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Rescuing Age-related Proteolysis Deficits with Methylene Blue

Shane E. Pullins
University of Wisconsin-Milwaukee

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by

Shane Pullins

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ABSTRACT

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by

Shane Pullins

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Under the Supervision of Professor Fred Helmstetter

The average lifespan is constantly increasing with the advent of new medical techniques, and age-related cognitive decline is becoming a prevalent societal issue. Even during healthy aging, humans and rats exhibit progressive deficits in episodic/declarative memory. In laboratory rats, age-related memory impairment can be assessed with trace fear conditioning (TFC). Recent research implicates ubiquitin proteasome system-mediated protein degradation in the synaptic plasticity supporting memory formation and retrieval. In rats, aging leads to decreased basal proteolytic activity in brain structures known to support the acquisition and retrieval of trace fear memories, and our preliminary data suggests activity-dependent proteasome activity declines in a similar fashion. The proposed experiments sought to rescue age-related decreases in plasticity-related protein degradation during memory consolidation via proteasome stimulation with the compound methylthioninium chloride (methylene blue [MB]). Intraperitoneal post-training MB administration at 1, 4, or 16mg/kg did not improve memory performance, chymotrypsin-like, or trypsin-like proteasome activity in young or aged rats in any of the four brain structures examined. Additionally, dietary treatment with MB for four months did not enhance memory, chymotrypsin-like, or trypsin-like proteasome activity in young or aged animals. These results suggest that MB may not be well suited to augment fear learning or to upregulate general proteasome activity. Future work should investigate other means of proteasome stimulation and subsequent rescue of cognitive decline during aging.

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INTRODUCTION

Normal Aging and Memory in Humans

Aging is common to all organisms; lifespans are finite, and typically a number of biological processes go awry with increasing age. The past century has yielded a remarkable increase in life expectancy, and one notable burden of this newfound longevity is a decline in cognitive ability (Hedden & Gabrieli, 2004). Senescence-related effects exist on a continuum ranging from pathological aging, wherein myriad pathologies including Alzheimer's and Parkinson's diseases may develop, to successful aging, wherein individuals do not display significant age-related deficits in cognitive ability (Rowe & Kahn, 1987). Most individuals qualitatively age somewhere between pathological and successful aging, in a pattern termed normal cognitive aging (Roberson et al., 2012). Even during normal cognitive aging, mnemonic function declines and these impairments often negatively affect societal function, independent living, and overall health (Petersen, Smith, Kokmen, Ivnik, & Tangalos, 1992). The proportion of the U.S. population aged over 65 years is projected to more than double by the year 2050 and one out of five individuals will be classified as aged (Ortman, Velkoff, & Hogan, 2014). With an increasing number of aged individuals, and subsequent increased prevalence of cognitive aging, our healthcare system will undoubtedly experience severe strain. As such, combatting age-related cognitive decline is one of the most important issues facing the neuroscientific community.

Normal aging does not globally affect mnemonic processes, rather, certain types of memory are more susceptible to age-related impairments (Drag & Bieliauskas, 2010). Memory can be broadly divided into four distinct memory systems including procedural memory, semantic memory, working memory, and episodic memory (Tulving, 1987). Procedural memory pertains to the acquisition and retention of behavioral skills, and seems to remain largely intact during normal aging, although age-related motor and coordination deficits may interfere with performance (Smith et al., 2005). Semantic memory, or "knowledge", involves the learning and retention of factual information, and results from the Berlin Longitudinal Study suggest that this

form of memory remains intact until ~90 years of age (Singer, Verhaeghen, Ghisletta, Lindenberger, & Baltes, 2003). Working memory, or the system that facilitates processing and mentally manipulating information over short periods of time, does seem to uniformly decline during normal aging (Gilinsky & Judd, 1994; Park et al., 2002). However, the focus of this proposal will be on episodic memory, which displays consistent and robust age-related deficits (L. Nilsson, Bäckman, & Erngrund, 1997).

The episodic memory system is responsible for the encoding of personal experiences and the conscious recollection of occurrences passed; as such, use of this memory system is largely dependent on contextual and spatial cues for proper recall of a previous episode (Nilsson, 2003; Tulving, 1987). Numerous reports suggest a global age-related decline in episodic memory performance, even during healthy aging (Craik & Rose, 2012; Koen & Yonelinas, 2014; reviewed in Rönnlund, Nyberg, Bäckman, & Nilsson, 2005). Specifically, healthy aging impairs episodic associative memory involving verbal, written, and pictorial cues (Cabeza et al., 1997; Craik & McDowd, 1987; Daselaar, Fleck, Dobbins, Madden, & Cabeza, 2006; Daselaar, Veltman, Rombouts, Raaijmakers, & Jonker, 2003; Monti et al., 1996; Naveh-Benjamin, 2000; Naveh-Benjamin, Hussain, Guez, & Bar-On, 2003; Provyn, Sliwinski, & Howard, 2007), and these deficits seem to consistently manifest after ~60 years of age. Some of the strongest evidence for a decline in spatial episodic memory in humans comes from an experiment in which participants of various ages were brought into a science exhibit center and allowed to examine a number of displays (Uttl & Graf, 1993). After exploring the center, one component of testing involved asking participants to place the various items they saw on a map. Performance on this component sharply declined after the 6th decade of life, adding support to the notion that, even in normally aging participants, episodic memory performance declines after age 60 (Uttl & Graf, 1993).

Collectively, it seems that normal aging is accompanied by an episodic, associative memory deficit wherein individuals have difficulty mentally binding elements or cues together in time (Golomb, Peelle, Addis, & Kahana, 2008; Monti et al., 1996) and/or space (Driscoll et al.,

2003; Newman & Kaszniak, 2000; Uttl & Graf, 1993). Together, these patterns of inability have problematic implications, which give impetus for appropriately modeling these forms of memory in laboratory animals for investigating and correcting age-related memory decline.

Animal Models of Memory and Aging

Fear conditioning is a powerful tool to model human episodic memory processes in nonhuman animals. As typically performed in rodents (which will henceforth be the focus of this proposal), this paradigm involves repeated pairings of an innocuous conditioned stimulus (CS) with an aversive unconditioned stimulus (UCS - footshock) in order to elicit a measurable fear response (e.g. freezing behavior). Following successful training, exposure to either the CS (a tone in the case of auditory fear conditioning) or the training context is sufficient to elicit freezing behavior in absence of the UCS allowing for an index of associative memory strength (Gilmartin & Helmstetter, 2010).

Numerous variants of Pavlovian fear conditioning have been characterized. Traditionally, in auditory fear conditioning the UCS is presented at the offset of the CS in a form of conditioning called "delay" fear conditioning (DFC). This training paradigm can model implicit memory in that it depends largely on subcortical structures like the amygdala (AMY) in rats and humans, and does not require sustained attention or contingency awareness in humans (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999; Clark & Squire, 1998; Knight, Nguyen, & Bandettini, 2006). In contrast, when the CS and UCS are separated in time by a stimulus-free "trace interval" (TI), in a paradigm deemed trace fear conditioning (TFC), the association between them is thought to weaken, as successful training requires a greater number of training trials (Beylin et al., 2001; Pavlov, 2010). Importantly, the inclusion of this interval is proposed to model explicit or episodic memory, as learning this association recruits additional brain structures that are known to support episodic associative memory in humans (Connor & Gould, 2016). Similar to DFC, successful TFC requires the AMY, but TFC also engages the hippocampus (HPC), prefrontal cortex (PFC), anterior cingulate cortex (ACC), and retrosplenial cortex (RSC) due to the presumed requirement for sustained attention during the trace interval

and contingency awareness (Gilmartin, Miyawaki, Helmstetter, & Diba, 2013; Han et al., 2003; Knight, Cheng, Smith, Stein, & Helmstetter, 2004; Kochli, Thompson, Fricke, Postle, & Quinn, 2015; Kwapis, Jarome, Lee, & Helmstetter, 2015; Kwapis, Jarome, Schiff, & Helmstetter, 2011; Quinn, Oommen, Morrison, & Fanselow, 2002; Steenland, Li, & Zhuo, 2012; Weike, Schupp, & Hamm, 2007). Finally, associative fear conditioning may also be conducted without the use of discrete cues in a paradigm called contextual fear conditioning (CFC), which engages a similar set of brain structures as TFC (Cowansage et al., 2014; Einarsson & Nader, 2012; Fanselow, 1980; Gilmartin & Helmstetter, 2010; Kwapis et al., 2015; Quinn, Loya, Ma, & Fanselow, 2005; Zelikowsky, Hersman, Chawla, Barnes, & Fanselow, 2014). These various forms of fear conditioning have yielded a greater understanding of normal memory formation and retrieval, but importantly, they have also been applied to the problem of age-related deficits in learning and memory.

The pattern of cognitive decline during healthy aging seems to be conserved across species (Erickson & Barnes, 2003). Moreover, this pattern seems to implicate dysfunction within brain structures critical to memory that comprise the medial temporal lobe (MTL - Barnes, 1998). Support for this comes from investigations of memory performance in tasks that are known to be MTL-dependent (Driscoll & Sutherland, 2005). Relative to young rats, aged rats exhibit deficits when tested on the Morris water maze in the fixed position task (which assesses spatial memory), the repeated acquisition task (which assesses spatial working memory), as well as in discrimination procedures (Driscoll et al., 2006; Frick, Baxter, Markowska, Olton, & Price, 1995; Gallagher, Burwell, & Burchinal, 1993). Importantly, these deficits are not due to general age-related performance effects, and seem to arise from specific hippocampal dysfunction (Driscoll et al., 2006; Gallagher et al., 1993). Similarly, age-related spatial memory impairments extend to deficits for contextual memory in CFC procedures, which are also sensitive to manipulations of the MTL (Houston, Stevenson, McNaughton, & Barnes, 1999; Moyer & Brown, 2006; Oler & Markus, 1998; Stoehr & Wenk, 1995; Ward, Oler, & Markus, 1999).

While aged rats seem to be impaired in navigation and fear conditioning paradigms that do not involve discrete cues (i.e. CFC procedures), an interesting dichotomy arises within cued auditory fear conditioning. Following DFC, aged rats display reduced memory for the training context, but normal memory for the training tone (Houston et al., 1999; Oler & Markus, 1998; Stoehr & Wenk, 1995; Ward et al., 1999). However, in TFC procedures, aged rats exhibit deficits to the training context *and* the training tone (McEchron, Cheng, & Gilmartin, 2004; Moyer & Brown, 2006; Villarreal, Dykes, & Barea-Rodriguez, 2004). Moyer and Brown (2006) examined this selective deficit in TFC and incorporated a control for differences in inter-stimulus interval lengths between the two training procedures. Aged rats were trained in either TFC, consisting of a 15s tone and a 30s TI prior to each footshock, or long-DFC, consisting of a 45s tone co-terminating with a footshock. They found that aged rats had no difficulty learning about the training tone in long-DFC procedures, relative to TFC. Additionally, they performed important control experiments to determine that this deficit in TFC was not due to decreased shock perception or differences in activity levels between young and aged rats (Moyer & Brown, 2006). Thus, aged rats display reduced associative memory for the tone and footshock only when the two are separated in time, a feature that makes this learning paradigm a good model of episodic memory. This finding corroborates the previously mentioned idea that MTL dysfunction in humans and rodents leads to deficits in memory processing.

The details of age-related MTL dysfunction are complicated but some specific cellular processes have been investigated. Numerous reports indicate a reduction in synaptic plasticity, especially within the HPC. One of the principle mechanisms underlying synaptic strengthening within the HPC is long-term potentiation (LTP). Hippocampal LTP is reduced with increasing age (reviewed in Burke & Barnes, 2006). Two main culprits in age-related aberrant LTP are imbalances in calcium homeostasis and N-methyl-D-aspartate receptor (NMDAR) dysfunction (reviewed in Foster & Kumar, 2002). Additionally, LTP impairments have been attributed to reduced surface expression of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subunit GluR1, reduced dendritic αCamKII expression, imbalances in

kinase/phosphatase activity, reduced spine density and atuophosphorylated CamKII, and reduced levels of CREB and phosphorylated CREB (Almaguer, Estupinan, Frey, & Bergado, 2002; Chen et al., 2015; Davis et al., 2000; Jouvenceau & Dutar, 2006; Long et al., 2009; Morris & Gold, 2012). Driscoll and colleagues (2006) examined the aging hippocampus in female rats crossed between F344 and Brown Norway hybrid strains. They found reduced hippocampal volume when normalized to intracranial volume and reduced neuron density in aged rats. Additionally, they quantified dividing cells with BrdU labeling, immature cells with DCX labeling, and cycling cells with Ki67 labeling – there were significant age-related reductions in all immunohistochemical analyses. Further, these reductions in cellular division and integrity of mitosis, correlated with age-related deficits in performance on Morris water maze tasks (described above). While their spectroscopy data examining metabolic health in the HPC suggests no age related decline in this particular strain of rat, other investigations using solid state high-resolution magic angle spinning nuclear magnetic resonance imaging have shown decreases in concentrations of various metabolites in Long Evans rats (Driscoll et al., 2006; Paban, Fauvelle, & Alescio-Lautier, 2010). However, it may be the case that metabolic deficits arise during periods of increased activity or stress, as would be the case during fear learning and subsequent memory retrieval (Galeffi, Shetty, Sadgrove, & Turner, 2015).

In conclusion, aged humans and aged rats exhibit memory deficits in tasks known to depend on the MTL. In tandem with declines in working memory, complex associative memory (episodic in humans, TFC in rats) seems to deteriorate with increasing age. Fear conditioning procedures like TFC in rats provide a good model for further investigation of aberrant cellular function in normal aging. Much is known about the neural mechanisms of HPC dysfunction with increasing age, but an exhaustive examination of age-related effects on cellular processing remains incomplete.

Protein Degradation and Memory

There is currently general agreement about the idea that synaptic plasticity related to forming new memories requires altered gene expression and *de novo* protein synthesis in neurons (Chen & Tonegawa, 1997; Helmstetter, Parsons, & Gafford, 2008; Kandel, 2001). More recently, attention has shifted to the role of protein degradation through the ubiquitin proteasome system (UPS) (Ehlers, 2003; Hegde et al., 1997; Mabb & Ehlers, 2010). Within this system, proteins are tagged with ubiquitin polypeptides and are subsequently degraded by the 26S proteasome complex, which is comprised of a proteolytic core (20S) and two 19S regulatory caps (Hegde, 2010). During periods of synaptic activity calcium influx through NMDARs increases CamKII signaling, which directly activates the proteasome by phosphorylating serine 120 on the RPT6 subunit of the 19S cap (Jarome, Kwapis, Ruenzel, & Helmstetter, 2013). This phosphorylation activates the proteasome in an ATP-dependent manner, and allows for the 26S complex to deubiquitinate, unfold, and degrade substrates (Jarome & Helmstetter, 2013).

Once the proteasome complex is activated, it contributes to synaptic destabilization which is critical for the consolidation of new memories and following the retrieval of old ones (Lee et al., 2008). Known targets of the UPS that contribute to synaptic destabilization include AMPAR-associated scaffolding proteins SHANK and GKAP (Hung, Sung, Brito, & Sheng, 2010; Lee et al., 2008). Once degraded, calcium permeable AMPARs can be dissociated from the post-synaptic density allowing for the insertion of calcium impermeable AMPARs, ultimately stabilizing long term potentiation and presumably memories (Henley & Wilkinson, 2013).

UPS-mediated proteolysis is required for the synaptic plasticity contributing to the formation of many different types of memories in structures including the AMY, DH, and PFC (reviewed in Jarome & Helmstetter, 2013). Within the AMY, fear conditioning leads to increased lysine 48 (K48)-linked ubiquitin tagging of SHANK and proteins involved in translational control, as well as increased proteasome activity evidenced chymotrypsin and trypsin-like 20S activity assays (Jarome et al., 2013; Jarome, Werner, Kwapis, & Helmstetter, 2011). Moreover,

post-training inhibition of the proteasome with the compound clasto-Lactacystin ß-lactone (ß-lac) impairs long-term memory formation in a manner similar to protein synthesis inhibition. The upregulation of UPS activity following consolidation occurs following auditory or contextual memory retrieval as well (Jarome et al., 2011). Interestingly, while inhibition of protein synthesis following memory retrieval (with anisomycin) normally produces amnesia, this effect can be rescued via co-inhibition of proteolytic activity – an effect that suggests UPS activity triggers the need for protein synthesis and directly regulates the destabilization of memory traces.

The UPS-mediated processes in the AMY seem to occur in other brain structures as well. Inhibitory avoidance training increases ubiquitination of high molecular weight proteins in the hippocampus four hours after training (Lopez-Salon et al., 2001). In line with this, proteasome inhibition in the CA1 of DH one hour, four hours, or 7 hours following inhibitory avoidance training impairs memory. Additionally, following memory retrieval amnestic effects of anisomycin may be mitigated via co-infusion of a proteasome inhibitor, suggesting there are conserved mechanisms of plasticity between the AMY and the HPC (Lee et al., 2008). Similar to the AMY and HPC, Reis and colleagues (2013) found that UPS involvement in mnemonic processes is not limited to subcortical structures, as proteolysis in the PFC is critical for trace fear memory formation. Following TFC, the PFC displays increased ubiquitination on a similar time scale as that of the AMY (Reis et al., 2013).

With regard to contributions of the UPS to the plasticity required for TFC in particular, there is only one investigation published to date Reis and colleagues (2013). However, given the previously documented role of the AMY and the HPC in TFC, it is likely that UPS-mediated degradation contributes TFC in these structures (reviewed in Jarome & Helmstetter, 2014). Additionally, pilot data from an experiment wherein ß-lac was infused into the AMY of F344 rats prior to TFC suggests that amygdalar UPS activity is necessary for learning in the trace procedure as well (Figure 1). There was no difference in pre-training freezing between the two groups ($t=0.436$ $df=7$, $p=0.67$), and there was a modest, but significant, increase in post-training freezing in animals that received ß-lac ($t=2.515$ $df=7$, $p=0.04$; Figure 1a). Proteasome inhibition

impaired memory formation as evidenced by a reduction in freezing at retrieval during the average of the two CS presentations (t=2.139 df=7, *p=0.06*), during the average of the two TIs (t=3.727 df=7, *p<0.01*), and during the average of the two ITIs (t=5.391 df=7, *p<0.01*; Figure 1b). Thus, it seems that neurons within these brain structures that are critical to TFC undergo plastic changes during learning, and these cell-level changes depend on the UPS.

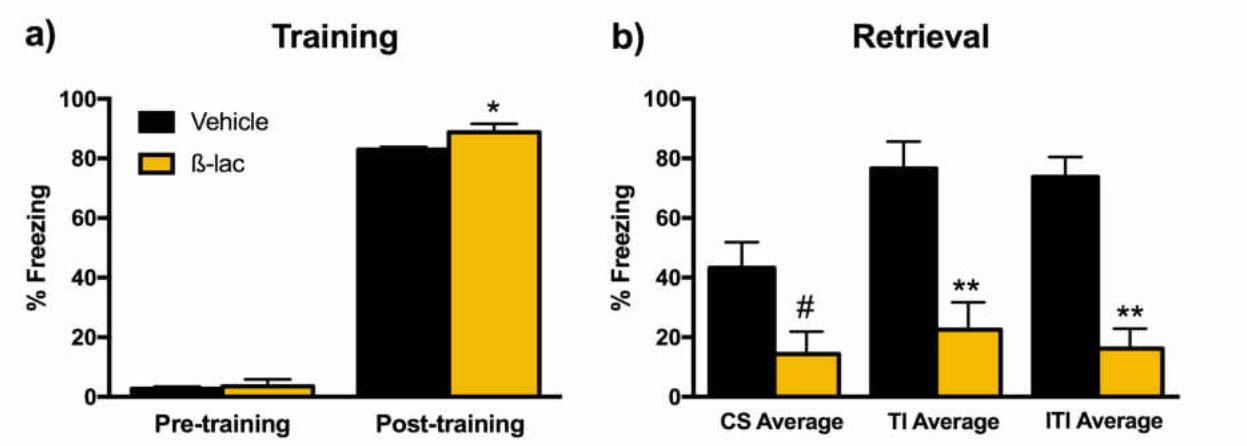

Figure 1. Proteasome inhibition in the BLA of young animals prior to TFC impairs memory for the training tone. **a)** There was no difference in pre-training freezing, and there was a modest, but significant, increase in post-training freezing in animals that received ß-lac. **b)** In animals that received ß-lac, there was a trend for a reduction in CS freezing, and a significant reduction in TI and ITI freezing. *#p=0.0697, * p<0.05, ** p<0.01*. CS, Conditioned Stimulus; TI, Trace Interval; ITI, Intertrial Interval.

Age-related Changes in UPS Activity

One of the hallmarks of aging is a loss of proteostasis, or an imbalance in the interplay between protein synthesis and protein degradation (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013; Martinez-Vicente, Sovak, & Cuervo, 2005; Saez & Vilchez, 2014). This imbalance, initially reviewed more than 30 years ago, usually tips towards an age-related decrease in clearance of damaged, misfolded, or normally short-lived proteins resulting in their accumulation (Ding & Zhu, 2015; Löw, 2011; Makrides, 1983). Age-related decreases in basal, or homeostatic, proteasome activity have been characterized in a number of bodily tissues including adipose, kidney, liver, heart, and lung (Baraibar & Friguet, 2012; Keller, Hanni, & Markesbery, 2000). These decreases in basal proteasome activity are not limited to somatic tissues; they also manifest in aging areas of the central nervous system including the cerebral

cortex, HPC, spinal cord, and cerebellum (Chondrogianni, Sakellari, Lefaki, Papaevgeniou, & Gonos, 2014; Chondrogianni et al., 2015; Giannini et al., 2013; Keller et al., 2000).

There is much debate regarding whether the *activity* of 20S or 26S proteasome complexes is reduced independently from the *capacity* for degradation. Some work from Giannini and colleagues (2013) suggests that, in Sprague Dawley rats, aged 20S and 26S proteasomes may be decreased in their activity when quantified via fluorogenic substrate degradation (e.g. chymotrypsin [Suc-LLVY-AMC] or trypsin [Bz-VGR-AMC]), but not when tested in a way that measures their capacity in requiring the 19S regulatory cap to deubiquitinate and unfold the substrate prior to degradation. However, it should be noted that the authors of that study did not sufficiently justify the reduction in aged proteasomal activity they observed when using the most commonly used proteasome activity assays in the field, and instead focused on the artificial substrate they designed to be more physiologically relevant. One other investigation of GFP reporter mice claims no age-related impairment in proteasome function, however the authors never directly test proteasome activity and instead quantify UPS-related mRNA and protein levels during aging (Cook et al., 2009). It may be the case that regulation of 20S core activity, either by means of 19S input or an age-related attenuation of 20S catalytic properties, underlies the frequently observed loss of proteostasis with increasing age. Indeed, transgenic mice engineered to display global reduced chymotrypsin proteasome activity have reduced life spans and develop age-related phenotypes suggesting that an age-related reduction in proteasome activity is likely involved in cellular and organismal senescence (Tomaru et al., 2012).

While substantial evidence exists for a reduction in homeostatic proteasome activity in the central nervous system with increasing age, no studies to date have investigated age-related changes in UPS function contributing to the synaptic plasticity underlying learning and memory, also known as *activity*-related UPS activity. If the deficits in basal proteolysis extend into activity-dependent proteolysis, then age-related impairments in UPS function could be responsible for memory decline during normal aging. In order to directly test this, we trained young (3 months) and aged (22 months) F344 rats in TFC and brought them back for a retrieval

session the following day. The two age groups showed no differences in freezing during the training session either before (t=0.1974 df=18, *p=0.84*) or after the tone-shock pairings (t=0.1845 df=18, *p=0.85*; Figure 2a). At retrieval, however, aged rats showed reduced CS freezing (t=2.678 df=18, *p=0.02*), TI freezing (t=9.246 df=18, *p<0.01*), and ITI freezing (t=6.968 df=18, *p<0.01*; Figure 2b). All rats were sacrificed 90 minutes following TFC retrieval for western blot analysis of activity-related UPS function, as this time point has been shown to maximally activate the UPS following auditory memory retrieval (Jarome et al., 2011). The amygdala and dorsal hippocampus were dissected and crude synaptosomal fractions were obtained from both structures as previously described (Jarome et al., 2011). Within the amygdala, western blotting revealed decreased levels of phosphorylated RPT6 at Serine 120 (t=2.381 df=17, *p=0.02*; Figure 2c), no differences in total RPT6 (t=2.002 df=17, *p=0.06*; Figure 2d), increased K48-linked ubiquitin levels (t=2.305 df=17, *p=0.03*; Figure 2e), and no differences in total actin (t=1.642 df=17, *p=0.12*; Figure 2f). This pattern of decreased UPS activation in aged rats following memory retrieval was also present in synapses of the dorsal HPC, where we observed decreased phosphorylated RPT6 (t=2.028 df=17, *p=0.05*; Figure 2g), no differences in total RPT6 (t=0.0076 df=17, *p=0.99*; Figure 2h), increased levels of K48-linked ubiquitin (t=2.704 df=17, *p=0.01*; Figure 2i), and no differences in actin (t=1.17 df=17, *p=0.25*; Figure 2j). These results suggest decreased activation of the UPS following memory retrieval, evidenced by decreased phosphorylated RPT6 and decreased clearance of K48-linked ubiquitin, accompany the age-related memory deficit observed in aged rats. Thus, stimulation of this activity-dependent UPS activity underlying memory formation and retrieval may serve as a novel and therapeutic way to treat cognitive decline in normal aging.

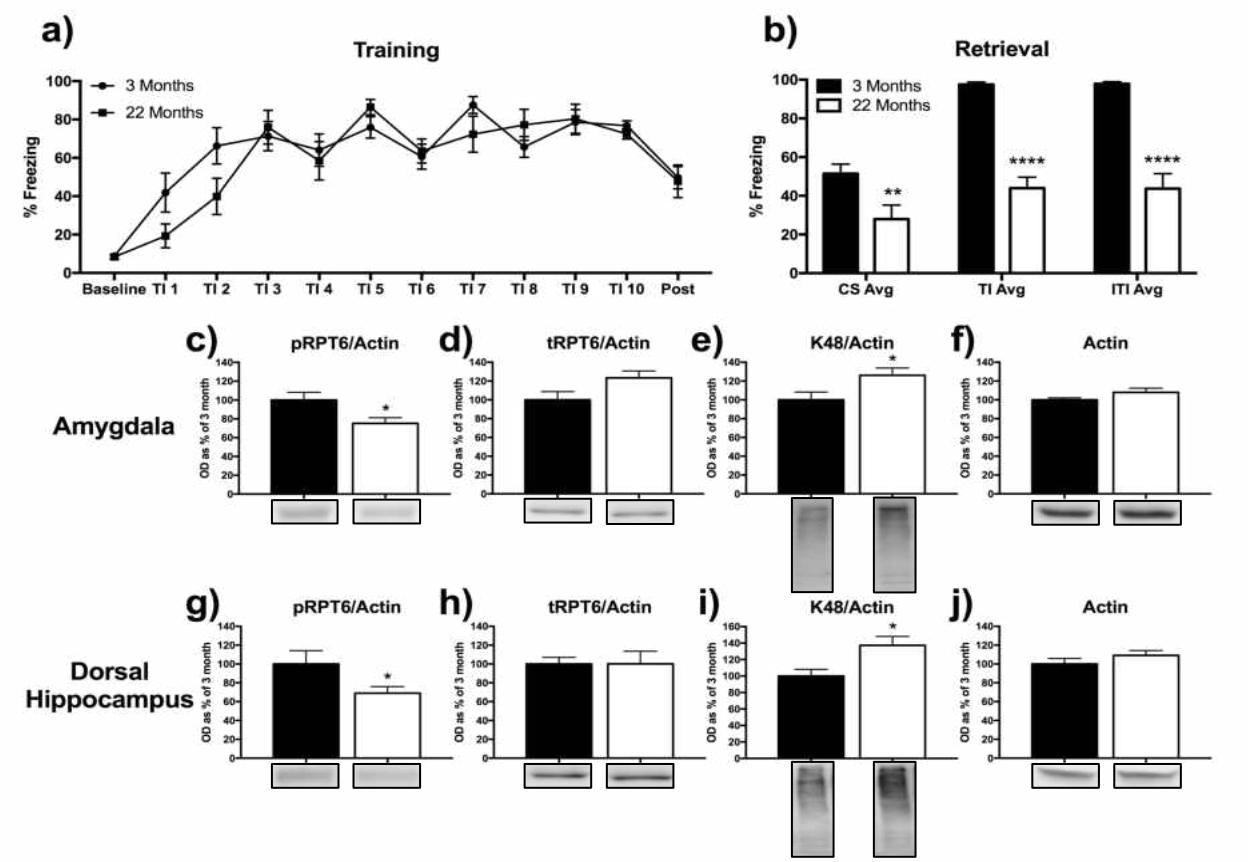

Figure 2. Aged rats are impaired in TFC and display reduced UPS activation following memory retrieval. **a)** There were no differences in freezing between age groups either before or after TFC. **b)** Aged rats displayed reduced CS freezing, TI freezing, and ITI freezing. **c-f)** In the synaptic fraction of the amygdala, aged rats show reduced levels of phosphorylated RPT6 (S120), no differences in total RPT6, increased levels of K48 linked ubiquitin, and similar levels of total actin relative to young rats. **g-j)** In the synaptic fraction of the dorsal hippocampus, aged rats have reduced levels of phosphorylated RPT6 (S120), no differences in total RPT6, increased levels of K48 linked ubiquitin, and similar levels of total actin relative to young rats. *$p<0.05$, **$p<0.01$, ****$p<0.0001$. CS, Conditioned Stimulus; TI, Trace Interval; ITI, Intertrial Interval.

Methylene Blue and Proteasome Activity Upregulation

One potential way of stimulating proteasome activity, and rescuing the aforementioned age-related decline, is via methylthioninium chloride or methylene blue (MB), administration (Medina, Caccamo, & Oddo, 2011). MB is an FDA-grandfathered tricyclic phenothiazine that was first synthesized in 1876 as a textile dye, and has since been used as a redox indicator in chemical reactions, a supravital/neuroanatomical stain, and a cancer chemotherapy agent (Peter, Hongwan, Kupfer, & Lauterburg, 2000; Wainwright & Crossley, 2002). Additionally, MB is effective in treating disorders ranging from methemoglobinemia, urinary tract infections, malaria, mental disorders (e.g. schizophrenia), and hypoxic effects on the central nervous system

resulting from cardiac arrest (Bruchey & Gonzalez-Lima, 2008; Howland, 2016; Oz, Lorke, Hasan, & Petroianu, 2010; Schirmer, Adler, Pickhardt, & Mandelkow, 2011).

A large portion of MB's therapeutic effects can be attributed to its unique auto-oxidizable properties conferred by the presence of a thiazine ring system and an imine group (Wainwright & Crossley, 2002). The thiazine ring system allows for a high reduction potential in the presence of oxygen, while the imine group confers antioxidant properties. Combined, these functional groups allow MB to participate in electron cycling without incurring any lasting changes in its net reduction (Schirmer et al., 2011). This participation in electron cycling allows MB to aid in aerobic respiration, particularly during periods of increased bioenergetic demands, stress, or disease (Rojas, Bruchey, & Gonzalez-Lima, 2012).

Medina and colleagues (2011) administered MB to 3xTg-AD transgenic mice, which develop age-related accumulation of Aß and tau and cognitive decline, for four months in their diet. Twenty-five milligrams of MB was mixed with every 100 milligrams of food powder, and the mice had access to food *ad libitum*. Unfortunately, this method lacks specificity in terms of dosage, but Rojas and colleagues (2012) calculated an approximate dose of 30mg/kg in this experiment. Nonetheless, MB treatment successfully prevented the strain-associated decline in performance on the spatial reference version of the Morris water maze task. Additionally, MB treatment reduced the accumulation of Aß, but not hyper-phosphorylated tau. While previous work suggests MB upregulates mitochondrial function, four months of dietary MB administration does not affect mitochondrial function in any way (Bruchey & Gonzalez-Lima, 2008; Medina et al., 2011). MB treatment did not alter amyloid precursor protein processing either, which lead the authors to hypothesize that MB-mediated prevention of Aß accumulation must occur after Aß has been produced, directly implicating the UPS in clearance of Aß. Interestingly, mice that were administered MB displayed higher levels of trypsin-like and chymotrypsin-like, but not caspase-like, 20S proteasome activity in whole brain homogenates (Medina et al., 2011). Thus, dietary MB administration significantly upregulates two of the three types of proteasome activity that are inherent to the UPS, and could possibly rescue the deficits

reported in Figure 2, although the brain structures and cellular compartments in which proteasome activity is upregulated remain to be elucidated.

While there is only one study to date on MB and proteasome upregulation, a number of other investigations have demonstrated a role for MB in enhancing mitochondrial function and general memory performance (reviewed in Rojas et al., 2012). Collectively, these studies revealed that MB displays hormetic properties wherein very low doses do not affect cellular or behavioral processes and high doses actually produce deficits in mitochondrial function and behavior. The vast majority of these investigations employed acute intraperitoneal (IP) MB administration. The first investigation to document MB-mediated memory enhancement showed a 1mg/kg IP injection immediately after inhibitory avoidance conditioning enhances retention of the avoidance memory (Martinez, Jensen, & Vasquez, 1978). A number of other studies show acute and low (i.e. 1-4mg/kg) doses of MB enhance memory in holeboard spatial search appetitive tasks, conditioned fear extinction, object recognition, open field habituation, and discrimination learning (Callaway, Riha, Bruchey, Munshi, & Gonzalez-Lima, 2004; Callaway, Riha, Wrubel, McCollum, & Gonzalez-Lima, 2002; Gonzalez-Lima & Bruchey, 2004; Riha, Bruchey, Echevarria, & Gonzalez-Lima, 2005; Riha, Rojas, & Gonzalez-Lima, 2011; Telch et al., 2014; Wrubel, Barrett, Shumake, Johnson, & Gonzalez-Lima, 2007a; Wrubel, Riha, Maldonado, McCollum, & Gonzalez-Lima, 2007b).

The acute and low dose manipulations that improve memory performance reliably upregulate cytochrome oxidase activity, and have contributed to the idea that MB participates in electron shuttling within the mitochondria and aids in upregulating the aerobic capacity of active neurons (Rojas et al., 2012). Unfortunately, no study to date has investigated the effects of acute, low dose MB on UPS function and it is possible that the field may have overlooked an additional effector for MBs unique memory enhancing properties. Indeed, there exists substantial interplay between the mitochondria and the UPS, wherein the UPS serves as a quality control mechanism for mitochondrial health especially in aging and disease (Lehmann, Udasin, & Ciechanover, 2016; Livnat-Levanon & Glickman, 2011; Ross, Olson, & Coppotelli, 2015). Thus, an

interaction between these two organelles when MB is administered at low doses still needs to be investigated.

Summary and Aims

An age-related decline in episodic memory performance in humans is evident, and this is analogous to the decline in TFC performance observed in aged rodents. While the UPS normally functions to aid in the formation and consolidation of memory, preliminary data suggests age-related aberrant UPS processing following memory retrieval. Moreover, preventing UPS activity in young rats mimics deficits observed in aged rats in TFC. The compound MB offers a novel and potentially therapeutic means to rescue age-related proteasome dysfunction, and subsequently abolish age-related impairments in TFC. Thus, the overarching aim of the present thesis was to address whether MB administration could improve memory in young rats and rescue memory impairments in aged rats via upregulation of activity-related UPS activity. Specific aims included determining age-appropriate intraperitoneal dosages of MB for peak proteasome activity upregulation and memory enhancement (Aim 1) and to use MB in a more translational and chronic dietary approach to enhance memory performance and prevent age-related cognitive decline (Aim 2). In Aim 1, it was predicted that aged rats would require a higher acute dose of MB (4 mg/kg) in order to stimulate UPS activity and rescue age-related memory impairments, and that young rats would require a lower dose of MB (1 mg/kg) for memory enhancement and UPS upregulation. In Aim 2, it was predicted that chronic dietary administration of MB would enhance memory and UPS function in young rats, and that aged rats receiving the MB diet would display enhanced memory and UPS function relative to aged rats receiving the control diet.

MATERIALS & METHODS

Subjects. Subjects were 100 male F344 rats obtained from Charles River (Raleigh, NC). Aim 1 consisted of 64 animals, 32 of them were 3 months old and 32 of them were 22 months old at the time of delivery. One aged rat died of natural causes after training but before retrieval, and thus

produced no data. **Aim 2** consisted of 36 animals, 18 of them were 3 months old and 18 of them were 18 months old at the time of delivery. Two aged rats died of natural causes during the four-month feeding period and thus no data was gathered from them. Animals were housed individually in shoebox cages with food and water available *ad libitum*. The colony room was maintained under a 14:10 hour light/dark cycle. The University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee approved all procedures prior to commencement of experimentation.

Conditioning Apparatus. Fear conditioning occurred in a set of four identical chambers (Context A). The floor of Context A was composed of stainless steel rods through which footshocks were delivered. Each chamber was illuminated by an overhead incandescent bulb and was connected to its own shock generator-scrambler (Coulbourn, Whitehall, PA). Ventilation fans provided constant background noise (~60 dB). Chambers in Context A were cleaned with a solution of 5% ammonium hydroxide between animals. A second set of chambers (Context B) was used to conduct auditory CS testing. Context B differed from Context A in a number of ways, including infrared lighting, a solid and opaque textured floor panel, and a different cleaning solution (5% acetic acid).

Behavioral Procedures. All animals were handled for three days prior to behavioral manipulation. This consisted of transport to the behavior room and gentle restraint in a towel. Fear conditioning was conducted in Context A while auditory CS testing was conducted in Context B. All animals were trace fear conditioned on day 1 with 10 CS-US pairings. The CS was a 10s white noise cue (72 dB) and the UCS was a 1s footshock (0.5 mA). Note: the weaker shock used in this paradigm was chosen to be sensitive to behavioral enhancements in freezing stemming from MB treatment. The CS and UCS were separated by an empty 30s trace interval, and CS-USC pairings were separated by an ITI of six minutes. One day following conditioning, rats received underwent a retrieval session consisting of two 15s CS presentations following a 2

minute baseline period. The two CS's were separated by 175 seconds allowing for measurement of freezing behavior.

Methylene Blue Administration. In Aim 1, USP-grade MB (Sciencelab.com, Houston, TX) was dissolved in 0.9% saline to create two stock concentrations, one at 2mg/ml and one at 10mg/ml. Rats were weighed the night before behavioral manipulations/injections and received the appropriate volume of MB solution corresponding to dosages of 1, 2, 4, and 16 mg/kg injected IP. Control rats in both age groups received a similar amount of saline as rats in other conditions (0.5mL) to control for volume of IP injections. In Aim 2, control rat chow was ground into a fine powder using a food processor and 2g of sucrose was added per 100g of food powder. The experimental rat chow was prepared in a similar manner, except 25mg of USP-grade MB was added per 100g of control diet. The control and experimental diets were placed in feeding dishes in rat cages and replaced every other day during the four-month feeding period.

Conditional Fear Responses. In all cases, the average percent time spent freezing was calculated using the FreezeScan 1.0 software (CleverSys, Reston, VA). In Aim 1, two-way ANOVAs (Dose x Age) were used to compare group means within each behavioral epoch analyzed (i.e. baseline, CS, TI, ITI). In Aim 2, two-way ANOVAs (Diet x Age) were used to compare group means within each behavioral epoch analyzed (i.e. baseline, CS, TI, ITI). In both aims, Dunnett's method was used to perform post-hoc comparisons of each group to the 3-month control group (Aim 1-control diet, Aim 2-0mg/kg MB) if the interaction term was significant ($p<0.05$).

Crude Synaptosomal Membrane Fractionation. Animals were sacrificed with an overdose of isoflurane. This occurred 90 minutes following TFC retrieval in all experiments. Brains were rapidly removed and flash frozen on dry ice. Using a rat brain matrix (Harvard Apparatus, MA) incubated on dry ice, structures of interest were dissected from the brains. Synaptosomal membrane fractions were obtained using methods previously described, but with minor

alterations (Jarome et al., 2011). Tissue samples were homogenized in TEVP buffer with 320 mM Sucrose and centrifuged at 1000 x g for 10-minutes at 4°C. The supernatant was collected and spun at 10,000 x g for 10-minutes at 4°C. The resulting pellet, containing the synaptosomal fraction, was resuspended in phospho-homogenization buffer (50 mM Tris-HCl, 6 mM sodium deoxycholate, 150 mM NaCl, 1mM NaF, two mini EDTA-free cOmplete protease inhibitor tablets (Roche), 0.1% SDS, 1 mM sodium orthovanadate) and measured using a 660nm protein assay (Pierce).

20S Proteasome Activity Assay. Brains were removed, frozen on dry ice, and stored at -80°C until use. Following dissection, synaptic fractionation, and protein measurement 10µg of sample was diluted in distilled water. Diluted samples were then mixed with reaction buffer (250 mM HEPES, pH 7.5, 5 mM EDTA, 0.5% NP-40, 0.01% SDS, 20 mM ATP). The fluorogenic peptides LLVY-AMC (Millipore, MA) and Bz-VGR-AMC (Enzo Life Sciences) were added to samples to assess chymotrypsin-like and trypsin-like activities of the proteasome complex. Reactions were incubated at 37°C for two hours and fluorescence was recorded every five minutes in a 96-well microplate reader (Synergy H1; Biotek, VA) at 360nm/460nm. Protein-free blanks were used to assess auto-hydrolysis of flourogenic peptides and activity was determined via interpolation to an AMC standard curve produced following manufacturer instructions. The normalized 20S activity assay data was then analyzed using two-way ANOVAs (Dose x Age in Aim 1, and Diet x Age in Aim 2). In both aims, Dunnett's method was used to perform post-hoc comparisons of each group to the 3-month control group (Aim 1-control diet, Aim 2-0mg/kg MB) only if the interaction term was significant ($p<0.05$).

Procedure: Aim 1. Male F344 rats aged 3 and 22 months were administered 0, 1, 2, 4, or 16 mg/kg of MB IP immediately after TFC. Twenty-four hours later, all rats underwent memory retrieval and were sacrificed 90 minutes following the retrieval session. Brains were collected and the PFC, AMY, DH, and RSC were dissected. After which, samples underwent

synaptosomal fractioning and subsequent trypsin-like and chymotrypsin-like 20S proteasome activity assays to measure raw UPS activation resulting from MB treatment and memory retrieval. Multiple doses of MB were used in order to determine the optimal MB dose for both UPS activation as well as for memory enhancement in acute procedures commonly employed in the field.

Procedure: Aim 2. This experiment attempted to recapitulate the findings from Medina and colleagues (2011) in order to determine if their effects extend to F344 rats and to determine in which brain structures and neuronal compartments proteasome activity is upregulated following dietary treatment with methylene blue. Male F344 rats aged 3 and 18 months were administered a control diet or a diet consisting of 25mg of MB per 100 grams of food powder for four months. After four months, all rats were trained in TFC procedures and underwent memory retrieval 24 hours after conditioning. Ninety minutes following retrieval, rats were sacrificed and brains were collected. The PFC, AMY, DH, and RSC were dissected and samples underwent crude synaptosomal fractionation prior to trypsin-like and chymotrypsin-like 20S proteasome activity assays. The aim of these procedures was to extend findings previously documented by Medina and colleagues, and importantly, to give better resolution into the effects of dietary administration of MB on memory performance and proteasome activity upregulation in both young and aged rats.

RESULTS

Aim 1

Behavior

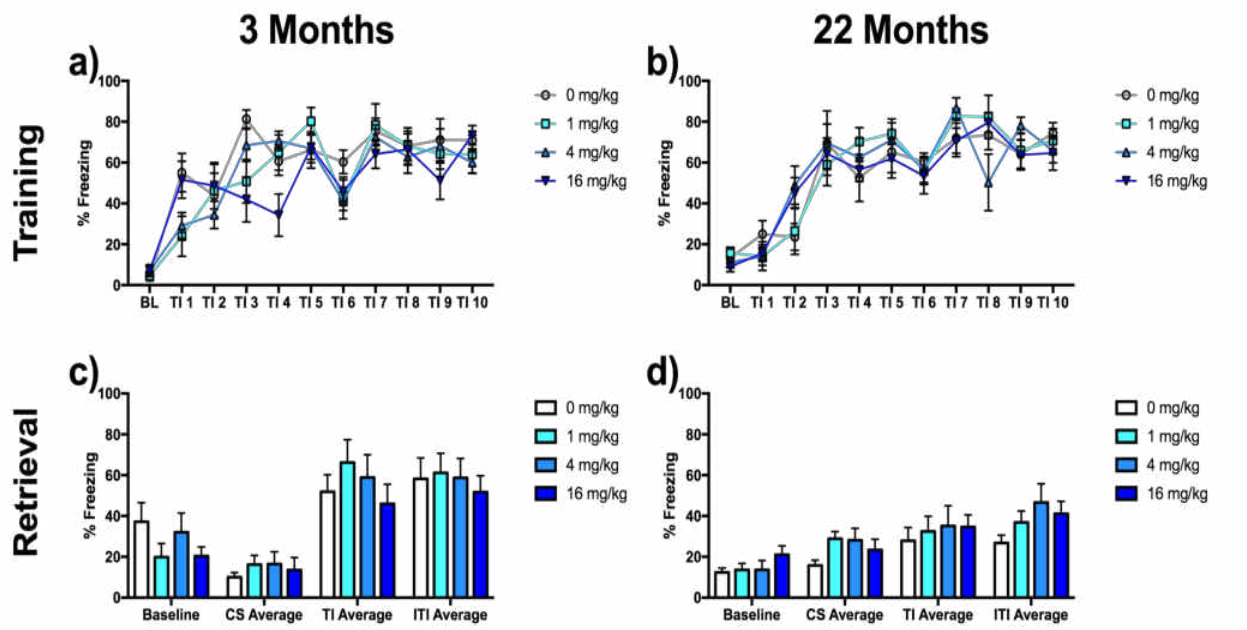

Figure 3. MB administered IP at 0, 1, 4, or 16 mg/kg immediately post-training does not enhance freezing behavior during memory retrieval in rats aged 3 months or 22 months. **a)** Rats aged 3 months froze similarly during the baseline period and during the last TI of training regardless of MB dose. **b)** Rats aged 22 months froze similarly during the baseline period and during the last TI of training regardless of MB dose. **c)** Rats aged 3 months froze similarly during all analyzed epochs of retrieval regardless of MB dose. **d)** Rats aged 22 months froze similarly during all analyzed epochs of retrieval regardless of MB dose. BL, Baseline; CS, Conditioned Stimulus; TI, Trace Interval; ITI, Intertrial Interval.

Rats aged 3 months (n=32) or 22 months (n=31) were split into groups to receive 0, 1, 4, or 16mg/kg MB IP immediately following TFC. All groups had eight subjects per group except for the 22-month 4mg/kg group, which had seven subjects. All rats were trained in TFC. During the baseline epoch of training there was a main effect of age on freezing behavior in that aged rats froze significantly higher than young animals ($F(1,55)=11.22$, $p<0.01$; Figure 3a-b). However, during the last TI of training there were no differences in freezing between young and aged rats ($F(1,55)=0.156$, $p=0.69$; Figure 3a-b). Thus, both age groups behaved similarly at the end of training, and there were no age-related deficits in displaying freezing behavior that could obfuscate interpretation of freezing behavior at retrieval. Main effects of dose and interaction terms were not examined as IP MB administration occurred post-training.

In order to determine the age- and dose-dependent effects of IP MB administration immediately following TFC on subsequent memory performance, all rats underwent memory retrieval 24 hours after training. During the baseline epoch of retrieval, there was a main effect of age on freezing behavior in that aged rats froze significantly less ($F(1, 55)=7.911$, $p<0.01$; Figure 3c-d). There was no main effect of dose of MB on freezing behavior ($F(3, 55)=0.6376$, $p=0.59$; Figure 3c-d), and no interaction between dose and age ($F(3, 55)=1.79$, $p=0.16$; Figure 3c-d). Average freezing during the CS presentations at retrieval was lower in aged animals ($F(1, 55)=9$, $p<0.01$; Figure 3c-d), but there was no effect of MB dose ($F(3, 55)=1.834$, $p=0.15$; Figure 3c-d) and no interaction ($F(3, 55)=0.2098$, $p=0.89$; Figure 3c-d). Additionally, aged rats froze significantly less than young rats during the two TIs ($F(1, 55)=13.51$, $p<0.001$; Figure 3c-d), but there was no effect of MB dose ($F(3, 55)=0.5744$, $p=0.63$; Figure 3c-d) and no interaction ($F(3, 55)=0.5402$, $p=0.66$; Figure 3c-d). Finally, freezing during the ITIs of retrieval was lower in aged rats regardless of MB dose ($F(1, 55)=11.95$, $p<0.01$; Figure 3c-d). However, there was no effect of dose on memory performance ($F(3, 55)=0.5612$, $p=0.64$; Figure 3c-d) and no interaction between age and MB dose ($F(3, 55)=0.7813$, $p=0.51$; Figure 3c-d).

20S Activity Assays

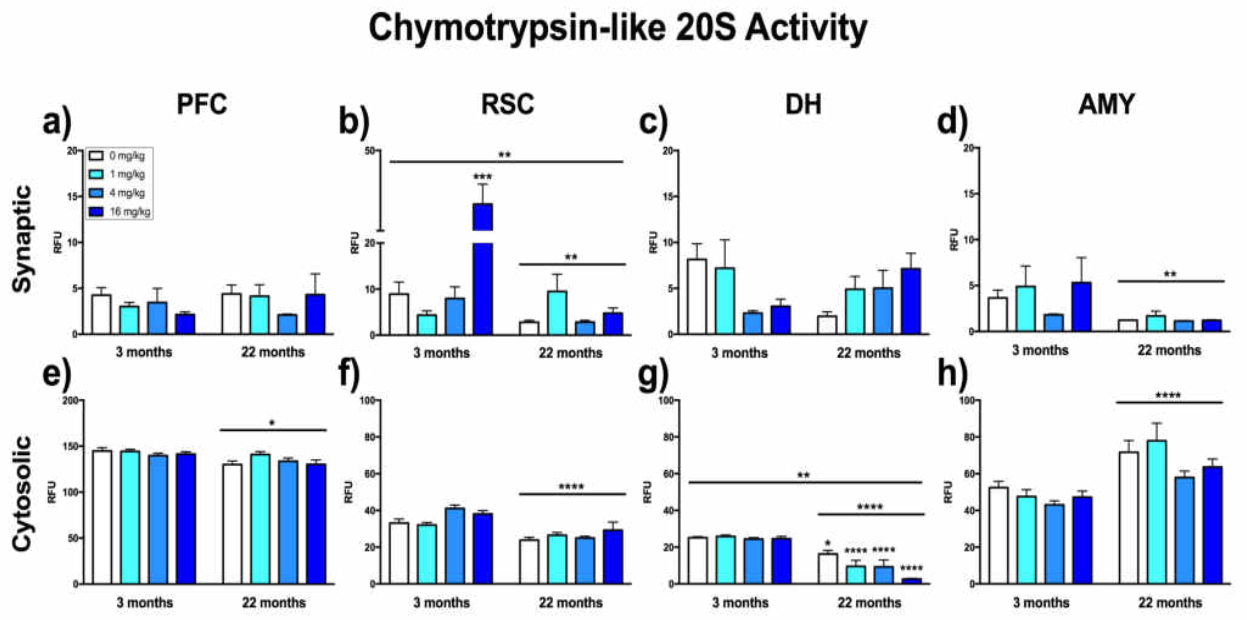

Figure 4. MB administered IP at 0, 1, 4, or 16 mg/kg immediately post-training does not consistently increase chymotrypsin-like 20S activity in rats aged 3 months or 22 months when measured 90 minutes post-retrieval. **a-d)** Chymotrypsin-like 20S activity in the synaptic fractions of tissue from the prefrontal cortex, retrosplenial cortex, dorsal hippocampus, and amygdala. **e-h)** Chymotrypsin-like 20S activity in the cytosolic fractions of tissue from the prefrontal cortex, retrosplenial cortex, dorsal hippocampus, and amygdala. Line across entire graph=main effect of dose, line across 22-month groups=main effect of age, symbols over individual bars=significant difference from 3-month 0mg/kg group in Dunnett's multiple comparisons after significant interaction. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. PFC, prefrontal cortex; RSC, retrosplenial cortex; DH, dorsal hippocampus; AMY, amygdala; RFU, relative fluorescent units.

In order to determine age- and MB dose-dependent differences in plasticity-associated 20S activity, tissue was collected 90 minutes post-retrieval. Chymotrypsin-like and trypsin-like 20S activities were measured in synaptic and cytosolic fractions from the PFC, RSC, DH, and AMY (Figures 4 and 5).

Chymotrypsin-like activity was not consistently upregulated after memory retrieval in response to post-training MB administration (Figure 4). In the synaptic fraction of the PFC there was no main effect of MB dose ($F(3,55)=0.5944$, $p=0.62$; Figure 4a), age ($F(1,55)=0.3783$, $p=0.54$; Figure 4a), or interaction between the two ($F(3,55)=0.7712$, $p=0.51$; Figure 4a). Aged rats displayed less chymotrypsin-like 20S activity in the cytosolic fraction of the PFC ($F(1,55)=13.77$, $p<0.001$; Figure 4e), but there was no effect of MB dose ($F(3,55)=1.631$, $p=0.19$; Figure 4e), or an interaction ($F(3,55)=1.129$, $p=0.35$; Figure 4e).

In the synaptic fraction of the RSC, there was an age related decrease in chymotrypsin-like activity ($F(1,55)=11.05$, $p<0.01$; Figure 4b), a main effect of dose ($F(3,55)=5.9$, $p<0.01$; Figure 4b), and an interaction between dose and age ($F(3,55)=7.345$, $p<0.001$; Figure 4b). Furthermore, 16mg/kg MB seems to maximally upregulate proteasome activity in young animals and this group was significantly higher than the 0mg/kg 3-month group ($p<0.001$). In the cytosolic fraction of the RSC, there was an age-related reduction in chymotrypsin-like activity independent of dose ($F(1,55)=39.68$, $p<0.0001$; Figure 4f). There was no main effect of MB dose ($F(3,55)=2.729$, $p=0.052$; Figure 4f) and no significant interaction between age and dose ($F(3,55)=1.918$, $p=0.14$; Figure 4f).

Within the DH, MB administration seems to differentially affect chymotrypsin-like 20S in the synaptic versus the cytosolic cellular compartments. In the synaptic fraction there was a significant interaction between dose and age ($F(3,55)=4.142$, $p=0.01$; Figure 4c). It seems that as the dose of MB increases, proteasome activity decreases in young animals and increases in aged animals, although no follow-comparisons of each group to the 0mg/kg 3-month group were significant. Additionally, there were no main effects of age ($F(1,55)=0.1292$, $p=0.72$; Figure 4c) or dose (($F(3,55)=0.6982$, $p=0.56$; Figure 4c). In contrast, cytosolic chymotrypsin-like activity changed in an age- and dose-dependent manner. There was a significant interaction between MB dose and age (($F(3,55)=3.918$, $p=0.01$; Figure 4g), and follow up comparisons revealed that, for aged animals, as the dose increased chymotrypsin-like activity decreased relative to the 0mg/kg 3-month group (22 month-0mg/kg, $p=0.01$; 22-month-1mg/kg, $p<0.0001$; 22-month-4mg/kg, $p<0.0001$; 22-month-16mg/kg, $p<0.0001$; Figure 4g). Additionally, there was a main effect of age ($F(1,55)=130.4$, $p<0.0001$; Figure 4g) and dose ($F(3,55)=4.708$, $p<0.01$; Figure 4g), although these effects are more difficult to interpret given the significant interaction between them.

There was a dichotomy in the pattern of 20S activation in the AMY between the synaptic and cytosolic fractions. In the synaptic portion of the AMY, there was an age-related decrease in 20S activity regardless of dose ($F(1,55)=7.802$, $p<0.01$; Figure 4d), but no main effect of dose

(F (3,55)=0.8212, *p=0.49*; Figure 4d) or interaction (F (3,55)=0.588, *p=0.63*; Figure 4d). In contrast, 20S activity in the cytosolic fraction was higher in aged animals regardless of MB dose ((F (1,55)=31.07, *p<0.0001*; Figure 4h). The main effect of dose approached significance (F (3,55)=2.53, *p=0.067*; Figure 4h) and there was no significant interaction (F (3,55)=0.9417, *p=0.43*; Figure 4h).

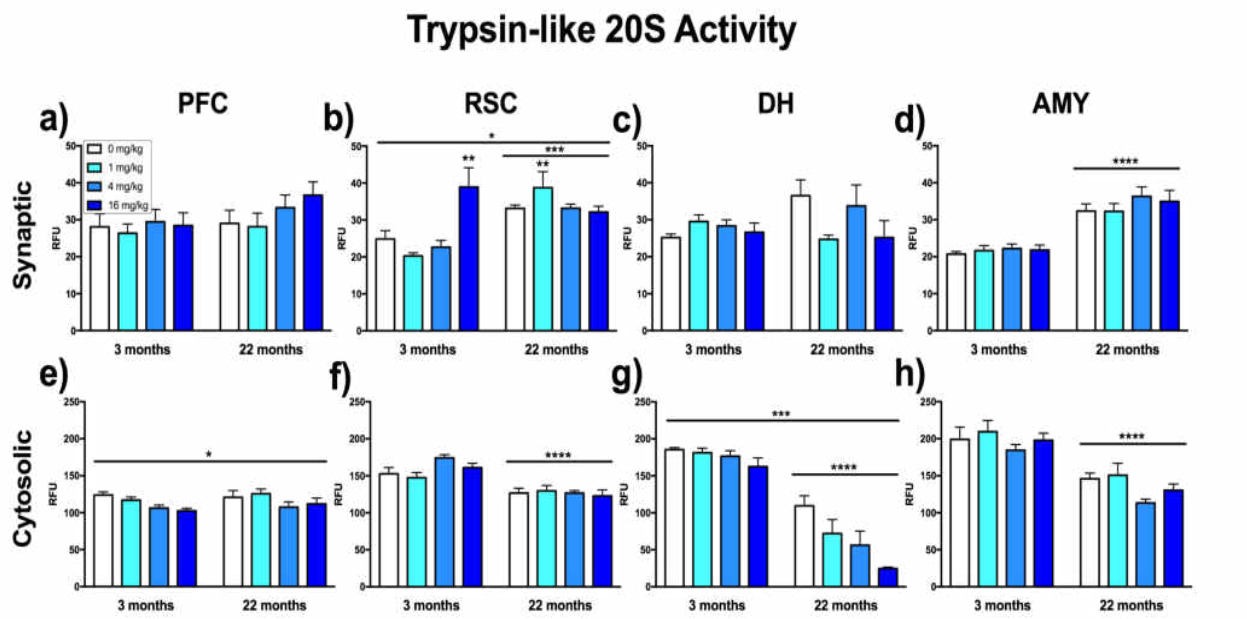

Figure 5. MB administered IP at 0, 1, 4, or 16 mg/kg immediately post-training does not consistently increase trypsin-like 20S activity in rats aged 3 months or 22 months when measured 90 minutes post-retrieval. **a-d)** Trypsin-like 20S activity in the synaptic fractions of tissue from the prefrontal cortex, retrosplenial cortex, dorsal hippocampus, and amygdala. **e-h)** Trypsin-like 20S activity in the cytosolic fractions of tissue from the prefrontal cortex, retrosplenial cortex, dorsal hippocampus, and amygdala. Line across entire graph=main effect of dose, line across 22-month groups=main effect of age, symbols over individual bars=significant difference from 3-month 0mg/kg group in Dunnett's multiple comparisons after significant interaction. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. PFC, prefrontal cortex; RSC, retrosplenial cortex; DH, dorsal hippocampus; AMY, amygdala; RFU, relative fluorescent units.

Similar to chymotrypsin-like activity, trypsin-like activity was not consistently upregulated following memory retrieval in response to post-training MB administration (Figure 5). In the synaptic fraction of the PFC, there was no effect of age (F (1,55)=2.32, *p=0.13*; Figure 5a), dose (F (3,55)=1.028, *p=0.39*; Figure 5a), or interaction between them (F (3,55)=0.4575, *p=0.71*; Figure 5a). However, in the cytosolic portion of the PFC, dose significantly influenced trypsin-like activity and higher doses of MB produced less 20S activity regardless of age (F (3,55)=3.829, *p=0.01*; Figure 5e). There was no main effect of age (F (1,55)=0.841, *p=0.36*;

Figure 5e), or interaction between dose and age (F (3,55)=0.4821, *p=0.70*; Figure 5e) on 20S activity.

MB administration greatly affected trypsin-like proteasome activity in the synaptic fraction of the RSC. An interaction between age and MB dose was evident (F (3,55)=7.297, *p<0.001*; Figure 5b), and follow-up comparisons to the 3-month 0mg/kg group revealed that 16mg/kg MB treatment in 3-month animals significantly upregulates 20S activity (*p<0.01*; Figure 5b), while 1mg/kg MB treatment in 22-month animals significantly upregulates 20S activity (*p<0.01*; Figure 5b). Aside from the interaction, there was a general age related increase in trypsin-like activity (F (1,55)=15.02, *p<0.001*; Figure 5b) and a main effect of MB dose (F (3,55)=3.406, *p=0.04*; Figure 5b). In the cytosolic portion of the RSC, there was only an age-related reduction in 20S activity that was independent of MB dose (F (1,55)=47.15, *p<0.0001*; Figure 5f), and there was no significant effect of MB dose alone (F (3,55)=1.26, *p=0.30*; Figure 5f) or an interaction (F (3,55)=1.928, *p=0.14*; Figure 5f).

Much like chymotrypsin-like activity, a similar pattern of trypsin-like activity emerged in the DH following MB administration. There were no significant main effects in the synaptic fraction (age: F (1,55)=1.353, *p=0.25*; Figure 5c; dose: F (3,55)=1.34, *p=0.27*; Figure 5c), or an interaction between them (F (3,55)=2.593, *p=0.06*; Figure 5c). In contrast, data from the cytosolic fraction of the DH indicate an age-related reduction (F (1,55)=177.8, *p<0.0001*; Figure 5g), and dose-related reduction (F (3,55)=7.378, *p<0.001*; Figure 5g) in trypsin-like 20S activity. However, because the interaction term was not significant (F (3,55)=2.494, *p=0.07*; Figure 5g), follow-up tests were not possible. Nonetheless, it appears that as dose increases, proteasome activity in the cytosolic fraction of the DH decreases.

Finally, the pattern of trypsin-like proteasome activity in the AMY was the opposite of that observed for chymotrypsin-like activity. In the synaptic fraction there was a general age-related increase in 20S activity (F (1,55)=84.71, *p<0.0001*; Figure 5d), but no effect of dose (F (3,55)=0.8653, *p=0.46*; Figure 5d) or interaction (F (3,55)=0.3271, *p=0.81*; Figure 5d). In stark contrast, the cytosolic fraction showed an age-related decrease in 20S activity that was

independent of MB dose (F (1,55)=57, *p<0.0001*; Figure 5h). However, dose alone (F (3,55)=2.536, *p=0.07*; Figure 5h) or an interaction between age and dose (F (3,55)=0.2416, *p=0.87*; Figure 5h) did not affect trypsin-like activity in the cytosolic fraction of the DH.

Aim 2

Behavior

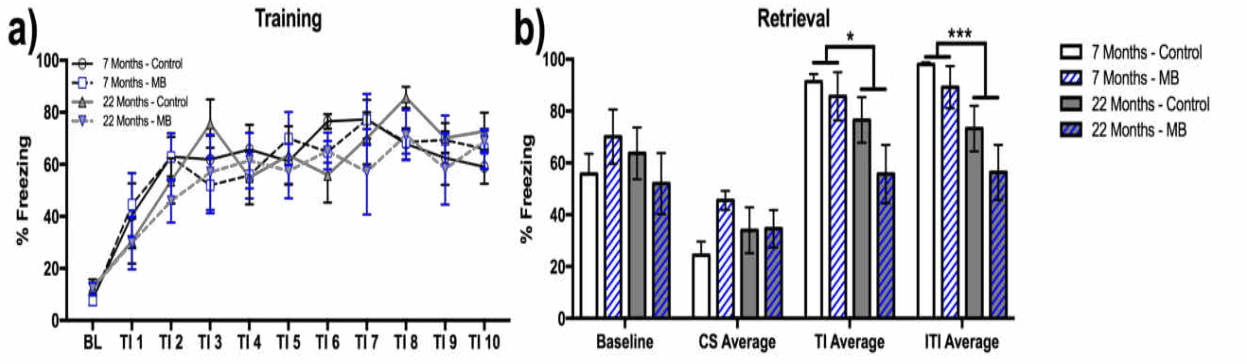

Figure 6. Dietary treatment with MB for four months does not enhance freezing behavior during training or retrieval in young or aged animals. **a)** All groups froze similarly during the baseline and last trace interval of training. **b)** All groups froze similarly during the baseline and conditional stimulus epochs of retrieval, but aged animals, regardless of diet, froze less during the trace interval and intertrial interval epochs of retrieval. BL, Baseline; CS, Conditioned Stimulus; TI, Trace Interval; ITI, Intertrial Interval. *$p<0.05$, ***$p<0.001$.

Rats aged 3 months (n=18) or 18 months (n=18) were split into groups to receive either a control diet or an MB-containing diet for a four-month period. All groups had nine subjects per group except for the 22-month MB group, which had seven subjects. In order to examine the mnemonic effects of dietary treatment with MB on young and aged rats and to extend the findings from Medina and colleagues (2011), all rats were trained in TFC at the end of the 4-month feeding period. During the baseline epoch of training, there were no effects of age (F (1,30)=3.681, *p=0.06*; Figure 6a), diet (F (1,30)=0.0396, *p=0.84*; Figure 6a), and no age by diet interaction (F (1,30)=0.0005, *p=0.98*; Figure 6a). Importantly, there were also no differences between groups in freezing during the last TI of training as a function of age (F (1,30)=1.477, *p=0.23*; Figure 6a), diet (F (1,30)=0.0475, *p=0.82*; Figure 6a), or an interaction between the two (F (1,30)=0.6396, *p=0.43*; Figure 6a). Thus, it can be concluded that there were no differences in

acquisition of freezing behavior between age groups or diet condition during training that may confound freezing behavior analysis at retrieval.

All rats underwent memory retrieval 24 hours post training in order to examine the effects of dietary MB administration on learning and memory processes. During the baseline period of retrieval there was no main effect of age ($F(1,30)=0.2518$, $p=0.62$; Figure 6b), diet ($F(1,30)=0.0191$, $p=0.89$; Figure 6b), or an interaction ($F(1,30)=1.696$, $p=0.20$; Figure 6b) on freezing behavior. Similarly, freezing behavior averaged across the two CS presentations did not differ as a function of age ($F(1,30)=0.0103$, $p=0.92$; Figure 6b), diet ($F(1,30)=2.771$, $p=0.11$; Figure 6b), or an interaction between the two ($F(1,30)=2.471$, $p=0.13$; Figure 6b). However, average freezing during the two TIs was lower in aged animals regardless of diet ($F(1,30)=7.117$, $p=0.01$; Figure 6b). Diet type did not influence TI freezing ($F(1,30)=2.49$, $p=0.13$; Figure 6b) and there was no significant interaction between age and diet ($F(1,30)=0.8256$, $p=0.37$; Figure 6b). ITI freezing was also lower in aged rats regardless of diet type ($F(1,30)=13.85$, $p<0.001$; Figure 6b), but there was no effect of diet independent of age ($F(1,30)=2.764$, $p=0.11$; Figure 6b) or significant interaction ($F(1,30)=0.2739$, $p=0.60$; Figure 6b).

20S Activity Assays

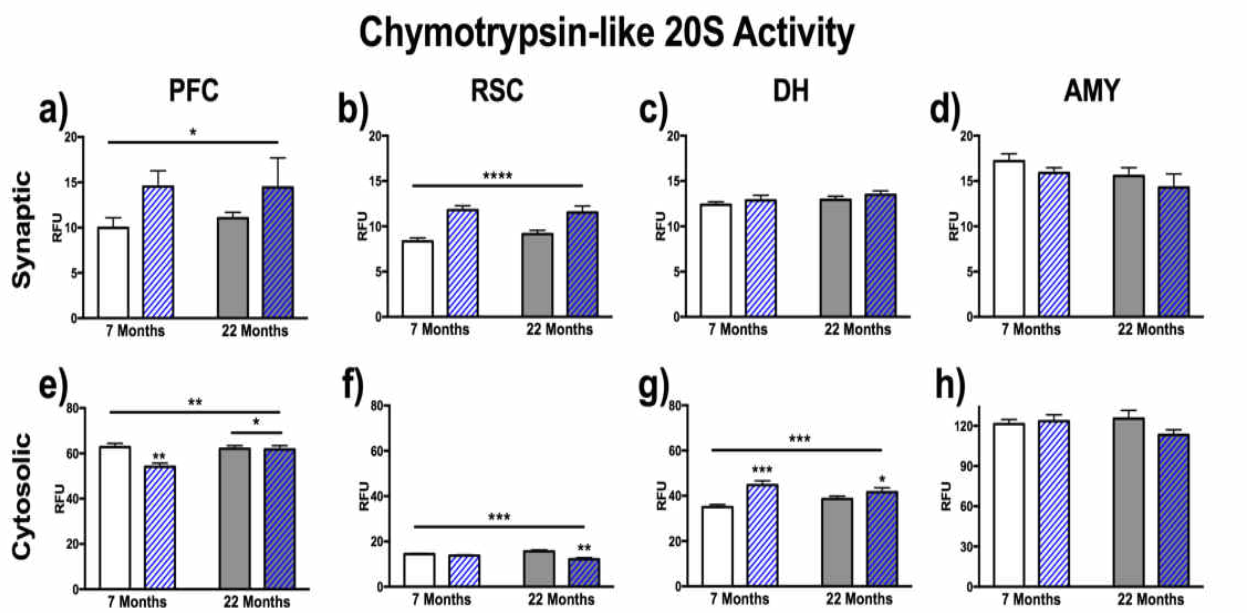

Figure 7. Dietary treatment with MB for four months does not consistently increase chymotrypsin-like 20S activity in rats aged 7 months or 22 months when measured 90 minutes post-retrieval. **a-d)** Chymotrypsin-like 20S activity in the synaptic fractions of tissue from the prefrontal cortex, retrosplenial cortex, dorsal hippocampus, and amygdala. **e-h)** Chymotrypsin-like 20S activity in the cytosolic fractions of tissue from the prefrontal cortex, retrosplenial cortex, dorsal hippocampus, and amygdala. Line across entire graph=main effect of diet, line across 22-month groups=main effect of age, symbols over individual bars=significant difference from 7-month control group in Dunnett's multiple comparisons after significant interaction. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. PFC, prefrontal cortex; RSC, retrosplenial cortex; DH, dorsal hippocampus; AMY, amygdala; RFU, relative fluorescent units.

In order to examine the effects of four months of dietary MB administration on plasticity-associated 20S proteasome activity in young and aged rats, brain tissue was collected 90 minutes post-retrieval. Chymotrypsin-like and trypsin-like 20S activities were measured in synaptic and cytosolic fractions from the PFC, RSC, DH, and AMY (Figures 7 and 8).

In a manner much similar to IP MB administration in Aim 1, dietary administration of MB did not consistently upregulate 20S proteasome activity throughout the brain regions and cellular compartments investigated. Dietary treatment with MB significantly upregulated chymotrypsin-like activity in the synaptic fraction of the PFC ($F(1,30)=5.111$, *$p=0.03$*; Figure 7a), but there was no main effect of age ($F(1,30)=0.0797$, *$p=0.78$*; Figure 7a) or an interaction between the two ($F(1,30)=0.106$, *$p=0.75$*; Figure 7a). In contrast, the cytosolic fraction of the PFC showed an interaction effect between diet and age on chymotrypsin-like activity ($F(1,30)=6.834$, *$p=0.014$*; Figure 7e), and follow up tests revealed that MB treatment in young

animals significantly reduces 20S activity ($p<0.01$; Figure 7e). Additionally, there were main effects of age ($F(1,30)=4.697$, $p=0.04$; Figure 7e) and diet ($F(1,30)=7.821$, $p<0.01$; Figure 7e), but these are likely driven by the sharp decrease in proteasome activity in young animals that received the MB-containing diet.

The pattern of chymotrypsin-like 20S activity in the RSC was different between the synaptic and cytosolic fractions. Animals that received the MB-containing diet showed higher chymotrypsin-like activity in the synaptic fraction of the RSC regardless of age ($F(1,30)=34.47$, $p<0.0001$; Figure 7b). There was, however, no age-related change in activity ($F(1,30)=0.2983$, $p=0.59$; Figure 7b) or interaction between age and diet ($F(1,30)=1.161$, $p=0.29$; Figure 7b). In the cytosolic fraction of the RSC there was an interaction between the effects of age and diet on chymotrypsin like activity ($F(1,30)=8.293$, $p<0.01$; Figure 7f), and follow up tests showed a significant reduction in the 22-month MB group relative to the 3-month control group ($p<0.01$; Figure7f). There was no main effect of age ($F(1,30)=0.1761$, $p=0.68$; Figure 7f), but there was a main effect of diet ($F(1,30)=17.94$, $p<0.001$; Figure 7f) that was likely driven by the sharp decrease in proteasome activity in the MB treated aged animals.

Dietary treatment with MB did not affect chymotrypsin-like 20S activity in the synaptic fraction of the DH. There was no main effect of diet ($F(1,30)=1.301$, $p=0.26$; Figure 7c), age ($F(1,30)=1.665$, $p=0.21$; Figure 7c), or an interaction between them ($F(1,30)=0.0045$, $p=0.95$; Figure 7c). In contrast, MB treatment significantly increased proteasome activity in the cytosolic fraction of the DH regardless of age ($F(1,30)=17.08$, $p<0.001$; Figure 7g). There was no main effect of age on 20S activity ($F(1,30)=0.0273$, $p=0.87$; Figure 7g), but there was an interaction between diet and age ($F(1,30)=4.82$, $p=0.04$; Figure 7g). Dunnett's follow up comparisons to the 3-month MB group showed significant increases in the 3-month MB group ($p<0.001$; Figure 7g) and the 22-month MB group ($p=0.02$; Figure 7g).

Tissue from the AMY, regardless of the cellular compartment, did not show any age-related or diet-related changes in chymotrypsin-like activity. In the synaptic fraction there was no effect of diet ($F(1,30)=1.836$, $p=0.19$; Figure 7d), age ($F(1,30)=2.939$, $p=0.10$; Figure 7d), or

age by diet interaction (F (1,30)<0.0001, *p=0.99*; Figure 7d). Similarly, in the cytosolic fraction of the AMY there was no main effect of diet (F (1,30)=1.095, *p=0.30*; Figure 7h), age (F (1,30)=0.4088, *p=0.53*; Figure 7h), or interaction between them (F (1,30)=2.216, *p=0.15*; Figure 7h) on chymotrypsin-like 20S activity.

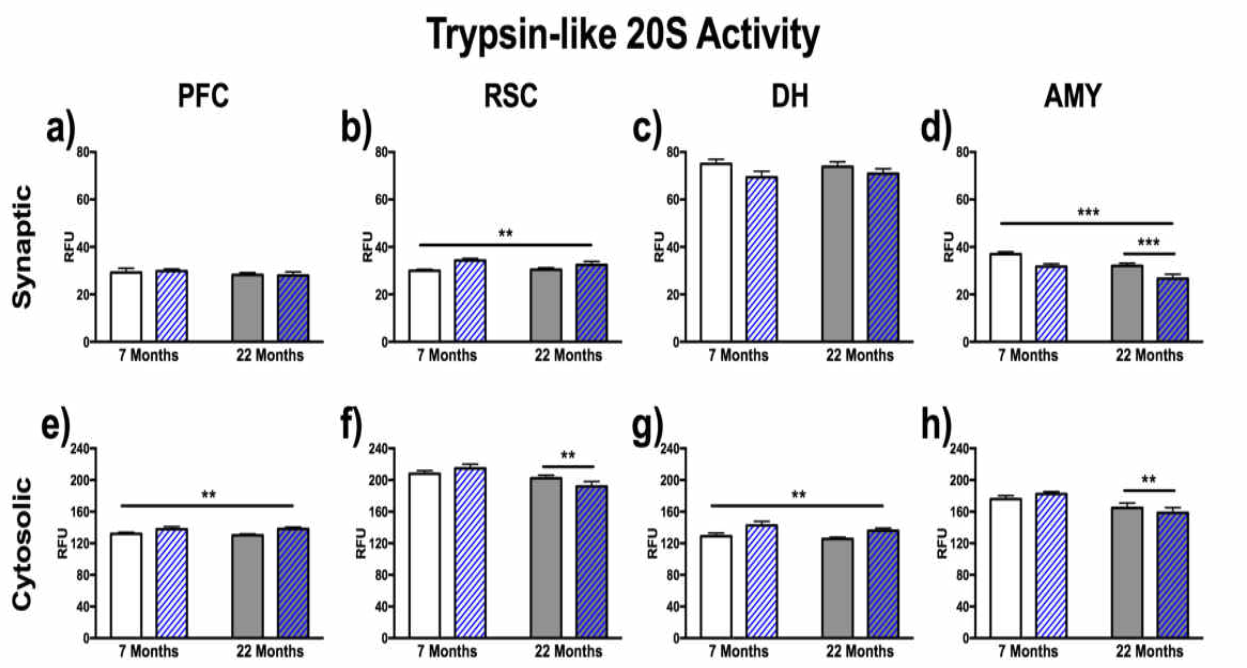

Figure 8. Dietary treatment with MB for four months does not consistently increase trypsin-like 20S activity in rats aged 7 months or 22 months when measured 90 minutes post-retrieval. **a-d)** Trypsin-like 20S activity in the synaptic fractions of tissue from the prefrontal cortex, retrosplenial cortex, dorsal hippocampus, and amygdala. **e-h)** Trypsin-like 20S activity in the cytosolic fractions of tissue from the prefrontal cortex, retrosplenial cortex, dorsal hippocampus, and amygdala. Line across entire graph=main effect of diet, line across 22-month groups=main effect of age, symbols over individual bars=significant difference from 7-month control group in Dunnett's multiple comparisons after significant interaction. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. PFC, prefrontal cortex; RSC, retrosplenial cortex; DH, dorsal hippocampus; AMY, amygdala; RFU, relative fluorescent units.

Similar to chymotrypsin-like activity, trypsin-like activity was not consistently upregulated following memory retrieval in response to four months of dietary MB administration (Figure 8). In the synaptic fraction of the PFC, there was no effect of diet (F (1,30)=0.0154, *p=0.90*; Figure 8a), age (F (1,30)=1.194, *p=0.28*; Figure 8a), or an interaction between them (F (1,30)=0.1148, *p=0.74*; Figure 8a) on 20S activity. In contrast, tissue from the cytosolic fraction of the PFC showed MB diet-related increases in trypsin-like activity (F (1,30)=7.778, *p<0.01*; Figure 8e). Age did not significantly affect 20S activity (F (1,30)=0.0584, *p=0.81*; Figure 8e), and there was not an interaction between diet and age (F (1,30)=0.1978, *p=0.66*; Figure 8e).

Patterns in trypsin-like 20S activity were not consistent between the synaptic and cytosolic cellular compartments of the RSC. In the synaptic fraction dietary MB significantly upregulated proteasome activity ($F(1,30)=11.43$, $p<0.01$; Figure 8b), while age ($F(1,30)=0.5833$, $p=0.45$; Figure 8b), or an interaction between age and diet ($F(1,30)=1.531$, $p=0.23$; Figure 8b) did not. Tissue from the cytosolic fraction of the RSC showed clear age-related decreases ($F(1,30)=8.622$, $p<0.01$; Figure 8f) that were independent of dietary treatment ($F(1,30)=0.1516$, $p=0.70$; Figure 8f) or an age by diet interaction ($F(1,30)=3.177$, $p=0.08$; Figure 8f).

Dietary treatment with MB has opposite effects on trypsin-like proteasome activity in the synaptic and cytosolic fractions of the DH. In the synaptic fraction, MB treatment produced a modest reduction in 20S activity that did not quite reach significance ($F(1,30)=3.806$, $p=0.06$; Figure 8c). However, age ($F(1,30)=0.0054$, $p=0.94$; Figure 8c) or an interaction between diet and age ($F(1,30)=0.3884$, $p=0.54$; Figure 8c) had no significant effect on proteolysis. In an opposite pattern, MB treatment increased proteolysis in the cytosolic fraction of the RSC ($F(1,30)=9.702$, $p<0.01$; Figure 8g). There were no significant effects of age ($F(1,30)=1.67$, $p=0.21$; Figure 8g) or a Diet x Age interaction ($F(1,30)=0.1705$, $p=0.68$; Figure 8g).

In the synaptic fraction of tissue from the AMY, dietary MB treatment significantly reduced trypsin-like 20S activity ($F(1,30)=17.41$, $p<0.001$; Figure 8d). Additionally, there was an age-related reduction in proteolysis regardless of diet type ($F(1,30)=15.2$, $p<0.001$; Figure 8d), but there was no interaction between dietary and age-related effects ($F(1,30)=0.0041$, $p=0.94$; Figure 8d). Contrastingly, the MB-containing diet had no effect on 20S activity in cytosolic 20S activity in the AMY ($F(1,30)=0.0005$, $p=0.99$; Figure 8h). There was, however, an age-related reduction in trypsin-like activity that was independent of diet type ($F(1,30)=11.79$, $p<0.01$; Figure 8d), but the interaction term was not significant ($F(1,30)=1.532$, $p=0.23$; Figure 8d).

DISCUSSION

Acute MB administration immediately post-training at doses of 1, 4, or 16mg/kg had no effect on subsequent memory performance at retrieval in young or aged rats. There was no clear MB effect in any of the analyzed epochs, which was unexpected given that we administered doses of MB that extended throughout its hormetic curve (Rojas et al., 2012). Our lack of an enhancement in young animals given 1mg/kg IP is in contrast to previously published memory enhancements with 1mg/kg of MB given IP to adult rats immediately post-inhibitory avoidance training (Martinez et al., 1978). Additionally, MB doses above 10mg/kg are known to exert deleterious effects on memory performance (for review see Rojas et al., 2012). However, we did not see decreased freezing in young or aged animals that received 16mg/kg. The lack of memory enhancement or impairment cannot be attributed to general performance deficits between groups, as all groups behaved similarly at the beginning and end of training (i.e. all rats displayed elevated freezing behavior over time). It is possible that memory enhancing effects of MB do not extend to TFC procedures, and may not extend to fear conditioning in general (Rojas et al., 2012). It is true that no study to date has reported MB-driven enhancement of classical auditory fear conditioning, and the majority of MB enhancements are in fear extinction retention, decreased fear renewal after extinction, better performance in spatial tasks, or better performance in object recognition tasks (Rojas et al., 2012).

Although there were no effects of acute MB administration on freezing behavior during retrieval in either age group, we did replicate our preliminary finding of an age-related deficit in TFC. Aged rats froze significantly less than young rats during all analyzed epochs of retrieval, and these results are congruent with previously published age-related deficits in TFC (Moyer & Brown, 2006; Villarreal et al., 2004).

The lack of a behavioral enhancement with IP MB administration makes the interpretation of post-retrieval 20S activity less meaningful. However, there were a few patterns of MB-driven upregulation of 20S activity that were conserved between the types of proteasome activity examined. Together, it appears the most consistent effects of post-training IP MB

administration on proteasome activity measured post-retrieval are in the synaptic fraction of the RSC and the cytosolic fraction of the DH. In synapses of the RSC, 16mg/kg seems to consistently upregulate general 20S (i.e. chymotrypsin- and trypsin-like) activity in young animals, whereas 1mg/kg upregulates general proteasome activity in aged animals. In the cytosolic fraction of the DH, general 20S activity is lower in aged animals and MB treatment lowers this activity linearly with increasing dose. Apart from these two clear patterns, data from the PFC and AMY do not show any consistencies between the types of proteasome activity examined.

Similar to results with acute MB administration, we did not see any memory enhancement with chronic MB administration. There were no age-related or diet-related differences in freezing behavior at the beginning or end of training that could have complicated our interpretation of freezing behavior at retrieval. During retrieval, the only group differences were age-related reductions in average freezing behavior during the TI and ITI periods. While it is important that the age-related deficit in TFC was reproduced once again, it is unfortunate that MB did not enhance memory performance as reported by Medina and colleagues (2011). There are few potential sources for our lack of replication. There were discrepancies in the type of memory task used; they saw enhancements in performance on the spatial references version of the Morris Water Maze, whereas we were seeking enhancements in fear conditioning. As previously mentioned, no study to date has demonstrated an MB-related enhancement in auditory fear conditioning and it's possible that fear conditioning procedures are too robust to observe an enhancement. It is also possible that enhancements they observed were species and strain specific; they used 3xTg-AD transgenic mice, whereas we used inbred F344 rats (Medina et al., 2011). Future work should examine if dietary treatment with MB, at the dose of 25mg per 100 grams of food powder, for four months can enhance spatial memory in non-transgenic rats in order to situate our results and extend their findings.

The lack of dietary MB-driven memory enhancement makes interpreting post-retrieval proteasome activity more difficult. We did not replicate the previously published global

upregulation in 20S activity in animals that received the MB-containing diet (Medina et al., 2011). They measured 20S activity in whole brain homogenates, whereas we had structural and cellular compartmental resolution. It is possible that by isolating certain brain structures, we may have overlooked other brain regions that drove their observed global increase. However, chronic MB administration did increase 20S activity in the synaptic fraction of RSC, much similar to acute MB administration. MB also increased 20S activity in the cytosolic fraction of DH when administered chronically, which opposed the reductions in 20S activity observed with acute MB administration. Nonetheless, given that learning during TFC procedures is dependent on the RSC and DH, it is unclear why upregulating proteasome activity in these structures did not enhance memory (Kwapis et al., 2015; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998). While there is evidence for proteasome activity in the DH being involved in CFC, no studies to date have investigated the role of the UPS in either the RSC or the DH during TFC (Lee et al., 2008). Though unlikely, it is possible that these structures do not depend on UPS function in TFC procedures.

Another possibility is proteasome upregulation does not necessarily correlate with better learning, and that previously documented memory enhancements with MB were attributable to enhanced mitochondrial function (Rojas et al., 2012). If this were true, MB should have affected electron transport in mitochondria, and resulted in some measurable change in behavior. By administering MB acutely at doses that cover the hormetic curve, and chronically at a ~30mg/kg dose known to be harmful to mitochondria, we should have either positively or negatively impacted cellular respiration. The lack of any change in behavior, positive or negative, suggests that the changes caused by MB (whether they be on proteolysis or cellular respiration) do not affect learning and memory processes in TFC.

Given that we have a clear age-related reduction in UPS activity following trace fear memory retrieval (Figure 2), work towards stimulating UPS activity should continue. Our results suggest that MB may not be well suited for rescue of age-related cognitive decline via proteasome activity upregulation. Another promising option for upregulating proteasome activity

is proteasome-activating peptide 1 (PAP1). PAP1 incubation in cell cultures from *C. elegans* and *H. sapiens* increases chymotrypsin-like proteasome activity (Vechio, Cerqueira, Augusto, Lopes, & Demasi, 2014). Additionally PAP1 confers resistance to oxidative stress, and attenuates protein accumulation in a cellular model of amyotrophic lateral sclerosis (Vechio et al., 2014). PAP1 could be locally infused into structures critical for TFC and could counteract the age-related decline in UPS function, ultimately rescuing age-related cognitive decline. Nonetheless, findings reported here rule out MB as a promising option and allow for advancement of the field towards more promising therapeutic options.

REFERENCES

Almaguer, W., Estupinan, B., Frey, J. U., & Bergado, J. A. (2002). Aging impairs amygdala-hippocampus interactions involved in hippocampal LTP. *Neurobiology of Aging*, *23*(2), 319–324. http://doi.org/10.1016/S0197-4580(01)00278-0

Bailey, D. J., Kim, J. J., Sun, W., Thompson, R. F., & Helmstetter, F. J. (1999). Acquisition of fear conditioning in rats requires the synthesis of mRNA in the amygdala. *Behavioral Neuroscience*, *113*(2), 276–282.

Baraibar, M. A., & Friguet, B. (2012). Changes of the proteasomal system during the aging process. In T. Grune (Ed.), *Progress in Molecular Biology and Translational Science* (Vol. 109, pp. 249–275). Progress in molecular biology and translational science. http://doi.org/10.1016/B978-0-12-397863-9.00007-9

Barnes, C. A. (1998). Memory Changes during Normal Aging. In R. Kesner & J. J. Martinez (Eds.), *Neurobiology of Learning and Memory* (pp. 247–287). Elsevier. http://doi.org/10.1016/b978-012475655-7/50008-3

Beylin, A. V., Gandhi, C. C., Wood, G. E., Talk, A. C., Matzel, L. D., & Shors, T. J. (2001). The role of the hippocampus in trace conditioning: temporal discontinuity or task difficulty? *Neurobiology of Learning and Memory*, *76*(3), 447–461. http://doi.org/10.1006/nlme.2001.4039

Bruchey, A. K., & Gonzalez-Lima, F. (2008). Behavioral, Physiological and Biochemical Hormetic Responses to the Autoxidizable Dye Methylene Blue. *American Journal of Pharmacology and Toxicology*, *3*(1), 72–79.

Burke, S. N., & Barnes, C. A. (2006). Neural plasticity in the ageing brain. *Nature Reviews Neuroscience*, *7*(1), 30–40. http://doi.org/10.1038/nrn1809

Cabeza, R., Grady, C. L., Nyberg, L., McIntosh, A. R., Tulving, E., Kapur, S., et al. (1997). Age-related differences in neural activity during memory encoding and retrieval: a positron emission tomography study. *Journal of Neuroscience*, *17*(1), 391–400.

Callaway, N. L., Riha, P. D., Bruchey, A. K., Munshi, Z., & Gonzalez-Lima, F. (2004). Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacology, Biochemistry, and Behavior*, *77*(1), 175–181. http://doi.org/10.1016/j.pbb.2003.10.007

Callaway, N. L., Riha, P. D., Wrubel, K. M., McCollum, D., & Gonzalez-Lima, F. (2002). Methylene blue restores spatial memory retention impaired by an inhibitor of cytochrome oxidase in rats. *Neuroscience Letters*, *332*(2), 83–86.

Chen, C., & Tonegawa, S. (1997). Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammalian brain. *Annual Review of Neuroscience*, *20*, 157–184. http://doi.org/10.1146/annurev.neuro.20.1.157

Chen, H.B., Wei, B., Wang, W., Zhou, P.L., Zhan, J.Q., Hu, M.R., et al. (2015). Cognitive decline is associated with reduced surface GluR1 expression in the hippocampus of aged rats. *Neuroscience Letters*, *591*, 176–181. http://doi.org/10.1016/j.neulet.2015.02.030

Chondrogianni, N., Sakellari, M., Lefaki, M., Papaevgeniou, N., & Gonos, E. S. (2014).

Proteasome activation delays aging in vitro and in vivo. *Free Radical Biology and Medicine*, *71*(C), 303–320. http://doi.org/10.1016/j.freeradbiomed.2014.03.031

Chondrogianni, N., Voutetakis, K., Kapetanou, M., Delitsikou, V., Papaevgeniou, N., Sakellari, M., et al. (2015). Proteasome activation: An innovative promising approach for delaying aging and retarding age-related diseases. *Ageing Research Reviews*, *23*(Pt A), 37–55. http://doi.org/10.1016/j.arr.2014.12.003

Clark, R. E., & Squire, L. R. (1998). Classical conditioning and brain systems: The role of awareness. *Science*, *280*(5360), 77–81.

Connor, D. A., & Gould, T. J. (2016). The role of working memory and declarative memory in trace conditioning. *Neurobiology of Learning and Memory*, *134*(Part B), 193–209. http://doi.org/10.1016/j.nlm.2016.07.009

Cook, C., Gass, J., Dunmore, J., Tong, J., Taylor, J., Eriksen, J., et al. (2009). Aging is not associated with proteasome impairment in UPS reporter mice. *PLoS ONE*, *4*(6), e5888. http://doi.org/10.1371/journal.pone.0005888

Cowansage, K. K., Shuman, T., Dillingham, B. C., Chang, A., Golshani, P., & Mayford, M. (2014). Direct reactivation of a coherent neocortical memory of context. *Neuron*, *84*(2), 432–441. http://doi.org/10.1016/j.neuron.2014.09.022

Craik, F. I. M., & Rose, N. S. (2012). Memory encoding and aging: a neurocognitive perspective. *Neuroscience and Biobehavioral Reviews*, *36*(7), 1729–1739. http://doi.org/10.1016/j.neubiorev.2011.11.007

Craik, F. I., & McDowd, J. M. (1987). Age differences in recall and recognition. *Journal of Experimental Psychology: Learning, Memory, and Cognition*, *13*(3), 474–479. http://doi.org/10.1037/0278-7393.13.3.474

Daselaar, S. M., Fleck, M. S., Dobbins, I. G., Madden, D. J., & Cabeza, R. (2006). Effects of healthy aging on hippocampal and rhinal memory functions: an event-related fMRI study. *Cerebral Cortex (New York, N.Y. : 1991)*, *16*(12), 1771–1782. http://doi.org/10.1093/cercor/bhj112

Daselaar, S. M., Veltman, D. J., Rombouts, S. A. R. B., Raaijmakers, J. G. W., & Jonker, C. (2003). Neuroanatomical correlates of episodic encoding and retrieval in young and elderly subjects. *Brain : a Journal of Neurology*, *126*(Pt 1), 43–56.

Davis, S., Salin, H., Helme-Guizon, A., Dumas, S., Stéphan, A., Corbex, M., et al. (2000). Dysfunctional regulation of alphaCaMKII and syntaxin 1B transcription after induction of LTP in the aged rat. *The European Journal of Neuroscience*, *12*(9), 3276–3282.

Ding, Q., & Zhu, H. (2015). Alteration of Proteasome System in Aging and Aging-Associated Disorders. *International Journal of Drug Development and …*, *7*(4), 27–30.

Drag, L. L., & Bieliauskas, L. A. (2010). Contemporary review 2009: cognitive aging. *Journal of Geriatric Psychiatry and Neurology*, *23*(2), 75–93. http://doi.org/10.1177/0891988709358590

Driscoll, I., & Sutherland, R. J. (2005). The aging hippocampus: navigating between rat and human experiments. *Reviews in the Neurosciences*, *16*(2), 87–121.

Driscoll, I., Hamilton, D. A., Petropoulos, H., Yeo, R. A., Brooks, W. M., Baumgartner, R. N., & Sutherland, R. J. (2003). The aging hippocampus: cognitive, biochemical and structural findings. *Cerebral Cortex (New York, N.Y. : 1991)*, *13*(12), 1344–1351. http://doi.org/10.1093/cercor/bhg081

Driscoll, I., Howard, S. R., Stone, J. C., Monfils, M. H., Tomanek, B., Brooks, W. M., & Sutherland, R. J. (2006). The aging hippocampus: a multi-level analysis in the rat. *Neuroscience*, *139*(4), 1173–1185. http://doi.org/10.1016/j.neuroscience.2006.01.040

Ehlers, M. D. (2003). Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nature Neuroscience*, *6*(3), 231–242. http://doi.org/10.1038/nn1013

Einarsson, E. Ö., & Nader, K. (2012). Involvement of the anterior cingulate cortex in formation, consolidation, and reconsolidation of recent and remote contextual fear memory. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *19*(10), 449–452. http://doi.org/10.1101/lm.027227.112

Erickson, C. A., & Barnes, C. A. (2003). The neurobiology of memory changes in normal aging. *Experimental Gerontology*, *38*(1-2), 61–69.

Fanselow, M. S. (1980). Conditional and unconditional components of post-shock freezing. *Pavlovian Journal of Biological Sciences*, *15*, 177–182.

Foster, T. C., & Kumar, A. (2002). Calcium dysregulation in the aging brain. *Neuroscientist*, *8*(4), 297–301.

Frick, K. M., Baxter, M. G., Markowska, A. L., Olton, D. S., & Price, D. L. (1995). Age-related spatial reference and working memory deficits assessed in the water maze. *Neurobiology of Aging*, *16*(2), 149–160.

Galeffi, F., Shetty, P. K., Sadgrove, M. P., & Turner, D. A. (2015). Age-related metabolic fatigue during low glucose conditions in rat hippocampus. *Neurobiology of Aging*, *36*(2), 982–992. http://doi.org/10.1016/j.neurobiolaging.2014.09.016

Gallagher, M., Burwell, R., & Burchinal, M. (1993). Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. *Behavioral Neuroscience*, *107*(4), 618–626.

Giannini, C., Kloß, A., Gohlke, S., Mishto, M., Nicholson, T. P., Sheppard, P. W., et al. (2013). Poly-Ub-substrate-degradative activity of 26S proteasome is not impaired in the aging rat brain. *PLoS ONE*, *8*(5), e64042. http://doi.org/10.1371/journal.pone.0064042

Gilinsky, A. S., & Judd, B. B. (1994). Working memory and bias in reasoning across the life span. *Psychology and Aging*, *9*(3), 356–371.

Gilmartin, M. R., & Helmstetter, F. J. (2010). Trace and contextual fear conditioning require neural activity and NMDA receptor-dependent transmission in the medial prefrontal cortex. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *17*(6), 289–296. http://doi.org/10.1101/lm.1597410

Gilmartin, M. R., Miyawaki, H., Helmstetter, F. J., & Diba, K. (2013). Prefrontal activity links nonoverlapping events in memory. *Journal of Neuroscience*, *33*(26), 10910–10914. http://doi.org/10.1523/JNEUROSCI.0144-13.2013

Golomb, J. D., Peelle, J. E., Addis, K. M., & Kahana, M. J. (2008). Effects of adult aging on utilization of temporal and semantic associations during free and serial recall. *Memory & Cognition*, *36*(5), 947–956. http://doi.org/10.3758/MC.36.5.947

Gonzalez-Lima, F., & Bruchey, A. K. (2004). Extinction memory improvement by the metabolic enhancer methylene blue. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *11*(5), 633–640. http://doi.org/10.1101/lm.82404

Han, C. J., OTuathaigh, C. M., van Trigt, L., Quinn, J. J., Fanselow, M. S., Mongeau, R., et al. (2003). Trace but not delay fear conditioning requires attention and the anterior cingulate cortex. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(22), 13087–13092. http://doi.org/10.1073/pnas.2132313100

Hedden, T., & Gabrieli, J. D. E. (2004). Insights into the ageing mind: a view from cognitive neuroscience. *Nature Reviews Neuroscience*, *5*(2), 87–96. http://doi.org/10.1038/nrn1323

Hegde, A. N. (2010). The ubiquitin-proteasome pathway and synaptic plasticity. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *17*(7), 314–327. http://doi.org/10.1101/lm.1504010

Hegde, A. N., Inokuchi, K., Pei, W., Casadio, A., Ghirardi, M., Chain, D. G., et al. (1997). Ubiquitin C-Terminal Hydrolase Is an Immediate-Early Gene Essential for Long-Term Facilitation in Aplysia. *Cell*, *89*(1), 115–126. http://doi.org/10.1016/S0092-8674(00)80188-9

Helmstetter, F. J., Parsons, R. G., & Gafford, G. M. (2008). Macromolecular synthesis, distributed synaptic plasticity, and fear conditioning. *Neurobiology of Learning and Memory*, *89*(3), 324–337. http://doi.org/10.1016/j.nlm.2007.09.002

Henley, J. M., & Wilkinson, K. A. (2013). AMPA receptor trafficking and the mechanisms underlying synaptic plasticity and cognitive aging. *Dialogues in Clinical Neuroscience*, *15*(1), 11–27.

Houston, F. P., Stevenson, G. D., McNaughton, B. L., & Barnes, C. A. (1999). Effects of age on the generalization and incubation of memory in the F344 rat. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *6*(2), 111–119.

Howland, R. H. (2016). Methylene Blue The Long and Winding Road from Stain to Brain: Part 1. *Journal of Psychosocial Nursing and Mental Health Services*, *54*(9), 21–24. http://doi.org/10.3928/02793695-20160920-04

Hung, A. Y., Sung, C. C., Brito, I. L., & Sheng, M. (2010). Degradation of Postsynaptic Scaffold GKAP and Regulation of Dendritic Spine Morphology by the TRIM3 Ubiquitin Ligase in Rat Hippocampal Neurons. *PLoS ONE*, *5*(3), e9842. http://doi.org/10.1371/journal.pone.0009842

Jarome, T. J., & Helmstetter, F. J. (2013). The ubiquitin-proteasome system as a critical regulator of synaptic plasticity and long-term memory formation. *Neurobiology of Learning and Memory*, *105*, 107–116. http://doi.org/10.1016/j.nlm.2013.03.009

Jarome, T. J., & Helmstetter, F. J. (2014). Protein degradation and protein synthesis in long-term memory formation. *Frontiers in Molecular Neuroscience*, *7*(61), 1–12. http://doi.org/10.3389/fnmol.2014.00061

Jarome, T. J., Kwapis, J. L., Ruenzel, W. L., & Helmstetter, F. J. (2013). CaMKII, but not protein kinase A, regulates Rpt6 phosphorylation and proteasome activity during the

formation of long-term memories. *Frontiers in Behavioral Neuroscience*, *7*, 115. http://doi.org/10.3389/fnbeh.2013.00115

Jarome, T. J., Werner, C. T., Kwapis, J. L., & Helmstetter, F. J. (2011). Activity dependent protein degradation is critical for the formation and stability of fear memory in the amygdala., *6*(9), e24349. http://doi.org/10.1371/journal.pone.0024349

Jouvenceau, A., & Dutar, P. (2006). A role for the protein phosphatase 2B in altered hippocampal synaptic plasticity in the aged rat. *Journal of Physiology, Paris*, *99*(2-3), 154–161. http://doi.org/10.1016/j.jphysparis.2005.12.009

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, *294*(5544), 1030–1038. http://doi.org/10.1126/science.1067020

Keller, J. N., Hanni, K. B., & Markesbery, W. R. (2000). Possible involvement of proteasome inhibition in aging: implications for oxidative stress. *Mechanisms of Ageing and Development*, *113*(1), 61–70.

Knight, D. C., Cheng, D. T., Smith, C. N., Stein, E. A., & Helmstetter, F. J. (2004). Neural substrates mediating human delay and trace fear conditioning. *Journal of Neuroscience*, *24*(1), 218–228. http://doi.org/10.1523/JNEUROSCI.0433-03.2004

Knight, D. C., Nguyen, H. T., & Bandettini, P. A. (2006). The role of awareness in delay and trace fear conditioning in humans. *Cognitive Affective & Behavioral Neuroscience*, *6*(2), 157–162. http://doi.org/10.3758/CABN.6.2.157

Kochli, D. E., Thompson, E. C., Fricke, E. A., Postle, A. F., & Quinn, J. J. (2015). The amygdala is critical for trace, delay, and contextual fear conditioning. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *22*(2), 92–100. http://doi.org/10.1101/lm.034918.114

Koen, J. D., & Yonelinas, A. P. (2014). The effects of healthy aging, amnestic mild cognitive impairment, and Alzheimer's disease on recollection and familiarity: a meta-analytic review. *Neuropsychology Review*, *24*(3), 332–354. http://doi.org/10.1007/s11065-014-9266-5

Kwapis, J. L., Jarome, T. J., Lee, J. L., & Helmstetter, F. J. (2015). The retrosplenial cortex is involved in the formation of memory for context and trace fear conditioning. *Neurobiology of Learning and Memory*, *123*, 110–116. http://doi.org/10.1016/j.nlm.2015.06.007

Kwapis, J. L., Jarome, T. J., Schiff, J. C., & Helmstetter, F. J. (2011). Memory consolidation in both trace and delay fear conditioning is disrupted by intra-amygdala infusion of the protein synthesis inhibitor anisomycin. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *18*(11), 728–732. http://doi.org/10.1101/lm.023945.111

Lee, S.H., Choi, J.H., Lee, N., Lee, H.R., Kim, J.I., Yu, N.K., et al. (2008). Synaptic Protein Degradation Underlies Destabilization of Retrieved Fear Memory. *Science*, *319*(5867), 1253–1256. http://doi.org/10.1126/science.1150541

Lehmann, G., Udasin, R. G., & Ciechanover, A. (2016). On the linkage between the ubiquitin-proteasome system and the mitochondria. *Biochemical and Biophysical Research Communications*, *473*(1), 80–86. http://doi.org/10.1016/j.bbrc.2016.03.055

Livnat-Levanon, N., & Glickman, M. H. (2011). Ubiquitin–Proteasome System and mitochondria — Reciprocity. *BBA - Gene Regulatory Mechanisms*, *1809*(2), 80–87. http://doi.org/10.1016/j.bbagrm.2010.07.005

Long, L.H., Liu, R.L., Wang, F., Liu, J., Hu, Z.L., Xie, N., et al. (2009). Age-related synaptic changes in the CA1 stratum radiatum and spatial learning impairment in rats. *Clinical and Experimental Pharmacology & Physiology*, *36*(7), 675–681. http://doi.org/10.1111/j.1440-1681.2008.05132.x

Lopez-Salon, M., Alonso, M., Vianna, M. R., Viola, H., Mello e Souza, T., Izquierdo, I., et al. (2001). The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. *The European Journal of Neuroscience*, *14*(11), 1820–1826.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The Hallmarks of Aging. *Cell*, *153*(6), 1194–1217. http://doi.org/10.1016/j.cell.2013.05.039

Löw, P. (2011). The role of ubiquitin-proteasome system in ageing. *General and Comparative Endocrinology*, *172*(1), 39–43. http://doi.org/10.1016/j.ygcen.2011.02.005

Mabb, A. M., & Ehlers, M. D. (2010). Ubiquitination in postsynaptic function and plasticity. *Annual Review of Cell and Developmental Biology*, *26*, 179–210. http://doi.org/10.1146/annurev-cellbio-100109-104129

Makrides, S. C. (1983). Protein synthesis and degradation during aging and senescence. *Biological Reviews of the Cambridge Philosophical Society*, *58*(3), 343–422.

Martinez, J. L., Jensen, R. A., & Vasquez, B. J. (1978). Methylene blue alters retention of inhibitory avoidance responses. *Physiological Psychiatry*, *6*(3), 387–390.

Martinez-Vicente, M., Sovak, G., & Cuervo, A. M. (2005). Protein degradation and aging. *Experimental Gerontology*, *40*(8-9), 622–633. http://doi.org/10.1016/j.exger.2005.07.005

McEchron, M. D., Bouwmeester, H., Tseng, W., Weiss, C., & Disterhoft, J. F. (1998). Hippocampectomy disrupts auditory trace fear conditioning and contextual fear conditioning in the rat. *Hippocampus*, *8*(6), 638–646. http://doi.org/10.1002/(SICI)1098-1063(1998)8:6<638::AID-HIPO6>3.0.CO;2-Q

McEchron, M. D., Cheng, A. Y., & Gilmartin, M. R. (2004). Trace fear conditioning is reduced in the aging rat. *Neurobiology of Learning and Memory*, *82*(2), 71–76. http://doi.org/10.1016/j.nlm.2004.06.002

Medina, D. X., Caccamo, A., & Oddo, S. (2011). Methylene blue reduces aβ levels and rescues early cognitive deficit by increasing proteasome activity. *Brain Pathology (Zurich, Switzerland)*, *21*(2), 140–149. http://doi.org/10.1111/j.1750-3639.2010.00430.x

Monti, L. A., Gabrieli, J. D. E., Reminger, S. L., Rinaldi, J. A., Wilson, R. S., & Fleischman, D. A. (1996). Differential effects of aging and Alzheimer's disease on conceptual implicit and explicit memory. *Neuropsychology*, *10*(1), 101–112. http://doi.org/10.1037/0894-4105.10.1.101

Morris, K. A., & Gold, P. E. (2012). Age-related impairments in memory and in CREB and pCREB expression in hippocampus and amygdala following inhibitory avoidance training. *Mechanisms of Ageing and Development*, *133*(5), 291–299. http://doi.org/10.1016/j.mad.2012.03.004

Moyer, J. R., & Brown, T. H. (2006). Impaired trace and contextual fear conditioning in aged rats. *Behavioral Neuroscience*, *120*(3), 612–624. http://doi.org/10.1037/0735-7044.120.3.612

Naveh-Benjamin, M. (2000). Adult age differences in memory performance: tests of an associative deficit hypothesis. *Journal of Experimental Psychology: Learning, Memory, and Cognition*, *26*(5), 1170–1187.

Naveh-Benjamin, M., Hussain, Z., Guez, J., & Bar-On, M. (2003). Adult age differences in episodic memory: further support for an associative-deficit hypothesis. *Journal of Experimental Psychology: Learning, Memory, and Cognition*, *29*(5), 826–837. http://doi.org/10.1037/0278-7393.29.5.826

Newman, M. C., & Kaszniak, A. W. (2000). Spatial Memory and Aging: Performance on a Human Analog of the Morris Water Maze. *Aging, Neuropsychology, and Cognition (Neuropsychology, Development and Cognition: Section B)*, *7*(2), 86–93. http://doi.org/10.1076/1382-5585(200006)7:2;1-U;FT086

Nilsson, L., BÄCkman, L., & Erngrund, K. (1997). The Betula prospective cohort study: Memory, health, and aging. *Aging*, *4*(1), 1–32.

Nilsson, L.-G. (2003). Memory function in normal aging. *Acta Neurologica Scandinavica*, *107*(s179), 7–13. http://doi.org/10.1034/j.1600-0404.107.s179.5.x

Oler, J. A., & Markus, E. J. (1998). Age-related deficits on the radial maze and in fear conditioning: Hippocampal processing and consolidation. *Hippocampus*, *8*(4), 402–415. http://doi.org/10.1002/(SICI)1098-1063(1998)8:4<402::AID-HIPO8>3.3.CO;2-U

Ortman, J. M., Velkoff, V. A., & Hogan, H. (2014). An aging nation: the older population in the United States. 2014 (pp. 1–28). U.S. Census Bureau (Ed.).

Oz, M., Lorke, D. E., Hasan, M., & Petroianu, G. A. (2010). Cellular and molecular actions of Methylene Blue in the nervous system. *Medicinal Research Reviews*, *31*(1), 93–117. http://doi.org/10.1002/med.20177

Paban, V., Fauvelle, F., & Alescio-Lautier, B. (2010). Age-related changes in metabolic profiles of rat hippocampus and cortices. *The European Journal of Neuroscience*, *31*(6), 1063–1073. http://doi.org/10.1111/j.1460-9568.2010.07126.x

Park, D. C., Lautenschlager, G., Hedden, T., Davidson, N. S., Smith, A. D., & Smith, P. K. (2002). Models of visuospatial and verbal memory across the adult life span. *Psychology and Aging*, *17*(2), 299–320. http://doi.org/10.1037//0882-7974.17.2.299

Pavlov, P. I. (2010). Conditioned reflexes: An investigation of the physiological activity of the cerebral cortex. *Annals of Neurosciences*, *17*(3), 136–141. http://doi.org/10.5214/ans.0972-7531.1017309

Peter, C., Hongwan, D., Kupfer, A., & Lauterburg, B. H. (2000). Pharmacokinetics and organ distribution of intravenous and oral methylene blue. *European Journal of Clinical Pharmacology*, *56*(3), 247–250.

Petersen, R. C., Smith, G., Kokmen, E., Ivnik, R. J., & Tangalos, E. G. (1992). Memory Function in Normal Aging. *Neurology*, *42*(2), 396–401.

Provyn, J. P., Sliwinski, M. J., & Howard, M. W. (2007). Effects of age on contextually mediated associations in paired associate learning. *Psychology and Aging*, *22*(4), 846–857. http://doi.org/10.1037/0882-7974.22.4.846

Quinn, J. J., Loya, F., Ma, Q. D., & Fanselow, M. S. (2005). Dorsal hippocampus NMDA receptors differentially mediate trace and contextual fear conditioning. *Hippocampus*, *15*(5), 665–674. http://doi.org/10.1002/hipo.20088

Quinn, J. J., Oommen, S. S., Morrison, G. E., & Fanselow, M. S. (2002). Post-training excitotoxic lesions of the dorsal hippocampus attenuate forward trace, backward trace, and delay fear conditioning in a temporally specific manner. *Hippocampus*, *12*(4), 495–504. http://doi.org/10.1002/hipo.10029

Reis, D. S., Jarome, T. J., & Helmstetter, F. J. (2013). Memory formation for trace fear conditioning requires ubiquitin-proteasome mediated protein degradation in the prefrontal cortex. *Frontiers in Behavioral Neuroscience*, *7*. http://doi.org/10.3389/fnbeh.2013.00150

Riha, P. D., Bruchey, A. K., Echevarria, D. J., & Gonzalez-Lima, F. (2005). Memory facilitation by methylene blue: Dose-dependent effect on behavior and brain oxygen consumption. *European Journal of Pharmacology*, *511*(2-3), 151–158. http://doi.org/10.1016/j.ejphar.2005.02.001

Riha, P. D., Rojas, J. C., & Gonzalez-Lima, F. (2011). Beneficial network effects of methylene blue in an amnestic model. *NeuroImage*, *54*(4), 2623–2634. http://doi.org/10.1016/j.neuroimage.2010.11.023

Roberson, E. D., Defazio, R. A., Barnes, C. A., Alexander, G. E., Bizon, J. L., Bowers, D., et al. (2012). Challenges and opportunities for characterizing cognitive aging across species. *Frontiers in Aging Neuroscience*, *4*, 6. http://doi.org/10.3389/fnagi.2012.00006

Rojas, J. C., Bruchey, A. K., & Gonzalez-Lima, F. (2012). Neurometabolic mechanisms for memory enhancement and neuroprotection of methylene blue. *Progress in Neurobiology*, *96*(1), 32–45. http://doi.org/10.1016/j.pneurobio.2011.10.007

Ross, J., Olson, L., & Coppotelli, G. (2015). Mitochondrial and Ubiquitin Proteasome System Dysfunction in Ageing and Disease: Two Sides of the Same Coin? *International Journal of Molecular Sciences*, *16*(8), 19458–19476. http://doi.org/10.3390/ijms160819458

Rowe, J. W., & Kahn, R. L. (1987). Human aging: usual and successful. *Science*, *237*(4811), 143–149.

Rönnlund, M., Nyberg, L., Bäckman, L., & Nilsson, L.-G. (2005). Stability, growth, and decline in adult life span development of declarative memory: cross-sectional and longitudinal data from a population-based study. *Psychology and Aging*, *20*(1), 3–18. http://doi.org/10.1037/0882-7974.20.1.3

Saez, I., & Vilchez, D. (2014). The Mechanistic Links Between Proteasome Activity, Aging and Age-related Diseases. *Current Genomics*, *15*(1), 38–51. http://doi.org/10.2174/138920291501140306113344

Schirmer, R. H., Adler, H., Pickhardt, M., & Mandelkow, E. (2011). "Lest we forget you--methylene blue...". *Neurobiology of Aging*, *32*(12), 2325.e7–16. http://doi.org/10.1016/j.neurobiolaging.2010.12.012

Singer, T., Verhaeghen, P., Ghisletta, P., Lindenberger, U., & Baltes, P. B. (2003). The fate of cognition in very old age: six-year longitudinal findings in the Berlin Aging Study (BASE). *Psychology and Aging*, *18*(2), 318–331. http://doi.org/10.1037/0882-7974.18.2.318

Smith, C. D., Walton, A., Loveland, A. D., Umberger, G. H., Kryscio, R. J., & Gash, D. M. (2005). Memories that last in old age: motor skill learning and memory preservation. *Neurobiology of Aging*, *26*(6), 883–890. http://doi.org/10.1016/j.neurobiolaging.2004.08.014

Steenland, H. W., Li, X. Y., & Zhuo, M. (2012). Predicting Aversive Events and Terminating Fear in the Mouse Anterior Cingulate Cortex during Trace Fear Conditioning. *Journal of Neuroscience*, *32*(3), 1082–1095. http://doi.org/10.1523/JNEUROSCI.5566-11.2012

Stoehr, J. D., & Wenk, G. L. (1995). Effects of age and lesions of the nucleus basalis on contextual fear conditioning. *Psychobiology*, *23*, 173–177.

Telch, M. J., Bruchey, A. K., Rosenfield, D., Cobb, A. R., Smits, J., Pahl, S., & Gonzalez-Lima, F. (2014). Effects of Post-Session Administration of Methylene Blue on Fear Extinction and Contextual Memory in Adults With Claustrophobia. *American Journal of Psychiatry*, *171*(10), 1091–1098. http://doi.org/10.1176/appi.ajp.2014.13101407

Tomaru, U., Takahashi, S., Ishizu, A., Miyatake, Y., Gohda, A., Suzuki, S., et al. (2012). Decreased proteasomal activity causes age-related phenotypes and promotes the development of metabolic abnormalities. *The American Journal of Pathology*, *180*(3), 963–972. http://doi.org/10.1016/j.ajpath.2011.11.012

Tulving, E. (1987). Multiple memory systems and consciousness. *Human Neurobiology*, *6*(2), 67–80.

Uttl, B., & Graf, P. (1993). Episodic spatial memory in adulthood. *Psychology and Aging*, *8*(2), 257–273.

Vechio, F. H. D., Cerqueira, F., Augusto, O., Lopes, R., & Demasi, M. (2014). Peptides that activate the 20S proteasome by gate opening increased oxidized protein removal and reduced protein aggregation. *Free Radical Biology and Medicine*, *67*(C), 304–313. http://doi.org/10.1016/j.freeradbiomed.2013.11.017

Villarreal, J. S., Dykes, J. R., & Barea-Rodriguez, E. J. (2004). Fischer 344 rats display age-related memory deficits in trace fear conditioning. *Behavioral Neuroscience*, *118*(6), 1166–1175. http://doi.org/10.1037/0735-7044.118.6.1166

Wainwright, M., & Crossley, K. B. (2002). Methylene Blue - a therapeutic dye for all seasons? *Journal of Chemotherapy*, *14*(5), 431–443. http://doi.org/10.1179/joc.2002.14.5.431

Ward, M. T., Oler, J. A., & Markus, E. J. (1999). Hippocampal dysfunction during aging I: Deficits in memory consolidation. *Neurobiology of Aging*, *20*(4), 363–372. http://doi.org/10.1016/S0197-4580(99)00045-7

Weike, A. I., Schupp, H. T., & Hamm, A. O. (2007). Fear acquisition requires awareness in trace but not delay conditioning. *Psychophysiology*, *44*(1), 170–180. http://doi.org/10.1111/j.1469-8986.2006.00469.x

Wrubel, K. M., Barrett, D., Shumake, J., Johnson, S. E., & Gonzalez-Lima, F. (2007a). Methylene blue facilitates the extinction of fear in an animal model of susceptibility to learned helplessness. *Neurobiology of Learning and Memory*, *87*(2), 209–217. http://doi.org/10.1016/j.nlm.2006.08.009

Wrubel, K. M., Riha, P. D., Maldonado, M. A., McCollum, D., & Gonzalez-Lima, F. (2007b).

The brain metabolic enhancer methylene blue improves discrimination learning in rats. *Pharmacology, Biochemistry, and Behavior*, *86*(4), 712–717. http://doi.org/10.1016/j.pbb.2007.02.018

Zelikowsky, M., Hersman, S., Chawla, M. K., Barnes, C. A., & Fanselow, M. S. (2014). Neuronal ensembles in amygdala, hippocampus, and prefrontal cortex track differential components of contextual fear. *Journal of Neuroscience*, *34*(25), 8462–8466. http://doi.org/10.1523/JNEUROSCI.3624-13.2014

Shane E. Pullins

Address: 224 Garland Hall
2441 E. Hartford Ave.
Milwaukee, Wisconsin 53211

414-229-4979
spullins@uwm.edu

EDUCATION:

University of Wisconsin – Milwaukee, Milwaukee, Wisconsin
Doctoral Student in Experimental Psychology | August, 2014 – Present
Major: Neuroscience
G.P.A.: 4.0

Miami University, Oxford, Ohio
B.S. & B.A. in Zoology | May, 2014
Minor in Neuroscience

RESEARCH EXPERIENCE:

Graduate Research Assistant | August, 2016 - Present
Principal Investigator: Fred Helmstetter, Ph.D.

Undergraduate Research Assistant | October, 2011 – July, 2014
Principal Investigator: Jennifer Quinn, Ph.D.

AWARDS AND FELLOWSHIPS:

University of Wisconsin-Milwaukee – Graduate Student Excellence Fellowship | May, 2017

University of Wisconsin-Milwaukee – Department of Psychology Summer Research Fellowship | May, 2015

Society for Neuroscience - Faculty for Undergraduate Neuroscience Travel Award | November, 2013

Miami University - Student Enrichment Funding Award | October, 2013

Miami University - Undergraduate Presentation Award | October, 2013

Miami University - Undergraduate Summer Scholarship | May, 2013

Miami University - Undergraduate Presentation Award | October, 2012

PUBLICATIONS:

Pullins, SE, Cullen PK, Ferrara NC, Helmstetter, FJ. *In Preparation*. Age-related deficits in trace fear conditioning and activity-dependent protein degradation.

Pullins, SE, Cullen PK, Ferrara NC, Helmstetter, FJ. *In Preparation*. Contributions of the retrosplenial cortex to event-related and contextual memory formation.

Cullen PK, **Pullins SE**, Ferrara NC, Hintz J, Helmstetter FJ. *In Preparation.* Optogenetic stimulation of central nucleus of the amygdala-ventral periaqueductal gray results in consummatory behavior.

Cullen PK, Ferrara NC, **Pullins SE**, Helmstetter FJ. *In Preparation.* Expression of fear memory is maintained across a distributed neural network.

Ferrara NC, Cullen PK, **Pullins SE**, Rotondo EK, Helmstetter FJ. 2017. Input from the medial geniculate nucleus modulates amygdala encoding of fear memory discrimination. *Learning and Memory.* 24(9): 414-421**.** doi: 10.1101/lm.044131

Cullen PK, Ferrara NC, **Pullins SE**, Helmstetter FJ. 2017. Context memory formation requires activity dependent protein degradation in the hippocampus. *Learning and Memory.* 24(11): 589-596. doi: 10.1101/lm.045443.117.

Pierson, JL, **Pullins, SE**, Quinn, JJ. 2015. Dorsal hippocampus infusions of CNQX into the dentate gyrus disrupt expression of trace fear conditioning. *Hippocampus.* doi:10.1002/hipo.22413

POSTER PRESENTATIONS:

Pullins, S.E., Cullen, P.K., Ferrara, N.F., Cruz, W.C., Helmstetter, F.J. 2017. Contributions of the retrosplenial cortex to event-related and contextual fear memory formation in trace fear conditioning. The Society for Neuroscience Annual Meeting, Washington D.C.

Pullins, S.E., Cullen, P.K., Ferrara, N.F., Cruz, W.C., Helmstetter, F.J. 2017. Contributions of the retrosplenial cortex to event-related and contextual fear memory formation in trace fear conditioning. The Pavlovian Society Annual Meeting, Philadelphia.

Cruz, W.C., **Pullins, S.E.**, Moyer, J.R. Jr., Helmstetter, F.J. 2017. The effects of methylene blue on trace fear memory and brain proteolytic activity in young and aged rats. The Society for Neuroscience Annual Meeting, Washington D.C.

Pullins, S.E., Cullen, P.K., Ferrara, N.F., Moyer, J.R. Jr., Helmstetter, F.J. 2016. Activity-dependent protein degradation and age-related memory impairment. The Society for Neuroscience Annual Meeting, San Diego.

Cullen, P.K., Ferrara, N.C., **Pullins, S.E.**, Hintz, J., Helmstetter, F.J. 2016. Behavioral expression of a fear memory is maintained by neural activity in a distributed brain network throughout CS presentation. The Society for Neuroscience Annual Meeting, San Diego.

Ferrara, N.C., Cullen, P.K., Rotondo, E.K., **Pullins, S.E.**, Helmstetter, F.J. Medial geniculate nucleus input modulates amygdala encoding of fear memory discrimination. The Society for Neuroscience Annual Meeting, San Diego.

Cullen, P.K., Ferrara, N.C., **Pullins, S.E.**, Helmstetter, F.J. 2015. Neural activity in the lateral amygdala driven by auditory CS onset critically determines memory retrieval and behavioral expression of fear. The Society for Neuroscience Annual Meeting, Chicago.

Beeman C.L., **Pullins, S.E.**, Hoogendoorn, J.J., Quinn, J.J. 2014. Complete hippocampal lesions disrupt recent and remote trace fear memories regardless of the lesion-to-test interval. The Pavlovian Society Annual Meeting, Seattle.

Pierson, J.L., **Pullins, S.E.**, Quinn, J.J. 2014. Dorsal hippocampus infusions of CNQX into the dentate gyrus disrupt expression of trace fear conditioning. The Pavlovian Society Annual Meeting, Seattle.

Pullins, S.E., Beeman, C.L., Hoogendoorn, J.J., Quinn, J.J. 2013. Temporally-graded retrograde amnesia following trace fear conditioning: Focal hippocampus damage or distal disruption? The Society for Neuroscience Annual Meeting, San Diego.

Quinn, J.J., Skipper, R.A., **Pullins, S.E.**, Damas-Vannucchi, I., Martin, K., Powers, L., Renda, M., Reser, K.M., Ronau, R., Schaefer, M., Wilber, J., Wilkins, B., Winfield, M. 2013. Growing neuroscientists: Sparking an interest in elementary students. The Society for Neuroscience Annual Meeting, San Diego.

Beeman, C.L., Hoogendoorn, J.J., **Pullins, S.E.**, Quinn, J.J. 2013. Characterizing the effects of complete hippocampal lesions on trace and contextual fear conditioning following a 30 day lesion-to-test interval. The Society for Neuroscience Annual Meeting, San Diego.

Pullins, S.E., Pierson, J.L., Burton, J.M., Quinn, J.J. 2012. Immunohistochemical analysis of the dorsal hippocampus during recent and remote fear memory retrieval. The Society for Neuroscience Annual Meeting, New Orleans.

Pierson, J.L., **Pullins, S.E.**, Quinn, J.J. 2012. Expression of trace fear conditioning requires the dentate gyrus, but not CA1 or CA3, of the dorsal hippocampus. The Society for Neuroscience Annual Meeting, New Orleans.

ORAL PRESENTATIONS AND INVITED TALKS:

Pullins, S.E., Cullen, P.K., Helmstetter, F.J. 2017. Contributions of the retrosplenial cortex to event-related and contextual memory formation. Invited symposium speaker at the Midwestern Psychological Association Meeting, Chicago, Illinois.

Pullins, S.E., Cullen, P.K., Helmstetter, F.J. 2017. Contributions of the retrosplenial cortex to event-related and contextual memory formation. Oral presentation at AGSIP Symposium, University of Wisconsin – Milwaukee.

Pullins, S.E., Cullen, P.K., Helmstetter, F.J. 2017. Contributions of the retrosplenial cortex to event-related and contextual memory formation. Oral presentation at UWM Spring Neuroscience Symposium, University of Wisconsin – Milwaukee.

Pullins, S.E. April 2016. Age-related deficits in trace fear conditioning and activity related protein degradation. Oral presentation at AGSIP Symposium, University of Wisconsin – Milwaukee.

TEACHING AND OUTREACH:

Association for the Advancement of University Women, University of Wisconsin – Milwaukee, Milwaukee, Wisconsin
Assistant Teacher | August 2016 – Present

Molecular Basis of Memory, University of Wisconsin – Milwaukee, Milwaukee, Wisconsin
President | August 2017 – Present

Guest Lecture: Psychopharmacology, Introduction to Neuropsychology - University of Wisconsin – Milwaukee, Milwaukee, Wisconsin | February 2017

Molecular Basis of Memory, University of Wisconsin – Milwaukee, Milwaukee, Wisconsin
Vice President | August 2016 – August 2017

Teaching Assistant, University of Wisconsin – Milwaukee, Milwaukee, Wisconsin
Behavioral Neuroscience Laboratory | Spring, 2016

Teaching Assistant, University of Wisconsin – Milwaukee, Milwaukee, Wisconsin
Behavioral Neuroscience Laboratory | Fall, 2015

Teaching Assistant, University of Wisconsin – Milwaukee, Milwaukee, Wisconsin
Introduction to Physiological Psychology | Spring, 2015

Teaching Assistant, University of Wisconsin – Milwaukee, Milwaukee, Wisconsin
Administrative Assistant | Fall, 2014

Nu Rho Psi Gamma in Ohio, Miami University, Oxford, Ohio
President | November 2012 – May 2014

Talawanda-Miami Science Week, Miami University, Oxford, Ohio
Assistant Teacher | Spring 2013, Spring 2014

Distinguished Undergraduate Teaching Fellowship, Miami University, Oxford, Ohio
Psychopharmacology | Spring 2014

Distinguished Undergraduate Teaching Fellowship, Miami University, Oxford, Ohio
Introduction to Biopsychology | Fall 2013

Teaching in Neuroscience Capstone, Miami University, Oxford, Ohio
Assistant Teacher | Spring, 2013

SKILLS/TECHNIQUES:

Rodent Behavior: Pavlovian context fear conditioning, Pavlovian cued fear conditioning, fear extinction, maternal separation, restraint stress, open field analysis, elevated plus maze analysis, forced swim testing, chronic variable stress

Biological: fluorescent *in situ* hybridization, surgical cannulation, tissue extraction and sectioning, fluorescent microscopy, western blotting, optogenetics, confocal microscopy, immunohistochemistry, immunofluorescence, retrograde tract tracing

Software: SPSS, GraphPad Prism, Freescan and Freeze Frame, ImageJ, GeneSys, MedPC/MedAssociates