Contextual Fear Conditioning Uncovers Dendritic Spine and Learning Deficits in the Retrosplenial Cortex in a Familial Alzheimer's Disease Mouse Model

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Abstract

While excitatory synapse loss is documented in the brains of Alzheimer's Disease (AD) patients, its role in episodic learning decline, one of the first evident AD symptoms, is unclear. The retrosplenial cortex (RSC), a cortical structure required for episodic learning, provides an ideal entry point to study the role of synapse loss in cognitive decline in AD since it is a site of high amyloid load and becomes dysfunctional early in AD progression. Further, excitatory synapse assembly, including both their turnover and clustered formation, is highly correlated with episodic learning performance in the RSC. We hypothesize that alterations in synapse assembly in the RSC of AD patients contribute to early cognitive decline in the disease. To address this hypothesis, we examined both excitatory synapse density in the RSC and contextual learning in a familial AD (fAD) mouse model, an early onset amyloid model of AD. Importantly, we find age-dependent excitatory synapse loss in the RSC of fAD mice, as measured by imaging and quantification of GFP-labeled dendritic spines. Further, prior to frank spine loss in 5-month-old fAD mice we found episodic learning deficits, as measured by contextual fear conditioning (CFC). Using this early time point, we examined spine dynamics in vivo with MP imaging in the RSC of fAD mice or controls while engaged in CFC learning. Interestingly, we find deficits both in spine turnover and clustered spine formation and a loss of correlations of these spine metrics with CFC performance, suggesting aberrant spine dynamics may be causal in episodic learning deficits in this AD model, consistent with our hypothesis. Future studies will pharmacologically target these aberrant spine dynamics in fAD mice in an effort to strengthen the causal link between alterations in synapse assembly and cognitive decline in AD as well as provide potential therapeutic approaches to reverse early stages of the disease.

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Introduction

Alzheimer's disease (AD) comprises roughly 80% of dementia cases worldwide and is currently ranked as the seventh leading cause of death in the United States of America (Crous-Bou et al., 2017; Ahmad et. al., 2020). Despite its prevalence, there are currently no therapeutics to cure or attenuate AD symptom onset. Memory deficits are among the first AD symptoms and the loss of synapses, the connections between neurons, is one of the first known brain structure alteration documented in AD patients (Maloney, 2005). While it is widely believed that synapse loss drives cognitive decline in AD, whether synapses are lost because of their excess elimination or failure to form at AD onset is unclear, limiting the establishment of effective therapeutics to reverse these initial symptoms.

Amyloid-beta peptide (Aβ) is among the earliest known biomarkers of AD. These peptides are enriched in amyloid plaques, a defining pathophysiological indicator of AD (Chen et al., 2017). When isolated from AD patients and introduced into mice, Aβ causes synapse loss and cognitive deficits (Zhao et.al., 2017). However, whether Aβ causes synapse loss by excess synapse elimination and/or inhibition of formation is unclear. Although numerous Aβ receptors have been identified, what aspects of Aβ pathology they mediate, such as cell death and dysregulated calcium signaling. This uncertainty persists because assays that can distinctly differentiate between various synaptic pathologies caused by Aβ hav

e yet been developed. However, a connection between amyloid or pTau dependent synapse alteration and aberrant learning has not been identified at this time, nor has there been research on a brain region with high amyloid load in AD.

Recent work has identified the retrosplenial cortex (RSC) as being regulated by spine-dependent learning and altered in AD (Frank et.al., 2018). The RSC is a secondary association cortical structure essential for spatial and contextual learning, types of memory compromised early in AD progression (Frank et.al., 2018). Further, the RSC is a site of high amyloid load in AD patients (Poirier et.al., 2011). Interestingly, the RSC becomes hypometabolic early in AD progression in patients and AD mouse models, a deficit that may impair learning and memory and potentially be driven by a loss of neural circuit connectivity (Nestor et al., 2003; Bero et al., 2012). These observations suggest the RSC may be a site where Aβ's synaptic pathologies drive AD-related cognitive decline, but this has not been formally tested.

Contextual learning deficits and aberrant RSC neural circuit activity develop early in AD patients and mouse models; yet how these neural circuit alternations might contribute to this cognitive decline is still unknown. Recently, classic experimental paradigms for studying contextual and episodic learning in mice, such as contextual fear conditioning (CFC), have been modified to enable tracking of learning performance with alterations in spine dynamics. Specifically, daily MP (multi-photon) imaging of GFP-labeled spines in the RSC of mice engaged in this modified CFC protocol revealed that rates of spine turnover and clustered spine formation are highly correlated with CFC performance, suggesting these spine dynamics drive learning (Heiss et.al., 2017). Still, the strength of this correlation has not been rigorously tested by examining animal models of disease characterized by spine and learning deficits.

Numerous AD mouse models show spine loss in brain regions known to contribute to learning. Despite this, studies in brain regions like the RSC implicated in spine-dependent learning have not thus far been undertaken. An important AD model that shows high amyloid load as well as early cognitive

decline and synapse loss in various brain regions is the APPswe;PSENdE9 mouse line (fAD), a strain genetically engineered to express two common mutant genes linked to heritable forms of AD (Sasaguri et. al., 2017). Study of this fAD model, although an overexpression paradigm, may define novel events driving cognitive decline in AD as well as aid in the development of tools to reverse these deficits (Sasaguri et. al., 2017).

Our study documents age-dependent spine loss in the RSC of ~7-month-old fAD mice. Interestingly, we find contextual fear conditioning (CFC) deficits in ~5-month-old fAD mice roughly 2 months prior to frank spine loss. These finds suggest frank spine loss does not, at least initially, drive cognitive decline in this AD mouse model. In vivo MP imaging of spines in the RSC of 5-month-old fAD mice reveal multiple changes in spine dynamics, including reduced spine formation and clustering, and increased turnover, that, at present, do not correlate with CFC learning, in contrast to control animals.

These findings highlight features of spine changes that may contribute to episodic learning deficits in AD and provide potential benchmarks for disease progression. Further, this study defines aberrant spine dynamics to target therapeutically to potentially reverse various aspects of cognitive decline in AD in future work.

Methods

Subjects

Adult (3-8 months) male and female fAD (APPswe;PSENdE9) mice and their WT (GFPm) controls were used in CFC and spine imaging experiments.

Cranial Window and Headbar Implantation

Cranial windows were inserted in mice approximately three weeks prior to any conditioning following the procedure laid out by Holtmaat et.al. (2009). Using isoflurane, mice were anesthetized and put into a stereotaxic frame and kept warm with a heating blanket. The scalp was sterilized with 70% ethanol and a flap of skin (~1cm2) was removed. The skull was then thinned using a dental drill and removed; a square window (~2mm in diameter) was created. The surgical site was cleaned with saline and a coverslip (2mm x 2mm) was placed on surface of the dura with a maximum distance of ~100 µm. The coverslip was affixed in position with adhesive. Following implantation, mice are given three weeks to recover and placed on antibiotics to prevent infections. A headbar was surgically attached to the skull 2 weeks prior to imaging using dental cement after animals were anesthetized using Ketamine/ Xylazine mix to provide stabilization during imaging.

Imaging

Confocal imaging was conducted utilizing a Nikon A1R confocal microscope using a 63x 1.4NA oil immersion lens at 2x zoom. MP live spine imaging was completed using a Thor lab microscope using a 60x 1.05NA water-dipping objective through cranial windows on apical dendrites of layer 5 pyramidal neurons on the following timeline: 2 days prior to CFC learning, on day 0 of CFC, and on days 2 and 5 of CFC learning. Key metrics for MP imaging include rates of spine turnover, clustered formation, and spine

elimination. Animals were imaged for ~30 minutes before being placed on a heating pad and allowed to recover. Repeated imaging was conducted at the same locales, using brain vasculature to relocate sites of interest, across experimental days, permitting a longitudinal analysis of spine dynamics. Animals were anesthetized using ketamine/ xylazine mix administered intraperitoneally.

Contextual Fear Conditioning (CFC)

CFC was conducted in a fear conditioning chamber (Med-Associates, 29.21 x 26.01 x 4.45 cm) with a stainless-steel grid floor and stainless-steel drop-pan. The chamber was connected to a voltage box, which controls the administered shock. Animals were subjected to CFC protocol once per day for 5 consecutive days at approximately the same time of day (+/- 30 minutes) due to previous evidence that the diurnal cycle of mice impacts spine-dependent learning in other cortical regions. In concert with spine imaging, this protocol allows for correlating learning performance (freezing) and changes in spine dynamics (rates of clustered formation and turnover).

Before testing, electric shock currents were checked by an amp-meter to ensure proper electrical conductivity through the metal floor of the chamber. The dispensed shock had a current of 0.5 mA. Animals were placed in the testing apparatus and the camera lens was situated to capture movement and freezing occurring in IR (infrared) light. A 3-minute interval was utilized per trial. The first minute was the test period for quantifying contextual learning (animal freezing), prior to shock administration. A low-voltage shock (0.5 mA) was administered at the 1-minute mark for approximately 1.5 seconds and followed by another shock 10 seconds later of the same intensity and duration. Following the second shock, animals were left in the conditioning chamber for the remainder of the 3-minute trial. Animals were removed following the end of the 3-minute trial and placed back into their home cages. Animals were conditioned in an identical manner for the span of 5 days. Animals were imaged by MP microscopy roughly 90 minutes after CFC learning entrainment.

The CFC chamber was cleaned just prior and directly after each trial with 70% ethanol and water to remove any pheromones of previous mice.

Image Analysis

Images of specific dendritic sections were analyzed via Imaris Image Analysis Software (Bitplane Inc) and labeled based on changing spine dynamics as per an already established criteria (Harris et al., 1992). With the analyzer blind to condition, dendritic sections were defined and the presence, loss, or gain of spines was quantified across multiple days for each section and all sections were inspected for every animal (Frank et. al., 2018). Clustered spines were defined as those forming within 5um of a preexisting spine.

Freezing Analysis

ezTrack software was downloaded through instructions provided by Pennington et.al. (2019) and run through iPython/Jupyter Notebook. The Freezing Analysis Module was utilized to detect freezing by analyzing pixels changes across frames (Pennington et.al., 2019). Freezing was indicated by little to no change in pixels from frame to frame; the threshold for what constitutes freezing is defined as the freezing

threshold (FT). Recorded videos were analyzed through the Freeze Analysis Module as a means to obtain freezing data. Results were provided in the form of a summary file which has both average motion and freezing during each trial.

Statistical Analysis

Summary files from CFC analysis using ezTrack software were further analyzed across the five-day training paradigm to discern the reaction of memory during CFC. General trends were established through analysis via Excel. GraphPad Prism allows for analyses such as two-way repeated measures ANOVA, paired t-test, and Pearson's correlation analyses. P-values all originated from the results of two-sided tests; any p-value less than 0.05 was considered to be statistically significant. Two-Way Repeated ANOVA inspects the impact of two different independent variables on one dependent variable. In our case, the independent variables were fAD v. CON and the dependent variable was percent freezing, indicative of contextual learning. Two-Way ANOVA allowed for the determination of an interaction between the two independent variables as well. Repeated measures indicated this data was measured twice. A paired t-test provided information about the mean difference between two data sets. Pearson's correlation measured linear relationships between two sets of data, spine turnover rates and percent freezing during CFC learning in this experiment. Results were provided as an R-value between -1 and +1, with -1 being a strongly negative relationship and +1 being a strongly positive relationship. An R-value of 0 indicates no relationship between the two data sets.

Results

Emergence of Spine Loss in the RSC of 7-Month-Old fAD mice

Increases in brain amyloid, learning deficits and excitatory synapse loss emerge in multiple brain regions in fAD mice between 5 and 7 months; thus, we examined dendritic spines in the RSC of fAD mice and controls at both these time points (Tsai et.al., 2004). fAD; thy1-GFPm mice and controls were fixed, vibratome sectioned, immunostained and secondary apical dendrites from layer 5 pyramidal neuron (PN) dendrites imaged by confocal microscopy (Heiss et.al., 2017). The density of mature and immature spine types was subsequently quantified using volumetric analyses in Imaris software (Bitplane Inc) and established criteria for distinct spine types (Harris et al., 1992). Mature spine types include mushroom (large head, small neck), stubby (large head, no neck), and long thin (small head, large neck); immature spine types include filopodia (large neck, no head) (Harris et al., 1992). Our analyses revealed that mature spines (mushroom and long) are reduced in density at 7.7 but not 5.3 months of age (Fig 1). These findings pinpoint this age range as a critical timeline in the emergence of synapse loss in the RSC of fAD mice and implicate a potential age-dependent deficit in spine dynamics.

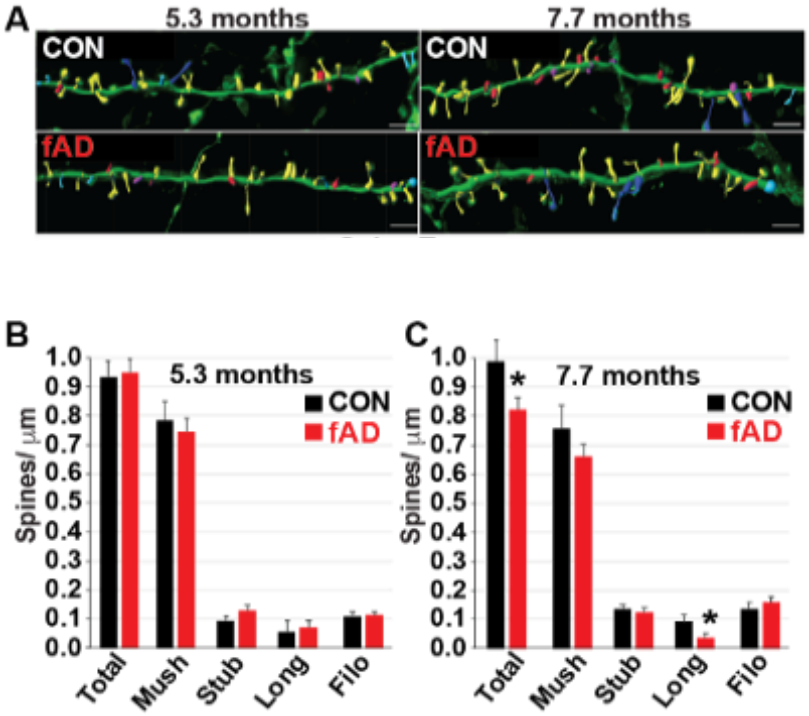

Figure 1: 7.7-Month-Old *fAD* Mice RSC Displays Spine Loss. (A) Imaris Images of GFP-labeled dendrites from specified mice and ages with labeled types of spines. N = 11-19 dendritic segments per genotype. Mature spines include mushroom (yellow), stubby (red), and long thin (light blue). Immature spines include filopodia (dark blue). Scale bar = 2.5µm. (B-C) Quantification of spine densities of specified mice and types of spines. *p<0.05 vs control (student t-test). Error bars equal SEM.

CFC Deficits Emerge in 5-Month-Old fAD mice

To determine if episodic learning deficits in fAD mice emerge prior to or in concert with spine loss in the RSC, we assessed contextual fear conditioning (CFC) learning in fAD mice and controls at ~5 months of age, just prior to frank spine loss in the RSC. Mice were placed in a novel chamber and their movements tracked using video recording under IR (infrared) light. Following a 1-minute control period animals were lightly shocked twice over 10 seconds (0.5 mA/2 seconds) and animals were imaged over the subsequent 3 minutes. Animals were subjected to 1 trial per day for 5 days. Animal freezing was quantified using ezTrack software.

Quantification of freezing during CFC training revealed a significant reduction in freezing in fAD mice after 4 days of training (Day 5) when combining data from males and females. Neither fAD males nor females alone showed significant differences in freezing, potentially because of the small number of animals in each of these groups. Interestingly, male and female controls showed significant learning over many of the trial days, while fAD mice show no such learning
in either sex. These findings suggest episodic learning deficits emerge in 5-month-old fAD mice, roughly two months prior to frank spine loss in the RSC, making clear that spine loss does not at least initiate CFC deficits in this AD model.

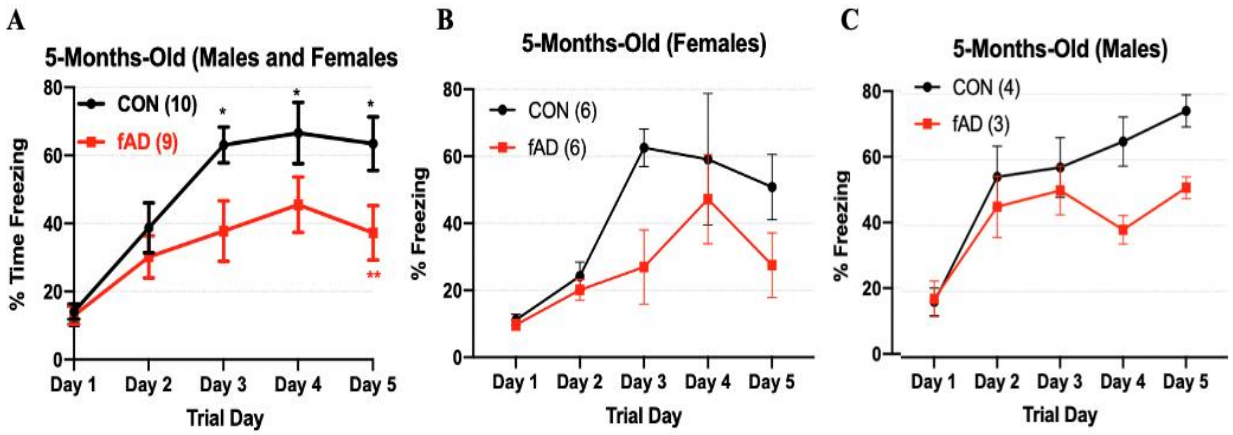

Figure 2: 5-month-old *fAD* Mice Show Learning Deficits during CFC Learning Entrainment in a Gender-Independent Manner
(A) 5-month-old mice freezing behavior during a 5-day CFC Learning paradigm without separation of genders, shows a difference in learning for control animals between day 1 and day 3, day 1 and day 4, and day 1 and day 5. An additional difference in learning occurs between fAD and CON mice on day 5 of paradigm. (B) 5-month-old female mice freezing behavior during 5-day CFC learning paradigm. (C) 5-month-old male mice freezing behavior during 5-day CFC learning paradigm. N is in parenthesis in each figure legend. * $p<0.05$ vs CON day 1 trial **$p<0.05$ CON vs fAD. Error bars equal SEM.

Aberrant Spine dynamics in the RSC Emerge in Concert with CFC Deficits in 5-Month-Old fAD mice

To determine if alterations in spine dynamics correlating with learning in the RSC might contribute to episodic learning deficits in fAD mice, we imaged dendritic spines in vivo in the RSC by MP microscopy in concert while animals were undergoing CFC learning entrainment. Cranial windows were inserted 3 weeks prior to experimentation in 5-month-old fAD mice and controls and apical dendrites of layer 5 pyramidal neurons were repeatedly imaged over 4 periods, once prior to training (day -2), at training onset (day 0) and during training (days 2 and 5), enabling a quantification of baseline spine turnover as well as learning-dependent spine changes. Consistent with previous work, we find spine dynamics (spine turnover and clustered spine formation) correlate with CFC performance in control mice, as determined by Pearson's correlation analyses comparing rates of spine turnover of clustered spine formation and percent freezing during CFC trials (Fig 3C-D).

Interestingly, we find in fAD mice significant increases in rates of spine turnover as well as a reduction in overall spine formation (Fig 3C, F). Further, correlations between spine turnover and clustered addition and CFC performance observed in control animals is lost in fAD mice. While altered spine turnover and formation have been observed in several AD mouse models (Heiss et. al., 2017; Zou et.al., 2015), loss of these normally correlating spine dynamic metrics with CFC learning highlight previously unrecognized synaptic alterations in AD, metrics that may be important in the emergence of the disease.

Discussion

While it is assumed that synapse loss might drive cognitive decline in AD, there has been little direct evidence to support this hypothesis, in either AD patients or mouse models. This is in part because of a lack of techniques to track changes in synapse characteristics and formation non-invasively, and then to correlate these changes with learning. In addition, previous imaging techniques did not allow live spine imaging of brain regions that exhibit large amyloid load.

Recent studies in the retrosplenial cortex (RSC) made clear it fulfilled these needs, being a site of high amyloid load and dysfunction early in AD progression, an associative cortical region required for episodic learning and governed by synaptic dynamics that correlate with learning performance. We therefore examined whether dysfunctional spine dynamics in the RSC might contribute to episodic learning deficits in a familial AD (fAD) mouse model.

Our analysis revealed that spine loss did not correlate with the emergence of learning deficits in fAD mice, as had been previously hypothesized. This suggests something more dynamic than a spine-

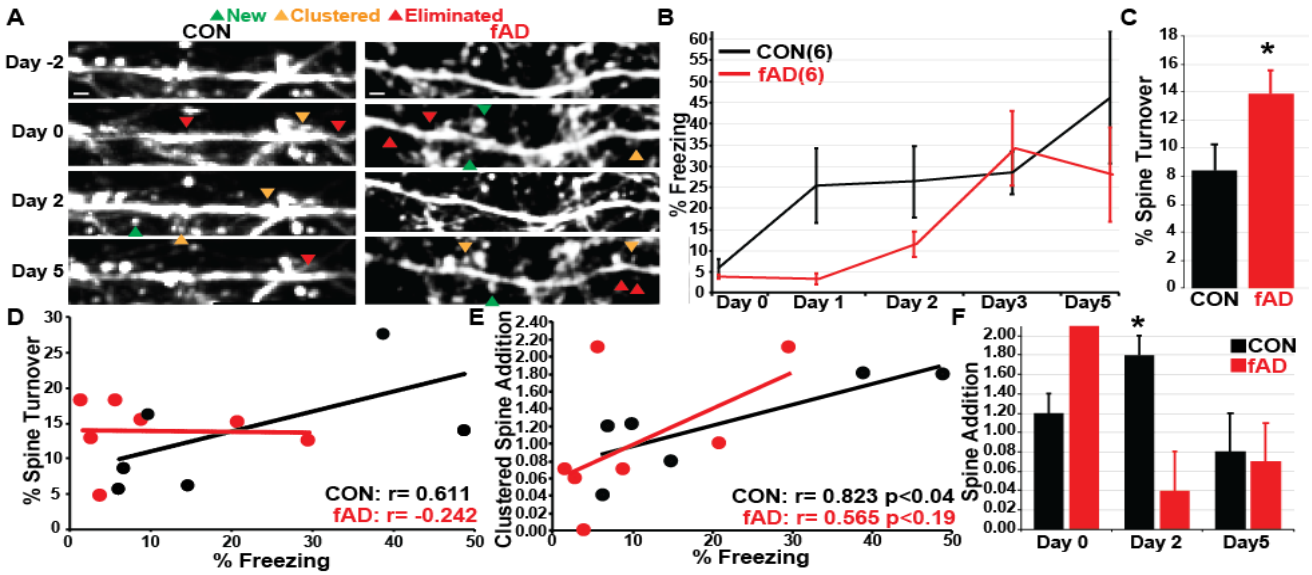

Figure 3: RSC Live Spine Imaging and Spine Analysis. (A) 7-month-old mice PN dendrite images with labeled spine categories (colored arrowheads). (B) CFC freezing quantification, N in parenthesis. (C) Spine turnover quantification. (D) Spine turnover and freezing correlation. (E) Spine clustering and freezing correlation. (F) Spine formation and quantification. *p<0.05 student t-test or One-Way ANOVA. N = 4 locales/ 2 animals /genotype for spine imagine experiments. r values derived from Pearson correlations.

dependent mechanism might be instrumental in AD-related cognitive decline. Spine loss emerged in 7.5-month-old animals but not in younger models (e.g., 5-months-old), despite other models indicating synaptic deficits could occur at this earlier timepoint (Jankowsky et.al., 2001). Strikingly, CFC deficits emerge in 5-month-old animals regardless of previous decreases in spine dynamics, supplementing the lack of correlation of aberrant learning with deficits in spine dynamics.

Given the emergence of CFC deficits in 5-month-old fAD mice, studies of spine dynamics in the RSC were undertaken in vivo in concert with CFC learning. Importantly, as previously observed, we find spine turnover and clustered formation in the RSC correlates with CFC performance in control mice. In contrast, spine turnover and formation are dramatically altered in the RSC of fAD mice. Increased spine turnover and reduced spine formation have been observed in other brain regions in fAD mice, such as the sensory cortex (Zou et. al., 2015); however, no prior studies had examined learning-dependent spine dynamics in AD mouse models.

Excitatory synapse loss was previously believed as a prime candidate to drive cognitive decline in AD (Scheff and Price, 2003). However, our findings refute this theory. Younger fAD mouse models show no connection between their cognitive deficits and spine loss. Rather, they exhibit cognitive decline independent of previous decreases in spine dynamics. Further analysis of learning deficits in fAD mouse models may provide an explanation and mechanism for AD-related cognitive decline that is unrelated to prior spine loss.

There remain limitations to our study. The sample size needs to be expanded to give a better indication of trends of learning and provide more evidence for AD-related cognitive and learning deficits. The fAD mouse model utilized in these studies is an overexpression model, which exaggerates some symptoms of AD in a manner that is not consistent with AD patients. However, it captures age-dependent amyloid-driven synaptic and cognitive pathology analogous to what is observed in AD patients. An important facet of further study is to analyze similar AD-related deficits in non-expression models and ones that capture Tau pathology. By providing a broader assessment of the role of spine dynamics in AD models, using other mouse models will more fully capture distinct aspects of the disease.

Through the pinpointing of these new synaptic pathologies in the RSC, there is potential for the development of tools to measure them in patients as well as establish therapeutic agents to specifically target them in the future. It is important to utilize the correlations found from these experiments to provide metrics for future therapeutical applications to combat AD-related cognitive decline.

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