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Apoaequorin Differentially Modulates Fear Conditioning in Adult and Aged Rats

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APOAEQUORIN DIFFERENTIALLY MODULATES FEAR CONDITIONING IN ADULT AND AGED RATS

by

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ABSTRACT

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Normal aging is associated with a number of changes in behavioral and cellular function, and is often linked to increased susceptibility to cognitive impairment. The hippocampus has been widely implicated in learning and memory, and many forms of learning that are hippocampus-dependent (e.g. trace fear conditioning) are impaired in aged animals. A proposed contributor to aging-related cognitive impairment is aging-related calcium (Ca^{2+}) dysregulation. This dysregulation is thought to result from changes in specific Ca^{2+}-regulatory mechanisms, including abnormal Ca^{2+} ion channel activity or expression, as well as reduced Ca^{2+}-binding protein (CaBP) expression, which is associated with cognitive and synaptic impairment. Previous data from our lab indicate that a single hippocampal infusion of the CaBP apoaequorin (AQ) is neuroprotective in the event of an ischemic insult, a process characterized by Ca^{2+}-induced excitotoxicity. However, the effect of AQ on fear memory in adult and aged animals has yet to be examined. The current experiments investigate the effect of AQ infusion on trace fear conditioning in adult and aged rats. We firstly demonstrate that a single infusion of AQ 24 h before trace fear acquisition fails to rescue an aging-related trace fear memory deficit. Second, we found that AQ infusion 1 h prior to trace fear acquisition reduces baseline freezing during a cue test in a novel context, suggesting pre-training AQ infusion may mitigate context fear

generalization. Furthermore, AQ infusion 1 h prior to trace fear acquisition and 1 h prior to testing results in a reversal of aging-related context fear memory impairment. The results of these studies suggest a possible role for AQ in modifying cognitive function in adult and aged rats.

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LIST OF ABBREVIATIONS

aCSF: artificial cerebral spinal fluid

AD: Alzheimer's disease

AMPA: α-amino-3-hydroxy-5 methyl-4-isoxazole propionate

AQ: apoaequorin

BAPTA-AM: 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetra acetoxymethyl ester

Ca^{2+}: calcium

CaBP: calcium-binding protein

CICR: calcium-induced calcium release

CaM: calmodulin

CB: calbindin

CR: calretinin

CBV: cerebral blood volume

CS: conditioned stimulus

DG: dentate gyrus

EGTA-AM: Ethyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetra acetoxymethyl ester

fEPSP: field excitatory post-synaptic potential

GABA: gamma-Aminobutyric acid

IL: infralimbic region of the medial prefrontal cortex

ITI: intertrial interval

K^{+}: potassium

LTD: long-term depression

LTP: long-term potentiation

L-VDCC: L-type voltage-dependent calcium channel

MGN: medial geniculate nucleus

PV: parvalbumin

RyR: ryanodine receptor

sAHP: slow afterhyperpolarization

SK3: small-conductance calcium-activated potassium channel

US: unconditioned stimulus

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Introduction

Normal aging versus aging-related neurodegeneration

While aging is associated with increased risk of developing neurodegenerative diseases, including Alzheimer's (AD), the neurobiological changes that accompany normal aging are distinct from neurodegenerative pathology. Despite early work that indicated substantial aging-related neuron loss in the absence of AD, review of this work uncovered a number of methodological confounds that likely contributed to these findings (Coleman & Flood, 1987) and the evidence suggesting normal aging is accompanied by a reduction in neuron number is no longer accurate (for review, see Morrison & Hof, 1997). Instead, more recent evidence suggests neuron loss is present in AD, but not in normal aging. While substantial neuron loss occurs within the entorhinal cortex of individuals with mild and severe AD, neuron loss in this region is not evident during normal aging (Gomez-Isla et al., 1996). Further, although Morris water maze memory deficits are present in aged rats, there is no evidence for a decrease in hippocampal neuron number in aged-impaired rats compared to aged-unimpaired rats or young rats (Rapp & Gallagher, 1996). This evidence suggests the cognitive decline that manifests in normal aging is not accompanied by neuron loss. Instead, there is abundant literature to indicate that normal aging is associated with specific changes in neuronal function and calcium (Ca^{2+})-regulatory mechanisms that likely contribute to performance on various cognitive tasks, many of which reveal a learning impairment in aged animals.

Aging-related behavioral impairments

Aged animals often exhibit a pronounced learning impairment that is revealed by several behavioral paradigms. These include spatial navigation tasks, such as the Barnes maze (Barnes, 1979), Y-maze (Pereira et al., 2014), and water maze (Guidi, Kumar, Rani, & Foster, 2014;

Tombaugh, Rowe, & Rose, 2005), as well as object recognition tasks (Burke, Wallace, Nematollahi, Uprety, & Barnes, 2010; de Lima et al., 2005). Additionally, aged animals exhibit disrupted associative learning, revealed by eyeblink conditioning (Moyer, Power, Thompson, & Disterhoft, 2000; Thompson, Moyer, & Disterhoft, 1996), contextual fear conditioning, and trace fear conditioning tasks (Houston, Stevenson, McNaughton, & Barnes, 1999; McEchron, Cheng, & Gilmartin, 2004; Moyer & Brown, 2006; Villarreal, Dykes, & Barea-Rodriguez, 2004). There is additional evidence to suggest an aging-related impairment of cognitive flexibility, demonstrated by impaired trace fear extinction in middle-aged and aged rats (Kaczorowski, Davis, & Moyer, 2012).

A common element linking these learning paradigms is the involvement of underlying brain structures, which most notably includes the hippocampus. Spatial learning in rodents is considered to be dorsal hippocampus-dependent (for review see Moser & Moser, 1998), and other evidence suggests lesions of the hippocampus disrupt object recognition (Broadbent, Gaskin, Squire, & Clark, 2010), trace eyeblink conditioning (Moyer, Deyo, & Disterhoft, 1990), as well as trace and context fear, but not delay fear conditioning (McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998). However, there is some disagreement regarding the specific contribution of dorsal versus ventral hippocampus to trace fear learning. While some studies have found successful trace fear learning is dependent on dorsal hippocampus (Pierson, Pullins, & Quinn, 2015; Quinn, Oommen, Morrison, & Fanselow, 2002), others suggest a prominent role for ventral hippocampus (Cox, Czerniawski, Ree, & Otto, 2013; Czerniawski, Yoon, & Otto, 2009; Rogers, Hunsaker, & Kesner, 2006). Hippocampal contribution to trace fear learning may also depend on the activity of particular subregions within the dorsal and ventral structures. While trace fear memory impairment is evident following dentate gyrus (DG) inactivation in

dorsal hippocampus (Pierson et al., 2015), another study found that such impairment is instead evident following lesions of CA1 in ventral hippocampus (Rogers et al., 2006). While the exact contribution of dorsal and ventral hippocampal subregions to trace fear learning remains to be further investigated, the hippocampus is clearly involved in several types of learning that are impaired in aged animals. Such learning impairments are thought to arise specifically from deterioration of synaptic structure and neuronal communication within the hippocampus.

Aging-related deficits in synaptic structure and neuronal function

Select aging-related learning deficits are linked to region-specific changes in hippocampal synaptic structure. Spatial memory deficits in aged rats are associated with decreased axospinous synapses in the DG, while no such reduction is evident in the DG of either aged-unimpaired or adult rats (Geinisman, de Toledo-Morrell, & Morrell, 1986). While the overall number of Schaffer collateral-CA1 synapses does not change with age (Geinisman et al., 2004), there is a reduction in perforated synapse postsynaptic density area in learning-impaired aged rats (Nicholson, Yoshida, Berry, Gallagher, & Geinisman, 2004). Since perforated synapse formation is indicative of enhanced synaptic transmission efficacy (Jones & Harris, 1995), these synapses may be important for successful learning in old age, and such a reduction in postsynaptic density area may reflect a transition of these synapses to a non-functional or silent state, thus contributing to aging-related cognitive decline (Burke & Barnes, 2006). Other studies suggest pharmacological treatment can lead to changes in morphology as well as overt behavior. Improved Y-maze performance is positively correlated with apical dendritic thin spine density in hippocampal CA1 of aged rats following riluzole treatment (Pereira et al., 2014), which inhibits glutamate release (Martin, Thompson, & Nadler, 1993) and facilitates astrocytic glutamate uptake (Frizzo, Dall'Onder, Dalcin, & Souza, 2004). Together, these studies highlight specific

changes in synaptic structure and dendritic morphology that are important factors to consider when addressing the mechanisms of aging-related cognitive decline.

There is a wealth of evidence linking physiological changes in neuronal function to overt behavioral deficits. While neurons from adult animals exhibit learning-related synaptic changes, such as enhanced synaptic transmission following spatial learning (Barnes, 1979; Boric, Munoz, Gallagher, & Kirkwood, 2008) and enhanced hippocampal long-term potentiation (LTP) that is positively correlated with trace fear learning (Song, Detert, Sehgal, & Moyer, 2012), advancing age alters the dynamics of basal, as well as learning-related, synaptic transmission. Aging is associated with an increased threshold for LTP induction, and a reduced threshold for induction of long-term depression (LTD) (see Foster, 1999). Neurons from CA1 of aged rats also exhibit a significant reduction of maximal field excitatory post-synaptic potential (fEPSP) amplitude (Ouanounou, Zhang, Charlton, & Carlen, 1999). As evidence of a link between memory deficits and dampened synaptic plasticity, impaired induction of synaptic potentiation is related to poor spatial memory in aged animals (Bach et al., 1999; Deupree, Turner, & Watters, 1991). Together, this evidence suggests that advancing age is accompanied by distinct changes in both synapse structure and morphology, as well as altered neuronal physiology that likely contributes to the aging-related manifestation of behavioral and cognitive decline. One mechanism that is proposed to lie at the heart of these aging-related behavioral and physiological deficits is Ca^{2+} dysregulation.

Aging-related calcium dysregulation

Aging-related cognitive decline is posited to arise from Ca^{2+} dysregulation (Khachaturian, 1987), in which several Ca^{2+}-regulatory mechanisms, including Ca^{2+} ion channels, ryanodine receptors (RyRs), and Ca^{2+}-binding proteins (CaBPs), undergo aging-related

changes in function or expression that lead to disruption of Ca^{2+} homeostasis. L-type voltage-dependent Ca^{2+} channel (L-VDCC) density and activity increase with age, and channel density is negatively correlated with performance on the Morris water maze in aged rats (Thibault & Landfield, 1996). When ryanodine is applied to aged hippocampal neurons in culture, it blocks caffeine-induced increases of Ca^{2+}, suggesting aging-related elevation of Ca^{2+} transients is RyR-dependent (Clodfelter, Porter, Landfield, & Thibault, 2002). There is additional evidence that these mechanisms are coupled, such that L-VDCC activity is modulated by Ca^{2+}-induced Ca^{2+} release (CICR) from RyRs, which is triggered by Ca^{2+} influx through L-VDCCs (Chavis, Fagni, Lansman, & Bockaert, 1996). Although there is evidence for increased endogenous Ca^{2+}-buffer capacity in neurons from aged rats, this may only be effective for the first few action potentials during a 100 Hz train, as Ca^{2+} concentrations increase thereafter (Oh, Oliveira, Waters, & Disterhoft, 2013), suggesting neuronal regulation of Ca^{2+} transients becomes more easily overwhelmed with age.

Calcium-binding proteins CaBPs are also involved in regulating cellular function by participating in Ca^{2+} homeostasis and various Ca^{2+}-signaling pathways. Some of the most ubiquitous CaBPs are part of a family of evolutionarily conserved proteins that contain an EF-hand binding domain. Named after the Ca^{2+}-binding domain of parvalbumin (PV), the EF-hand binding domain consists of a helix-loop-helix motif assembled in a spatial arrangement that resembles the spread index finger and thumb of the human hand (Kretsinger & Nockolds, 1973). Although there is some overlap, these proteins can be roughly grouped according to their function. For example, proteins that act as Ca^{2+} sensors, such as calmodulin (CaM), undergo a conformational change and modulate downstream targets following Ca^{2+} binding, while Ca^{2+}

buffers, including calbindin (CB) and calretinin (CR), regulate the amplitude and duration of Ca^{2+} signals (Barinka & Druga, 2010).

These CaBPs are commonly expressed in mammalian systems, but some CaBPs are not endogenous to these animals. One of these is the photoprotein aequorin, expressed in the jellyfish *Aequorea victoria*, which has traditionally been used as an autofluorescent indicator of cytoplasmic Ca^{2+} (Shimomura, Kishi, & Inouye, 1993). Data from our lab demonstrate that the Ca^{2+}-binding component of this protein, apoaequorin (AQ), is neuroprotective when administered prior to an ischemic insult (Detert, Adams, Lescher, Lyons, & Moyer, 2013), which is characterized by Ca^{2+}-induced excitotoxicity (Choi, 1992). However, the role of AQ as a possible neurotherapeutic tool in aging and cognitive decline has yet to be investigated, despite the following evidence suggesting CaBP expression is reduced in aged animals.

With increasing age there is a corresponding decrease in CaBP expression, which is evident in several species and in various brain regions. In humans, aging is associated with reduced CB and CR expression in the cortex (Bu, Sathyendra, Nagykery, & Geula, 2003). Aged rat and rabbit DG exhibit decreased CB expression (de Jong et al., 1996), and rat perirhinal CB levels are decreased beginning as early as middle age (Moyer, Furtak, McGann, & Brown, 2011). Findings from our lab have also demonstrated an aging-related reduction of CB within the dorsal hippocampus and the infralimbic region of the medial prefrontal cortex (IL), as well as a reduction of CaM in both dorsal and ventral hippocampus, and IL (Detert, 2011). This reduction of CaBP expression within brain regions implicated in various learning tasks suggests that aging-related learning impairments could arise from dysregulation of Ca^{2+} within these regions.

Several studies suggest a link between CaBP reduction and cognitive and neuronal dysfunction in animals of various ages. In adult mice, CB-deficiency results in impaired LTP

induction (Jouvenceau et al., 2002), and both adult and middle-aged CB-knockout mice demonstrate impaired active place avoidance learning relative to wild-type mice (Moreno et al., 2012). In aged mice, impaired object recognition is linked to reduced hippocampal CB protein expression (Soontornniyomkij et al., 2012). In addition to these synaptic and behavioral impairments, cerebral blood volume (CBV), an indicator of cellular metabolism, can also be affected by CaBP expression. CBV in the DG and CA1 of CB- and PV-knockout mice is reduced, and for CB-knockout mice this reduction is age-dependent (i.e. middle-aged mice demonstrate a greater reduction compared to young) (Moreno et al., 2012). Interestingly, homeostatic mechanisms that could potentially counteract such observed abnormalities in CaBP-deficient animals are not evident, as CB-deficient mice do not demonstrate a compensatory upregulation in the expression of other CaBPs, including CR or PV, relative to wild-type mice (Airaksinen et al., 1997). Together, these data emphasize the importance of Ca^{2+} ion channels, intracellular Ca^{2+} stores, and CaBPs in maintaining intact neuronal and behavioral function through the regulation of intracellular Ca^{2+}, and suggest not only that dysfunction of these mechanisms leads to cognitive decline, but also that restoring their function will benefit cognition and neuronal activity in aged animals.

Restoring calcium regulation improves cognitive and neuronal function

Evidence suggests that restoring Ca^{2+} regulation can mitigate aging-related physiological and cognitive dysfunction. The fEPSP in hippocampal CA1 from aged animals is enhanced by the Ca^{2+} chelators BAPTA-AM and EGTA-AM (Ouanounou et al., 1999). In aged animals, blockade of L-VDCCs facilitates trace eyeblink conditioning (Deyo, Straube, & Disterhoft, 1989) and radial arm water maze learning (Veng, Mesches, & Browning, 2003), while RyR antagonism reduces the latency to find the hidden platform on the Morris water maze (Hopp et

al., 2014). Additionally, there appears to be a link between physiology and behavioral performance in aged animals following enhanced Ca^{2+} regulation. Fear memory and synaptic plasticity are enhanced in aged mice as a result of reducing small-conductance Ca^{2+}-activated potassium (K^+) channel (SK3) expression (Blank, Nijholt, Kye, Radulovic, & Spiess, 2003). Enhanced spatial memory is associated with reduced Ca^{2+}-dependent slow afterhyperpolarization (sAHP) in aged rats that overexpress FK506-binding protein 12.6/1b (Gant et al., 2015), which is normally involved in regulating sarcoplasmic reticulum Ca^{2+} release in cardiac muscle (Zalk, Lehnart, & Marks, 2007). Thus, added regulation of neuronal Ca^{2+} in aged animals may be necessary for restoring physiological and cognitive function.

Proposed Study

The current experiments aim to investigate the role of the CaBP AQ in hippocampus-dependent fear learning in adult and aged rats. Although several studies suggest aged rodents exhibit impaired trace fear memory (Blank et al., 2003; McEchron et al., 2004; Moyer & Brown, 2006; Villarreal et al., 2004), there has been little investigation as to how this impairment might be mitigated. Because sufficient literature suggests aging is accompanied by Ca^{2+} dysregulation, and learning impairments in aged animals are linked to dysfunction of select Ca^{2+}-regulatory mechanisms (see above discussion), a reasonable prediction is that restoring Ca^{2+} regulation will rescue cognitive function in aged animals. This is supported by the evidence that learning is improved in aged animals following L-VDCC blockade (Deyo et al., 1989; Veng et al., 2003), blockade of Ca^{2+} release from intracellular stores (Gant et al., 2015; Hopp et al., 2014), and SK3 channel reduction (Blank et al., 2003). In addition, a single intrahippocampal infusion of the CaBP AQ reduces cell death following an ischemic insult (Detert et al., 2013), suggesting AQ mitigates Ca^{2+}-induced excitotoxicity, which is a major part of ischemic cell death (Choi, 1992). If aging is also accompanied by Ca^{2+} toxicity via dysregulation of Ca^{2+} homeostasis (Khachaturian, 1987), then the neuroprotection afforded by AQ in an ischemic model may also translate to aging and cognitive decline.

The effects of dorsal hippocampal AQ infusion were tested in adult and aged rats to determine the potential for AQ to modulate aging-related hippocampus-dependent fear memory impairment. Additionally, because state-dependent learning is evident for trace fear (Hunt & Barnet, 2015; Reich, Mohammadi, & Alger, 2008) as well as context fear (Jovasevic et al., 2015) we examined the potential for AQ to modify hippocampus-dependent fear memory state-dependently. This allowed us to dissociate effects of AQ on hippocampus-dependent fear from

those dependent on the drug- or emotion-related brain state that may be induced by AQ infusion. Understanding the role of the CaBP AQ in hippocampus-dependent fear memory will reveal its potential for neuroprotection of cognitive function, and will aid in the overall understanding of aging-related cognitive decline.

The role of AQ in modification of hippocampus-dependent fear memory will be addressed in two aims:

Aim 1: Determine the effect of a single dorsal hippocampal AQ infusion 24 h prior to trace fear acquisition on trace fear memory in adult and aged rats.

Aim 2: Determine whether aging-related fear memory deficits are dependent on order of test presentation, and whether AQ induces state-dependent modification of fear memory in adult and aged rats.

Methods

Subjects

Adult (3-6 months, mean age = 4.2 ± .1 months) and aged (22-26 months, mean age = 23.2 ± .16 months) male F344 rats were maintained on a 14 h light/10 h dark cycle (lights on at 7am) in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility. Rats were housed individually with free access to food and water, and were handled for at least 1 week prior to behavioral training. All procedures were conducted in accordance with the University of Wisconsin-Milwaukee animal care and use committee (ACUC).

Surgery

Rats were given Carprofen (5 mg/kg/day) prior to surgery, and after surgery for pain management. While anesthetized with isoflurane, rats were mounted on a stereotaxic apparatus. Under aseptic conditions, the scalp was incised and retracted to the side, and the head leveled between bregma and lambda. Bilateral stainless steel guide cannula (26-gauge) were then lowered into the dorsal hippocampus using stereotaxic coordinates (3.5 mm posterior, 2.6 mm lateral, 3.0 mm ventral) relative to bregma (Detert et al., 2013). Cannula were secured to the skull with stainless steel screws and acrylic cement. To prevent cannula occlusion, plastic caps were screwed onto the guide cannula. Rats were allowed to recover for at least 7 days prior to infusion.

Drugs and infusions

A 4% dose of AQ (w/v; CalciGenix) was infused into the dorsal hippocampus in zero Ca^{2+} artificial cerebral spinal fluid (aCSF; in mM: 124.00 NaCl, 2.80 KCl, 2.00 MgSO4, 1.25 NaH2PO4, 26.00 NaHCO3, 10.00 D-glucose, and 0.40 Na-ascorbate); zero Ca^{2+} aCSF served as a control for AQ. To facilitate neuronal uptake of AQ, 6% DMSO was added to both vehicle and

AQ. The 33-gauge infusion cannula extended 0.5 mm beyond the guide cannula. Rats received infusions (0.5 µl/side) over 60 sec, and infusers remained in place for an additional 2 min to ensure diffusion away from the tip. Infusions were conducted in a room isolated from both the colony room and the behavioral training and testing room. Rats were transported to the infusion room at least one day before training began, in order for habituation to infusion procedures (e.g. the sound of the infusion pump). One day before the first infusion, infusion cannula were lowered into the guide cannula and remained in place for 60 sec, as part of a mock infusion procedure.

Conditioning and testing chambers

Trace fear conditioning was conducted in a Plexiglas and stainless steel chamber (30.5 × 25.4 × 30.5 cm; Coulbourn Instruments), located in a sound-attenuating box. The chamber used a standard grid floor consisting of 26 parallel steel rods (each with a diameter of 5 mm, and 6 mm spacing). The floor was connected to a precision adjustable shock generator (Coulbourn Instruments) for delivery of a scrambled footshock US. Within the sound-attenuating box, a ventilation fan provided a constant background noise of about 58 dB (measured by a sound level meter, A scale; model: Digital 2055, RadioShack). The chamber was illuminated by a miniature incandescent white lamp (28V, type 1819, illumination 1.1 lux) and was wiped with a 5% ammonium hydroxide solution prior to each training session. During training, the room lights were left on (illumination 20.9 lux) for the entire session.

A separate Plexiglas chamber served as a novel context for the test session. This chamber was located within a separate sound-attenuating box in the same room as the training chamber. The test chamber was physically different from the training chamber in that the floor was a panel of black-painted Plexiglas (instead of grid bars) with holes drilled into it, the walls consisted of 6

panels of clear Plexiglas arranged in an octagon shape, and infrared lighting was used for illumination. In addition, the tray below the test chamber floor contained clean bedding, and the test chamber was wiped with 2% acetic acid prior to each test session to provide a different olfactory stimulus from that used during training. The room lights were turned off (illumination 0.2 lux) for the entire testing session. Stimulus delivery during training and testing was controlled by FreezeFrame 4.01 (Actimetrics Software, Coulbourn Instruments). The original training context served as a test chamber for all context tests. Room lights remained on throughout the context test session.

Experimental design

We first asked whether a single dorsal hippocampal AQ infusion would mitigate aging-related trace fear memory deficits. To address this question, we included the following experimental groups: 1) Adult-Veh (n = 5); 2) Adult-AQ (n = 5); 3) Aged-Veh (n=7); 4) Aged-AQ (n = 6). Rats were handled for at least 7 days prior to behavioral training. On day 1 of the experiment, rats received bilateral infusions of either AQ or vehicle. On day 2, rats were transported in metal cages to the behavioral training and testing room, where they underwent trace fear conditioning. This consisted of 10 trials of conditioned stimulus (CS) – unconditioned stimulus (US) pairings, with a 5.2 min (±20%) intertrial interval (ITI) (Detert, Kampa, & Moyer, 2008). The CS was a 15 sec 80 dB white noise, and the US was a 1 sec, 1 mA scrambled footshock. The CS and US were separated by a 30 sec silent trace interval. On day 3, rats were placed in the novel test chamber, and the CS was presented according to the same schedule as training, but without the US. Only the average freezing during the first two trials of the test was used to assess fear memory.

For our second set of experiments, we asked whether AQ would differentially affect fear memory when infused 1 h prior to training or testing, and whether AQ infusion induced state-dependent modifications of fear memory. We included a context test in the original training context in addition to the cue test as part of our assessment of whether AQ affects hippocampus-dependent fear memory. However, because our hypothesis was that AQ would facilitate fear memory, we needed to determine the test order that was least likely to result in ceiling effects in order to observe this hypothesized enhancement. Thus, we first explored whether test order differentially affected behavioral outcome. We included the following experimental groups: rats that were tested in the original training context first (Adult-Training First, n = 7; Aged-Training First, n = 6); and rats that underwent cue testing in the novel context first (Adult-Novel First, n = 4; Aged-Novel First, n = 4). All rats received vehicle infusions 1 h prior to training and 1 h prior to testing.

Handling was conducted for at least 7 days prior to the start of training. On day 1, rats were transported to the conditioning and testing room in metal cages, and trained using a trace fear conditioning paradigm similar to that of the first experiment. However, due to the observation of robust baseline freezing during the cue test in an initial cohort (data not shown), we reduced the number of trials from 10 to 6 so as to minimize the likelihood of fear generalization during the cue test. On day 2, rats were tested for cued fear in a novel context as well as context fear in the original training context in a counterbalanced manner. Additionally, rats were transported in different cages that consisted of black painted Plexiglas to minimize fear generalization induced by contextual transport cues. The cue test consisted of a 120 sec stimulus-free period (baseline), followed by two CS presentations (15 sec, 80 dB), separated by a 2.9 min ITI. The context test consisted of 10 min of exposure to the original training context, without CS

or US presentations. Tests were separated by a 30 min interval, to allow for cleaning of the first test chamber and setup of the second test chamber.

To assess whether AQ induced state-dependent modification of hippocampus-dependent fear memory in our last experiment, we included the following groups: vehicle infusion prior to training and prior to testing (Veh-Veh; Adult n = 9, Aged n = 9); vehicle infusion prior to training, AQ infusion prior to testing (Veh-AQ; Adult n = 5; Aged n = 4); AQ infusion prior to training, vehicle infusion prior to testing (AQ-Veh; Adult n = 5; Aged n = 4); AQ infusion prior to training and testing (AQ-AQ; Adult n = 5; Aged n = 4). A subset of the Veh-Veh rats were previously part of the assessment of whether test order differentially affects behavioral outcome. Data from this subset of Veh-Veh rats were included in the current experiment, as their behavioral performance was not different from Veh-Veh rats that were part of state-dependent effects cohorts.

On day 1, 1 h following bilateral infusion, rats underwent trace fear conditioning using the same 6 trial paradigm used to assess test order effects. On day 2, 1 h following bilateral infusion, rats underwent a cue test in a novel context, followed by a context test in the original training context 30 min later. Parameters for each test were identical to those used in the assessment of test order effects. Following testing, rats were returned to their home cages.

Analysis of behavioral data

For behavioral experiments, a remote CCD video camera (model #STC-MB33USB; Sensor Technologies America, Inc. Carrollton, TX), mounted to the top of each behavioral chamber, was used to record the activity of each rat during training and testing. The video data were fed to a PC running FreezeFrame 4.01 (Actimetrics Software, Coulbourn Instruments). Data were analyzed using FreezeView 4.01 (Actimetrics Software) where a 1 sec bout of

immobility was scored as freezing. The absence of all movement except that required for respiration was used to define freezing (Blanchard & Blanchard, 1969).

Statistical analyses

Overall treatment effects were examined using Student's t-test, mixed ANOVA, or two-way ANOVA where appropriate using SPSS 23.0 (IBM Corp., Armonk, NY). A Greenhouse-Geisser correction was used if Mauchly's test of Sphericity indicated the assumption of sphericity had been violated. For significant main effects ($\alpha = .05$), *post hoc* analysis was performed using Fisher's LSD. Bonferroni adjustment was used for multiple comparisons. Data are expressed as mean ± SEM.

Results

A single dorsal hippocampal infusion of AQ 24 h prior to trace fear conditioning does not affect trace fear memory in adult or aged rats

In our initial experiment, data were collected to investigate the hypothesis that a single dorsal hippocampal infusion of AQ 24 h prior to trace fear acquisition would facilitate trace fear learning aged rats (see Fig. 1*A* for experimental design). On day 1 adult and aged rats received bilateral dorsal hippocampal infusions of either vehicle (adult: n = 5; aged: n = 7) or AQ (adult: n = 5; aged: n = 6) 24 h prior to trace fear conditioning. This time point was chosen because a previous study from our lab demonstrates that administering AQ either 24 or 48 h prior to an *in vitro* ischemic insult is neuroprotective (Detert et al., 2013). Trace fear conditioning on day 2 consisted of 10 pairings of a 15 sec, 80 dB white noise CS, and 1 sec, 1 mA footshock US, separated by a 30 sec stimulus-free trace interval. On day 3, rats were placed in a novel context, and the CS was presented without the US. Percent freezing during the average trace interval for the first two test trials (defined as the first 30 sec after CS offset) was used as a measure of trace fear memory.

During trace fear conditioning, performance was similar between groups (Fig. 1*B*). A mixed ANOVA of percent freezing during blocks of two trace interval trials revealed a significant effect of trial block [$F(4, 76) = 21.909$, $p < .001$], but no significant interaction between age and trial block [$F(4, 76) = .668$, $p = .616$], no infusion by trial block interaction [$F(4, 76) = .233$, $p = .919$], and no age by infusion by trial block interaction [$F(4, 76) = 1.233$, $p = .304$]. Additionally, there was no main effect of age [$F(1, 19) = 1.779$, $p = .198$], no main effect of infusion [$F(1, 19) = .153$, $p = .7$], and no age by infusion interaction [$F(1, 19) = .488$, $p = .493$].

A two-factor ANOVA was used to analyze the effect of infusion and age on behavioral performance during the cue test. There was no effect of age [F(1, 19) = .239, p = .630] or infusion [F(1, 19) = .352, p = .560], nor was there an age by infusion interaction effect [F(1, 19) = .000, p = .983] on baseline freezing. Similarly, CS freezing did not differ between age groups [F(1, 19) = .915, p = .351] or infusion groups [F(1, 19) = 2.184, p = .156], and there was no interaction effect [F(1, 19) = .489, p = .493]. However, analysis of trace interval freezing revealed a significant main effect of age [F(1, 19) = 5.033, p < .05], but no main effect of infusion [F(1, 19) = .023, p = .880], and no age by infusion interaction [F(1, 19) = .263, p = .614]. When baseline freezing was subtracted from trace freezing (Trace-B) to normalize for generalized baseline freezing observed in all groups, there was a significant main effect of age [F(1, 19) = 9.622, p < .01], but no main effect of infusion [F(1, 19) = .124, p = .728] and no age by infusion interaction [F(1, 19) = .347, p = .563] (Fig. 1*B*). Overall, these data suggest that a single dorsal hippocampal infusion of AQ does not alter trace fear learning in adults, and that there is a learning impairment in aged rats that is not rescued by a single infusion of AQ.

Test order differentially affects fear memory

Before we could address the effects of pre-training or pre-testing AQ infusion on trace and context fear memory, we first needed to determine whether test order affected behavioral outcome. Because our hypothesis was that AQ would rescue aging-related fear memory deficits, our goal was to identify the test order that would result in the most pronounced impairment. This would increase the likelihood of observing beneficial effects of AQ, while minimizing the possibility of ceiling effects. Thus, adult and aged rats underwent trace fear conditioning, followed by an auditory cue test in a novel context, as well as a context fear memory test in the original training context. Each age group was subdivided based on test order to yield the

following four groups: Adult-Training First (n = 7), Adult-Novel First (n = 4), Aged-Training First (n = 6), Aged-Novel First (n = 4). Tests were separated by 30 min, and test order was counterbalanced (see Fig. 2 for experimental setup).

To determine whether test order differentially affected behavioral outcome, behavioral performance of aged rats was compared to that of adults within each test order group. That is, Aged-Training First was compared with Adult-Training First, and Aged-Novel First was compared with Adult-Novel First. For rats tested in the novel context first, there was a significant effect of trial on trace interval freezing during trace fear acquisition on day 1. A mixed ANOVA revealed a significant effect of trial [$F(5, 30) = 7.330$, $p < .001$], and a significant age by training trial interaction [$F(5, 30) = 3.281$, $p < .05$]. There was no significant main effect of age [$F(1, 6) = 1.588$, $p = .254$]. For rats tested in the original training context first, analysis of trace interval freezing during acquisition on day 1 revealed a significant effect of training trial [$F(5, 55) = 30.039$, $p < .001$], and a significant age by training trial interaction [$F(5, 55) = 2.461$, $p < .05$]. The overall main effect of age was not significant [$F(1, 11) = .108$, $p = .749$].

For rats that were tested in the novel chamber first on day 2, unpaired t-tests (one-tailed) indicated aged rats displayed significantly reduced freezing to the CS [$t(6) = 2.006$, $p < .05$], to the trace interval when baseline freezing was subtracted (Trace-B) [$t(6) = 2.114$, $p < .05$] as well as to the original training context [$t(6) = 2.409$, $p < .05$]. The effect of age on baseline freezing was trending but not significant [$t(6) = -1.814$, $p = .06$], while trace freezing was not different [$t(6) = .353$, $p = .368$].

In contrast, rats that were tested in the original training context first displayed a different pattern of results. For this group, analysis of freezing during the novel context test revealed that

aged rats displayed greater freezing during the baseline period [t(11) = -2.458, $p < .05$], but reduced Trace-B freezing [t(11) = 3.631, $p < .01$]. Interestingly, aged rats tested in the original training context first failed to display impaired context fear memory [t(11) = .914, $p = .212$], unlike their counterparts that were tested in the novel chamber first. Additionally, CS freezing was not different [t(11) = .374, $p = .358$], nor was trace freezing [t(11) = 1.3, $p = .11$] (Fig 3*A*). These observations suggest that when baseline freezing is subtracted from trace freezing, aged rats exhibit disrupted trace fear memory regardless of test order, but they only display impaired context fear memory when they are tested in the novel context first.

In order to determine whether test order also affected discrimination ability, average freezing during the first two min of the original context test (A) was compared with freezing during the first two min of the novel context test (B). A paired t-test of average freezing during the first two min of each test was performed for each group. Adult-Training First rats displayed significantly reduced freezing during B relative to A [t(12) = -8.326, $p < .001$], while Aged-Novel First rats exhibited increased freezing during B relative to A [t(6) = 2.776, $p < .05$]. No differences were evident for the Adult-Novel First group [t(6) = .443, $p = .337$] or the Aged-Training First group [t(10) = 1.323, $p = .108$]. Finally, a discrimination ratio was calculated by subtracting the average freezing during the first two min of the novel test (B) from the average freezing during the first two min of the original training context test (A), and dividing the difference by the sum of A and B (i.e. (A-B)/(A+B)). Aged rats displayed significantly reduced discrimination ratios when tested in the novel context first [t(6) = 2.366, $p < .05$] and when tested in the original training context first [t(11) = 2.863, $p < .01$] (Fig. 3*B*).

AQ infusion prior to training and testing rescues an aging-related context fear memory deficit

The findings from the previous experiment indicate that presentation of the cue test in the novel context first reveals an aging-related context fear memory deficit that is otherwise absent. Thus, in order to address whether AQ is capable of mitigating either aging-related trace or context fear memory deficits, rats in the present experiment underwent cue testing in the novel context first, followed by testing for context fear memory in the original training context second. We included four infusion groups within each age group to assess the effect of pre-training and pre-testing AQ infusion on hippocampus-dependent fear memory, and to address whether any effects of AQ were state-dependent: 1) vehicle infusion before training and testing (Veh-Veh; adult: n = 9, aged: n = 9); 2) vehicle infusion before training, and AQ infusion before testing (Veh-AQ; adult: n = 5; aged: n = 4); 3) AQ infusion before training, and vehicle infusion before testing (AQ-Veh; adult: n = 5; aged: n = 4); 4) AQ infusion before training and testing (AQ-AQ; adult: n = 5; aged: n = 4) (see Fig. 4 for experimental setup).

A mixed ANOVA was used to analyze the effect of trial, age, and infusion on trace interval freezing during training on day 1. Overall, there was a significant effect of training trial [$F(3.533, 130.707) = 41.097$, $p < .001$] and a significant main effect of age [$F(1, 37) = 12.092$, $p < .01$]. There was no interaction between training trial and age [$F(3.533, 130.707) = 2.366$, $p = .064$], between training trial and infusion [$F(10.598, 130.707) = .804$, $p = .632$], nor between trial, age, and infusion [$F(10.598, 130.707) = 1.082$, $p = .381$]. There was also no main effect of infusion [$F(3, 37) = .179$, $p = .910$], and no age by infusion interaction [$F(3, 37) = 1.358$, $p = .271$].

Several effects became apparent during the cue test in the novel context and the context test in the original training context on day 2. Analysis of baseline freezing revealed a significant

main effect of age [$F(1, 37) = 7.128$, $p < .05$], and a significant main effect of infusion [$F(3, 37) = 3.431$, $p < .05$], but no age by infusion interaction [$F(3, 37) = .182$, $p = .908$]. *Post hoc* analysis of infusion indicated percent freezing for Veh-Veh was significantly higher than that of AQ-Veh ($p < .05$) and AQ-AQ ($p < .01$). Freezing to the CS was not different between age groups [$F(1, 37) = 3.177$, $p = .083$] or infusion groups [$F(3, 37) = 1.627$, $p = .200$], and there was no interaction effect [$F(3, 37) = .625$, $p = .603$]. Trace interval freezing was significantly reduced among aged rats [$F(1, 37) = 6.047$, $p < .05$], but there was no effect of infusion [$F(3, 37) = .221$, $p = .881$], and there was no age by infusion interaction [$F(3, 37) = .367$, $p = .777$]. When baseline freezing was subtracted from trace freezing (Trace-B), aged rats displayed reduced freezing [$F(1, 37) = 33.652$, $p < .001$], and there was a significant main effect of infusion [$F(3, 37) = 2.952$, $p < .05$], but no infusion by age interaction [$F(3, 37) = .665$, $p = .579$]. *Post hoc* analysis of Trace-B freezing revealed Veh-Veh rats froze significantly less when compared with rats from AQ-Veh ($p < .05$) and AQ-AQ ($p < .01$) groups. Additionally, Veh-AQ rats froze significantly less when compared to AQ-AQ ($p < .05$). Finally, analysis of average context freezing during the context test revealed a significant main effect of age [$F(1, 37) = 25.196$, $p < .001$], but there was no effect of infusion [$F(3, 37) = .721$, $p = .546$], and no age by infusion interaction effect [$F(3, 37) = .885$, $p = .458$].

Additional pairwise comparisons were conducted to determine the extent of aging-related fear memory impairment, and whether such impairment was mitigated by AQ infusion (Fig. 5). During the cue test in the novel context, aged rats displayed significantly reduced CS freezing relative to adults within the Veh-Veh group only ($p < .05$). Interestingly, while there was no aging-related reduction of trace interval freezing for any infusion group, subtraction of baseline freezing from trace freezing (Trace-B) revealed an aging deficit among all groups (all *p*-values, *p*

< .05). Finally, pairwise analysis of average freezing during the context test revealed an aging-related impairment for every group except AQ-AQ (AQ-AQ *p*-value, $p = .258$; all others $p < .01$).

Together, these data suggest aged rats exhibit impaired trace and context fear memory, and that pre-training AQ infusion reduces baseline freezing during the cue test in a novel context. This baseline reduction likely accounts for overall increase in Trace-B freezing observed when AQ was infused prior to training. Because pre-training AQ infusion reduced baseline freezing regardless of whether a pre-testing infusion of AQ also occurred, this suggests AQ infusion does not induce state-dependent modification of this measure. Additionally, pairwise comparisons revealed an aging-related impairment of context fear memory within every infusion group except AQ-AQ. This suggests AQ may state-dependently mitigate an aging-related context fear memory deficit.

Discussion

Trace fear memory is not affected by a single dorsal hippocampal infusion of AQ 24 h prior to training

The purpose of these experiments was to determine whether AQ mitigates aging-related fear memory impairment, and whether AQ induces state-dependent modification of fear memory. We first asked whether aging-related trace fear memory deficits could be mitigated following a single dorsal hippocampal infusion of AQ. Our data suggest that while aged rats exhibit impaired trace fear memory relative to adults, a single infusion of AQ does not rescue this impairment when it is infused 24 h prior to trace fear acquisition (see Fig. 1).

While AQ is neuroprotective when infused 24 h or 48 h prior to *in vitro* ischemia (Detert et al., 2013), AQ infusion 24 h prior to trace fear conditioning may not effectively target critical aspects of learning, such as acquisition or consolidation, in this behavioral model. Further, western blot analysis indicates the AQ protein is present in hippocampal tissue 1 h and 1 d following infusion (Detert et al., 2013). This suggests there may be a disconnect between the neuroprotection that AQ confers in an ischemic model and the presence of the protein in brain tissue, as the temporal contingency between protein presence and neuroprotection is not identical. Thus, the neuroprotective effects that AQ may afford in an ischemic model may not map directly onto a behavioral model like that used in the current study. The effects of AQ on overt cognitive function may instead only be observable if behavioral manipulations occur when protein presence is greatest (i.e. 1 h after infusion) (Detert et al., 2013).

Additionally, the possibility remains that chronic AQ administration may be required to effect observable behavioral changes in aged animals. Following 3 weeks of treatment with nimodipine, an L-VDCC blocker, aged rats display fewer working memory errors on a water

maze task, and exhibit reduced protein expression of $Ca_v1.3$, an L-VDCC subunit (Veng et al., 2003). An additional study found that chronic RyR blockade in aged rats improves water maze performance (Hopp et al., 2014). However, pilot data from our lab suggest limited feasibility of performing multiple infusions in aged rats, as chronic restraint that is part of our standard infusion protocol leads to increased baseline freezing among old rats during a cue test in a novel context (data not shown). The possibility that chronic AQ administration may effectively mitigate aging-related fear memory deficits remains to be investigated, with additional experiments required to optimize compatibility between drug administration protocols and behavioral protocols.

Test order and age differentially affect fear memory and discrimination ability

Because the aim for our next set of experiments was to determine the effect of AQ infusion on trace as well as context fear memory, it was necessary to first determine whether test order differentially affected behavioral outcome in adult and aged rats. To address this, a subset of adult and aged rats underwent trace fear conditioning, followed 1 d later by a cue test in a novel context to assess CS and trace fear memory, as well as a test in the original training context to assess context fear memory. Test order was counterbalanced within each age group, so that four separate experimental groups were included: Adult-Novel First, Adult-Training First, Aged-Novel First, Aged-Training First (see Fig. 2 for experimental setup). Our data suggest aged rats display impaired trace fear memory regardless of test order. However, there is an aging-related context fear memory deficit only among rats that underwent the cue test in the novel context first (see Fig. 3*A*).

One possible contributing factor to these observed order effects may be an aging-related disruption of discrimination ability that is also dependent on test order. To address this in the

current study, we compared percentage freezing during the first two min of the cue test in the novel context (B) to percentage freezing during the first two min of the context test in the original training context (A) for each experimental group. Because the novel context was not previously paired with shock, we predicted that initial freezing in this environment would be minimal compared to that of the original training context, and that any substantial freezing may be indicative of fear generalization. We found that while Adult-Training First rats displayed significantly less freezing during B compared to A, Aged-Novel First rats displayed significantly more freezing in B compared to A. Further, while Aged-Training First rats exhibit reduced freezing during B, this reduction was not significant, as it was for Adult-Training First rats. These data suggest that aging is accompanied by an impaired ability to distinguish between a context that was previously paired with shock and a completely novel context. Interestingly, Adult-Novel First rats failed to exhibit an increase of freezing in A relative to B, which also might be indicative of impaired discrimination, since exposure to the original training context should theoretically evoke higher levels of freezing than exposure to a novel context (Blanchard & Blanchard, 1969; Bolles & Collier, 1976; Fanselow, 2000).

To better understand discrimination ability, we calculated a discrimination ratio by dividing the difference between A and B by the sum of A and B. We observed an aging-related reduction of discrimination ratio for both the Novel First and Training First test order groups. Like our findings with context fear memory, aged rats exposed to the cue test in the novel environment first exhibited the poorest performance, indicated by the observed negative discrimination ratio (see Fig. 3*B*). These data suggest that the combination of old age and exposure to a cue test in a novel environment first may reveal an underlying deficit in hippocampus-dependent context fear memory that otherwise may not be evident if testing for

context fear memory in the original training context occurred first. Additionally, our observation that discrimination is disrupted in aged rats, particularly for those that undergo the cue test in the novel context first, lends further support to the notion that behavioral performance among aged rats is especially susceptible to the effects of test order.

Other studies that have assessed fear memory using two distinct contexts do not always address whether test order affects behavioral outcome. In two separate investigations of the effects of *E. coli* and aging on fear memory, vehicle-treated aged rats display similar context and tone fear memory compared to vehicle-treated adults (Barrientos et al., 2009; Barrientos et al., 2006). In both studies, fear to the original training context was tested before cued fear in a novel context. The authors speculate this order of testing extinguishes fear to the training context to some degree, minimizing generalized fear that might otherwise be expressed if the first test consisted of a cue presentation in a novel context (Barrientos et al., 2009). In contrast, when rats undergo a cued fear test in a novel context prior to testing in the original training context, aged rats do exhibit impaired context fear memory relative to adults (Kaczorowski et al., 2012; Moyer & Brown, 2006). This supports findings from the current experiments that suggest aged rats exhibit a context fear memory impairment only when they undergo a cue test in a novel context first.

Other studies that have counterbalanced test order do not necessarily find evidence for differential fear memory. These investigations demonstrate that fear memory is unaffected by test order following delay fear conditioning (Baldi, Lorenzini, & Bucherelli, 2004), and context fear conditioning (Migues et al., 2016). However, tests were conducted 1 d apart, unlike the 30 min used in the current experiments, and the effect of test order on fear memory in aged rats was not addressed. The finding that test order affects behavioral outcome in the current experiments

may be specific to shorter intervals between tests, and the inclusion of aged rats in the experimental design.

Previous work investigating discrimination ability following fear learning in adult animals suggests specific mechanisms contribute to context discrimination as well as discrimination between distinct auditory stimuli. For example, lipopolysaccharide (LPS) – induced neuroinflammation increased hippocampal expression of proinflammatory cytokines, disrupted context discrimination following context fear conditioning, and increased the overlap of cell network activity in both CA1 and CA3 subregions of the dorsal hippocampus in adult rats (Czerniawski & Guzowski, 2014). In a separate study that utilized discriminative auditory fear conditioning, which included a CS paired with shock (CS^+) and a CS paired with absence of shock (CS^-), medial geniculate nucleus (MGN) lesions resulted in impaired discrimination of the CS^+ and CS^- during the test (Antunes & Moita, 2010). Thus, while discrimination of different auditory stimuli may require an intact MGN, context discrimination likely depends upon distinct hippocampal neuronal ensembles, suggesting the impaired context discrimination observed in the current experiments could stem from a similar mechanism.

Successful context fear discrimination is also dependent on specific temporal parameters. The ability to discriminate between two distinct contexts following context fear conditioning was intact in adult rats 24 h following conditioning, but was impaired two weeks later, suggesting context fear generalization is time-dependent. Successful discrimination at the two week remote time point likely requires the presence of synaptic GluA2 AMPARs, as blocking endocytosis of these receptors in the dorsal hippocampus reversed context fear generalization (Migues et al., 2016). A separate study that also employed context fear conditioning found that generalized fear to a novel context three weeks following training was reversed by inactivation of the anterior

cingulate cortex and ventral hippocampus. Additionally, inactivation of the dorsal hippocampus disrupted fear memory to the original training context at this three week remote time point (Cullen, Gilman, Winiecki, Riccio, & Jasnow, 2015), lending further support for a role of the dorsal hippocampus in maintaining context specificity. Interestingly, while adult rats exhibited increased fear responses over time to various stimuli, including the original training context, a novel context, and the tone CS, this incubation effect was diminished in aged rats (Houston et al., 1999). These findings suggest that in adult animals, context generalization manifests at greater training-to-test intervals.

In contrast to adults, fear generalization in aged animals is often evident at more immediate time points. Initial freezing following placement in a novel environment (i.e. baseline freezing) can be robust for aged animals, and may serve as direct evidence of fear generalization that accompanies aging. For example, a study investigating the effect of hippocampal DNA methyltransferase overexpression on fear learning found that aged mice displayed robust baseline freezing when tested in an altered context 24 h after trace fear conditioning, and freezing remained unchanged from baseline following presentation of the CS. Baseline freezing was subtracted from CS freezing to normalize for this aging-related increase of fear generalization, and only then were aging-related deficits evident (Oliveira, Hemstedt, & Bading, 2012). In a separate study using delay fear conditioning, both adult and aged mice exhibited substantial freezing during the baseline of an extinction session in a novel context (~53% and ~58% freezing, respectively) 1 d following training. Although extinction learning was intact in aged mice, they failed to display renewal of extinguished fear in a third context, unlike adult controls (Sanders, 2011). This study suggests that while both age groups display some degree of fear generalization upon initial exposure to a new context, the observation that aged mice also

fail to exhibit renewed fear to the tone when the context is shifted a third time may indicate generalized extinction learning. Thus, not only have aged animals been found to display increased fear generalization closely after the time of training, there is also evidence that suggests they may generalize their learning of extinction to different contexts.

In summary, our data suggest that context fear and discrimination ability are particularly susceptible to disruption among aged rats when a cue test in a novel environment occurs before a context test in the original training context. This is supported by other literature that suggests aged animals display increased fear generalization and disrupted discrimination. However, it is possible that this effect is specific to shorter intervals between tests. The only interval used in the current experiment was 30 min, while other studies have typically separated their tests by one day or longer. Because our goal for the following experiment was to address the effect of AQ infusion shortly before behavioral manipulation, we needed to use the shortest test-test interval that was still practical. Our data suggest that testing in a novel context first leads to poor behavioral performance for aged rats. Therefore, this test order was used in the following experiment to investigate whether AQ-induced modification of fear memory could be attributed to state-dependent effects.

Aging-related context fear memory impairment is mitigated by pre-training and pre-testing AQ infusion

We next sought to determine the effect of pre-training and pre-testing AQ infusions on hippocampus-dependent fear memory, and whether any effects could be attributed to state-dependent learning. Adult and aged rats were divided into four groups based on infusion schedule: vehicle both before training and testing (Veh-Veh); vehicle before training and AQ

before testing (Veh-AQ); AQ before training and vehicle before testing (AQ-Veh); AQ both before training and testing (AQ-AQ) (see Fig. 4 for experimental setup).

Aged rats demonstrated impaired performance on a number of measures. Despite overall increased trace interval freezing across training trials, aged rats displayed reduced freezing during acquisition compared to adults. The following day, aged rats overall exhibited increased baseline freezing and reduced trace interval freezing during the cue test in the novel context, as well as reduced freezing to the original training context. Additionally, AQ infusion resulted in differential behavioral performance overall. When AQ was infused prior to training, baseline freezing during the cue test was reduced, while Trace-B freezing was increased. Importantly, because both the AQ-Veh and AQ-AQ groups displayed similar performance for these measures, these effects are likely due to pre-training AQ infusion, and not to any state-dependent effects of AQ (Poling & Cross, 1993). Additional pairwise comparisons within each infusion group were used to assess whether AQ infusion mitigated impaired learning among aged rats. While there was no aging-related reduction of trace interval freezing for any infusion group, subtraction of baseline freezing from trace freezing (Trace-B) revealed a significant aging-related impairment within each infusion group that was not rescued by AQ infusion. Despite the evidence for an aging-related impairment of context fear memory for the Veh-Veh, Veh-AQ, and AQ-Veh groups, the AQ-AQ group failed to display this impairment (see Fig. 5). Thus, pre-training AQ infusion resulted in an overall reduction of baseline freezing during the cue test, while pre-training and pre-testing AQ infusions reversed an aging-related context fear memory deficit.

The Ca^{2+} dysregulation and aging literature suggests that restoring some aspect of Ca^{+} regulation is beneficial for cognitive function. Most of these studies utilize substances that impede Ca^{2+} entry into the cytosol, either from the extracellular space via L-VDCCs (Deyo et al.,

1989; Veng et al., 2003), or from intracellular stores like RyRs (Gant et al., 2015; Hopp et al., 2014). Regulating Ca^{2+}-dependent physiological mechanisms, such as the Ca^{2+}-dependent sAHP, for example, may also effectively restore cognitive function in aged animals (Blank et al., 2003). However, few studies have examined whether sequestration of intracellular free Ca^{2+} effectively mitigates aging-related deficits of cognitive function. While the Ca^{2+} chelators BAPTA-AM and EGTA-AM enhanced synaptic plasticity in aged hippocampal CA1 (Ouanounou et al., 1999), it remains unclear if overexpression of these or other Ca^{2+}-buffering mechanisms mitigate aging-related cognitive deficits.

We found that pre-training AQ infusion reduced overall baseline freezing during the cue test in a novel context, which suggests that fear generalization was evident among animals that received vehicle infusions prior to training and testing. Minimizing generalized fear during a cue test in a novel context is critically important for obtaining an accurate measurement of learned fear to conditioned stimuli, and others have attempted to determine specific factors that contribute to fear generalization following training. In an assessment of the effects of nicotine on fear learning, nicotine-treated mice displayed increased baseline freezing during a test in a shifted context 1 d following training. The test chamber was the same as the training chamber, except the grid floor used during training was replaced with a plastic floor, and the chamber area was decreased by inserting a divider. The authors found that increased fear generalization in nicotine-treated mice could be mitigated if the test chamber was located in a different room from that of the training chamber, and if training and testing were conducted by different experimenters (Gould & Wehner, 1999). Although we attempted to minimize generalization by conducting the cue test in a different chamber from that used during training, with different olfactory, tactile, and light conditions (see methods), the cue test was conducted in the same

room as behavioral training, and the same experimenter conducted both behavioral training and testing. The generalization we observed in the current experiments could be due to the fact that the cue test was conducted in the same room used during training, the same experimenter conducted both training and testing, or some other unknown factor or combination of factors.

However, because rats displayed robust baseline freezing during the cue test, we were able to observe a reduction in generalized fear following pre-training AQ infusion. This suggests that perhaps there is a role for Ca^{2+} or Ca^{2+}-dependent mechanisms in generalized learning. One possible contributor may include Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), which is normally activated following increases of intracellular Ca^{2+}. CaMKIV knockout mice exhibited reduced baseline freezing in an altered context 28 d after auditory fear conditioning, suggesting a potential role for Ca^{2+}-induced activation of this enzyme in generalized fear expression (Takao et al., 2010). A separate investigation of downstream regulatory element antagonistic modulator (DREAM), a Ca^{2+}-dependent transcriptional repressor, revealed that mice lacking this protein spent more time exploring a novel object following object recognition training, while those expressing this protein showed a generalized preference for a novel and familiar object (Fontan-Lozano et al., 2009). Thus, it may be the case that disrupting these Ca^{2+}-dependent signaling mechanisms facilitates discrimination learning, and sequestration of intracellular Ca^{2+} by AQ may reduce the fear generalization observed in the current experiment via similar mechanisms. Given that the links between Ca^{2+}-dependent signaling and generalized learning are indirect and few in number, future work will be needed to determine the specific role of AQ in modifying generalized fear.

Additionally, we found that aged rats fail to exhibit a context fear memory deficit when AQ is infused prior to training and testing. Context fear memory is dependent upon dorsal

hippocampal function (Chowdhury, Quinn, & Fanselow, 2005; McEchron et al., 1998; Misane et al., 2005) and others have found that aging is associated with impaired context fear memory following trace fear conditioning (Kaczorowski & Disterhoft, 2009; Moyer & Brown, 2006). Further, CaBP expression is reduced with advancing age in the DG of the hippocampus (de Jong et al., 1996) and within both the dorsal and ventral hippocampal subregions (Detert, 2011). Given the evidence suggesting the dorsal hippocampus underlies context fear, and that aging is associated with reduced hippocampal CaBP expression, our data suggest that AQ-induced rescue of aging-related context fear memory impairment may be due to Ca^{2+} sequestration in this region of the brain.

Our finding that AQ did not significantly affect trace fear memory may reflect differential involvement of underlying neural circuitry that mediates the recall of trace fear memory versus context fear memory. While the hippocampus seems to be one of the main contributors to context fear, the circuitry underlying trace fear memories appears to be more complex. In support, while hippocampal lesions disrupt context and trace fear memory (McEchron et al., 1998; Quinn et al., 2002), disrupted entorhinal cortex-hippocampal synaptic transmission impairs trace fear memory, but leaves context fear memory intact (Suh, Rivest, Nakashiba, Tominaga, & Tonegawa, 2011). However, other evidence suggests both trace fear and context fear memory are disrupted when NMDAR transmission is blocked in the medial prefrontal cortex, and when this region is inactivated by the $GABA_A$ agonist muscimol (Gilmartin & Helmstetter, 2010). Additional findings suggest NMDA-induced excitotoxic lesions of perirhinal cortex disrupt trace as well as context fear memory (Kholodar-Smith, Boguszewski, & Brown, 2008). It remains unclear whether AQ infusion targeted to these or

other brain regions that are also known to support trace fear memory would result in a rescue of aging-related trace fear memory deficits.

Another possible contributing factor to the observation that AQ differentially affects context versus trace fear memory is the evidence that these two forms of memory may be disrupted at different points in the lifespan. For instance, context fear memory is disrupted as early as middle-age, while disruption of trace fear memory is not as severe in this age group (Kaczorowski & Disterhoft, 2009; Kaczorowski, Sametsky, Shah, Vassar, & Disterhoft, 2011; Moyer & Brown, 2006). Although the current experiments did not include middle-aged rats, the effect size was much more robust for the overall aging-related context fear deficit compared to the overall aging-related trace fear deficit (partial eta squared: .405 vs .14, respectively). Perhaps in order for AQ to elicit an observable behavioral effect on trace fear memory, a more robust aging-related trace fear memory deficit is required. This could potentially be accomplished by including older rats than what was used in the current study, or modifying our behavioral protocol so as to reduce the valence of conditioning stimuli, thus increasing the difficulty of the behavioral paradigm.

Finally, our finding that aging-related context fear memory impairment is mitigated following pre-training and pre-testing AQ infusion begs the question of whether these effects of AQ are state-dependent. Symmetrical state-dependent learning is traditionally thought to occur when behavioral performance following a change of state from training to testing is different from behavioral performance when the state of the animal remains unchanged from training to testing (Overton, 1974; Poling & Cross, 1993). In the current experiments, only the aged rats that received pre-training and pre-testing AQ infusion displayed unimpaired context fear memory, unlike the other infusion groups that all displayed an aging-related context fear memory

impairment. These results suggests symmetrical state-dependent learning is not evident. Instead, our findings may reflect a state-dependent facilitation of context fear retrieval that requires the presence of AQ, not vehicle, during training and testing.

Others have also found evidence to support state-dependent modification of fear memory. In adolescent rats, ethanol administration prior to training or prior to testing disrupted fear memory following trace fear conditioning, but failed to impair fear memory when administered prior to both training and testing (Hunt & Barnet, 2016). Context fear memory was also found to be state-dependent following administration of gaboxadol, a selective extrasynaptic $GABA_A$ receptor agonist (Jovasevic et al., 2015). In both of these studies, behavioral performance for groups that received drug treatment prior to both training and testing was similar to that of controls that received no drug treatment prior to training or testing, suggesting the occurrence of symmetrical state-dependent effects. Interestingly, fear memory was enhanced following pre-training and pre-testing CB1 antagonism relative to pre-training CB1 antagonism only, as well as relative to pre-training and pre-testing vehicle administration (Reich et al., 2008). Because the group that received drug prior to training and testing performed differently from the group receiving vehicle prior to training and testing, this suggests the occurrence of state-dependent enhancement of fear memory following CB1 antagonism. Taken together, the current experiments suggest that the reversal of aging-related context fear memory impairment may be due to state-dependent effects, however, future studies are required to determine the role of other factors, such as multiple AQ administrations, in the observed behavioral modification.

Limitations and Future Directions

In summary, our findings suggest the CaBP AQ reduces fear generalization in a novel context when infused 1 h prior to training, and rescues aging-related context fear memory impairment when infused 1 h prior to training and 1 h prior to testing. However, there are several limitations that need to be addressed in future studies. The 4% concentration of AQ used in the current experiments is based on a separate study that found this dose to be sufficient to mitigate ischemic cell death (Detert et al., 2013), but it may not be the most effective dose to counteract the Ca^{2+} dysregulation that can accompany aging-related cognitive decline. Our finding that AQ infusion mitigates aging-related context fear memory impairment, but not trace fear memory impairment, could be due to an ineffective dose. The current experiments also failed to address the effect of chronic AQ infusion on behavioral performance. Other studies found that cognitive deficits in aged animals could be rescued following chronic blockade of L-VDCCs or RyRs (Hopp et al., 2014; Veng et al., 2003). Perhaps chronic AQ infusion is required to effectively mitigate aging-related trace fear memory deficits. Future studies are needed to assess the dose-dependent as well as time-dependent effects of AQ infusion on behavioral outcomes.

Future studies that investigate physiological effects of AQ also may provide insights into appropriate administration protocols that will effectively alter cognitive function. Increased magnitude of the Ca^{2+}-dependent, K^{+}-mediated sAHP has invariably been linked to aging-related reductions of neuronal excitability, and is thought to contribute to learning impairment (Disterhoft, Thompson, Moyer, & Mogul, 1996; Landfield & Pitler, 1984; Tombaugh et al., 2005). Blockade of L-VDCCs reduces AHP magnitude (Moyer, Thompson, Black, & Disterhoft, 1992) and improves eyeblink conditioning in aged rabbits (Deyo et al., 1989), while blockade of Ca^{2+} release from RyRs reduces the sAHP amplitude and facilitates spatial learning in aged rats

(Gant et al., 2015). Thus, future experiments should also address the effect of AQ on Ca^{2+}-dependent physiological mechanisms such as the post-burst AHP.

Additionally, downstream neuroimmunomodulatory effects of AQ may contribute to changes in cognitive function. Previous data from our lab suggest AQ is capable of modulating mRNA expression of various cytokines and chemokines (Detert et al., 2013). Cytokines can directly modify various Ca^{2+}-regulatory mechanisms, including NMDARs, L-VDCCs, IP_3Rs, and RyRs (see Sama & Norris, 2013). Further investigation of the link between AQ and the neuroimmune response may reveal an indirect path by which AQ is capable of modulating Ca^{2+}-dependent mechanisms that contribute to learning and memory.

Ultimately our goal was to investigate the effect of an acute AQ infusion on hippocampus-dependent fear learning to understand its capacity for ameliorating aging-related cognitive deficits. We found that AQ infusion 24 h prior to trace fear acquisition failed to mitigate trace fear memory deficits in aged rats. However, when AQ was infused 1 h prior to trace fear acquisition, generalized freezing during a cue test the following day was mitigated independently of age. Pre-training and pre-testing AQ infusion in aged rats also lead to a reversal of aging-related reduction of freezing during the context test, suggesting AQ induces state-dependent enhancement of context fear memory in aged rats. Other factors, such as the number of AQ infusions, could also play a role in the observed enhancement of context fear memory in aged rats. We conclude that AQ infusion 1 h prior to trace fear conditioning modifies generalized fear, and that aging-related context fear memory impairment is mitigated in a state-dependent manner. Further studies will be needed to address the underlying mechanisms of AQ-induced reduction of generalized fear, and whether the reversal of an aging-related context memory deficit is truly due to state-dependent modification.

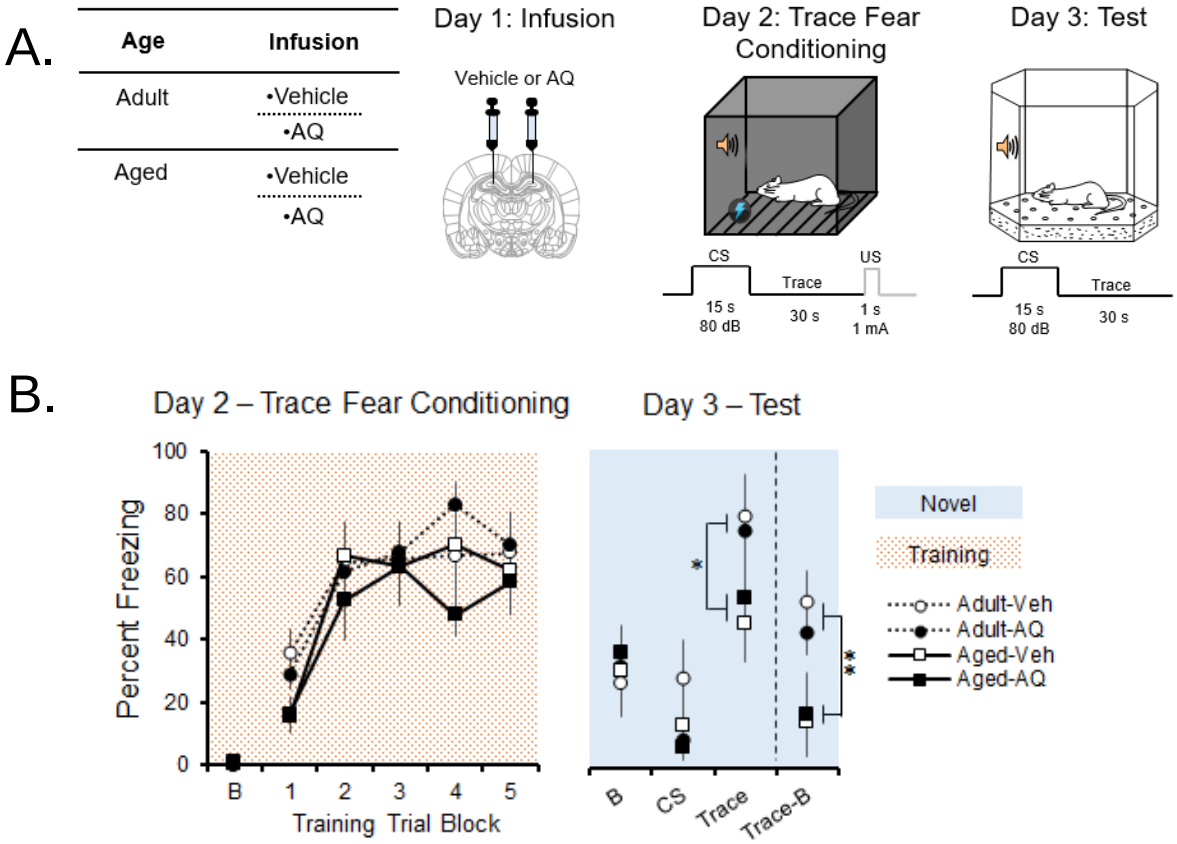


Figure 1. A single AQ infusion 24 h prior to trace fear conditioning does not affect trace fear memory in adult or aged rats. ***A.*** Experimental setup. On day 1 adult and aged rats received bilateral dorsal hippocampal infusions of vehicle or AQ (Adult-Veh (n = 5), Adult-AQ (n = 5), Aged-Veh (n = 7) and Aged-AQ (n = 6)). On day 2 rats underwent trace fear conditioning, which consisted of 10 trials of a white noise CS (15 s, 80 dB) paired with a scrambled footshock US (1 s, 1 mA) using a 5.2 min ITI (± 20%). The CS and US were separated by 30 s. On day 3 rats were placed in a novel context where the CS was presented alone. Average freezing during the first two trials was used to assess CS and trace fear memory. ***B.*** There was no effect of age or infusion on acquisition of trace fear conditioning. During the test, analysis of trace freezing revealed a significant main effect of age. Baseline freezing during the test was subtracted from trace freezing (Trace-B) to normalize for generalized fear. Analysis of Trace-B freezing revealed a main effect of age, but no effect of infusion. There was no effect of age or infusion on baseline or CS freezing during the test. **p* < .05; ***p* < .01.

A.

Age	First Test
Adult	•Training Context •Novel Context
Aged	•Training Context •Novel Context

B.

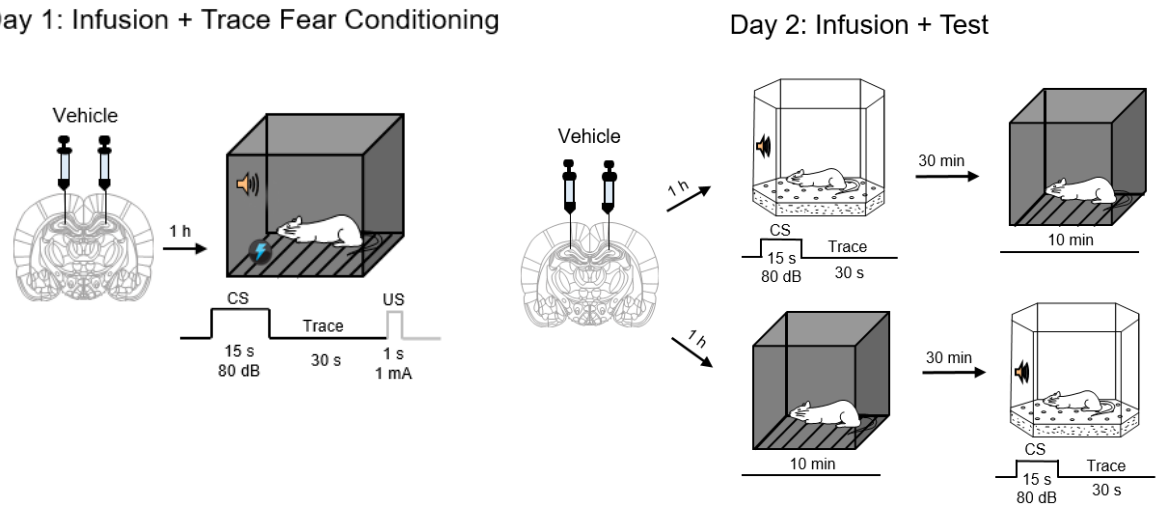
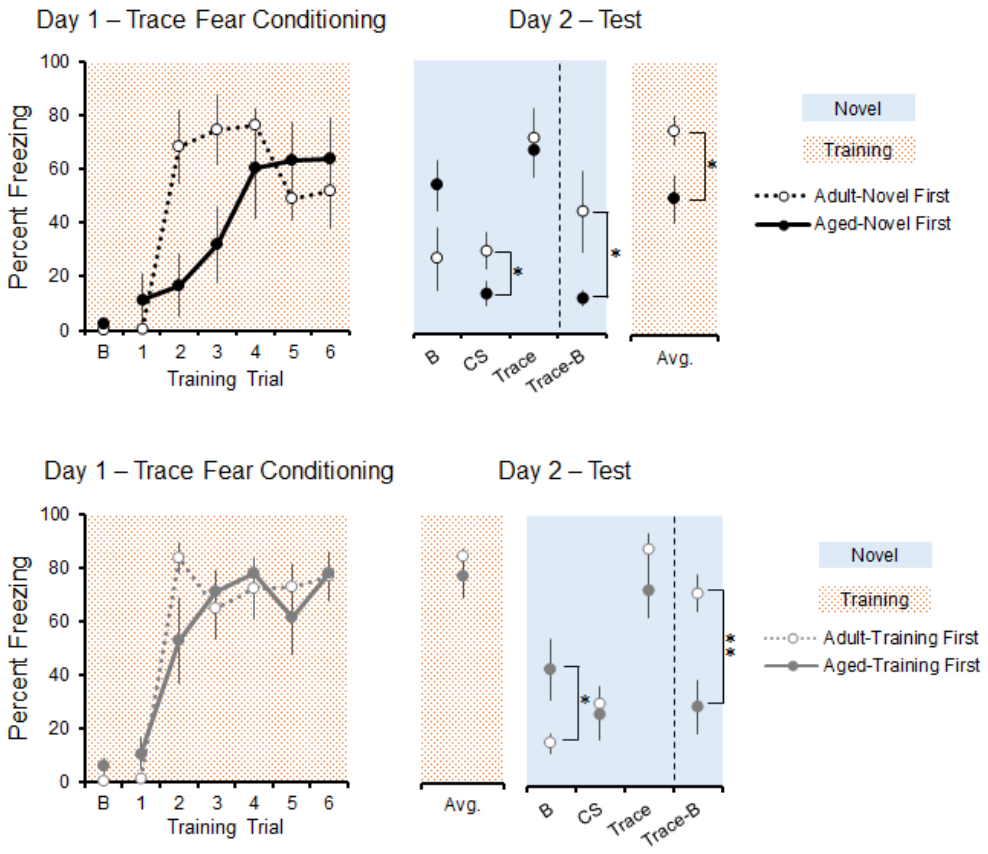


Figure 2. Experimental setup: Does test order alter hippocampus-dependent fear memory? ***A.*** Table of group assignments. Each age group was subdivided based on order of test presentation, which yielded four groups: Adult-Training First (n = 7), Adult-Novel First (n = 4), Aged-Training First (n = 6), Aged-Novel First (n = 4). To assess the effect of test order and age on fear memory, only vehicle-infused rats were included. ***B.*** On day 1, adult and aged rats received bilateral dorsal hippocampal infusions of vehicle 1 h before trace fear conditioning. This training paradigm consisted of 6 trials of CS – US pairings, with the CS and US separated by a 30 s silent trace interval. The CS was a 15 s, 80 dB white noise, and the US was a 1 s, 1 mA scrambled footshock. Trials were separated by a 5.2 min ITI (± 20%). On day 2, rats again received vehicle infusions, and 1 h later were tested in two different contexts. Test order was counterbalanced. The test in the original training context consisted of a 10 min stimulus-free period, and average freezing during the entire session was used to assess fear memory to the training context. The cue test in the novel context consisted of two CS-alone presentations, and was used to assess fear memory to the CS and trace interval.

A.


Day 1 – Trace Fear Conditioning
Day 2 – Test
Percent Freezing
Training Trial
B
CS
Trace
Trace-B
Avg.
Novel
Training
Adult-Novel First
Aged-Novel First
Adult-Training First
Aged-Training First

B.

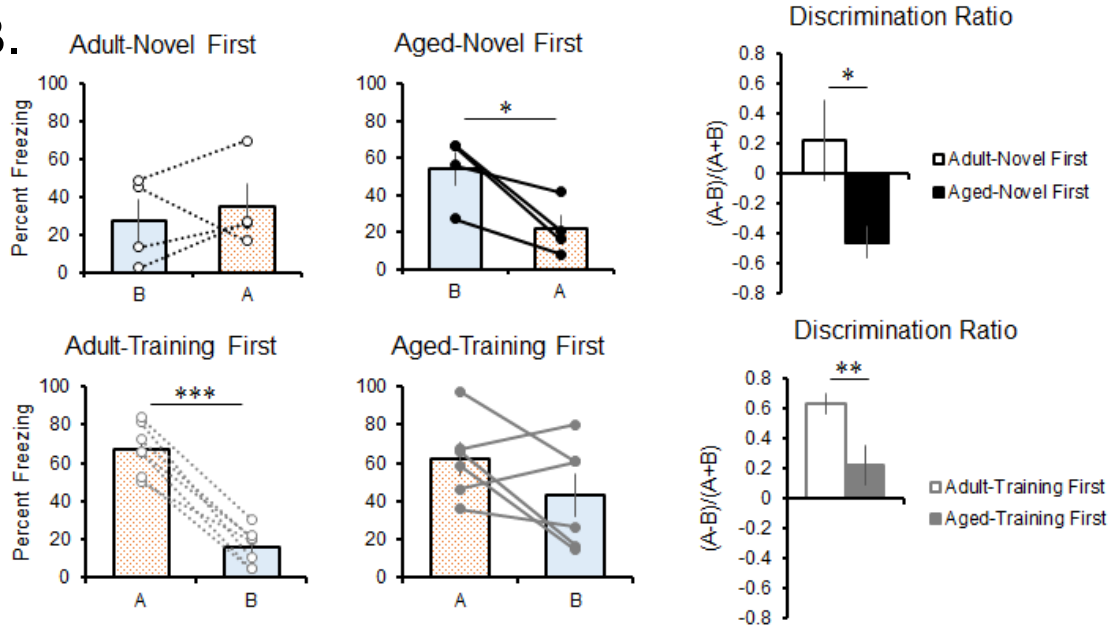

Adult-Novel First
Aged-Novel First
Discrimination Ratio
Percent Freezing
(A-B)/(A+B)
Adult-Training First
Aged-Training First

Figure 3. Aging and test order alter hippocampus-dependent fear memory. ***A.*** Each group readily acquired trace fear conditioning on day 1. On day 2, rats underwent a cue test in a novel context to assess CS and trace fear memory, and a context test in the original training context to assess context fear memory. Test order was counterbalanced. Among rats that underwent testing in the novel context first, aged rats displayed significantly reduced freezing during the CS as well as to the original training context. While there was not an aging-related reduction of trace interval freezing, there was a difference between age groups when baseline freezing was subtracted from trace freezing (Trace-B). Among rats that were tested in the original training context first, aged rats displayed significantly increased freezing during the baseline period of the cue test in the novel context, as well as reduced Trace-B freezing. However, aged rats failed to exhibit a context fear memory deficit when they were tested in the original training context first. ***B.*** To assess fear discrimination, freezing during the first two min of the test in the original training context (A) and the first two min of the cue test in the novel context (B) were compared within each experimental group. Adults that underwent the context test first exhibited significantly reduced freezing during B relative to A, suggesting good discrimination. Aged rats that were tested in the novel context first displayed significantly reduced freezing during A relative to B, suggesting poor discrimination. A discrimination ratio was calculated by subtracting freezing during B from freezing during A, then dividing the difference by the sum of freezing during B and A. For both test order groups, aged rats displayed significantly reduced discrimination ratios relative to their respective adult controls. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

A.

Age	Pre-Training Infusion	Pre-Testing Infusion
Adult	•Vehicle	•Vehicle
	•Vehicle	•AQ
	•AQ	•Vehicle
	•AQ	•AQ
Aged	•Vehicle	•Vehicle
	•Vehicle	•AQ
	•AQ	•Vehicle
	•AQ	•AQ

B.

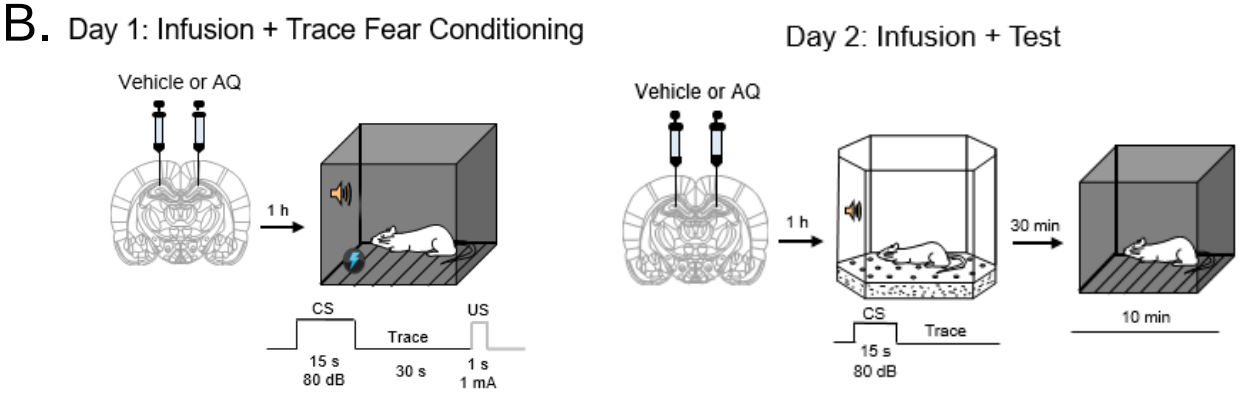


Figure 4. Experimental setup: Does AQ differentially affect fear memory in a state-dependent manner in adult and aged rats? ***A.*** Table of experimental groups. Adult and aged rats were divided into four infusion groups to assess the effects of AQ on fear learning: 1) vehicle pre-training and pre-testing (Veh-Veh, Adult: n = 9; Aged n = 9); 2) vehicle pre-training, AQ pre-testing (Veh-AQ, Adult: n = 5; Aged: n = 4); 3) AQ pre-training, vehicle pre-testing (AQ-Veh, Adult: n = 5; Aged: n = 4); 4) AQ pre-training and pre-testing (AQ-AQ, Adult: n = 5, Aged = 4). ***B.*** On day 1 rats received bilateral dorsal hippocampal infusions of either vehicle or AQ 1 h prior to trace fear conditioning. This paradigm consisted of 6 trials of a white noise CS (15 s, 80 dB) paired with a scrambled footshock US (1 s, 1 mA), using a 5.2 min ITI (± 20%). The CS and US were separated by a stimulus-free 30 s trace interval. The next day, rats were tested for fear memory beginning 1 h following infusions of either vehicle or AQ. Rats were tested for CS and trace fear memory in a novel context, followed 30 min later by a test for context fear memory in the original training context.

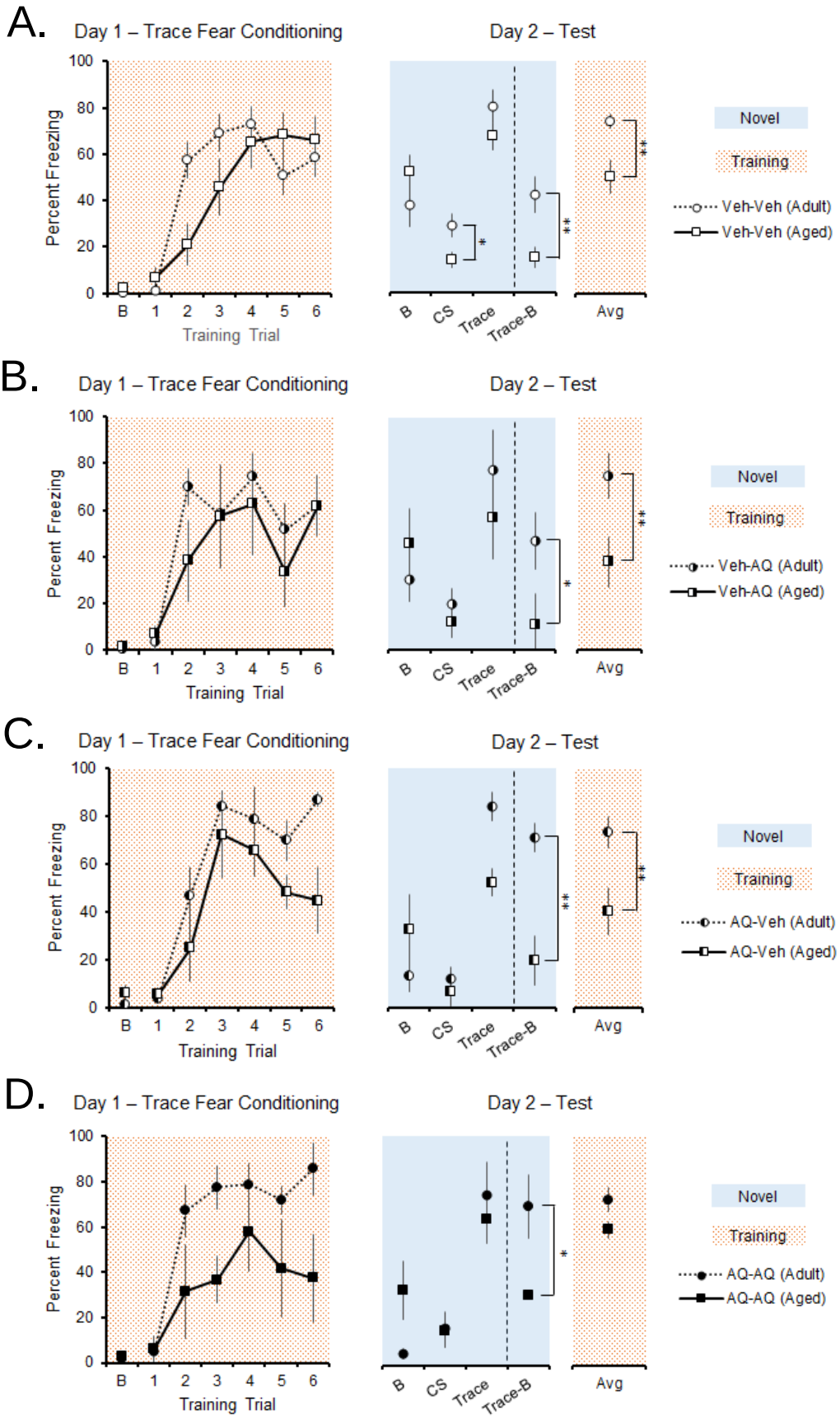

A.
Day 1 – Trace Fear Conditioning
Day 2 – Test
Percent Freezing
Training Trial
B
CS
Trace
Trace-B
Avg
Novel
Training
Veh-Veh (Adult)
Veh-Veh (Aged)
B.
Veh-AQ (Adult)
Veh-AQ (Aged)
C.
AQ-Veh (Adult)
AQ-Veh (Aged)
D.
AQ-AQ (Adult)
AQ-AQ (Aged)

Figure 5. AQ infusion prior to training and testing mitigates an aging-related context fear memory deficit. Each infusion group was plotted separately: ***A.*** Veh-Veh; ***B.*** Veh-AQ; ***C.*** AQ-Veh; ***D.*** AQ-AQ. Overall, there was a significant increase of trace interval freezing across training trials on day 1 ($p <$ .001), however, aged rats displayed reduced trace interval freezing relative to adults ($p < .01$). During the cue test in the novel context on day 2, there was a significant effect of infusion on baseline freezing. Pre-training AQ infusion (AQ-Veh and AQ-AQ) resulted in reduced freezing relative to Veh-Veh (Veh-Veh vs. AQ-Veh: $p < .05$; Veh-Veh vs. AQ-AQ: $p < .01$). Additional comparisons were conducted within each infusion group to determine the extent of aging-related deficits. Aged Veh-Veh rats displayed reduced freezing to the CS relative to the adult Veh-Veh group ($p < .05$). While there was no aging-related reduction of trace freezing for any infusion group, an aging-related deficit became apparent for all groups when baseline freezing was subtracted from trace freezing (all p-values, $p < .05$). Analysis of average freezing during the context test revealed that aged rats that received AQ infusion prior to training and prior to testing (AQ-AQ) failed to exhibit a context fear memory deficit ($p = .258$), suggesting pre-training and pre-testing AQ infusion mitigates an aging-related context fear memory impairment. *$p <$ 0.05; **$p < 0.01$.

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