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Disease-Modifying Effects of M₁ Muscarinic Acetylcholine Receptor Activation in an Alzheimer's Disease Mouse Model

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscemneur-o.6b00278. Supplementary methods; contextual fear conditioning in 5XFAD mice with VU0364572; effect of VU0364572 on A β _{40/42} levels in primary cortical and hippocampal neurons; effect of VU0364572 on A β ₄₀ neuropathology in neocortex and hippocampus of 5XFAD mice; A β ₄₀ and A β ₄₂ immunohistochemistry in 5XFAD WT littermate control sections; correlation of A β ₄₀ levels with memory impairment in 5XFAD mice; effects of VU0364572 on A β _{40/42} ratios in 5XFAD mice; satellite pharmacokinetic study with VU0364572 (PDF)

Notes

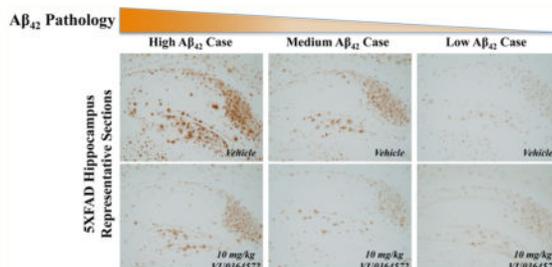
E.P.L. performed experiments comprising this study while a student at Emory University, prior to his current employment by Pfizer, Inc.

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Abstract

Alzheimer's disease (AD) is the leading cause of dementia worldwide, and currently no disease-modifying therapy is available to slow or prevent AD, underscoring the urgent need for neuroprotective therapies. Selective M₁ muscarinic acetylcholine receptor (mAChR) activation is an attractive mechanism for AD therapy since M₁ mediates key effects on memory, cognition, and behavior and has potential for disease-modifying effects on A β formation and tau phosphorylation. To validate M₁ as a neuroprotective treatment target for AD, the M₁-selective agonist, VU0364572, was chronically dosed to 5XFAD mice from a young age preceding A β pathology (2 months) to an age where these mice are known to display memory impairments (6 months). Chronic M₁ activation prevented mice from becoming memory-impaired, as measured by Morris water maze (MWM) testing at 6 months of age. Additionally, M₁ activation significantly reduced levels of soluble and insoluble A $\beta_{40,42}$ in the cortex and hippocampus of these animals, as measured by ELISA and immunohistochemistry. Moreover, soluble hippocampal A β_{42} levels were strongly correlated with MWM memory impairments and M₁ activation with VU0364572 abolished this correlation. Finally, VU0364572 significantly decreased oligomeric (oA β) levels in the cortex, suggesting one mechanism whereby VU0364572 may be exerting its neuroprotective effects is by reducing the available oA β pool in the brain. These findings suggest that chronic M₁ activation has neuroprotective potential for preventing memory impairments and reducing neuropathology in AD. M₁ activation therefore represents a promising avenue for preventative treatment, as well as a promising opportunity to combine symptomatic and disease-modifying effects for early AD treatment.

Graphical Abstract



Keywords

Muscarinic; acetylcholine; Alzheimer's; amyloid; memory; hippocampus

INTRODUCTION

Alzheimer's disease is a progressive, neurodegenerative disease, which is the most common cause of dementia worldwide and accounts for 60–70% of all cases of dementia.^{1,2} At present, there is not a single disease-modifying available therapy for AD patients that slows or prevents the course of the disease.^{2–4} AD is characterized by the presence of insoluble A β plaques and neurofibrillary tangles composed of hyperphosphorylated tau.^{5–15} While many anti-amyloid therapies have shown promising effects in mouse models of AD, so far none

have translated into an effective therapy in human AD patients.²⁻⁴ Data from recent human biomarker studies now clearly suggest that asymptomatic AD cascades begin up to two decades prior to symptom onset; this has refocused therapeutic efforts from symptomatic treatment to disease prevention and underscores the urgent need for developing neuroprotective therapies that slow or prevent AD onset.^{2,16,17} Moreover, there is an unmet need for therapies with both symptomatic and disease-modifying effects for individuals with mild cognitive deficits or early stage dementia.

The only currently United States Food and Drug Administration (FDA)-approved therapies for AD are AChEIs (e.g., donepezil) and the NMDAR antagonist memantine, both of which only provide modest symptomatic effects.^{2-4,18,19} Cholinergic neurotransmission is complex, whereby ACh acts on multiple subtypes of both nicotinic and muscarinic ACh receptors to mediate its effects, many of which functionally oppose one another.^{3,4,20-22} Thus, it is not surprising that nonselective activators such as AChEIs suffer from modest efficacy and dose-limiting side effects.^{23,24} Selective M₁ activation is a strategy considered to be highly attractive for AD therapy given the importance of M₁ in mediating crucial effects on memory, cognition, and behavior.^{3,4} The M₁ receptor is a G_q-coupled G-protein-coupled receptor (GPCR) expressed heavily throughout the hippocampus and neocortex, and M₁ knockout animals show memory deficits.²⁵⁻³³ Previous studies have shown that selective M₁ activators can both improve memory as measured behaviorally as well as modulate hippocampal synaptic plasticity.³⁴⁻³⁸ The mechanism underlying the disease-modifying effects of M₁ activation for AD has been well-established through both pharmacological and genetic studies, as M₁ activation shifts the balance of amyloid precursor protein (APP) processing toward nonamyloidogenic sAPP formation and decreases A β production by modulating cellular secretase machinery.^{21,22,33,39} The ability of M₁ to directly modulate APP processing has important implications for AD therapy, as the accumulation of A β into insoluble neuritic plaques and soluble pools of oligomeric A β (oA β) is widely believed to play a key role in AD pathogenesis.⁴¹⁻⁵⁶ Additionally, activating M₁ receptors decreases tau pathology by signaling through GSK3 β .⁴⁰ Taken together, the available data indicate that selective M₁ activators have the potential to simultaneously bolster the function of memory circuitry while protecting against the development of AD neuropathology.

The current study sought to evaluate M₁ as a neuroprotective target for AD therapy. The failure of all disease-modifying clinical trials to date, along with the recognition that disease pathology begins 20 or more years prior to the onset of clinical symptoms, has produced a paradigm-shift in AD treatment efforts. Specifically, the field is increasingly focused on earlier intervention, including in asymptomatic individuals with pathology already present (i.e., secondary prevention), and individuals with mild cognitive impairment due to AD. Therefore, we took a similar view in the current study by designing a preventative treatment trial that models exactly what is now being pursued in the leading NIH and industry-sponsored human trials. 5XFAD mice were chronically dosed with the orally bioavailable M₁-selective agonist, VU0364572, from an age before mice display A β pathology (2 months) to an age where mice are known to display memory impairments (6 months).^{37,38,57} Subsequent behavioral and neuropathological analyses confirmed that chronic M₁ activation by VU0364572 prevented the development of memory impairments and significantly decreased levels of both soluble and insoluble A β _{40,42} in the neocortex and hippocampus of

5XFAD mice. These results validate M₁ activators as a promising neuroprotective strategy to stem AD progression.

RESULTS

Chronic M₁ Activation by VU0364572 Preserves Hippocampal Memory in 5XFAD Transgenic Alzheimer's Mice

Given the potentially disease-modifying role of M₁ activation for AD, we designed a preclinical proof-of-mechanism trial to assess the neuroprotective efficacy of chronic M₁ activation and simulate the design of a prevention trial in humans (Figure 1A). We dosed the M₁ agonist, VU0364572, to selectively drive M₁ activation in the aggressive 5XFAD mouse model of AD, taking the view that such a model would provide a rigorous test of predictive validity for possible translation to clinical efficacy in humans.⁵⁷ 5XFAD animals were dosed with placebo (regular drinking water) or M₁ agonist (approximately 10 mg/kg/day VU0364572 in drinking water) chronically from 2 months of age to 6 months (Figure 1A). Because M₁ activators like VU0364572 have symptomatic effects on memory, we incorporated a drug-washout period of at least 24 h at the end of the treatment phase to avoid confounding the detection of disease-modifying effects. The 24 h washout period was deemed sufficient based upon previously published pharmacokinetic data demonstrating that VU0364572 has a half-life of 45 min.³⁷

Following placebo treatment and behavioral testing at 6 months of age, 5XFAD mice displayed a significant deficit (mean probe trial performances of 26.7%; $**p < 0.01$, $t = 2.93$, $df = 26.17$) in Morris water maze probe trial performance relative to WT littermate controls (42.6% for WT; Figure 1B). These results are in good agreement with previous results of the magnitude and rate of cognitive decline in 5XFAD mice.⁶³ Following chronic drug treatment, the performance of VU0364572-treated animals on the memory task was significantly improved (mean probe trial performance of 36.8%; $*p < 0.05$, $t = 2.11$, $df = 24.49$) relative to 5XFAD vehicle-treated animals, and not significantly different from WT animals (Figure 1B). Swim speed was not impacted relative to WT littermate controls by either genotype or treatment (Figure 1C), indicating no effect of drug treatment on motor behavior. All mice were also tested on cued and contextual fear conditioning tasks, which are amygdalo-hippocampal memory tests. While 5XFAD mice were found to be impaired in contextual fear conditioning, no impairment was observed in tests of cued fear conditioning and no drug effect was noted for either task (Figure S1). Measures of freezing between vehicle-treated and drug-treated 5XFAD animals were not found to be different between groups on either task.

VU0364572 Decreases Soluble and Insoluble A β ₄₀ and A β ₄₂ Levels in 5XFAD Mice

A truly effective disease-modifying treatment for AD would delay cognitive decline by reducing the neuropathological burden, including A β accumulation. In chronically treated 5XFAD mice, A β ₄₀ and A β ₄₂ levels were analyzed separately in both the SDS-soluble and formic-acid-insoluble fractions for hippocampus and total cortex (neocortex + entorhinal cortex) by ELISA following chronic treatment with VU0364572 or placebo. There was a significant VU0364572-mediated decrease in soluble hippocampal A β ₄₀ levels (40.4%; $*p <$

0.05, $t = 2.15$, $df = 24.55$), but no significant effect noted on cortical $A\beta_{40}$ levels versus vehicle-treated 5XFAD mice (n.s., $p > 0.05$, $t = 1.30$, $df = 23.84$) (Figure 2A). Insoluble $A\beta_{40}$ levels were found to be significantly decreased in both the hippocampus (43.3%; $*p < 0.05$, $t = 2.69$, $df = 22.35$) and cortex (38.9%; $*p < 0.05$, $t = 2.47$, $df = 19.60$) (Figure 2B). Furthermore, there was a trend toward a decrease in soluble $A\beta_{42}$ levels in the hippocampus following VU0364572 treatment (23.4%; $p < 0.1$, $t = 1.87$, $df = 25.66$) and a significant decrease observed in the cortex (34.2%; $*p < 0.01$, $t = 2.54$, $df = 20.68$) (Figure 2C). Insoluble $A\beta_{42}$ levels were also significantly decreased in the cortex (34.2%; $p = 0.05$, $t = 1.98$, $df = 25.62$) with no significant effect observed in the hippocampus (n.s., $p > 0.05$, $t = 1.30$, $df = 25.21$) following VU0364572 treatment (Figure 2D). $A\beta_{40}$ and $A\beta_{42}$ levels were also found to be decreased by VU0364572 treatment in ex vivo studies in primary hippocampal and cortical neurons from WT mice transduced with hAPP^{695WT} (Figure S2), suggesting the amyloid-modifying effects are directly mediated by neurons. Thus, chronic M₁ activation by VU0364572 robustly decreased $A\beta_{40}$ and $A\beta_{42}$ levels in the soluble and insoluble fractions cortex and hippocampus.

M₁ Activation by VU0364572 Reduces $A\beta_{40}$ and $A\beta_{42}$ Neuropathology in 5XFAD Mice

To determine if the strong effects of VU0364572 on insoluble $A\beta_{40}$ and $A\beta_{42}$ levels translated into reduced amyloid plaque burden, brain sections from VU0364572- and vehicle-treated 5XFAD mice (Figures 3 and S3), and WT littermate controls (Figure S4) were stained with antibodies against human $A\beta_{42}$ (Figure 3) and $A\beta_{40}$ (Figure S3). Qualitatively, there was a striking decrease in $A\beta_{42}$ plaque immunoreactivity throughout the hippocampus and cortex of VU0364572-treated 5XFAD mice (Figure 3D–F, J–L) relative to vehicle-treated 5XFAD mice (Figure 3A–C, G–I). In the hippocampus, substantial decreases were noted across all hippocampal subfields: subiculum, dentate gyrus, CA3, and CA1 (Figure 3J–L). In order to quantify VU0364572-induced changes, $A\beta_{42}$ immunoreactivity was measured in a blinded manner in equivalent regions of cortex and hippocampus. 5XFAD vehicle-treated animals showed a heavy burden of $A\beta_{42}$ pathology that was significantly mitigated by VU0364572 treatment in both the hippocampus (14.9% decrease; $****p < 0.0001$, $t = 4.82$, $df = 24.88$) and cortex (36.1% decrease; $***p < 0.001$, $t = 4.58$, $df = 24.18$) (Figure 3M). Similarly, VU0364572 globally decreased $A\beta_{40}$ immunoreactivity in plaques across hippocampus and cortex in 5XFAD mice (Figure S3D–F, J–L) relative to vehicle-treated 5XFAD mice (Figure S3A–C, G–I). Quantification of $A\beta_{40}$ immunoreactivity in a similar fashion yielded a substantial decrease in $A\beta_{40}$ pathology following VU0364572 treatment in both the neocortex (32.8% decrease; $***p < 0.001$, $t = 3.81$, $df = 24.49$) and hippocampus (28.1% decrease; $**p < 0.01$, $t = 3.87$, $df = 17.26$) (Figure S3M). Representative sections stained from WT littermate controls with the $A\beta_{42}$ and $A\beta_{40}$ antibodies used to assess neuropathology in this study showed no evidence of positive immunoreactivity, indicating that the observed $A\beta_{42}$ (Figure 3) and $A\beta_{40}$ (Figure S3) staining is highly specific (Figure S4).

Soluble $A\beta_{42}$ Levels are Strongly Correlated with Memory Impairment in 5XFAD Animals

Since higher soluble $A\beta_{42}$ levels are known to correlate with worse memory performance in human AD patients, we assessed the correlation between $A\beta_{40}$ or $A\beta_{42}$ pathology in 5XFAD mice and memory performance in the Morris water maze.^{41–53} To this end, pairwise

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correlations were performed between $A\beta_{40}$ and $A\beta_{42}$ fractions (soluble and insoluble, cortical and hippocampal) and Morris water maze probe trial performance for all mice (Figures 4 and S5). The strongest correlation was obtained between soluble $A\beta_{42}$ levels in the hippocampus and memory performance, with increasing levels of soluble hippocampal $A\beta_{42}$ highly correlated ($R^2 = 0.44$; $***p < 0.001$) to worsening behavioral performance (Figure 4C). The correlation of soluble $A\beta_{42}$ levels with memory impairment is consistent with a similar correlation in APP/PS1 mice and with the notion that $A\beta_{42}$ is the predominant species giving rise to soluble $A\beta$ oligomers which profoundly disrupt synaptic plasticity and memory.⁶³

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Apart from soluble $A\beta$ species, levels of insoluble $A\beta_{40}$ ($R^2 = 0.36$; $**p < 0.01$) and $A\beta_{42}$ ($R^2 = 0.37$ $***p < 0.001$) in the cortex were found to correlate significantly with Morris water maze probe trial performance (Figures 4B, D and S5B, D). Treatment with VU0364572 abolished both insoluble $A\beta_{40}$ ($R^2 = 0.089$; n.s.) and $A\beta_{42}$ ($R^2 = 0.069$; n.s.) correlations with probe trial performance. There were no significant perturbations in soluble or insoluble $A\beta_{40/42}$ ratios in VU0364572-treated samples relative to controls (Figure S6).

Since soluble $oA\beta$ levels have been linked most directly to the memory-deficits and impairments in synaptic plasticity, we also evaluated the effects of chronic M_1 treatments on soluble $oA\beta$ in the brain. VU0364572 significantly lowered $oA\beta$ levels in the cortex (20.1% decrease; $*p < 0.05$, $t = 3.04$, $df = 11.77$), with a similar but nonsignificant trend observed in the hippocampus (Figure 5). These observations suggest that VU0364572 may improve memory, in part, by decreasing the size of the available $oA\beta$ pool in the brain.

DISCUSSION

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The M_1 receptor has long been regarded as a promising target for disease modification in AD. Many studies have described the ability of M_1 stimulation to exert nonamyloidogenic effects on APP processing in vitro, and M_1 knockout mice have demonstrated the importance of the M_1 receptor for regulating amyloid levels in vivo. However, a lack of truly selective M_1 activators that display druggable characteristics acceptable for in vivo dosing has hampered the investigation into whether chronic M_1 activation can prevent AD pathology and preserve memory by modifying underlying $A\beta$ pathology. While key proof of concept studies with M_1 activators to-date have demonstrated the disease-modifying effects of M_1 activation on $A\beta$ and tau pathologies, these studies were conducted with nonselective M_1 receptor ligands and were performed in mouse models of disease with a very mild phenotype (e.g., in 3xTg mice).^{39,64,65} Many treatments have advanced to the clinical trials after showing improvements in such animal models with mild disease phenotypes and so far none have translated into an effective treatment that slows or blocks the clinical progression of AD. Since the overproduction and accumulation of $A\beta$ species is widely viewed as a proximal pathological event in the course of AD, preventative efficacy in an aggressive rodent AD model may offer higher translational potential.

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The present work shows for the first time that chronic activation of M_1 with the selective M_1 agonist VU0364572 protects against neuropathology and prevents memory impairments from forming in an aggressive 5XFAD mouse model of AD. Specifically, chronic M_1

activation by VU0364572 from 2 to 6 months of age prevented development of memory impairments in 5XFAD transgenic AD mice, as measured by Morris water maze testing. Importantly, at 2 months of age, 5XFAD mice display little detectable amyloid, whereas at 6 months these mice are known to display significant amyloid accumulation along with impairments in the Morris water maze. The Morris water maze is a sensitive test of hippocampal-dependent memory function and deficits in this task are thought to model the disruption of memory encoding and recognition memory processes that occurs in humans with AD.

Chronic M₁ activation with VU0364572 was found to exert sustained and lasting benefits on lowering soluble and insoluble A β ₄₀ and A β ₄₂ levels and pathology in the cortex and hippocampus of 5XFAD mice, which translated to the prevention of hippocampal memory impairments in animals tested on the Morris water maze. The significant correlation of higher soluble A β ₄₂ levels with worse memory performance is consistent with recent findings on the role of soluble oA β species in AD. A number of studies have attributed the memory impairments that accompany early AD to the accumulation of oA β that has been shown to profoundly disrupt synaptic plasticity. The significant decrease in both cortical oA β levels as well as hippocampal A β ₄₂ pathology observed in our studies and the finding that VU0364572 abolishes the correlation between soluble A β ₄₂ levels and memory performance highlights the potential of M₁ activation by VU0364572 to lower the circulating levels of oA β that disrupt synaptic plasticity.

While improved performance was observed in the MWM, we observed no evidence of a VU0364572-mediated benefit in reversing contextual fear conditioning deficits at the dose tested. Most parsimoniously MWM, cued fear conditioning, and contextual fear conditioning are all distinct behavioral paradigms that depend upon partially overlapping but distinct brain circuitry, including the hippocampus. While the MWM is a sensitive test of hippocampal memory and cued fear conditioning is a sensitive test of amygdala function (with involvement of hippocampus also), it has become increasingly clear that interaction between hippocampus, amygdala and prefrontal cortex are crucial for other varieties of fear learning such as contextual (tested in the current study) and trace conditioning.⁶⁶ Although we do not have mechanistic data that directly speaks to engagement of these individual regions, it is possible that 5XFAD mice may exhibit greater prefrontal-hippocampal pathology that impairs contextual fear learning that the current degree of M₁ activation is unable to rescue at the dose and age tested. While M₁ receptors are expressed across all of the above brain regions, the levels are substantially higher in the amygdala and hippocampus than in the prefrontal cortex. In this vein, previous studies have demonstrated hippocampal efficacy of VU0364572 at doses below those required to elicit prefrontal efficacy.³⁸ Therefore, since the doses in the present study were based upon previously observed hippocampal MWM effects it may be that the dose was simply too low to result in beneficial effects on PFC-dependent processes. Future work in an independent cohort may help to confirm the present results and clarify these findings.

The observed efficacy of VU0364572 together with its novel pharmacological properties offer several advantages for clinical translation. Unlike conventional M₁ agonists that were extensively developed by the pharmaceutical industry to target the orthosteric acetylcholine

binding site, M₁ allosteric and bitopic agonists, including VU0364572, do not appear to couple as efficiently to the intracellular β -arrestin signaling cascades involved in desensitization of M₁ receptor signaling.^{22,38,67,68} This property is beneficial, as receptor desensitization limits the ability to dose treatments chronically, as is necessary for AD. Also, extensive molecular pharmacological characterization of VU0364572 reveals this compound has excellent selectivity for M₁, but rather low intrinsic activity. Thus, the present findings indicate that modest but selective M₁ activation with allosteric or bitopic ligands may be therapeutically beneficial. The lower intrinsic activity of a partial M₁ agonist would also reduce seizure liability and off-target side effects elicited by peripheral mAChR activation.

Our findings support further development of M₁ activators for early intervention for presymptomatic and prodromal AD. There is increasing recognition that the decades-long preclinical period of AD offers a large window for disease-modifying treatment. Unlike anti-amyloid treatments, which have substantial risk of amyloid-related vasogenic edema (ARIA-E), chronic M₁ treatment would not be expected to have this risk—an important consideration given that preventive treatments will be given to large numbers of asymptomatic individuals. Individuals with subjective memory loss, i.e., memory symptoms but no objective deficits on neuropsychological testing, and mild cognitive impairment, are also excellent groups to target for clinical translation of M₁ activators. Given there are currently no FDA-approved drugs with symptomatic benefits on memory for age-associated memory loss or for mild cognitive impairment, M₁ activators would provide urgently needed symptom relief and disease modification.

METHODS

Subjects for Transgenic AD Mouse Studies

5XFAD transgenic AD mice from Jackson Laboratories were utilized for this study.⁵⁷ The 5XFAD strain is a double transgenic APP/PS1 strain that expresses five AD mutations. These mice bear Florida, London, and Swedish familial AD mutations in the gene coding for APP. Additionally, these mice bear M146L and L286 V familial AD mutations in the PS1 gene. These mutations result in higher overall levels of A β , as well as increased production of A β ₄₂, the major plaque forming species in AD. The 5XFAD mice show intraneuronal A β ₄₂ and amyloid deposition beginning around 2 months of age, and memory deficits at 6 months.⁵⁸ All procedures involving mice were approved by the Emory University Institutional Animal Care and Use Committee. Mice were chronically dosed with VU0364572 ad libitum in drinking water for 4 months (from age 2 to 6 months). At 6 months, a 24 h drug washout was performed followed by behavioral testing. The 24 h washout period was deemed sufficient based upon previously published pharmacokinetic data demonstrating that VU0364572 has a half-life of 45 min.³⁷ Thus, no drug was onboard for any behavioral testing performed. Immediately after testing, the mice were perfused and brains were harvested, as described below.

A previously published study treated wild type animals with VU0364572 and showed a beneficial effect on memory in the Morris water maze,³⁸ indicating that indeed VU0364572 has procognitive symptomatic effects. Since this experiment has already been performed and

published, our primary goal was to model a treatment regimen as would be performed in humans. The prevention trials that seek to modify the course of disease begin treatment in individuals with existing amyloid pathology and/or at high risk for the disease (e.g., carrying genetic mutations). Thus, we only pursued drug treatment in transgenic animals carrying autosomal dominant mutations.

Drugs for Transgenic AD Mouse Studies

VU0364572 was synthesized as a mono-HCl salt and provided as a jet-milled powder to aid in drug solubilization and systemic absorption. VU0364572 was kindly provided by P. Jeffrey Conn and Craig Lindsley in the Vanderbilt Center for Neuroscience Drug Discovery (Vanderbilt University, Nashville, TN), and central penetrance verified as described in the Supporting Information and detailed in Table S1. Due to the documented oral bioavailability of VU0364572 in rat, drug was delivered to transgenic mice ad libitum in their drinking water (0.075 mg/mL concentration) from 2 months of age to 6 months of age. Mice had continuous access to drug-treated water at all times during this 4 month dosing window. Mouse cages were coded so that experimenters testing transgenic 5XFAD mice were blinded to treatment.

Determination of CNS VU0364572 Concentrations for Chronic Dosing

VU0364572 was delivered ad libitum to a group of six wild type B6SJL (same genetic background of 5XFAD mice) mice for 5 days. Calculation of the administered dose (approximately 10 mg/kg/day) was based upon the average weight of mice (30 g) and the average volume of drinking water a mouse was found to consume during a given 24 h period (4 mL; compound concentration = 0.075 mg/mL). With regard to the behavioral readouts in the current study, previously published data demonstrates that doses up to 56.6 mg/kg VU0364572 have no effect on baseline locomotor activity.³⁸ Following dosing to steady-state, mice were decapitated, brains removed and immediately washed with ice-cold phosphate-buffered saline, and brains then immediately frozen on dry ice until analysis. Trunk blood was immediately collected in EDTA Vacutainer tubes and plasma was separated by centrifugation and stored at -80°C until analysis. Bioanalysis of plasma and tissue samples to quantitate VU0364572 concentration was as described previously.^{36,37}

Biochemical Tissue Fractionation

Fresh-frozen sagittal hemibrains were removed from -80°C storage after which neocortex and total hippocampus were microdissected from each hemisphere. Wet tissue weight for each cortex and hippocampus sample was recorded and tissue homogenized using a Konte's Dounce tissue grinder in phosphate-buffered saline with $1\times$ protease inhibitor cocktail (Roche, Indianapolis, IN). Cortical samples were homogenized to a concentration of 150 mg/mL, whereas hippocampal samples were homogenized to a concentration of 100 mg/mL. Total homogenate was then sonicated for ~ 30 s using a microtip sonicator at 20% total amplitude. 2% SDS was then added to the homogenate in order to enable soluble amyloid extraction. Homogenates were spun for 1 h at $53\,000g$ at -4°C (Optima TLX Ultracentrifuge, Beckman-Coulter, Fullerton, CA) to separate soluble from insoluble amyloid species. The supernatant containing soluble amyloid was then collected and the pellet containing insoluble amyloid resuspended in 70% formic acid. Once resuspended, the

insoluble fraction was resonicated for ~30s at 20% total amplitude. Individual tissue fractions were analyzed fresh and never subjected to more than one freeze–thaw cycle. All brains were randomized prior to microdissection so that experimenters were blinded during downstream biochemical analyses (e.g., A β ₄₀ and A β ₄₂ ELISAs).

Immunohistochemistry

Sagittal hemibrains were removed from storage in 30% sucrose and serially sectioned at a thickness of 50 μ m on a freezing stage sliding microtome. Sections were immediately submerged in cryoprotectant and placed at –20 °C until analysis. For immunohistochemical staining, six consecutive tissue sections were taken from equivalent depths across all mice enrolled in the 5XFAD chronic dosing trial.

For immunohistochemical analysis, free-floating brain sections were then rinsed in 0.1 M phosphate buffer pH 7.2 (PB) 5 times \times 5 min. Next, PB was used to dilute 30% H₂O₂ (Sigma) to 3%. Tissue was washed with 3% H₂O₂ for 15 min in order to remove any endogenous peroxidase activity, after which the sections were again rinsed with PB 5 \times 5 min. Sections were then blocked with a solution of 10 μ g/mL avidin (1:1), 8% normal serum, and 0.1% Triton-X in TBS. Sections were blocked for 1 h at 4 °C, then rinsed with TBS 3 \times 5 min. Primary antibody incubation then took place in a solution of 50 μ g/mL biotin, 2% normal serum, and α -hA β _{1–40} (rabbit polyclonal, BioSource Invitrogen, Carlsbad, CA, 1:5000) or α -hA β _{1–42} (rabbit polyclonal, BioSource Invitrogen, Carlsbad, CA, 1:1000) in TBS. Incubation in the primary antibody solution took place for 48 h at 4 °C with shaking.

Following primary incubation, tissue was rinsed with TBS 4 \times 5 min. Sections were then incubated with a biotinylated secondary antibody (bG α Rb) for 3 h at 4 °C with shaking and washed again with TBS 4 \times 5 min. Secondary signal was then visualized using the avidin–biotin–peroxidase complex (ABC) method (ABC kit; Vector Laboratories, Burlingame, CA). ABC reagent was prepared according to the manufacturer’s instructions in TBS and allowed to stand on ice for 30 min prior to use. Sections were then incubated in the ABC solution for 1 h with shaking at 4 °C. Sections were then rinsed 4 \times 5 min with TBS and stained with diaminobenzidine (DAB). Following DAB staining, tissue was removed and rinsed with TBS 4 \times 5 min. Brain sections were then mounted on precleaned Superfrost Plus slides (Fisher) in 0.1 M NaNO₃. Slides were then allowed to air-dry after which they were then immersed in dH₂O 3 min, 70% ethanol 3 min, 95% ethanol 2 \times 3 min, 100% ethanol 2 \times 3 min, and Histoclear 3 \times 3 min. Slides were then coverslipped with DPX and allowed to dry overnight.

To quantify hA β ₄₀ and hA β ₄₂ immunoreactivity, cortex and hippocampus were photographed at low power (4 \times) from each of six consecutive sections stained for either hA β ₄₀ or hA β ₄₂. Total A β ₄₀ and A β ₄₂ immunoreactive surface area was then measured in a blinded manner using MetaMorph 5.0 software (Molecular Devices).

A β ₄₀, A β ₄₂, and oA β ELISAs

hA β ₄₀ and hA β ₄₂ levels in conditioned media from primary neuronal cultures as well as soluble and insoluble A β ₄₀ and A β ₄₂ tissue homogenates from biochemically fractionated

mouse brains were measured using human A β ₄₀ and A β ₄₂ ELISA kits according to the manufacturer's protocols (Biosource, Invitrogen). Insoluble amyloid fractions from mouse tissue homogenates containing 70% formic acid were first neutralized by performing a 1:100 dilution in a solution of 1.0 M Tris (pH 11) prior to performing dilution series in ELISA diluent buffer supplied with ELISA kits. Soluble amyloid fractions were diluted as normal directly in ELISA diluent buffer. Plates were read at 450 nm using a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA). ELISA-based detection of oA β was performed as described previously.⁵⁹ For detection of oA β , total tissue homogenates in PBS were utilized for oA β ELISAs, as the addition of detergent is known to induce artifactual A β oligomerization. Moreover, albuminized tubes were used for the handling of total tissue homogenates to block nonspecific oA β binding sites on the plastic tubes.

Statistical Analyses

For all behavioral, primary culture data and ELISA data, graphs were generated using GraphPad Prism 5.0. Due to the small and unequal sample sizes of the study, as well as to account for the likelihood of different population variances, Welch's unequal variance *t*-test was used.^{60,61} All *p*-values reported for these tests are two-tailed, Welch's-corrected, *p*-values. Due to the small number of subjects in the drug treatment trial of 5XFAD mice, outlier detection/exclusion was performed using a test of Median Absolute Deviation (MAD), which is less biased by any outliers themselves than tests which rely upon standard deviations from the mean.⁶² Samples with a MAD ≥ 2.0 were excluded as outliers. For MWM data, fear-conditioning data, and immunohistochemistry data, one animal was excluded from the vehicle-treated WT group and one animal from the vehicle-treated 5XFAD group. For the soluble and insoluble A β ₄₀ and A β ₄₂ ELISA data and subsequent correlations with MWM data, two animals from the Ctx-Veh group were excluded, four animals from the Ctx-M₁ group, two animals from the Hp-Veh group, and four animals from the Hp-M₁ group. For oA β data, three animals were excluded from the Ctx-Veh group, three animals from the Ctx-M₁ group, three animals from the Hp-Veh group, and two animals from the Hp-M₁ group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Aβ	amyloid beta
AChEI	acetylcholinesterase inhibitor
ACh	acetylcholine
AD	Alzheimer's disease
APP	amyloid precursor protein
Ctx	cortex
ELISA	enzyme-linked immunosorbent assay
GPCR	G protein-coupled receptor
Hp	hippocampus
mAChR	muscarinic acetylcholine receptor
MWM	Morris water maze
oAβ	oligomeric amyloid beta
PS1	presenilin 1
sAPP	soluble amyloid precursor protein
WT	wild type

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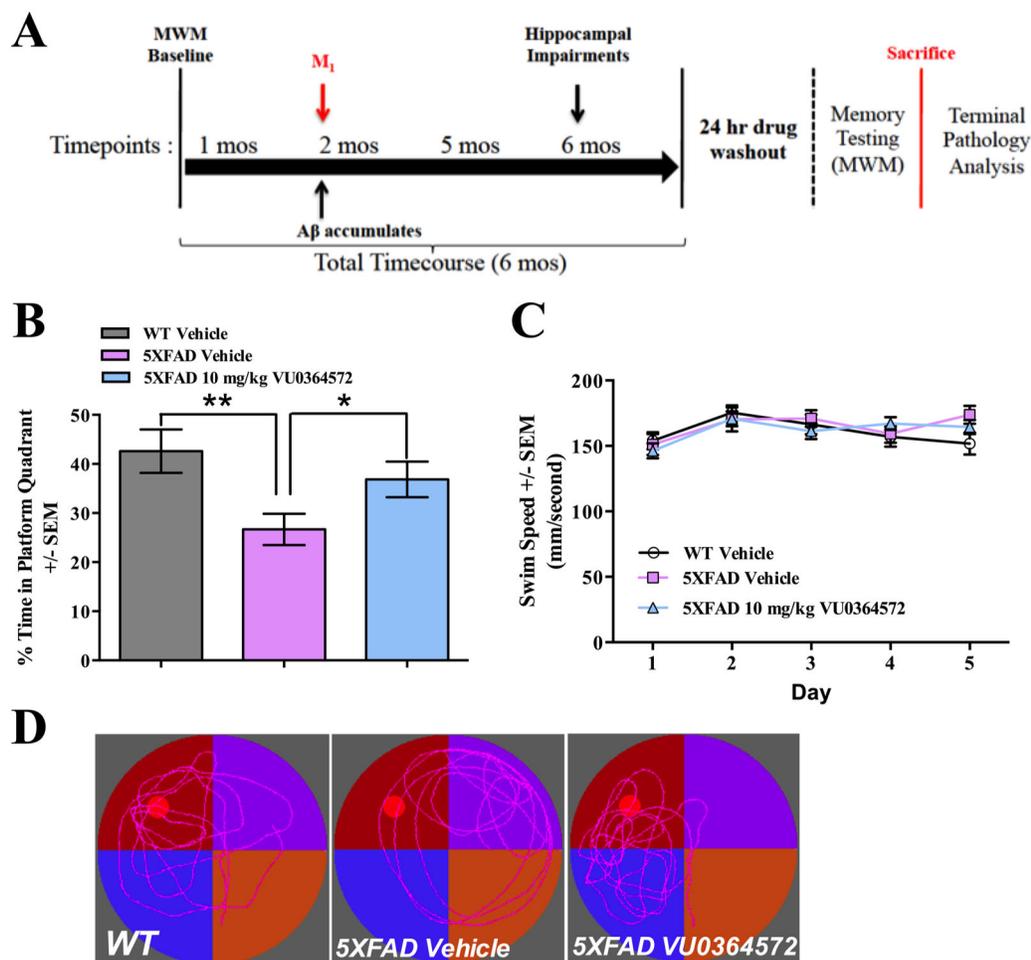


Figure 1.

M₁ Activation by VU0364572 preserves hippocampal memory when dosed chronically in 5XFAD transgenic Alzheimer's mice. VU0364572 preserves memory when dosed chronically prior to the initiation of Morris water maze training, but does not benefit nonmemory measures of Morris water maze performance, such as swim speed. (A) Schematic depicting design of chronic treatment trial of 5XFAD mice with M₁ agonist, VU0364572. (B) Following dosing VU0364572 at 10 mg/kg to 5XFAD mice for 4 months, drug-treated mice were significantly improved in their performance (36.7% time in probe quadrant) on the probe trial of the Morris water maze relative to vehicle-treated (26.7% time in probe quadrant) 5XFAD mice (**p* < 0.05, *t* = 2.11, *df* = 24.49). 5XFAD mice were found to be significantly impaired relative to vehicle-treated WT littermate controls (***p* < 0.01, *t* = 2.93, *df* = 26.17). (C) VU0364572 does not influence swim speed of animals during training. No statistically significant difference was observed during training in swim speed for either WT mice (*N* = 13), 5XFAD vehicle-treated mice (*N* = 17), or 5XFAD mice receiving 10 mg/kg VU0364572 (*N* = 12). Error bars show ± SEM across all mice within a treatment group. (D) Representative swim paths depicting probe trial performance of WT littermate controls (left), 5XFAD vehicle-treated mice (center), and drug-treated 5XFAD mice (right) with maze quadrants depicted as red, purple, blue, and orange quadrants,

respectively, and the submerged platform location (platform not present for probe trial) depicted as a red dot.

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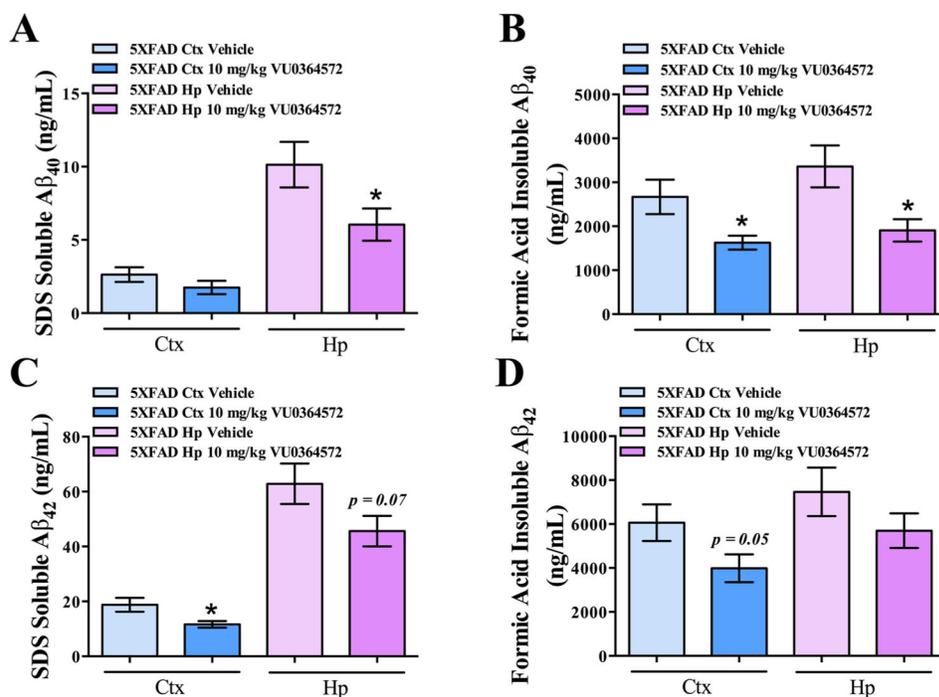


Figure 2.

M₁ activation by VU0364572 decreases soluble and insoluble Aβ₄₀ and Aβ₄₂ levels in cortex and hippocampus of 5XFAD mice. Total cortex and hippocampus were microdissected from 5XFAD animals and WT littermate controls and biochemically fractionated. Fractions were then subjected to Aβ₄₀ and Aβ₄₂ ELISA analysis. (A) VU0364572 significantly decreases soluble Aβ₄₀ levels in the hippocampus of 5XFAD mice by 40.4% relative to vehicle-treated 5XFAD animals (**p* < 0.05, *t* = 2.15, *df* = 24.55). No significant decrease of soluble Aβ₄₀ was observed in the cortex. (B) VU0364572 significantly decreases insoluble Aβ₄₀ levels in both the neocortex (38.9%; **p* < 0.05, *t* = 2.47, *df* = 19.60) and hippocampus (43.3%; **p* < 0.05, *t* = 2.69, *df* = 22.35) of 5XFAD mice relative to vehicle-treated 5XFAD animals. (C) VU0364572 significantly decreases soluble Aβ₄₂ levels in both the cortex (34.2%; **p* < 0.01, *t* = 2.54, *df* = 20.68) with a trend toward a decrease in the hippocampus (23.4%; *p* < 0.1, *t* = 1.87, *df* = 25.66) of 5XFAD mice relative to vehicle-treated 5XFAD animals. (D) VU0364572 significantly decreases insoluble Aβ₄₂ levels in the cortex of 5XFAD mice relative to vehicle-treated 5XFAD animals (34.2%; *p* = 0.05, *t* = 1.98, *df* = 25.62), with no significant decrease in hippocampus (23.4%; n.s., *p* > 0.05, *t* = 1.30, *df* = 25.21). Error bars show ± SEM across all mice within a treatment group.

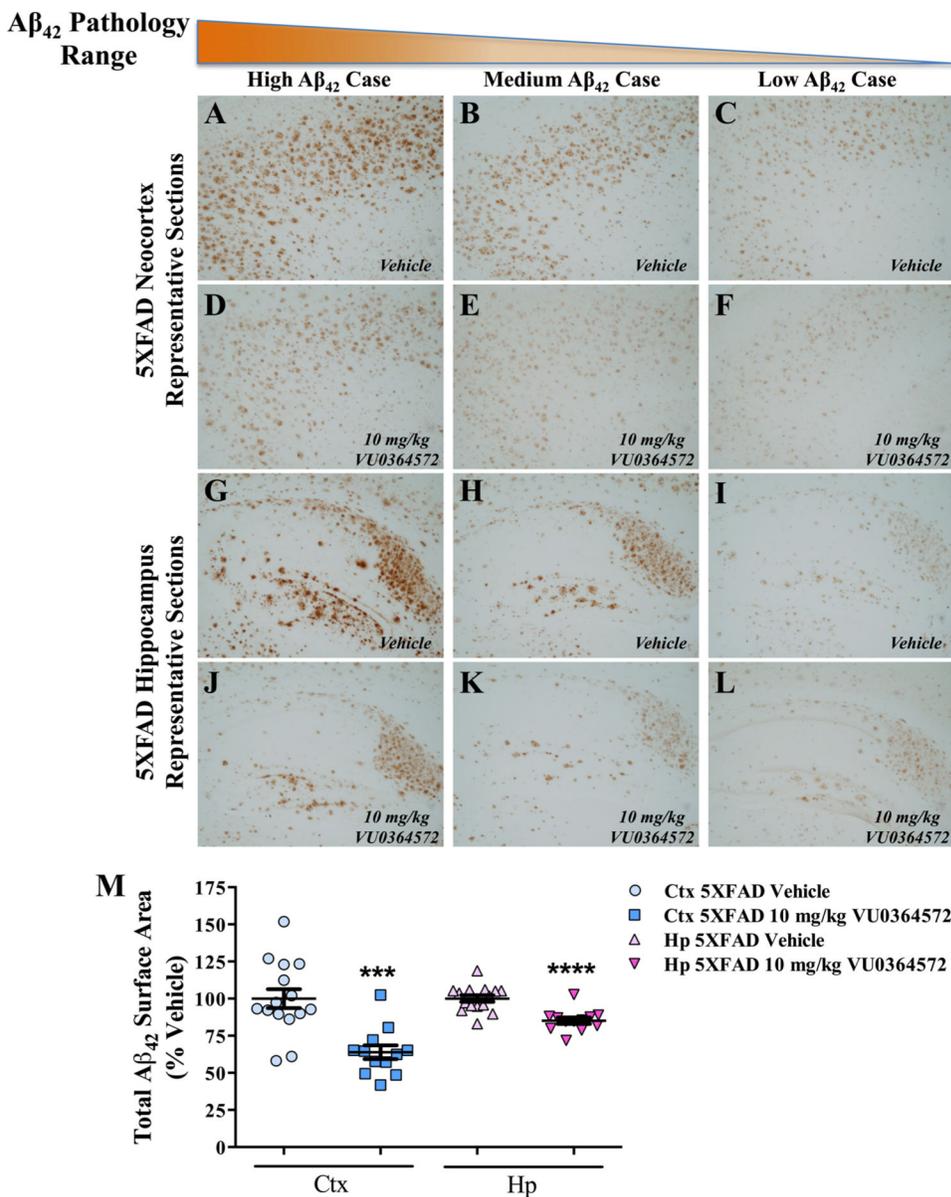


Figure 3.

M₁ Activation by VU0364572 reduces A β_{42} neuropathology in the brains of 5XFAD mice. Serial sections at 50 μ m were taken from 5XFAD mice and WT littermate controls, and six sections per animal were then subjected to A β_{42} immunohistochemical analysis with anti-human A β_{42} antibody (1:1000; Biosource) in order to examine A β_{42} pathology in the cortex and hippocampus. Vertical columns show representative sections for cases displaying high (A, D, G, J), medium (B, E, H, K), and low (C, F, I, L) A β_{42} pathology. Horizontal rows denote cortical (A–F) and hippocampal (G–L) sections for both vehicle-treated (A–C, G–I) animals and VU0364572-treated animals (D–F, J–L). 5XFAD vehicle-treated animals show marked cortical (A–C) and hippocampal (G–I) A β_{42} pathology that is substantially mitigated by VU0364572 treatment across the neocortex (D–F) and all hippocampal subfields (J–L). (M) Total A β_{42} immunoreactive surface area was measured for 6

consecutive slices taken at the same anterior-posterior coordinates per animal for cortical and hippocampal regions, with the mean immunoreactive surface area plotted for individual animals. 5XFAD vehicle-treated animals showed robust $A\beta_{42}$ pathology that is significantly mitigated by VU0364572 treatment in both the neocortex (36.1% decrease; *** $p < 0.001$, $t = 4.58$, $df = 24.18$) and hippocampus (14.9% decrease; **** $p < 0.0001$, $t = 4.82$, $df = 24.88$). Error bars show \pm SEM across all mice within a treatment group.

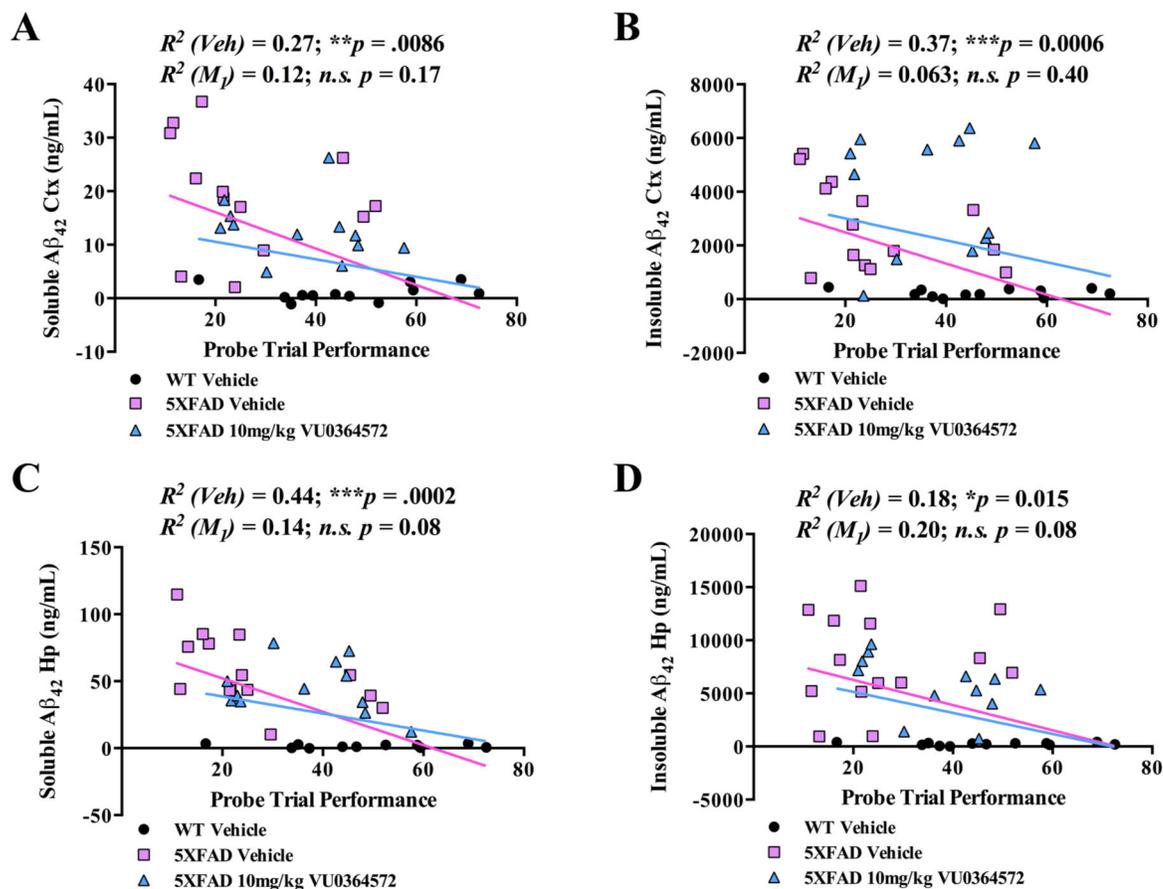


Figure 4.

$A\beta_{42}$ Levels in the hippocampus and neocortex are correlated with memory performance. Behavioral performance of 5XFAD mice on the probe trial of the Morris water maze was correlated with soluble (A, C) and insoluble (B, D) $A\beta_{42}$ levels from the cortex (A, B) and hippocampus (C, D) of these mice. (A) The strongest correlation was observed between soluble hippocampal $A\beta_{42}$ levels and probe trial performance in the Morris water maze (C), which was very highly significant and the strongest correlation observed in the study for any $A\beta_{40}$ or $A\beta_{42}$ species with memory performance ($R^2 = 0.44$; $***p < 0.001$). (B) There was also a strong correlation (B) noted between insoluble cortical $A\beta_{42}$ levels and probe trial performance ($R^2 = 0.37$; $***p < 0.001$). While significant, correlations between soluble cortical $A\beta_{42}$ levels and insoluble hippocampal $A\beta_{42}$ levels were substantially less robust (A, D). All correlations reflect the inclusion of WT animals that received vehicle alone.

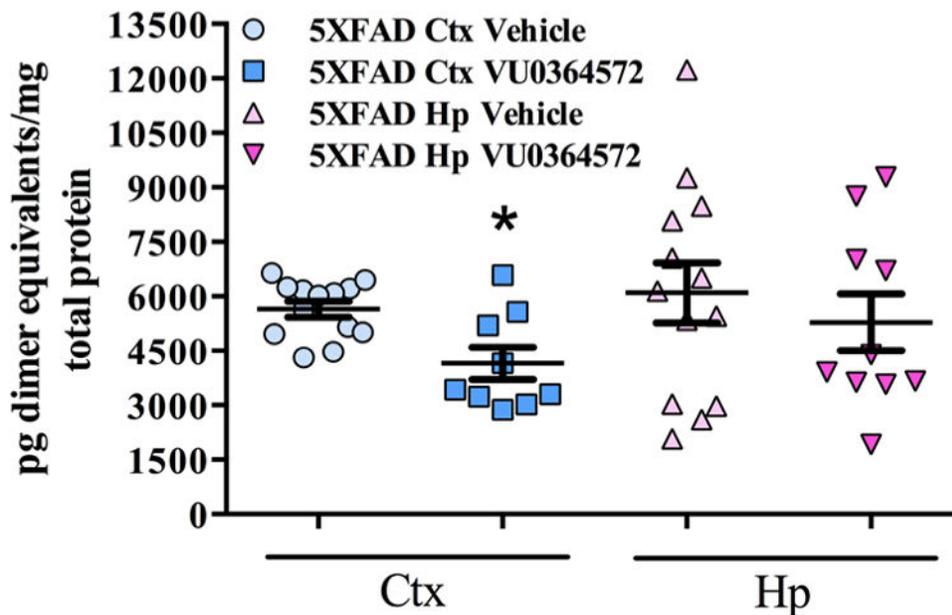


Figure 5.

M₁ Activation by VU0364572 reduces oligomeric $\alpha\beta$ neuropathology in the cortex of 5XFAD mice. Total cortex (neocortex + entorhinal cortex) and hippocampus were microdissected from 5XFAD animals and WT littermate controls and biochemically fractionated. Soluble A β fractions were then subjected to ELISA analysis for oA β detection as described previously.⁵⁹ VU0364572 was found to significantly decrease oA β levels in the cortex (20.1% decrease; * $p < 0.05$, $t = 3.04$, $df = 11.77$), however, hippocampal samples did not reach significance, as much more variability was present. Error bars show \pm SEM across all mice within a treatment group.