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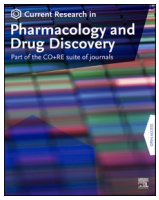

A PAM of the α_{1A}-Adrenergic receptor rescues biomarker, long-term potentiation, and cognitive deficits in Alzheimer's disease mouse models without effects on blood pressure

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A B S T R A C T

α_1-Adrenergic Receptors (ARs) regulate the sympathetic nervous system by the binding of norepinephrine (NE) and epinephrine (Epi) through different subtypes (α_{1A}, α_{1B}, α_{1D}). α_{1A}-AR activation is hypothesized to be memory forming and cognitive enhancing but drug development has been stagnant due to unwanted side effects on blood pressure. We recently reported the pharmacological characterization of the first positive allosteric modulator (PAM) for the α_{1A}-AR with predictive pro-cognitive and memory properties. In this report, we now demonstrate the *in vivo* characteristics of Compound 3 (Cmpd-3) in two genetically-different Alzheimer's Disease (AD) mouse models. Drug metabolism and pharmacokinetic studies indicate sufficient brain penetrance and rapid uptake into the brain with low to moderate clearance, and a favorable inhibition profile against the major cytochrome p450 enzymes. Oral administration of Cmpd-3 (3–9 mg/kg QD) can fully rescue long-term potentiation defects and AD biomarker profile (amyloid β-40, 42) within 3 months of dosing to levels that were non-significant from WT controls and which outperformed donepezil (1 mg/kg QD). There were also significant effects on paired pulse facilitation and cognitive behavior. Long-term and high-dose *in vivo* studies with Cmpd-3 revealed no effects on blood pressure. Our results suggest that Cmpd-3 can maintain lasting therapeutic levels and efficacy with disease modifying effects with a once per day dosing regimen in AD mouse models with no observed side effects.

1. Introduction

Alzheimer's Disease (AD) is characterized by neuritic plaques and neurofibrillary tangles, composed mostly of β-amyloid (Aβ) and tau (Selkoe, 2001). Clinical trials focused on amyloid immunotherapies or amyloid precursor protein (APP) secretase inhibitors have failed to show clinical and cognitive efficacy. Acetylcholinesterase inhibitors, such as donepezil (i.e., Aricept), which prevent the breakdown of the neurotransmitter, acetylcholine, are currently approved drugs for AD. This mode of action is hypothesized to improve the symptoms of dementia from the deterioration of cholinergic neurons in the brain and the decrease in neurotransmission that contribute to the decline in cognitive function in AD (Bartus et al., 1982). However, the efficacy of these drugs is limited and short-acting, failing to arrest the progression of the disease. At best, donepezil only temporarily delays but does not arrest symptomatic deterioration as assessed by a review of over 36 controlled, multi-centered, double-blinded, independent clinical studies with 8257 patients (Birks and Harvey, 2018; Jia et al., 2017; Li et al., 2019). The recent FDA approval of Aduhelm, the first AD drug approval in 20 years, works by clearing amyloid plaques but is not yet proven to be effective at improving cognition and may lead to significant side effects of brain bleeding and swelling.

Loss of neuronal synaptic density is another invariant feature of AD that precedes neuronal loss (DeKosky and Scheff, 1990). Thus, synaptic dysfunction is likely the most significant factor contributing to the initial and progressive stages of AD memory loss (Dickson et al., 1995; Flood and Coleman, 1990; Masliah et al., 2001; Cummings, 2021). This is where cognitive enhancers, which also neuroprotect, boost neurogenesis, increase long-term potentiation (LTP) and synaptic plasticity, may benefit AD patients.

α_1-Adrenergic Receptors (ARs) are a G-Protein Coupled Receptor (GPCR) within the largest division of the Class A superfamily of "rhodopsin-like" neurotransmitters and hormones. α_1-AR subtypes (α_{1A}, α_{1B}, α_{1D}) belong to the smaller family of ARs which also contain α_2-and β-AR subtypes with each subtype regulating distinct but commonly overlapping functions. Within the α_1-AR subtypes, there are also distinct functions, notably with the α_{1A}-AR being cardioprotective, neurogenic, and pro-cognitive (Perez, 2021a). The use of genetically-modified mouse models of the α_{1A}-AR subtype confirms learning and memory enhancement via increased synaptic plasticity as α_{1A}-ARs are highly expressed in key memory centers (Doze et al., 2011; Perez, 2020).

Decreased NE levels is associated with cognitive decline in AD

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(Gottfries et al., 1983; Chalermpalanupap et al., 2013) and theorized to be etiological in AD onset (Gannon et al., 2015; Ponnusamy et al., 2019). NE enhancement can improve memory formation and executive functions in healthy humans (Kuo et al., 2021). NE mediates synaptic plasticity and cognition through the cAMP signaling of α_1-ARs in addition to β-ARs (Ferry et al., 1999a; Harley et al., 2006; Ramasamy et al., 2020; Perez, 2020) via the phosphorylation of cAMP Response Element-Binding Protein (CREB) and its transcriptional activity (Kabitzke et al., 2011; Kandel, 2012; Huang et al., 2017).

The high homology of the orthosteric agonist binding site in the AR family, as they all bind catecholamines with similar affinity, results in difficulty in the design of drugs with high degree of selectivity. In addition, for α_1-AR agonists, the canonical inositol phosphate (IP)-calcium release signaling pathway also drives vascular smooth muscle contraction, inducing increased blood pressure. However, the advance of positive allosteric modulators (PAMs) has alleviated some of these design problems resulting in agonist-like functions without basal activity and with greater selectivity than previously achievable. Allosteric modulators also possess additional properties of both ligand and signaling bias resulting in potentially lesser side effects (Christopoulos, 2014; Wold et al., 2019). We previously published that Cmpd-3 potentiates NE-mediated cAMP signaling with no effects on NE-mediated IP release. There are also no effects on EPI-mediated signaling pathways (Papay et al., 2023). This signal bias would target effects to the brain with reduced effects in peripheral functions, particularly in blood pressure. Cmpd-3 is a pure PAM having no basal activity and with at least 1000-fold higher affinity compared to other AR subtypes. In this report, we confirm effects of Cmpd-3 via α_{1A}-AR activation in learning and memory functions that rescues these deficits in two genetically-different AD mouse models and with disease-modifying outcomes as evidenced by the rescue of AD biomarkers.

2. Materials and methods

2.1. Animals

All animal studies, including those at Contract Research Organizations (CROs) were performed according to the NIH Guide for the Care and Use of Laboratory Animals and experimental protocols were reviewed by the Cleveland Clinic animal care and use committee. 3XTG mice (APP(swe), PS1(M146V), tau(P301L) were obtained from Jackson Laboratory and bred in house at The Cleveland Clinic. hAPP[V717I] transgenic mice (FVB/N x C57Bl/6J background) (hAPP-Tg) were genotyped and used by the CRO ReMYND (Leuven-Heverlee, Belgium). WT controls are non-transgenic littermates on the same genetic background. hAPP-Tg has a point mutation in the human amyloid precursor protein (hAPP) at Valine 717 to Isoleucine and was first identified in an English family, referred to as the London mutation (*lon*)(Goate et al., 1991). Only female mice were used in studies for cognitive and electrophysiological measurements as male mice display increased aggression which can interfere with data interpretation (Moechars et al., 1998; Lalonde et al., 2012). Water and feed were available ad libitum. Rodents were housed in temperature-controlled rooms with a 12-h light/dark cycle and examined daily for health and weighed throughout the study. On average, the body weight was 20–35 g, depending on the age of the mice and the duration of the dosing.

2.2. Compounds

Cmpd-3 was synthesized by The Center of Therapeutic Discovery at the Cleveland Clinic and verified by LC-MS and NMR at 98^{+}% purity. Details on synthesis and characterization is published (Papay et al., 2023).

2.3. In vivo drug metabolism/pharmacokinetic (DMPK) studies

Studies were performed by Alliance Pharma (Malverm, PA), or Q2 Solutions (Morrisville, NC) in B6V8F1/J mice or rats (20–25g, 8 weeks) obtained from Jackson Laboratories. Rodents were dosed with Cmpd-3, formulated in 0.9% saline, using a single dose at either 20 mg/kg (PO), 10 mg/kg (PO) or 1 mg/kg (IV) and blood drawn and tissue collected at 0.25, 0.5, 1, 2, 4, 8, or 24 h. Brains were removed, rinsed in cold saline, weighed and flash frozen. Three rodents were used for each time point. Cmpd-3 concentrations were assessed using UPLC (Shimadzu) and MS (Triple Quad 5500, AB Sciex) and calibrated with internal and compd-3 standards. All bioanalysis and calculation of PK parameters was carried out by the CROs and a final report issued upon study completion.

2.4. Administration of Cmpd-3

Our first test of Cmpd-3 on cognitive function was in the 3XTG mouse model and 16 mice per treatment group were first randomly separated into two groups: vehicle (saline) and Cmpd-3 (approximate final dose 2.6 mg/kg QD). Drugs were administered in the drinking water of the cage and the dose calculated based upon the average mouse consumption of 5 ml of drinking water per day. Prior studies utilizing LC-MS established that Cmpd-3 was stable for at least 14 days in cage-derived drinking water. The water bottles were monitored daily and refilled as needed, then completely changed out every 14 days with freshly made Cmpd-3. Mice were continuously dosed starting at 2 months of age, then analyzed for LTP and cognitive behavior at 12 M of age by AfaSci, Inc. For studies with the hAPP-Tg mice, ReMYND administered drugs by oral gavage and mice were randomly assigned into treatment groups. In the first experiment, we determined the effects of 2 weeks twice-daily treatment (BID, 20 mg/kg) with Cmpd-3 or vehicle (saline), beginning at 7.6 months of age. In the second experiment, mice were orally dosed with either Cmpd-3 (0.2, 2, or 9 mg/kg QD) or vehicle (saline) for 12 weeks starting at 6 months of age. In a third experiment, mice were dosed with either Cmpd-3 (3, 6, 9 mg/kg QD), donepezil (1 mg/kg QD) or vehicle (saline) for 12 weeks starting at 6 months of age. For all *in vivo* experiments, the mice were letter and/or color-coded blindly to the technician based upon drug administration and sacrificed the day of or after the final treatment.

2.5. Locomotor activity

These studies were performed by the CRO AfaSci, Inc. Mice (3XTG) are assessed individually in a SmartCage™ (inner dimensions: L 12.5" x W 8." x H 5") which included a thin layer of wood bedding. Mice are acclimatized for 48 h prior to testing with free access to food and water. The mouse is continuously recorded under the 12:12 h light:dark cycle. The active time, travel distance, and velocity is assessed for each mouse via Infrared photobeam breaks (x, y, z) in 2-h time-bins.

2.6. Electrophysiology

LTP was performed on the 3XTG mice by AfaSci, Inc. (San Francisco, CA) and on the hAPP-Tg mice by ReMYND at its testing facility, E-PHY-SCIENCE (Biot, France). LTP measurements were randomized over treatment groups. Operators were blinded to treatment for all experiments. Mice were anesthetized with isoflurane and then decapitated. Mouse brains were collected approximately 30 min after the last dose and between 90 and 120 min after excision of the brain. Brains were extracted and slices prepared immediately, followed by incubation of the slices in aCSF as described below. No Cmpd-3 was added to the artificial cerebrospinal fluid (aCSF) in which the brain slices were incubated. At least 2 validated slices were performed for each mouse.

Acute slices (400 μm thick) were prepared with a vibratome (VT 1000S; Leica Microsystems, Bannockburn, IL) in ice-cold gassed aCSF enriched with sucrose. Sections were incubated in aCSF at 34 °C for 20

min and then kept at room temperature for at least 1 h before recording. Recordings were performed in a submerged chamber continuously flowed with aCSF at 2 ml/min. A monopolar electrode was placed in the Schaffer collaterals, and stimulation was applied at 0.066 Hz (every 15 s) with various ranges of stimulus intensity yielding evoked field Excitatory Post Synaptic Potentials (fEPSPs) of approximately 0.2–0.5 mV. fEPSPs were recorded in the stratum radiatum using a borosilicate micropipette filled with aCSF. After a 10 min stable baseline period, LTP was induced by a High Frequency Stimulation (HFS) protocol (3 trains of 100 stimulations at 100 Hz, each train separated by 20 s) at baseline stimulation intensity. Following this conditioning stimulus, a 1 h test period was recorded where responses were again elicited by a single stimulation every 10 s (0,1Hz) at the same stimulus intensity.

Signals were amplified with an Axopatch 200B amplifier (Molecular Devices, Union City, CA) digitized by a Digidata 1322A interface (Axon Instruments, Molecular Devices, US) and sampled at 10 kHz. Recordings were acquired using Clampex (Molecular Devices) and analyzed with Clampfit (Molecular Devices). LTP was quantified by comparing the mean fEPSP slope over the post-tetanus period with the mean fEPSP slope during the 10 min baseline period. The initial fESPS slope in mV/ms was determined by the software. The average of all fEPSP slopes during the baseline measurement was taken as the baseline response to which all other measurements were normalized.

Input-output (I–O) curves were obtained using stimulus intensity from the threshold to a strength evoking the maximum response. The I–O curve was constructed by plotting the fEPSP slope against the different intensities of stimulation. Group effects were assessed by changes in fEPSP slope, expressed as the percentage of the maximal value. For Paired Pulse Facilitation experiments, the ratio of the amplitude of the second to first response was used. If the PPR is higher than 1 it concerns PPF, if the ratio is lower than 1 it concerns PPD.

Table 1
DMPK Parameters after oral administration of Cmpd-3.

Characteristic	Administration Dose	Species	Result
Solubility		In hand	11 mg/ml
T ½ -Plasma	20 mg/kg PO Alliance	Mouse	1.36 (h)
T ½ -Brain	Pharma		2.11 (h)
T max - Plasma			0.25 (h)
T max -Brain			0.5 (h)
MRT - Plasma			1.55 (h)
MRT - Brain			2.77 (h)
C_{max} Plasma	20 mg/kg PO Alliance Pharma	Mouse	754.4 (ng/mL)
C_{max} Brain			47.8 (ng/mL)
AUC_{Inf} Plasma			870 (ng/mL)
AUC_{Inf} Brain			236 (ng/mL)
Brain/Plasma		Mouse	0.27
Plasma Binding	Alliance Pharma	Mouse	86 (% free)
Plasma Binding	Q2 Solutions	Human	58 (% free)
		Rat	90 (% free)
Intrinsic Clearance Mouse	Alliance Pharma	Mouse Liver Microsomes	30.8 (ml/min/mg)
Intrinsic Clearance Rat		Rat Liver Microsomes	65 (ml/min/mg)
Intrinsic Clearance Human	Q2 Solutions	Human Liver Microsomes	7.2 (ml/min/mg)
Permeability Potential	Alliance Pharma	Caco-2 cells	Efflux Ratio = 7.68
Permeability Potential	Alliance Pharma	MDR1-MDCK cells	Efflux Ratio = 9.38
CYP inhib profile	Q2 Solutions		(% inhibition)
3A4			0
2D6			9
2C9			5

AUC, area under the curve; Cmax, concentration maximum; CYP, cytochrome P450.
Enzymes; MDCK, Madin-Darby canine kidney; MDR1, Multidrug Resistance gene-1.
MRT, mean resident time.

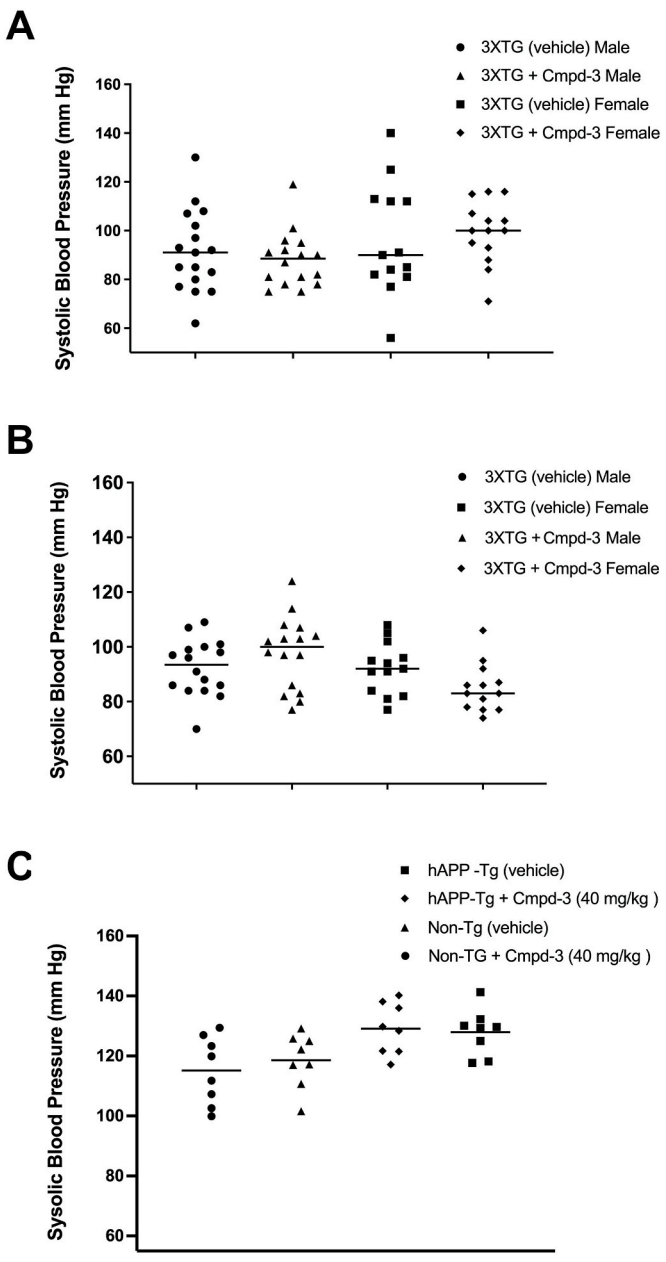

Fig. 1. Cmpd-3 treated mice under long-term or high dose treatment does not increase blood pressure. Systolic blood pressure in 3XTG mice after (**A**) 1 month or (**B**) 6 months dosing of 2.6 mg/kg PO, QD of Cmpd-3 or vehicle. 3XTG vehicle males (circles); 3XTG vehicle females (squares); 3XTG + Cmpd-3 males (triangles); 3XTG + Cmpd-3 females (diamonds). Each data point represents an average of ten cycles per mouse. There was no statistical difference between the groups. N = 13–16 mice, each group. (**C**) Mean arterial blood pressure of hAPP-Tg and non-Tg mice after 2 weeks (BID, PO) with Cmpd-3 (20 mg/kg) or vehicle controls. hAPP-Tg vehicle (squares); WT vehicle (triangles); hAPP-Tg + Cmpd-3 (diamonds); non-Tg + Cmpd-3 (circles). Blood pressure was measured using a tail cuff method the day before LTP. Each data point represents an average of ten cycles. 2-way ANOVA analysis revealed a significant difference for genotype ($p = 0.0013$), Tukey's post hoc test: $p = 0.0261$ for comparison of hAPP-Tg and Non-Tg controls dosed Cmpd-3 but no statistical difference between Compd-3 and saline. N = 8 mice per group.

2.7. Blood collection

Blood was collected at the start of the study and before first dosage to assess baseline conditions. After 3 months of dosing, blood was collected before the mice were sacrificed for the LTP measurement. Blood was

collected using Microvette CB 300 tubes with K_2EDTA (Sarstedt; cat 16.444). Conscious mice were restrained either manually or using a restraint tube and 150 μl sample was collected from the lateral saphenous vein. Tubes were stored at 4 °C for no more than 1 h. Samples were centrifuged in Microvette tubes down at 2000 g at 4 °C for 15 min. 20 μl of plasma was transferred to labelled tubes and color and letter-coded to treatment conditions. Samples were frozen in liquid N_2 and then stored at −80 °C till analysis of AD biomarker profile.

2.8. AD biomarkers

We analyzed the plasma samples for a series of AD biomarkers using the CRO, Quanterix, and their Simoa© Assay which has ultra-sensitivity using the 4plexE assay. Plasma obtained by cardiac puncture and immediately frozen at −80 °C were shipped to Quanterix to determine levels of AD biomarkers using the 4plexE multiplex assay. The assay was performed within a 2 month time frame of the samples being frozen. As the hAPP mice express a mutation of the human APP protein, the assay must be able to detect human Aβs in a mouse blood matrix. The 4plexE multiplex assay can simultaneously measure human Nf-l, Aβ40, Aβ42, and GFAP in one sample. There is also cross-reactivity with mouse for Nf-l but not for GFAP. The lower limits of detection of the assay for Nf-l, Aβ40, Aβ42, and GFAP are 1.6 pg/mL, 4.08 pg/mL, 1.51 pg/mL, and 11.6 pg/mL, respectively. The inter-lot and inter-instrument coefficient of variation (CV) in most instances (>90%) for all of the proteins were <10%.

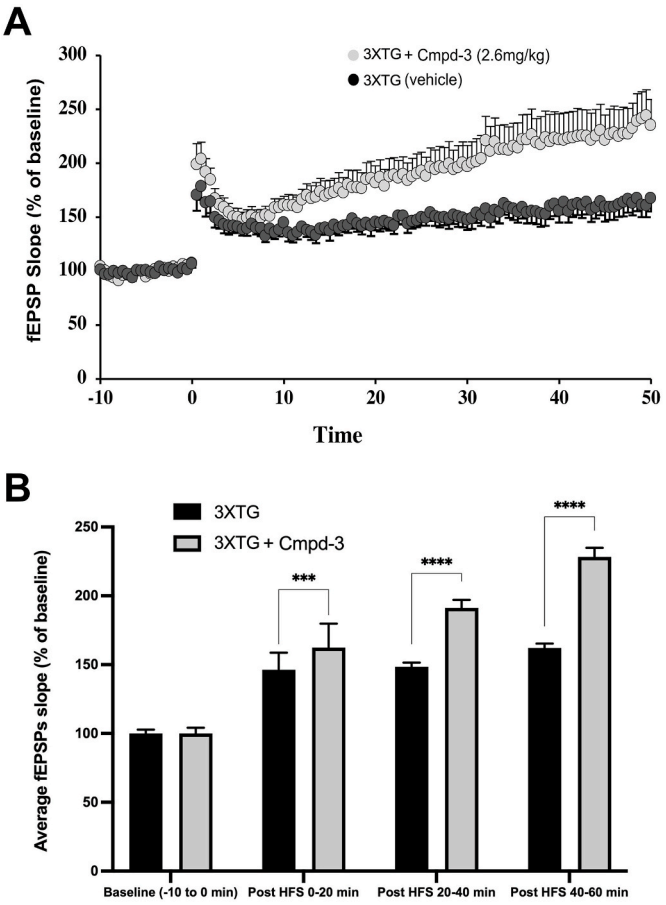

Fig. 2. Cmpd-3 treatment rescued LTP deficits in 3xTG (2.6 mg/kg). (A) LTP improved in Cmpd-3-treated AD female mice (grey circles) compared to vehicle control AD female mice (lower curve, black circles) assessed at 12 months of age. **(B)** Cmpd-3 statistically rescued the LTP deficit compared in the 3XTg mice from 0 to 20 min (***$p < 0.001$), 20–40 min (****$p < 0.000001$) and at 40–60 min post LTP induction (****$p < 0.000001$) using an unpaired Student's t-test (Fig. 2B). N = 8 mice per group.

2.9. Barnes Maze

A shortened Barnes maze was performed by AfaSci, Inc., according to the protocol as originally published for the 3XTG mice (Attar et al., 2013). Twenty holes with a diameter of 1.75" were made on the perimeter at a distance of 1" from the edge. This circular platform was then mounted on top of a rotating stool, 35" above the ground and balanced. The maze was placed in the center of a dedicated room and two 120 W lights were placed on the edges of the room facing towards the ceiling about 3/4 of the way up from the floor and about 3–5 feet away from the maze. Eight simple colored-paper shapes (squares, triangles, circles) were mounted around the room as visual cues, in addition to the asymmetry of the room itself. After testing each mouse, the cleaning of the quadrant of the maze around the target hole was alternated with cleaning the whole maze, using 70% ethanol. The maze was rotated clockwise after every 3 mice to avoid intra-maze odor or visual cues. Acquisition is assessed as a decrease in latency and/or in the number of erroneous holes searched before finding the target hole, but not necessarily going into the escape cage.

2.10. Tone-induced fear conditioning

These studies were performed by AfaSci, Inc. Mice were subjected to fear-conditioning using the foot shock module according to their protocols. The module is composed of a metal grid connected to an electric shock generator. Each mouse's activity was recorded for 5 min followed by five mild electric foot shocks to the paws (70 ± 2 V, for 2 s, at 60 s intervals). Each foot shock (unconditioned stimulus) was paired with a tone (conditioned stimulus). The tone preceded the shock by 2 s and lasted for a total of 6 s. The baseline for each mouse was determined during the training period. Freezing behavior, which indicates a fear response, was assessed by measuring velocity, travel distance, and active time (Radulovic et al., 1998; Hicks et al., 2016).

2.11. Blood pressure (BP)

BP measurements were performed in our laboratory for the 3XTG mice or by ReMYND for the hAPP-Tg mice. Both mouse models were assessed using Volume Pressure Recording (VPR)-tail-cuff approach (Krege et al., 1995), using the CODA Non-Invasive Blood Pressure System from Kent Scientific (Torrington, CT). To obtain reliable measurements 10 data points were averaged per mouse. To accustom the mice to the procedure, the mice were trained 3 days before the measurement of blood pressure using the same procedure as the actual measurement, performed at the same time of the day as the actual measurement. Each animal was placed in a holder at least 10–15 min before pressure measurements began and pre-warmed using a thermal blanket. Upon deflation of the cuff, a VPR sensor was placed distal to the occlusion cuff and was used to monitor the blood pressure. Ten cycles of blood pressure measurements were obtained for each mouse with 5 min rest between measurements.

2.12. Statistics

All data are presented as mean ± SEM and were subjected to statistical tests using GraphPad Prism (version 9.5.1). For multiple groups, statistical testing is performed using a one-way or two-way ANOVA and the Turkey's, Bonferroni's, or Sidak post hoc multiple comparison test to determine significant differences. For two groups, a paired or unpaired Student's *t*-test was used. Statistical significance was determined to be $p < 0.05$. Trend was defined as $p < 0.1$.

3. Results

3.1. Cmpd-3 DMPK profile indicates rapid uptake and brain permeability

While α_1-AR agonists are mostly brain impervious due to a protonated amine on the phenylethylamine structure (i.e. norepinephrine), imidazoline-based α_1-AR agonists, such as cirazoline, display increased brain penetrance due to the dissipation of the positive charge resulting from resonance within the imidazoline ring (Davies and Wellman, 1992). Cmpd-3 is also an imidazoline-based chemotype like cirazoline but behaves pharmacologically as a PAM. To determine the dose of Cmpd-3 to use *in vivo*, we performed tissue distribution studies to establish brain to plasma exposure levels after a single dose of Cmpd-3 administered PO in mice. We also examined potency and efficacy in both rat and human cell lines (Table 1). Cmpd-3 has a low molecular weight, high solubility, and a rapid uptake into the brain. Cmpd-3 penetrates the blood brain barrier with a brain to plasma ratio of 0.27, has longer half-life in the brain than plasma, with low to moderate clearance, low plasma binding, and a clean profile against the major cytochrome p450 enzymes. The DMPK profile and high binding affinity (pM) of Cmpd-3 results in a drug that can maintain lasting therapeutic levels and efficacy in the brain with a once per day dosing regimen. A 3 mg/kg daily dose of Cmpd-3 produces a C_{max} of 7.6 ng/g in the brain or 24 nM, well within the peak nanomolar range (affinity & potency) of our compound as assessed in our primary PAM assay (cAMP)(Papay et al., 2023).

3.2. Cmpd-3 does not increase blood pressure in vivo

As α_1-AR agonists potently increase blood pressure (BP), we tested the effects of Cmpd-3 in 3XTG mice (2.6 mg/kg/day PO) after 1 (Fig. 1A) and 6 months (Fig. 1B) of dosing on the mean systolic BP (mmHg). There was no statistical difference between the groups based on sex or treatment (M: 97 + 3; F: 85 + 2.4) vs untreated groups (M: 92 + 2.5; F: 92 + 2.6) as hypothesized. In separate studies performed independently at ReMYND, Cmpd-3 at a much higher dose (40 mg/kg/day, PO) for 2 weeks in 9 M hAPP-Tg mice also did not change BP between treated and vehicle-treated mice (Fig. 1C), nor had effects on general health or weight. However, there was a significant difference for genotype (*p = 0.026) between hAPP-Tg and WT mice with hAPP-Tg mice displaying higher mean arterial BP, a phenotype not previously reported in this AD mouse model.

3.3. Cmpd-3 rescues LTP deficit in both the 3XTG and hAPP-Tg AD mouse models

It is widely accepted that hippocampal LTP is a cellular correlate of memory, producing long-lasting increases in synaptic plasticity and efficiency (Bliss and Collingridge, 1993). We chose to begin our studies with the 3XTG mouse model because of its documented synaptic dysfunction, LTP deficits, and cognitive impairment manifesting in an age-related manner beginning from 6 months of age (Sterniczuk et al., 2010; Romberg et al., 2011). As 2–3 months of dosing of cirazoline, a mildly-selective orthosteric α_{1A}-AR partial agonist, resulted in enhancement of cognition (Doze et al., 2011) and adult neurogenesis (Gupta et al., 2009), the 3XTG mice were dosed with Cmpd-3 (2.6 mg/kg/day) or placebo for 10 M, starting at age 2 months. The mice were then assessed for LTP at age 12 months to maximize the manifestations of AD symptoms in control mice. Differences in the I/O curves were not statistically significant (Fig. S1). However, LTP was statistically improved in the Cmpd-3-treated 3XTG mice (Fig. 2A). Cmpd-3 statistically rescued the LTP deficit compared in the 3XTg mice from 0 to 20 min (***p < 0.001), 20–40 min (****p < 0.000001) and at 40–60 min post LTP induction (****p < 0.000001) using an unpaired student's T-test (Fig. 2B).

The hAPP-Tg AD mouse model displays LTP deficits as early as 6–8

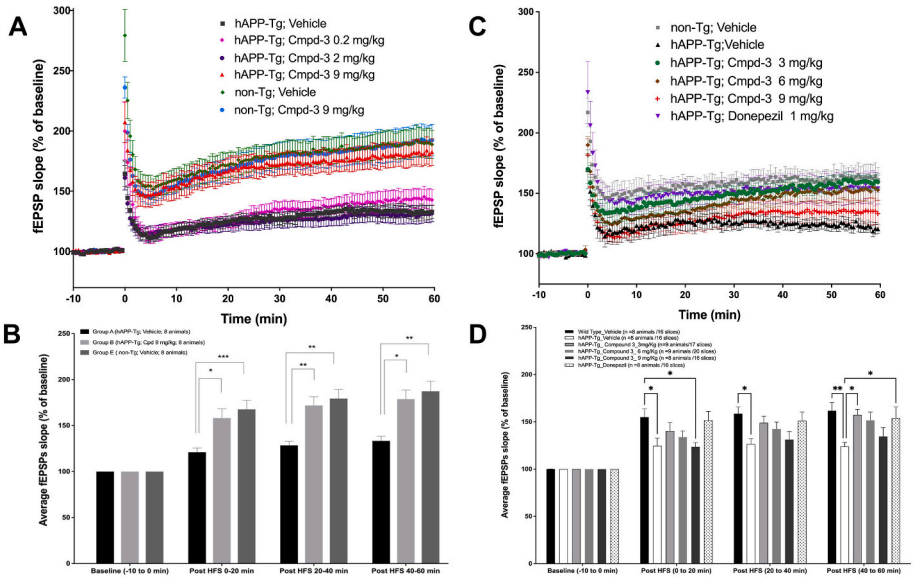

Fig. 3. Cmpd-3 dose-dependently rescued LTP deficits in hAPP-Tg mice at (0.2, 2, 9 mg/kg) (AB) or at (3, 6, 9 mg/kg)(CD). (A) Cmpd-3 at 9 mg/kg completely rescued LTP deficits in hAPP-Tg mice. hAPP-Tg, vehicle (black squares); hAPP-Tg + Cmpd-3, 0.2 mg/kg (pink diamonds); hAPP-Tg + Cmpd-3, 2 mg/kg (dark blue circles); hAPP-Tg + Cmpd-3, 9 mg/kg (orange triangles); non-Tg, Vehicle (green diamonds); non-Tg + Cmpd-3, 9 mg/kg (light blue circles). **(B)** Columns represent the average slope response during baseline (−10 to 0 min), 0–20 min post LTP induction, 20–40 min post LTP induction and 40–60 min post LTP induction. A significant deficit in hippocampal LTP of hAPP-Tg mice (black bars) compared with non-Tg (dark grey bars) mice was observed at (0–20 min post LTP induction: ***p = 0.0008; 20–40 min post LTP induction: **p = 0.001; 40–60 min post LTP induction: **p = 0.0026). In addition, there was a significant increase in hippocampal LTP of hAPP-Tg mice with Cmpd-3 at 9 mg/kg (light grey bars) compared with hAPP-Tg vehicle at (0–20 min post LTP induction: *p = 0.0125; 20–40 min post LTP induction: **p = 0.0078; 40–60 min post LTP induction: *p = 0.018) which was non-significant to the non-Tg vehicle-treated animals. **(C)** hAPP-Tg + Cmpd-3 at 3 mg/kg (green circles) completely rescued LTP compared to hAPP-Tg vehicle (black triangles). hAPP + Cmpd-3 at 6 mg/kg (brown diamonds) or at 9 mg/kg (red plus) also increased LTP. **(D)** Columns represent the average slope response during baseline (−10 to 0 min), 0–20 min post LTP induction, 20–40 min post LTP induction and 40–60 min post LTP induction. There was a significant deficit in hippocampal LTP of hAPP-Tg mice compared with non-Tg mice with vehicle at (0–20 min post LTP induction; *p = 0.0413; 20–40 min post LTP induction; *p = 0.0233; 40–60 min post LTP induction; **p = 0.030). There was a significant increase in hippocampal LTP of hAPP-Tg mice with Donepezil compared with hAPP-Tg vehicle at (40–60 min post LTP induction; *p = 0.0466). There was also a significant increase in hippocampal LTP of hAPP-Tg with Cmpd-3 at 3 mg/kg compared with hAPP-Tg vehicle at (40–60 min post LTP induction; *p = 0.0111). N = 8–9 mice per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

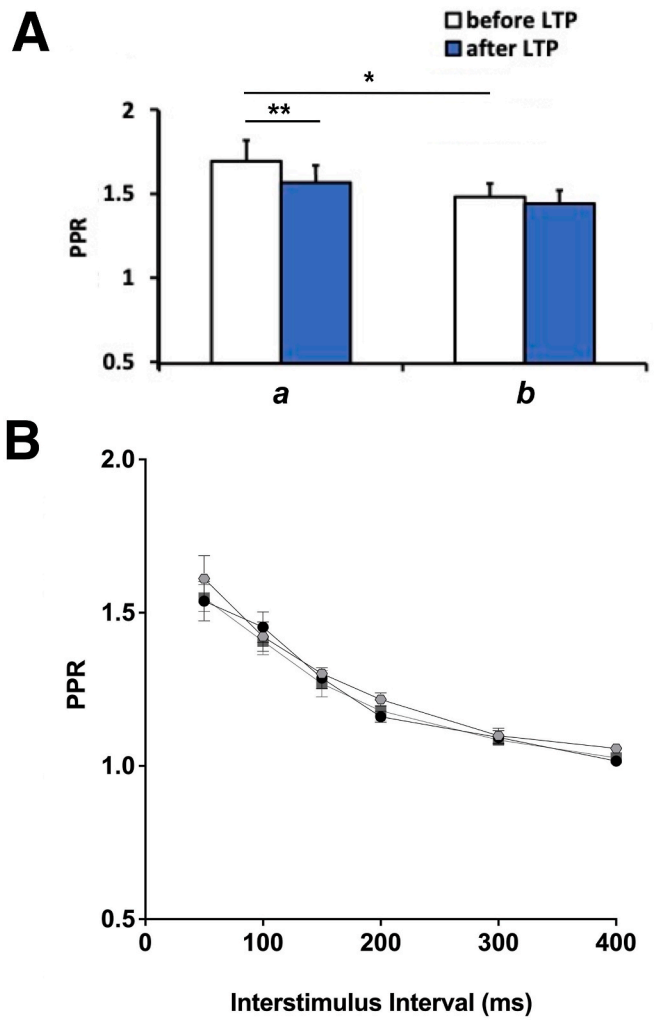

Fig. 4. Effects of Cmpd-3 on paired-pulse facilitation in 3xTG mice (A) or hAPP-Tg (B). (A) Cmpd-3 (2.6 mg/kg) treated 3xTG mice (*a*) significantly increased the paired pulse ratio (PPR) at the 50ms interstimulus interval over vehicle-treated 3xTg control mice (*b*)(*p < 0.05, one-tailed) before LTP induction. After LTP induction, there was a decrease in PPR in the Cmpd-3-treated mice, suggesting that short-term plasticity was pre-synaptic (**p < 0.03, paired, one-tailed). **(B)** There was no significant change in PPR at any interstimulus interval between hAPP-Tg, Vehicle (dark grey circles), Cmpd-3 (9 mg/kg, grey squares)-treated hAPP-Tg or non-Tg, Vehicle (light grey hexagon). N = 8 mice per group.

months of age (Dewachter et al., 2000, 2009). In hAPP-Tg mice (9 month) treated with Cmpd-3 (40 mg/kg) for 2 weeks, there was no significant changes in LTP compared to controls (Fig. S2A). However, Cmpd-3 treated hAPP-Tg mice did display a significant increase in short-term potentiation during the first 10 min of post LTP induction when compared to vehicle-treated ones (*p < 0.001) (Fig. S2B). We then hypothesized that mediated changes in cognition by the α_{1A}-AR are likely post-transcriptional; therefore, we increased dosing length to 3 months starting with mice that are 6 months of age to analyze effects at 9 months of age.

In hAPP-Tg mice with 3 months dosing of Cmpd-3 (0.2, 2, 9 mg/kg/day), there were no changes in the I/O curves at any of the three doses administered compared to controls (data not shown). While there were significant differences in LTP between the hAPP-Tg and non-Tg controls (*p < 0.0026), Cmpd-3 at 0.2 or 2 mg/kg dose did not alter LTP in the hAPP-Tg mice However, a 9 mg/kg dose of Cmpd-3 completely rescued the LTP deficits in hAPP-Tg mice to levels that were non-significant from non-Tg (vehicle) controls (*p < 0.018) (Fig. 3AB). In hAPP-Tg mice with

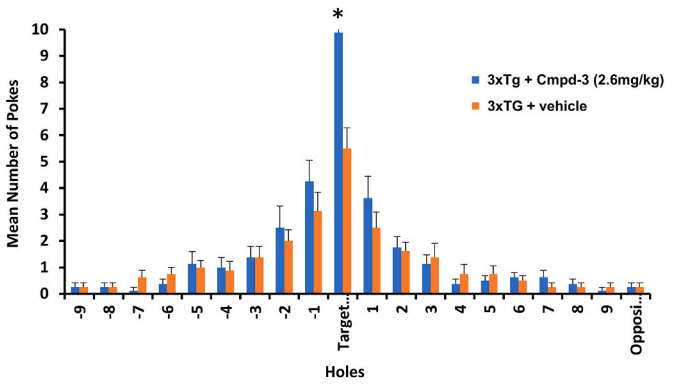

Fig. 5. Cmpd-3 treated 3xTg mice improved memory performance in the Barnes maze cognitive test. Tests were performed blinded to the CRO, AfaSci. The number of hole pokes for female 3xTg mice during day 5 memory probe test of the shortened Barnes Maze protocol. Cmpd-3 treated mice (2.6 mg/kg) (blue bars) significantly identified the correct target hole compared to 3xTg (vehicle) control (orange bars) (*p < 0.037). N = 8 mice per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3 months dosing of Cmpd-3 (3, 6, 9 mg/kg/day) and benchmarked to donepezil (1 mg/kg), all Cmpd-3 dosing regimens produced a dose-dependent rescue on LTP deficit with 3 mg/kg producing 100% rescue, and better than donepezil (at 80% rescue)(Fig. 3CD). Cmpd-3 at 3 mg/kg had 100% rescue of LTP deficit compared with hAPP-Tg vehicle control at 40–60 min post LTP induction (*p < 0.01), better than donepezil (*p < 0.046) (Fig. 3CD) and was non-significant from WT control.

3.4. Cmpd-3 increases paired pulse facilitation in 3xTG mice

We next examined short-term plasticity by measuring paired pulse facilitation using the paired pulse ratio (PPR). When a presynaptic terminal is stimulated by two rapid pulses in succession, the second post-synaptic terminal will display either a larger or smaller response attributed to the amount of calcium in the synapse. The ratio of the amplitude of the second to first response is called the PPR. Cmpd-3 (2.6 mg/kg) treated 3xTG mice (A) significantly increased the PPR at the 50ms interstimulus interval over vehicle-treated 3xTg control mice (B) (*p < 0.05) before LTP induction. After LTP induction, there was a decrease in PPR in the Cmpd-3 -treated mice (**p < 0.03)(Fig. 4A). As PPR decreases with increased neurotransmitter release (Saviane et al., 2002), the observed changes in short-term plasticity after LTP mediated by Cmpd-3 suggests a pre-synaptic mechanism. There was no statistically significant alterations of the PPR in the untreated 3XTG control mice before or after LTP. The hAPP-Tg mice did not display statistically significant alteration of the PPR at any stimulation frequency (Fig. 4B) as was previously reported in this mouse model (Moechars et al., 1999).

3.5. Cmpd-3 increases cognitive functions in the 3XTG mouse model

Changes in locomotor activity can affect cognitive tests and indicate neurological side effects such as pain or stress. Therefore, we analyzed Cmpd-3 treated and untreated AD 3XTG mice for changes in velocity (Fig. S3A), travel distance (Fig. S3B), and active time (Fig. S3C) using the Smart Cage system which allows the mice to freely roam for 48 h with free access to food and water. There were no significant changes in any measure of locomotion activity in Cmpd-3 treated mice.

We next performed the Barnes Maze cognitive test which is a measure of spatial learning and memory on the 3xTG mice and is reported to be the most sensitive measure of cognitive deficits in this AD mouse model (Stover et al., 2015). Comp-3-treated mice (Fig. 5) improved cognitive function that was statistically significant (*p < 0.037)

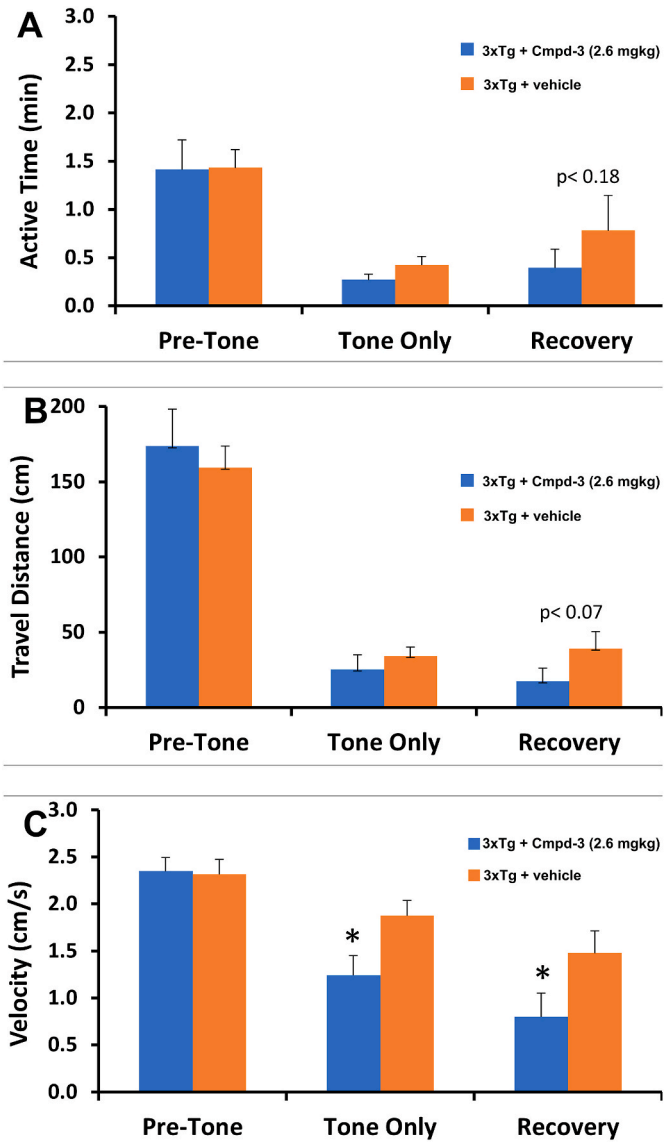

Fig. 6. Cmpd-3 treated 3xTg mice improved memory retrieval during fear conditioning cognitive test. Tests were performed blinded to the CRO, AfaSci. Amount of active time **(A)**, travel distance **(B)** or velocity **(C)** measured in 3xTg mice after tone-induced fear conditioning (tone & foot-shock) during the Pre-Tone, Tone Only and Recovery Periods for Cmpd-3 treated (blue bars) and vehicle-treated (orange bars) AD mice. Cmpd-3 treated 3XTG mice significantly remembered conditioning during tone and recovery phases when velocity was measured (*p < 0.03) and trended towards significance (p < 0.07) when travel distance was measured. N = 8 mice per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

compared to vehicle-treated control mice on the number of correct hole pokes at the target. While the latency to escape was decreased for Cmpd-3 treated mice, it was not significant from control (data not shown).

Fear Conditioning is a type of associative learning task in which mice learn to associate a particular stimulus (i.e. tone) with an aversive stimulus (i.e. foot shock). After repeated pairings of the stimuli, the animal learns and remembers to fear the tone as assessed by a conditional response (freezing, i.e. less activity). 3XTG mice have been shown to have memory retrieval deficits in this behavior test (Pairojana et al., 2021). While Cmpd-3 treated mice had reduced active time during tone and recovery (Fig. 6A), this was not statistically significant. However, Cmpd-3 treated mice had statistically significant (*p < 0.03) improved memory retrieval during tone and recovery when measuring velocity (Fig. 6C) and trended for significance (p < 0.07) when the distance traveled was measured (Fig. 6B) when compared to vehicle treated controls. Due to cost restraints, we could not analyze the hAPP-Tg mice for cognitive tests.

3.6. Cmpd-3 rescues AD biomarker profile in hAPP-Tg mouse model

As previous reports of plasma detection of Aβ are variable due to low sensitivity, we first performed a separate optimization study with the CRO, Quanterix using the ultrasensitive (pg/ml) Simoa© Assay with the multiplex 4PlexE plate to determine hAβ40 and hAβ42 levels using 8.4 month WT and hAPP mouse plasma. Through trial and error, we found that mouse plasma could be frozen for 2 months that yields consistent data as the amyloid peptides quickly degrade under longer storage times. Dilution factors also needed to be adjusted as human proteins were being measured in a mouse plasma matrix. Under optimized conditions, we found that Aβ40 was highly statistically elevated in the hAPP mice (*p < 0.0001)(Fig. S4). While Aβ42 was also elevated as expected, it was not statistically significant.

We next determined if dose-efficacy in the LTP rescue (Fig. 3CD) corroborated with any changes in the AD biomarker profile that were also benchmarked to donepezil. Blood samples were collected from the study in Fig. 3A drawn prior to sacrifice for the LTP studies. We also found a dose-efficacy effect of Cmpd-3 at reducing Aβ to levels that were non-significant from normal controls. Cmpd-3 at 9 mg/kg was the most efficacious at significantly reducing Aβ40 (*p < 0.004) (Fig. 7A) and Aβ42 (*p < 0.02) (Fig. 7B) from 9 months hAPP-Tg controls, followed by significant reductions also with the 6 mg/kg dose (*p < 0.017 and *p < 0.034, respectively). While donepezil (1 mg/kg) significantly rescued the LTP deficit, it did not significantly reduce any of the Aβ AD biomarkers. For Nf-l, a promising non-invasive AD biomarker for neuron damage (Lewczuk et al., 2018), there was a significant increase in the hAPP mice compared to non-Tg controls (*p < 0.008). However, Cmpd-3 at 9 mg/kg dose was trending near significance at reducing Nf-l levels (p < 0.12) and may be able to become significant if more animals are used. As the Nf-l assay is based upon detecting the human form of Nf-l, the assay may not be as sensitive.

4. Discussion

Previous studies in transgenic and KO mouse models indicated that α_{1A}-AR activation would evoke enhancement in learning, memory, and cognition (Doze et al., 2011), but the use of orthosteric agonists leads to increases in BP that decrease interest in therapeutic development. We have now overcome this limitation with the use of allosteric modulators of the α_{1A}-AR. We previously reported the synthesis and characterization of a series of PAMs for the α_{1A}-AR. Cmpd-3 is receptor and ligand-specific in addition to being signal-biased. It is α_{1A}-AR subtype selective that can induce conformational changes only in the NE-bound receptor and is signaling-biased to potentiate the NE-mediated cAMP signal involved in memory formation but does not affect the IP pathway that causes increases in BP. There is no detectable effect of Cmpd-3 on either binding or signaling with the EPI-bound receptor. The NE conformational-specificity of Cmpd-3 will cause effects to be targeted to the brain while the lack of effect on EPI and having no basal activity on its own will decrease possible side effects in the periphery. In this report, four different *in vivo* studies in two different AD mouse models demonstrated restorative cognitive and/or memory effects with no side effects on BP and with decreased AD biomarker levels, indicating a disease modifying effect with potential disease reversal with Cmpd-3, which has not been reported with donepezil.

There were notable differences in the dosage of Cmpd-3 needed to achieve 100% rescue in the LTP experiments, resulting in an inverted U-shaped dose effect in our second dose-efficacy study (Fig. 3CD). Each experiment was performed about 1–2 years apart from different broods

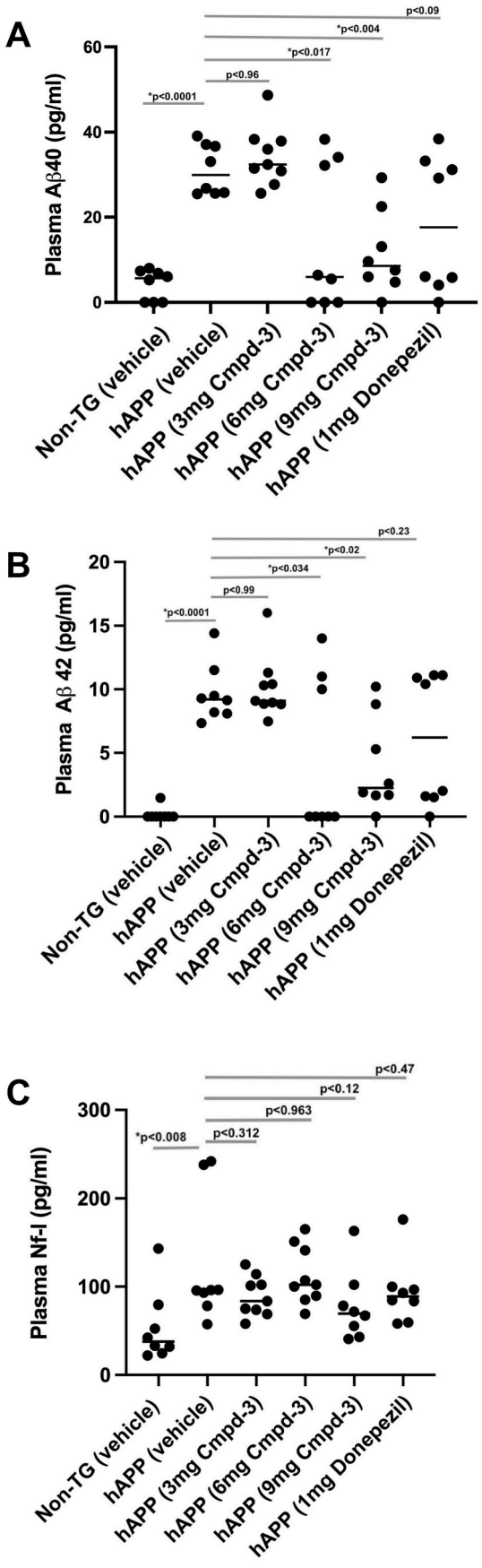

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Fig. 7. Cmpd-3 dose-dependently reversed plasma AD biomarkers in hAPP-Tg mice. Plasma samples from hAPP-Tg or non-Tg mice dosed for 3 months with vehicle or Cmpd-3 (3, 6, 9 mg/kg) or donepezil (1 mg/kg) were analyzed to determine levels of AD biomarkers using the 4plexE multiplex assay. There was a dose-efficacy effect of Cmpd-3 at reducing Aβ to levels that were non-significant from normal controls. Cmpd-3 at 9 mg/kg was the most efficacious at significantly reducing Aβ40 (*$p < 0.004$)(**A**) and Aβ42 (*$p < 0.02$) (**B**), followed by significant reductions also with the 6 mg/kg dose at Aβ40 (*$p < 0.017$) and Aβ42 (*$p < 0.034$). Donepezil (1 mg/kg) while significantly the rescuing the LTP deficit did not produce any significant changes in reducing any of these AD biomarkers. For Nf-l, there was a significant increase in the hAPP-Tg vehicle mice compared to non-Tg controls (*$p < 0.008$)(**C**). Cmpd-3 at 9 mg/kg dose was trending near significance at reducing Nf-l levels ($p < 0.12$). N = 8 mice per group.

which may account for some differences. Inverted U-shaped dose response relationships in various behavior and cognitive functions mediated by neuromodulators are common (Cools and Arnsten, 2022), including NE and Epi (Baldi and Bucherelli, 2005), dopamine (Monte-Silva et al., 2009), NMDA (Uslaner et al., 2009), glucocorticoids (Roozendaal, 2002), cannabinoids (Nedelescu et al., 2022), a PAM for the GluR5 (Parmentier-Batteur et al., 2012), and the currently prescribed drugs for Alzheimer's Disease, anticholinesterases such as donepezil (Cohen et al., 2002; Wezenberg et al. 2005; Bentley et al., 2011; Reches et al., 2014). Biological non-linear relationships are common and most inverted U-shaped dose effects are described in rodent models but limited examples have been documented in humans (Zuardi et al., 2017; Fresnoza et al., 2014). While inverted dose effects are poorly understood, the leading theory is at higher activation levels, stress and emotional arousal levels become elevated (i.e. induced by training conditions in rodents) to the point that inhibit memory processes (Baldi and Bucherelli, 2005). It is worthy to note that the rescue of the amyloid biomarkers did not display an inverted U-shaped dose (peaking at 9 mg/kg; Fig. 7) and consistent with the 2nd LTP study at 9 mg/kg (Fig. 3A) which displayed 100% rescue but did not have inverted U-shaped dosage. 10 month dosing at 2.6 mg/kg as in the 3XTG mouse (Fig. 2) produced highly significant results on LTP which suggests that long-term dosing is safe and effective. As inverted U-shaped dosing was not consistent but disease modification was dose-dependent, Cmpd-3 is likely to display a wide enough therapeutic index to modify Alzheimer's Disease under chronic use.

There are profound effects on the NE system during AD due to loss of the locus coeruleus neurons (Haglund et al., 2006) resulting in the loss of neuroprotection and cognitive reserve (Plini et al., 2021) with decreases in α_{1A}-AR mRNA (Szot et al., 2006). The α_{1A}-AR subtype is highly expressed in the human and rodent hippocampus (Szot et al., 2005; Papay et al., 2006), a major center of learning and memory, and in the amygdala, prefrontal, and entorhinal/perirhinal cortices (Papay et al., 2006; Graebenitz et al., 2011; Perez, 2020). These are also domains affected in AD (Berron et al., 2020). α_{1A}-AR activation increases both long-term and short-term synaptic plasticity (Pussinen and Sirviö, 1998; Doze et al., 2011; Perez, 2020) and increases spatial, working, reward, and fear-conditioned memory formation, storage, and recall (Perez, 2020; Ferry et al., 1999b). Cognitive and memory enhancement by Cmpd-3 is hypothesized to be through its potentiation of the NE-mediated cAMP signal and p-CREB transcription through activation of the α_{1A}-AR (Papay et al., 2023; Lin et al., 1998; Thonberg et al., 2002). A requirement for cAMP in presynaptic LTP is well known and demonstrated in mice lacking the genes encoding adenylyl cyclase or PKA (Yang and Calakos, 2013) as well as for learning, memory, and consolidation (Bito et al., 1996; Kandel, 2012). Increased p-CREB is considered a molecular marker of late LTP in the hippocampus after learning (Barco et al., 2002), which also requires transcription and new protein synthesis (Nguyen et al., 1994) and/or adult neurogenesis which is increased by α_{1A}-AR agonists in the subventricular and subgranuar zones (Gupta et al., 2009). This may be why dosing of Cmpd-3 requires at least 2–3

months before LTP effects can be measured.

NE may influence amyloid accumulation and clearance (Kong et al., 2010). NE deficiency increases amyloid plaque burden by decreasing clearance (Kalinin et al., 2007). Similarly, α_{2A}-AR agonists which inhibit NE release from sympathetic nerve endings also increase amyloidogenesis and plaque burden (Chen et al., 2014). β_2-AR activation is neuroprotective against amyloid toxicity (Tamano et al., 2018; Kawano et al., 2022; Chai et al., 2022). There are no prior reports concerning effects of α_1-ARs in amyloid biology. The familial hAPP[V717I] mutation was the first discovered and the most common mutation for AD (Cruts et al., 2012) and is hypothesized to alter APP processing by both β- and γ-secretase (Muratore et al., 2014). The hAPP-Tg mice do not develop plaques until 12–15 months of age, but increase Aβ 40 and 42 levels as they age (Dewachter et al., 2000, 2001). Cmpd-3 caused a reduction in Aβ40 and Aβ42 suggesting clinically-relevant changes in the disease process and manifests *in vivo* target engagement. Cmpd-3 outperformed donepezil in the reduction of AD biomarkers, suggesting that changes in LTP alone should not be used for AD drug discovery. Acetylcholinesterase inhibitors, such as donepezil, while efficacious for LTP, has limited and short-acting effects on AD symptoms, suggesting that it is not disease-modifying as evidenced by the lack of efficacy in reducing AD biomarkers. The use of AD biomarkers for both diagnosing as well as validating promising therapeutics for effectiveness and target-engagement will accelerate clinical trials (Cummings and Kinney, 2022).

α_{1A}-AR selective agonists have been previously shown to be efficacious in relieving animal models of stress urinary incontinence (Taniguchi et al., 1996; Modiri et al., 2000). Partial imidazoline and not phenethylamine agonists such as RO-115-1240 (Dabuzalgron) were shown to improve stress urinary incontinence without increasing BP (Blue et al., 2004; Musselman et al., 2004) but these agonists were pulled early in clinical trials for lack of efficacy (Bishop, 2007), suggesting that imidazoline-based orthosteric α_{1A}-AR agonists have a narrow therapeutic window over blood pressure effects. α_{1A}-AR selective imidazoline agonists (Beak et al., 2017; Montgomery et al., 2017) have also been previously shown to be efficacious in the treatment of heart failure in animal models. There is a well-known association of cardiovascular disease in AD patients, particularly with ischemia and heart failure, as they share genetic profiles (Tublin et al., 2019). Cmpd-3 may be useful to treat these other conditions without effects on BP.

Cmpd-3 has a low molecular weight, high solubility, and rapid uptake into the brain. While the efflux ratio is > 3 and indicates that it is a substrate for P-glycoprotein, Cmpd-3 penetrates the brain with a brain to plasma ratio of 0.27 and a slightly longer half-life in the brain than in plasma. In addition, Cmpd-3 has low to moderate plasma clearance which allows for good efficacy in a once per day dosing regimen as shown in our studies. By virtue of its allosteric profile, Cmpd-3 is predicted to have less side effects than traditional orthosteric drugs**.** Cmpd-3 also has a clean profile against the major cytochrome 450 enzymes. There were no observable side effects in any of the *in vivo* studies and previous studies suggest that α_{1A}-AR agonists are cardio and neuroprotective (Perez and Doze, 2011; Perez, 2021b) and increase longevity (Doze et al., 2011). Based upon our results, Cmpd-3 has the potential to fully treat and modify AD and may become the treatment of choice to also treat cardiovascular problems in AD patients.

CRediT authorship contribution statement

Robert S. Papay: Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Visualization. **Shaun R. Stauffer:** Conceptualization, Validation, Resources, Writing – review & editing. **Dianne M. Perez:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors have filed the following patents: PCT/US2020/029583, filed 4/23/2020. European Patent (No. 20794530.4) filed 10/15/2021. US utility (17/605,801) filed 10/21/2021.Inventors: PEREZ, Dianne M.; STAUFFER, Shaun R.; MACDONALD, Jonathan. Allosteric Activators of the Alpha1A-Adrenergic receptor. European Patent (No. 20794530.4) filed June 10, 2022.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphar.2023.100160.

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