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RESEARCH ARTICLE

Chronic GCPII (glutamate-carboxypeptidase-II) inhibition reduces pT217Tau levels in the entorhinal and dorsolateral prefrontal cortices of aged macaques

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Abstract

Introduction: Current approaches for treating sporadic Alzheimer's disease (sAD) focus on removal of amyloid beta 1-42 ($A\beta_{1\text{-}42}$) or phosphorylated tau, but additional strategies are needed to reduce neuropathology at earlier stages prior to neuronal damage. Longstanding data show that calcium dysregulation is a key etiological factor in sAD, and the cortical neurons most vulnerable to tau pathology show magnified calcium signaling, for example in dorsolateral prefrontal cortex (dlPFC) and entorhinal cortex (ERC). In primate dlPFC and ERC, type 3 metabotropic glutamate receptors (mGluR3s) are predominately post-synaptic, on spines, where they regulate cAMP-calcium signaling, a process eroded by inflammatory glutamate carboxypeptidase II (GCPII) actions. The current study tested whether enhancing mGluR3 regulation of calcium via chronic inhibition of GCPII would reduce tau hyperphosphorylation in aged macaques with naturally-occurring tau pathology.

Methods: Aged rhesus macaques were treated daily with the GCPII inhibitor, 2-MPPA (2-3-mercaptopropyl-penanedioic acid (2-MPPA)),

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Aged rhesus macaques were treated daily with the GCPII inhibitor, 2-MPPA (2-3-mercaptopropyl-penanedioic acid (2-MPPA)),

Results: Aged macaques that received 2-MPPA had significantly lower pT217Tau levels in dlPFC and ERC, and had lowered plasma pT217Tau levels from baseline. pT217Tau levels correlated significantly with GCPII activity in dlPFC. Both 2-MPPA- and vehicle-treated monkeys showed cognitive improvement; 2-MPPA had no apparent side effects. Exploratory CSF analyses indicated reduced pS202Tau with 2-MPPA administration, confirmed in dlPFC samples.

Discussion: These data provide proof-of-concept support that GCPII inhibition can reduce tau hyperphosphorylation in the primate cortices most vulnerable in sAD. GCPII inhibition may be particularly helpful in reducing the risk of sAD caused by inflammation. These data in nonhuman primates should encourage future research on this promising mechanism.

KEYWORDS
calcium, CSF, dorsolateral prefrontal cortex, entorhinal cortex, macaque, mGluR3, plasma, pT217Tau

Highlights

- Inflammation is a key driver of sporadic Alzheimer's disease.
- GCPII inflammatory signaling in brain decreases mGluR3 regulation of calcium.
- Chronic inhibition of GCPII inflammatory signaling reduced pT217Tau in aged monkeys.
- GCPII inhibition is a novel strategy to help prevent tau pathology at early stages.

1 | INTRODUCTION

Novel therapeutic strategies are needed to reduce the risk, and possibly prevent, the neuropathology of sporadic Alzheimer's disease (sAD) by intervening at early stages prior to neuronal damage. Dysregulated calcium signaling is well established as an early etiological factor in sAD,[1,2] related to inflammatory mechanisms[3]; thus treatments that restore calcium regulation may help to prevent or slow pathological cascades. However, these interventions need to be tested in animal models with naturally-occurring calcium dysregulation and sAD-like pathology, as mouse models employ genetic modifications based on autosomal dominant disease that produce pathology *downstream* from calcium dysregulation. Aged rhesus macaques provide a unique opportunity to test upstream approaches, as they are all apolipoprotein E (*APOE*) ε4 homozygotes,[4] and they naturally develop calcium dysregulation, amyloid plaques, and tau pathology, including tangles in the oldest animals.[5,6] Higher cortical circuits in primates require type 3 metabotropic glutamate receptor (mGluR3) regulation of calcium signaling[7] and loss of calcium regulation with age and/or inflammation contribute to tau hyperphosphorylation.[6] The current study used the aged macaque model to test the hypothesis that inhibiting glutamate carboxypeptidase II (GCPII) inflammatory signaling to restore mGluR3 regulation of calcium would reduce tau hyperphosphorylation in primate cortex.

Tau pathology selectively afflicts the glutamatergic neurons in association/limbic cortices that subserve memory and higher cognition,[8,9] correlating with cognitive symptoms in AD.[10–12] Thus reducing tau hyperphosphorylation may be especially helpful in protecting higher brain circuits and cognitive state. Tau pathology first appears in layer II perirhinal and entorhinal cortices (ERCs), and later in the association cortices and hippocampus, but only afflicts primary visual and auditory cortices at end-stage disease.[13] Tau pathology in ERC correlates with recent memory deficits,[14] whereas that in dorsolateral prefrontal cortex (dlPFC) correlates with general cognitive deficits on the Clinical Dementia Rating (CDR) scale,[15] consistent with its role in working memory and higher cognition.[7] Within the dlPFC, the layer III pyramidal cells that express the calcium-binding protein, calbindin, when young, become most vulnerable to tau pathology and neurodegeneration in sAD[16] when calbindin is lost with age.[17] A similar pattern and sequence of tau pathology is seen in aging rhesus monkeys, beginning in layer II ERC, and later in dlPFC, but not in aged V1,[5,18] with loss of calbindin in layer III dlPFC pyramidal cells with advancing age making them vulnerable to calcium dysregulation and tau hyperphosphorylation.[6]

Thus restoring calcium regulation may be beneficial in reducing tau pathology.

As summarized in Figure 1, post-synaptic mGluR3s are positioned to regulate calcium signaling in the dlPFC layer III circuits that subserve higher cognition.[19] These recurrent excitatory circuits express the molecular machinery for cyclic adenosine monophosphosphate-protein kinase A (cAMP-PKA) signaling to magnify internal calcium release near glutamate synapses on dendritic spines, needed to maintain neuronal firing during working memory.[7] In the young, healthy dlPFC, feedforward cAMP-calcium signaling is tightly regulated by calbindin, the phosphodiesterases PDE4A and PDE4D, and by mGluR3 on dendritic spines.[7] However, these are reduced with age and/or from inflammation,[5–7,18,20] which can lead to calcium dysregulation, activation of calpain-2, the disinhibition of GSK3β and cdk5, and the hyperphosphorylation of tau[21,22] (Figure 1B), for example, at pT217Tau, a new fluid biomarker for AD.[23–25] Recent data show similar mechanisms to magnify calcium in the layer II ERC cell islands (Datta and Arnsten, in press). However, these ERC cells never express calbindin,[26] which may render them especially vulnerable to tau pathology at an early age. There are few available pharmacological strategies to restore regulation of cAMP-calcium signaling in the aging cortex, but one promising avenue is to boost endogenous regulation by mGluR3s, which perform a large, beneficial role in primate cortex.[19]

mGluR3s play a powerful, post-synaptic role in primate dlPFC, where they regulate cAMP-calcium opening of K^+ channels and enhance neuronal firing and working memory performance[19] (Figure 1A). It is important to note that mGluR3s are stimulated not only by glutamate, but by *N*-acetylaspartylglutamate (NAAG), which is co-released with glutamate and is selective for mGluR3s.[27] However, NAAG is catabolized by GCPII,[27] an enzyme synthesized in glia, especially under conditions of inflammation.[28,29] Inhibiting GCPII restores neuronal firing and working memory performance in aged macaques with naturally-occurring GCPII expression,[30] and improves object recognition in AD model mice.[31]

The current study tested whether chronic treatment with a GCPII inhibitor might also reduce tau hyperphosphorylation in aged rhesus monkeys with naturally-occurring GCPII and tau pathology. Given that aged rhesus monkeys are a rare resource, the current study necessarily employed a small number of subjects, and focused on pT217Tau, as this pTau species is an emerging blood biomarker for sAD that heralds future disease,[23–25] and is prominently expressed in aged macaque dlPFC and ERC,[6,32] including seeding between neurons where it is exposed to the extracellular fluid for potential transport to blood and cerebrospinal fluid (CSF) (Datta and Arnsten, unpublished data). The current study shows that pT217Tau can be used as a fluid biomarker in this animal model, similar to in humans. We tested whether chronic inhibition of GCPII would reduce tau pathology in brain and plasma, using the selective GCPII inhibitor, 2-MPPA (2-(3-mercaptopropyl)pentanedioic acid), as it is currently the only orally bioavailable, brain-penetrant GCPII inhibitor that has progressed to clinical trials,[33] and has been shown to improve working memory and dlPFC neuronal firing in aged macaques with acute administration.[30] We tested whether chronic 2-MPPA treatment would reduce pT217Tau levels and GCPII activity in cortex, and pT217Tau in plasma. We also performed exploratory assays of drug effects on amyloid beta 1-42 ($A\beta_{1\text{-}42}$) levels in cortex and plasma, and mass spectrometry (MS) assays of phosphorylated tau species in macaque CSF. We found that 6 months of daily treatment with 2-MPPA reduced levels of pT217Tau in dlPFC, ERC, and plasma, as well as in an exploratory analysis showing a reduction in CSF pS202Tau.

RESEARCH IN CONTEXT

1. **Systematic Review**: The authors reviewed the literature using traditional (e.g., PubMed, conference abstracts) sources, focusing on type 3 metabotropic glutamate receptors (mGluR3s), glutamate carboxypeptidase II (GCPII), inflammation, and tau hyperphosphorylation.
2. **Interpretation**: The findings that (1) GCPII activity in the primate association cortex highly correlated with levels of pT217Tau, and (2) that chronic treatment with a GCPII inhibitor reduced levels of pT217Tau, are consistent with the hypothesis that GCPII erosion of mGluR3 calcium regulation contributes to tau hyperphosphorylation in the cognitive circuits most vulnerable to tau pathology in sporadic Alzheimer's disease (sAD). These data suggest a novel mechanism to reduce early stage tau pathology.
3. **Future Directions**: Currently available GCPII inhibitors have poor brain penetration. The current data encourage the development of GCPII inhibitors with superior brain penetration for future testing. As brain GCPII expression is increased by inflammation, this mechanism may be particularly helpful in reducing the risk posed by neuroinflammation in the etiology of sAD.

2 | METHODS

All research was approved by the Yale Institutional Animcal Care and Use Commitee (IACUC) and performed under National Institutes of Health (NIH) guidelines. Please see the Supplementary Materials for more detailed Methods.

2.1 | Subjects

This study utilized eight, aged rhesus macaques (20–27 years; 6 female) acquired from a retired cognitive pharmacology lab at Yale. The monkeys were housed under standard laboratory conditions with pair-housing, and daily environmental enrichment and veterinary care. The 2-MPPA ($n = 4$, 1 male, mean$\pm$SEM age 23.3 $\pm$ 0.28 years) versus vehicle ($n = 4$, 1 male, mean age 22 $\pm$ 0.51 years) groups were matched for sex and age.

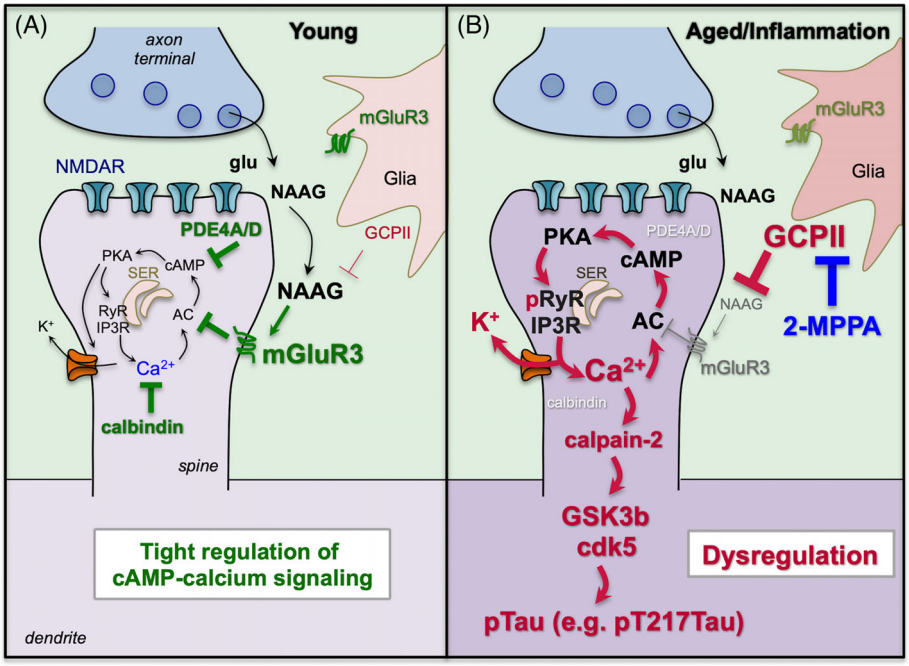

FIGURE 1 Schematic illustration of mGluR3-GCPII influence on cAMP-calcium intracellular signaling in primate dlPFC. (A) mGluR3s are concentrated on dendritic spines as well as on astrocytes in macaque dlPFC and ERC. These spines express the molecular machinery for feedforward cAMP-calcium signaling needed to maintain persistent firing in working memory, but also capable of opening nearby K^+ channels as negative feedback. In young, healthy dlPFC, cAMP-calcium signaling is tightly regulated by PDE4A/D and calbindin, and by glutamate and NAAG stimulation of mGluR3s. (B) Under conditions of inflammation/advancing age, glia increase their expression of GCPII, which catabolizes NAAG and reduces mGluR3 regulation of cAMP-calcium signaling. Aging/inflammation also reduce the expression of PDE4A/D and calbindin, resulting in elevated levels of cAMP-PKA-calcium signaling, including PKA phosphorylation of ryanodine receptors to cause calcium leak from the SER. Excessive cAMP-PKA-calcium signaling opens K^+ channels, which reduce neuronal firing and induce calpain activation of kinases such as GSK3b to hyperphosphorylated tau, for example, at pT217Tau. dlPFC, dorsolateral prefrontal cortex; ERC, entorhinal cortex; GCPII, glutamate-carboxypeptidase II; NAAG, *N*-acetylaspartylglutamate; PKA, protein kinase A.

2.2 | Experimental Design

The monkeys were retrained on the delayed response task (see Supplemental Methods; note that lab closure due to coronavirus disease 2019 [COVID-19] interrupted training), followed by 6 months of daily treatment with 2-MPPA (0.1 mg/kg diluted in sterile saline, p.o.) or vehicle; twice a week they were tested on the delayed response task of spatial working memory, 2 hours following treatment. In rats, 2-MPPA has a rapid terminal elimination half-life of 1 hour following intravenous administration.[33] Monkeys were tested by technicians who were highly familiar with their normative behavior but blind to drug-treatment conditions. Monkeys were rated for any changes in sedation, agitation, or aggression, with notice of any unusual changes, for example, in pallor or appetite. Because 2-MPPA can be unstable in solution, drug was diluted fresh each day immediately prior to administration. On the last day of the study, monkeys were treated with vehicle or 2-MPPA as appropriate and were humanely sacrificed by Yale veterinarians to rapidly acquire brains for assessment of phosphorylated tau levels; the average time post-treatment to brain extraction was approximately 3 hours. As nonfibrillar phosphorylated tau rapidly dephosphorylates post-mortem, great care was taken to remove and freeze brains as quickly as possible (under 15 min). Blood and CSF were drawn at baseline and on the day of sacrifice under anesthesia by Yale veterinarians. Details of brain extraction are in the Supplementary Methods.

2.3 | GCPII activity and 2-MPPA assays in dlPFC tissue

Frozen dlPFC samples were sent to Johns Hopkins University where assays were performed blind to drug-treatment conditions. The levels of 2-MPPA in the dlPFC were below the limit of quantification, likely due to the brief half-life of this compound. Enzymatic assays of GCPII activity in dlPFC tissue were performed as previously described [34] and in the Supplementary Methods; there was insufficient ERC tissue to send for this additional analysis.

2.4 | Protein extraction, western blot, and A$\beta_{1\text{-}42}$ assays and statistics

Biochemical assays of pT217Tau and A$\beta_{1\text{-}42}$ levels in brain (dlPFC and ERC), and A$\beta_{1\text{-}42}$ in plasma were performed by the Nairn lab, blind to drug treatment. Details can be found in the Supplemental Methods.

2.5 | Nanoneedle plasma assays for pT217Tau

Levels of pT217Tau in plasma were analyzed by a novel, nanoneedle technology by the Xie lab at Harvard, blind to drug treatment. Baseline and post-treatment samples were all run at the same time at the end of the study to allow direct comparison. Details can be found in the Supplemental Methods.

2.6 | CSF assays

The Barthelemy/Bateman group utilized MS to assay pTau species in cerebral spinal fluid (CSF)[23]; assays were performed blind to drug treatment. A baseline sample was unable to be acquired from one aged monkey in the 2-MPPA group with extensive spinal arthritis, which limited the numbers of before- versus after-treatment comparisons.

2.7 | Statistics

Data were analyzed by between-subject independent *t*-tests for between-group comparisons, and with Pearson's *r* correlation for relationships between factors. Given the small number of subjects, only large, consistent drug effects reached statistical significance.

3 | RESULTS

3.1 | Chronic 2-MPPA effects on working memory performance

Although all of the 2-MPPA-treated monkeys showed improved working memory performance compared to baseline levels, there was no significant difference from vehicle, as three of the four control animals improved as well (Figure S1). As described in the Methods, this was likely due to the COVID-19 lab closures, which prevented the extensive training period needed to achieve a stable baseline performance prior to the onset of the 6-month drug/vehicle treatment. It is notable that no side effects were evident in 2-MPPA-treated monkeys, that is, there were no alterations in measures of sedation, agitation, aggression (all measures "0", i.e., similar to normative behavior for that animal), with no changes noted in motivation or pallor.

Chronic 2-MPPA effects on pT217 in dlPFC and ERC and plasma

The primary hypothesis tested in the current study is whether chronic treatment with a GCPII inhibitor would reduce tau hyperphosphorylation, potentially by increasing regulation of calcium signaling. The study focused on pT217Tau, given its increasing relevance to sAD diagnosis. Aged monkeys treated daily with 2-MPPA for 6 months had significantly lower pT217Tau levels in both the dlPFC (Figure 2A; $p = 0.016$) and ERC (Figure 2B; $p = 0.04$) compared to vehicle-treated controls.

Plasma pT217Tau levels are under development as a potential blood biomarker in human studies. Thus the current study also examined pT217Tau levels in the plasma of the aged monkeys and compared them to levels in brain. The baseline levels of plasma pT217 were similar in the vehicle-treated (5.0 ± 0.51) versus 2-MPPA-treated (5.2 ± 0.69) groups (5.0 ± 0.51 vs 5.2 ± 0.69, respectively, $p = 0.74$). Daily treatment with 2-MPPA for 6 months reduced pT217Tau levels in plasma from baseline levels in three of the four aged monkeys (Figure 2C; $p = 0.06$ for baseline vs treatment for all four monkeys). The one aged monkey whose levels were unchanged following 2-MPPA had the lowest baseline pT217Tau levels, suggesting potential floor effects. Of interest, plasma levels of pT217Tau in the control group showed a very positive trend for correlation with pT217 levels in brain ($r = 0.88$, $p = 0.1$, $n = 4$; Figure 3A).

3.2 | Chronic 2-MPPA reduced GCPII activity in dlPFC tissue

The 2-MPPA-treated group showed a strong trend for reduced GCPII enzyme activity in dlPFC compared to the vehicle-treated controls ($p = 0.07$; Figure 4A). Analysis showed that dlPFC levels of GCPII enzyme activity highly correlated with dlPFC levels of pT217Tau ($r = 0.96$, $p =$ 0010; Figure 4B), consistent with the hypothesis that GCPII inhibition reduces tau hyperphosphorylation.

3.3 | Exploratory analyses

3.3.1 | $A\beta_{1\text{-}42}$ plasma assays

There were no significant effects of chronic 2-MPPA treatment on assays of brain or plasma $A\beta_{1\text{-}42}$ levels. Enzyme-linked immunosorbent assay (ELISA) measures of $A\beta_{1\text{-}42}$ from the ERC showed a mean of 2.8 ± 0.2 pg/mL for the vehicle-treated group, and 3.6 ± 1.1 pg/mL for the 2-MPPA-treated group. ELISA measures of $A\beta_{1\text{-}42}$ from the dlPFC showed a mean of 2.2 ± 0.1 pg/mL for the vehicle-treated group, and 2.9 ± 0.7 pg/mL for the 2-MPPA-treated group. Plasma levels before and after treatment were 14.9 ± 2.3 pg/mL before, and 13.7 ± 1.7 pg/mL after, for the vehicle-treated group, and 10.6 ± 1.5 pg/mL before, and 10.0 ± 0.4 pg/mL after, for the 2-MPPA-treated group. It is possible that treatment longer than 6 months may lead to changes in $A\beta_{1\text{-}42}$ as well as tau phosphorylation.

3.4 | CSF data

The CSF data were considered exploratory based on the inability to collect baseline CSF from one of the aged monkeys in the 2-MPPA group due to extensive spinal arthritis, and because the MS approach used

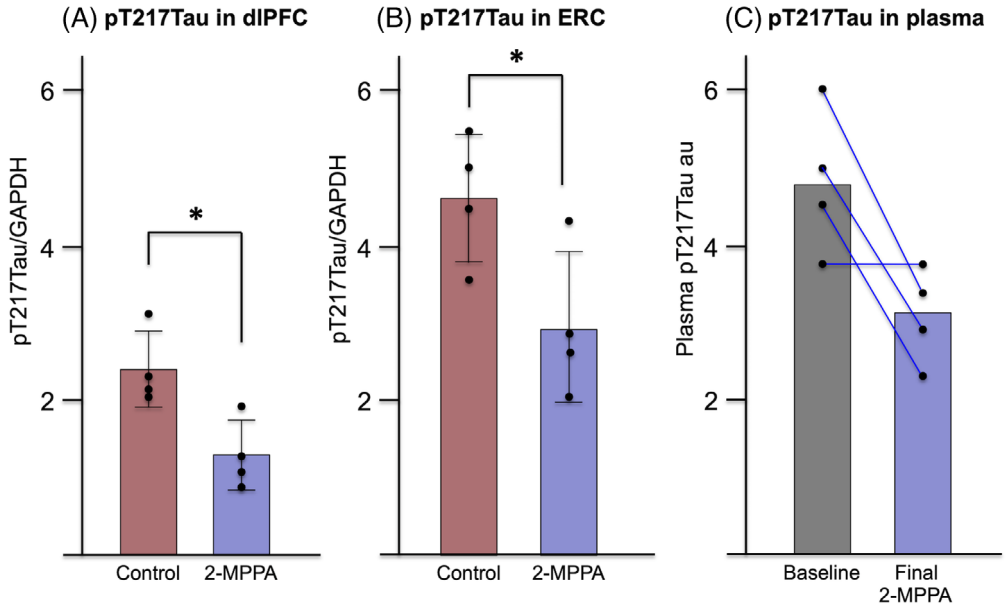

FIGURE 2 Chronic daily treatment with the GCPII inhibitor, 2-MPPA, was associated with reduced levels of pT217Tau in brain and plasma. (A) Aged macaques receiving 2-MPPA treatment had significantly lower levels of pT217Tau in dlPFC compared to aged macaques treated with vehicle (*$p = 0.016$; $n = 4$/group). pT217Tau levels were assayed by western blot and are expressed as a ratio of GAPDH expression. (B) Aged macaques receiving 2-MPPA treatment also had significantly lower levels of pT217Tau in ERC compared to aged macaques treated with vehicle (*$p = 0.04$; $n = 4$/group). (C) Plasma levels of pT217Tau measured by nanoneedle assays were reduced from baseline levels after 6 months of daily treatment with 2-MPPA in three aged macaques; the fourth animal with the lowest levels at baseline was unchanged after treatment, resulting in an overall trend level reduction from baseline in the group ($p = 0.1$; $n = 4$/group). dlPFC, dorsolateral prefrontal cortex; GCPII, glutamate-carboxypeptidase II.

allows for analysis of a large number of pTau species in addition to pT217Tau, which would require a much larger number of subjects to have sufficient statistical power. The data for all pTau species assayed are shown in Table S1. CSF assays of pT217Tau showed that the 2-MPPA group had higher levels of CSF pT217Tau at baseline ($p = 0.02$) than in control animals, and a large decrease in CSF pT217Tau levels in two of the three aged monkeys treated with 2-MPPA (Figure 5; $p = 0.27$, $n = 3$). However, there was a reduction in three of the four control monkeys as well (Figure 5). As with the plasma experiment, the aged monkey in the 2-MPPA group with the lowest baseline levels was unchanged following drug treatment, suggesting that there may have been floor effects in this animal.

The monkey with the lowest CSF pT217Tau was not the same animal as the monkey with the lowest plasma levels of pT217Tau; and there was no correlation between CSF and plasma levels of pT217Tau in either the entire sample or the controls ($r = -0.59$, $r = -0.57$, respectively). CSF pT217Tau levels in control monkeys did not correlate with brain levels (Figure 3B); an apparent negative correlation was due to one outlier aged monkey with high CSF pT217Tau levels and relatively low pT217Tau levels in dlPFC+ERC.

An exploratory analysis of the CSF data found two additional tau species of interest: pT111Tau, which was significantly decreased by 2-MPPA treatment (Figure 6A), and pS202Tau, where levels tended to increase in the control group but decrease in the 2-MPPA treated monkeys (Figure 6B). Based on these CSF data, we analyzed remaining dlPFC tissue by western blot for pS202Tau (there are no antibodies yet available for pT111Tau), and we found that the monkeys treated chronically with 2-MPPA had reduced pS202Tau levels in dlPFC compared to controls (Figure 6C; $p = 0.022$).

4 | DISCUSSION

The current study tested a proof-of-concept hypothesis that enhancing mGluR3 regulation of cAMP-calcium signaling through the inhibition of GCPII would reduce tau hyperphosphorylation in aged macaque cortex.[6,18,19] Although the number of animals was necessarily small, the data are highly encouraging, showing that aged macaques treated daily for 6 months with the GCPII inhibitor, 2-MPPA, had significantly lower levels of pT217Tau in the dlPFC and ERC compared to vehicle-treated controls, with levels of GCPII activity in dlPFC highly correlating with dlPFC levels of pT217Tau. Three of the four aged macaques also showed a reduction in plasma pT217Tau from baseline, encouraging the use of this fluid biomarker in monkeys. The CSF data showed trends for reductions in pT217Tau from baseline in 2-MPPA-treated monkeys, but was hampered by complications in acquiring samples resulting in a smaller n, and likely floor effects in one animal. The strong correlation between GCPII activity and pT217Tau levels in the dlPFC is consistent with GCPII inhibition as an effective mechanism to reduce tau pathology, especially to reduce the increased risk from inflammation. Because post-synaptic mGluR3 regulation of cAMP-calcium signaling appears to expand in the primate circuits mediating cognition that are especially vulnerable to tau pathology, this novel therapeutic strategy deserves further research.

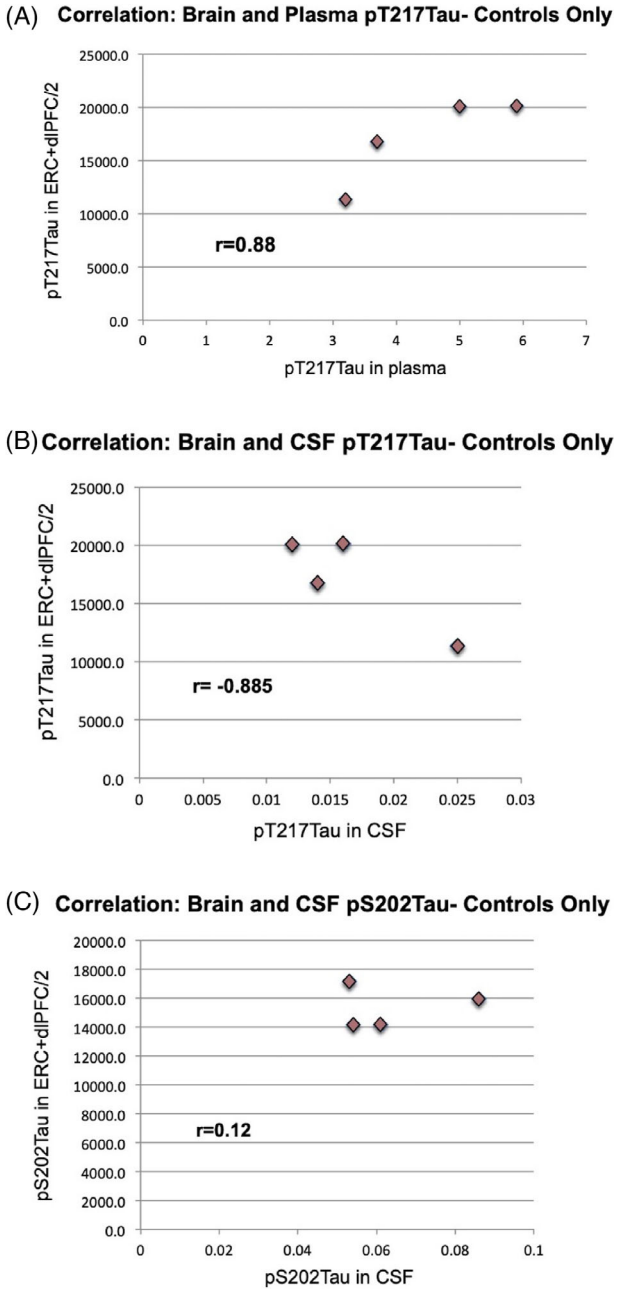

FIGURE 3 Correlations between fluid biomarkers and brain levels of pTau in vehicle-treated aged macaques. (A) Levels of pT217Tau in plasma versus brain (dlPFC+ERC/2 raw values) showed a nonsignificant, but highly positive correlation ($r = 0.88$, $p = 0.12$ two-tailed; $n = 4$/group). The pT217Tau levels from both dlPFC and ERC were combined to compare to plasma levels, as immunoEM shows evidence of pTau trafficking between neurons in both of these cortical areas, where it is exposed to the extracellular space and may be transported to blood.[22] (B) Levels of pT217Tau in CSF versus brain (dlPFC+ERC/2 raw values) showed a nonsignificant negative correlation ($r = 0.885$, $p = 0.12$ two-tailed; $n = 4$/group) that was strongly affected by one aged monkey with high CSF and low brain levels. Removal of this animal resulted in no correlation ($r = 0.025$, $p = 0.98$). dlPFC, dorsolateral prefrontal cortex; ERC, entorhinal cortex. (C) Levels of pS202Tau in CSF did not correlate with levels in brain ($r = 0.12$, $p = 0.88$).

4.1 | Weaknesses of the study

There are several weaknesses that are important to highlight: (1) The most obvious weakness is the small number of subjects due to the great expense and scarcity of very old rhesus macaques. Although this makes statistics challenging, only large, consistent, drug effects reach the threshold for significance, and it is large effects that are most likely to translate to human. (2) We were unable to interpret the effects of chronic 2-MPPA on cognitive performance, as the control group also improved over the course of the 6 months. This was likely due to the lab shut-down during the early months of the COVID-19 pandemic, which interrupting baseline training. However, our previous study showed that acute 2-MPPA treatment improved working memory in aged monkeys where stable baseline had been established.[30] (3) A baseline CSF sample was not possible in one aged rhesus from the 2-MPPA group due to extensive spinal arthritis, thus lowering the *n* for these analyses. (4) 2-MPPA, although currently the best available GCPII inhibitor, is not ideal given its limited brain penetration and short half-life. Thus the creation of superior GCPII inhibitors may have even greater effects. (5) The difficulties in deriving CSF samples from very old monkeys lengthened the time between drug administration and eventual sacrifice to ~3 hours. Given the relatively short half-life of 2-MPPA,[33] it is likely that levels of GCPII activity in the brains of 2-MPPA-treated monkeys would have been even lower if assayed at this earlier time. Despite these challenges, the primary analysis showed a significant decrease in pT217Tau levels in 2-MPPA-treated aged monkeys, including a very high correlation between GCPII activity and pT217Tau levels in aged macaque dlPFC.

4.2 | Correlations between brain and fluid biomarker levels of pTau

The current study showed that pT217Tau levels in dlPFC+ERC correlated with levels in plasma, but not in CSF. This was surprising, but may be related to how pTau species enter these differing fluid compartments. It is likely that CSF is enriched in pTau species from structures that reside near the ventricular system, for example, hippocampus and locus coeruleus, which were not measured in our biochemical analyses. In contrast, plasma levels of pT217Tau may be populated from pTau seeding between neurons, where it is exposed to the extracellular space. pT217Tau seeding is extensive in dlPFC and especially ERC (Datta and Arnsten, unpublished data), and thus plasma levels of pT217Tau may be better correlated with dlPFC+ERC levels. However, it is noteworthy that trends in CSF pS202Tau predicted differences in dlPFC levels using an unbiased approach. Antibodies for pT111Tau have yet to be developed, highlighting the power of the MS approach to capture novel tau species. As both pT111Tau and pT217Tau are specifically hyper-phosphorylated in AD,[35] it is of interest that they appear to be effective targets of 2-MPPA treatment.

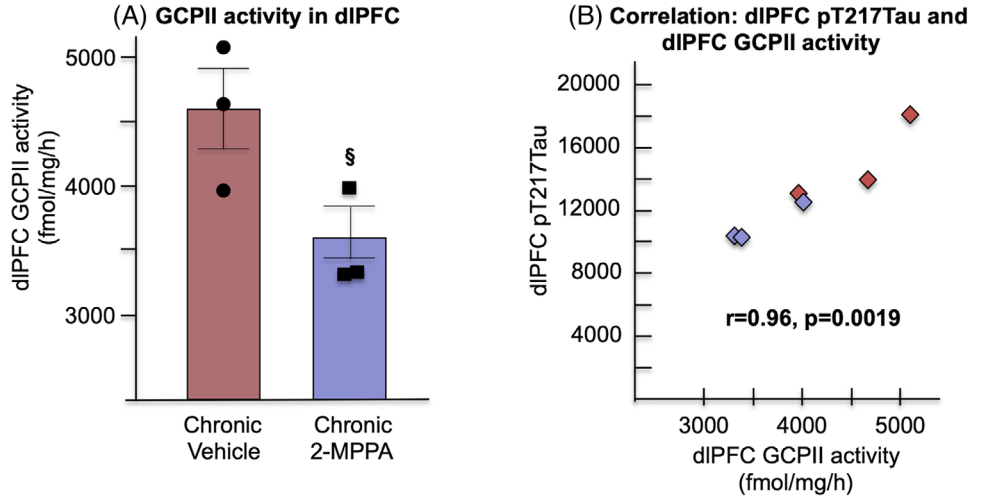

FIGURE 4 GCPII activity in the dlPFC of aged macaques treated with 2-MPPA ($n = 3$) or vehicle ($n = 3$), and correlation with pT217Tau levels. Only tissue from Herpesvirus B–negative monkeys could be shipped to Johns Hopkins University for assays of fresh frozen tissue; drug or vehicle was administered ~3 hours prior to sacrifice and rapid brain removal. (A) Levels of GCPII activity were decreased in the aged monkeys that had received chronic 2-MPPA compared to those that had received chronic vehicle ($p = 0.07$, $n = 3$). (B) The levels of GCPII activity correlated highly with levels of pT217Tau in aged macaque dlPFC ($r = 0.96$, $p = 0.0019$); blue = 2-MPPA-treated monkeys; red = vehicle-treated monkeys. dlPFC, dorsolateral prefrontal cortex; GCPII, glutamate-carboxypeptidase II.

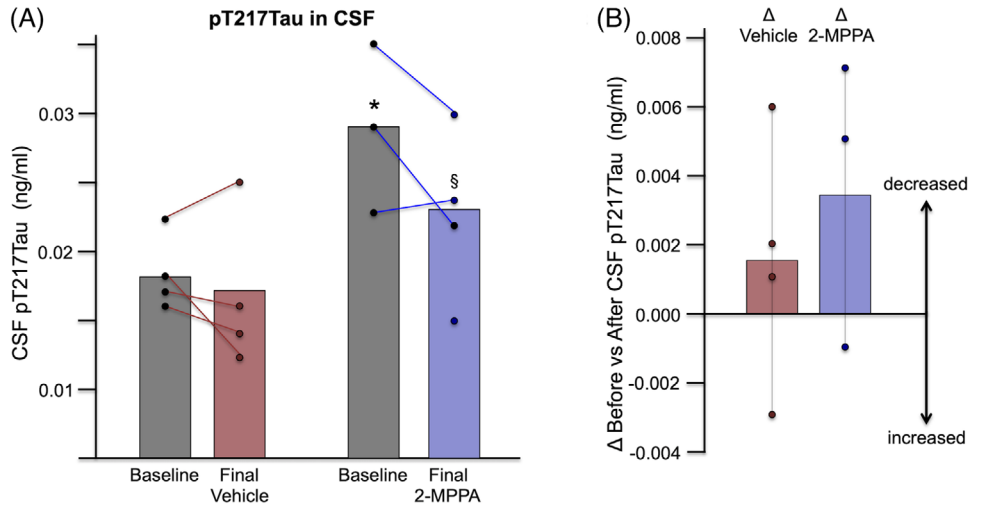

FIGURE 5 Differences in levels of pT217Tau in CSF assayed by MS between baseline and after 6 months of daily treatment with 2-MPPA or vehicle. (A) Differences from baseline to 6 months post-treatment for vehicle-treated versus 2-MPPA-treated animals, showing the change for each individual aged macaque. Note that one baseline draw failed from an aged macaque in the drug group and thus only the "after" data point is shown for this monkey. *$p = 0.02$ compared to baseline levels for vehicle group; $p = 0.27$, baseline versus following 6 months of treatment for 2-MPPA group only ($n = 3$). (B) The same data plotted as the difference between baseline and after 6 months of treatment.

4.3 | The aging macaque as a useful animal model

The aged macaque model can provide several unique advantages particularly important for assessing treatment strategies for sAD. Because the etiology of sAD likely differs in some important ways from autosomal dominant disease (e.g., the key roles of aging and inflammation), it is vital to have an animal model that allows testing of treatments that might act on these etiological factors, which are presumably upstream from the mutations that cause autosomal dominant disease. Thus mouse models that rely on autosomal dominant AD mutations may be inappropriate when testing mechanisms that act upstream in the pathological cascade, especially because mouse models do not recapitulate the changes in NAAG seen in human sAD.[36] In contrast, aged macaques naturally express *APOE* ε4,[4] and develop calcium dysregulation, inflammation, synapse loss, and amyloid and tau pathology in the same circuits, and in the same sequence as that seen in aging humans.[32] Although the aged macaque does not develop the same large amounts of fibrillated tau pathology as in human AD, it allows detection and analyses of early stage, soluble tau pathology, for example, at a time when pTau is seeding between neurons.[5] In contrast, human studies

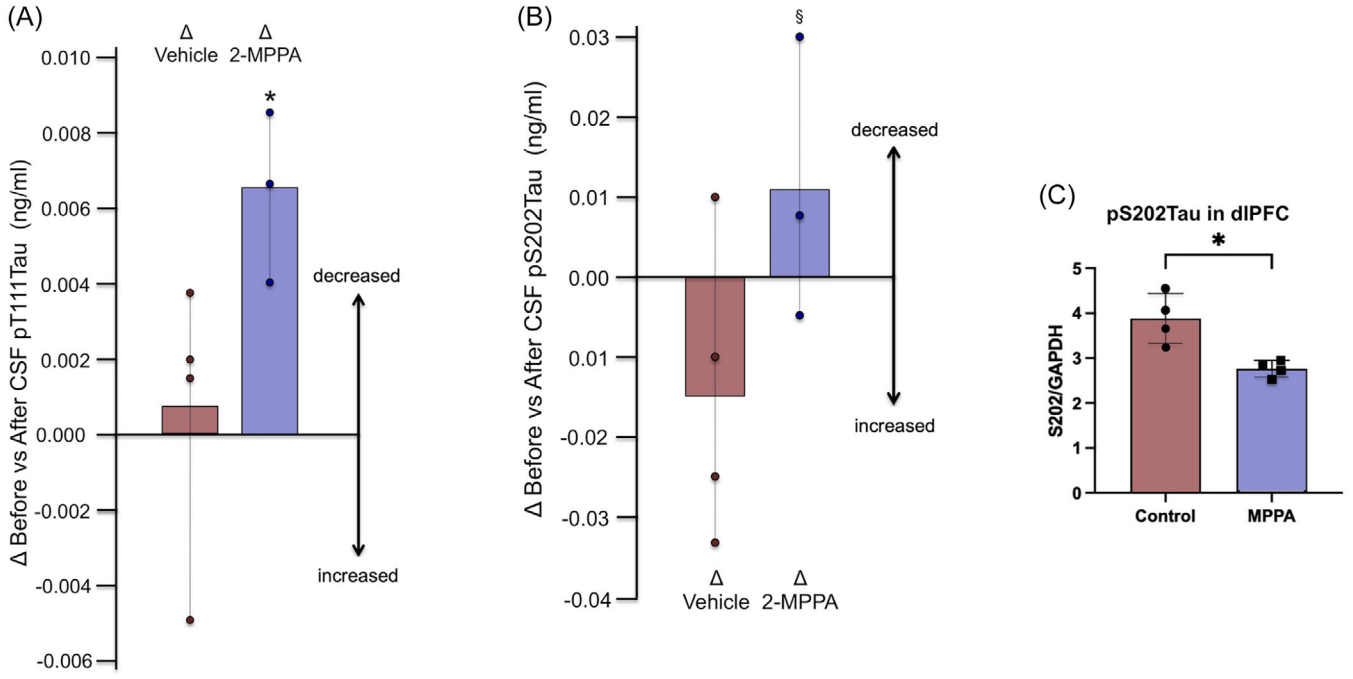

FIGURE 6 Exploratory analyses of CSF pTau levels. (A) Exploratory analysis of CSF pTau levels showed that 2-MPPA treatment significantly reduced pT111Tau levels from baseline (*$p = 0.038$, $n = 3$), whereas levels in control animals were unchanged ($n = 4$). (B) Exploratory analysis indicated potential drug-related differences in pS202Tau, where vehicle-treated monkeys generally showed increases over the 6 months, whereas 2-MPPA-treated monkeys generally showed decreases (§ differences in vehicle- vs 2-MPPA-treated monkeys: Tind, $p = 0.13$). (C) Based on the CSF data, pS202Tau levels were then tested in dlPFC. Western blotting showed decreased levels of pS202Tau in the dlPFC of 2-MPPA-treated monkeys compared to vehicle-treated controls; *$p = 0.022$. As there are currently no antibodies that recognize pT111Tau, this analysis of brain tissue could not be performed.

rely on positron emission tomography (PET) imaging of later stage, fibrillated tau in order to compare pTau levels in fluid biomarkers with those in brain,[37] and yet fibrillation is likely occurring at a more advanced stage where irreversible damage is already being done to neurons, for example, with autophagic degeneration of dendrites.[5] As soluble pTau dephosphorylates very rapidly post-mortem,[38] reliable assays of early stage tau pathology require either brain biopsies from human brain, or an animal model that naturally develops tau pathology, and from which brains can be harvested quickly, as in the current study. The challenge for this field is the small numbers of aged rhesus monkeys available, especially since the extensive use of rhesus monkeys to create vaccines for the COVID-19 pandemic. This may be an arena where the National Primate Centers can be instrumental, preserving aged monkeys to assess important potential therapeutic strategies. If plasma levels of pT217Tau continue to correlate with brain levels, plasma could eventually be used as the primary measure, without the need to sacrifice animals, especially if animals could be pre-screened to ensure sufficient pT217Tau levels at baseline.

4.4 | GCPII inhibition as a novel therapeutic mechanism

The current study presents a novel, potential therapeutic mechanism for decreasing risk of sAD. The idea of using GCPII inhibitors to treat sAD was raised by Neale and Olszewski[31]; however, the sAD field has been more focused on mechanisms that directly remove $A\beta_{1\text{-}42}$ or fibrillated tau. Because mGluR3s are predominantly presynaptic in rodent PFC, much of the focus has been on their beneficial presynaptic role in inhibiting glutamate release under conditions of excitotoxicity.[39] However, their role appears to have expanded with brain evolution, with mGluR3s playing a major role in human cognition,[40] and post-synaptic regulation of cAMP-calcium signaling expanding in the primate cognitive circuits that are so vulnerable in sAD[19] (Figure 1B). Given that GCPII expression increases under conditions of inflammation,[28,29] the reduction in mGluR3 regulation of cAMP-calcium signaling may contribute to increased risk for sAD in multiple inflammatory conditions. For example, GCPII inhibition or knockout is beneficial in animal models of diabetes/insulin resistance,[41] traumatic brain injury (TBI),[42] and hypoxia,[29,43] all of which increase calcium dysregulation and are risk factors for sAD.[44–48] Recent data indicate that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection also increases risk for sAD,[49] and post-mortem data show large increases in both GCPII and tau hyperphosphorylation in patients who died from SARS-CoV-2 infection.[50] The current study shows a striking correlation between GCPII activity and pT217Tau expression in the primate dlPFC, suggesting that GCPII inhibition can be a fruitful mechanism for reducing tau pathology. The apparent absence of side effects suggests that GCPII inhibitors could be taken early in the aging process over a long time frame as a potential preventive strategy. These data encourage

the further development of brain penetrant GCPII inhibitors to restore calcium regulation and reduce tau pathology at early stages.

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CONFLICT OF INTEREST STATEMENT

The authors have no financial conflicts of interest to disclose.

CONSENT STATEMENT

There were no human subjects in this research. The animal research was conducted under National Institutes of Health (NIH) and Yale Institutional Animal Care and Use Committee (IACUC) guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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