THE ROLE OF EXOGENOUSLY ADMINISTERED MURINE AMYLOID-BETA IN DISRUPTING DIFFERENT PHASES OF THE LEARNING PROCESS: FROM CONSOLIDATION TO EXTINCTION

by

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CHAPTER 1: GENERAL INTRODUCTION

An integral part of survival is the process of learning. Learning is a biological process that enables adjustment to one's environment (Domjan, 2003). These learned adjustments cannot only exist in the present; they must be stored and able to affect one's future behavior. The ability of an organism to respond to or recount information that was previously experienced is often referred to as memory (Domjan, 2003). As such, the processes of acquiring new information and remembering that information are unequivocally linked and crucial for survival. While physiological systems have evolved to accomplish basic biological functions like breathing, digestion, and pathogen resistance, nonhuman animals and humans alike are forced to make behavioral adjustments to changes in their environment. Throughout development, this is exactly what humans do; humans learn to produce and withhold specific behaviors, in order to thrive. Unfortunately, as one ages, many of the behaviors that once promoted independence and success become compromised. This reality is readily apparent in neurodegenerative diseases, and more specifically, in the onset and progression of Alzheimer's disease (AD). AD is a slow, progressive and irreversible process that interferes with one's ability to remember past events, learn new information, and eventually affects core biological processes like speaking, swallowing, and breathing (Huang & Mucke, 2012). The specific mechanisms through which AD acts to disrupt both learning and memory processes are not fully understood, despite being heavily explored for more than one hundred years.

In 1906, Alios Alzheimer was introduced to a 51-year old woman diagnosed with pre-senile dementia, but unlike other patients with her diagnosis, she showed extreme

personality changes, complete disorientation, and her physical health rapidly deteriorated (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995). Upon post-mortem analysis, Alzheimer noticed severe cortical atrophy of the brain along with tangled fibrils where neurons should be, "minute miliary foci… caused by the deposition of a special substance" (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995). The lesions that Alzheimer originally documented in the early 20th century have come to be known as neurofibrillary tangles and senile plaques (Miklossy, 2008). These senile plaques were made of aggregated amyloid-beta (Aβ) fibrils and the intracellular tangles were comprised of detached, hyperphosphorylated tau, a microtubule-associated protein (Lee et al., 2008). For the purposes of this document, most attention will be placed on Aβ production and Aβ-induced pathologies as they relate to AD.

Aβ Production

Glenner and Wong (1984), using high performance liquid chromatography, isolated and purified the composition of these senile plaques. The plaques contained an aggregation of a hydrophobic polypeptide, 40 or 42 amino acids in length, which had a strong tendency to form stable dimers, trimers, oligomers, and eventually the insoluble plaques seen in AD brains (Glenner & Wong, 1984; Delacourte, 2006; Masters & Selkoe, 2012). This polypeptide is now referred to as amyloid-beta ($A\beta_{1-42}$ or Aβ for short). Aβ is derived through the enzymatic breakdown of the amyloid precursor protein (APP). APP is a single-pass membrane spanning protein with a large extracellular domain and a short cytoplasmic tail (O'Brien & Wong, 2011; Müller & Zheng, 2012). Alternate splicing of the APP transcript creates multiple isoforms of the protein, but the 695-long amino acid form is predominately expressed in neurons and accounts for the primary

source of APP in the brain (Sisodia et al., 1993; Bayer et al., 1999). Neurons produce large amounts of APP throughout the lifespan (Lee et al., 2008), and when delivered to the cell surface, APP can be directly cleaved by α-secretase and then γ-secretase, a process that does not result in the production of Aβ. Alternatively, APP can be internalized into endosomal compartments where the enzymatic complexes beta-site APP cleaving enzyme 1 (BACE1 also known as the neuronal β-secretase) and γ-secretase make cuts at their respective cleaving sites (Cai et al., 2001; McGeer & McGeer, 2001). The successive cleavage of APP by β-secretase and γ-secretase creates an aberrant, 42-amino-acid long peptide (Aβ), which is then released into the extracellular space and is capable of aggregating (O'Brien & Wong, 2011).

BACE1 and γ-secretase, are responsible for the amount of Aβ that is produced, but BACE1 activity is the rate-limiting factor (Zhang, Thompson, Zhang, & Xu, 2011). BACE1 protein levels and enzymatic activity are elevated in the brains of patients with sporadic AD (R. Li et al., 2004), and, in BACE1 knock-out animals (*BACE1*$^{-/-}$), aggregated Aβ plaques never form (Huang & Mucke, 2012; O'Brien & Wong, 2011). While BACE1 is highly relevant in Aβ generation, mutations to sub-units of γ-secretase, i.e. presenilins (PS)1 or 2, increase γ-secretase activity and up-regulate Aβ production (Harris et al., 2010; Huang & Mucke, 2012; R. Li et al., 2004). Patients with mutations to *PSEN1*, the gene that encodes PS1, have the largest amount of neurodegeneration, as marked by increased aggregation of Aβ and severe tissue loss (Snyder et al., 2005; Sokolova et al., 2009). Mutations to the APP protein itself can also increase AD symptomatology. For example, in animals that overexpress mutant APP, there is increased expression of Aβ plaque deposition throughout the hippocampus and cortex (Harris et al., 2010). Overall,

there are roughly 32 APP, 179 *PSEN1*, and 14 *PSEN2* gene mutations that have been linked to early-onset AD, and the common feature in all of these mutations is the increased production of the less soluble $A\beta_{1-42}$ species relative to its $A\beta_{1-40}$ relative (Shen & Kelleher, 2007). Additionally, specific inherited alleles like apolipoprotein E-e4 (*APOE4*) have been linked to late-onset, sporadic AD (Holtzman et al. 1999; Tiraboschi et al., 2004). The genetic contributions to AD development were further supported when APP was found to be encoded on the 21st chromosome in humans that, when triplicated, produces Down syndrome. Down syndrome produces remarkably similar neurological pathologies to those found in AD, e.g., the accumulation of Aβ (Haass & Selkoe, 1993; Masters & Selkoe, 2012; Selkoe, 1991). Since these findings, genetic models of AD constitute the majority of research efforts in the field (Kitazawa, Medeiros, & LaFerla, 2012).

Mouse Models of AD

Several different AD models in mice have emerged that contain many of the mutations discussed previously. For the sake of brevity, a few of the more heavily studied models will be discussed here. The Tg2576 mouse strain expresses human APP at a level five times higher than endogenous mouse APP, produces elevated $A\beta_{1-40}$ and $A\beta_{1-42}$ levels and plaque deposition in an age-dependent fashion, and demonstrates impaired cognition by 9–10 months of age (Elder, Sosa, & Gasperi, 2010). There are also mouse strains that have targeted *PSEN1* mutations, without accompanying *APP* mutations. Although these mice do not develop plaques per se, they still demonstrate elevated $A\beta_{1-42}$ production and cognitive deficits (Elder et al., 2010). When these mutations are combined, as in the 5xFAD mouse strain that contains 3 *APP* and 2 *PSEN1*

familial AD-linked (FAD) mutations, severe neuronal loss is seen in conjunction with the development of plaques and high $A\beta_{1-42}$ production and resultant cognitive decline (Elder et al., 2010). Overall, most animal strains developed to study AD involve mutations that are associated with Aβ production and/or deposition, but there are a few strains with modifications to tau as well (Elder et al., 2010)

Neurodegenerative Effects of Aβ

Communication from one neuron to the next occurs at the synapse, and full-length APP appears to play an important role in the formation and repair of these synapses, as APP knockout animals have impaired synaptic function and display marked memory disruptions without neuronal loss (see Hoe, Lee, & Pak, 2012). This beneficial effect stands in contrast to Aβ peptides, derivatives of APP, which can alter synaptic structure and function in deleterious ways (Harris et al., 2010; Ribrault, Sekimoto, & Triller, 2011). Indeed, the presence of Aβ oligomers at the synapse is more neurotoxic than the presence of Aβ plaques (Huang & Mucke, 2012; Kamenetz et al., 2003), and in the presence of these oligomers, there is a 60% loss of excitatory synapses (Koffie et al., 2009), with synapses containing acetylcholine, glutamate, and serotonin in the neocortex and hippocampus most affected (Grutzendler & Morris, 2001). Furthermore, Aβ in the synapse can lead to the endocytosis of NMDA receptors and decreased signaling through cAMP response element binding protein (CREB), a transcription factor crucial in neuronal plasticity and survival (Ittner & Götz, 2011; Snyder et al., 2005). Aβ oligomers have also been associated with decreased spine density, inhibited synapse remodeling, potent inhibition of long-term potentiation (LTP), and enhanced long-term depression (LTD) (Li et al., 2009 & 2014; Borlikova et

al., 2013; Freir et al., 2011; Snyder et al., 2005; Shankar et al., 2008; Hu et al., 2008; Freir et al., 2001; Dineley et al., 2010).

LTP is a form of synaptic plasticity that is considered a molecular and electrophysiological correlate of learning and memory. One of the first notions that molecular mechanisms could mediate synaptic plasticity came from Donald Hebb (1949) who argued that neural connections could be strengthened when two adjacent cells are activated at the same time. This idea was supported by the work conducted by Bliss and Lømo in 1973. With the understanding that electrically stimulated neurons in the perforant path lead to neural activity in the mossy fibers of the dentate gyrus, Bliss and Lømo examined the specifics of this activity and how it affected post-synaptic neurons. Eventually they noted that when a strong high-frequency electrical stimulation was used, subsequent stimulation of the neurons of the perforant pathway resulted in exaggerated post-synaptic excitability in neurons found in the dentate gyrus of the hippocampus. This phenomenon is now called LTP. Briefly, LTP occurs when there is a brief, high frequency train of action potentials in the same axon that causes physiological changes to the pre- and post-synaptic neuron. At the molecular level, when a neuron is simultaneously depolarized and has glutamate bound to the N-methyl-D-aspartate (NMDA) receptor, calcium ions (Ca^{2+}) flood into the cell, activate calcium-calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC), which phosphorylate existing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) channels, making them more permeable to sodium ions (Na^{+}). Additionally, new AMPA receptors are inserted into the membrane (Mucke & Selkoe, 2012). Furthermore, LTP leads to structural changes in the synapse. For example, there is an increase in the

dendritic spine density of the post-synaptic neuron (Kasai et al., 2010), along with a significantly higher frequency of multiple-spine synapses (Toni et al., 1999). The overall effect of LTP is to make the neuron more sensitive to subsequent release of neurotransmitters; the long-lasting changes in synaptic strength are thought to involve some of the molecular underpinning of memory storage. The importance of NMDA receptors and LTP in learning and memory was further supported in work conducted by Morris and colleagues in 1986. They noted that treatment with the NMDA antagonist, 2-amino-5-phosphonovaleric acid (AP5), decreased *in vivo* induction of LTP and selectively impaired place learning. These data are merely some of the multitude of data strengthening the case that hippocampal LTP may be particularly important in spatial learning. When Aβ is present at the synapse, NMDA receptor expression is lower, NMDA-mediated currents are reduced, and CREB signaling is reduced, all of which result in a net reduction in LTP (Snyder et al. 2005). Freir and colleagues (2001) also demonstrated that LTP could be blocked *in* vivo through intracerebroventricular (i.c.v.) administration of short Aβ peptides, namely, $A\beta_{15–25}$ and $A\beta_{25–35}$. Furthermore, Shankar and colleagues (2008) demonstrated reduced excitatory postsynaptic potential (EPSP) frequencies in Aβ-treated hippocampal slices in addition to reduced dendritic spine density.

The functional opposite of LTP is LTD, which can be triggered by prolonged periods of low-frequency stimulation, and results in the decreased synaptic response of neurons to stimulation of their afferents, and a weakening or loss of the synapse itself (for review, see Collingridge et al., 2010). This molecular process is enhanced in the presence of Aβ oligomers (Wang et al., 2002; Shankar et al., 2008). The neurotoxic role

of Aβ in the functional decrease in LTP and enhancement of LTD is further supported in studies utilizing antibodies against Aβ, which have restored baseline functioning (Rosenblum, 2014; Shankar et al., 2008) following induction of LTD. Thus, Aβ appears to be modifying molecular cascades, either by enhancing LTD or disrupting LTP, to disrupt memory processes. Furthermore, these molecular manipulations correlate with synaptodendritic rarefaction, or the diminished density of synapses on dendritic spines, an effect which is more predictive of cognitive decline and AD than plaque load (Huang & Mucke, 2012; Rosenblum, 2014; Snyder et al., 2005). Further, learning and memory deficits have been seen long before the formation of plaques or neuronal loss, when there is only diffuse Aβ accumulation (Lee et al., 2008; Kahn et al., 2012; White et al., 2016).

Although the cognitive decline seen in transgenic models is important to understand, these models represent severe and combined pathologies; therefore, it is difficult to tease apart the relative contribution that Aβ oligomers alone have on cognitive processes. This is where studies involving exogenous administration of Aβ peptides come into play. Over the past twenty-years, scientists have been developing alternate ways to study Aβ-induced impairments in cognition, synaptic health, oxidative stress, and tau pathologies (see Selkoe, 2008). Many of these experiments have utilized stereotaxic infusions of various species of Aβ peptides and allowed long recovery times before investigating cognitive function. For the sake of brevity, only a sampling of studies conducted in mice and rats will be discussed here. First, some studies that have used $A\beta_{1-42}$ or shorter-length Aβ peptides to "seed" endogenous production of $A\beta_{1-40}$

and $A\beta_{1-42}$ (Asle-Rousta et al., 2013; Zussy et al., 2013; Villard et al., 2008; Stepanichev et al., 2003; Meunier et al., 2013; Maurice et al., 2016, 1996; Detrait et al., 2014; Chavant et al., 2010; Chen, Wright, & Barnes, 1996; Telegdy et al., 2010). In these studies, rodents are infused with synthetic human Aβ and allowed to recover anywhere from one to twenty-five weeks before behavioral and biological assessments begin. Overall, these studies show that oligomeric infusions of human Aβ result in a marked decline in both short- and long-term memory, decreased spatial and working memory, decreased hippocampal acetylcholine levels, increased levels of plasma corticosterone, increased oxidative stress, gliosis and neuronal loss. Finally, Aβ infusions lead to enhanced pro-inflammatory cytokine production and the increased production and deposition of the neurotoxic peptides $A\beta_{1-40}$ and $A\beta_{1-42}$.

Many laboratories have also utilized single i.c.v. infusions of $A\beta_{1-40}$ or $A\beta_{1-42}$ to investigate the role of Aβ in cognitive decline and synaptic dysfunction, and like the seeding experiments, recovery times between infusions and behavioral testing is highly variable, ranging from three days to fifteen months. Winkler and colleagues (1994) utilized the longest recovery time. They found that rats infused with murine $A\beta_{1-42}$ and allowed to recover for fifteen months displayed impaired ability to acquire platform location in the Morris water maze (MWM), but, once learned, these animals did not show impairments on probe trials. In other words, animals infused with Aβ had a more difficult time learning to utilize the extra-maze cues to locate the platform, but once they could reliably locate the platform, these animals performed equal to saline-infused controls when the platform was removed on probe trials, i.e. they spent equal time in

the area where the platform used to be as control animals. This study found that, when the peptide (or vehicle) was infused into the hippocampus, as compared to infusion in the lateral ventricle, there was marked decline in spatial memory at the probe trial, indicating that infusions directly into the hippocampus may be confounded by the structural damage that can be caused (Winkler et al., 1994). In contrast, O'Hare and colleagues (1999) demonstrated that behavioral detriments following bilateral hippocampal injections of Aβ can occur as early as one month following infusions and can persist until three months, which was the longest they tested in this particular experiment. The idea that Aβ infusions can have persistent effects was further supported by Nakamura and colleagues (2001) who demonstrated that following three days of continuous synthetic human $A\beta_{1-42}$ infusions into the lateral ventricles, animals display marked cognitive decline in spatial working and reference memory tasks. Interestingly, these authors found that the deficits were more pronounced in animals tested eighty days after the infusion compared to animals tested only twenty days following Aβ infusion. Furthermore, they found severe brain atrophy and neuronal damage in the hippocampus of Aβ-treated animals compared to vehicle controls, suggesting that seeding with $A\beta_{1-42}$ may serve as a partial model of AD, as there was no evidence of aggregated Aβ in this study. Ali and Kim (2015) used i.c.v. infusions of a synthetic human $A\beta_{1-42}$ peptide into the lateral ventricle and tested cognitive performance in the MWM and Y-maze three weeks later. Animals administered Aβ spent significantly less time in the target quadrant during the probe trial, made fewer crossings of the zone where the platform had been, and had a significantly lower

spontaneous alteration percentages in the Y-maze. Shorter-term studies, with recovery times ranging from three to sixteen days have found that i.c.v. or intrahippocampal administration of $A\beta_{1-42}$ or $A\beta_{1-40}$ lead to increased trials to criterion in maze learning, decreased alternation behavior in a Y-maze paradigm, reduced reference memory in a radial-arm maze, increased escape latencies and time spent in the target quadrant in MWM tests, reduced step-through latencies in passive avoidance paradigms, decreased recognition indexes in novel object paradigms, and impaired choice behavior in an aversive Y-maze test (Li et al., 2014; Fu et al., 2006; Jhoo et al., 2004; Huang et al., 2007; Furukawa-Hibi et al., 2011; Wang et al., 2014; Prediger et al., 2007; Zhang et al., 2015; Cho et al., 2014; McDonald et al., 1994; Li et al., 2014; Singh & Kumar, 2016; Tsukuda et al., 2009; Hashimoto et al., 2005; Poling et al., 2008; Yan et al., 2001; Bagheri et al., 2011). Interestingly, Huang and colleagues (2010), found that $A\beta_{1-40}$ infusions into the dorsal hippocampus did not alter light-dark transition latencies or escape latencies in MWM testing, but they did find that stress (in the form of foot shocks) interacted with Aβ infusions to significantly decrease cognitive function in both tasks.

While these studies show that "seeding" the endogenous production of toxic species of Aβ or the exogenous infusion of synthetic Aβ can mimic multiple features of AD pathology, this procedural timeline, Aβ infusions followed by extended recovery time prior to cognitive testing, limits one's ability to determine the involvement of $A\beta_{1-42}$ in different phases of learning. Specifically, these models lead to the presence of Aβ during the acquisition of a task, consolidation of the experience, and during the recall of relevant information during testing; therefore, the precise role that Aβ plays in memory

disruption is difficult to determine and interpret from these designs. Moreover, all but one of the studies discussed above utilized synthetic forms of human Aβ to disrupt murine cognitive processes. While the utilization of human Aβ may seem appealing, considering AD is a human disorder, human Aβ is slightly different from rodent Aβ peptides. Murine $A\beta_{1-42}$ (mAβ) has three amino acid differences (R5G, Y10F, H13R) compared to human $A\beta_{1-42}$ (hAβ); all of which reside in the metal ion-binding region (Yamada et al., 1987; Eury et al., 2011). These differences affect aggregation efficacy; while mAβ is still capable of aggregating (Fung et al., 2004), it does so at a much slower rate. Specifically, Lv and colleagues (2013) demonstrated that the percentage of β-sheet content in mAβ was decreased by 20% compared to that in hAβ and that mAβ had reduced hydrophobic surface tendencies. Murine Aβ also induces less neuronal cytotoxicity than hAβ, as a result of decreased generation or reactive oxygen species (ROS), thought to be due to the tyrosine mutation in mAβ (Lv et al., 2013). Furthermore, efflux analysis in CD-1 mice demonstrates that mAβ is cleared more rapidly than hAβ (Banks et al., 2003). Therefore, it is possible that the structural and functional differences in mAβ may also modify the way in which it alters synaptic plasticity and function. For instance, zinc (Zn), which binds to both mAβ and hAβ, is known to be released at the synapse (Ketterman & Li, 2008), but its effects on the two species are different, i.e. hAβ in the presence of Zn reduces cell viability to roughly 60% whereas mAβ/Zn+ milieus maintains cell viability near 90% (Lv et al., 2013). This study indicates that mAβ is less neurotoxic alone, and is less susceptible to the deleterious effects of metal binding; thus, mAβ in the synapse may be less effective at disrupting the

various synaptic signaling cascades mentioned previously, e.g. NMDA receptor function and LTP. Furthermore, human Aβ in a murine model may lead to an erroneous understanding of how Aβ oligomers impact cognitive processes, as there is evidence to suggest that mAβ interacts with the hAβ to interfere with typical assembly rates, metal binding capabilities, and/or plaque compositions (Eury et al., 2011). In other words, injecting hAβ into mice may result in unintended molecular interactions that complicate the interpretation of results.

Our laboratory has previously helped establish a non-transgenic, murine Aβ-driven AD model in which repeated peripheral immune stimulation leads to increased production of endogenous Aβ (Kahn et al., 2012; White et al., 2016). It is known that activation of the innate immune system up-regulates pro-inflammatory cytokines, and that it is these cytokines that have been shown to transcriptionally up-regulate BACE mRNA, protein, and enzymatic activity, thus increasing the cleavage of APP into Aβ (Lee et al., 2008). Specifically, our model utilizes multiple injections of lipopolysaccharide (LPS), a fragment of a gram-negative bacterial cell wall that activates toll-like receptor-4 (TLR4). Upon activation, TLR4 signals through the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), to initiate pro-inflammatory cytokine production (Kamer et al., 2008). Following a single intraperitoneal injection of LPS, levels of IL-1β, IL-6, and TNF-α, (MCP)-1 peak peripherally and centrally roughly 4 h post-injection, and return to basal levels around 24 h post-injection (Kahn et al., 2012; Kranjac et al., 2012; Nahid, Satoh, & Chan, 201l). The levels and timing of pro-inflammatory mediators are slightly different following multiple LPS injections. Erickson and Banks (2011) found that a three-injection protocol of LPS resulted in

prolonged elevations in peripheral cytokines. Our laboratory specifically utilizes seven peripheral injections of LPS or polyinosinic:polycytidylic acid (poly I:C; a synthetic viral double-stranded RNA that also consistently produces pro-inflammatory cytokines; Konat et al., 2009) to reliably produce elevations of Aβ in the hippocampus of non-transgenic mice while also disrupting contextual-fear-conditioning (Kahn et al., 2012; Weintraub et al., 2013; Weintraub et al., 2014; White et al., 2016). This model leads to the presence of murine Aβ at all phases of cognition, making it impossible to disentangle the impact of Aβ oligomers on each phase of learning. Therefore, it is the purpose of this dissertation to evaluate how the administration of exogenous mAβ at different time-points impacts different phases of cognition in a contextual-fear-conditioning paradigm. Specifically, we will evaluate how mAβ treatment impacts the consolidation, retrieval, and extinction of a learned context-shock association.

CHAPTER 2: THE EFFECT OF Aβ ON CONSOLIDATION OF CONTEXTUAL MEMORY

Abbreviated Introduction

While learning and memory are often assessed in temporally distinct ways, the process of learning and the subsequent encoding, storage, and retrieval of that information are fluid and both the process of acquiring the information and the consolidation of that information are subject to disruption. As previously mentioned, disrupted memory processes are readily apparent in neurodegenerative diseases like AD. In fact, patients with AD are prone to difficulties in a number of various types of memories, i.e. episodic, semantic, working memory, and to some extent procedural memory (see Bastin & Salmon, 2014 or Gold & Budson, 2008). Furthermore, studies in humans have demonstrated that patients with mild cognitive impairment (MCI) or AD also have severe deficits specifically in consolidation (Moulin et al., 2004; Genon et al., 2012).

George Elias Müller and his student Alfonso Pilzecker first introduced consolidation in 1900; their groundbreaking work proposed that learning does not induce instantaneous, permanent memories but that memories change over time from a fragile, labile form to a 'fixed' form; they called this process consolidation (see McGaugh, 2000). This hypothesis meant that memories are vulnerable to disruption for a period of time after learning. Hebb (1949) furthered this concept and postulated that structural changes at the level of the synapse must occur to allow for a permanently stored memory. This structural alteration meant that there must be some sort of *de novo* protein synthesis occurring during the phase of consolidation; a theory that was confirmed by multiple researchers using protein synthesis inhibitors following training

procedures (see Davis & Squire, 1984). As a field, we now have a firmer grasp on the cascade of biological changes in neurons that occur during the short time period following learning. In an attempt to be succinct, only some of the relevant details will be discussed here; for a more in-depth review on the biological underpinnings of consolidation see Kandel, Dudai, and Mayford (2014).

There is general consensus that during the consolidation of contextual fear memories, there are two waves of protein synthesis: one immediately after conditioning, and another four hours later (Bourtchouladze et al., 1998). Interestingly, this phenomenon was only demonstrated when there was a weak training, i.e., 1 conditioned stimulus-unconditioned stimulus (CS-US) pairing; whereas, when there were 3 pairings, the period sensitive to disruption was found to be between 1 and 3 h following training. Over the years, researches have noted that different signal transduction pathways are activated at various time points. For instance, Bernabeu and colleagues (1997) found that increases in hippocampal protein kinase A (PKA) activity and phosphorylated-CREB levels can be found immediately, three and six hours after contextual fear-conditioning training. Brain-derived neurotropic factor (BDNF) mRNA, on the other hand, has been shown to be significantly elevated as early as 30 min following contextual fear conditioning in the dorsal hippocampus (Hall, Thomas, & Everitt, 2001), but has also been shown to be elevated 1, 3, and 6 h after training (Ma et al., 1998).

The concept that consolidation occurs in 'waves' links nicely with the distinction between early and late-phase LTP. Brain slice experiments have demonstrated that a single, high-frequency, stimulation can produce an 'early' phase of LTP (E-LTP) that

lasts for several minutes, is independent of protein or RNA synthesis, and is thought to involve modifications of existing proteins. By contrast, multiple high-frequency trains of stimulation will produce a 'late' phase of LTP (L-LTP) that lasts minutes to several hours or weeks and depends on protein and RNA synthesis (see, Kandel, 1997). Thus, L-LTP, unlike E-LTP, is thought to involve structural modifications of the synapse during consolidation. This L-LTP has been demonstrated to require BDNF, but not necessarily the immediate early transcription factor Zif268 (Pang et al., 2004; Lee, Everitt, & Thomas, 2004), as antibodies against the BDNF receptor, TrkB, impairs LTP maintenance (Korte, et al., 1998), and recombinant BDNF is able to rescue hippocampal LTP in BDNF knockout mice (Patterson et al., 1996). Furthermore, BDNF is considered critical for the consolidation of both short and long-term fear-related memories in the hippocampus; for example, intrahippocampal infusions of an antibody against BDNF or a TrkB-Fc chimera protein prior to inhibitory avoidance training impairs long-term retention (Chen et al., 2012; Alonso et al., 2002).

Hippocampal slice experiments have also demonstrated that BDNF treatment increases the expression of vesicle proteins, e.g., synaptophysin, and synaptotagmin, in different ways; synaptophysin levels are elevated within 6 hours after BDNF application, but peak increases in synaptotagmin did not occur until 24 hours later (Tartaglia et al., 2001), indicating an important role for BDNF in the formation of new synapses. Other *in vitro* work has shown that BDNF treatment elevates synaptophysin protein levels within 30 minutes, with peak expression 2 hours later (Coffey, Akerman, & Courtney, 1997). The discrepancy in the temporal pattern of BDNF-induced changes in synaptophysin between these studies is most likely due to the use of brain slices

verses cultured neurons. Bamji and colleagues (2006) also demonstrated that three days of BDNF treatment resulted in increased synapse numbers in cultured hippocampal neurons. Additionally, BDNF has been implicated in enhancing synaptic transmission, through increased neurotransmitter release and synapse sprouting (Jovanovic et al., 2000), and evidence from research in mice lacking BDNF or TrkB indicate that there is a dramatic decrease in the number of docked vesicles within the synapse, deficits in synaptic sprouting, reduced synapse numbers overall, and impaired LTP (Genoud et al., 2004, Otal et al., 2005, Nagappan & Lu, 2005, Zakharenko et al., 2003). Overall, the converging research suggests that BDNF plays a critical role in consolidation processes, and that this may be due to its impact on synaptic plasticity.

Given that consolidation is decreased in AD and that consolidation involves both synapse modification and BDNF signaling, it would seem likely that Aβ oligomers may lead to dysregulation of BDNF in such a way as to block consolidation. In fact, in a study comparing multiple transgenic AD models, large Aβ oligomers, and high $A\beta_{1-42}/A\beta_{1-40}$ ratios were correlated with significant reductions in cortical BDNF mRNA (Peng et al., 2009). Reduced BDNF protein levels have also been found in preclinical and early stage AD patients (Peng et al., 2005), and impaired BDNF signaling causes AD-like impairments in synaptic plasticity (Xu et al., 2000). Furthermore, *in vitro* models have shown that cultured neurons from the Tg2576 AD mouse model have impaired BDNF signaling and trafficking. Additionally, neurons from wild-type animals (WT) exposed to synthetic Aβ oligomers show reduced BDNF trafficking to the soma (Poon et al., 2011). This group later demonstrated that this deficit was mediated by the down regulation of ubiquitin C-terminal hydrolase, UCH-L1, which is a deubiquitinating enzyme that

functions to regulate cellular ubiquitin (Poon et al., 2013). Interestingly, Curcumin treatment, which increases hippocampal BDNF levels, has also been used to rescue Aβ-induced deficits in working memory and spatial reference memory (Zhang et al., 2015). In this experiment, $hA\beta_{1-42}$ was infused into the lateral ventricles of rats 5 days prior to training in a y-maze or MWM paradigm; when animals received multiple curcumin infusions, their spontaneous alteration and escape latencies returned to normal. Furthermore, multiple studies have also demonstrated that i.c.v. oligomer infusions lead to reduced synapse numbers in the hippocampus (Freir et al., 2011; Borlikova et al., 2013; Kuperstein et al., 2010; Prediger et al., 2007; Ali & Kim, 2015), thus one could hypothesize that the Aβ oligomers are altering BDNF signaling in a way that results in reduced synapse formation.

The studies discussed above point to a clear dysregulation of key biological processes underpinning L-LTP and consolidation, but direct examination of how hAβ oligomers impact consolidation processes *in vivo* is less substantial. There are multiple ways in which researchers have attempted to tease out this relationship, and a subset of studies have utilized Aβ-containing medium from 7PA2 cells. These cells are from a Chinese Hamster Ovary line, that stably express hAPP containing a particular mutation which leads to the production of low-n Aβ oligomers (Podlisny et al., 1995). The media from these cells has been used in a number of papers, and their neurotoxic capabilities well characterized (see Selkoe, 2008). For example, Aβ-containing medium was infused into the lateral ventricle of mice at various time points following passive avoidance training, and learning was assessed 24 and 48 h later. In this experiment, Freir and

colleagues (2011) found that consolidation was only significantly altered when infusions were given either 6 or 9 h post training and learning assessed after 48 h. The authors attributed this deficit to a reduced number of synapses in the Aβ-treated animals. These natural hAβ oligomers have also been shown to impair the formation of contextual fear memories in a CFC paradigm when i.c.v. infusions are delivered 1 h prior to contextual training, but not 2 h prior (Kittelberger et al., 2012). Furthermore, the 7PA2-conditioned media, containing hAβ oligomers, have been used to potently disrupt memory behaviors in animals responding under the operant alternating-lever cyclic-ratio (ALCR) schedule of food reinforcement, a deficit that can be rescued by blocking the fibrilization of Aβ *in vivo* (O'Hare et al., 2010).

Other studies have relied on synthetic preparations of hAβ to explore how consolidation is affected. For example, Balducci and colleagues (2010) found that synthetic hAβ oligomers, and not monomers or fibrils, were able to impair recognition memory in a novel object task when infused two hours prior to training and tested 24 h later. The authors argued that this infusion protocol demonstrated an impairment in consolidation of the long-term recognition memory, but since Aβ was onboard during the familiarization phase, when the animal is freely exploring two similar objects, interpretation of the behavioral outcomes is limited. Specifically, it is possible that hAβ impaired the animal's ability to perceive certain qualities of the object and therefore made discrimination between a novel and familiar object more difficult. This ambiguity is persistent in several experiments in which it is difficult to determine whether there was a performance deficit or impaired consolidation. In other words, the deficits may

have resulted from impaired perception, motivation, altered motor function, or increased emotionality, and not as a result of a learning deficit per se.

For instance, day-old chicks were injected with low-n hAβ 5–45 min prior to training with deficits appearing in testing sessions as early as 30 min after training (Gibbs & Gibbs, 2013). Additionally, bilateral infusions of synthetic $hA\beta_{1-42}$ into the hippocampus of mice one hour prior to training in a contextual-fear-conditioning paradigm has been shown to significantly reduce freezing behavior when tested 24 h later (Granic et al., 2010). Furthermore, when synthetic $hA\beta_{1-40}$ is infused i.c.v. into mice two days prior to y-maze or passive avoidance training, significant deficits are observed (Jeong et al., 2014). Finally, hAβ infusion 24 h prior to training results in impaired freezing behavior in a contextual-fear-conditioning paradigm, but not in a cued-conditioning paradigm (Dineley et al., 2010).

Alternatively, some researchers have infused various lengths of hAβ during the consolidation window. For example, immediate post-training infusions of a shorter Aβ peptide, 1–28, resulted in impaired consolidation of active avoidance learning in male mice when assayed 7 days post training (Flood, Morley, & Roberts, 1991). Human Aβ has also been infused following 4 days of training in the MWM, and resulted in significantly longer escape latencies when measured 24 h later (Lee et al., 2009). Furthermore, $A\beta_{25-35}$ infused into the lateral ventricle of mice and rats immediately and 30 min following training in passive avoidance resulted in reduced step-through latencies when assessed 24 h later (Telegdy, Tanaka, & Schally, 2010, 2011). Interestingly, Tucci and colleagues (2014) attempted to distinguish between the deficit

in learning and consolidation when Aβ is infused prior to training by using a modified version of the novel object test. In this experiment, i.c.v. administration of $hA\beta_{1-42}$ occurred 2 h prior to training. When the inter-trial interval (ITI) was 1 min, both sham and Aβ-treated animals spent significantly more time exploring the novel object, but when the ITI was extended to 24 hours, Aβ-treated animals explored both objects equally, indicating they did not remember the familiar object. The authors claim that because there was no deficit in the 1-min ITI condition, Aβ was only disrupting consolidation, but they point out that it is possible that retrieval was also being impacted. Thus, even when distinguishing between training and consolidation, one cannot fully rule out the possibility that Aβ is interfering with the process of retrieval.

Finally, some laboratories have used Aβ derived from the tissue of patients with AD in their attempts to understand how consolidation is impacted. For example, Borlikova and colleagues (2013) utilized water-soluble Aβ-containing extracts from AD brains in their model of consolidation. Rats underwent passive avoidance training followed by an i.c.v. infusion of Aβ-containing tissue extracts at either 1, 6, or 9 h post-training. The authors found no significant differences between Aβ and control infused animals when tested 24 h later, but 48 h post-training testing revealed significant deficits only in animals infused 1 h after training with Aβ. This deficit was attributed to a reduced synapse density and altered synaptic structure in Aβ treated animals. Additionally, work conducted by Reed and colleagues (2011) compared the effects of 7PA2-derived Aβ, synthetic hAβ, and brain-derived soluble Aβ from Tg2576 APP over-expressing transgenic mice on the ALCR cognitive assay; the authors demonstrated that

while synthetic and 7PA2-derived Aβ oligomers were capable of disrupting cognition, brain-derived Aβ from transgenic mice did not alter cognitive performance at any of the doses tested (0.4–1.3 μM, ~5.4–17.6 μg/ml).

Overall, there seems to be consensus that hAβ, synthetic or otherwise, can alter the consolidation of associative learning, but the temporal properties of this effect are far from consistent and whether this pattern extends to murine Aβ is unknown. The following experiments investigated how i.c.v. infusions of murine $A\beta_{1-42}$, immediately, 2, and 6 h after training in a contextual fear-conditioning (CFC) paradigm, impacted the consolidation of the contextual memory and the subsequent freezing behavior assessed in the same context 48 h after training. We hypothesized that $A\beta_{1-42}$ infusions administered during both early and late-phase consolidation would detrimentally impact freezing behavior when assessed 48 h later. Furthermore, we hypothesized that these effects would correspond to reductions in mRNA levels for genes associated with synaptic transmission and formation.

Methods

Male 4–6 month-old experimentally-naïve C57BL/6J mice, bred in the Texas Christian University vivarium from a breeding stock purchased from Jackson Laboratory (Bar Harbor, ME) were utilized in all experiments. Animals were reared in groups of three or four in standard polycarbonate mouse cages (31.75 × 17.78 × 16.51 cm) at ambient temperature (22˚C), and were maintained on a 12h light/dark schedule with food and water available ad libitum. Following cannulation, all animals were individually housed. All behavioral paradigms occurred between 0800 h and 1100 h.

Animals were housed and cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee at Texas Christian University (TCU 15/04).

Stereotactic Surgery Method

Mice were food and water deprived for two hours prior to surgery, and then deeply anesthetized by vaporizing isoflurane (5%) in an induction chamber. Surgical anesthesia was confirmed via an absence of response following tail, and hind limb pinch. Following confirmed anesthesia, the animal's head was shaved and then placed on the stereotaxic apparatus, in a skull-flat position. The head position was maintained by blunted ear bars and anesthesia maintained with 1–2% isoflurane. The scalp was cleaned with Betadine (Amazon.com LLC, Seattle, WA) and a small midline incision was made. The fascia was retracted to expose the skull, and bregma (a landmark used to identify stereotaxic "0") was identified. Three burr holes (.96mm in diameter) were made into the skull and self-tapping jeweler's screws (PlasticsOne, Roanoke, VA) were positioned in two of the holes (See Figure 1). A guide cannula, measuring 9 mm in length and constructed from 23-gauge stainless steel tubing was inserted 0.5 mm posterior to bregma, 0.5 mm lateral to midline, and 1.9 mm ventral from the surface to the brain (See Figure 2). Cannulae (custom-constructed from stainless steel tubing purchased from Amazon.com LLC, Seattle, WA) were secured to the skull using Teet's cold cure dental cement (A-M Systems, Sequim, WA). A 26-gauge stainless steel occluder was placed inside the guide cannula to prevent tissue from solidifying and blocking the internal opening of the cannula. Buprenorphine (Sustained-release, 3 mg/kg s.c.) was administered immediately after surgery as a post-surgical anesthetic.

Animals were allowed seven days to recover before onset of behavioral testing. During this seven-day period, the animals were monitored for health, e.g., fur quality, eating, and movement, and the occluder was removed and reinserted daily.

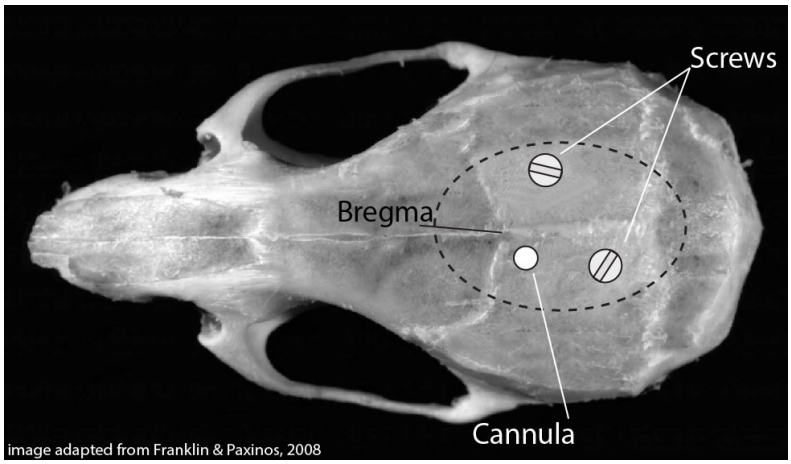

Figure 1. Visual Representation of the configuration of cannula and screws utilized in all experiments

Aβ Preparation and Verification

Murine Aβ peptide was purchased from Bachem Americas, Inc., CA and stored at -20°C until reconstituted. Peptide was dissolved in sterile-filtered phosphate buffered saline (PBS; pH 7.4) to a 1 mg/500 µl or 1 mg/mL stock solution depending on experiment. To clarify, 6 µl was utilized from the 1 mg/mL stock solution and 3 µl was utilized from the 1 mg/500 µl stock solution; thus, the same dose was given, but in different volumes[1]. Aliquots of 25 µl were made and frozen at -80°C until infusion. An aliquot from each preparation was examined by sodium dodecyl sulfate (SDS)-PAGE Western blot to verify the presence of low-n oligomers. Samples were diluted in sample buffer to a working concentration of 1 µg/µl. Proteins were then separated by size using

[1] This difference was methodological in order to reduce the time it took to perform the infusion.

Mini-Protean® TGXTM precast gradient gels (4–15%; BioRad, Hercules, CA). 200 V was applied across the gel for 40 min, followed by removal of the gel and equilibration in Towbin transfer buffer. Proteins were transferred onto Immobilon® P^{SQ} Transfer Membrane (Millipore, Billerica, MA) at 0.15 amps per gel for 40 min using a semi-dry transfer unit (BioRad). After transfer, membranes were blocked using 5% bovine serum albumin in Tris buffered saline with 0.3% Tween® 20 (TBST) for 2 h. Primary antibodies were diluted in TBST and applied to the membrane overnight at 4°C. The following day, membranes were washed in TBST followed by peroxidase- conjugated AffiniPure secondary antibody application (Jackson ImmunoResearch Laboritories, Inc., West Grove, PA) for 2h at room temperature. Membranes were washed and coated with SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Wiltham, MA), and Chemiluminescence was imaged using a Syngene G:Box (Syngene, Fredrick, MD).

ICV Infusion Protocol

Animals were briefly restrained, the occluder removed and an injection cannula (26-gague stainless steel tubing, 1 mm beyond the end of the guide cannula) was inserted into the cannula. Using a Hamilton syringe (10µl, Sigma, St. Louis, MO) attached to an infusion pump, 3–6 µl of Aβ peptide, depending on stock solution (Bachem Americas, Inc., CA), or an equal volume of sterile saline (pH 7.4) was infused into the third ventricle at a rate of 1µl/min. The third ventricle was chosen as it allows for diffusion into limbic structures (Lee et al., 2009; Flood, Morley, & Roberts, 1991; see Figure 2). The injection cannula remained in place for an additional 2 min to allow time for diffusion. The occluder was then replaced, and the animal was returned to its home cage.

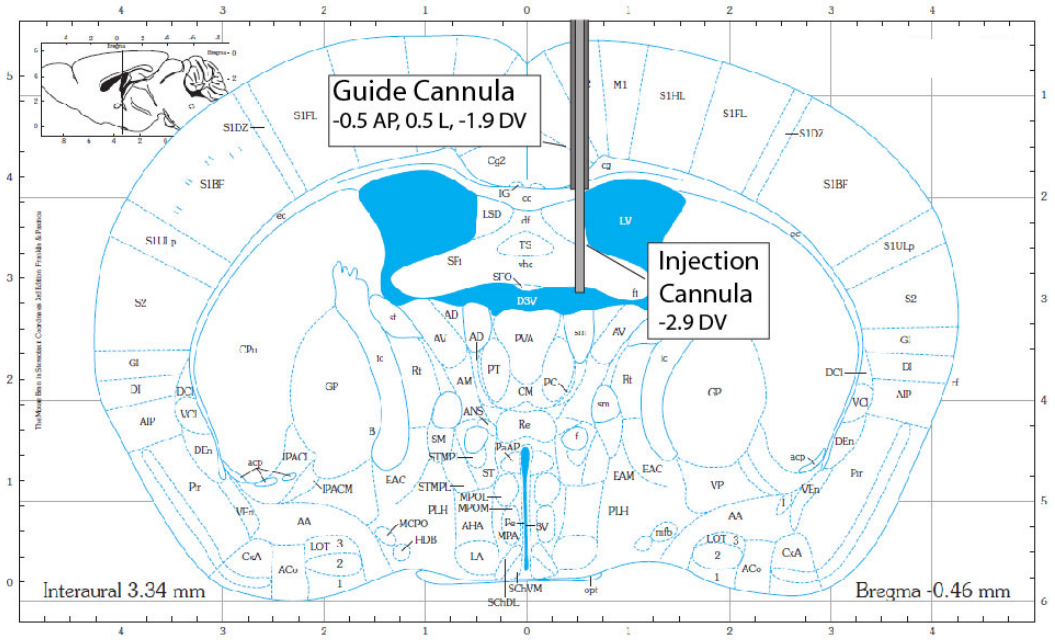

Figure 2. Diagram of guide cannula and injection cannula location. Picture adapted from Franklin and Paxinos, 2008.

Injection Verification

At the end of all behavioral experiments, animals were euthanized and India ink was infused to verify cannula location; see Figure 3 for an example of what this looks like. Any animals with improper cannula location were removed from behavioral data analysis.

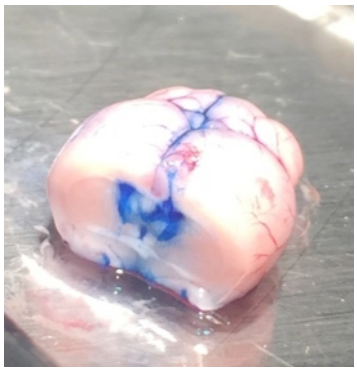

Figure 3. Example of the injection verification utilized following behavioral experiments. The blue found in the ventricles demonstrates proper position of the cannula.

CFC Protocol

Testing apparatus

Fully automated fear-conditioning units (Coulbourn Instruments, Whitehall, PA, 7W × 7D × 12H) and FreezeFrameTM software (ActiMetrics Software, Wilmette, IL) were used to assess learning and memory in a contextual fear-conditioning (CFC) paradigm. The context included a dotted wall pattern and a peppermint olfactory cue (1:10 in deionized water) to enhance context salience, as validated and discussed in previous reports (Kranjac et al 2012), and used an electrified grid floor to deliver a mildly aversive stimulus (0.7 mA shock). Movement of the animal was monitored and recorded continuously using FreezeFrameTM software (ActiMetrics Software, Wilmette, IL).

Consolidation

Seven days following cannulation, animals were trained in a CFC paradigm. Animals were placed into the chambers and allowed a 120 s acclimation period followed by a single 2 s, 0.7 mA foot-shock. Animals remained in the chamber an additional 60 s and were then, either immediately transported to a different room to be infused with 6 μl Aβ or Saline or returned to their home cage until time for an infusion. Following the infusion, animals were placed back in their home cages. Animals were returned to the testing chambers 48 hrs following training and freezing behavior was monitored for 120 s. The behavioral timeline can be found in Figure 4.

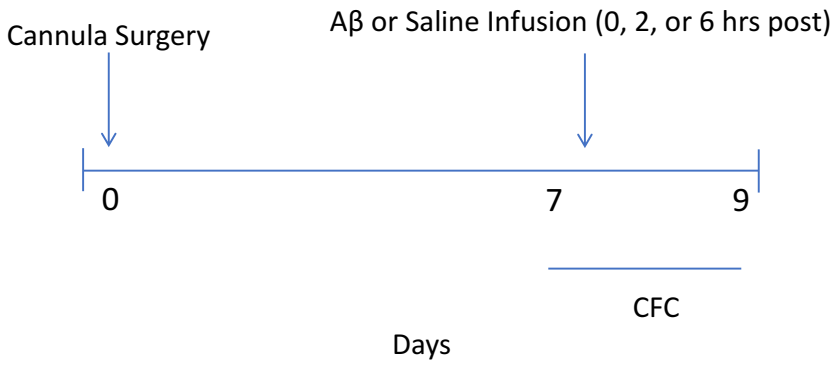

Figure 4. Experimental Timeline for Consolidation Behavior. CFC: Contextual fear conditioning paradigm. Aβ = amyloid-beta; CFC = contextual fear-conditioning

mRNA Analysis

Tissue Collection and RT-PCR

A second batch of animals underwent cannulation, and Aβ or saline infusions occurred immediately post training in a CFC paradigm. Six hours after the 0 hr infusion, animals were rapidly euthanized via CO_2 asphyxiation and the dorsal hippocampus was dissected out under RNAse-free conditions and stored in RNAlater® (Sigma-Aldrich Co., St. Louis, MO.) at -20°C until processing, see Figure 5 for experimental timeline. Samples were then processed according to the Maxwell® 16 LEV simplyRNA Tissue kit (Promega Corporation, Madison, WI.) instructions and total RNA yield was quantified (NanoDrop, ThermoFisher Scientific, Wilmington, DE) prior to being diluted to a uniform concentration for RT-qPCR analysis. The RT portion of the procedure was conducted with a 7500 RealTime PCR Thermal Cycling System (Applied Biosystems, Foster City, CA) using iScript™ reverse transcription supermix (BioRad, Hercules, CA), and the qPCR portion was conducted using the CFX Connect™ System (BioRad, Hercules, CA). All probes and primers were obtained from BioRad (Hercules, CA), and

were amplified in accordance with manufacture recommended protocols. Regression quantification cycle determination was used to evaluate amplification data before target gene expression was normalized relative to β-actin and analyzed using the CFX Manager Gene Study software (BioRad).

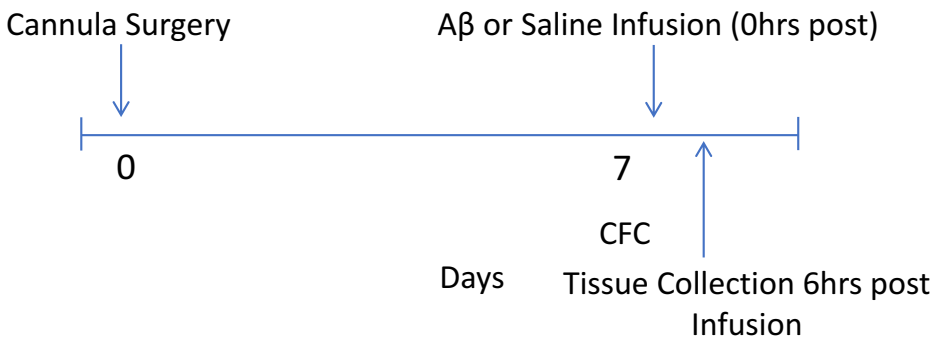

Figure 5. Experimental Timeline for tissue collection. CFC: Contextual fear conditioning. Aβ = amyloid-beta; CFC = contextual fear-conditioning

Statistical Protocol

All statistics were performed with Prism (Version 6.07, GraphPad, La Jolla, CA). Student's t-Tests were conducted between treatment groups (Aβ or Saline) in all Consolidation experiments, with $\alpha \leq 0.05$.

Results

The presence of Aβ oligomers were verified via SDS-PAGE Western blot

Samples from each preparation of mAβ were run through SDS-PAGE Western blot procedures to ensure that the drug infused in all experiments did contain low-n mA**β** oligomers; see Figure 6. As indicated in the figure, there are some larger proteins present in lanes 1 and 3, but the vast majority of protein sizes seen correspond with low-n oligomers.

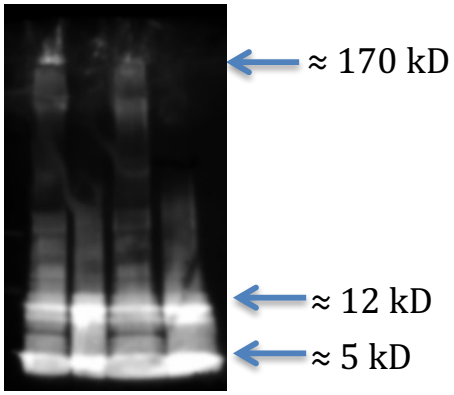

Figure 6. Western blot analysis of Aβ preparations used in infusions throughout all experiments. Size of proteins found in solution are consistent with those of low-n oligomers of the Aβ peptide.

Murine Aβ has differential effects on cognition based on time of infusion post-training

In all experiments described in this chapter, there were no significant differences in freezing behavior between groups on the day of training, either in the 120 s prior to the shock, or the 60 s after the shock (0 hr pre: t (29) = 0.4188, p = 0.678, 0 hr post: t (29) = 0.8266, p = 0.415; 2 hr pre: t (27) = 0.1135, p = 0.912, 2 hr post: t (27): 1.241, p =0 .225; 6 hr pre: t (28) = 2.019, p = 0.053, 6 hr post: t (28) = 0.091, p = 0.928; Figure 7). In order to assess whether mAβ could disrupt consolidation, three injection time-points were chosen: immediately after training (0 hr), two hours after training, or six hours after training. Animals that received an infusion of mAβ immediately after training (0 hr) or two hours after training froze significantly less on the day of testing (48 or 46 hrs later, respectively) than animals given an infusion of saline, 0 hr.: t (16) = 2.456, p = 0.026, 2 hr.: t (19) = 2.399, p = 0.027. Animals that received an infusion of mAβ six hours after training show no significant differences in freezing behavior from

saline-infused animals on the day of testing (42 hrs later), t (12) = 0.176, p = .863; see Figure 7.

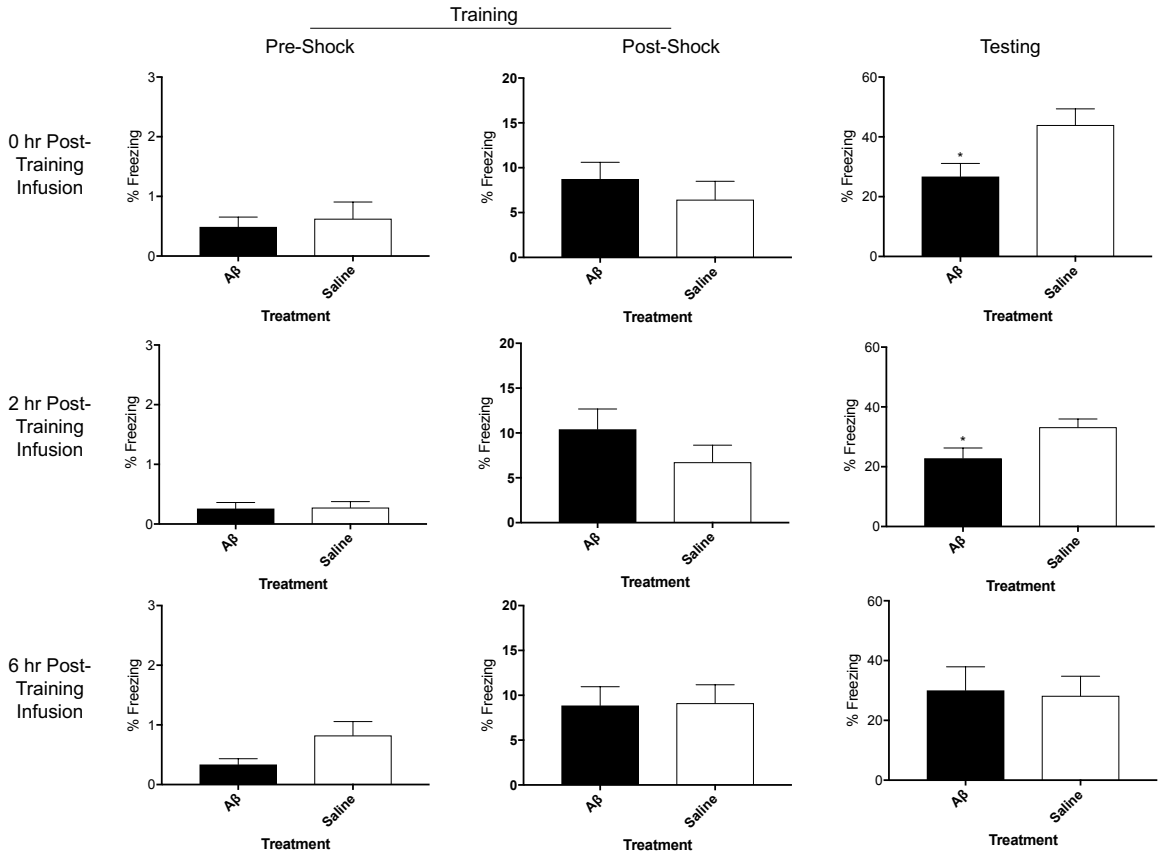

Figure 7. Percentage freezing in a CFC paradigm during training and testing phases for three infusion time-points. Bars represent mean ± SEM *represents $p < 0.05$. Aβ = amyloid-beta; CFC = contextual fear-conditioning

Murine Aβ administered following training elevates IL-1β mRNA, but suppresses synaptophysin mRNA expression

Following significant findings from the 0 hr consolidation experiment, an additional batch of animals was utilized to explore gene transcription during the consolidation window. Immediately following training, animals were infused with either mAβ or sterile saline. Six hours after the infusion, the dorsal third of the

hippocampus from both hemispheres was collected, RNA was isolated, and analyzed for levels of gene expression for BDNF, Syn-1, GAP-43, IL-1β and Syp. Contrary to our hypothesis, there were no significant differences in gene expression for BDNF, Syn-1, or GAP-43 between groups 6 hours after the infusion, BDNF: t (14): 0.1215, $p = 0.905$; Syn-1: t (14) = 0.6411, $p = 0.532$; GAP-43: t (13): 1.22, $p = 0.244$. Animals treated with mAβ did have significantly less Syp expression, t (12) = 2.272, $p = 0.042$, while showing significant elevations in IL-1β mRNA expression, t (14) = 3.356, $p = 0.005$, Figure 8. To further asses the stability of this pattern in gene expression, a second batch of tissue from the dorsal hippocampus was collected immediately after testing and analyzed for gene expression.[2] At the time of testing, there were no differences in BDNF mRNA levels, t (15) = .7314, $p = 0.48$, between treatment groups, but IL-1β expression levels are marginally upregulated in Aβ-treated animals, t (18): 1.914, $p = 0.072$, and synaptophysin levels are significantly elevated over saline-treated controls, t (18): 2.332, $p = 0.032$, Figure 9.

[2] The tissue collected for this analysis came from the animals used in the behavioral experiment for the 2 hr consolidation time-point.

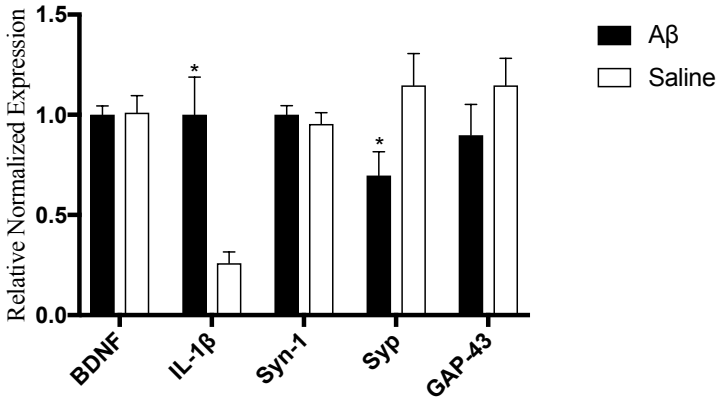

Figure 8. Gene expression six hours after an infusion of mAβ or Saline. β-actin = loading control. Bars represent mean ± SEM *represents $p < 0.05$. Aβ = amyloid-beta; BDNF = brain derived neurotrophic factor; IL-1β = interleukin 1β; Syn-1 = synapsin; Syp = Synaptophysin; GAP-43 = growth associated protein 45.

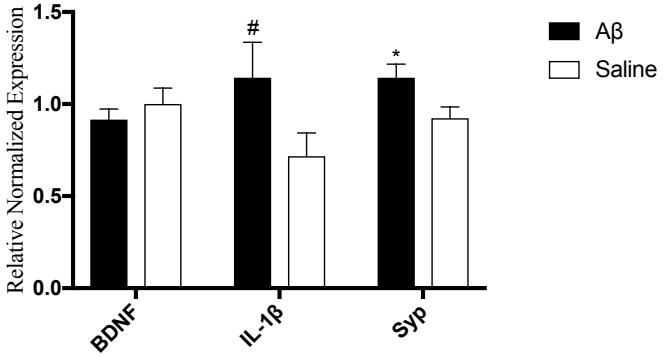

Figure 9. Gene expression forty-six hours after an infusion of mAβ or Saline. β-actin= loading control. Bars represent mean ± SEM *represents $p < 0.05$, #represents $p = 0.072$ BDNF = brain derived neurotrophic factor; IL-1β = interleukin 1β; Syp = Synaptophysin

Discussion

The present study tested the hypothesis that mAβ would disrupt the consolidation of contextual learning, and that this disruption would correspond to dysregulated mRNA production of genes associated with synaptic formation and function. These hypotheses were partially confirmed. First, mAβ does disrupt

consolidation, but this effect appears to be temporally limited. More specifically, i.c.v. infusions of mAβ within two hours of training, but not six hours after training, detrimentally impacts freezing behavior during testing. Second, mAβ appears to both cause central inflammation and dysregulate certain markers of synaptic plasticity.

We were able to replicate several experiments in which hAβ was able to disrupt consolidation when infusion occurs within two hours of training (Borlikova et al., 2013, Balducci et al., 2010, Garcia-Osta & Alberini, 2009, Telegdy et al., 2010); however, our results add to the ambiguity surrounding the role of Aβ on later phases of consolidation. More specifically, our results are consistent with Bolikova and colleagues (2013) who found no differences in performance in a passive avoidance task when hAβ was infused six hours following training, but are in contrast to the findings from Frier and colleagues (2011) which found that hAβ infusions only disrupted consolidation when administered either six or nine hours after training procedures. In the experiments discussed above, several efforts were made to understand the mechanism through which hAβ was causing consolidation errors, but these investigations were mostly limited to synapse number or neuronal viability. For example, infusions of synthetic $hA\beta_{1\text{-}42}$ and/or 7PA2 cell-derived Aβ lead to decreased neuronal viability, reduced total synapse number within the hippocampus, and increased caspase-3 levels and oxidative stress (Balducci et al., 2010, Frier et al., 2011, Borlikova et al., 2013, Kuperstein et al., 2010, Jeong et al., 2014).

Interestingly, none of the experiments that utilized tissue collections within 48 hours of infusions investigated the role of inflammation, which given the relationship

between inflammation and AD pathology, is a gross oversight. This issue was partially addressed in a study that investigated the role of microglia cell function in relation to Aβ-induced cognitive deficits, as microglial cells contribute to neuroinflammation (Meda et al., 1995). In this study, administration of minocycline, a microglial activation blocker, eliminated the negative effects of Aβ when administered concurrently (Gibbs & Gibbs, 2013), but the authors did not further investigate the original role that the microglial cells were performing to disrupt cognition. Based on the data from this dissertation, we propose that the infusion of Aβ leads to the activation of microglial cells and the subsequent production of the pro-inflammatory cytokine IL-1β. Further, we suggest that the induction of IL-1β may directly drive the reduction in synaptophysin mRNA levels seen in this study, and that this reduction may account, at least in part, for the cognitive dysfunction. This hypothesis is supported by several *in vitro* and *in vivo* studies documenting the negative relationship between IL-1β and Synaptophysin levels (Han et al., 2017; Hajjar et al., 2013; Disdier et al., 2017). For example, microglial cells treated with secreted β-amyloid precursor protein or LPS show elevated levels of IL-1β mRNA, and when these cells are co-cultured with primary neurons, there is a significant reduction in synaptophysin protein levels, an effect that can be attenuated by treatment with an IL-1 receptor antagonists (IL-1ra; Li et al., 2003). Additionally, elevated levels of IL-1β protein are related to decreased Synaptophysin mRNA levels *in vivo* (Disdier et al., 2017). It should be noted that in the present experiment, immediately following testing, synaptophysin mRNA levels were significantly elevated in Aβ-treated animals despite moderately elevated levels of IL-1β

at this time, which appears in contrast to negative relationship between the two discussed above. This rebound in synaptophysin mRNA may be a compensatory mechanism driven by the additional learning experience induced by the testing session. Future work should address this question by including an additional control group, without the testing experience, to elucidate whether the testing session itself is capable of driving increases in synaptophysin processing. Either way, the possibility that mAβ is altering synaptic functioning through IL-1β needs further inquiry.

To elucidate whether the cognitive dysfunction found in these experiments is IL-1β-dependent, several studies could be conducted. It would be important to verify that IL1ra treatment rescues both cognitive function and synaptophysin levels. Additionally, whether mAβ is directly activating microglial cells to produce IL-1β, or if the mAβ within the synapse is altering other cellular processes which then leads to the activation of microglial cells needs to be determined. This may be accomplished by further exploring how mAβ impacts neuronal viability and health *in vitro* to determine if the neurons are releasing other mediating signals which could account for microglial activation. Finally, it is possible that the cognitive dysfunction described in the present studies is due to the combined activity of microglial cells and the mAβ peptide itself. This possibility could be explored by removing microglial cells from the hippocampus prior to mAβ administration through the administration of the CSF1R/c-Kit inhibitor, PLX3397, which can reliably eliminate 99% of microglial cells from the CNS (Elmore et al., 2015). If proper cognitive function is restored, then this suggest that the mAβ-

induced dysfunction is microglial-dependent, but, if the dysfunction remains, then it suggests that the mAβ is acting through multiple pathways.

One of those pathways could be through the actions of BDNF. Although we did not find differences in gene expression for BDNF in this experiment, it is possible that the actions of the existing protein are still relevant. Specifically, several studies have shown that Aβ can disrupt BDNF signaling and trafficking (Xu et al., 2000, Poon et al., 2011, Zhang et al., 2015), and that increasing BDNF signaling could overcome cognitive deficits, as administration of a TrkB agonist, 7, 8-Dihyroxyflavone, improves recognition and working memory in both the 5xFAD and APPswe/PS1dE9 mouse models of AD (Bollen et al., 2013; Devi & Ohno, 2012). Signal efficacy of BDNF was not explored in this study, so, despite no changes in BDNF mRNA following mAβ infusions, we cannot comment on whether the functioning of the BDNF present was disrupted in any way. Future work should explore this possibility, and address the question as to whether mAβ interferes with pre-synaptic TrkB receptors. This is especially important to elucidate, as hAβ treatment of primary neurons in culture leads to significant increases in mRNA for the truncated TrkB receptor isoform, which leads to decreased BDNF functionality despite no changes in the mRNA for the full-length receptor isoform (Jeronimo-Santos et al., 2015). This supports the idea, that mAβ could be acting through an indirect mechanism to disrupt BDNF signaling.

Overall, the present experiment demonstrates that the infusion of murine Aβ, in the absence of hAβ, is sufficient to disrupt performance in a hippocampus dependent task and that this disruption is likely related to mAβ-induced inflammation and disrupted

synaptic plasticity within the hippocampus. The design of the current experiment is such that we cannot rule out the possibility that the dysfunction found is related to the retrieval of the learned information and not its consolidation. The next study was conducted in order to investigate this possibility.

CHAPTER 3: THE EFFECT OF Aβ ON THE RETRIEVAL OF A CONTEXTUAL MEMORY

Abbreviated Introduction

Experiment 1 explored whether mAβ could disrupt consolidation of contextual memories when centrally infused immediately, 2, or 6 h following training in a contextual fear-conditioning paradigm. The purpose of Experiment 2 was to verify that the deficits found in Experiment 1 were related to problems with consolidation and not with problems in retrieving information already learned. Retrieval is considered the third phase of memory, in which previously acquired information is recovered or reactivated in order to guide current behavior (Domjan, 2003). Typically, retrieval tests are conducted in the absence of the unconditioned stimulus, so as to evaluate memory while minimizing further learning.

During memory assessments, there are often two expected outcomes: either the subject behaves in a way that suggests learning occurred or the subject behaves in such a way that suggests learning has not occurred. The problem with this dichotomy stems from stark difference between two theories, the memory consolidation theory and the retrieval-failure hypothesis. The memory-consolidation theory postulates that poor performance on a testing trial is due to a disrupted transfer of newly acquired information from short- to long-term memory storage; more specifically, the process of consolidation was interrupted in such a way that the new memory was never fully stored (Domjan, 2003). Alternatively, the retrieval-failure hypothesis, discussed by Miller and Springer (1973), states that poor performance in a testing trial may be attributed to a failure to reactivate the memory trace, in order to behave accordingly. Therefore, in order to understand the unique systems involved in recall versus

consolidation, studies utilizing pharmalogical manipulations during or immediately prior to testing, when original consolidation processes are complete, are extremely important.

Unlike in acquisition and consolidation (Shimizu et al., 2000), NMDA receptors do not appear to play a role in the retrieval of previously learned spatial information, as pharmacological blockade of these receptors in the hippocampus has no impact on retrieval (Steele & Morris, 1999). However, it should be noted that blockade of NMDA receptors in the retrosplenial cortex has been shown to abolish contextual retrieval in CFC (Corcoran et al., 2011). Similarly, protein synthesis, which is critical for acquisition, consolidation, reconsolidation, and extinction, is not required for the retrieval of conditioned fear (Bourtchouladze et al., 1998; Lattal & Abel, 2000), just as PKA, PKC, and CamKII are not implicated in retrieval processes, despite their importance in acquisition and consolidation (Bourtchouladze et al., 1998; Goosens, Holt, & Maren, 2000; Kim et al., 2012; Sakurai, Yu, & Tan, 2007). This would indicate that retrieval, to some extent, is directed through distinct cellular pathways from acquisition and consolidation. For example, ERK phosphorylation in CA3 appears to be critical in retrieval as reduced ERK phosphorylation at the retention trial is associated with decreased step through latencies (Kim et al., 2012).

At the time of retrieval, when assessed a few days after training, the hippocampus, basolateral amygdala, entorhinal, anterior and posterior retrosplenial cortices, and parietal cortex create an integrated network to reactivate previously stored information (Izquierdo et al., 1997; Barros et al., 2003; Anderson et al., 2004). This retrieval appears to require AMPA receptor activation, as administration of the

antagonist 6-cyano-7-nitroquinoxaline-2,3-dione(CNQX) into the hippocampus or amygdala two hours prior to testing sessions impairs inhibitory avoidance (Izquierdo et al., 1997). Furthermore, β-noradrenergic, D1-dopaminergic, and muscarinic cholinergic receptor activation within the hippocampus have been shown to facilitate retrieval processes, whereas, serotonin-1A receptor activation hinders retrieval (Barros et al., 2001). In fact, the blockade of cholinergic receptors or β-adrenergic receptors can ablate retrieval completely (Barros et al., 2001; Otis & Mueller, 2011). However, the blockade of $GABA_A$ receptor activation in the CA3 will enhance memory retrieval (Kim et al., 2012); indicating, in part, that the complex activation or deactivation of various receptors in different regions of the hippocampus is critical for proper retrieval. For instance, optogenetic work conducted by Andrews-Zwilling and colleagues (2012), demonstrated that optogenetically inhibiting hilar GABAergic interneuron activity impaired spatial memory retrieval in the MWM paradigm. Additionally, excitatory neurons of the CA1 are not required for retrieval, but play a critical role in the retrieval of spatial memories, as optogenetic inhibition of these neurons blocks retrieval in a CFC paradigm (Goshen et al., 2011). The importance of the hippocampus in early retrieval processes is further supported by recent work conducted by Tanaka and colleagues (2014) and Tayler et al. (2013) which demonstrated that the hippocampus reactivates specific memory representations during retrieval processes, and that inactivation of the hippocampus, via infusions of an AMPA receptor antagonist immediately prior to testing, results in abrogated retrieval of recent events (Wiltgen et al., 2010). Furthermore, the retrieval of recent and remote contextual memories leads to activity in both the hippocampus and the medial prefrontal cortex, as indicated by fos and Arc

staining, (mPFC; Lopez et al., 2012; Wiltgen et al., 2010; Tayler et al., 2013). This is in line with the early work of Riedel *et al.* (1999), which found that temporary inactivation of the hippocampus, blocked retrieval in a spatial preference test. Overall the literature suggests that there is a complex signaling pathway that allows for successful retrieval, and the proper functioning of the hippocampus is particularly important in that process.

Early studies in humans with mild cognitive impairment (MCI) or AD, which have deteriorated hippocampal function, have demonstrated impairments in the retrieval of episodic memories (Bäckman et al., 1999). In fact, compromised recall is considered a universal early symptom of Alzheimer's disease (Hodges, Erzinçlioğlu, & Patterson, 2006), and recent work by Dhanjal and Wise (2014) demonstrated that free recall, or effortful recall that occurs in the absence of retrieval cues, is highly disrupted in AD patients. Dhanjal and Wise (2014) argue that this is due to decreased and imbalanced neural activity in a variety of cortical areas, an idea also explored by Buckner and colleagues (2005). Most notably, the recent work by Roy and colleagues (2016) suggested that the cognitive deficits found in early stages of AD are related more to a failure in retrieval processes than that of consolidation errors. Specifically, direct optogenetic stimulation of memory engram cells in the hippocampus of APP/PS1 and microtubule associated protein tau (MAPT) AD mice lead to the restoration of fear memory, an effect that was paralleled by improved spine density in the hippocampus (Roy et al., 2016). This effect was replicated by Perusini et al. (2017) who also found that APP/PS1 animals have impaired retrieval that corresponds to decreased dentate gyrus memory engram traces, which, when optogenetically stimulated improved

retrieval performance in CFC tasks. The human and rodent literature on AD suggest that there are problems in both consolidation and retrieval, but the direct influence of Aβ on retrieval processes is less clear, despite clear roles for Aβ in the disruption of consolidation. Thus, the current study explored this relationship further.

Aβ and Retrieval

Treating rodent fetal cortical neurons with synthetic $A\beta_{23-35}$ and $A\beta_{1-40}$ results in reduced muscarinic cholinergic receptor (mAChR) activation of G proteins and subsequent calcium release that ultimately leads to reduced cholinergic transmission (Kelly et al., 1996); an effect that may result in impaired retrieval processes as mAchR activation has been found to facilitate retrieval (Barros et al., 2001). Additional studies have demonstrated that $A\beta_{1-40}$ can also inhibit alpha7 nicotinic acetylcholine receptors (Nery et al., 2013), and that nicotine can protect against these Aβ-induced synaptic impairments, an effect that translates to improved memory in young APP/PS1 animals (Inestrosa et al., 2013). For a more detailed review of how Aβ interacts with nicotinic receptors, see a review by Dineley, Pandya, and Yakel (2015). Aβ has also been shown to disrupt AMPA signaling and receptor integrity, which, like receptor blockade (Izquierdo et al., 1997), may interfere with proper retrieval. Specifically, AMPA receptors containing the GluA3 subunit are vulnerable to Aβ-driven receptor loss, which functionally weakens synaptic integrity (Reinders et al., 2016). Additionally, AMPA receptor removal underlies Aβ-driven synaptic depression and dendritic spine loss (Hsieh et al., 2006). Thus, the evidence presented here suggests that the presence

of Aβ oligomers may specifically alter retrieval processes in rodent behavioral paradigms, a topic rarely addressed in the literature.

In a study discussed previously, Balducci and colleagues (2010) attempted to demonstrate that synthetic hAβ was actively disrupting consolidation processes in a novel object paradigm. To do so, they had three treatment groups, (1) vehicle-infused animas, animals that received an i.c.v. infusion of hAβ pre-training, and a group that received hAβ two hours prior to testing. The authors reported that the hAβ oligomers were acutely disrupting memory storage but not retrieval, as animals administered hAβ prior to testing performed like that of vehicle injected control animals. Thus, under the conditions described by Balducci *et al.,* (2010), hAβ oligomers did not interfere with object recognition retrieval.

In Experiment Two, we replicated the two-hour pre-testing infusion timeline utilized by Balducci *et al.,* (2010) for two reasons: (1) if no differences at the time of testing were found then the results obtained in Experiment 1 could more easily be attributed to disrupted consolidation and not retrieval interference, (2) to directly compare the effect of hAβ and mAβ on retrieval performance in two hippocampus-dependent tasks. We hypothesize that, like synthetic hAβ, mAβ will be unable to disrupt the recall of previously learned information in the paradigm utilized here. More precisely, the freezing of animals administered mAβ two hours prior to testing will not differ from that of saline-infused controls.

Methods

Unless otherwise stated, all subjects, stereotactic surgery methods, infusions, and post-behavioral verification protocols were identical to those described above.

CFC protocols

Using the same CFC protocol described above, we investigated how i.c.v infusions of Aβ can disrupt the recall of learned information. Seven days following cannulation, all animals underwent training procedure as those described above, but 46 h following training, animals were infused with 3µl Aβ (prepared from a stock concentration of 2mg/mL, and verified as stated above) or sterile saline. Subjects were then returned to their home cage, and testing occurred 2 hours later. Animals were returned to the original chamber and freezing behavior was monitored for 120 s, see Figure 10.

RT-PCR for inflammatory cytokines

In order to verify that any group differences, or lack of differences, could be attributed to the Aβ infusions and not the presence of central cytokines, we also assessed central mRNA expression levels for pro-inflammatory cytokines at the time of testing between groups. Immediately following testing, animals were rapidly euthanized, tissue collected, RNA purified and RT-PCR performed as described for Experiment1.

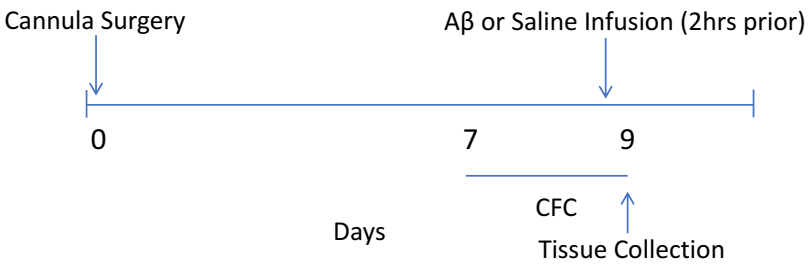

Figure 10. Experimental Timeline for recall behavior and tissue collection.

Statistical Protocol

Student's t tests were conducted between treatment groups (Aβ or Saline), with $\alpha \leq 0.05$ using Prism (Version 6.07, GraphPad, La Jolla, CA).

Results

Infusions of mAβ two hours prior to testing does not alter freezing behavior

As expected, there were no significant differences between groups on the training day, either in the 120 s prior to the shock, t (33) = 0.91, p = 37, or the 60 s after the shock, t(33) = 0.74, p = 0.47. Further, on the day of testing, animals administered mAβ two hours prior to the testing session froze indiscriminately from animals administered saline, t(18) = 1.30, p = 0.21, see Figure 11.

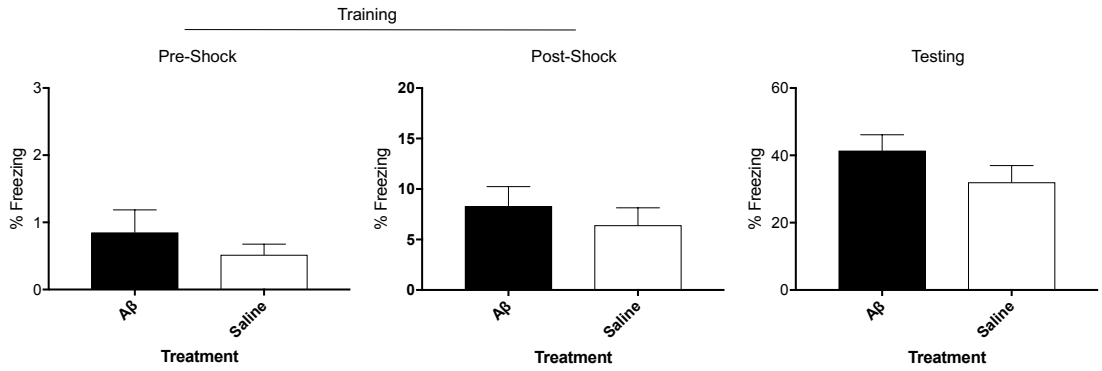

Figure 11. Percentage freezing in a CFC paradigm. No significant differences were found between groups for any time-point analyzed. Bars represent mean ± SEM.

IL-1β mRNA levels at the time of testing are consistent between treatment groups

Immediately after testing, RNA was collected from all animals and gene expression for IL-1β was analyzed. There were no significant differences in mRNA expression for IL-1β between Aβ and saline-treated animals, $t(11) = 1.992$, $p = 0.072$. This suggests that the freezing behavior produced by animals in the Aβ infusion group was not related to pro-inflammatory cytokine-induced immobility. It should be noted that there is a marginally significant elevation of IL-1β mRNA in the saline-treated animals, an effect that, if correct, would alter the interpretation of the behavioral results.

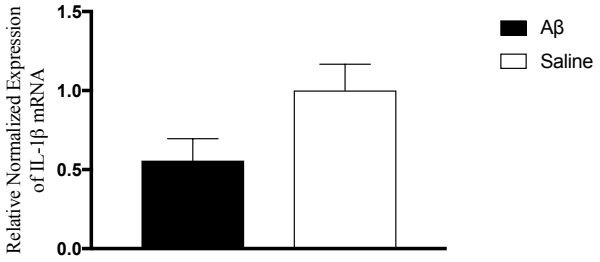

Figure 12. IL-1β mRNA levels at the time of testing. There were no significant differences in gene expression between animals administered mAβ and saline. Bars represent mean ± SEM.

Discussion

The present study tested the hypothesis that mAβ would not alter the retrieval of previously learned information. This hypothesis was confirmed, as central infusions of mAβ two hours prior to testing had no effect on freezing behavior (Figure 11). This finding is in accordance with previous work conducted in mice utilizing synthetic human Aβ in a novel object paradigm (Balducci et al., 2010) and in an active avoidance paradigm (Flood et al., 1991), and further demonstrates that Aβ, whether human or

murine, administered two hours prior to a testing session does not influence the retrieval of previously stored information. Furthermore, we verified that the null findings in this experimental paradigm could not otherwise be accounted for by inflammation-induced sickness behaviors. More specifically, at the time of testing, quantification of IL-1β mRNA was not significantly different between treatment groups.

Although these findings do support the conclusions drawn from Experiment 1, i.e., mAβ oligomers impair the consolidation of contextual fear-conditioning, they do not correspond with the literature supporting retrieval deficits in AD populations or AD models in mice (Hodges, Erzinçlioğlu, & Patterson, 2006; Dhangal & Wise, 2014; Buckner et al., 2005; Roy et al., 2016; Perusini et al., 2017). There are a number of probable explanations for this inconsistency.

To begin with, the patients tested in the human literature on AD and retrieval would have marked cellular loss, as noted by medial temporal lobe atrophy (Buckner et al., 2005; Dhanjal & Wise, 2014), and there is substantial literature describing the cell death in AD rodent models (For review, see Wirths & Bayer, 2010). Therefore, it is not entirely surprising that studies conducted in these late-stage scenarios would be more predisposed to find errors in retrieval than studies administering Aβ alone, as neuronal loss would have more widespread effects. Furthermore, while the literature utilizing central Aβ infusions notes changes to dendritic spines and synapse formation (Frier et al., 2011; Borlikova et al., 2013), there is no indication, that ventricular Aβ infusions leads to neuronal loss within the time frame investigated here. It is also possible that the protocol utilized in the present study was flawed. Specifically, given the literature suggesting that Aβ does, in fact, alter muscarinic and nicotinic cholinergic transmission

in addition to disrupting AMPA receptor signaling (Barros et al., 2001; Otis & Mueller, 2011; Izquierdo et al., 1997), it is possible that the timing of the infusion was not well suited to answer the question asked in this experiment, i.e. does mAβ block retrieval? If this is true, then it suggests that the results presented here and in Balducci *et al.* (2010) may not tell the whole story, and had infusions occurred either farther or closer to testing, a different result would have been found. This explanation, while possible, has several problems. First, had infusions occurred more than six hours prior to testing, then the results presented here would be confounded by inflammation and ensuing sickness behaviors, as Experiment 1 demonstrates that there is a significant increase in the gene expression of pro-inflammatory cytokines six hours following infusions. Second, AMPA receptor antagonists administered two hours prior to testing has been previously shown to block retrieval (Izquierdo et al., 1997), suggesting that if the centrally infused Aβ is acting on AMPA receptors, this time frame should have been sufficient.

It seems more plausible that the lack of a retrieval deficit found in this study can be attributed to a dosing effect, i.e. the amount of Aβ delivered to the hippocampus was not sufficient to produce substantial disruptions in AMPA receptor signaling and function and/or to produce neuronal loss. For example, while treatment with an AMAPR antagonist has been used to abrogate retrieval, antagonists are much more selective in their binding sites, unlike Aβ which, due to its structure, is known to bind to a variety of biomolecules, including lipids, proteoglycans and proteins (For review, see Verdier & Penke, 2004). Thus, while the selective targeting of AMPA is sufficient to disrupt retrieval processes, the more diffuse actions of Aβ may require higher levels of

Aβ be achieved before noticeable changes in behavioral outcomes can be found. Additionally, neuronal loss occurs in much later stages of AD, well after the initial burst in extracellular Aβ monomers and oligomers. Furthermore, early research indicates that Aβ levels are not directly correlated with neuronal loss (Irizarry et al., 1997; Gomez-Isla, et al., 1997; Terry et al., 1991), so it is possible that without an intervening factor, Aβ infusions are unlikely to lead to neuronal loss in the short term. Thus, we believe that the model utilized in this experiment demonstrates that retrieval may be less susceptible to oligomeric Aβ levels than is consolidation. This interpretation is buttressed by another experiment recently conducted in our laboratory which also found no Aβ-induced deficits in retrieval, utilizing a different paradigm. In this experiment, animals, C57BL/6J mice between the ages of 4–6 months, were trained in a CFC paradigm in which they received two 0.5 mA shocks. Twenty-four hours following training, animals began an injection series. Each morning for seven days, animals were administered LPS (250μg/kg; *Escherichia coli*, serotype 055:B5 Sigma-Aldrich, St. Louis, Missouri) or sterile saline via intraperitoneal injections. This injection series has been shown to reliably elevate hippocampal Aβ and disrupt cognition in a CFC task (Kahn et al., 2012; Weintraub et al., 2013); additionally, pro-inflammatory cytokine levels are not elevated 24 h after the last injection (Kahn et al., 2012), removing sickness behaviors as an alternative explanation for any results. Twenty-four hours after the last injection, animals were returned to the training context and freezing behavior was monitored for 214 s, as previously described (Kahn et al., 2012). Results from this study demonstrate that, in the presence of significantly elevated Aβ in the LPS-treated animals, freezing

behavior between treatment groups is not altered (t(40) = 1.25, p = 0.22; Figure 13). This suggests that endogenously produced Aβ, that is onboard at the time of testing, does not impact the retrieval of previously consolidated contextual information. Again, this finding, utilizing LPS-induced production of Aβ, is in accordance with the findings of this study and may suggest that Aβ levels in both paradigms are insufficient to produce the necessary signaling abnormalities typically associated with retrieval errors in AD populations and AD rodent models.

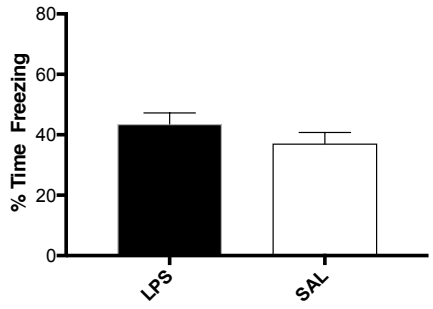

Figure 13. Percentage Freezing behavior in a CFC paradigm. Student's t-tests revealed no significant difference in freezing behaviors on testing day between animals administered LPS, and saline-treated controls. Bars represent mean ± SEM.

Future research should investigate this further. Particularly, research should examine whether the LPS and infusion models utilized in the two studies discussed here alter AMPAR, mAChR, or nAChR activation and signaling, and if they do, to what extent. Additionally, the lack of neuronal loss in these models should be confirmed through histology analysis, and more work should be done to investigate the minimum dose of Aβ needed to sufficiently disrupt retrieval in CFC.

Overall, under the conditions utilized in this experiment, murine Aβ, whether endogenously produced or exogenously administered, does not disrupt the retrieval of previously consolidated information in a hippocampal-dependent task.

CHAPTER 4: THE EFFECT OF Aβ ON THE EXTINCTION OF A CONTEXTUAL MEMORY

Abbreviated Introduction

Procedurally, extinction refers to the repeated presentation of a previously conditioned stimulus (CS, or the context, in our paradigm) without the unconditioned stimulus (US, in our paradigm, the shock). This procedure typically results in a decrease in conditioned responding (CR, or freezing; Pavlov, 1927; Bouton, 1993). Furthermore, extinction occurs in the wake of a violation of the expected contingency between the CS and the US, in which the CS loses its predictive quality in such a way as to diminish its ability to elicit the CR. This is distinct from forgetting, in which the decrease in conditioned responding could be attributed to the passage of time and memory decay (Domjan 2003). There are many theories as to how to categorize extinction. Perhaps one of the simpler mechanisms involves the idea of 'unlearning' in which the excitatory association between the CS and US is ultimately broken by extinction training; this idea was first introduced by Rescorla and Wagner (1972), but this theory of extinction does not easily explain CR recovery, i.e., spontaneous recovery or renewal, phenomenon that demonstrate that forgetting did not actually occur. Extinction has also been described as new learning in which the original associative memory remains intact, but a secondary association is formed that inhibits or competes with the original US-CS relationship expression in some way (Rescorla & Cunningham, 1978; Bouton, 1993). It should not be overlooked, however, that CR recovery is seldom complete (Rescorla, 2001) and many have now found evidence for both new learning and erasure of previous learning depending on the interval between training and extinction sessions (Myers et al., 2006; Cammarota et al., 2005; Maren & Chang, 2006; Cavallo, Hamilton, &

Farley, 2014). This information, paired with the multitude of nonassociative theories of extinction (for review, see Myers & Davis, 2007), indicates that extinction involves both associative and non-associative mechanisms that have important temporal properties.

The neurobiological literature on extinction is vast; therefore, only a sampling of relevant brain structures and signaling pathways will be discussed here. To begin with, extinction, in the context of the fear conditioning task, requires the interplay of the basolateral amygdala (BLA), hippocampus, and the infralimbic prefrontal cortex (IL; for review, see Quirk and Mueller, 2008), in which disruptions in the signaling cascade in any one of these areas can alter extinction learning. Pharmalogical blockade of the BLA during extinction has led to mixed results, with some citing interference, others facilitation, and still others, no effect at all (Akirav et al., 2006; Berlau & McGaugh, 2006; Lalumiere and McGaugh, 2005). The same pattern of inconsistent results is also found in studies looking at the hippocampus (For review, see Myers & Davis, 2007). Thus, it may be more useful to look at specific signaling systems to understand extinction processes.

For example, BDNF plays a role in the extinction of fear memories, as humans and rodents with a specific BDNF polymorphism display impaired extinction learning (Soliman et al, 2010). Furthermore, BDNF appears to link the IL, BLA, and the hippocampus. This is demonstrated in studies that knock down BDNF or its signaling in either the ventral hippocampus or the BLA and find impaired extinction learning (Heldt et al, 2007, 2014; Psotta et al., 2013), or in studies demonstrating that BDNF expression is induced, via extinction training, in the ventral hippocampus and the IL, which

enhances the firing rate of IL neurons thereby supporting extinction memories (Bredy et al., 2007; Rosas-Vidal et al., 2014).

GABA signaling is also important in the facilitation of extinction learning. Briefly, many studies indicate in important role for GABAergic transmission in fear extinction. Specifically, animals treated with a $GABA_A$ agonist, muscimol into the hippocampus show impaired extinction retention (Corcoran et al., 2005; Hobin et al., 2006); whereas picrotoxin and bicuculline ($GABA_A$ antagonists) administration after extinction training facilitate extinction retention (McGaugh et al., 1990; Berlau & McGaugh, 2006). For a detailed review on how $GABA_A$ signaling disrupts the acquisition and consolidation of fear extinction memories, see Makkar, Zhang, and Cranney (2010). Finally, conditioning and extinction training have differential and, most often, opposing, effects on dendritic spine density in a variety of brain regions (For review, see Flavell et al., 2013), indicating a particular role for synapse remodeling in both processes. Furthermore, synaptic restructuring is critical in extinction learning, as preventing synaptic protein degradation, through proteasome actions, can disrupt this process (Lee, S. H. et al., 2008; Mao et al., 2008; Pick et al., 2013; Ren et al., 2013). Specifically, proteasome inhibitors impair extinction retention (Lee, S. H. et al., 2008; Ren et al., 2013) and block $_D$-cycloserine-mediated enhancement of extinction learning (Mao et al., 2008).

As previously discussed, AD patients have stark difficulties with the consolidation and retrieval of emotional memories, but research in transgenic mouse models of AD suggest that deficits in the extinction of conditioned fear may precede other cognitive deficits. For example, four-month old female APP/PS1 mice were able to acquire the context shock pairing indiscriminately from their WT littermates, and

showed equal retention of the association 24 hours later, but failed to extinguish the association when tested again 24 hours later. More specifically, WT littermates froze significantly less than APP/PS1 animals on the extinction retention test, and in fact, APP/PS1 mice showed no freezing decrement between test 1 and test 2 (Bonardi et al., 2011). This fear extinction deficit has also been found in 4-month-old male TASTPM mice that overexpress APP and PS1 genes (Rattray et al., 2009). It should be noted that by 3.5 months, these APP/PS1 mice have significant increases in oligomeric Aβ and synaptic deficits in the hippocampus (Shemer et al., 2006; Hu et al., 2010; Howlette et al., 2004), hinting at a potential relationship between oligomeric Aβ and disruptions in extinction learning. Interestingly, in a different AD model, that causes the hyperphsphorylation of tau and leads to tau aggregates and neurofibrillary tangles by 6 months, exhibited *enhanced* extinction in a conditioned taste aversion paradigm (Pennanen et al., 2004); indicating that tau and amyloid may differentially regulate extinction learning. This view is supported by work from Lo and colleagues (2013), who found that Aβ transgenic mice APPPS1-21 had deficits in fear extinction whereas Tau22 mice did not.

Almost no studies have explored the effect of Aβ on extinction learning in the absence of other pathologies found in animal models of AD, i.e. cell death, neuroinflammation, or gliosis. In the only example of such a study, of which the authors are aware, direct hAβ administration to adult snails *Helix lucorum L*. (Crimea population) before training and again three days after training resulted in accelerated decay of the learned behavior (Korshunova, Bravarenko, & Balaban, 2008). The authors argue that this demonstrates an Aβ-induced enhancement of extinction, but this effect is

likely more related to decreased acquisition than any effect on extinction, based on the timing of the administration. Outside of transgenic models of AD and hAβ infusion models, researchers have shown age-dependent impairments of context-dependent extinction learning, object recognition, and object-place learning in aged animals (Wieschollеck, Andre, & Manahan-Vaughan, 2013). Unpublished data from our laboratory indicates that aged animals have increases in basal mAβ in the hippocampus; thus, the problems in context-extinction noted in the aforementioned study may be related to changes in oligomeric Aβ. Overall, there appears to be a link between Aβ levels and impaired extinction learning that warrants further investigation.

The purpose of the following study was to explore whether central infusions of mAβ could alter extinction of fear memories in non-transgenic animals. We hypothesized that infusions of mAβ would prevent or impair extinction learning; more specifically, we expected that mAβ-treated animals would continue to show high levels of freezing behavior across extinction trials, while saline-treated animals would show significantly reduced freezing behavior over the course of extinction trials.

Methods

Unless otherwise stated, all subjects, stereotactic surgery methods, infusion and post-behavioral verification protocols were identical to those described above.

CFC protocols

Using the same CFC training protocol described above, we investigated whether ICV infusions of mAβ could disrupt the extinction of previously learned associations. Five days following cannulation, all animals underwent training procedure as those

described above and were then returned to their home cage. A 120 s testing session occurred 24 h later.[3] Immediately following the first testing session, animals were infused with 5 µl of Aβ (prepared and verified as stated above) or sterile saline. They were then returned to their home cage, and a second testing session occurred 24 h later, see Figure 14.

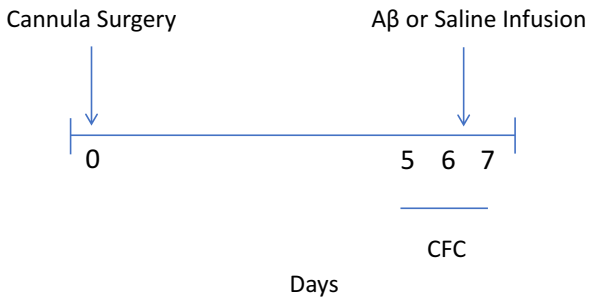

Figure 14. Experimental Timeline for extinction behavior.

Statistical Protocol

Repeated Measures ANOVA procedures were used to identify differences between groups across testing days, $p < .05$ (SPSS, IBM, Armonk, New York). Any significant omnibus effects were followed up by Fischer's post-hoc comparisons.

Results

On the day of training, prior to any manipulation, there was a significant difference in the variance of freezing behavior in the 120 s prior to the onset of the foot shock between the two groups. Thus, a Welch correction was applied, but baseline freezing behavior between groups was significantly different, $t(11.02) = 3.301$, $p = 0.007$. It should be noted that both groups froze less than 1% in the 120 s prior to the

[3] This training-testing ITI is reduced from 48 hours in order to minimize the chance that implants would become compromised over the course of multiple infusions.

shock, suggesting that this significant difference may be artificial. In support of this, in the 60 s after the end of the foot shock, there was no significant differences between groups, t (17): 0.683, p = 0.5039. Repeated measures ANOVA procedures were utilized to examine the relationship between the two treatment groups across extinction trials. Unfortunately, no significant effects were found for either within-subjects' tests or between-subjects' tests. More specifically, no differences emerged between extinction trials (F(1, 17): 0.001, p = 0.97) or the interaction between extinction trial and treatment (F(1, 17): 0.103, p = 0.75. Furthermore, there was not a significant main effect for treatment (F(1, 17): 1.141, p = 0.3, see Figure 15).

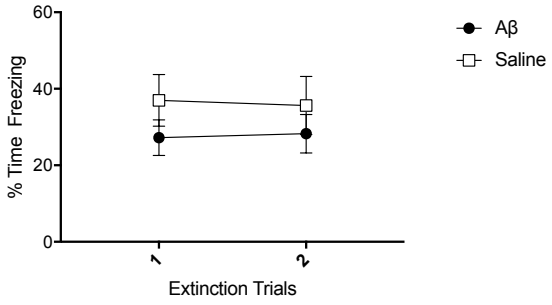

Figure 15. Freezing behavior over two days of extinction procedures. Markers represent mean ± SEM.

Discussion

The purpose of the current study was to investigate whether central infusions of mAβ could disrupt the extinction of previously learned fear memories. Unfortunately, the present study was unable to answer this question.

In order to assess the effectiveness of mAβ at disrupting extinction learning, it is critical that the control animals exhibit the expected reduction in freezing behavior over extinction trials. Unfortunately, in the results presented here, saline-infused control

animals did not show any decrements in freezing behavior between the first and second day of testing. There are several possible explanations for this effect. First, it is possible that additional testing session are needed to show extinction in the saline-infused control animals. Although this is a strong theoretically possibility, it creates practical complications. Primarily, there is the question of implant stability over time. In the current experiment, there were originally four testing sessions, with an i.c.v. infusion of mAβ or saline occurring immediately after the first and second sessions (24 h apart). Unfortunately, under these circumstances, a third of the animals were removed from the study following the second infusion due to implant loss, making analysis of the freezing behavior among the remaining animals extremely underpowered. For this reason, analysis was confined to the first two days of extinction. This suggests that the cannula implant becomes less stable over time, and is especially susceptible to damage arising from the infusion protocol utilized in the present study. Thus, additional animals would be needed in order to properly assess whether mAβ, centrally infused immediately following testing session, impairs extinction learning across multiple testing sessions.

Additionally, failure to demonstrate extinction in control animals may be a result of the stress that is induced by the i.c.v. injection procedure itself. In fact, Kim and colleagues (1998, 2003) demonstrated that i.c.v. infusion of sterile saline leads to increased plasma corticosterone levels in the mouse within thirty minutes of the infusion. Although the injection protocol utilized in the aforementioned studies are slightly different from the one used in the present study, they all perform the infusion on a mouse that is immobilized without the use of anesthesia. Thus, our protocol is

likely to result in a stress response and concordant increases in corticosterone levels as well. This increase in corticosterone may have attenuated new learning in the testing session, as studies in both humans and animals have demonstrated that cortisol injections or inescapable stressors can impede or delay the process of extinction learning (Merz et al., 2014; Baratta et al., 2007; Hartley et al., 2014; Miracle et al., 2006; Izquierdo, Wellman, & Holmes, 2006). It should be noted that, at least in the experiments described by Izquierdo, Wellman, and Holmes (2006) and Baratta and colleageus (2007), the stressors were applied prior to the onset of extinction learning, and thus may not be generalizable to the results presented here. Interestingly, cortisol injections have also been shown to enhance fear reconsolidation (Drexler et al., 2015), introducing another possible explanation for the lack of extinction over the two days.

Overall, between the instability of the cannula and its vulnerability to the manipulations which occur during the infusion protocol, in addition to the possible interactions between the extinction sessions, the mAβ treatment, and the infusion-induced stress response, i.c.v. administration of mAβ may prove impractical when trying to understand the intricacies of extinction learning within the context of AD. Thus, it is important to consider alternative procedures.

To this end, we utilized a different method to ensure that mAβ was on board at the time of extinction learning, while removing the possibly confounding effects that a stressor may have on the extinction process. Instead of infusing mAβ directly into the CNS, we utilized a paradigm previously used in our laboratory to reliably induce the endogenous production of Aβ in C57BL/6J animals (Kahn et al., 2012; Weintraub et al, 2013). In this experiment, animals were trained in a similar CFC paradigm to that

discussed in chapter 2. Briefly, animals were placed into a novel context for 120 s, followed by two, two second, 0.05 mA foot shocks, with a 30 s ITI. Sixty seconds after the last foot shock, animals were returned to their home cages. Twenty-four hours after training, a seven-day injection protocol was begun. Each morning animals were administered either LPS (250μg/kg; *Escherichia coli*, serotype 055:B5 Sigma-Aldrich, St. Louis, Missouri) or sterile saline via intraperitoneal injections. While i.p. injections are a mild stressor and can result in elevated plasma corticosterone levels (Stuart & Robinson, 2015), the injections in this study design are not directly linked to the behavioral paradigm, and thus less likely to serve as a confound. Twenty–four hours after the last injection, animals were returned to the testing chamber, and their freezing behavior was monitored for 214 s, after which they were returned to their home cages. Animals were tested again 24 h later.

Repeated measures ANOVA procedure revealed a significant interaction between treatment groups over extinction trials, $F(1, 52)$: 6.18, $p = 0.016$. Pairwise comparisons revealed that both LPS and Saline-treated animals froze indiscriminately on day 1 of extinction, $p = 0.71$, replicating findings from the LPS model discussed in chapter 3. While both treatment groups froze significantly less on day 2 of extinction compared with day 1 ($ps < 0.01$), LPS-treated animals froze significantly more than saline-treated animals on day 2 ($p = 0.037$; Figure 16). This suggests that the saline-treated animals extinguished faster than the LPS-treated animals.

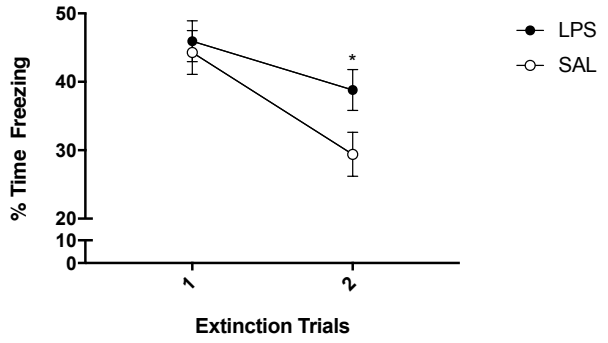

Figure 16. Freezing behavior over two days of extinction procedures. Markers represent mean ± SEM. *represents $p < 0.05$.

Based on previous research from our laboratory, this injection procedure reliably leads to significant increases in hippocampal Aβ (Kahn et al., 2012); thus, the finding above suggests that endogenously produced Aβ is capable of disrupting, or at least delaying, the extinction of learned fear in a CFC paradigm. This finding is in line with our original hypothesis, and warrants additional investigation. For instance, it would be important to investigate how long Aβ continues to suppress extinction, and if there are ways to change the parameters of the extinction training in ways to overcome this deficit.

Based on the two studies discussed in this chapter, we argue that mAβ appears to be capable of disrupting extinction learning. Additionally, this effect appears to be small, as extinction does occur in the presence of mAβm, but at a slower rate. Overall, extinction learning appears to be less susceptible to the deleterious effects of mAβ, or more susceptible to the stressful effects of the infusion procedure.

CHAPTER 5: GENERAL CONCLUSION

Current projections estimate that the scale of the coming dementia epidemic will be driven by population aging, and that less developed regions will suffer more acutely, housing roughly 79% of the 1.25 billion people expected to be living with dementia by 2050 (Prince et al., 2013). While there is no cure for AD, projections in developed countries indicate a very small decline in prevalence by 2050, <2%, that is being attributed with increased public education and efforts to control cardiovascular disease, obesity, and diabetes (Prince et al., 2016). Despite increased efforts to promote overall health, the scientific community still lacks a full understanding of the pathophysiology of AD, and even less understanding about discrete aspects of the disease, i.e. early cognitive complications arising from increased presence of Aβ oligomers in the interstitial space. The focus of this dissertation has been to elucidate the specific effects of Aβ on different phases of memory storage and retrieval within a closed system, i.e. non-transgenic mice, with the hopes of better characterizing the cognitive deficits present in the early stages of sporadic AD.

The decision to utilize non-transgenic animals in these experiments rests on multiple factors. To begin with, over the past ten years, there have been a number of studies exploring the extent to which transgenic models of AD in mice recapitulate the human disease. The general consensus has been that these models fail to fully replicate pathologies seen in humans, i.e. models may include plaques and tangles without neuronal death, or vice versa (for review, see Platt et al., 2013). Furthermore, many of the transgenic models that have been developed have severe

pathologies due to the addition of multiple transgenes, and the plaques seen in these models are distinct from those seen in humans (Fung et al., 2004), a phenomenon attributed to the interaction between the two species of peptides (Eury et al., 2011). Additionally, these peptide interactions have been shown alter the extent of amyloid deposition and route of pathogenesis in hAPP- transgenic animals; more specifically, when the human APP gene is introduced into a mAPP knockout animal, Aβ plaque load is significantly increased, higher levels of the toxic $A\beta_{1-42}$ peptide are found within the cortex, and there is decreased microglial cell convergence around plaques (Steffen et al., 2017). This suggest that the presence of the murine peptide is significantly altering the presentation of pathology, although, the authors failed to explore how the lack of mAβ alters plaque characteristics or whether cognitive function is altered in any measurable way. Moreover, the authors note several abnormalities associated with the mAPP mouse strain which may confound its usability, e.g. decreased brain weight, decreased locomotion, and age dependent astrogliosis (Steffen et al., 2017).

The use of C57BL/6J animals was also due to the results of previous research in our laboratory documenting the use inflammation to increase the endogenous production of the murine Aβ peptide, and how this increase in Aβ is related to cognitive deficits in a contextual learning environment (Kahn et al., 2012). Furthermore, this cognitive deficit has been shown to be highly related to Aβ levels, as blockade of Aβ production via the γ-secretase inhibitor, imatinib, results in restored cognitive function (Weintraub et al., 2013). Moreover, Aβ levels have been

shown to account for a significant proportion of the variance in freezing behavior (White et al., 2016), indicating that the cognitive dysfunction seen in the inflammation-induced Aβ model used in our lab is, at least partially a result of Aβ. Thus, based on our previous work, and the work demonstrating the problems associated with and artificial nature of transgenic models of AD, we chose to utilize murine Aβ to further elucidate the precise phases of learning and memory that are being disrupted in our inflammation-induced Aβ model.

Our general hypothesis was that mAβ, in the absence of hAβ, would be sufficient to disrupt cognition, and this hypothesis was generally supported. In chapter 1, we were specifically interested in how mAβ impacts the consolidation of a context-shock association. Our results demonstrate that when mAβ is centrally infused within two hours following training, freezing behavior during the retrieval test is significantly impaired (Figure 7), suggesting that mAβ does in fact disrupt the early phase of memory consolidation. Additionally, to the authors best knowledge, we are the first to propose that the deficits in consolidation seen following central infusion of mAβ are related to the mAβ-induced elevations in inflammation, marked by increased mRNA expression of IL-1β, and subsequent depression of synaptophysin mRNA levels within the hippocampus (Figures 8). This link to inflammation is especially important within the context of cognition, as there is a general lack of attention paid to the short-term inflammatory properties of hAβ infusions in the literature. For example, most of the studies investigating consolidation deficits as a result of hAβ infusions attribute the deficits to reduced

synaptic plasticity or synapse number (Frier et al., 2011; Borlikova et al., 2013; Prediger et al., 2007; Ali & Kim, 2015), but do not elucidate how synaptic plasticity is being suppressed. Thus, the finding that mAβ induces central inflammation within six hours of the infusion may provide a novel mechanism partially underlying the deleterious effects of Aβ on synaptic function; a conclusion that warrants further investigation in other AD-like models. Additionally, we provide evidence that researchers utilizing central infusions of Aβ, whether human, murine, or synthetic, should pay particular attention to the role that inflammation may have on the interpretation of their results.

Furthermore, the assertion that consolidation is particularly sensitive to the negative effects of mAβ, is supported by the findings from chapter 2 which demonstrate that when animals are allowed 46 hrs between training and the infusion on mAβ, freezing behavior is not impaired (Figures 11). This suggests that memory retrieval is not impaired in the presence of mAβ, at least under the parameters of the current experiment. Deficits in consolidation, but not retrieval are consistent with research conducted in other non-transgenic animals infused with the hAβ peptide (Borlikova et al., 2013; Balducci et al., 2010), and suggests that models utilizing either hAβ or mAβ may be a useful tool to mirror consolidation errors that are found in AD patients (Moulin et al., 2004; Genon et al., 2012). However, unlike some work done in transgenic AD models and AD patients (Hodges et al., 2006; Roy et al., 2016; Dhanjal & Wise, 2014), our results demonstrate that mAβ alone is not sufficient to disrupt the retrieval of previously learned

information, an effect that we argue is related to the diffuse actions of Aβ and the probable lack of neuronal cell death.

Finally, results from chapter 4 suggests that extinction learning may be impaired in the presence of mAβ. This finding is in agreement with the results seen in transgenic models of AD in mice (Bonardi et al., 2011; Rattray et al., 2009; Lo et al., 2013), but, unlike work conducted in these transgenic models, our model relies solely on mAβ. Thus, we are the first, to the best of the authors knowledge, to find that mAβ, in the absence of hAβ, significantly impairs extinction learning. It is important to note that this conclusion is drawn based on the LPS-induced production of mAβ, and thus, the authors cannot rule out the possibility that there are other contributing factors outside of the mAβ alone that accounts for the reduced extinction learning in the LPS-treated animals (Figure 16).

Conclusion

Based on the results discussed in this dissertation, mAβ is sufficient initiate central immune activation, disrupt synaptic plasticity, and cause deficits in both consolidation and extinction processes. Overall, these data support the notion that models utilizing endogenous or exogenous mAβ in non-transgenic animals can be useful in examining the role of Aβ in memory processes.

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VITA

PERSONAL BACKGROUND

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EDUCATION

Diploma, Creekview High School, Carrollton, Texas, 2007
Bachelor of Science, Psychology, Texas Christian University, 2010
Master of Education, Community Counseling, University of Oklahoma, 2013
Master of Philosophy, Experimental Psychology, Texas Christian University, 2015

EXPERIENCE

Assistant instructor in Special Education, Lewisville Independent School District, 2010-2011
Resident Director of Graduate Housing, University of Oklahoma, 2011-2013
Teaching Assistantship, Texas Christian University, 2013-2017

VOLUNTEER WORK

Crisis interventionist at Dallas Area Rape Crisis Center, 2010-2011

PROFESSIONAL MEMBERSHIPS

Society for Neuroscience
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ABSTRACT

THE ROLE OF EXOGENOUSLY ADMINISTERED MURINE AMYLOID-BETA IN DISRUPTING DIFFERENT PHASES OF THE LEARNING PROCESS: FROM CONSOLIDATION TO EXTINCTION

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The presence of soluble Aβ oligomers alters synaptic function and is implicated in cognitive dysfunction. Furthermore, established research in rodents indicates that intracerebroventricular (ICV) injections of human Aβ alters both the acquisition and consolidation of associative memories, but little is understood about how oligomeric Aβ impacts the retrieval of those memories, or their extinction. Murine and human Aβ differ by three amino acids (R5G, Y10F, H13R) which alter aggregation efficacy and diminish toxicity. The purpose of the present experiments was to determine how oligomeric murine Aβ impacts the consolidation, retrieval, and extinction of associative memories. Using a contextual fear-conditioning (CFC) paradigm, five experiments were carried out to disentangle which phase of learning, consolidation and/or retrieval is impacted in the presence of murine Aβ oligomers. In Experiments 1, 2, and 3, animals received an injection of Aβ or sterile saline immediately following training, or 2 or 6 hours post-training, and were tested in the same context 42-48 hours later. Results indicate that Aβ infusions within 2 hours of training lead to decreased freezing behavior, indicating that murine Aβ disrupted the consolidation and possibly the retrieval of the context-shock pairing. In Experiment 4, animals were trained in CFC and received injections of Aβ or sterile saline 46

hours later. Two hours after infusions, freezing behavior was assessed in the same context. Results from Experiment 3 revealed that Aβ infusions 2 hours prior to testing had no impact on freezing behavior. Together these results indicate that Aβ is disrupting the consolidation of new memories, but is not impacting the recovery of previously consolidated information. Experiment 5 investigated the ability of murine Aβ to disrupt the extinction of learned fear. This study utilized a LPS-induced Aβ production protocol previously utilized in our laboratory. Findings revealed that murine Aβ is also capable of disrupting, or at least delaying extinction learning. This research suggests that despite differences between murine and human Aβ, the functional outcomes on memory are strikingly similar. Thus, models utilizing endogenous or exogenous mAβ in non-transgenic animals can be useful in examining the role of Aβ in memory processes and the subtle ways in which Aβ oligomers alter synaptic functioning.