can begin to induce an aged-like brain profile in the young (Sapolsky, 1999). In addition to cognition and hormones, allostatic load can also influence sleep (Karatsoreos and McEwen, 2011) and body temperature (McEwen, 2003). Despite the extensive research already performed in this area, there still remains

a deficit in studies looking at the effects of new-onset stress on existing allostatic load as hypothesized in aging.

Sleep plays a role in memory processing (Kushida, 2012; Marshall and Born, 2007; Sejnowski and Destexhe, 2000; Tononi and Cirelli, 2006, 2012; Walker, 2009), energy conservation (Berger and Phillips, 1995; Walker and Berger, 1980), metabolism (Leproult and Van Cauter, 2010; Spiegel et al., 1999) and physical restoration (Adam and Oswald, 1977; Kushida, 2012). Additionally, deep sleep is thought to play a role in stress resiliency, as it inhibits the HPA axis and the sympathetic system (Basta et al., 2007; Brand et al., 2014; Meerlo et al., 2008; Sadeghi Bahmani et al., 2016). Unfortunately, gerontology research shows that elderly individuals have disrupted sleep architecture (Espiritu, 2008; Foley et al., 1995; Wolkove et al., 2007) and a loss of deep sleep (Carskadon and Dement, 2005; Mathias et al., 2001b), suggesting a dampened ability to manage stress.

5.2 Summary of Methods

5.2.1 Psychosocial Stress

Psychosocial stress results from a non-noxious stimulus (Fink, 2009), therefore any method I used to stress the animals could not be classified as a physical stressor (e.g. exercise, cold stress, foot shock, etc.). While there are several methods to induce psychosocial stress (e.g. strobe, water avoidance), I chose

restraint stress because this method was used in prior studies (Buechel et al., 2014) from my lab and I wanted to reduce variability between studies.

Compared to rats, humans have a highly developed prefrontal cortex, giving them the ability to reminisce and anticipate their stressors (Ongur and Price, 2000; Uylings et al., 2003), turning an acute stressor into chronic stress. To model chronic psychosocial stress in rodents, the restraint protocol was repeated daily, always avoiding the rats' inactive period. However, in the literature, there is a variation in the duration (Buynitsky and Mostofsky, 2009; Jackson and Moghaddam, 2006; Kang et al., 2007; Luine et al., 1996) of the restraint protocols. I chose a duration of three hours as an optimal duration because the animals have plenty of time to mount a response to the stressor and the duration is short enough to minimize other stressors like food/ water/sleep deprivation. I made sure to stress all animals during their active period to reduce the contributions of sleep deprivation.

5.2.2 Cognitive Measures

The Morris water maze was used in both studies to assess cognition in the animals. This method requires the animals to use external cues to find a submerged platform (Morris, 1984) and has been previously used in aging studies (Carter et al., 2009; Frick et al., 2003; Latimer et al., 2014; Ma et al., 2014; van Praag et al., 2005; Yau et al., 2002; Zyzak et al., 1995). While other cognitive tasks such as delayed match to sample and avoidance are appropriate

to measure cognition, the amount of time to train animals, the introduction of a noxious stimuli, and methods for motivation to learn and perform the task were not feasible for my studies.

5.2.3 Sleep Monitoring

Surgically implanted wireless telemetry devices (DSI International) were used to monitor sleep architecture and body temperature in both studies. Because animals were not tethered by wires, I could easily restrain them to model stress as well as put them in the water maze to analyze cognitive abilities. I also had the capability of continuously collecting temperature data without introducing an additional stressor. The continuity of data collection provided a more accurate picture of the temperature response to psychosocial stress throughout the entire inactive period instead of a couple of time points.

5.2.3 Stress Hormone Measures

Studies have demonstrated sustained elevation of corticosterone after the termination of stress (Akerstedt, 2006; Buechel et al., 2014; Kuhlmann et al., 2005; Lupien et al., 2009). I was more interested in the implications of prolonged elevation of corticosterone, especially in terms of allostatic load. Therefore, trunk blood was collected at the end of the study and corticosterone levels were analyzed.

I had two aims in this work: 1) to investigate the influence of acute psychosocial stress on sleep, cognition, body temperature, and blood hormone levels in mid-aged male rats and 2) to investigate the influence of chronic psychosocial stress on the same measures in young and aged male rats. To complete this, I used the approaches described above to develop a thorough understanding of how psychosocial stress was influencing the animals.

5.3 Summary of Hypotheses

In the first study, I hypothesized that the stress response of the mid-aged animals would be intermediate between the response of young and aged animals. The main focus of this project was to determine the influence of psychosocial stress in different age groups. Prior work (Buechel et al., 2014) provided a good foundation of the acute stress response in young and aged animals, however there is a lack of research of the acute stress response in mid-aged animals. Therefore, in the first study, I aimed to fill that knowledge gap by investigating this response in mid-age animals. Mid-age is a key transition period from young to aged and this age population could hold important information about the transition from healthy to unhealthy brain aging.

While acute stress gave us a nice snapshot of the stress response in all age groups, stressors humans typically encounter are thought to be more chronic in nature. This is mainly due to the anticipation and/or rumination of a singular stressor. Thus, the second study investigated the influence of chronic

psychosocial stress in young and aged rats. Here, I hypothesized that aged animals would continue to be hyporesponsive to chronic psychosocial stress.

5.4 Summary of Hypothesis Tests

Previous work in our lab provided a foundation of the acute psychosocial stress response in young and aged animals (Buechel et al., 2014). Our lab found that while young animals demonstrated a response to acute stress, resulting in cognitive deficits, deep sleep loss, hyperthermia, and elevated corticosterone, aged animals were hyporesponsive. I tested the same parameters in mid-aged subjects. Just like the young, the mid-aged experienced cognitive deficits and hyperthermia. Similar to the aged, the mid-aged showed no changes to their inactive period deep sleep. Interestingly, we did note that transport before end measurement collection actually stressed our animals (see Supplemental Data 4.2). This provided very useful information for future studies about the necessity of a “cooling off” period before blood collection.

In our second study, we chronically stressed young and aged animals to model the influence of chronic psychosocial stress. While aged animals maintained their hyporesponsiveness to chronic stress as they did with acute stress, interestingly, the young animals developed a blunted response in some classic measures of the stress response. The young still suffered from cognitive deficits, but by the termination of the chronic stress, their sleep architecture, body temperature, and corticosterone levels were unaltered.

5.5 Theoretical Implications

The results of my work have raised several questions, as well as have implications that are outside the original scope of the studies.

5.5.1 Age-related sleep changes may be early critical changes in the hyposensitive stress phenotype of age

I demonstrated mid-aged animals did not show changes to their sleep architecture following acute psychosocial stress (see Mid-aged Acute Stress 2.3.2), when compared to young animals (Buechel et al., 2014). On the other hand, the mid-aged animals did show post-stress hyperthermia and cognitive deficits. Hyperthermia is an established consequence of stress (Kataoka et al., 2014; Morimoto et al., 1993), however the exact mechanism and reason why this effect is lost in the aged population is still unclear.

It is that possible stress-induced hyperthermia occurs via prostaglandin E_2 (PGE_2) signaling in the preoptic area of the hypothalamus from stress-induced elevations of glucocorticoids (Morimoto et al., 1991; Oka et al., 2001; Vellucci and Parrott, 1995). It has been demonstrated that increased c-Fos expression in the median preoptic nucleus can cause significantly elevated temperature. However, the rise in temperature can be blocked by administering indomethacin, which prevents PGE2 synthesis (Morimoto et al., 1991; Vellucci and Parrott, 1995). While we did not observe sustained elevations in corticosterone (18 hrs post-stress), it is probable that glucocorticoids were elevated during and

immediately following the restraint stress, leading to the increase in c-Fos expression.

Another possibility is through stimulation of brown adipose tissue (BAT) via stress-induced increases of corticotropin releasing factor (CRF) (Morimoto et al., 1993; Nakamori et al., 1993; Oka et al., 2001; Watanabe et al., 1990). CRF activates the SNS, leading to increases in norepinephrine, epinephrine, and glucose (De Souza, 1995; Fisher and Brown, 1991). Interestingly, consistent with our findings, the response to CRF in aged subjects is blunted (De Souza, 1995; Hylka et al., 1984); researchers point towards a decrease in the CRF receptor density to explain the dampened response. While controversial, some studies show elevated basal levels of glucocorticoids in the aging population (Lupien et al., 1998; Sapolsky, 1992). Sapolsky et al (1983) suggests that aged-related increases in glucocorticoids may contribute to the blunted CRF response.

Given these possible mechanisms to explain the hyperthermia, the young and mid-aged rodents were the only age groups to experience hyperthermia and it was only present during acute stress. Brown adipose tissue has been shown to not only decrease with age, but to also have impaired function with increasing age (McDonald et al., 1988; Norman et al., 1985; Saely et al., 2012). Another possibility is that the temperature elevation seen in the young and mid-aged

animals is working through a completely different mechanism and that mechanism becomes impaired/ blunted with increased age.

5.5.2 Aging acts as a stressor and occludes additional stressors

Stress (McEwen, 1998a; Sapolsky, 1996) and aging (Lupien et al., 1998; Porter and Landfield, 1998; Roth, 1974) can alter the concentration of glucocorticoids. Long-term exposure to stress stimuli leads to dendritic atrophy and cognitive impairments. These impairments were seen in aged control and young stressed animals in my study, indicating that the additional exogenous stress does not further impact aged animals' cognition, but does so in the young. Our work supports the hyporesponsiveness McEwen (1998a) described in the allostatic load hypothesis.

This hypothesis shows that chronic stress can result in remodeled dendrites in tree shrews (Magarinos et al., 1996), rats (Conrad et al., 1996; Galea et al., 1997; Watanabe et al., 1992), and primates (Uno et al., 1994). Mechanistically, the increased glucocorticoids cause an increase in extracellular glutamate (Moghaddam et al., 1994). This could potentially cause a downregulation in the glutamate receptors and contribute to decreased dendritic branching (Magarinos and McEwen, 1995). While we did not specially focus on dendritic branching, it is tempting to speculate that this happened to our chronically stressed young animals.

5.5.3 Stress-induced cognitive dysfunction could be a result of leaky neuronal ryanodine receptors

Ryanodine receptors (RyR) help regulate the release of intracellular calcium stores (Kostyuk and Verkhratsky, 1994; Spacek and Harris, 1997). Mechanistically, calstabin2 (FKBP1b/FKBP12.6) binds to and stabilizes RyR2 channels and prevents calcium leak from intracellular stores. PKA mediated phosphorylation and oxidation/nitrosylation (Yuan et al., 2014) modifications to the RyR2 channel after stress reduce the RyR2 channel's affinity for FKBP12.6, resulting in 'leaky' RyR2 channels and increased intracellular Ca^{2+} (Bellinger et al., 2008; Liu et al., 2012; Shan et al., 2010). RyR1 and RyR2 are also found in the hippocampus, with RyR2 found to be significantly upregulated after cognitive training (Cavallaro et al., 1997; Zhao et al., 2000).

It has been documented that the ryanodine receptor channels of chronically stressed mice showed significantly higher open probabilities than the non-stressed mice or the RyR2-S2808A$^{+/+}$ mice (Liu et al., 2012). This also led to significantly worsened cognition in the stress animals compared to the controls, suggesting a role for these channels in the cognitive deficits of our chronically stressed young animals. Interestingly, mice treated with a compound S107, which has been found to prevent a leaky RyR2 channels (Bellinger et al., 2008), have reduced stress-induced calcium leak and improved cognition (Liu et al., 2012). We did not specifically investigate the influence of chronic stress to the

ryanodine receptor channels. It is possible we are seeing this happen in the young animals and it would be a possible avenue in the future to investigate mechanistically the role chronic stress has in ryanodine receptor channel modifications (if any).

The long-standing calcium hypothesis of aging posits that neuronal calcium becomes dysregulated with age, leading to an increase in intracellular calcium associated with cognitive deficits in aging and neurodegeneration (Berridge, 2010; Khachaturian, 1994; Landfield, 1987a; Landfield and Pitler, 1984). Considering RyR2's role in leaky calcium channels in chronically stressed animals, RyR2 could play a role in the increased intracellular calcium in aged subjects. In fact, it has previously been reported that disrupting calstabin2 with small interfering RNA induces the calcium dysregulation seen in the calcium hypothesis of aging (Gant et al., 2014; Gant et al., 2011). If ryanodine receptor channels are already disrupted with age then it could not only explain the age-related cognitive deficits, but also explain why the aged animals were hyporesponsive to new onset stressors in my studies.

5.5.4 The timing of the stressor is important to memory recall

There are three phases of memory: acquisition (acquiring the information), consolidation (processing/storing the information), and recall (the ability to remember the information) (Marshall and Born, 2007). Activation of the HPA axis, and thus elevated glucocorticoids, at the appropriate time in a physiologically

normal system has been shown to contribute to the learning and memory consolidation process (Wolf, 2003). For instance, exposure to a stressor immediately following a training exercise can actually enhance consolidation (Cordero and Sandi, 1998; Ferry et al., 1999; Flood et al., 1978; Roozendaal and McGaugh, 1996), indicating that acute stress in the proper context can be beneficial.

Whilst beneficial, glucocorticoids can also have a negative effect on the same process. Stress prior to a memory recall task hinders the subject's ability to recall the information, both in humans (Domes et al., 2004; Kuhlmann et al., 2005; Sandi and Pinelo-Nava, 2007) and in animals (de Quervain et al., 1998; Diamond et al., 2006; Sandi and Pinelo-Nava, 2007). Our animals were stressed immediately prior to acquisition as well as recall (Chapters 2 and 3). While the young animals did demonstrate the ability to learn the spatial cue task (based off the improvement each day), the elevated glucocorticoid levels they experienced during the restraint immediately prior to recall clearly impacted their ability to recall the position of the platform. This does raise a very good question about the impact that stress immediately prior to acquisition has on recall. While the animals were stressed during the acquisition period, we cannot comment about the effects other than the animals still possessed the ability to learn the task.

5.5.5 Aging has Benefits

While aging does have many negative consequences, there are some benefits to getting older. For example, Salthouse points out that older individuals score higher on vocabulary and comprehension tests (Salthouse, 2004). Carstensen et al., (1999) argue that the older population have higher emotional reasoning skills than their younger counterparts. To add to this, other researchers have indicated that older adults have better understanding of various emotional states (Carstensen et al., 1999; MacKay and James, 2001), allowing them to more successfully control their negative emotions (Carstensen et al., 1999; Lawton et al., 1992). Thus, one challenge may be to address the cognitive losses associated with aging, while retaining aging's benefits.

5.6 Limitations and Future Directions

While these studies accomplished the aims I originally set out to complete, the results opened up the realm to further investigate different aspects of stress, aging, cognition, and sleep. I was also able to determine how to optimize the execution of future studies using similar techniques, as well as new approaches (e.g. selective sleep stage suppression) to test the molecular underpinnings of these age-related changes.

5.6.1 Surgical implants

The wireless telemetry devices provided us with a wealth of data and a lot of possibilities for measurements (e.g. temperature, sleep architecture, sleep

power) that we would not normally be able to measure. Additionally, because they are wireless, we could subject our animals to restraint stress and water maze behavior tasks. That said, these implants did lengthen our study and limited the number of animals we had in each cohort. Due to surgery and recovery periods, an additional three weeks had to be added to each cohort's timeline. Also, because each animal had to have its own receiver for the emitter, we could only have eight animals per cohort.

To combat this in the future, I have been working with SignalSolution, LLC, a small biotech company co-founded by Dr. Bruce O'Hara, to investigate using piezo electronics to monitor sleep and activity non-invasively. This method uses a pad embedded with piezo electronics that is placed under the animals' cage. It is able to monitor the animals' breathing with such a degree of sensitivity that it may be possible to distinguish between the stages of sleep. Currently, I have used implanted animals together with the piezo pads to record sleep data. After a recording period of 24h, I analyze the data collected from the telemetry devices and send the results to the company so they can determine how well the data has matched up and make adjustments for future recordings. While the number of animals per cohort would be limited to the number of piezo pads, the time saved by eliminating surgery and recovery would afford us more time, as well as give us the ability to stack our cohorts more tightly and reduce surgery stress on the animals.

5.6.2 Sleep Stage Intervention

Though the telemetry devices add additional time to a study, they can be an asset for targeting specific stages of sleep for sleep stage interventions/ studies. I had the opportunity to work with biomedical engineers, specifically Dr. Sridhar Sunderam, to attempt to suppress deep sleep in young male F344 rats. Briefly, we used a vibrating pad that was programmed to vibrate whenever the computer detected the animal in deep sleep. The pad vibrated just enough to bring the animal out of deep sleep without waking the animal. The reasoning was to study seizures brought on by deep sleep suppression, but there is an application in aging as well. Because deep sleep is lost with aging (Bliwise, 1993; Espiritu, 2008; Zepelin et al., 1972), we could potentially manipulate the sleep architecture in young animals to model the consequences of deep sleep loss without using sleep deprivation.

5.6.3 Blood Sampling

In my studies, I collected trunk blood to measure sustained circulating levels of corticosterone 18-20h following stress. The reason behind this was because it was previously documented that corticosterone could remain elevated even after acute stress (Buechel et al., 2014). Having a baseline corticosterone measurement along with additional sampling throughout the study could provide valuable information, such as when the young animals began to show a blunted biochemical response to the restraint. Cannulas would allow for long-term blood sampling; however, these are not compatible with the water maze. However,

other hippocampal-dependent assessments, such as radial arm or Y-mazes could be used in place of the water maze to allow the use of cannulas.

5.6.4 Intervention Strategies

Sleep is important for learning and cognition (Borbely, 2001; Goerke et al., 2013; Marshall and Born, 2007; Stickgold, 2005) and can be disrupted by stress (Akerstedt, 2006). More specifically, deep sleep is thought to play a crucial role in not only memory processing (Marshall et al., 2006), but also stress resiliency (Brand et al., 2014; Sadeghi Bahmani et al., 2016). Pharmacologically promoting deep sleep may help preserve the young animals' ability to mount a proper stress response and therefore sustain cognition while facing an exogenous stressor. In the older animals, promoting deep sleep may help restore the ability to process new information as well as potentially alleviate some of the allostatic load they've accumulated from aging. This was recently studied in my lab using gaboxadol as a deep-sleep promoting agent. The data has been collected and the analysis is ongoing.

Other than promoting deep sleep, investigating the in RyR2 channel in aged subjects may help determine if these leaky calcium channels could help explain much of the negative consequences seen with age. The use of ryanodine receptor stabilizers or Rycals (e.g. S107) to help stabilize calstabin2's binding to RyR channels, or over-expressing calstabin 2 via viral injection, in young chronically stressed animals may help to elucidate their role. It would be

interesting to determine if promoting stable binding in these channels helps prevent or reverse the cognitive deficits and stress hyposensitivity seen with aging. It would also be beneficial to see the interplay between sleep and leaky RyR2 channel activity, as changes in sleep architecture are associated with both stress and aging.

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Vita

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EDUCATION

University of Kentucky
Ph.D. in Pharmacology and Nutritional Sciences **2016**

Eastern Kentucky University
B.S. in Forensic Science **2012**

AWARDS

SfN Travel Award, 2014
Graduate student fellowship, 2012
Three Minute Thesis Finalist- University of Kentucky College of Medicine 2014
Outstanding Poster Award 2015
Three Minute Thesis Participant- University of Kentucky College of Medicine 2015
SfN Travel Award, 2016

PRESENTATIONS

Abstract: Staggs K, Buechel HM, Popovic J, Blalock EM (2014) Acute psychosocial stress response in mid-aged male f344 rats. Soc. Neurosci. Abs #310.09. *This poster was also presented at the 2015: UK Research Day, Bluegrass SFN Research Day, IBS Orientation Research Day*

Abstract: Huffman D, Hargis K, Yaghouby F, Blalock E, and Sunderam S (2015) Feasibility of Selective Deep Sleep Restriction in Rats Using Mild Somatosensory Stimulation. Center for Clinical and Translational Science, Spring Conference UKCOM.

Abstract: Staggs K, Popovic J. Blalock EM (2015) Effects of chronic restraint on cognition and stress hormone level in young and aged rats. Soc. Neurosci. Abs.

Presentation: Acute psychosocial stress response in mid-aged male F344 rats (May 2015) Department of Pharmacology and Nutritional Sciences, UKCOM

Abstract: Staggs K, Popovic J. Blalock EM (2015) Effects of chronic restraint on cognition and stress hormone level in young and aged rats. Markesbery Symposium on Aging and Dementia

Abstract: Staggs K, Popovic J. Blalock EM (2016) Effects of chronic restraint on cognition and stress hormone level in young and aged rats. Midwest Graduate Research Symposium

Abstract: Staggs K, Popovic J, Qutubuddin Q, Blalock EM (2016) Aged Animals Appear Cognitively and Behaviorally Hyporesponsive to Chronic Restraint (Psychosocial Stress) Compared to Young. Bluegrass Society for Neuroscience

Presentation: Staggs K, Popovic J, Qutubuddin Q, Blalock EM (May 2016) Aged Animals Appear Cognitively and Behaviorally Hyporesponsive to Chronic Restraint (Psychosocial Stress) Department of Pharmacology and Nutritional Sciences, UKCOM

Presentation: Staggs K, Popovic J, Qutubuddin Q, Blalock EM (May 2016) Aged Animals Appear Cognitively and Behaviorally Hyporesponsive to Chronic Restraint (Psychosocial Stress) Department of Pharmacology and Nutritional Sciences, Barnstable Brown Diabetes and Obesity Center

Presentation: Staggs K, Popovic J, Qutubuddin Q, Blalock EM (June 2016) Aged Animals Appear Cognitively and Behaviorally Hyporesponsive to Chronic Restraint (Psychosocial Stress) Department of Pharmacology and Nutritional Sciences, 2nd Annual Postdoctoral Symposium, University of Kentucky

Presentation: Staggs K, Popovic J, Qutubuddin Q, Blalock EM (November 2016) Influence of drug intervention on acute stress (psychosocial stress) in young and aged rats. Markesbery Symposium on Aging and Dementia, University of Kentucky

PUBLICATIONS AND PAPERS

Buechel HM, Popovic J, Staggs K, Anderson KL, Thibault O, Blalock EM (2014) Aged rats are hyporesponsive to acute restraint: implications for psychosocial stress in aging. Front. Aging Neurosci. 6(13): 1-16

Hargis K, Blalock EM (2016) Transcriptional signatures of brain aging and Alzheimer's disease: What are our models telling us? Behav Brain Res [epub ahead of print] DOI: 10.1016/j.bbr.2016.05.007

Huffman* DM, Staggs* K, Yaghouby F, Agarwal A, O'Hara BF, Donahue KD, Blalock EM, Sunderam S (2016) Selective deep sleep deprivation without wake enrichment in F344 rats. EMBC DOI: 10.1109/EMBC.2016.7591028

Hargis K, Popovic J, Blalock EM (in review) Effects of acute restraint on cognition and stress hormone levels in mid-aged rats. Neurobiology of Aging

Hargis K, Popovic J. Blalock EM (in review) Effects of chronic restraint on cognition and stress hormone level in young and aged rats. Behavioral Brain Research

Hargis* K, Qutubuddin* S, Popovic J, Blalock EM (in prep) Influence of the deep sleep-promoting agent gaboxadol after acute restraint in young and aged rats.

Hargis K, Kim, H, Blalock EM (in prep) Effects of acute restraint during learning on cognition and stress hormone levels in young and aged rats.