

## RESEARCH COMMUNICATION

# Selective inhibition of phosphodiesterase 4D increases tau phosphorylation at Ser214 residue

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## Abstract

Tau is a protein that normally participates in the assembly and stability of microtubules. However, it can form intraneuronal hyperphosphorylated aggregates that are hallmarks of Alzheimer's disease and other neurodegenerative disorders known as tauopathies. Tau can be phosphorylated by multiple kinases at several sites. Among such kinases, the cAMP-dependent protein kinase A (PKA) phosphorylates tau at Ser214 (pTAU-S214), an event that was shown to reduce the pathological assembly of the protein. Given that the neuronal cAMP/PKA-activated cascade is involved in synaptic plasticity and memory, and that cAMP-enhancing strategies demonstrated promising therapeutic potential for the treatment of cognitive deficits, we investigated the impact of cAMP on pTAU-S214 in N2a cells and rat hippocampal slices. Our results confirm that the activation of adenylyl cyclase increases pTAU-S214 in both model systems and, more interestingly, this effect is mimicked by GEBR-7b, a phosphodiesterase 4D inhibitor with proven pro-cognitive efficacy in rodents.

## KEYWORDS

cyclic adenosine monophosphate, GEBR-7b, phosphodiesterase inhibitors, tau protein

## 1 | INTRODUCTION

The Microtubule-Associated Protein Tau (MAPT), commonly known as tau, plays a critical role in tubulin

assembly and cytoskeleton stabilization. In humans, tau gene is located on chromosome 17 q21-22 and its alternative splicing leads to the expression of six main isoforms with a length ranging from 352 to 441 amino acids.<sup>1</sup>

**Abbreviations:** AC, adenylyl cyclase; AD, Alzheimer's disease; A $\beta$ , beta-amyloid; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CoE, Compound E; DMSO, dimethyl sulfoxide; N2a, mouse Neuro-2a cells; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase A; pTAU-S202, tau phosphorylated at Ser202; pTAU-S214, tau phosphorylated at Ser214.

Viviana Villa and Giulia Montalto contributed equally to this study.

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Although tau is present in a variety of tissues, it is highly expressed in the brain and especially in neurons, where it becomes predominantly axonal during maturation.<sup>2</sup>

Under physiological conditions, the interaction of tau with the microtubule system is regulated by phosphorylation/dephosphorylation dynamics at different serine, threonine, and tyrosine residues that overall represent 85 putative sites in the longest protein isoform.<sup>3,4</sup> Upon phosphorylation, tau has a lower affinity for tubulin and detaches from the cytoskeleton. Aberrant alterations of this physiological process, together with the propensity of tau to aggregate, can lead to the neuronal accumulation of hyperphosphorylated tau inclusions that are characteristic of neurodegenerative disorders known as tauopathies.<sup>5,6</sup> Among these, Alzheimer's disease (AD), the most common cause of dementia, represents a so-called secondary tauopathy, because its neuropathological diagnosis requires both the aggregation of tau and the deposition beta-amyloid (A $\beta$ ) peptides in plaques. Tau lesions in AD include intraneuronal paired helical filaments (PHF), which finally evolve in insoluble neurofibrillary tangles (NTF).<sup>7</sup>

In the last few years, tau phosphorylation mechanisms have aroused renewed interest as (i) tau aggregates show a time-dependent correlation with disease progression undoubtedly better than A $\beta$  plaques, and (ii) all anti-A $\beta$  therapeutic strategies tested to date have not shown relevant symptomatic or disease-modifying effects, including aducanumab, the anti-A $\beta$  monoclonal antibody that has been controversially approved by FDA.<sup>8</sup> Therefore, a more detailed understanding of the molecular determinants that modulate the physiological processes of tau phosphorylation can provide key information for alternative therapeutic interventions capable of preventing or slowing cognitive decline in AD.

Our previous studies have shown that cyclic guanosine monophosphate (cGMP), a classic second messenger crucially involved in memory formation and consolidation, induces tau phosphorylation by activating protein kinase G (PKG).<sup>9</sup> It is of possible translational impact the evidence that PKG phosphorylates tau at Ser214 but not at Ser202, a condition that would favor a conformational switch of the protein from a neurotoxic pro-aggregant structure to a neuroprotective anti-aggregant one.<sup>10,11</sup>

Interestingly, increased levels of cyclic adenosine monophosphate (cAMP), another second messenger deeply involved in cognitive processes, have recently been shown to improve memory deficits in AD mice, an effect that was associated with a significant reduction of tau and phosphorylated tau in hippocampal tissue.<sup>12,13</sup>

Based on these premises, in the present work we evaluated whether, and to what extent, drugs that increase

the levels of cAMP can influence the phosphorylation of tau at Ser214 and S202. To raise intracellular cAMP we used both the adenylyl cyclase activator forskolin and GEBR-7b, a selective inhibitor of phosphodiesterase 4D (PDE4D) able to improve memory in physiological and pathological conditions.<sup>14,15</sup> We found that, similar to what previously observed with cGMP enhancers, cAMP elevation increases tau phosphorylation at Ser214 leaving Ser202 unaffected, both in N2a neuronal cells and in rat hippocampal slices.

Although preliminary, our data suggest that pharmacological interventions aimed at enhancing the cAMP signaling system may be of therapeutic relevance to reduce tau aggregation and its synapto/neurotoxic effects.

## 2 | EXPERIMENTAL PROCEDURES

### 2.1 | Cell culture and treatments

The cells used in this study [mouse Neuro-2a (N2a)] were grown in 50% Dulbecco modified Eagle medium (DMEM), 50% OptiMEM with 0.1 mM nonessential amino acids, 1% penicillin–streptomycin mixture, and 5% fetal bovine serum. Forskolin (Sigma-Aldrich, Italy), the  $\gamma$ -secretase inhibitor Compound E (Adipogen, USA), and the PDE4D inhibitor GEBR-7b (Millipore, USA) were dissolved in dimethyl sulfoxide (DMSO), stored at  $-20^{\circ}\text{C}$ , and diluted to the indicated final concentrations immediately before use.

### 2.2 | Rat hippocampal slices

Transverse hippocampal slices (250  $\mu\text{m}$ ) from adult Sprague–Dawley rats (250–300 g) were obtained using a McIlwain tissue chopper, incubated at  $37^{\circ}\text{C}$  into 2 ml of a physiological solution (125 mM NaCl, 3 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM CaCl<sub>2</sub>, 22 mM NaHCO<sub>3</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM glucose, pH 7.2–7.4) continuously aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and treated as indicated.

Prior to sacrifice, rats were maintained in the animal facility of the University of Genoa, housed on a regular light/dark cycle and kept at constant temperature ( $22 \pm 1^{\circ}\text{C}$ ) and relative humidity (50%) with free access to water and food. The experimental procedures and animal care complied with the European Communities Parliament and Council Directive of 22 September 2010 (2010/63/EU) and with the Italian D.L. n. 26/2014. Hippocampi were obtained from animals used in the project approved by the Italian Ministry of Health (protocol number 30/11/2016-OPBA of November 2016), in accordance with Decreto Ministeriale 116/1992.

## 2.3 | Immunoblot analysis

N2a cells were processed for total protein extraction as previously reported.<sup>16</sup> Hippocampal slices were homogenized in 0.6 ml of ice-cold buffer containing 1% complete protease inhibitor, 1% Triton X-100, 25 mM Tris HCl, 25 mM NaF, 1 mM EDTA, 0.5 mM EGTA, and centrifuged at 10,000g for 10 min at 4°C. The supernatant was centrifuged a second time at 10,000g for 10 min and then used for immunoblot assays, which were performed according to standard methods using the following antibodies purchased from abcam (UK): rabbit monoclonal anti-tau (phospho S202) [EPR2402], rabbit monoclonal anti-tau (phospho S214) [EPR1884 (2)], and rabbit polyclonal anti-total tau (ab64193). Anti-rabbit secondary antibodies were coupled to horseradish peroxidase (Amersham, Bucks, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's instructions (Amersham, Bucks, UK). Chemiluminescence was monitored by exposure to film and the signals were analyzed under the non-saturating condition with an image densitometer (Biorad, Hercules, CA).

## 2.4 | cAMP enzymatic immunoassay

Quantification of intracellular cAMP was performed with DetectX<sup>®</sup> Direct Cyclic AMP Enzyme Immunoassay Kit (Arbor Assay, USA), following the manufacturer's protocol. cAMP levels were calculated according to the standard curves prepared on the same EIA plates.

## 2.5 | A $\beta$ ELISA

The A $\beta$ <sub>X-42</sub> ELISA kit (Wako Chemicals GmbH, Germany) was used to determine the levels of A $\beta$  peptides released into supernatant media from cultured cells, as described previously.<sup>17</sup> Briefly, at the end of treatments, the conditioned media were collected, spun at 1000 g for 10 min at 4°C to remove cell debris, and stored at -80°C until use. ELISA tests were carried out following the manufacturer protocols and the levels of A $\beta$  peptides were calculated according to the standard curves prepared on the same ELISA plates.

## 2.6 | Statistical analyses

All data are expressed as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc (GraphPad software). The level of significance was set at  $P < 0.05$ .

## 3 | RESULTS

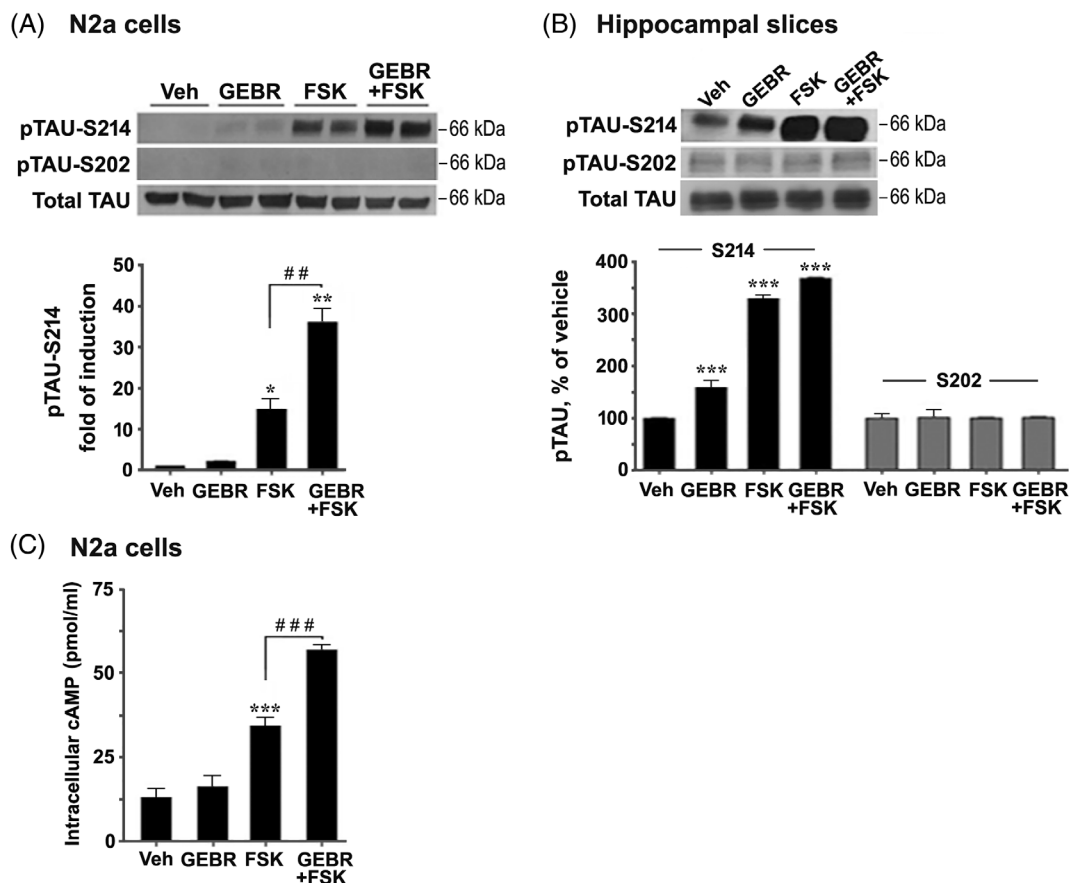
### 3.1 | cAMP enhancers stimulate tau phosphorylation at Ser214

To verify the effect of cAMP enhancers on tau phosphorylation, we incubated N2a cells and rat hippocampal slices with GEBR-7b and forskolin (FSK). GEBR-7b is a specific inhibitor of the enzyme phosphodiesterase 4D (PDE4D) that hydrolyses cAMP,<sup>14</sup> while FSK is an activator of adenylyl cyclase, the enzyme that synthesizes cAMP from adenosine triphosphate (ATP). At the end of treatments, cells and tissues were processed for total protein extraction and immunoblot analyses, which revealed that both cAMP enhancers increased tau phosphorylation at Ser214 (pTAU-S214), albeit to different degrees. Specifically, GEBR-7b alone showed a trend towards an increase of pTAU-S214 that, however, did not reach statistical significance in N2a cells. On the other hand, FSK induced a significant 16.03-fold increase of pTAU-S214 in these cells, which was further augmented by the combination with GEBR-7b (Figure 1A). In hippocampal slices, both GEBR-7b and FSK induced pTAU-S214 levels of 1.6 and 3.31-fold, respectively, an effect that was potentiated by the two drugs applied together (Figure 1B). Using the same samples, we also analyzed the phosphorylation of tau at Ser202 (pTAU-S202), since it is one of the earliest markers of tau aggregation into PHFs<sup>18</sup> and PKA has been involved in the phosphorylation of tau at this residue.<sup>19</sup> However, we did not observe detectable levels of the phosphopeptide in N2a cells (Figure 1A), and none of the treatments increasing cAMP was able to promote pTAU-S202 in hippocampal slices (Figure 1B).

Furthermore, using a cAMP-specific enzymatic immunoassay on N2a extracts, we verified the ability of the treatments to increase the intracellular concentration of cAMP. As shown in Figure 1C, the results obtained indicate that GEBR-7b significantly enhanced the FSK-induced accumulation of cAMP, in perfect agreement with the effects observed on pTAU-S214 (Figure 1A).

### 3.2 | The enhancement of pTAU-S214 is not mediated by A $\beta$ peptides

Since the increase in cAMP stimulates the production of A $\beta$  peptides,<sup>20-22</sup> we tested whether the effect of cAMP on tau phosphorylation was somehow mediated by A $\beta$ . To this end, we treated N2a cells with compound E (CoE), a  $\gamma$ -secretase inhibitor that precludes A $\beta$  formation,<sup>23</sup> prior to exposure to GEBR-7b and FSK. As confirmed by specific ELISA performed on the conditioned media, CoE inhibited the production of A $\beta$  peptides even in the presence of the cAMP enhancers



**FIGURE 1** cAMP enhancers increase pTAU-S214 but not pTAU-S202. (A) N2a cells were pretreated for 1 h with 10  $\mu$ M GEBR-7b (GEBR) and then incubated with 1  $\mu$ M FSK or an equal volume of vehicle (Veh, DMSO) for 15 h. At the end of treatments, cells were processed for immunoblot analyses. (B) Hippocampal slices were pretreated for 15 min with 100  $\mu$ M GEBR, then incubated for 45 min with 100  $\mu$ M FSK or an equal volume of vehicle and processed for immunoblot analyses. Total tau signals represent the internal loading control. (C) Where indicated, N2a cells were pre-treated for 10 min with 10  $\mu$ M GEBR-7b (GEBR) or an equal volume of vehicle (Veh, DMSO). Then, 1  $\mu$ M FSK was added for 20 min. At the end of the incubation periods, intracellular cAMP was measured with a cAMP-specific EIA kit, according to the manufacturer's instructions. Graphed data show mean  $\pm$  SEM for at least three independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.001, and \*\*\* $P$  < 0.0001 versus vehicle-treated group, ## $P$  < 0.01, ### $P$  < 0.0001 (one-way ANOVA, Bonferroni post-test)

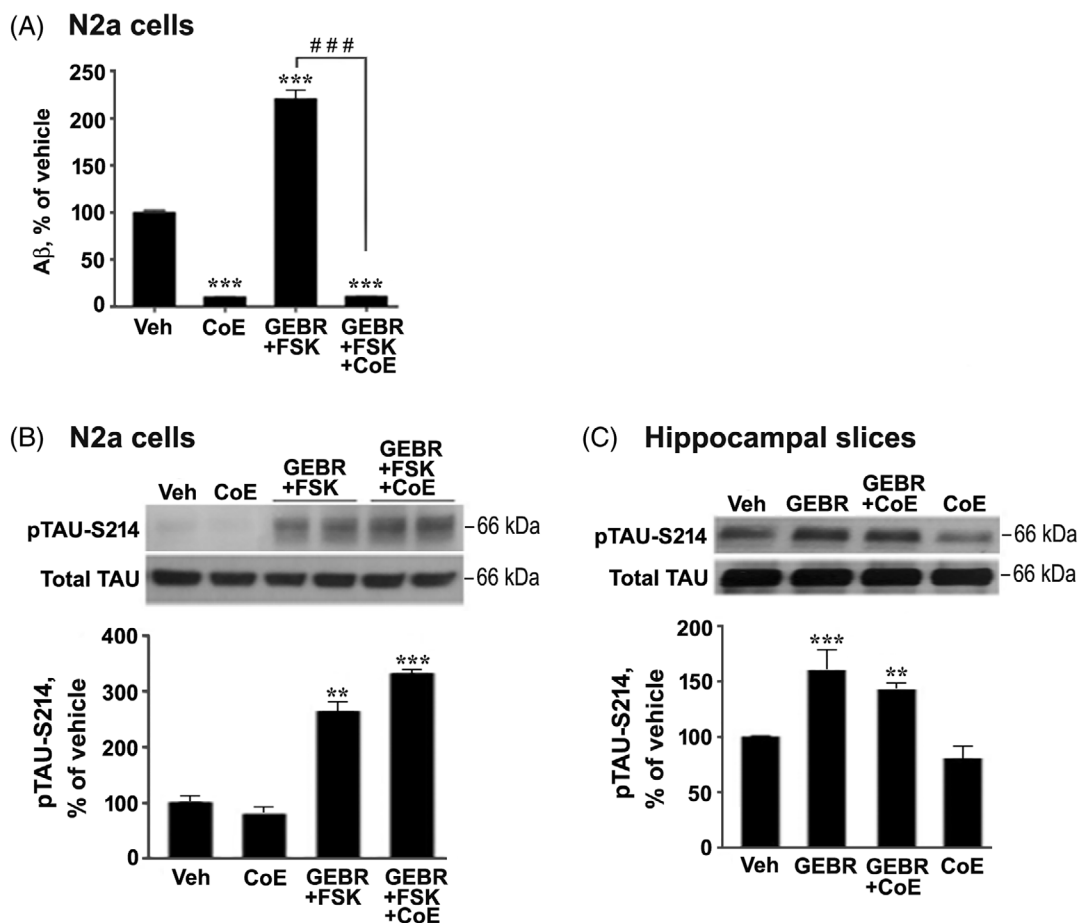
(Figure 2A). However, the lack of A $\beta$  did not prevent the effect of cAMP on tau phosphorylation, as indicated by immunoblot analyses performed on cell extracts (Figure 2B). To further confirm this evidence, we tested the same treatments on hippocampal slices. The results obtained, shown in Figure 2C, allowed us to conclude that A $\beta$  is not required for the cAMP-dependent phosphorylation of tau at S214.

## 4 | DISCUSSION

Although highly phosphorylated tau characterizes AD and other tauopathies such as frontotemporal dementia, Pick's disease, supranuclear palsy, and corticobasal degeneration,<sup>5,6</sup> it is not necessarily a pathogenic event as

it has been described in physiological processes, such as neuronal development and during sleep.<sup>24-26</sup>

Over the past 30 years, several in vitro studies have identified a number of enzymes (e.g., glycogen synthase kinase 3 $\beta$ , cyclin-dependent kinase 5, casein kinase 1, protein kinase A and Fyn kinase) capable of phosphorylating tau at different serine, threonine and tyrosine residues.<sup>27,28</sup> Still, we have recently discovered that tau can also be phosphorylated by the cGMP-dependent protein kinase G (PKG).<sup>9</sup> Specifically, we have shown that increasing cGMP levels by selective inhibition of PDE5 with vardenafil results in the phosphorylation of tau at Ser214 but not at Ser202. This effect may inhibit the interaction of the protein with the microtubule system, but it has also been proposed to promote neuroprotection by reducing the aggregation of tau into PHF.<sup>10,11</sup> Since



**FIGURE 2** A $\beta$  does not mediate the increase of pTAU-S214. (A) Where indicated, N2a cells were pretreated with 1  $\mu$ M CoE, then incubated for 1 h with 10  $\mu$ M GEBR-7b (GEBR) and finally for 15 h with 1  $\mu$ M FSK or an equal volume of vehicle (Veh, DMSO). At the end of the incubation periods, conditioned media were subjected to specific A $\beta$  ELISA. (B) N2a cells were treated as described in (A) and processed for total protein extraction and immunoblot analyses. (C) Hippocampal slices were pretreated with 10  $\mu$ M CoE for 15 min, then incubated with 100  $\mu$ M GEBR-7b (GEBR) or an equal volume of vehicle (Veh, DMSO) for 45 min, and finally processed for immunoblot analyses. The total tau signal represents the internal loading control. Graphed data show mean  $\pm$  SEM for at least three independent experiments. \*\* $P$  < 0.001 and \*\*\* $P$  < 0.0001 versus vehicle-treated group, ### $P$  < 0.0001 (one-way ANOVA, Bonferroni post-test)

selective PDE5 inhibitors are considered promising drugs for the treatment of memory deficits in AD,<sup>29</sup> such anti-aggregant property of vardenafil, if confirmed in vivo, could help to achieve a better therapeutic outcome in this neurodegenerative disease.

In addition to cGMP, also cAMP is instrumental in the process of memory formation and consolidation.<sup>30</sup> Indeed, a large number of preclinical studies have shown that selective inhibitors of the PDE4 family, the enzymes degrading cAMP, could be therapeutically useful for different pathological conditions characterized by cognitive deficits, including AD.<sup>31</sup> Therefore, to gain more insight into the relationships between cAMP and tau, here we have examined the levels of pTAU-S214 and pTAU-Ser202 under cAMP stimulation. Since pan PDE4 inhibitors (i.e., rolipram) have important emetic effects that prevent their clinical use, we used GEBR-7b, a selective inhibitor of PDE4D isoforms which are considered more

favorable targets for the development of procognitive drugs with minimized side-effects.<sup>32</sup> Furthermore, it is worth noting that increased expression of different PDE4D isoforms has recently been observed in the brain of AD patients compared to gender-matched control subjects.<sup>33</sup>

Our results clearly show that FSK, a potent adenylyl cyclase (AC) activator, and GEBR-7b are able of increasing pTAU-S214 in both N2a neurons and hippocampal slices. Notably, in N2a cells, GEBR-7b alone did not induce a statistically significant increase of pTAU-S214, in line with its lack of effect on intracellular cAMP levels. However, activation of AC by FSK resulted in a robust elevation of both cAMP and pTAU-S214, which were further increased by inhibiting the degradation of cAMP with GEBR-7b. These results suggest that, under basal conditions, N2a cells have low levels of cAMP and inhibiting its degradation is not sufficient to activate

PKA. Consistent with this hypothesis, when cAMP production was increased by FSK, the effects of GEBR-7b on cAMP and pTAU-S214 were observed. Conversely, in hippocampal slices, where synaptic circuits are likely more active and cAMP levels higher, GEBR-7b increased pTAU-S214 even in the absence of FSK stimulation.

Regarding pTAU-S202, we could not detect the phosphopeptide in N2a cells neither in basal conditions nor under cAMP stimulation, but it was clearly detectable in hippocampal slices, where GEBR-7b, alone or in combination with FSK, did not modify its levels. This may appear in contrast to the study by Liu et al.,<sup>19</sup> which involves PKA in pTAU-S202. Indeed, those experiments were conducted in vitro with purified proteins and demonstrated that PKA does not directly phosphorylate tau in Ser202, but activates a kinase that, in turn, does so. It is likely that in our experimental models (i.e., living cells and hippocampal tissue) this pathway is not activated.

Here we also show that, similarly to what was previously observed with the cGMP-enhancer vardenafil,<sup>9</sup> the cAMP-induced elevation of pTAU-S214 is not affected by  $\gamma$ -secretase inhibition which, on the other hand, effectively prevented the production of A $\beta$  triggered by the cyclic nucleotide. Hence, this result indicates that the cAMP-mediated phosphorylation of tau at Ser214 occurs independently of A $\beta$  peptide, at least under physiological conditions.

Interestingly, using an in vitro approach, Zheng-Fischhöfer et al.<sup>34</sup> have shown that a priming phosphorylation of Ser214 by PKA protects other sites of tau (i.e., Thr212 and the ser-pro motifs around residue 200) from phosphorylation by glycogen synthase kinase 3 $\beta$ , thus preventing the PHF-like conformation of tau.

Although further investigation is needed, our previous and present results indicate that PDE5 and PDE4D inhibitors, which have consistently demonstrated memory-enhancing effects in AD models, increase tau phosphorylation at a serine residue that could prevent protein aggregation into PHFs, an effect that, if confirmed in vivo, could endow disease-modifying properties to these drugs.



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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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