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Calcineurin/NFAT Signaling in Activated Astrocytes Drives Network Hyperexcitability in Aβ-Bearing Mice

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Hyperexcitable neuronal networks are mechanistically linked to the pathologic and clinical features of Alzheimer’s disease (AD). Astrocytes are a primary defense against hyperexcitability, but their functional phenotype during AD is poorly understood. Here, we found that activated astrocytes in the 5xFAD mouse model were strongly associated with proteolysis of the protein phosphatase calcineurin (CN) and the elevated expression of the CN-dependent transcription factor nuclear factor of activated T cells 4 (NFAT4). Intrahippocampal injections of adeno-associated virus vectors containing the astrocyte-specific promoter Gfa2 and the NFAT inhibitory peptide VIVIT reduced signs of glutamate-mediated hyperexcitability in 5xFAD mice, measured in vivo with microelectrode arrays and ex vivo brain slices, using whole-cell voltage clamp. VIVIT treatment in 5xFAD mice led to increased expression of the astrocytic glutamate transporter GLT-1 and to attenuated changes in dendrite morphology, synaptic strength, and NMDAR-dependent responses. The results reveal astrocytic CN/NFAT4 as a key pathologic mechanism for driving glutamate dysregulation and neuronal hyperactivity during AD.

Key words: Alzheimer’s disease; astrocytes; calcineurin; dementia; glutamate; hyper excitability

Significance Statement

Neuronal hyperexcitability and excitotoxicity are increasingly recognized as important mechanisms for neurodegeneration and dementia associated with Alzheimer’s disease (AD). Astrocytes are profoundly activated during AD and may lose their capacity to regulate excitotoxic glutamate levels. Here, we show that a highly active calcineurin (CN) phosphatase fragment and its substrate transcription factor, nuclear factor of activated T cells (NFAT4), appear in astrocytes in direct proportion to the extent of astrocyte activation. The blockade of astrocytic CN/NFAT signaling in a common mouse model of AD, using adeno-associated virus vectors normalized glutamate signaling dynamics, increased astrocytic glutamate transporter levels and alleviated multiple signs of neuronal hyperexcitability. The results suggest that astrocyte activation drives hyperexcitability during AD through a mechanism involving aberrant CN/NFAT signaling and impaired glutamate transport.

Introduction

Alzheimer’s disease (AD) is the most common cause of dementia. Similar to other neurodegenerative diseases, AD is accompanied by profound glial activation (Bouvier and Murai, 2015; Heneka et al., 2015). Though most conspicuous around amyloid-β (Aβ) deposits at late disease stages, activated glial cells (i.e., microglia and astrocytes) also appear at the outset of clinical symptoms (Carter et al., 2012; Schöll et al., 2015; Yokokura et al., 2016), suggesting that glial modulation strategies could slow the progression of AD. However, the phenotype of activated glial cells is very complex (Oberheim et al., 2012; Prokop et al., 2013; Pekny et al., 2014; Malm et al., 2015; Andreasson et al., 2016; Liddelow et al., 2017), and more work is required to elucidate their functional impact in neurodegeneration.
In healthy brain, astrocytes fine-tune synaptic function and preserve neurite integrity by removing excitotoxic glutamate from the extracellular milieu (Huang and Bergles, 2004; Schousboe et al., 2004; Sattler and Rothstein, 2006). Genetic deletion or inhibition of astrocyte glutamate transporters in rodents causes hyperexcitability and/or excitotoxicity (Rothstein et al., 1996; Rao et al., 2001; Selkirk et al., 2005; Petr et al., 2015; Moidunny et al., 2016), while the promotion of glutamate-uptake imparts neuroprotection (Prow and Irani, 2008; Harvey et al., 2011; Zumkehr et al., 2015; Karlik Fontana et al., 2016). Network hyperexcitability is commonly observed in AD brain and AD mouse models (Palop et al., 2007; Busche et al., 2008; Minkevičienė et al., 2009; Noebels, 2011; Putcha et al., 2011; Bakker et al., 2012; Grienberger et al., 2012; Bomben et al., 2014; Kellner et al., 2014; Šíková et al., 2014; Vossel et al., 2016; Fontana et al., 2017) where it is thought to contribute to excitotoxic damage and cognitive loss. Diminished expression and/or functional impairment of astrocyte glutamate transporters is similarly associated with AD and AD-related pathology (Maslia et al., 1996, 2000; Abdull et al., 2009; Mookherje et al., 2011; Schallier et al., 2011; Scimemi et al., 2013; Meeker et al., 2015; Audrain et al., 2016; Hefendehl et al., 2016; Xu et al., 2016), suggesting that impaired glutamate regulation is a key phenotypic trait of activated astrocytes and a primary mechanism for AD-related neurodegeneration.

Numerous intracellular mechanisms, including the Ca\(^{2+}/\)calmodulin-dependent phosphatase calcineurin (CN), have been implicated in the phenotypic switching of glial cells with injury and disease (Burman and Norris, 2014; Pekny et al., 2016). CN is rapidly activated/inactivated by fluctuating Ca\(^{2+}\) levels within healthy cells but can become proteolyzed and irreversibly activated following neural damage (Wu et al., 2004, 2010; Huang et al., 2005; Liu et al., 2005; Shiota et al., 2006; Mohammadi et et al., 2011). Proteolysis of CN is especially pronounced in astrocytes associated with Aβ pathology (Pleiss et al., 2016), leading to the hyperactivation of key substrates, such as the nuclear factor of activated T cells (NFATs; Mohammadi et al., 2011). NFAT activation is increased at early stages of AD-related cognitive decline (Abdull et al., 2009) and linked to altered glial phenotypes and neuroinflammation (Fernandez et al., 2007; Nagamoto-Combs and Combs, 2010; Serrano-Perez et al., 2011; Rojanathammannae et al., 2015). Selective inhibition of astrocytic CN/NFATs normalizes the basal function and plasticity of glutamatergic synapses in mouse models of AD and brain injury (Burman et al., 2012; Burman et al., 2016), suggesting that at least some detrimental phenotypic traits of activated astrocytes arise from aberrant CN/NFAT signaling.

Here, we used in vivo and ex vivo measures to determine whether astrocytic CN/NFATs underlie glutamate dysregulation and possibly excitotoxicity in a highly aggressive AD mouse model. Adeno-associated virus (AAV)-mediated expression of an NFAT inhibitory peptide in hippocampal astrocytes of 5xFAD mice reduced the frequency of spontaneous glutamate transients and synaptic events, increased GLT-1 expression, prevented dendritic derangement, and normalized NMDA-to-AMPA receptor activity ratios. The results suggest that hyperexcitability in AD arises from the loss of critical glutamate regulatory properties in activated astrocytes due to aberrant CN/NFAT signaling.

Materials and Methods

**Animals.** 5xFAD mice (Oakley et al., 2006) and wild-type (B6/SJL) littermates were bred in a pathogen-free environment in accordance with University of Kentucky guidelines. Mice of either sex were used in this study and held in standard laboratory cages under 12 h light/dark cycles. The animals had access to food and water *ad libitum*. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by University of Kentucky Institutional Animal Care and Use Committees.

**Hippocampal protein measures.** For CN protein measures, fresh-frozen hippocampus was thawed on ice for 10 min and then homogenized in sucrose buffer (0.25 M sucrose, 20 mM EDTA, pH 8.0, 20 mM EGTA, pH 8.0, 100 mM Tris, pH 7.4), supplemented with a protease and phosphatase inhibitor mix (catalog #Calbiochem 524625, catalog #Calbiochem 208733, and catalog #Calbiochem 539134, EMD Millipore). Resulting homogenate was aliquoted (150 μl) and then combined and gently mixed with an equal volume of RIPA buffer (Sigma-Aldrich) and incubated on ice for 45 min. Samples were then centrifuged at 13,000 rpm for 10 min, and the supernatant was stored at −80°C until use for Western blot analyses. For GLT-1 protein measures, hippocampal tissue was homogenized in ice-cold PBS supplemented with protease inhibitor mixture, phosphatase inhibitor mixture, and calpain inhibitor (all from Calbiochem) and then centrifuged at 20,800 × g for 30 min at 4°C. The resultant pellet was re-extracted by sonication in 2% SDS containing protease, phosphatase, and calpain inhibitors and centrifuged at 20,800 × g for 30 min. The supernatant was stored at −80°C until use.

Approximately 10–60 μg of protein (dependent on the antigen) were resolved on 4–20% Criterion gradient gels (Bio-Rad) and transferred to Immobilon-FL PVDF membranes (Millipore). After washing and blocking in Odyssey protein blocking reagent (LI-COR), the primary antibodies were incubated with the blots overnight at 4°C as follows: calcineurin (anti-CN-A, catalog #07-1492, Millipore; RRID: AB_10563965; GAPDH (catalog #13-0300, Invitrogen; RRID: AB_2532984); EAAT2/GLT-1 (catalog #ab41621, Abcam; RRID: 941782); and GAPDH (catalog #ab9484, Abcam; RRID: AB_307274). Following washes and incubating with secondary antibodies to mouse (800) and rabbit (700) primaries, blots were imaged on an Odyssey Scanner (LI-COR) and the quantification was performed using Image Studio 3.1 Software (LI-COR; RRID: SCR_013715).

**Brain section preparation.** Brains were collected and fixed in 4% paraformaldehyde in phosphate buffer, pH 7.4, and then saturated with 30% sucrose buffer.Coronal sections at 40 μm thickness were prepared using a microtome (Leica) and kept at −20°C in cryoprotectant solution containing 25% ethylene glycol and 25% glycerin in 0.5 M phosphate buffer.

**Immunofluorescent labeling and NeuN immunohistochemistry.** Brain sections were labeled following the free-floating protocol using the following primary antibodies: anti-GFAP (1:500; catalog #12389, Cell Signaling Technology; RRID: 28066914), anti-NFAT4 (1:50; catalog #sc-8405, Santa Cruz Biotechnology; RRID: AB_628041); anti-NeuN (1:1000; catalog #MAB377, EMD Millipore; RRID: AB_2298772); anti-MAP2 (1:500; catalog #ab8408, Abcam; RRID: AB_652014); and GAPDH (catalog #ab9484, Abcam; RRID: AB_307274). Following washes and incubating with secondary antibodies to mouse (800) and rabbit (700) primaries, blots were imaged on an Odyssey Scanner (LI-COR) and the quantification was performed using Image Studio 3.1 Software (LI-COR; RRID: SCR_013715).
NeuN-positive cells were counted within a 300 × 300 pixel window and positive pixels were visualized in ImageScope Software (Aperio). Slides were mounted on glass slides, dehydrated in ethanol, cleared in Safeclear (Cascade Scientific, Inc.), and coverslipped with Permount (Fisher Scientific). Stained sections were placed in a stereotaxic frame and anesthetized with isoflurane (2.5%) in a 95% O2/5% CO2 mixture. Body temperature was maintained at 37°C throughout recording using a water pad connected to a circulating water bath. Final MEA placement coordinates were as follows: −2.3 mm anteroposterior and ±1.7 mm mediolateral relative to bregma, and −1.5 mm dorsoventral relative to dura. Once MEAs were in place, a stable 20–30 min baseline was collected at a final recording display rate of 4 Hz. Basal glutamate levels were calculated and averaged during the last 10 min of the baseline. Spontaneous glutamate spikes were then recorded over an additional 5 min window. Transients recorded at glutamate-sensing sites were subtracted from current activity at the sentinel sites. Maximal amplitude for each transient (in micrometers) was calculated by taking the difference between the transient peak and the baseline glutamate level immediately before the transient. After glutamate recording, brain tissues were collected and cresyl violet staining was used to confirm the location of the MEA for each mouse.

Data acquisition was controlled by the Fast Analytical Sensing Technology (FAST-16) mkIII System (Quanteon) to verify glutamate selectivity, limit of detection, and sensitivity. Mice were anesthetized and placed in a stereotaxic frame and a 3- to 4-mm-diameter cranialotomy was performed over both hemispheres. Under continuous inhalation isoflurane (2%) anesthesia, MEAs were lowered into the CA1 region of the hippocampus and a miniature Ag/AgCl reference electrode was placed into the frontal cortex contralateral to the glutamate MEA. Body temperature was maintained at 37°C throughout recording using a water pad connected to a circulating water bath. Final MEA placement coordinates were as follows: −2.3 mm anteroposterior and ±1.7 mm mediolateral relative to bregma, and −1.5 mm dorsoventral relative to dura. Once MEAs were in place, a stable 20–30 min baseline was collected at a final recording display rate of 4 Hz. Basal glutamate levels were calculated and averaged during the last 10 min of the baseline. Spontaneous glutamate spikes were then recorded over an additional 5 min window. Transients recorded at glutamate-sensing sites were subtracted from current activity at the sentinel sites. Maximal amplitude for each transient (in micrometers) was calculated by taking the difference between the transient peak and the baseline glutamate level immediately before the transient. After glutamate recording, brain tissues were collected and cresyl violet staining was used to confirm the location of the MEA for each mouse.

Soluble Aβ peptide measures. Methods for quantifying soluble Aβ(1–42) peptide levels using ELISA were nearly identical to those used in our previous work (McGowan et al., 2005; Murphy et al., 2007; Furman et al., 2012). Tissue was homogenized in ice-cold PBS containing protease and phosphatase inhibitor cocktails and calpain I and II inhibitors (Calbiochem) and then centrifuged at 20,800 × g for 30 min at 4°C. The pellet was extracted by sonication in 2% SDS and spun again at 20,800 × g for 30 min. The supernatant was collected and diluted in antigen capture (AC) buffer that contained 20 mM NaHPO4, 0.4% Block Ace (AbD Serotec), 0.05% NaN3, 2 mM EDTA, 0.4 mM NaCl, 0.2% BSA, and 0.05% CHAPS, pH 7. Immuno 4HBX plates were coated with 2.13 (end specific for Aβ(1–42)) capture antibody (0.5 μg/well) and blocked with Syn-block (AbD Serotec), as per the instructions of the manufacturer. Synthetic human Aβ(1–42) was diluted in AC buffer and used to construct a standard curve. Protein standards and sample extracts were loaded at least in duplicate, and antigen was detected with 4G8 (Aβ(17–24); Covance) biotinylated antibody. Reactions were developed with 3′,5′-tetramethylbenzidine reagent, stopped with 6% H2O2, and read at 450 nm using a multwell plate reader. Aβ levels were calculated relative to the standard curve.

Glutamate transport currents in primary astrocytes. Primary astrocyte cultures were prepared from P7 Sprague Dawley rat pups. Cerebral cortices were collected and washed in HBSS then trypsinized and triturated. Isolated cells were washed and seeded in culture flasks in MEM completed with NaHCO3, l-glutamine, 1% antibiotics/antimycotics, and 10% fetal bovine serum. These cultures were grown for 10–12 d and then trypsinized and plated on plastic inserts within 35 mm culture dishes
until electrophysiological recordings. Approximately 48 h before electrophysiological recording, cultures were infected with adenoviruses vectors 100 multiplicity of infection (MOI) expressing LacZ-IRES-GFP (control (CT)), ACN-IRES-DsRed2, and VIVIT-EGFP, as described previously (Sama et al., 2008).

Astrocyte-containing inserts were washed once in recording solution and then transferred to a Siskiyou recording chamber and perfused (3–5 ml/min) with additional recording solution containing the following (in mM): 150 NaCl, 5 KCl, 2 MgCl₂, 10 HEPES, 2 CaCl₂, and 10 mM glucose, pH 7.4. Whole-cell astrocyte patch clamp was performed as described previously (Dallas et al., 2007). Briefly, high-resistance membrane seals were achieved with a pulling pipette of series resistance (1–3 GΩ) on individual astrocytes. The pipette solution contained (in mM): 120 CH₃CsO₃S, 10 CsCl, 5 NaCl, 10 HEPES, 0.5 MgCl₂, and 1.0 M CsOH, pH 7.4. The whole-cell recording configuration was obtained with gentle suction, and the astrocyte membrane potential was held at −70 mV. To induce excitatory amino acid transporter (EAAT)-dependent inward currents, we applied 10 μM l-glutamate to the perfusion media. To confirm that inward currents were mediated by EAATs, some astrocyte-containing inserts were perfused with the glutamate transport inhibitor TBOA (100 μM; Tocris Bioscience) 15 min before and during perfusion with glutamate. Data acquisition was controlled using a Multichamp 700B amplifier, Digitida 1332a, and pClamp software (Molecular Devices; RRID: SCR_011323). All currents were filtered at 2 kHz and digitized at 10 kHz.

Acute brain slice preparation. Brain slices were prepared as described in our previously published work (Mathis et al., 2011; Bachstetter et al., 2012; Furman et al., 2012). Mice were deeply anesthetized with CO₂ and decapitated. Brains were removed and immersed briefly in Ca²⁺-free, ice-cold, oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (aCSF) containing the following (in mM): 124 NaCl, 2 KCl, 1.25 KH₂PO₄, 2 MgSO₄, 26 NaHCO₃, and 10 dextrose, pH 7.4. Four hundred-micrometer-thick sections from one hemisphere, chosen at random, were cut on a vibratome (Leica). Slices were then quickly transferred to netting in a custom Plexiglas holding chamber and maintained in CaCl₂-containing (2 mM) aCSF at an interface with warm (32°C), humidified air. Slices were permitted to equilibrate for at least 1.5 h before beginning electrophysiological analysis. These slices were used for whole-cell patch-clamp and synaptic strength analyses.

Spontaneous EPSC measures in brain slices. Slices were transferred to an RC-27 recording chamber (Warner Instruments) and continuously perfused with aCSF (2 ml/min) heated to 32°C and saturated with 95% O₂/5% CO₂. Whole-cell patch-clamp recordings were obtained from individual CA1 pyramidal neurons visualized under infrared microscopy using a Nikon El600 microscope. The patch pipette solution contained the following (in mm): 120 CH₃CsO₃S, 10 CaCl₂, 5 NaCl, 10 HEPES, 0.5 EGTA, 5 TEA-Cl, 4 Mg-ATP, and 0.3 NaGTP, pH 7.35 adjusted with CsOH, to maintain osmolarity 290 mOsm. The pipette solution also contained QX-314 (1 mM) to block voltage-gated Na⁺ channels. The whole-cell recording configuration was obtained using gentle suction, and the astrocyte membrane potential was held at −70 mV. To induce excitatory amino acid transporter (EAAT)-dependent inward currents, we applied 10 μM l-glutamate to the perfusion media. To confirm that inward currents were mediated by EAATs, some astrocyte-containing inserts were perfused with the glutamate transport inhibitor TBOA (100 μM; Tocris Bioscience) 15 min before and during perfusion with glutamate. Data acquisition was controlled using a Multichamp 700B amplifier, Digitida 1332a, and pClamp software (Molecular Devices; RRID: SCR_011323). All currents were filtered at 2 kHz and digitized at 10 kHz.

Isolation of NMDAR-dependent field potentials. The CA3 region was dissected away with a scalpel blade, and slices were transferred to a Kerr recording system and perfused (1–2 ml/min) with oxygenated prewarmed (−30°C) aCSF as described above. Field potentials were recorded in CA1 stratum radiatum in response to electrical stimulation of CA3 Schaffer collaterals at a rate of 0.033 Hz. After a stable baseline was obtained (−20 min), the AMPAR antagonist CNQX (10 μM) and the GABA[sub A] receptor antagonist picrotoxin (50 μM) were added to the perfusion media to isolate NMDAR-dependent EPSPs. The NR2B-dependent component was further isolated by adding 3-phenoxy-3-phenylprop-1-amine (PDDA; 0.5 μM), while the NR2A component was isolated using Ro-25-6981 (1 μM). Ten consecutive EPSPs were collected at 20–30 min post-drug washin and averaged, and then compared with EPSPs averaged immediately before drug wash. Field potentials were amplified 100× and digitized at 10 kHz using the Kerr Tissue Recording System amplifier and a 4/35 PowerLab analog-to-digital converter (ADInstruments). Stimulus timing and data acquisition were controlled by LabChart 8 Software.

Statistics. ANOVA, repeated-measures ANOVA (rMANOVA), Fisher’s exact test, and Kolmogorov–Smirnov test for parametric tests, post hoc comparisons were performed using Fisher’s LSD. All statistical comparisons were made with GraphPad Prism version 7 software (RRID: SCR_002798), except for three-way repeated-measures ANOVAs, which were performed with StatView version 5 software. Statistical significance for all comparisons was set at p ≤ 0.05.
Results

CN/NFAT4 expression/activity in 5xFAD mice is associated with astrocyte activation

The CN/NFAT pathway becomes excessively activated in astrocytes in multiple forms of injury and disease. Figure 1A–C shows hippocampal protein levels for full-length CN (60 kDa) and a high-activity CN proteolytic fragment (48 kDa) in 8-month-old WT (n = 15) and 5xFAD (n = 16) mice. Although levels for full-length CN did not differ significantly between genotype groups (Fig. 1B), ΔCN appeared almost exclusively in 5xFAD mice (Fig. 1C; t(29) = 7.35, p = 4.3 × 10⁻⁸) characterized by significantly elevated GFAP expression (Fig. 1D; t(29) = 14.4, p = 1.0 × 10⁻¹⁴), indicative of astrocyte activation. Within the 5xFAD group, ΔCN levels increased directly in proportion to GFAP levels (Fig. 1E; r = 0.82, p = 0.0001). A similar ΔCN fragment has been previously linked to neuronal damage associated with excitotoxicity (Wu et al., 2004), acute injury (Shioda et al., 2006; Furman et al., 2016), and Aβ pathology (Wu et al., 2010; Mohmmad Abdul et al., 2011). The direct association of ΔCN with activated astrocytes was further demonstrated using confocal microscopy and a custom-made antibody that detects ΔCN, but not full-length CN (Pleiss et al., 2016). As shown in Figure 1, F and G, ΔCN labeling was associated with numerous GFAP-positive astrocytes throughout the hippocampus of 5xFAD mice but did not appear at high levels in the neuronal cell layers. Similar to ΔCN, the NFAT4 isoform, previously identified in activated astrocytes of acute injury models (Serrano-Pérez et al., 2011; Furman et al., 2016), was also colocalized to GFAP-positive astrocytes in 5xFAD mice (Fig. 1H). NFAT4 was found at elevated levels in astrocytes (t₁₁₄ = 2.94, p = 0.01) and astrocyte nuclei (t₁₁₄ = 2.84, p = 0.01) of 5xFAD mice (n = 8), relative to WT mice (n = 8), which is consistent with increased NFAT activation (Fig. 1I, J). Together, these results confirm the presence of aberrant CN/NFAT signaling in activated astrocytes of the 5xFAD mouse model.
AAV-Gfa2-VIVIT inhibits NFAT nuclear localization and improves cognition in 5xFAD mice

At 1 to 2 months of age, before 5xFAD mice develop extracellular Aβ deposits (Oakley et al., 2006), mice in both transgene groups received intrahippocampal injections of AAV-Gfa2-EGFP control or AAV-Gfa2-VIVIT-EGFP at 1.5–2 months of age. Behavioral, glutamate signaling, and/or electrophysiology endpoint measures were collected at 6–8 months of age. A transverse hippocampal section from an AAV-Gfa2-EGFP–treated mouse at 8 months of age showing extensive EGFP labeling. D, Confocal micrographs of EGFP (green), NFAT4 (red), and DAPI in 5xFAD mice treated with AAV-Gfa2-EGFP or AAV-Gfa2-VIVIT-EGFP. Left panels are 2-D confocal micrographs, and right panels are 3-D reconstructed images of ~15 μm z-stacks (0.5 μm sections). E, Mean ± SEM NFAT4 activity levels (ratio of nuclear NFAT4 to total NFAT4 labeling) in 5xFAD mice treated with AAV-Gfa2-EGFP (n = 6) or AAV-Gfa2-VIVIT-EGFP (n = 6). Nuclear NFAT4 levels were compared across AAV treatment groups using Student’s t test. F, G, Mean ± SEM error rate (percentage of total errors on block 1) on the 2 d RAWM task in WT mice (F) and 5xFAD mice (G) treated with AAV-Gfa2-EGFP (CT) or AAV-Gfa2-VIVIT-EGFP. Genotype and AAV treatment effects on learning rates were determined with ANOVA and Fisher’s LSD test. H, Mean ± SEM total errors committed on days 1 and 2 of the RAWM task. WT-CT group, 28 mice/group; WT-VIVIT group, 15 mice/group; 5xFAD-CT group, 22 mice/group; 5xFAD-VIVIT group, 10 mice/group. Genotype, AAV, and training day effects (along with significant interactions) were determined with a three-way rmANOVA and follow-up one-way rmANOVAs within each experimental group.

Figure 2. AAV-Gfa2-VIVIT reduces astrocytic NFAT activation and improves cognition in 5xFAD mice. A, B, Experimental treatments and timeline. Mice received intrahippocampal injections of AAV-Gfa2-EGFP control or AAV-Gfa2-VIVIT-EGFP at 1.5–2 months of age. Behavioral, glutamate signaling, and/or electrophysiology endpoint measures were collected at 6–8 months of age. C, Transverse hippocampal section from an AAV-Gfa2-EGFP–treated mouse at 8 months of age showing extensive EGFP labeling. D, Confocal micrographs of EGFP (green), NFAT4 (red), and DAPI in 5xFAD mice treated with AAV-Gfa2-EGFP or AAV-Gfa2-VIVIT-EGFP. Left panels are 2-D confocal micrographs, and right panels are 3-D reconstructed images of ~15 μm z-stacks (0.5 μm sections). E, Mean ± SEM NFAT4 activity levels (ratio of nuclear NFAT4 to total NFAT4 labeling) in 5xFAD mice treated with AAV-Gfa2-EGFP (n = 6) or AAV-Gfa2-VIVIT-EGFP (n = 6). Nuclear NFAT4 levels were compared across AAV treatment groups using Student’s t test. F, G, Mean ± SEM error rate (percentage of total errors on block 1) on the 2 d RAWM task in WT mice (F) and 5xFAD mice (G) treated with AAV-Gfa2-EGFP (CT) or AAV-Gfa2-VIVIT-EGFP. Genotype and AAV treatment effects on learning rates were determined with ANOVA and Fisher’s LSD test. H, Mean ± SEM total errors committed on days 1 and 2 of the RAWM task. WT-CT group, 28 mice/group; WT-VIVIT group, 15 mice/group; 5xFAD-CT group, 22 mice/group; 5xFAD-VIVIT group, 10 mice/group. Genotype, AAV, and training day effects (along with significant interactions) were determined with a three-way rmANOVA and follow-up one-way rmANOVAs within each experimental group.

AAV-Gfa2-VIVIT inhibits NFAT nuclear localization and improves cognition in 5xFAD mice

At 1 to 2 months of age, before 5xFAD mice develop extracellular Aβ deposits (Oakley et al., 2006), mice in both transgene groups received intrahippocampal injections of vehicle or AAV vectors expressing EGFP (control AAV) or the NFAT inhibitor VIVIT (tagged to EGFP). Transgene expression was limited to astrocytes using a human GFAP promoter (Gfa2; Fig. 2A–C), as described previously (Furman et al., 2012, 2016). Similar to our previous work, vehicle-treated and AAV-Gfa2-EGFP–treated mice were statistically comparable on every biomarker investigated (data not shown) and were therefore combined into a single control (CT) group for each transgene group (i.e., WT-CT or 5xFAD-CT). Mice were then examined at 6–8 months post-AAV injection, when Aβ pathology and glial activation are widespread in 5xFAD mice (Oakley et al., 2006).

To assess the impact of VIVIT on astrocytic NFAT activation, we quantified the nuclear localization of NFAT4 exclusively in individual EGFP-positive astrocytes of 8-month-old 5xFAD mice (EGFP control, n = 6; VIVIT-EGFP, n = 6) using confocal microscopy. Representative three-dimensional reconstructions of NFAT4 labeling in EGFP- and VIVIT-EGFP-positive astrocytes are shown in Figure 2D. NFAT4 present in DAPI-labeled nuclei was significantly reduced by ~30% in VIVIT-EGFP-positive astrocytes (Fig. 2E; t(10) = 2.3, p = 0.04), demonstrating VIVIT-mediated inhibition of NFAT4. Figure 2F–H shows the effects of genotype and AAV on relative learning rates (Figs. 2F, G) and the total number of errors committed (Fig. 2H) on the 2 d version of a radial arm water maze task (WT-CT group, 28 mice/group; WT-VIVIT group, 15 mice/group; 5xFAD-CT group, 22 mice/group; 5xFAD-VIVIT group, 10 mice/group). ANOVA detected a significant genotype × AAV treatment interaction for learning rate (F(1,71) = 5.261, p = 0.02), with post hoc tests showing a reduced learning rate in 5xFAD-CT mice versus WT-CT mice (p = 0.02, Fisher’s LSD) and in 5xFAD-CT mice versus 5xFAD-VIVIT mice (p =...
0.04, Fisher’s LSD). No differences were observed between 5xFAD-VIVIT mice and either WT AAV treatment group. By the last training block on day 2, 5xFAD-VIVIT mice made significantly fewer errors than 5xFAD-CT mice (Fig. 2G; p = 0.03, Student’s t test). When the total number of errors was compared across training days using a three-way rmANOVA, a significant genotype by AAV treatment by training day interaction was observed (Fig. 2H; F(1,21) = 6.2, p = 0.02). Within both genotype groups, there was a significant effect of training day, indicating that errors were reduced on day 2 for both genotype groups (WT mice: F(1,141) = 35.28, p < 0.0001; 5xFAD mice: F(1,139) = 20.49, p < 0.001). However, for the 5xFAD group, a significant AAV treatment by training day interaction was also found (F(1,39) = 5.46, p = 0.03). A follow-up rmANOVA in each AAV treatment condition within the 5xFAD group revealed a significant training effect only for 5xFAD-VIVIT mice (F(1,9) = 20.76, p = 0.002). Thus, all groups exhibited significant improvement on day 2 of the RAWM task except for 5xFAD-CT mice. Together, the results demonstrate that blockade of astrocytic CN/NFAT signaling before AD pathology helps stabilize cognitive function in 5xFAD mice.

AAV-Gfa2-VIVIT normalizes spontaneous glutamate transients in 5xFAD mice

Signs of neuronal hyperexcitability and/or excitotoxicity have been reported in AD (Putcha et al., 2011; Bakker et al., 2012) and can occur in conjunction with increasing amyloid pathology in transgenic mice (Palop et al., 2007; Busche et al., 2008; Grienberger et al., 2012; Bomben et al., 2014; Kellner et al., 2014; Šišková et al., 2014; Busche and Konnerth, 2015; Fontana et al., 2017). Though glutamate dysregulation is key to excitotoxic damage, dynamic levels of glutamate have only rarely been directly examined in common AD mouse models. Using ceramic-based, enzyme-coated (i.e., glutamate oxidase) MEAs (Hascup et al., 2007; Miller et al., 2014; Hunsberger et al., 2015; Fig. 3A–C), we measured multiple glutamate signaling parameters in CA1 of anesthetized WT and 5xFAD mice treated with AAV vectors (WT-CT group, 10 mice/group; WT-VIVIT group, 4 mice/group; 5xFAD-CT group, 8 mice/group, 5xFAD-VIVIT group, 5 mice/group). Similar to previous work (Matveeva et al., 2012), basal glutamate levels were highly variable (Fig. 3D) and no significant effects of genotype or AAV were found. Mice also exhibited spontaneously generated glutamate transients (Fig. 3E) that varied in frequency, amplitude, and duration (Fig. 3F–I). The average glutamate transient amplitude did not differ significantly across groups (Fig. 3G), although VIVIT tended to have opposing effects in WT and 5xFAD mice (i.e., transient amplitude was increased in WT mice but was decreased in 5xFAD mice treated with VIVIT).

In contrast to amplitude, both the frequency and duration of glutamate transients differed significantly depending on genotype and AAV treatment (Fig. 3F–I). For transient frequency, ANOVA detected a significant genotype by AAV treatment interaction (Fig. 3F; F(1,22) = 3.92, p = 0.05), which was characterized by a greater response rate in 5xFAD-CT mice relative to WT-CT mice (p = 0.02, Fisher’s LSD test) and to 5xFAD-VIVIT mice (p = 0.02, Fisher’s LSD). No differences were found among WT-CT, WT-VIVIT, and 5xFAD-VIVIT groups. To calculate transient duration (measured in seconds), glutamate levels were calculated along the descending phase of the transient at three different points: T50 (50% decay from peak amplitude), T80 (80% decay), and T100 (100% decay; Fig. 3H). Decay times were then compared across genotype and AAV treatments using a three-way rmANOVA, which detected a significant AAV treatment by time point interaction (Fig. 3I; F(2,20) = 3.71, p = 0.03).

A two-way rmANOVA performed within each genotype group revealed a similar AAV by time point interaction within 5xFAD mice (F(2,10) = 3.78, p = 0.04) but not within the WT group. Post hoc tests showed significantly longer decay times for 5xFAD-CT mice relative to both WT-CT mice (T100; p = 0.02, Fisher’s LSD) and 5xFAD-VIVIT mice (T100; p = 0.02, Fisher’s LSD). The results demonstrate an increase in the frequency and duration of spontaneous glutamate transients in 5xFAD mice due, in part, to elevated astrocytic CN/NFAT activity.

AAV-Gfa2-VIVIT reduces Aβ pathology and increases GLT-1 expression

Previous work has shown that the levels and/or function of EAA12 (rodent analog, GLT-1), the most abundantly expressed glutamate transporter in hippocampal astrocytes (Maragakis and Rothstein, 2006) are reduced in conjunction with Aβ pathology and/or glial activation (Masliah et al., 1996; Abdul et al., 2009; Simpson et al., 2010; Tian et al., 2010; Scimemi et al., 2013; Heffendehl et al., 2016). AAV-mediated delivery of VIVIT to 5xFAD mice reduced both Aβ plaque load in CA1 (Fig. 4A, B; 5xFAD-CT group, 6 mice/group; 5xFAD-VIVIT group, 7 mice/group; t(10) = 2.41, p = 0.04) and soluble Aβ42 peptide levels in whole hippocampus (Fig. 4C; 5xFAD-CT group, 4 mice/group, 5xFAD-VIVIT, 5 mice/group; t(7) = 3.1, p = 0.02). Furthermore, GFAP labeling around Aβ deposits was significantly reduced in VIVIT-treated 5xFAD mice (Fig. 4A, D; t(10) = 2.28, p = 0.04). GLT-1 showed diffusely labeling across the hippocampus (Fig. 4E, F) as reported in other studies (Heffendehl et al., 2016). VIVIT increased GLT-1 labeling in 5xFAD mice (Fig. 4E, G; 5xFAD-CT group, 6 mice/group; 5xFAD-VIVIT group, 6 mice/group; t(10) = 2.29, p = 0.04), especially around Aβ deposits (Fig. 4F, H; t(10) = 5.8, p = 0.0002), and was associated with an overall increase in hippocampal GLT-1 protein (Fig. 4I; 5xFAD-CT group, 3 mice/group; 5xFAD-VIVIT group, 3 mice/group; t(10) = 5.1, p = 0.007).

To determine whether hyperactive astrocytic CN/NFAT signaling directly affects glutamate uptake, we used whole-cell voltage clamp (Fig. 4K) to measure glutamate transport currents in primary rat astrocytes expressing an activated CN proteolytic fragment (ΔCN), similar to that found in 5xFAD astrocytes (Fig. 1C, F, G). Astrocytes were infected with control adenovirus (Ad-LacZ-GFP, n = 8) or adenoviruses expressing ΔCN or VIVIT (Ad-CMV-ΔCN-Ds-Red2, n = 9; Ad-CMV-VIVIT-EGFP, n = 4) at an MOI of 100, as described previously (Sama et al., 2008). Perfusion of primary astrocytes with 1-glutamate (10 μM) resulted in an inward current that was largely mediated by TBOA-sensitive glutamate transporters (Fig. 4L). ANOVA detected a significant effect of virus treatment (F(2,18) = 6.25, p = 0.009). Inward currents were significantly reduced in astrocytes expressing ΔCN (p = 0.03, Fisher’s LSD vs CT), but not VIVIT (Fig. 4M). The results suggest that glutamate dysregulation in 5xFAD mice is attributable to the CN/NFAT-mediated downregulation of GLT-1-dependent glutamate uptake in astrocytes.

AAV-Gfa2-VIVIT normalizes spontaneous synaptic activity in 5xFAD mice

We next determined whether astrocytic CN/NFATs were involved in hyperexcitability at the synaptic level. Brain slices were prepared from AAV-treated WT and 5xFAD mice, and spontaneous EPSCs were recorded across a 3 min period from individual CA1 neurons held at −80 mV using whole-cell voltage clamp (Fig. 5A, B; WT-CT group, 39 cells from 16 mice; WT-VIVIT group, 28 cells from 10 mice; 5xFAD-CT group, 33 cells from 11 mice; 5xFAD-VIVIT group, 14 cells from 7 mice). Under these
conditions, neurons exhibited highly variable activity rates (Fig. 5B), which could be sorted into four broad categories: low (0–499 events), low-normal (500–999 events), high-normal (1000–1499 events), and high (≥1500 events). As shown in Figure 5C, a significantly greater proportion of cells in the 5xFAD-CT group exhibited normal-high to high activity rates compared with WT-CT mice (p = 0.0007, Fisher’s exact test) and 5xFAD mice (p = 0.004, Fisher’s exact test). Amplitude histograms (Fig. 5D) showed that the majority of EPSCs occurred within the 10–20 μA range across all groups, suggesting that miniature EPSC amplitudes were unaffected by genotype or virus treatment. However, relative to both WT groups, 5xFAD-CT mice exhibited a higher peak and a broader distribution, which is indicative of overall greater activity. Similarly, the cumulative EPSC frequency distribution (Fig. 5E) for 5xFAD-CT mice exhibited a significant rightward shift compared with WT-CT mice (p < 1 × 10−15, Kolmogorov–Smirnov test) and 5xFAD-VIVIT mice (p < 1 × 10−15, Kolmogorov–Smirnov test). When the total number of EPSCs was counted (regardless of amplitude), ANOVA detected a significant AAV treatment effect (Fig. 5F; $F_{(1,36)} = 7.11$, $p =$...
Post hoc tests showed a significantly higher activity rate in 5xFAD-CT mice relative to WT-CT mice (p < 0.05, Fisher’s LSD test) and 5xFAD-VIVIT mice (p < 0.03, Fisher’s LSD test). EPSC distributions (Fig. 5D) and frequency (Fig. 5E, F) in 5xFAD-VIVIT mice were statistically comparable to those of WT-CT mice. Together, the results suggest that astrocytic CN/NFAT helps to drive hyperactive spontaneous synaptic activity associated with AD-like pathology.

Figure 4. AAV-Gfa2-VIVIT-EGFP reduces Aβ levels and increases GLT-1 labeling in 5xFAD mice. A, Confocal micrographs of CA1 in CT- and VIVIT-treated 5xFAD mice showing immunolabeling of Aβ (red) and GFAP (blue). B–D, Mean ± SEM Aβ plaque load (B), Aβ peptide levels (C), and GFAP labeling (volume × μm$^3$) in CA1 of AAV-treated 5xFAD mice. 5xFAD-CT group, 6 mice/group; 5xFAD-VIVIT group, 7 mice/group. E, F, 3-D reconstructions of GLT-1 labeling (blue) in hippocampal area CA1. Aβ deposits are shown in red at low (E) and high (F) magnification. G, H, Mean ± SEM GLT-1 labeling intensity (arbitrary units (A.U.)/tissue volume × μm$^3$) across total hippocampus (G) and in the immediate vicinity (H) of Aβ deposits of 5xFAD-CT and 5xFAD-VIVIT mice. I, J, Representative Western blots (I) and mean ± SEM hippocampal protein levels (J) for GLT-1 in CT mice and VIVIT-treated 5xFAD mice. (n = 3/group).
AAV-Gfa2-VIVIT prevents dendritic degeneration and improves basal synaptic strength

CA1 neuronal loss and dendritic abnormalities commonly arise with AD (Spries and Hyman, 2004; Padurariu et al., 2012), similar to what is observed following glutamate dysregulation associated with excitotoxic insults (Andrew and MacVicar, 1994; Hasbani et al., 1998; Greenwood et al., 2007). To determine whether changes in the number and/or structural integrity of hippocampal neurons is altered in 5xFAD mice, we counted NeuN-positive CA1 pyramidal neurons (Fig. 6A), the number of CA1 neurons held at −80 mV. Traces are from cells that showed low, normal, and high (hyperactive) levels of spontaneous synaptic activity. Pie charts of the percentage of cells in each treatment group sorted by activity levels (i.e., number of EPSCs) during the 3 min recording window: low (0–499, dark green), low-normal (500–999, light green), high-normal (1000–1499, yellow), and high (>1500, red). The proportion of cells at each activity level was compared across genotype/AAV groups using Fisher’s exact test. B, Mean EPSC amplitude histograms from WT (left) and 5xFAD mice (right) treated with AAV-Gfa2-EGFP (CT) or AAV-Gfa2-VIVIT-EGFP (VIVIT). C, Mean cumulative frequency distributions for WT mice (left) and 5xFAD mice (right) under CT and VIVIT treatment conditions. Significant shifts in the frequency distributions between 5xFAD-CT vs WT-CT cells (*) and 5xFAD-VIVIT vs 5xFAD-CT cells (#) were determined using the Kolmogorov–Smirnov test. D, Mean EPSC frequency (events/min) in AAV-treated WT and 5xFAD mice. WT-CT group, 39 cells from 16 mice; WT-VIVIT group, 39 cells from 16 mice; WT-CT group, 28 cells from 10 mice; 5xFAD-CT group, 33 cells from 11 mice; 5xFAD-VIVIT group, 14 cells from 7 mice. Genotype and AAV effects in F were determined with ANOVA and Fisher’s LSD test.

In WT mice, VIVIT treatment was associated with a small downward shift in the synaptic strength curve (Fig. 6E), which was attributable to a modest reduction in the CA3 FV amplitude (data not shown). However, maximal EPSP/FV ratio in WT-VIVIT-treated mice was statistically comparable to that in WT-CT mice, suggesting that VIVIT effects on basal synaptic strength in WT mice were minimal (Fig. 6G). While FV amplitudes were similar in WT-CT and 5xFAD mice, 5xFAD-CT mice exhibited a marked downward shift in the synaptic strength curve.
amplitude (Fig. 6F). For the EPSP/FV ratio, ANOVA detected a significant interaction between genotype and AAV treatment ($F_{(1,39)} = 6.8, p = 0.001$). Post hoc tests revealed a significant (>60%) reduction in 5xFAD-CT mice relative to WT-CT mice ($p = 0.0002$, Fisher's LSD test). In contrast, the EPSP/FV ratio for 5xFAD-VIVIT mice did not differ from that for WT-CT mice but was significantly elevated by >90% compared with that for 5xFAD-CT mice ($p = 0.007$, Fisher's LSD test). A significant transgene by AAV treatment interaction was also observed for the evoked PS threshold, which is a measure of overall neuronal excitability (Fig. 6H; $F_{(1,39)} = 9.9, p = 0.003$). In most slices, an upward-going PS (indicative of a synchronous population action potential in CA1) appeared in the ascending limb of the field potential with increasing stimulus intensities (Fig. 6H, inset, arrow). Relative to WT-CT mice, the PS for 5xFAD-CT mice occurred in response to significantly lower levels of postsynaptic activation (i.e., smaller EPSP slopes; $p = 1.1 \times 10^{-5}$, Fisher's LSD test), suggesting that CA1 neurons from 5xFAD-CT mice are hyperexcitable, even though evoked synaptic responses are reduced. Although the PS threshold was also reduced in 5xFAD-VIVIT mice relative to WT-CT mice ($p = 0.02$, Fisher's LSD), VIVIT treatment resulted in a significant elevation in the PS threshold relative to the 5xFAD-CT control group ($p = 0.04$, Fisher's LSD test), indicative of reduced excitability. Together, the results suggest that inhibition of astrocytic CN/NFAT signaling helps protect against neurite abnormalities and basal synaptic strength deficits in 5xFAD mice.

**AAV-Gfα2-VIVIT normalizes NMDAR/AMPA activity in CA1 pyramidal neurons in 5xFAD mice, but does not prevent synapse silencing**

Reductions in the CA1 field potential in 5xFAD mice may be attributable to functional deficits at individual synapses and/or to the silencing of individual synapses. To test these possibilities,
EPSC amplitudes and synaptic failure rates were investigated using a minimal stimulation paradigm and whole-cell voltage clamp (see Materials and Methods; Isaac et al., 1995; Liao et al., 1995). Inward-going EPSCs were orthodromically elicited from CA1 pyramidal neurons held at −80 mV (i.e., 120 stimulus pulses, five second interpulse interval). Stimulation intensity was lowered until transmission failures were observed on at least 50% of stimulus trials. Because of the voltage-dependent Mg\(^{2+}\)/ block, NMDA receptor (NMDAR) currents are negligible at −80 mV, resulting in a purely AMPA-driven EPSC. The membrane potential was then stepped to −40 mV and outward-going EPSCs, consisting of both AMPA and NMDAR-mediated currents (due to relief of the voltage-dependent Mg\(^{2+}\) block), were elicited across 120 additional trials at the same stimulus intensity.

Figure 7A shows a representative time plot of the minimal stimulation paradigm. For some cells, the NMDAR antagonist, Ro-25-6981, or APV, was washed in 10–20 min after stepping to −40 mV to confirm that outward-going EPSCs were dependent on functional NMDARs. No effects of genotype or AAV were observed for the average amplitude of evoked inward-going EPSCs recorded at −80 mV (Fig. 7B, C; WT-CT group, 16 cells from 15 mice; WT-VIVIT group, 10 cells from 9 mice; 5xFAD-CT group, 16 cells from 11 mice; 5xFAD-VIVIT group, 9 cells from 7 mice). This result suggests that unitary AMPAR currents are intact at surviving synapses of 5xFAD mice and insensitive to astrocytic CN/NFAT activity. In contrast, ANOVA detected a significant genotype by AAV treatment interaction for outward-going EPSC amplitudes recorded at −40 mV (Fig. 7B, C; F(1,47) = 4.08, p = 0.04).

Post hoc
tests showed that 5xFAD-CT exhibited elevated outward currents relative WT-CTs ($p = 0.005$, Fisher’s LSD test) and 5xFAD-VIVIT mice ($p = 0.04$, Fisher’s LSD test) consistent with a proportional increase in NMDAR function. The 5xFAD-VIVIT group was statistically comparable to both WT groups.

ANOVA also detected a genotype effect for synaptic transmission failure rate at $+40 \text{ mV vs } -80 \text{ mV}$ (Fig. 7D; E; $F_{(1,47)} = 7.82$, $p = 0.008$). Post hoc tests showed a significant decrease in the failure rate for both 5xFAD-CT and 5xFAD-VIVIT mice relative to WT-CT mice (5xFAD-CT vs WT-CT $p = 0.03$; 5xFAD-VIVIT vs WT-CT $p = 0.03$, Fisher’s LSD test) indicative of a higher proportion of functionally silent synapses (Isaac et al., 1995; Liao et al., 1995) in 5xFAD mice. No effect of VIVIT treatment within the 5xFAD group was observed. The results suggest that population synaptic deficits in 5xFAD-CT mice are at least partially due to the silencing of functional synapses, but, this silencing appears to be independent of hyperactive CN/NFAT signaling in astrocytes.

The increased amplitude of outward-going EPSCs in 5xFAD-CT, but not 5xFAD-VIVIT mice (Fig. 7C), suggests that NMDAR function at surviving synapses is proportionally increased in a CN/NFAT-dependent manner. To test this possibility, field EPSCs were recorded in CA1 stratum radiatum under normal perfusion conditions and 10–20 min after the pharmacological isolation of NMDAR potentials (Fig. 7F–J; WT-CT, 11 mice/group; WT-VIVIT, 10 mice/group; 5xFAD-CT, 12 mice/group; 5xFAD-VIVIT, 9 mice/group). NMDAR-dependent EPSPs were isolated by including AMPAR and GABAR blockers (CNQX and picrotoxin) in the perfusion media. The NR2A antagonist (PPPA) or the NR2B antagonist (Ro-25-6981) were also added to further isolate the NR2B (Fig. 7F, G) and NR2A (Fig. 7H, I) components of the NMDAR-mediated EPSP. For WT mice, both the NR2B and NR2A components were ~20% of the baseline field EPSP (Figs. 7G, I). There were no genotype or AAV treatment effects for NR2B-dependent EPSPs (Fig. 7G). In contrast, there were significant effects of both genotype and AAV treatment on the isolated NR2A-mediated EPSPs (Fig. 7F; genotype: $F_{(1,38)} = 6.7, p = 0.01$; AAV: $F_{(1,38)} = 3.9, p = 0.05$). Post hoc tests revealed a proportional increase in the NR2A-dependent EPSP in 5xFAD-CT mice relative to WT-CT mice ($p = 0.009$, Fisher’s LSD test). In contrast, the NR2A EPSP in 5xFAD-VIVIT mice was statistically comparable to that in WT-CT mice and was significantly reduced relative to that in the 5xFAD-CT group ($p = 0.04$, Fisher’s LSD test). Thus, astrocytic CN/NFAT signaling appears to alter the balance of NMDAR sensitivity in CA1, which could further contribute to excitotoxic damage.

**Discussion**

Our results directly implicate activated astrocytes in the emergence of pathologic glutamate signaling in AD. Neuronal hyperexcitability in 5xFAD mice was revealed using several independent approaches, including in vivo glutamate sampling, in situ whole-cell patch-clamp analysis of spontaneous EPSCs, and in situ field recordings of NMDA-dependent EPSPs. For each approach, the blockade of astrocytic CN/NFAT activity normalized glutamate signaling, providing critical insights into the mechanisms that drive neuronal dysfunction/deterioration during the progression of AD.

**Activated astrocytes are permissive for hyperactive CN signaling**

Although featured prominently in nearly every neurodegenerative disorder, activated astrocytes have a complex phenotype, and their roles in pathophysiology remain unclear. However, the appearance of activated astrocytes at early stages of AD (Carter et al., 2012; Schöll et al., 2015) suggests an important role in the emergence of later pathophysiological changes. We have exploited specific changes in the CN/NFAT pathway to determine how activated astrocyte signaling specifically affects neural function (Furman et al., 2012, 2016; Pleiss et al., 2016). Several lines of evidence in human AD and AD mouse models suggest that CN/NFAT signaling is elevated during disease progression (Liu et al., 2005; Norris et al., 2005; Reese et al., 2008; Abdul et al., 2009; Wu et al., 2010; Lim et al., 2013). Levels of cytosolic Ca$^{2+}$, the primary endogenous activator of CN, show higher and more rapid fluctuations in astrocytes of amyloidogenic mice (Kuchibhotla et al., 2009). Moreover, Ca$^{2+}$-dependent proteases, which show increased expression in activated astrocytes (Gray et al., 2006; Kim et al., 2016), can cleave CN into highly active proteolytic fragments (Wu et al., 2004). Thus, activated astrocytes seemingly provide a very permissive environment for hyperactive CN signaling. In the present study, CN proteolysis occurred almost exclusively in 5xFAD mice and increased in direct proportion with GFAP levels. Similar observations were recently made for activated astrocytes associated with Aβ deposits and microinfarcts in humans (Pleiss et al., 2016). NFAT4 expression in 5xFAD mice paralleled that of ACN, confirming that the NFAT4 isoform is an excellent marker for activated astrocytes (Lim et al., 2013; Furman et al., 2016). NFATs are well known for their pivotal roles in phenotype switching in a variety of tissues (Furman and Norris, 2014). The present results suggest that ΔCN/NFAT4 is central to a deleterious astrocyte phenotype that is responsible for glutamate dysregulation.

**Activated astrocytes, CN/NFATs, and network excitability**

Glutamate is an essential neurotransmitter but can cause hyperexcitability and excitotoxic damage if key regulatory mechanisms are disrupted. Symptoms of neuronal hyperexcitability develop in up to 40% of patients in whom AD has been diagnosed (e.g., subclinical epileptiform activity), and patients with AD and epileptiform comorbidities show accelerated cognitive decline (Vossel et al., 2016). Moreover, therapeutics that reduce excitability, including the NMDAR blocker memantine, slow the progression of dementia when administered to subjects with mild, moderate, or severe AD (Reisberg et al., 2003; Tariot et al., 2004; Peskind et al., 2006). Hyperexcitability is also a key phenotypic feature of many common AD mouse models (Palop et al., 2007; Busche et al., 2008; Grienberger et al., 2012; Bomben et al., 2014; Kellner et al., 2014; Šíšková et al., 2014; Hefendehl et al., 2016; Fontana et al., 2017). Although we did not see genotype- or AAV-dependent changes in basal glutamate in CA1, other dynamic properties of glutamate regulation/signaling, including glutamate transient rate and duration in vivo and spontaneous synaptic activity ex vivo, showed significant elevations in 5xFAD mice. Further signs of hyperexcitability included a reduced PS threshold and a proportional increase in NMDAR function. The delivery of VIVIT to astrocytes significantly ameliorated nearly all of these changes, suggesting that alterations in astrocyte signaling play a major role in the development of hyperactive neuronal circuits during AD.

Critical glutamate regulatory mechanisms in astrocytes are lost and/or disrupted with AD, in parallel with astrocyte activation (Masliah et al., 1996; Abdul et al., 2009; Simpson et al., 2010). Levels and/or function of the astrocytic glutamate transporter EAAT2/GLT-1 decline in human hippocampus at the onset of cognitive dysfunction (Abdul et al., 2009) and are similarly disrupted in numerous AD mouse models (Masliah et al., 2000; Mookherjee et al., 2011; Schallier et al., 2011; Scimemi et al., 2013;
Astrocytic CN/NFAT and synaptic deficits

Excess glutamate causes dendritic degeneration and synapse loss in numerous injury and disease models. Consistent with glutamate toxicity, 5xFAD mice showed signs of dendritic degeneration, in parallel with reduced population EPSPs in CA1 stratum radiatum. While the amplitude of miniature AMPAR currents was not reduced in 5xFAD mice, we did observe a significant drop in the synaptic transmission failure rate when EPSCs were minimally evoked at +40 vs. −80 mV, suggesting a higher proportion of functionally silent synapses (Isaac et al., 1995; Liao et al., 1995). To our knowledge, this evidence is the first to directly implicate synapse silencing as a mechanism for synapse loss/dysfunction with AD. Astrocytic CN/NFATs do not appear to be involved in the conversion of functional-to-silent synapses in 5xFAD mice because transmission failure rates were not significantly affected by AAV treatment. The beneficial effects of AAV-Gfa2-VIVIT on evoked population EPSPs, therefore, most likely reflect the structural preservation of dendrites and synapses.

Interestingly, 5xFAD-CT mice exhibited a proportional increase in NR2A function, and this effect was significantly reduced by VIVIT. This increase may reflect a compensatory response to synapse loss or impaired synaptic efficacy (Nudnamand-Thanoi et al., 2006; Lacey et al., 2012), which nonetheless could ultimately lead to synapse instability. For instance, earlier work reported that Aβ triggers dendritic spine loss and synapse dysfunction specifically through the increased activation of NR2A receptors (Tackenberg et al., 2013). NR2A receptors also appear to be highly sensitive to glutamate transport at the synapse and exhibit greater function when GLT-1-mediated transport is reduced (Armbruster et al., 2016), which is consistent with the present findings. Together, the results suggest that the CN/NFAT-dependent loss of GLT-1, and the impaired uptake of synaptic glutamate, could adversely augment NR2A function, leading to dendritic damage and synapse dysfunction.

Effects of VIVIT in other cell types during progression of AD-related pathology

In addition to astrocytes, hyperactive NFAT signaling linked to AD pathology has also been targeted in microglia and neurons using VIVIT. In primary microglia, where NFATs 1 and 2 play a predominant role, VIVIT reduced the expression of several cytokines linked to chronic neuroinflammation, including TNF-α and monocyte chemoattractant protein-1 (Nagamoto-Combs and Combs, 2010). Follow-up studies from the Combs laboratory showed that intraventricular delivery of VIVIT peptide suppressed microglial activation and reduced Aβ plaque load in intact APP/PS1 mice (Rojanathammanee et al., 2015), similar to what we have observed following VIVIT delivery to astrocytes (Furman et al., 2012; Fig. 4A–C). However, unlike the present findings and our earlier work, intraventricular delivery of VIVIT did not improve cognition in the study by Rojanathammanee et al. (2015), perhaps because of the timing (post-Aβ pathology vs pre-Aβ pathology) and/or duration of treatment (1 month treatment vs multiple months). In neurons, targeted delivery of a constitutively active form of NFAT3, which is upregulated with human AD (Abdul et al., 2009; Wu et al., 2010), recapitulated dendritic spine loss and dendritic degeneration, typically associated with elevated Aβ levels (Hudry et al., 2012). Conversely, AAV-mediated delivery of VIVIT directly to neurons of an intact mouse model of AD reduced dendritic spine loss, particularly in the vicinity of Aβ deposits. Thus, VIVIT appears to protect the integrity of neurites whether targeted to astrocytes or neurons. Together, these results suggest that pharmacologic agents specifically targeting NFAT activity—regardless of cell type—could provide clinical efficacy in cases of human AD, either as an individual therapy or when given in conjunction with newly developed Aβ-inhibiting treatments.

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