Supplementary Information

Materials and Methods

Animal Perfusion

Mice were deeply anesthetized by delivering an overdose of sodium pentobarbital i.p. and monitored until no longer responsive to a toe pinch. Animals were then transcardially-perfused with ice-cold normal saline for 4 minutes at a flow rate of 10 mL/min. Following perfusion, the brain was cut sagittally along the midline. One hemisphere was immediately snap-frozen on dry ice for biochemical analysis and placed at -80 ^{o}C until analysis. The other hemisphere was immersion-fixed in 4% paraformaldehyde for 4 hr followed by cryoprotection in 30% sucrose until tissue sectioning.

Morris Water Maze

Morris water maze training took place in a round, water-filled tub (52 inch diameter) in an environment rich with ambient cues in the testing room visible to animals navigating the maze. These ambient cues help facilitate the ability of animals to navigate the maze. Mice were placed in the water maze with their paws touching the wall from 4 different starting position (N, S, E, W) in water that started at 25 °C and typically declined to 22 °C by the time a whole group of mice was tested. An invisible escape platform was located in the same spatial location 1 cm below the water surface independent of a subject's start position on a particular trial. In this manner subjects are able to utilize ambient testing room cues to determine the submerged platform's location. Each subject was given 4 trials/day for 5 days with a 15-min intertrial interval. The maximum trial length was 60 s and if subjects did not reach the platform in the allotted time, they were manually guided to it. At the end of each day of testing, water was drained and the tank was cleaned with quatracide.

Upon reaching the invisible escape platform, subjects were left on it for an additional 5 s to allow for survey of the spatial cues in the environment to guide future navigation to the platform. After each trial subjects were dried and kept in a dry plastic holding cage filled with paper towels, which allowed the subjects to dry off. Following the 5 days of task acquisition, a probe trial was presented during which time the platform was removed and the amount of time and distance swam in the quadrant which previously contained the escape platform during task acquisition was measured over 60 s. All trials will be videotaped and performance analyzed by means of MazeScan (Clever Sys, Inc.).

Cued and Contextual Fear Conditioning

Cued and contextual fear conditioning are tasks which measure the ability of a rodent to form and retain an association between an aversive experience and environmental cues. This task requires a slightly different set of sensory and motor abilities than the water maze and can be performed in a much more time efficient manner. While contextual fear conditioning tests primarily hippocampal circuitry, cued fear conditioning tests both hippocampal and amygdala circuitry.

Fear conditioning took place over a period of three days. Animals were placed in the fear conditioning apparatus (Colbourn) and allowed to explore the enclosure for 3 min. Following this habituation period 3 conditioned stimulus (CS)-unconditioned stimulus (US) pairings were presented with a 1 min intertrial interval. The CS consisted of a 20 second 85 db tone and US consisted of 2 seconds of a 0.5 mA foot shock which co-terminated with each CS presentation. One minute following the last CS-US presentation animals were returned to their home cage. On day 2 the animals were presented with a context test during which subjects were placed in the same chamber used during conditioning on Day 1, and the amount of freezing was

recorded via a camera and the software provided by Colbourn. On day 3, a tone test was presented during which time subjects were exposed to the CS in a novel compartment. Initially animals were allowed to explore the novel context for 2 min. Following this habituation period the 85 db tone was presented for 6-min and the amount of freezing behavior recorded. Data was binned into 60s time bins and analyzed via a two-way repeated measures ANOVA with a Bonferroni correction for multiple comparisons applied.

Primary Neuron Culture

Cortical and hippocampal neurons were isolated from mouse pups harvested from an E18 pregnant C57Bl/6 dam. Embryos were dissected and then cortical hemispheres and hippocampi were isolated in dissection buffer (Hanks Balanced Salt Solution (HBSS), 10 mM HEPES, 1 penicillin/streptomycin). The cortices and hippocampi were then pooled in separate tubes, whereupon they were digested with 0.25% trypsin (Gibco) and 0.01% deoxyribonuclease in dissection buffer for 15 min at 37^{o}C. Following digestion, the tissue was rinsed gently twice with dissection buffer and twice with plating media (buffered MEM (Gibco), 0.6% glucose (Gibco), 2 mM L-glutamine (Cellgro), 10% heat-inactivated horse serum, and 1% penicillin/streptomycin). After rinsing, the tissue was then mechanically-dissociated with large bore and small bore fire-polished Pasteur pipettes. The tissue was triturated 10 times with a large bore pipette, followed by another 10 times with a small bore pipette.

Once dissociated, the neurons were seeded on 12-well poly-L-lysine plates in a volume of 1 mL high-glucose plating media at 80,000-100,000 cells/cm^2 for 2.5 hours[1,2]. Following seeding, an additional 2.5 mL of neurobasal (Gibco) growth media was added gently to the wells containing B-27 growth supplement (Gibco), 2 mM L-glutamine, and 1% penicillin/streptomycin at 37^{o}C, 5% CO_2. On day 2 in vitro, the neurons were then transduced with a human amyloid

precursor protein (hAPP) cassette using lentiviral delivery for 72 hours. Following transduction, the cells were treated with a vehicle and a dose response of VU0364572 (10μM, and 30μM). These cells were incubated in neuronal growth media containing drug for 16 hours and then cell lysates and conditioned media containing extracellular Aβ were collected, as previously described[1,2].

Lentiviruses expressing $hAPP^{695}$ were packaged by calcium phosphate triple transfection of HEK293FT cells with the transgene/FUW cassette Δ8.9 HIV-1 packaging vector and pVSVG envelope glycoprotein. High titer (~1 x 10^9 infectious virus particles/mL) virus was then used to transduce primary neurons.

Figure S1. M_1 Activation by VU0364572 Does not Rescue Deficits in Contextual Fear Conditioning in 5XFAD Mice.

5XFAD mice dosed with VU0364572 show no benefit or decrement in either cued or contextual fear conditioning. **A)** At 6 months of age a significant deficit was observed in vehicle-treated 5XFAD animals in contextual fear conditioning test of memory, however, no significant improvement was observed following 4 months of dosing VU0364572 at approximately 10 mg/kg/day. Drug was allowed to completely wash out for a period of at least 24 hours prior to behavioral testing so there was no drug on board the animals during testing. **B)** On the other hand, no significant deficit was observed in cued fear conditioning in 5XFAD vehicle-treated transgenic animals at 6 months of age. There was similarly no significant effect of VU0364572 treatment on 5XFAD animals relative to WT littermate controls. Error bars show ± SEM across 60-second time bins for all mice within a treatment group.

Figure S2. M_1 Activation by VU0364572 Lowers $A\beta_{40/42}$ Levels in Mouse Primary Cortical and Hippocampal Neurons.

M_1 activation with VU0364572 was shown to decrease markers of both $A\beta_{40}$ and $A\beta_{42}$ pathology in primary rat cortical and hippocampal neurons *in vitro* to provide proof-of-concept for subsequent *in vivo* experiments. **A)** M_1 activation by VU0364572 at 30 μM was found to dose-dependently decrease $A\beta_{40}$ in conditioned media harvested from E18 primary mouse hippocampal neurons transduced with $hAPP^{695WT}$ (* $p < 0.05$). **B)** 10 μM and 30 μM VU0364572 were found to dose-dependently decrease $A\beta_{40}$ in conditioned media harvested from E18 primary mouse cortical neurons transduced with $hAPP^{695WT}$ (* $p < 0.05$; ** $p < 0.01$). **C)** 30 μM VU0364572 was found to dose-dependently decrease $A\beta_{42}$ in conditioned media harvested from E18 primary mouse cortical neurons transduced with $hAPP^{695WT}$ (** $p < 0.01$), but not from hippocampal neurons **(D)**. Error bars show ± SEM across triplicate samples for each drug condition.

Figure S3. M_1 Activation by VU0364572 Reduces $A\beta_{40}$ Neuropathology in the Neocortex and Hippocampus of 5XFAD Mice.

Serial sections at 50 μm were taken from 5XFAD mice and WT littermate controls and 6 sections per animal were then subjected to $A\beta_{40}$ immunohistochemical analysis with anti human $A\beta_{40}$ antibody (1:5000; Biosource) in order to examine $A\beta_{40}$ 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)** $A\beta_{40}$ pathology. Horizontal rows denote cortical **(A-F)** and hippocampal **(G-L)** sections for both vehicle-treated animals **(A-C**, **G-I)** and VU0364572-treated animals **(D-F**, **J-L)**. 5XFAD vehicle-treated animals show marked cortical

(A-C) and hippocampal **(G-I)** $A\beta_{40}$ pathology that is substantially mitigated by VU0364572 treatment across the neocortex **(D-F)** and all hippocampal subfields **(J-L)**. **(M)** Total $A\beta_{40}$ 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 $A\beta_{40}$ pathology that is significantly mitigated by VU0364572 treatment in both the cortex (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). Error bars show ± SEM across all mice within a treatment group. Taken together, these results strongly indicate that VU0364572 exerts sustained, disease-modifying effects on underlying Aβ neuropathology in 5XFAD mice and that these beneficial effects occur in all areas of the hippocampus and cortex known to be impacted by AD.

Figure S4. Representative sections from 5XFAD WT control animals show no detectable $A\beta_{40}$ or $A\beta_{42}$ pathology as compared to 5XFAD vehicle-treated animals or VU0364572-treated animals.

Serial sections at 50 μm were taken from 5XFAD mice and WT littermate controls and 6 sections per animal were then subjected to $A\beta_{40}$ immunohistochemical analysis with **A)** anti human $A\beta_{40}$ antibody (1:5000; Biosource) or **B)** $A\beta_{42}$ antibody (1:1000; Biosource) in order to examine $A\beta_{40}$ pathology in the cortex and hippocampus (representative sections from hippocampus shown). In both cases, all tissue sections from wild-type littermate controls was completely devoid of immunoreactivity for either $A\beta_{40}$ or $A\beta_{42}$.

Figure S5. $A\beta_{40}$ levels are correlated with memory impairment in 5XFAD mice.

The behavioral performance of 5XFAD mice on the probe trial of the Morris water maze was correlated with soluble and insoluble $A\beta_{40}$ levels from the cortex and hippocampus of mice treated with 10 mg/kg VU0364572 versus vehicle-treated 5XFAD mice and WT littermate controls. The strongest correlation was observed between insoluble hippocampal $A\beta_{40}$ levels and probe trial performance **(B)**, where higher insoluble cortical $A\beta_{40}$ levels correlated with worse probe trial performance ($R^2 = 0.36$; $^{**}p < 0.01$). Furthermore, the correlation in **B** was abolished by drug treatment with VU0364572, providing further evidence for the ability of M_1 activation to guard against the onset of disease pathology and memory impairments ($R^2 = 0.089$; n.s.). While significant, correlations between soluble $A\beta_{40}$ cortical levels, soluble hippocampal $A\beta_{40}$ levels, and insoluble hippocampal $A\beta_{40}$ levels were all substantially less robust **(A, C, D)**. All correlations reflect the inclusion of WT animals that received vehicle alone.

Figure S6. $A\beta_{40/42}$ ratios are unchanged by VU0364572 administration as tested by ELISA.

Therapeutic effects appeared evenly split across $A\beta_{40}$ and $A\beta_{42}$ species and thus, no significant perturbations in $A\beta_{40/42}$ ratios were noted in VU0364572-treated samples relative to vehicle-treated 5XFAD animals, as expected. Error bars show ± SEM across all mice within a treatment group.

Table S1. Satellite pharmacokinetic (PK) study to verify compound exposure from ad lib. oral dosing.

VU0364572 was delivered ad lib. to wild type (N = 6) B6SJL mice for 5 days. Calculation of the administered dose (approximately 10 mg/kg/day) was based upon the average weight of

mice (30 grams) and the average volume of drinking water a mouse was found to consume during a given 24 hour period (4 mL; compound concentration = 0.075 mg/mL). Average brain levels of VU0364572 were found to be 57 ng/mL. Since the effective $t_{1/2}$ of VU0364572 in rat is rather short (46 min), it bears mentioning that animals 1-3 were housed in one cage for dosing and animals 4-6 in a separate cage. Thus, cage-specific activity levels of animals close to the time point of sacrifice is likely to heavily influence the concentration of drug detected on-board the animals, suggesting animals 4-6 more accurately approximate the maximal daily brain levels of VU0364572 achieved by ad lib. chronic dosing. Taking the data for animals 4-6 yields a 103 ng/mL brain concentration of VU0364572. Bioanalysis of plasma and tissue samples to quantitate VU0364572 concentration was as described previously[3,4].

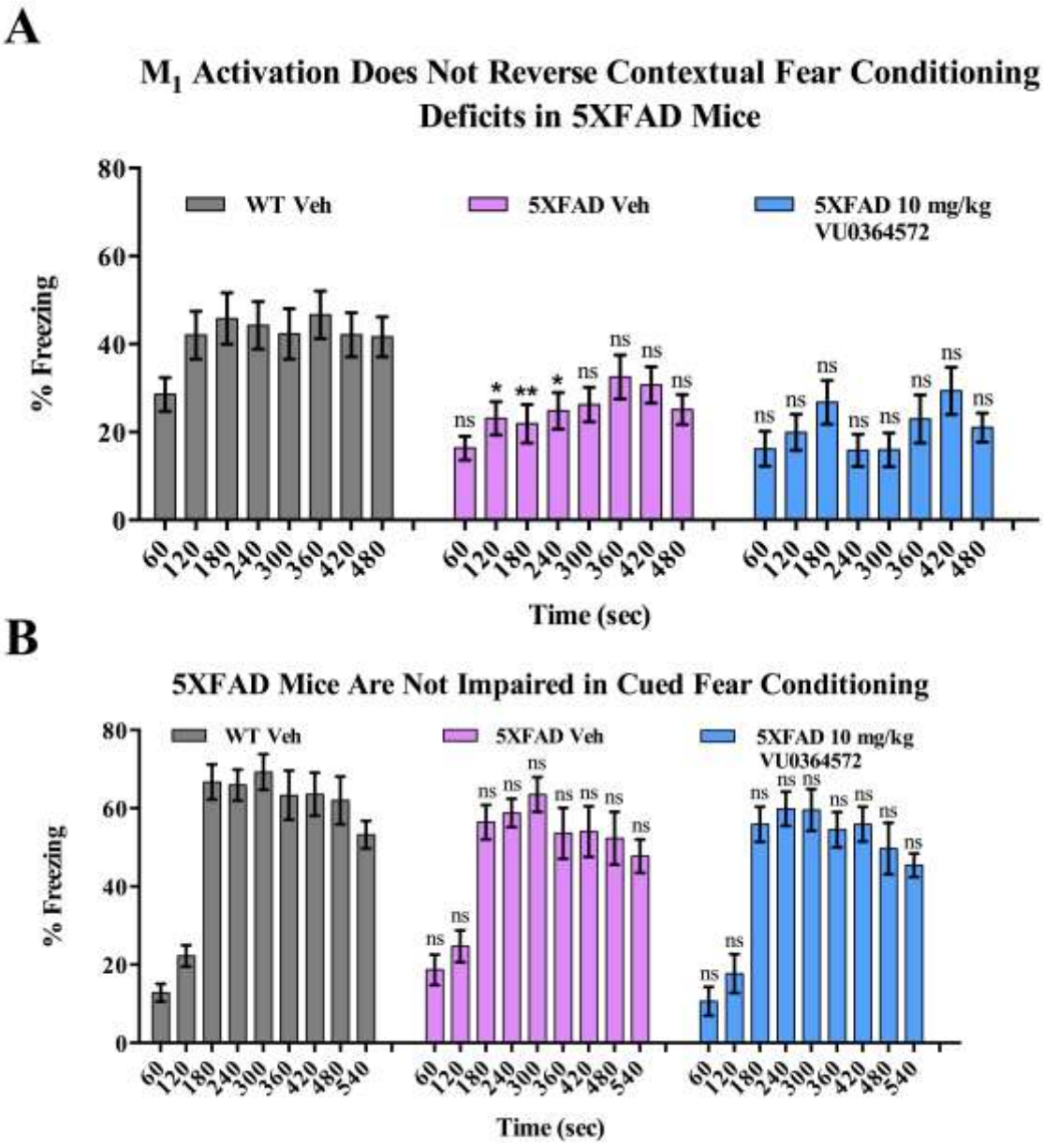
A
M1 Activation Does Not Reverse Contextual Fear Conditioning Deficits in 5XFAD Mice
WT Veh
5XFAD Veh
5XFAD 10 mg/kg VU0364572
% Freezing
Time (sec)
B
5XFAD Mice Are Not Impaired in Cued Fear Conditioning
WT Veh
5XFAD Veh
5XFAD 10 mg/kg VU0364572
% Freezing
Time (sec)

Figure S1.

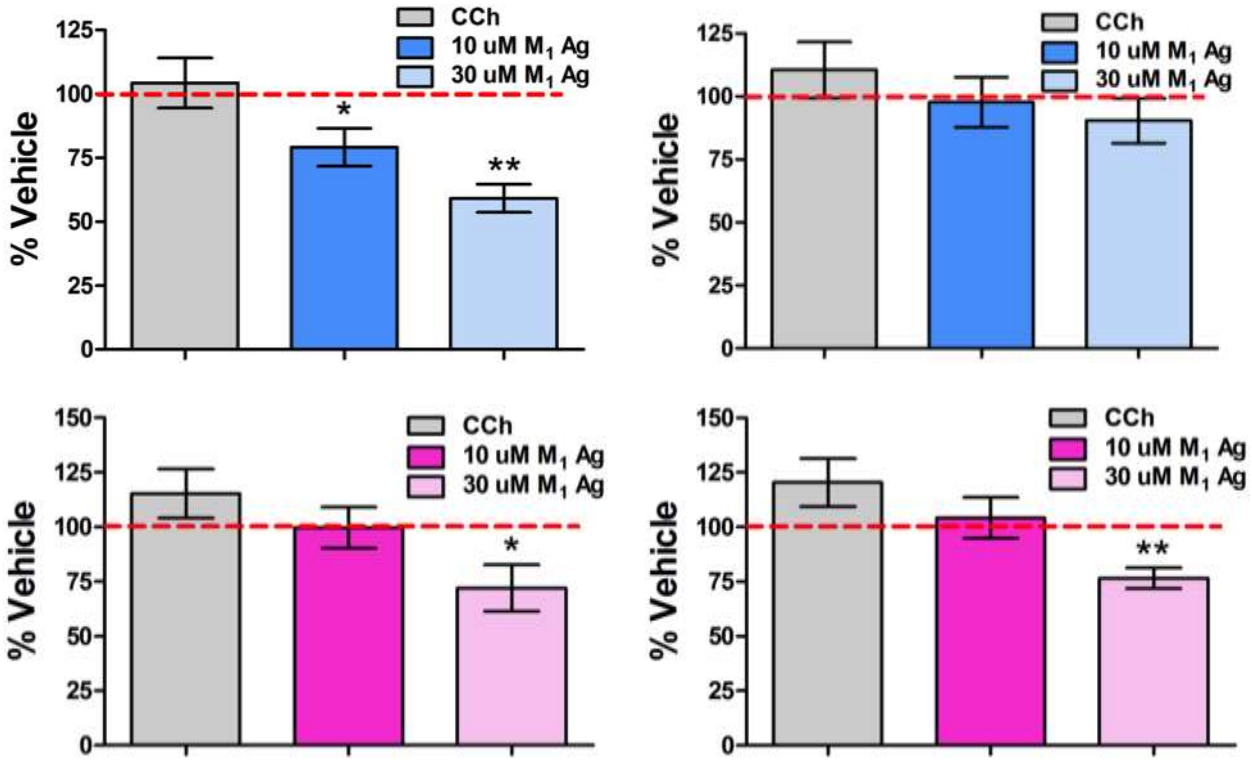

Figure S2.

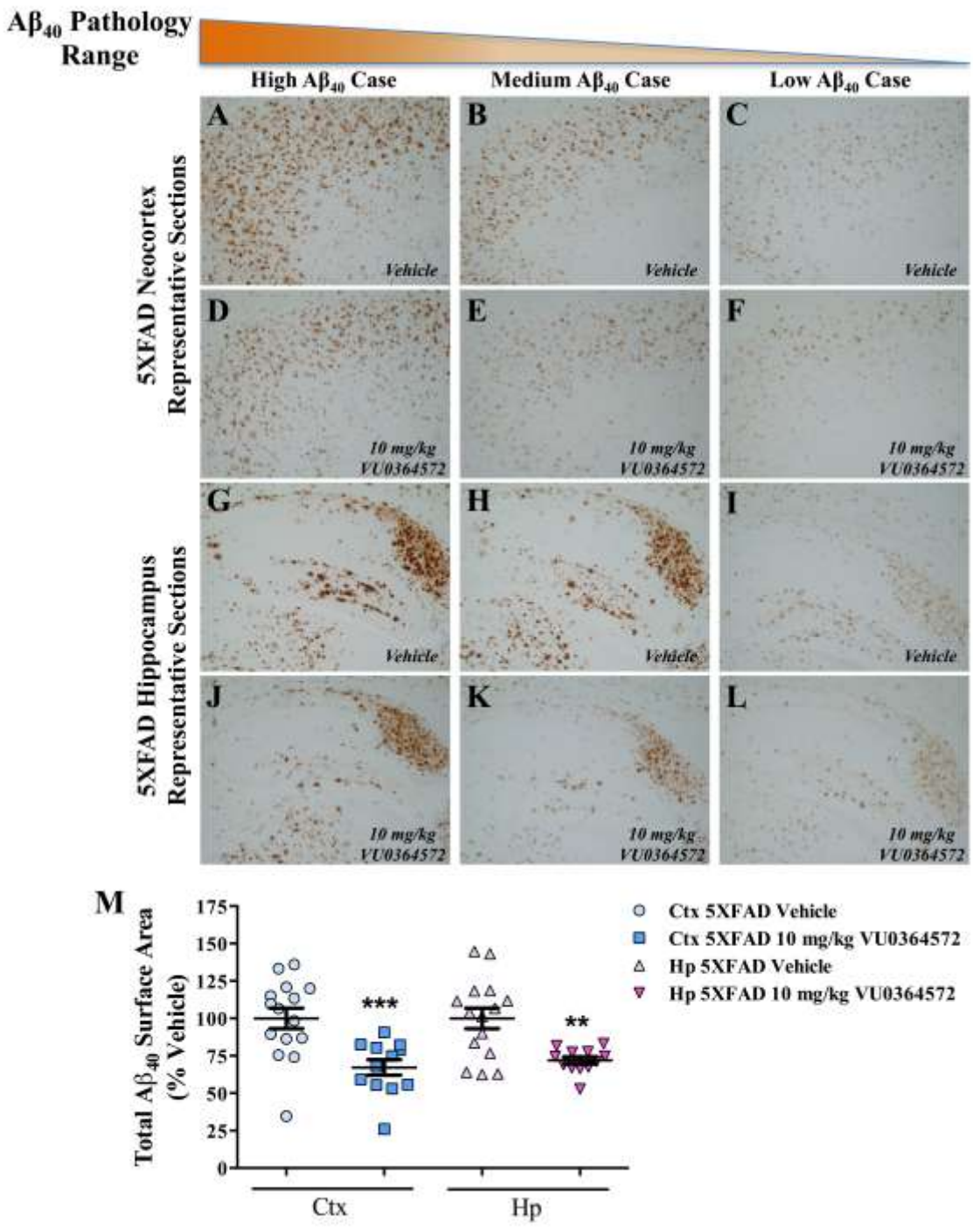
Aβ40 Pathology Range
High Aβ40 Case
Medium Aβ40 Case
Low Aβ40 Case
5XFAD Neocortex Representative Sections
5XFAD Hippocampus Representative Sections
A
B
C
D
E
F
G
H
I
J
K
L
Vehicle
10 mg/kg VU0364572
M
Total Aβ40 Surface Area (% Vehicle)
175
150
125
100
75
50
25
0
***
**
Ctx
Hp
Ctx 5XFAD Vehicle
Ctx 5XFAD 10 mg/kg VU0364572
Hp 5XFAD Vehicle
Hp 5XFAD 10 mg/kg VU0364572

Figure S3.

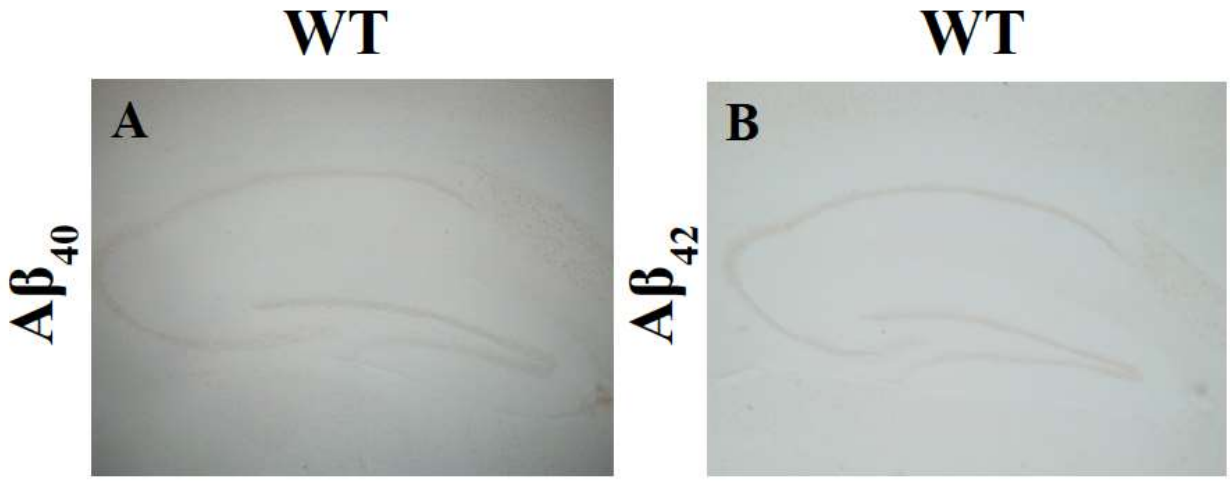

Figure S4.

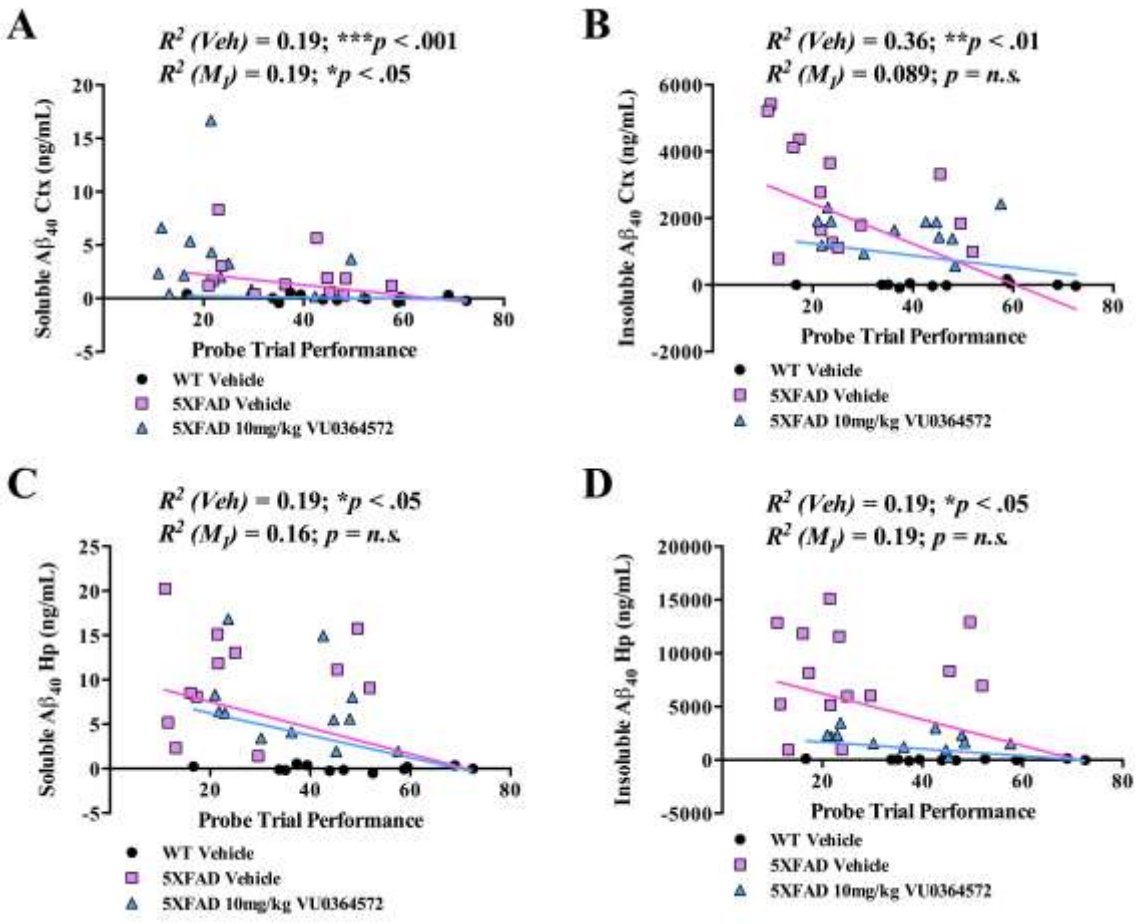
A
R^2 (Veh) = 0.19; ***p < .001
R^2 (M_1) = 0.19; *p < .05
Soluble $A\beta_{40}$ Ctx (ng/mL)
Probe Trial Performance
WT Vehicle
5XFAD Vehicle
5XFAD 10mg/kg VU0364572
B
R^2 (Veh) = 0.36; **p < .01
R^2 (M_1) = 0.089; p = n.s.
Insoluble $A\beta_{40}$ Ctx (ng/mL)
Probe Trial Performance
WT Vehicle
5XFAD Vehicle
5XFAD 10mg/kg VU0364572
C
R^2 (Veh) = 0.19; *p < .05
R^2 (M_1) = 0.16; p = n.s.
Soluble $A\beta_{40}$ Hp (ng/mL)
Probe Trial Performance
WT Vehicle
5XFAD Vehicle
5XFAD 10mg/kg VU0364572
D
R^2 (Veh) = 0.19; *p < .05
R^2 (M_1) = 0.19; p = n.s.
Insoluble $A\beta_{40}$ Hp (ng/mL)
Probe Trial Performance
WT Vehicle
5XFAD Vehicle
5XFAD 10mg/kg VU0364572

Figure S5.

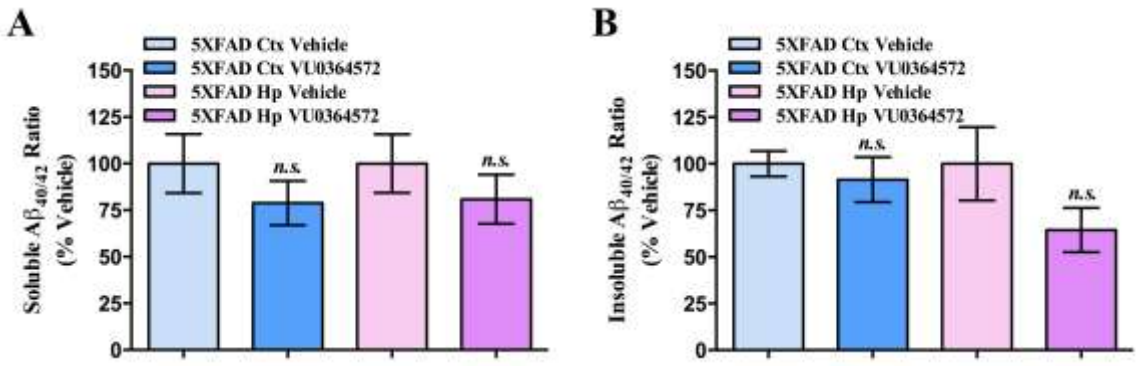
A
5XFAD Ctx Vehicle
5XFAD Ctx VU0364572
5XFAD Hp Vehicle
5XFAD Hp VU0364572
Soluble Aβ40/42 Ratio
(% Vehicle)
150
125
100
75
50
25
0
n.s.
n.s.
B
5XFAD Ctx Vehicle
5XFAD Ctx VU0364572
5XFAD Hp Vehicle
5XFAD Hp VU0364572
Insoluble Aβ40/42 Ratio
(% Vehicle)
150
125
100
75
50
25
0
n.s.
n.s.

Figure S6.

Dose (mg/kg/day)	Animal	Plasma (ng/mL)	Brain (ng/mL)	Brain:Plasma (ng/mL)
10	1	99.3	16.8	0.17
10	2	22.9	5.8	0.25
10	3	15.1	9.5	0.63
10	4	142.3	77	0.54
10	5	99.7	99	0.99
10	6	332.7	133	0.40

Table S1.

SI References

1. Davis AA, Fritz JJ, Wess J, Lah JJ, & Levey AI. Deletion of M1 muscarinic acetylcholine receptors increases amyloid pathology in vitro and in vivo. *J. Neurosci.* 2010; **30(12):** 4190-4196.
2. Davis AA, Heilman CJ, Brady AE, Miller NR, Fuerstenau-Sharp M, Hanson BJ, et al. Differential effects of allosteric M1 muscarinic acetylcholine receptor agonists on receptor activation, arrestin 3 recruitment, and receptor downregulation. *ACS Chem. Neurosci.* 2010; **1(8):** 542-551.
3. Lebois EP, Bridges TM, Lewis LM, Dawson ES, Kane AS, Xiang Z. et al. Discovery and characterization of novel subtype-selective allosteric agonists for the investigation of M1 receptor function in the central nervous system. *ACS Chem. Neurosci.* 2009; **1(2):** 104-121.
4. Lebois EP, Digby GJ, Sheffler DJ, Melancon BJ, Tarr JC, Cho HP, et al. Development of a highly selective, orally bioavailable and CNS penetrant M1 agonist derived from the MLPCN probe ML071. *Bioorg. Med. Chem. Lett.* 2011; **21(21):** 6451-6455.