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"Study of a cyclic nucleotide-mediated mechanism that could favor an antiaggregant conformation of tau protein"

Ph.D. Candidate: Dr. Giulia Montalto

Tutor: Prof. Roberta Ricciarelli

Ph.D. Coordinator: Prof. Giambattista Bonanno

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CONTENTS

1. ABBREVIATIONS

AD: Alzheimer’s disease

AGD: Argyrophilic grain disease

ApoE: Apolipoprotein E

APP: Amyloid precursor protein

ARTAG: Ageing-related tau astrogliopathy

Aβ: Amyloid-β

BCA: Bicinchoninic acid

CaMKII: Ca2+/calmodulin-dependent protein kinase II

CBD: Corticobasal degeneration

Cdk5: Cyclin-dependent kinase 5

CK1: Casein kinase 1

CoE: Compound E

DMSO: Dimethyl sulfoxide

EIA: Enzymatic immunoassay

ELISA: Enzyme-linked immunosorbent assay

FAD: Familial Alzheimer’s disease

FDA: Food and drug administration

FSK: Forskolin

FTDP-17: Frontotemporal dementia with parkinsonism linked to chromosome 17

FTLD: Frontotemporal lobar degeneration

GGT: Globular glial tauopathy

GOI: globular oligodendroglial inclusion

GSK3β: Glycogen synthase kinase 3β

HRP: Horseradish peroxidase

JNK: JUN N-terminal kinase

LTP: Long-term potentiation

MAPK: Mitogen-activated protein kinase

MAPT: Microtubule-associated protein tau

MCI: Mild cognitive impairment

MT: Microtubule

MTBD: Microtubule-binding domain

N2a: Neuro-2a

NCLK: Neuronal Cdc2-like kinase

NFT: Neurofibrillary tangle

PBS: Phosphate buffered saline

PDE: Phosphodiesterase

PDPK: Proline-directed enzyme

PET: Positron emission tomography

PHF: Paired helical filament

PKA: Cyclic-AMP-dependent kinase

PSP: Progressive supranuclear palsy

PVDF: Polyvinylidene difluoride

RISC: RNA-induced silencing complex

SAD: Sporadic Alzheimer's disease

SDS: Sodium dodecyl sulfate

SiRNA: Small (or short) interfering RNA

ThT: Thioflavin T

TMB: Tetramethylbenzidine

TsA: Thorn shaped astrocyte

UPS: Ubiquitin–proteasome system

VDF: Vardenafil

WB: Western blot

WHO: World health organization

2. ABSTRACT

Tau is a protein which normally participates in the assembly and stability of microtubules. However, under pathological conditions, it can form aggregated and hyperphosphorylated structures that are associated with the pathogenesis 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 exerts a protective effect against the assembly of the protein. The activation of PKA by cAMP also sustains long-term potentiation (LTP), a form of synaptic plasticity, through the stimulation of Aβ production. In a similar manner, cGMP was found to boost Aβ levels and to favour LTP and memory, but an effect of cGMP on tau phosphorylation has never been reported. To investigate this issue, we first verified the effect of cAMP-enhancing treatments on pTAU-S214 and then evaluated the possibility of a PKG-induced phosphorylation of tau.

Different cAMP and cGMP enhancing strategies were used in different model systems: neuro N2a cells, rat hippocampal slices and adult male C57BL/6 mice. Phosphorylation of tau was analyzed by immunoblotting, gene silencing, in vitro enzymatic assays and nano-HPLC mass spectrometry. Aggregation of tau was evaluated by gel electrophoresis and thioflavin T-binding assay.

Our data confirm that cAMP stimulates the phosphorylation of tau at Ser214, as expected, but not at Ser202, which is considered one of the earliest markers of tau aggregation. Furthermore, we report for the first time that the cGMP-activated PKG phosphorylates tau at Ser214 and other 7 Ser/Thr tau residues, but not Ser202. Also, preliminary results indicate that PKG phosphorylation could reduce the aggregating capacity of tau.

Taken together, our results demonstrate the existence of a PKG-mediated mechanism that might shift tau from a pro-aggregant to an anti-aggregant conformation, which has been reported to exert neuroprotective functions.

3. INTRODUCTION

3.1. Biochemistry of tau protein

Tau was discovered in 1975 by Weingarten and colleagues, who described it as *"A heat stable protein essential for microtubule assembly"* [1].

Today we know that tau is encoded by the microtubule-associated protein tau (MAPT) gene, which is located on chromosome 17q21-22 [2] and is expressed in a variety of tissues. In fact, relatively high levels of tau can be found in the heart, skeletal muscle, lung, kidney, and testis. It is present at low levels in the adrenal gland, stomach, and liver; nevertheless, the tissue where the protein is most expressed is the brain. Here, tau is commonly found within axons [3], but it is also present in neuronal somatodendritic compartments and in oligodendrocytes [4, 5]. Abnormal accumulation of tau in somatodendritic compartments is associated with Alzheimer's disease (AD) and other tauopathies [6].

There are six different isoforms of tau, generated by alternative splicing of exons 2, 3 and 10 [7] (Figure 1). To a lesser extent, alternative splicing may also involve exon 6, but this type of isoform is weakly expressed [8]. Exons 2 and 3, which both encode a 29-residue insert near the N-terminus, undergo coupled splicing, with exon 2 inclusion being dependent on exon 3 inclusion. The presence or absence of these two exons in the protein is indicated by the name of the isoform itself (0N, neither included; 1N, exon 3 included; 2N, both exons included).

Exon 10 belongs to the microtubule-binding domain (MTBD); it encodes the second of four highly conserved repeat regions and is 31 residues long. Among the six isoforms of tau, three of them contain exon 10 and are called four repeat (4R) tau, whereas the other three isoforms, which lack exon 10, are known as three repeat (3R) tau [7, 9, 10].

Depending on the presence or absence of exons 2, 3 and 10, the six tau isoforms have different lengths, ranging from 352 to 441 amino acids (0N3R and 2N4R, respectively).

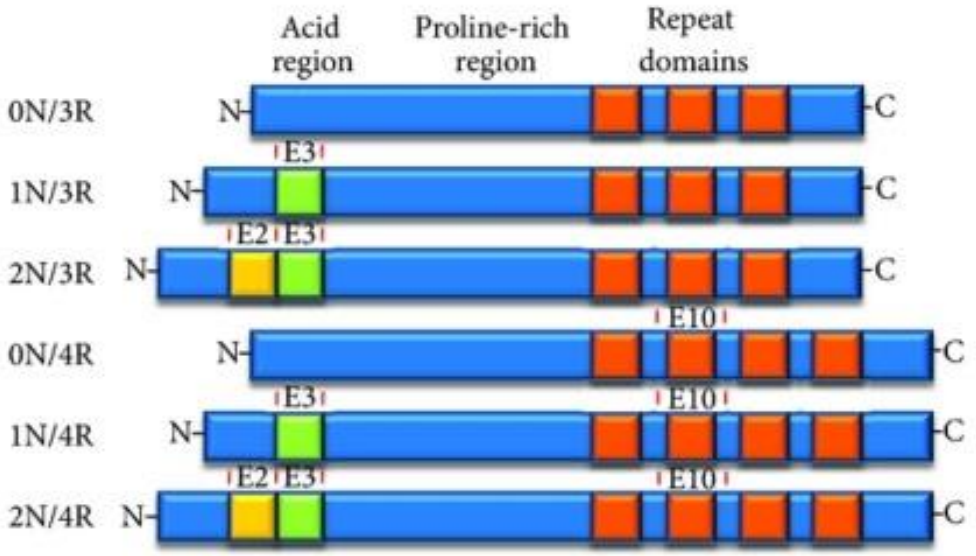


Figure 1: Splice variant isoforms of tau.

Each of the six isoforms has shown a peculiar physiological role since they are differentially expressed during development and are distributed in different neuronal subpopulations. For instance, only the tau isoform 0N/3R is present during fetal stages, while the adults express all six isoforms [10, 11]. Functional differences have been suggested for 4R and 3R tau, as 4R isoforms are more efficient in promoting microtubule (MT) assembly than the 3R ones [11, 12].

In general, the protein structure consists of three parts: one assembly domain, one proline-rich domain, and one projection domain (Figure 2). The assembly domain is responsible for MT binding and tau aggregation [13, 14], and comprises the carboxy-terminal region and the repeat domain. The amino-terminal fragment of tau does not bind to MTs and is called projection domain because it projects away from the MT surface [15]. These two regions are separated by a proline-rich domain that contains multiple Thr-Pro or Ser-Pro motifs, which are targets of kinases such as glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (Cdk5), mitogen-activated protein kinase (MAPK), and JUN N-terminal kinase (JNK) [16].

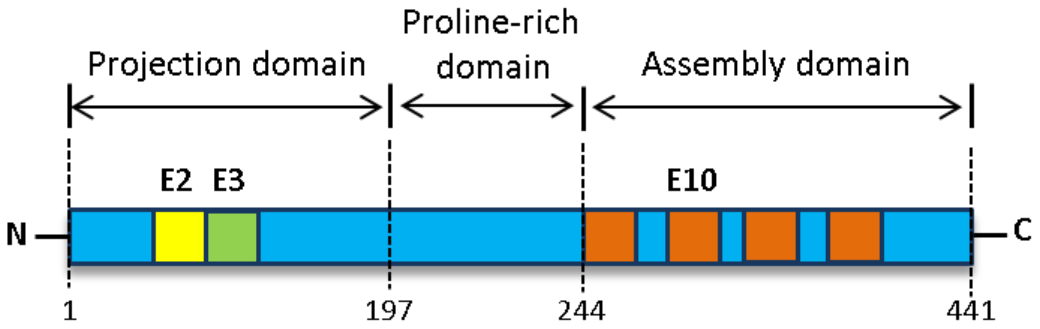


Figure 2: Domains of tau. The isoform represented in the picture is the 2N/4R, which is the longest tau isoform.

From a chemical point of view, tau is overall a basic protein, but the N-terminal residues are predominantly acidic, while the C-terminus is neutral. The different charges affect the association of tau with MTs and other molecules, as well as the folding and aggregation of the protein itself [17].

Tau is stable under acidic conditions and at high temperatures; being a highly soluble molecule, it is unfolded in the cytosol, unlike most cytosolic proteins [18]. Due to its "natively unfolded" nature, tau is a flexible and elongated protein, with no extensive alpha-helical or beta-sheet domains [13, 19].

3.2. Physiology of tau and interaction with microtubules

Since its discovery, it was clear that tau had a role in the assembly and stability of MTs, which are tubulin polymers involved in important features of the cell, such as mitosis, intracellular transport, and maintenance of cell shape. In the brain, these structures are vital for neuronal survival, since they provide stability to the axons and support the transport of materials to and from the distant synapses.

Tau protein mainly distributes along the axons, where it provides stabilization of MTs and promotes their assembly [16]. Interestingly, the residues involved in binding with the MTs have been also classified as essential for the pathological aggregation of tau, thus implying that these two phenomena are in competition [20]. Even if the regulation of MT dynamics is considered its main function, tau is also involved in axonal transport. As a matter of fact, it can compete with the motor proteins dynein and kinesin (which are deputed to retrograde and anterograde transport of cargoes, respectively) for binding sites on the MT surface, resulting in the accumulation of cargoes in the soma [21]. Tau has also been observed in the nuclei of neuronal and non-neuronal cells, where it seems to maintain DNA and RNA integrity under physiological conditions and under ROS-producing conditions such as hyperthermia [22].

Early studies reported that tau knockout mice do not exhibit a pathological phenotype, possibly due to a compensatory increase in microtubule-associated MAP1B protein. [23]. However, more recently, worsening of neuronal function in tau-deficient mice has been described [16].

3.3. Post-translational modifications

3.3.1. Phosphorylation

Among all the post-translational modifications that tau protein undergoes, phosphorylation is certainly the prevalent one, and the most intensely studied for its pathological implications.

The longest tau isoform (2N4R) contains 85 potential phosphorylation sites, 45 of which have been characterized using mass spectrometry, sequencing and phosphorylation-dependent monoclonal antibodies. Among the 85 putative phosphorylation sites, 45 are serines (53% of tau phosphorylation sites), 35 are threonines (41%) and 5 are tyrosines (6%) (Figure 3) [24, 25].

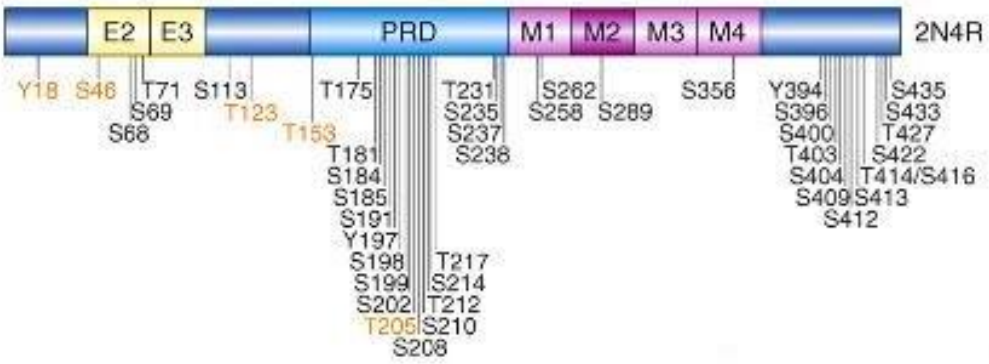


Figure 3: Positioning of phosphorylation sites on tau from Alzheimer brain.

Phosphorylation reduces tau's affinity for tubulin, allowing the protein to detach from the cytoskeleton. This ensures the dynamics of the system in healthy neurons [26]. Although the loss of affinity for MTs is a physiological event, an abnormal phosphorylation of tau could have detrimental effects, since hyperphosphorylated tau can entry into postsynaptic terminals causing synaptic dysfunction [27]. Furthermore, phosphorylation may decrease tau degradation through proteasome or autophagy, thus triggering aberrant accumulation or changing the way the protein interacts with partners (for instance, phosphorylated

but not unphosphorylated tau is able to interact with JIP1 protein, impairing the formation of kinesin complex and affecting axonal transport) [28]. But most of all, hyperphosphorylated tau is the main component of neurofibrillary tangles (NFTs), which are cerebral lesions found in AD and other neurodegenerative disorders, implying that excessive phosphorylation of tau is a prerequisite for NTF formation. [29]. In effect, some tau residues are considered critical, as when phosphorylated they make tau more prone to aggregation and possibly pathogenic. The combination of Thr212, Thr231, and Ser262 phosphorylation, for example, has been shown to cause disruption of the MT network and cell death [30]. Moreover, the epitope of the monoclonal antibody AT8 (phospho-Ser199/-Ser202/-Thr205) was found to correlate with remodelling and instability of MTs, diminished mitochondrial transport, cell death and neurodegeneration [31].

However, although excessive tau phosphorylation is considered a key pathogenic event, phosphorylation of some residues has been shown to reduce the protein aggregation. In particular, the phosphorylation of Ser214 and Thr231 causes tau to lose its ability to interact with MTs, leading to the detachment of the protein [16, 32]. At the same time, this modified tau has been shown to resist aggregation, so these residues are of particular interest as their phosphorylation could counteract the formation of NFTs [33].

The phosphorylation of tau is carried out by several kinases, each of which can act on more than one residue [34]. However, it is still a matter of debate whether all the phosphorylations observed during *in vitro* experiments actually take place *in vivo*.

Protein kinases acting on tau can be divided into proline-directed enzymes (PDPKs), which phosphorylate Ser or Thr residues followed by a proline, and non-PDPKs [31].

PDPK enzymes include:

- **Glycogen synthase kinase 3 beta (GSK3β)**. This is the kinase that phosphorylates the largest number of tau residues, which at present are more than 40 [24], though the predominant are Ser199, Ser202, Thr231, Ser396, Ser400, and Ser413 [35]. The phosphorylation of these sites generates an

epitope of PHFs (paired helical filaments), which are the main component of NFTs [36]. The overactivation of GSK3β can abnormally phosphorylate tau, leading to its detachment from MTs with subsequent alterations in axonal transport and neurodegeneration, and eventually memory impairment [37, 38]. For instance, Thr231 is one of the most important sites for the regulation of tau activity and when it is phosphorylated by GSK3β tau undergoes a conformational change affecting its stability and capacity to bind MTs [39-41].

- **Mitogen activated protein kinases (MAPKs)**. This is a group of kinases that regulate important cellular mechanisms such as proliferation, stress responses, apoptosis and immune defense [42]. There are three well-known MAPK pathways in mammalian cells: the ERK1/2, the c-JUN N-terminal kinase 1, 2 and 3 (JNK1/2/3), and the p38 MAPK-α,-β,-δ, and-γ. The overactivation of p38 and JNK (both of which phosphorylate tau) has been observed, in several neurodegenerative diseases, in cells containing the pathogenic filamentous form of the protein [43-45].
- **Cyclin-dependent kinase (Cdk5)**. It is an inactive enzyme, *per se*, and is activated after binding the regulatory subunits p39 and p35 in the cytosol [46]. P35 and p39 are proteins with a short half-life; under stress conditions, however, the protease calpain cleaves p35 into p25, which has a longer half-life and, unlike p35, is considered pathological [47-49]. *In vitro,* Cdk5 is able to phosphorylate 9–13 tau residues [24, 50]. Uncertainty about the exact number, and whether phosphorylation can occur *in vivo*, could be attributed to an overlap in the phosphorylation of many (Ser/Thr)-Pro sites in tau by different PDPKs and to the lack of specific methods to discriminate them. Among the residues that have been assigned as phosphosites, Ser202, Thr205, Ser235, and Ser404 appear to be major sites, whereas Thr153 and Thr212 are minor sites in Cdk5-p25 *in vitro* assays [51]. Since these sites appear to be phosphorylated in AD brains, a pathogenic role for Cdk5 has been hypothesized [52]. On the other hand, Cdk5 can act as a modulator of tau hyperphosphorylation through the inhibitory regulation of GSK3β [53].

Non PDPK enzymes include:

- **Cyclic-AMP-dependent kinase (PKA)**. It is an ubiquitous tetrameric protein consisting of two regulatory and two catalytic subunits. Its activity is regulated by intracellular concentrations of the second messenger cAMP. Once activated, PKA phosphorylates many substrates, such as CREB, Raf, Bad and GSK3β, with consequences on gene expression, cell survival and migration. In particular, phosphorylation by PKA inactivates both isoforms of GSK3 (α and β) [54]. Moreover, both proapoptotic and antiapoptotic effects of PKA were reported in nucleated cells [55]. On tau, PKA was found to phosphorylate residues Ser214, Ser217, Ser262, Ser396/404, and Ser416 [56].

  As already mentioned, phosphorylation at Ser214 is an event of particular interest because it exerts a protective effect against the assembly of the protein into PHFs [33].
- **Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)**. It is one of the most abundant protein kinases in the brain, regulating a wide range of neuronal functions, including neurotransmitter synthesis and release, modulation of ion channel activity, synaptic plasticity, and gene expression [57]. This protein is capable of phosphorylating tau in several residues that have been found hyperphosphorylated in AD brains, such as Ser262, Ser356, Ser409 and Ser416. Phosphorylation of Ser262 and Ser356 seems to particularly affect the ability of tau to bind MTs and promote their assembly [58].
- **Casein kinase 1 (CK1)**. It is a family of Ser/Thr–specific protein kinases that counts at least seven isoforms (Ck1-α, -β, -γ1, -γ2, -γ3,-δ, and -ε) [59, 60]. By phosphorylating different substrates, these kinases regulate diverse cellular processes, including circadian rhythms, cell signalling, vesicular trafficking, cell division, and DNA repair [61]. CK1 is considered one of the most important tau kinases, as it is capable of phosphorylating more than 40 sites on the protein [62]. As with other previously described kinases, CK1 seems to have pathogenic roles: levels of CK1-α, CK1-δ and CK1-ε are highly increased in hippocampi of Alzheimer brains, compared to controls [63]; moreover, the protein colocalizes with pathogenic tau aggregates during the disease progression [64]. Overexpression of CK1-ε suppresses tau exon 10 inclusion and imbalances 3R-tau/4R-tau ratio, which also contributes to the development of several

tauopathies, such as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and Down syndrome [65].

- **Tyrosine kinases.** Human tau has five tyrosines in its longest sequence, and all of them are possible phosphorylation sites. Compared with the ser/thr kinases, tyrosine kinases involved in tau phosphorylation were less investigated. However, phosphorylation of tyrosines 18, 197, and 394 has been demonstrated in PHF-tau. FYN (proto-oncogene tyrosine-protein kinase), Syk (spleen tyrosine kinase), Met (a receptor tyrosine kinase), and c-Abl (a non-receptor tyrosine kinase) were found to phosphorylate tau at Tyr18, Tyr197, and Tyr394, respectively [66, 67].

3.3.2. Acetylation

Tau acetylation occurs at lysine residues and is operated by the P300 acetyltransferase and the CREB-binding protein [68]. Tau can also catalyse autoacetylation at some lysine sites, such as Lys280 [69], which is of particular importance because, when acetylated, it decreases MT binding, increases fibrillation *in vitro* [70] and has been detected in AD and in other tauopathies [71].

3.3.3. Glycosylation

Glycosylation consists in the covalent attachment of oligosaccharides to polypeptides. There are two kinds of glycosylation: O-glycosylation, in which sugars are linked to the hydroxyl group of serine or threonine residues, and N-glycosylation, in which sugars are linked to the amide group of asparagine. Aberrant glycosylation of tau is an event that leads to the hyperphosphorylation of the protein, both by promoting its phosphorylation and by inhibiting its dephosphorylation. [72].

Abnormal glycosylation positively modulates phosphorylation of tau by Cdk5, GSK-3β, and PKA [73, 74]. However, O-GlcNacylation, a type of O-glycosylation obtained from engraftment of N-acetyl-glucosamine, protects tau from excessive phosphorylation (as it occupies the kinase substrates), and is impaired in AD [75, 76].

3.3.4. Glycation

Glycation consists of a non-enzymatic reaction between a carbohydrate and a lysine residue of a protein. Since glycated tau is less susceptible to degradation, its accumulation in cells could promote aggregation, although this is not a direct consequence of glycation [77, 78]. To date, twelve glycation sites have been attributed to tau [79-81].

3.3.5. Ubiquitination

Ubiquitination of proteins is the linkage of a small peptide, ubiquitin, which acts as a tag for degradation by the UPS (ubiquitin–proteasome system). Tau is mostly polyubiquitinated through Lys48 linkages [82], but some other sites have been identified, such as Lys6, Lys11, and Lys63 [83, 84]. However, the effect of ubiquitination on tau degradation has yet to be elucidated. In AD, PHF-tau is ubiquitinated and not degraded, possibly explaining its accumulation in NFTs [85].

3.3.6. Nitration

Nitration is the introduction of a nitro group ($-NO_2$) into a tyrosine residue. Although four out of five tyrosine residues on tau may be nitrated, normal brains show only tau-nTyr197, while in AD and other tauopathies Tyr18, Tyr29 and Tyr394 also appear to be nitrated. Nitration reduces the affinity of tau for MTs and has pro- or anti-aggregating effects depending on the residue involved [86].

3.3.7. Methylation

Methylation occurs physiologically at the lysine residues on tau. *In vitro* studies have shown that high stoichiometric methylation is able to suppress tau aggregation without affecting its function on MT assembly [87].

3.4. Tau in pathology

3.4.1. Aggregation

Under normal conditions, tau is a natively unfolded protein, but its aggregation is the common thread to several neurodegenerative diseases known as tauopathies. Upon aggregation, the protein can form two different structures, PHFs and NFTs: PHFs are double helical stacks with a half-periodicity of about 80 nm (Figure 4), while NFTs are bundles of PHFs in the cytosol of neurons.

Tau is a hydrophilic and unstructured protein, thus its aggregation in ordered fibers may appear counterintuitive. Indeed, the solubility of tau *in vitro* is in the millimolar range of concentrations, whereas in the brain it is at least 1000 times lower, likely explaining why the aggregation of tau was reproduced *in vitro* long after its discovery [88].

The two hexapeptide sequences Val-Gln-Ile-Ile-Asn-Lys and Val-Gln-Ile-Val-Tyr-Lys, at the beginning of R2 and R3, respectively, are responsible for the organization of tau into β-sheet structures and are essential for tau aggregation [89]. It has been shown both *in vitro* and *in vivo* that the disruption of these motifs can suppress the aggregation of tau [90], which is instead accelerated by the polyanions that compensate for the positive charges in the central part of the molecule. The compounds most largely used to obtain the aggregation *in vitro* are heparin, nucleic acids, arachidonic acid micelles, acidic peptides and carboxylated microbeads [88, 91]. Two proteins, 14-3-3ζ and immunophilin, have also been shown to induce the aggregation of tau, probably stabilizing a conformation that is more prone to aggregation [92, 93].

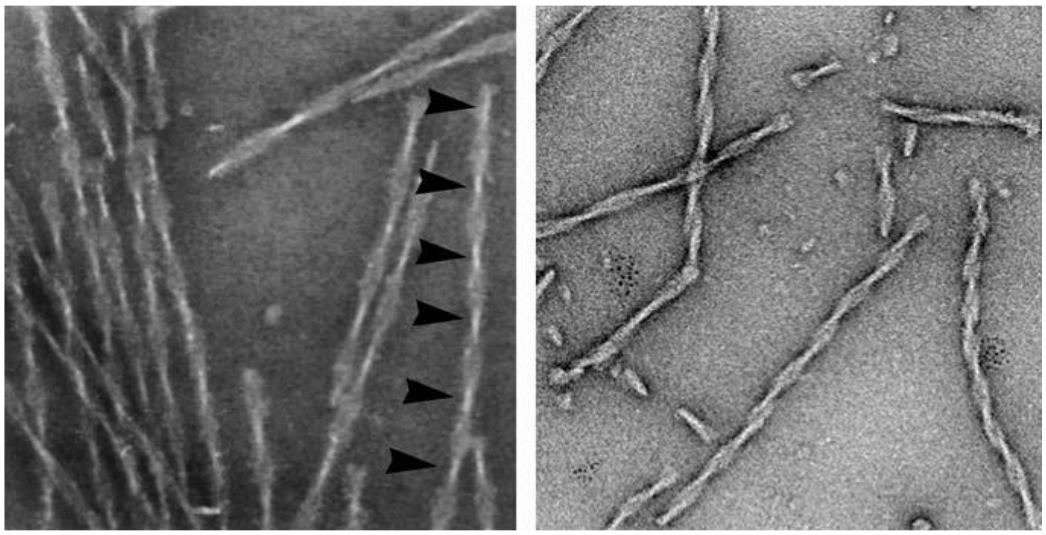


Figure 4: Tau fibers. On the left: PHFs isolated from an AD brain (the arrows indicate the periodicity of ~80 nm). On the right: PHFs formed in vitro.

Despite all the interest aroused, it is still unclear whether it is the phosphorylation of tau that induces its aggregation or whether it is the aggregation that favors the phosphorylation of the protein. However, abnormally phosphorylated tau isolated from human AD brains can self-assemble into PHFs *in vitro* [94], and aggregated tau from patients with tauopathy, or transgenic mice, invariably show hyperphosphorylation [16]. Nevertheless, hyperphosphorylation of tau can occur also in non-pathological conditions, such as animal hibernation and anaesthesia-induced hypothermia [95, 96].

Taken together, the available evidence suggests that tau hyperphosphorylation is necessary but not sufficient by itself to justify pathological aggregation of the protein.

3.4.2. Gain or loss of function?

Although tau dysfunction has been shown to cause neurodegeneration, the precise pathogenic mechanisms involved have yet to be established.

One of the main questions is whether tau becomes pathogenic due to a loss of function or due to a gain of cytotoxic conformation/aggregation. In fact,

hyperphosphorylation and aggregation are both events that affect the ability of tau to bind the MTs, leading to the disassembly of these structures, deficits in axonal transport and neurodegeneration. Moreover, tau is involved in other cellular processes (e.g., DNA protection [22]) and, therefore, its modification may cause additional impairments.

On the other hand, NFT distribution in AD brains correlates with the severity of cognitive deficits, strongly suggesting a pathogenic role of tau when it aggregates perhaps acquiring neurotoxic properties. However, an experiment on transgenic mice expressing the human tau carrying the P301L mutation (linked to FTDP-17) demonstrated that switching off the expression of the mutant protein improves memory and rescues neuronal loss, even in the presence of NFTs, indicating that these structures alone are not sufficient to induce neurodegeneration [97].

3.4.3. Tauopathies

Tauopathy is a term used for a heterogeneous group of sporadic or familial disorders characterized by the deposition of tau in the brain [98]. A large number of neurodegenerative diseases are grouped under this definition, and there are several classification criteria:

- Primary and secondary tauopathies

  A tauopathy is defined as primary when tau is the main contributing factor to neurodegeneration [99]. On the other hand, it is considered secondary when pathological tau is associated with another disease. It should be noted that in the latter case the tau pathology coexists, but it is not necessarily the cause of the other pathology. Table 1 summarizes the most common primary and secondary tauopathies.

  Primary tauopathies are part of frontotemporal lobar degeneration (FTLD), a group of different neurodegenerative diseases characterized by predominant destruction of the frontal and temporal lobes [100]. People affected by FTLD can present different clinical symptoms, such as cognitive, behavioural, language and motor deficits, which, in some cases, may overlap with parkinsonism or amyotrophic lateral sclerosis/motor neurons disease [101].

A subclassification of primary tauopathies is based on whether they are sporadic or familial. PSP, CBD, argyrophilic grain disease (AGD), and Pick's disease are the most common sporadic primary tauopathies.

Most familial cases of FTLD-tau are due to tau mutations, but at least other 20 genes have been shown to be associated with this pathology [102-104].

Secondary tauopathies are a broad group of different neuropathologies [99]. The most prominent secondary tauopathy is AD, which could be a true tauopathy, were it not for the presence of amyloid plaques [99, 105].

Some secondary tauopathies appear to have an environmental etiology, as in chronic traumatic encephalopathy [106], while others have a genetic component, as in Down syndrome.

Table 1: Principal primary and secondary tauopathies.

Predominant tau pathology "Primary tauopathies"	Associated with other types of pathology "Secondary tauopathies"
Progressive supranuclear palsy	Alzheimer's disease
Argyrophilic grain disease	Down's syndrome
Corticobasal degeneration	Lewy body disorders
Pick's disease	Prion disease
Frontotemporal dementia and parkinsonism linked to chromosome 17	Familial British dementia and familial Danish dementia
Postencephalitic Parkinsonism	Chronic traumatic encephalopathy
Parkinson's dementia complex of Guam	Myotonic dystrophy
Guadeloupean parkinsonism	Niemann–Pick disease type C
Globular glial tauopathies	Subacute sclerosing panencephalitis
Ageing-related tau astrogliopathy	Frontotemporal lobar degeneration with C9orf72 hexanucleotide repeat expansion
	Diffuse neurofibrillary tangles with calcification
	Neurodegeneration with brain iron accumulation
	Mutation affecting the sodium/proton exchanger, SLC9A6
	Cerebrotendinous xanthomatosis
	TARDBP mutation p.Ile383Val associated with semantic dementia

- Classification according to the tau isoform pattern

  As already mentioned, exon 10 of tau is alternatively spliced to form tau 3R or 4R. One way to classify tauopathies is based on which of these species is prevalent in NFTs, and identifies 4 different classes.

  In class I (comprising AD, Down's syndrome, parkinsonism-dementia complex of Guam, Niemann-Pick disease type C, chronic traumatic encephalopathy, and some cases of FTDP-17), 3R and 4R tau are both present approximately in equal amounts.

  In class II tauopathies, 4R isoforms are prevalent. In this group, we can find PSP, CBD, AGD, globular glial tauopathy (GGT), ageing-related tau astrogliopathy (ARTAG), and some cases of FTDP-17 [107-111].

  Pick's disease and some cases of FDTP-17 belong to class III, in which 3R tau is the prevalent isoform [107].

  Finally, in class IV, tau pathology is mainly due to isoforms lacking sequences encoded by exons 2, 3 and 10, as in myotonic dystrophy [112].

- Classification according to the cellular pattern of tau pathology

  Another way to classify tauopathies is by the distribution of the pathological tau deposits. Tau can accumulate in neurons (Pick's disease, AD), glial cells (GGT, ARTAG), or in both types of cells (PSP, CBD, AGD) [113] .

  Depending on the disease and the type of cells involved, different immunopositive tau lesions are found. In neurons, the main tau inclusions are pretangles, tangles, Pick bodies, spherical cytoplasmic inclusions, dystrophic neurites, threads, and grains [114].

  Glial cells where tau accumulates are astrocytes and oligodendrocytes. Astroglial phenotypes are usually specific to one disease, such as astrocytic plaques in CBD, tufted astrocytes in PSP, and thorn shaped astrocytes (TsAs) in AGD [113]. Globular glial tauopathies are characterized by coiled bodies and globular oligodendroglial inclusions (GOIs) [115, 116]. It has been demonstrated that the expression of FTDP-17 mutant tau in oligodendrocytes of transgenic mice results in age-related disruption of myelin integrity, impaired axonal transport and axonal degeneration [117, 118].

It is worth mentioning that, although microglia does not express tau protein, tau disease has been observed in this cell type [119], possibly due to a prion-like diffusion mechanism [120, 121].

3.5. Alzheimer's disease

Among the tauopathies, AD is the most studied. According to the World Health Organization (WHO), AD and other forms of dementia are among the top 10 causes of death worldwide.

In 2005, there were approximately 24.3 million people with dementia, with 4.6 million new cases each year. The number of people affected doubles every 20 years, reaching 81 million by 2040, with uneven growth rates: while in developed countries a 100% increase is projected, in China, India and other countries in Southeast Asia and the Western Pacific the increase could exceed 300%.

The disease is named after the neuropathologist who first described it in 1906, Alois Alzheimer. He reported the case of a 50-year-old woman who exhibited paranoia, progressive sleep, memory impairment, aggression and confusion, and died after a 5-year stay at the Community Psychiatric Hospital in Frankfurt. *Post mortem*, histological examination of the brain revealed to Alzheimer the presence of two types of lesions, now known as amyloid (or senile) plaques and NFTs.

AD is a progressive and unremitting pathology, with long preclinical and prodromal phases (20 years) and an average clinical duration of 8–10 years [122].

The course of the disease is usually divided in three different stages [123]:

- Preclinical Alzheimer's disease. In this phase, the brain and cerebrospinal fluid start showing the biomarkers associated to AD, but the patients do not manifest symptoms yet. Notably, the presence of the biomarkers in the body does not automatically mean that the subsequent two stages will occur [124, 125].
- Mild cognitive impairment (MCI). People with MCI start showing cognitive decline, but this decline does not significantly interfere with everyday activities and may be noticed only by people close to the subject, such as familiars and friends. Approximately 15-20% of people aged 65 and over have MCI from any cause, but not all cases of MCI are due to AD, and in some cases MCI remains stable or reverts to normal cognition [126].
- Dementia. This last stage is characterized by a marked cognitive decline. People show symptoms that interfere with their daily life activities, such as memory

loss or confusion, depression, personality changes, and loss of interest in activities they previously loved. These symptoms reflect the degree of neuronal damage in different parts of the brain.

With the progression of the disease, patients require increasing assistance, and in the severe stage they need help with activities of daily living. Eventually, the brain areas involved in movement are damaged, and individuals become bedridden and cannot swallow properly, with a reduced ability to eat and drink as well. AD is ultimately fatal [127].

3.5.1. Genetics and risk factors

There are two different types of AD, the familial (FAD) and the sporadic (SAD) form.

FAD represents less than 1% of total cases [128] and is related to the mutation of genes encoding for the amyloid precursor protein (APP) and presenilin 1/presenilin 2, all of which are involved in the production of amyloid-β (Aβ) peptides [127]. Individuals with FAD show an early-onset disease, with symptoms that appear before the age of 65. The fact that all FAD-related mutations are on proteins involved in the production of Aβ strongly supported the "amyloid cascade hypothesis", which considers Aβ accumulation as the primary cause of AD [129-131].

SAD includes over 99% of all AD cases [132]. Unlike FAD, symptoms generally appear around age 65 or older, and the etiology of this form is still unknown. However, it is believed that rather than a single cause, the onset of SAD is due to multiple factors, both modifiable and non-modifiable.

Non-modifiable risk factors are:

- Age
- Apolipoprotein E (ApoE), a protein encoded by the APOE gene, which exists in three different alleles (ε2, ε3, ε4). Individuals carrying the ε4 allele have a higher risk of developing AD than people who express the ε3 allele [133]. Conversely, ε2 carriers showed protection against the onset of the disease [134].

- Family history. Individuals with a close relative (such as a parent or sibling) who have suffered from AD are more likely to develop the disease, not only because of genetics, but also because they share the same environment and habits (such as diet or exercise) that can make people prone to AD.
- Gender. AD is more prevalent in women than in men [135].

Along with non-modifiable risk factors, others can be changed to reduce the risk of cognitive decline [136]. These factors are:

- Smoking
- Obesity
- Hypertension
- Diabetes mellitus
- Education and sociability. Higher education is believed to help build a “cognitive reserve”, which is the brain’s ability to make flexible and efficient use of cognitive networks [137]. Additionally, having an active social life and engaging in mental activities can help keep the brain healthy, thus reducing the risk of AD [138].

3.5.2. Brain changes

The brain of people suffering from AD is characterized by macroscopic and microscopic alterations.

At the macroscopic level, the most evident feature is cortical atrophy due to cell loss. Neuronal degeneration, which leads to the reduction of synaptic junctions and ultimately to brain shrinkage, is associated with the two microscopic lesions already described by Alois Alzheimer: extracellular Aβ plaques and intraneuronal NFTs composed of tau.

Aβ plaques, also known as senile or neuritic plaques, are roundish structures formed by the aggregation of Aβ, a 40-42 amino acids peptide. According to the "amyloid hypothesis" of AD, Aβ plaques are thought to interfere with neuron-neuron communication in synapses, leading to cell death. Aβ accumulation starts in the frontal and temporal lobes, hippocampus and limbic system.

NFTs spread in AD brains following a typical pattern that was first described by Braak and Braak and is now a widely accepted staging system used to study the disease progression. Braak's six stages can be summarized in three: entorhinal (stages I-II), limbic (stages III-IV) and isocortical (stages V-VI) [139] (Figure 5).

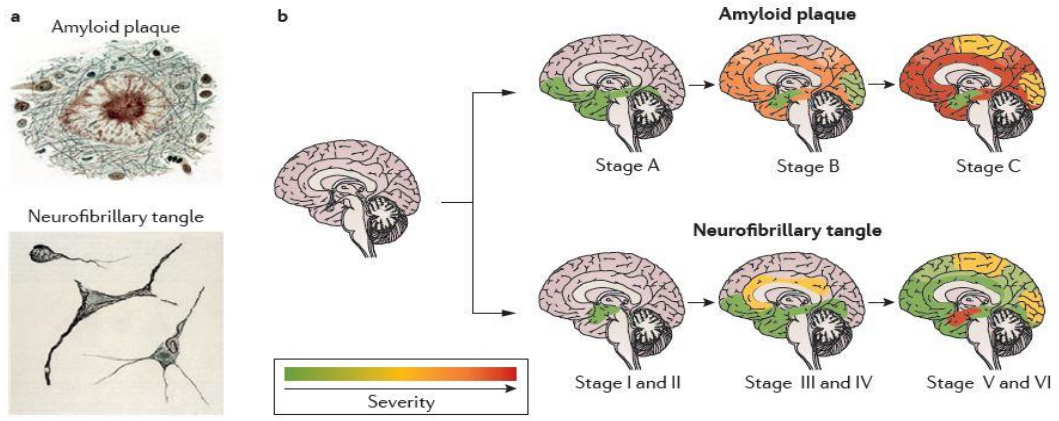


Figure 5: The pathological evolution of Alzheimer's disease. (A) Amyloid plaques and neurofibrillary tangles visualized using the Bielschowsky method of silver impregnation. (B) In typical cases of Alzheimer's disease, Aβ deposition has an apparent origin in the frontal and temporal lobes, hippocampus and limbic system (top row). The neurofibrillary tangles and neuritic degeneration start in the medial temporal lobes and hippocampus, and progressively spread to other areas of the neocortex (bottom row).

Another important feature of the AD brain is inflammation. The microglia is activated by the accumulation of Aβ and tau, and tries to eliminate proteins and cell debris. At some point, the toxic burden is believed to exceed the immune system's capacity to eliminate it, and this event results in chronic inflammation [127, 140].

3.5.3. Etiology

Although the main alterations that occur in AD brains were identified more than a century ago, the true cause of the disease is yet to be established. Over the years,

scientists have proposed some theories, and the two most important identify Aβ and tau, respectively, as the triggering factor of neurodegeneration.

1.5.3.1. The Aβ hypothesis

In 1992 Hardy and Higgins stated *"Our hypothesis is that deposition of amyloid β protein (AβP), the main component of the plaques, is the causative agent of Alzheimer's pathology and that the neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition"* [130].

As mentioned above, this hypothesis has long been supported by important evidence:

- Mutations of APP and presenilins, which are associated with FAD, increase the production of Aβ [141]. $Aβ_{42}$, in particular, is considered the most toxic Aβ peptide, as it aggregates more readily than $Aβ_{40}$.
- All AD patients have amyloid plaques, especially in brain regions involved in memory and learning [142].
- The early development of AD in individuals suffering from Down syndrome is believed to be a consequence of an enhanced production of the Aβ-precursor protein APP, whose gene maps to chromosome 21 [143].
- The genetic risk factor APOEε4 impairs the clearance of Aβ favouring its accumulation [144-146]. Additionally, more recent studies showed that ApoE4 protein stimulates the transcription of APP [147].
- Aβ is neurotoxic *in vitro* [142, 148].

However, in the past 30 years, scientists have not been able to address the main weaknesses of this theory, which are as follows:

- Transgenic mice overexpressing human $Aβ_{42}$ show no cognitive impairment and NFTs, despite plaque formation [149].
- Many people with Aβ-positive PET scan do not show memory impairment [150].
- Cognitive deficits correlate with NFTs and not with the amount of plaques [151].
- NFTs and Aβ plaques do not have the same distribution in AD brains [152].

Furthermore, Aβ-targeting therapies have so far not met the predetermined endpoints and, at best, just a short delay in cognitive decline has been achieved.

1.5.3.2. The tau hypothesis

According to this hypothesis, the formation of NFTs is the key event in the pathogenesis of AD. Toxic tau aggregates would be the cause of neuronal damage, while the accumulation of Aβ would represent a later phenomenon.

The evidence supporting this hypothesis is:

- The spread of tau disease is related to neuronal loss and the severity of dementia [151].
- Tau lesions precede Aβ accumulation [153].
- Mice that are knockout for the endogenous tau but overexpress human tau develop NFTs in the absence of Aβ [154].
- Tau mutations cause tauopathies, such as FTDP-17, which lack senile plaques or other disease-specific inclusions [155].

However, as with the amyloid hypothesis, this theory also has its cons.

As already mentioned, in transgenic mice carrying a pathogenic tau mutation, suppression of the transgene led to memory improvement and rescue of neuronal loss, despite the presence of NFTs [97]. This could imply that the toxic effect of tau on neurons is given by some forms of the protein that precede the formation of tangles. In this context, soluble tau oligomers could be likely candidates, as they increase in brains with AD and other tauopathies and can be detected prior to NFT formation [156, 157]. Furthermore, in mice, tau oligomers have been found to cause misfolding and spread of endogenous tau to unaffected brain regions [157, 158], thus suggesting that tauopathies could progress by a prion-like mechanism that depends on tau oligomers [159, 160]. On the other hand, the results obtained with the suppression of the mutated gene could support the hypothesis of a loss of function in the pathogenesis of AD and other tauopathies.

4. AIMS

Because knowing the physiological function of a molecule is extremely important for understanding its pathological implications, the laboratory in which I worked on this thesis has long focused on the physiology of Aβ.

As a matter of fact, since the late 1990s a growing body of evidence had suggested a promnesic role of the peptide, and in 2008 it was definitively demonstrated that picomolar amounts of $Aβ_{42}$ (similar to those produced physiologically in the healthy brain) enhance hippocampal long term potentiation (LTP), a form of synaptic plasticity [161]. More recent results from our laboratory have also indicated that Aβ is one of the downstream effectors of the two cyclic nucleotides cAMP and cGMP to sustain LTP and memory [162, 163]. In particular, it was found that both cyclic nucleotides stimulate the production of Aβ through different mechanisms: cAMP acts via PKA, which stimulates the synthesis of APP and consequently of Aβ [162, 164], whereas cGMP increases Aβ production by favouring the convergence of APP and its cleaving enzyme BACE-1 in endo-lysosomal compartments [163, 165]. Once produced, Aβ is released from the presynaptic terminals and sustains LTP in postsynaptic neurons with a mechanism that is yet to be established (Figure 6).

The study that allowed the drafting of this thesis arises from the fact that PKA is also one of the kinases that phosphorylate tau protein. As described in the Introduction, PKA phosphorylates tau at Ser214 (pTAU-S214) [166], an event of particular interest because it exerts a protective effect against the assembly of the protein into PHFs [33]. This notion prompted the idea that cAMP and, more interestingly, cAMP enhancing strategies, may have a double protective effect against AD, stimulating the physiological production of Aβ that is necessary for synaptic plasticity, and preventing the pathological aggregation of tau. Furthermore, it has suggested the hypothesis that cGMP can also act in a similar manner, although no one has ever shown that PKG phosphorylates tau.

Given these premises, the objectives of this study were to verify the effect of cAMP-enhancing treatments on pTAU-S214 and to investigate whether the second messenger cGMP could also play a role in tau phosphorylation and aggregation.

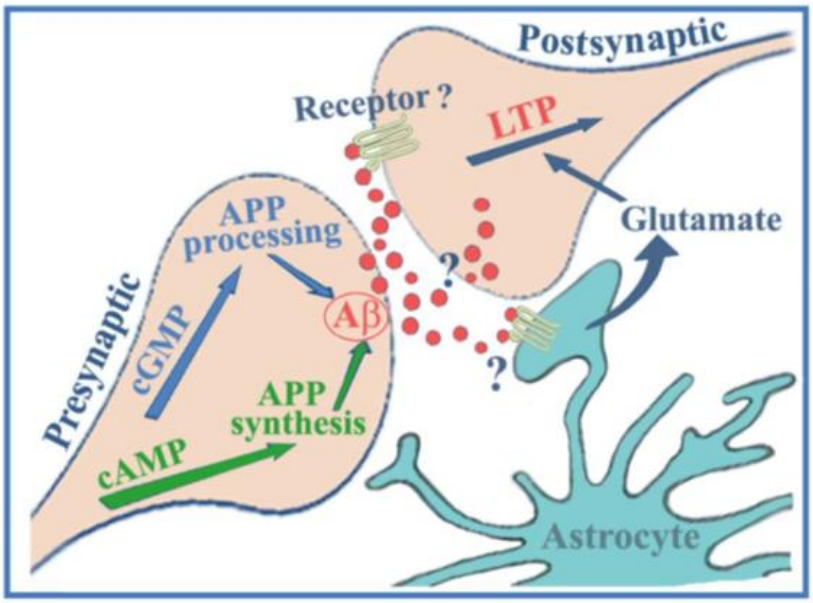


Figure 6: Theoretical model explaining how cGMP and cAMP modulate Aβ leading to the enhancement of LTP. At presynaptic region, cAMP (green) stimulates Aβ production by inducing APP synthesis, whereas cGMP (blue) increases Aβ levels by modulating the processing of APP. In particular, cGMP favors the approximation of APP and BACE-1 in endo-lysosomal compartments. Once secreted, Aβ (red) might influence LTP by activating postsynaptic receptors and/or the astrocytic α7 nicotinic acetylcholine receptor. It is also possible that Aβ acts by entering the postsynaptic intracellular compartment [167].

5. RESULTS

5.1. 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 [168], while FSK is an activator of the enzyme adenylyl cyclase. At the end of treatments, cells and tissues were processed for total protein extraction and immunoblot analyses, which revealed that the levels of pTAU-S214 were increased by both cAMP enhancers, albeit to different degrees. Specifically, GEBR-7b increased pTAU-S214 by 2.45 and 1.60-fold in N2a cells and hippocampal slices, respectively, while FSK produced an induction of 15.03 and 3.31-fold, respectively. As expected, the combination of GEBR-7b and FSK was the most effective in both systems (Figure 7). Using the same samples, we also analysed tau phosphorylation at Ser202 (pTAU-S202), as it is one of the earliest markers of tau aggregation into PHFs [169], and PKA has been shown to phosphorylate this residue [170]. However, we did not observe detectable levels of the phosphopeptide in N2a cells (Figure 7A), and none of the treatments increasing cAMP was able to promote pTAU-S202 in hippocampal slices (Figure 7B).

We then measured $A\beta_{42}$ peptides in conditioned media of N2a cells, confirming that they were increased by the cAMP enhancers (Figure 8), as previously shown by this lab in both cell cultures and hippocampal slices [162, 164].

Next, using a cAMP-specific enzymatic immunoassay (EIA) on N2a extracts, we verified that the treatments actually increased intracellular cAMP. The results obtained confirmed that GEBR-7b significantly enhanced the FSK-induced cAMP accumulation without affecting the basal levels of the cyclic nucleotide (Figure 9).

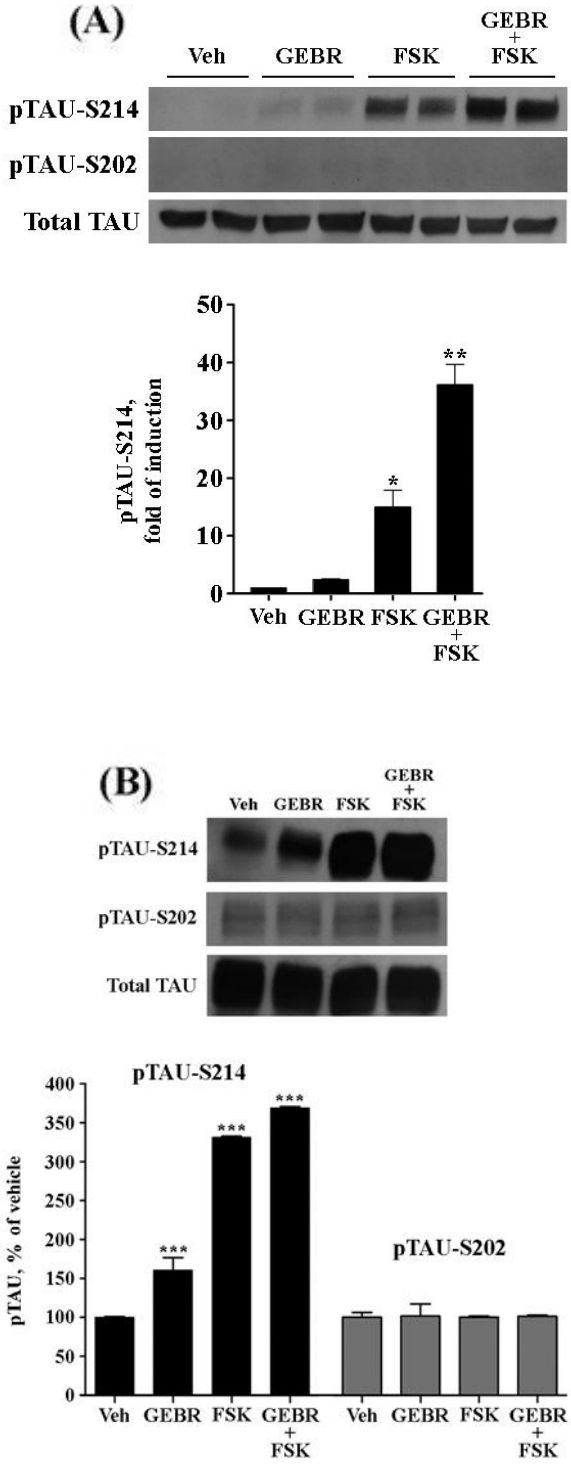


*Figure 7: cAMP enhancers increase pTAU-S214 but not pTAU-S202. (A) N2a cells were pretreated for 1 hour with 10 µM GEBR-7b (GEBR) and then incubated with 1 µM FSK or an equal volume of vehicle (Veh, DMSO) for 15 hours. At the end of treatments, cells were processed for immunoblot analyses. (B) Hippocampal slices were pretreated for 15 minutes with 100 µM GEBR, then incubated for 45 minutes with 100 µM FSK or an equal volume of vehicle and processed for immunoblot analyses. Total tau signals represent the internal loading control. Graphed data show mean ± SEM for three independent experiments. *p < 0.05, **p < 0.001, and ***p < 0.0001 vs vehicle-treated group (one-way ANOVA, Dunnett post-test).*

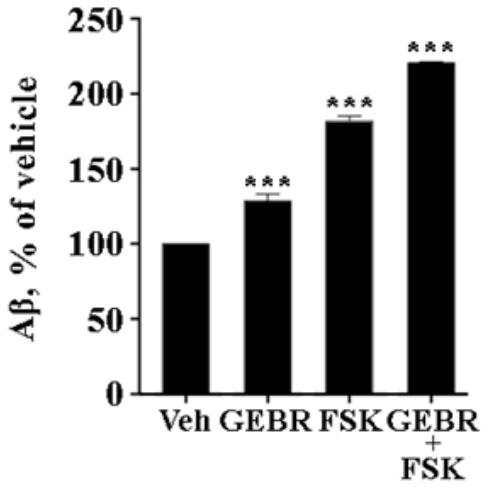


*Figure 8: cAMP enhancers increase Aβ levels in N2a cells. Conditioned media from the experiments described in Figure 7A were subjected to specific $Aβ_{42}$ ELISA. Graphed data show mean ± SEM for three independent experiments. ***p < 0.0001 vs vehicle-treated group (one-way ANOVA, Dunnett post-test).*

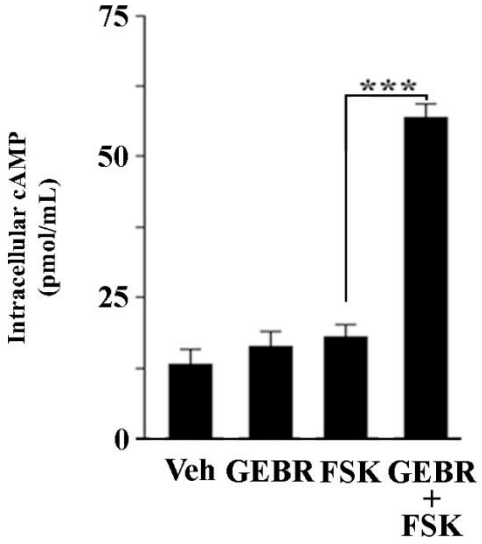


*Figure 9: Quantification of intracellular cAMP. Where indicated, N2a cells were pre-treated for 10 minutes with 100 µM GEBR-7b (GEBR) or an equal volume of vehicle (Veh, DMSO). Then, 1 µM FSK was added for 20 minutes. At the end of the incubation periods, intracellular cAMP was measured with a cAMP-specific EIA kit, according to the manufacturer's instructions. Graphed data show mean ± SEM for three independent experiments. ***p < 0.0001 (one-way ANOVA, Bonferroni post-test).*

5.2. Aβ does not mediate the increase of pTAU-S214

Since the increase in cAMP stimulates the production of Aβ peptides, we tested whether the effect of cAMP on tau phosphorylation was somehow mediated by Aβ.

To this end, we treated both N2a cells and rat hippocampal slices with compound E (CoE), a γ-secretase inhibitor that precludes Aβ formation [171], prior to exposure to GEBR-7b and FSK. As confirmed by specific ELISA performed on the conditioned media of both models, CoE inhibited the production of Aβ peptides even in the presence of the cAMP enhancers (Figure 10). However, the lack of Aβ did not prevent the effect of cAMP on tau phosphorylation, as indicated by immunoblots performed on cell (Figure 11A) and slice extracts (Figure 11B).

These results allowed us to conclude that Aβ is not required for the cAMP-dependent phosphorylation of tau at S214.

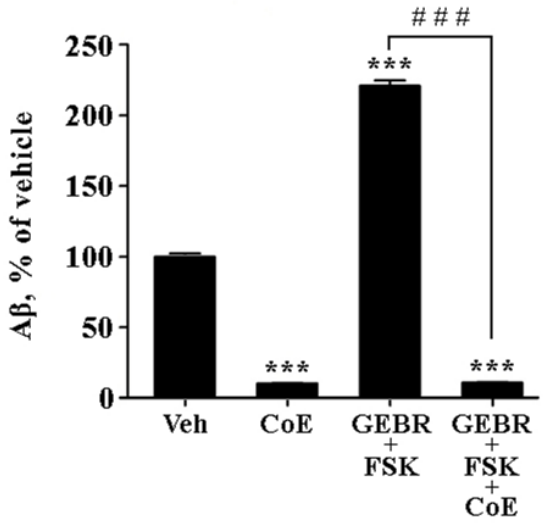


*Figure 10: CoE inhibits the production of Aβ peptides. Where indicated, N2a cells were pretreated with 1 µM CoE for 1 hour, then incubated for 1 hour with 10 µM GEBR-7b (GEBR) and finally for 16 hours with 1 µM FSK or an equal volume of vehicle (Veh, DMSO). At the end of the incubation periods, conditioned media were subjected to specific $Aβ_{42}$ ELISA. Graphed data show mean ± SEM for three independent experiments. ***p < 0.0001 vs vehicle-treated group, ###p < 0.0001 (one-way ANOVA, Dunnett post-test).*

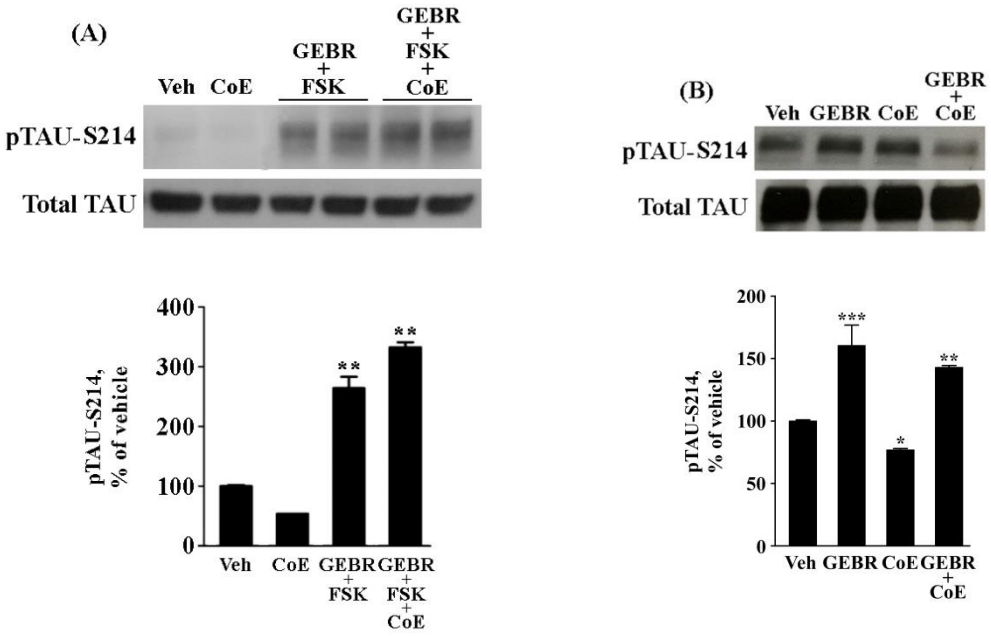


*Figure 11: The enhancement of pTAU-S214 is not mediated by Aβ peptides. (A) N2a cells were treated as described in Figure 10. At the end of the incubation period, cells were processed for total protein extraction, and immunoblot analyses were performed with anti-pTAU-S214 antibody. (B) Hippocampal slices were pretreated with 10 µM CoE for 15 minutes, then incubated with 100 µM GEBR-7b (GEBR) or an equal volume of vehicle (Veh, DMSO) for 45 minutes, and finally processed for immunoblot analyses. The total tau signal represents the internal loading control. Graphed data show mean ± SEM for three independent experiments. *p < 0.05, **p < 0.001, and ***p < 0.0001 vs vehicle-treated group (one-way ANOVA, Dunnett post-test).*

5.3. Vardenafil enhances tau phosphorylation at S214 but not at S202

In light of the results obtained, and given that cAMP and cGMP have similar effects on Aβ production and synaptic plasticity [162, 164], we wondered if cGMP could also play a role in tau phosphorylation.

To this end, we performed immunoblot-based analyses of pTAU-S214 in rat hippocampal slices treated with vardenafil (VDF), a selective PDE5 inhibitor that enhances intracellular cGMP by inhibiting its degradation. We found a significant and concentration-dependent increase of pTAU-S214 (1.65-fold induction and 5.28-fold induction with 10 and 100 μM VDF, respectively) (Figure 12). On the same samples, we also analysed the levels of pTAU-S202, but no effect of cGMP was detected (Figure 12).

In addition, similarly to what observed with the cAMP enhancers (Figure 11), we found that the effect of VDF on pTAU-S214 was not mediated by Aβ, since it was not counteracted by CoE (Figure 13A, B).

Most importantly, immunoblot analysis of hippocampi from mice acutely treated with VDF (0.3 mg/kg) confirmed that the selective and cGMP-induced increment of pTAU-S214 also occurs *in vivo* (+44.9% vs vehicle treated animals) (Figure 14).

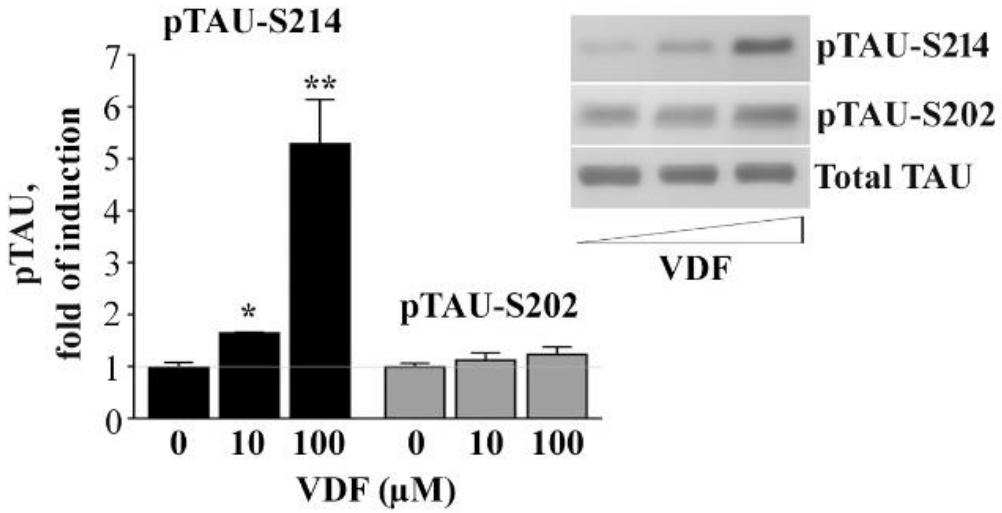


*Figure 12: In rat hippocampal slices, VDF increases pTAU-S214 but not pTAU-S202. Slices were treated with VDF (10, 100 µM) or an equal volume of vehicle (Veh, DMSO) for 45 minutes and then processed for immunoblot analyses. The total tau signal represents the internal loading control. Graphed data show mean ± SEM for three independent experiments. *p < 0.05 and **p < 0.001 vs vehicle-treated group (one-way ANOVA, Dunnett post-test).*

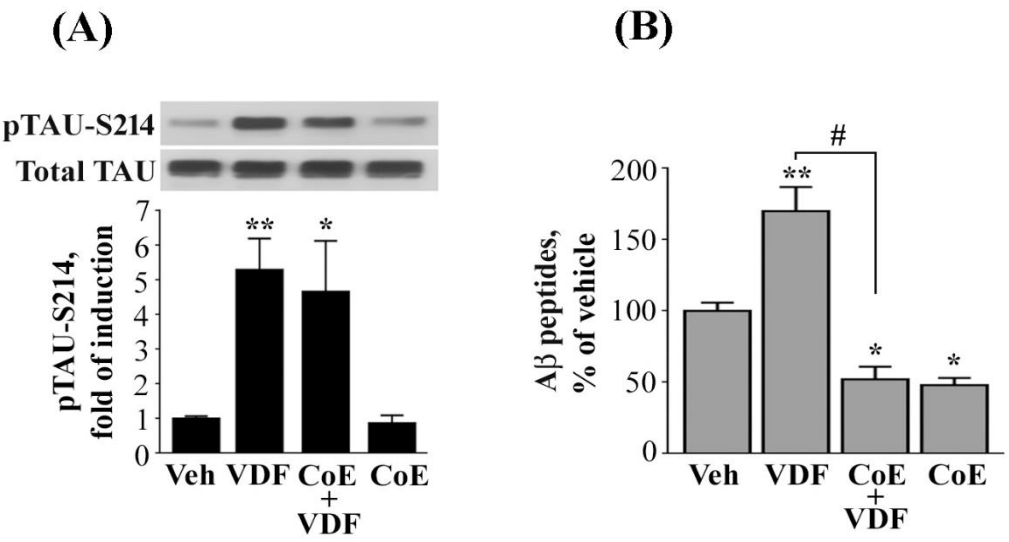


*Figure 13: The enhancement of pTAU-S214 is not mediated by Aβ. (A) Where indicated, hippocampal slices were pretreated with 10 µM CoE for 15 minutes and then exposed to 100 µM VDF or an equal volume of vehicle (Veh, DMSO) for 45 minutes. At the end of the incubation periods, samples were processed for total protein extraction followed by immunoblot analyses. The total tau signal represents the internal loading control. (B) Conditioned media from the experiments described in (A) were subjected to specific $Aβ_{42}$ ELISA. Graphed data show mean ± SEM for three independent experiments. *p < 0.05 and **p < 0.001 vs vehicle-treated group, #p < 0.05 (one-way ANOVA, Dunnett post-test).*

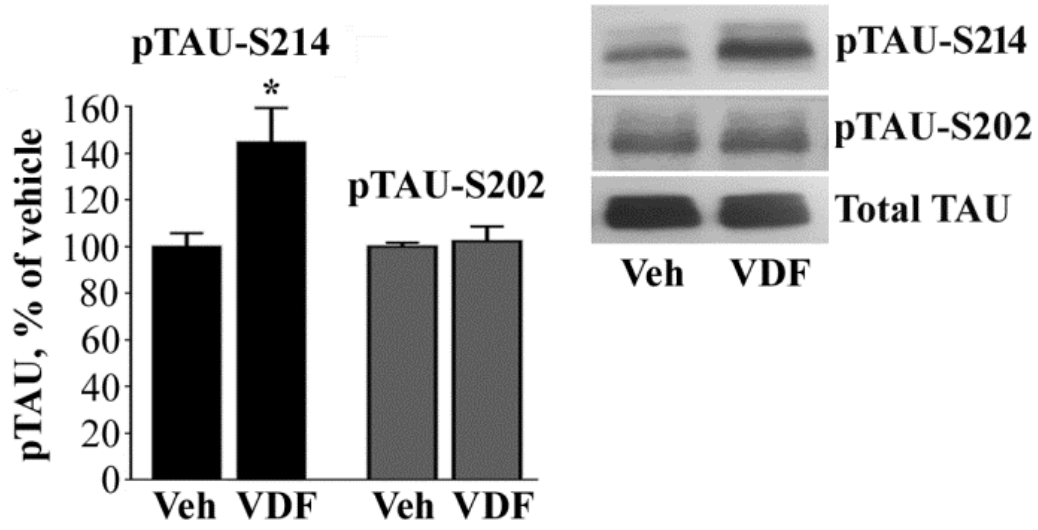


*Figure 14: In vivo effect of VDF on tau phosphorylation. One hour after i.p. injection of VDF (0.3 mg/kg) or vehicle (Veh, DMSO), mice were sacrificed and hippocampi were processed for total protein extraction and immunoblot analyses. The total tau signal represents the internal loading control. Graphed data show mean ± SEM (n= 3 mice for each group of treatment). *p < 0.05 vs vehicle-treated group (two-tailed student t test).*

5.4. PKG mediates the phosphorylation of tau at Ser214

Next, for a better understanding of the mechanisms underlying the observed phenomenon, we performed immunoblot analyses of N2a cells incubated with VDF for different time periods. This experiment revealed that pTAU-S214 is strongly increased already after 10 minutes of VDF treatment (6.55-fold induction compared to the vehicle-treated cells) and returns close to baseline levels after 60 minutes (Figure 15A). We also confirmed, by enzyme immunoassay, that the intracellular concentration of cGMP is increased after 10 minutes of treatment and continues to increase in a time-dependend manner (Figure 15B). It is likely to assume that, once activated, the cGMP-responsive kinase become insensitive to further stimuli.

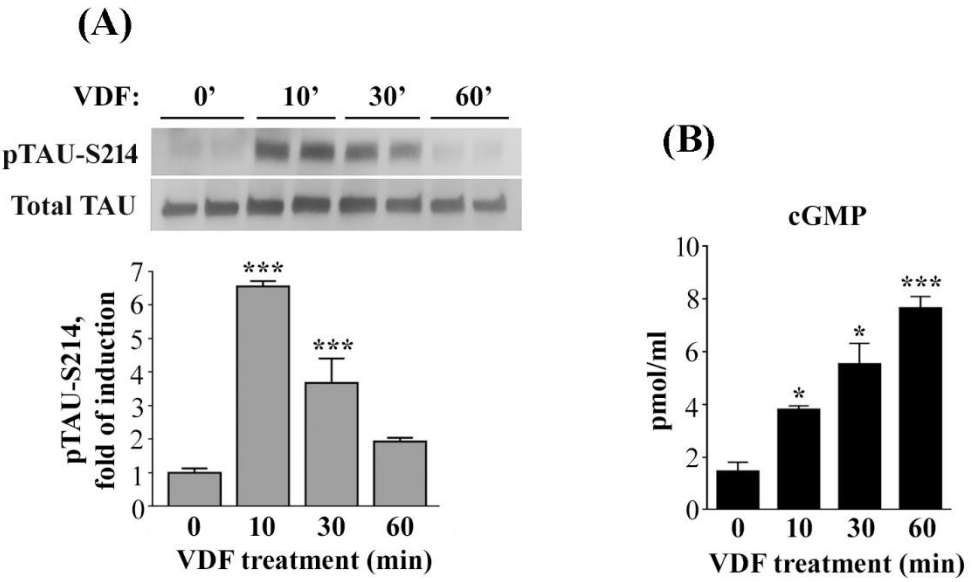


*Figure 15: (A) Time course of VDF in N2a cells. Cells were treated with 100 µM VDF or an equal volume of vehicle (DMSO) for the indicated times and then processed for immunoblot analyses. The total tau signal represents the internal loading control. (B) Intracellular cGMP levels in VDF-treated cells. At the end of VDF treatment (100 µM), N2a cells were processed for cGMP evaluation. Graphed data show mean ± SEM for three independent experiments. *p < 0.05 and ***p < 0.0001 vs vehicle-treated group (Time 0) (one-way ANOVA, Dunnett post-test).*

Given that cGMP acts as a second messenger primarily by activating PKG, we used RNA interference technology to test whether the effect of VDF on pTAU-S214 is mediated by the cGMP-dependent kinase. We observed that, in N2a cells, the knockdown of PKG by siRNA not only prevents the effect of VDF (−70% compared to VDF treated control cells) but it also reduces the pTAU-S214 basal levels by 60%, indicating that there is an endogenous PKG-mediated phosphorylation of tau that occurs also independently from VDF stimulation (Figure 16).

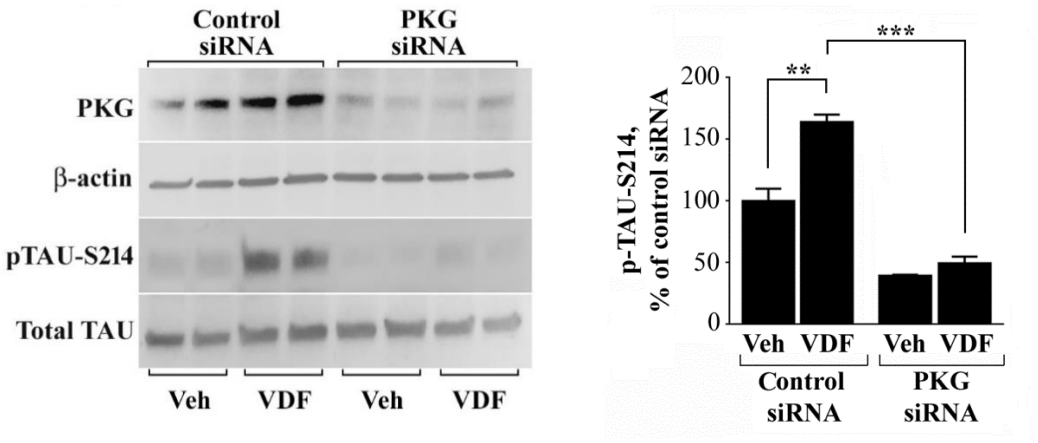


*Figure 16: Effect of PKG silencing on pTAU-S214. N2a cells were transfected with PKG siRNA or non-targeting siRNA (control siRNA), treated with 100 µM VDF or an equal volume of vehicle (Veh, DMSO) for 30 minutes, and then processed for immunoblot analyses. β-actin and total tau signals represent internal loading controls. Graphed data show mean ± SEM for three independent experiments. **p < 0.01 and ***p < 0.001 (one-way ANOVA, Dunnett post-test).*

5.5. Tau is directly phosphorylated by PKG at distinct sites

We next sought to determine whether tau is a direct substrate of PKG. To this aim, human recombinant tau 2N4R was incubated with purified PKG for 1 hour and then subjected to SDS-PAGE and immunoblotting with anti-pTAU-S214, anti-pTAU-S202, and anti-total tau antibodies. As shown in Figure 17, PKG directly phosphorylated tau at Ser214 but not at Ser202.

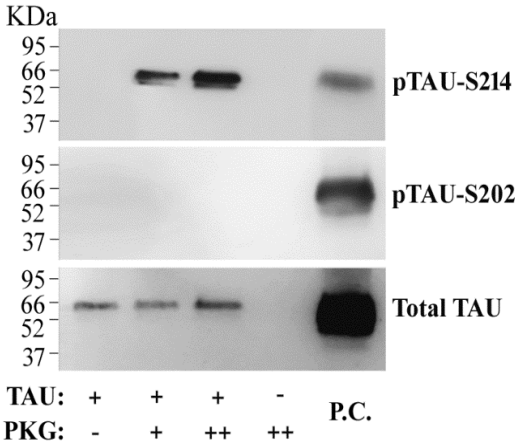


Figure 17: In vitro phosphorylation assay. Recombinant human tau (1 µg) was incubated with 0.1 (+) or 0.2 (++) µg of recombinant PKG for 1 hour. The reaction mixture was then subjected to immunoblot analyses. Protein extract from rat hippocampal slices was used as a positive control (P.C.). Blots are representative of three independent experiments with similar results.

To further confirm these data and to investigate whether other Ser/Thr residues on tau were phosphorylated by PKG, following incubation with the kinase, SDS-PAGE and in-gel digestion, tau samples were analyzed by nano-HPLC-ESI-MS/MS. The obtained results were submitted to the SEQUEST search engine against the Uniprot Human protein database using the phosphoRS tool, which allowed for the specific identification of tau 2N4R and confirmed its phosphorylation at Ser214. Additionally, other seven tau residues, but not Ser202, were unambiguously assigned as phosphosites: Ser409, Ser235, Thr111, Ser356, Ser412, Thr212, and Thr245 (Table 2). As expected, no phosphopeptides were detected when tau was incubated in the absence of PKG (data not shown).

Table 2: PKG-phosphorylated tau residues identified by nano-LC mass spectrometry.

Peptide sequence	Phospho -site	pRS peptide score	pRS isoform probability	pRS site probabilities	q-value	PEP Score	Missed Cleavages
HLSNVSSTGSIDMVDSPQLATLADEVSASLAK	S409	235	100,0%	S(3):100.0; S(6):0.0; S(7):0.0; T(8):0.0; S(10):0.0; S(16):0.0; T(21):0.0; S(27):0.0; S(29):0.0	0	0,00001	0
TPPKSPSSAK	S235	124	92,2%	T(1):0.0; S(5):92.2; S(7):7.7; S(8):0.1	0	0,0361	1
QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQAR	T111	103	84,5%	T(8):0.0; T(14):0.1; T(15):0.1; T(24):84.5; S(26):15.2; T(36):0.0	0	0,00001	0
IGSLDNITHVPGGGNK	S356	115	100,0%	S(3):100.0; T(8):0.0	0	0,0010	0
HLSNVSSTGSIDMVDSPQLATLADEVSASLAK	S409/S412	85	87,6%	S(3):100.0; S(6):87.6; S(7):3.8; T(8):3.8; S(10):3.8; S(16):0.9; T(21):0.1; S(27):0.0; S(29):0.0	0	0,0009	0
SRTPSLPTPPTREPK	T212/S214	89	86,7%	S(1):13.3; T(3):86.8; S(5):99.9; T(8):0.0; T(11):0.0	0	0,0462	2
TPSLPTPPTR	S214	62	99,4%	T(1):0.6; S(3):99.4; T(6):0.0; T(9):0.0	0	0,0496	0
LQTAPVPMPDK	T245	66	100,0%	T(3):100.0	0	0,0121	0

For each peptide, the following details are reported: amino acid sequence, phosphorylation site(s); pRS peptide score (cumulative binomial probability that the observed match is a random events); pRS isoform probability (probability that the isoform is correct); pRS site probability (probability of each site being truly phosphorylated); q-value (minimal false discovery rate at which the identification is considered correct); PEP score (probability that the peptide spectrum match is incorrect); number of missed-cleavages.

5.6. Effect of PKG on tau *in vitro* aggregation

Finally, we investigated whether the phosphorylation operated by PKG could affect tau aggregation. To this purpose, after incubation with PKG, recombinant tau 2N4R was triggered to aggregate by the polyanionic aggregation inducer heparin [172]. Tau aggregation was allowed to proceed for 24 h and then monitored by SDS-PAGE. As shown in Figure 18, the monomeric form of unphosphorylated tau runs as a single band at around 66 kDa, with a slight upward shift occurring in the presence of PKG, most likely as an effect of phosphorylation. An additional band appears in the range of 175 kDa and presumably represents SDS-stable aggregation products [173]. Densitometric analyses of this high molecular weight tau species indicated that, as expected, it increases in the presence of heparin (+90% vs untreated tau) and that, more interestingly, such increase is reduced by 56% in the presence of PKG (Figure 18, right panel).

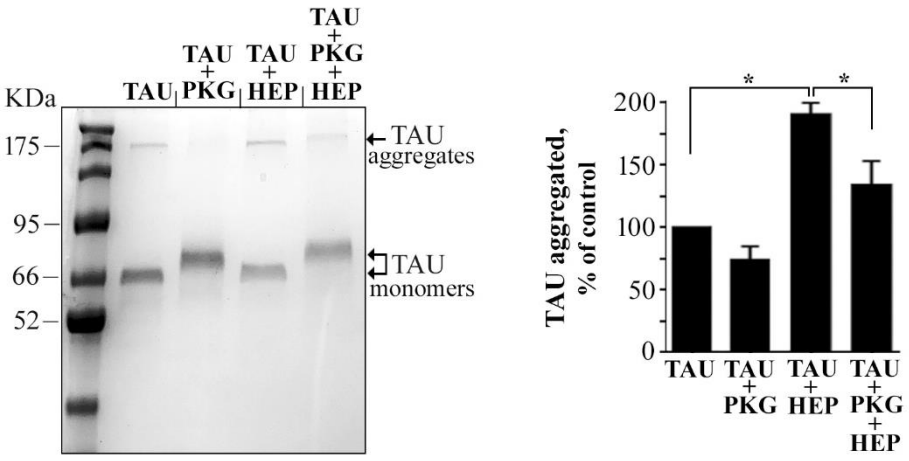


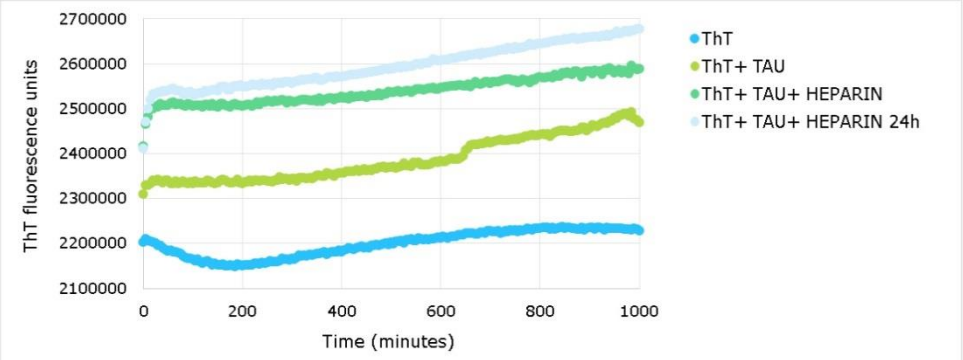
*Figure 18: Aggregation of PKG-phosphorylated tau. Recombinant human tau (1.3 µg) was incubated with or without heparin (HEP, 0.2 µM) and recombinant PKG (0.2 µg) for 24 hours. The reaction mixture was then subjected to 10% sodium dodecyl sulfate (SDS)-PAGE, followed by staining with Coomassie Brilliant Blue R250. Graphed data show mean ± SEM for three independent experiments. *p < 0.05 (one-way ANOVA, Dunnett post-test).*

To confirm this result, we performed a fluorescence assay using thioflavin T (ThT), a molecule that becomes fluorescent when bound to protein aggregates, allowing their detection and quantification [174].

To set the experimental conditions and to verify whether the aggregation of tau could be effectively monitored by ThT fluorescence, we first analysed tau in the presence and absence of heparin. Heparin was added to tau 24 hours prior to or immediately prior to analysis, which was conducted for 16 hours. Both samples showed a greater fluorescence than heparin-free tau, indicating that the method was able to distinguish the aggregated form from the monomeric form of tau (Figure 19A).

We then repeated the experiment by adding PKG-phosphorylated tau, to see if phosphorylation could affect the protein aggregation. However, despite numerous attempts, we have obtained too variable and inconclusive results (Figure 19B).

(A)



(B)

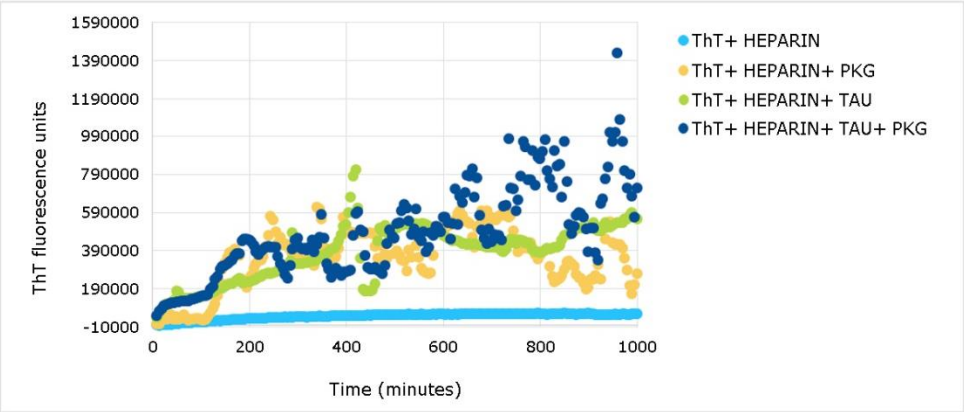


Figure 19: Tau aggregation assay in the presence of ThT. Fluorescence intensity was measured every 5 minutes until the indicated times (excitation 450 nm, emission 482 nm). (A) For the analysis, samples were incubated in a 96-well plate at a final volume of 39.7 µL. Where indicated, samples contained 5 µM ThT as a probe, 0.18 µM heparin as aggregation inducer, 1.32 µg tau, and/or 0.135 µg PKG, in a 20 mM Tris–HCl buffer (pH 7.4). (B) Total volume was increased to 100 µL and ThT to 12.5 µM concentration, while other conditions remained unchanged. The ThT assay was performed independently multiple times.

6. DISCUSSION

The fight against AD is a compelling challenge of modern medicine. The etiology of this syndrome is still a matter of debate, so a deeper knowledge of the molecular events leading to tau and Aβ dysregulation is of key importance for the development of effective therapies.

Over the past 20 years, several clinical trials have been conducted aimed at reducing Aβ levels, but although most of drugs have successfully reduced the amyloid load, no cognitive improvement has been achieved [175]. In 2020, this therapeutic approach seemed to have been definitively abandoned, but a few months ago FDA approved aducanumab, yet another antibody targeting Aβ, for the treatment of AD. Curiously, approval was given despite the opposition of the medical advisory committee, as clinical trials of the drug had been stopped due to futility [176].

Most probably, Alzheimer's scientific research has failed so far because the body of evidence indicating that Aβ is needed for synaptic plasticity and memory was not taken into account. Among the most recent publications, an important study conducted by Sturchio and colleagues revealed that in individuals with cerebral amyloidosis, the highest amounts of soluble $A\beta_{42}$ are detected in subjects with normal cognition and not in patients with MCI or AD. This further evidence strengthens the hypothesis that the pathogenicity of Aβ could be due to the loss of its normal function rather than the acquisition of toxic properties. From this point of view, it is likely that when Aβ is sequestered in the plaques, it is no longer available in a soluble form to carry out its physiological functions.

Similarly, tau may lose its functionality when hyperphosphorylated and aggregated in NFTs, thus leading to pathological effects. Therefore, focusing on the molecular mechanisms that govern tau phosphorylation could help to reveal important elements for future therapeutic strategies.

In the present study, we demonstrate that both cAMP and cGMP are involved in tau phosphorylation regardless of their amyloidogenic capacity. This evidence is important because indicates that Aβ is not required for the process of tau phosphorylation, unlike what appears to occur under pathological conditions [177].

Another interesting evidence of this study is that enhancing cAMP by treatments with GEBR-7b or FSK increases the phosphorylation of tau at Ser214, as expected, but not at the proaggregant S202. Given that the cAMP-activated PKA has been shown to promote tau phosphorylation at Ser202 [170], our results may appear contradictory. Indeed, it must be considered that PKA does not phosphorylate tau directly, but through activation of GSK-3β, a pathway that may not be involved in our system.

Most importantly, this study is the first showing that, under physiological conditions or VDF stimulation, PKG phosphorylates tau both *in vitro* and *in vivo*. Moreover, we provide evidence that PKG directly phosphorylates Ser214 and, at least *in vitro*, other 7 tau residues, but not Ser202.

Under our experimental conditions, phosphorylation of tau by PKG seems to reduce its heparin-induced aggregation, although we do not know, at this stage of the study, whether the other phospho-residues identified, beside Ser214, may contribute to this effect. Indeed, Goedert et al. showed that if tau is phosphorylated by p42 MAP kinase or neuronal Cdc2-like kinase (NCLK) it is still able to bind heparin, concluding that the heparin-induced aggregation is not significantly affected by phosphorylation [91]. However, this observation does not exclude that the PKG-operated phosphorylation may instead inhibit the effect of heparin on tau aggregation.

Although further investigation is needed, our findings suggest that PKG activation may shift tau from a proaggregant to an anti-aggregant conformation, which has been reported to exert neuroprotective functions [16, 17, 24]. As a matter of fact, the conditional transgenic expression of pro-aggregant tau mutants causes neuronal loss, decreases LTP and impairs learning ability and memory in mice [178, 179]. When the transgenic expression is switched off and tau aggregates moderately decrease, less neuronal damage is observed and LTP and memory recover [180]. In accordance with this, in mice overexpressing the anti-aggregant form of tau, NFTs and neuronal loss have not been observed, with LTP being even slightly enhanced, and memory being normal.

The hypothesis that tau may play a physiological role in learning and memory has been recently confirmed in a study showing that tau knockout mice manifest age-

dependent LTP alterations and cognitive deficits. cGMP and cAMP, by activating their respective effectors PKG and PKA, take part in both early and late-LTP, which are generally recognized as the electrophysiological correlates of short- and long-term memory, respectively. Indeed, inhibitors of enzymes that degrade cyclic nucleotides, such as PDE5 and PDE9 for cGMP and PDE4D for cAMP, have been consistently reported to enhance LTP, learning and memory formation/consolidation under physiological and pathological conditions [181-183]. Intriguingly enough, in senescence-accelerated and AD mice models, both sildenafil and tadalafil, two selective PDE5 inhibitors, were shown to reduce PHF and to rescue memory dysfunctions [184, 185].

In conclusion, our results not only indicate the existence of a novel cGMP/PKG-mediated mechanism that might regulate the conformational state of tau favouring its phosphorylation at specific sites, but could also further explain how cAMP and cGMP participate in the molecular processes of memory formation, as summarized in figure 20.

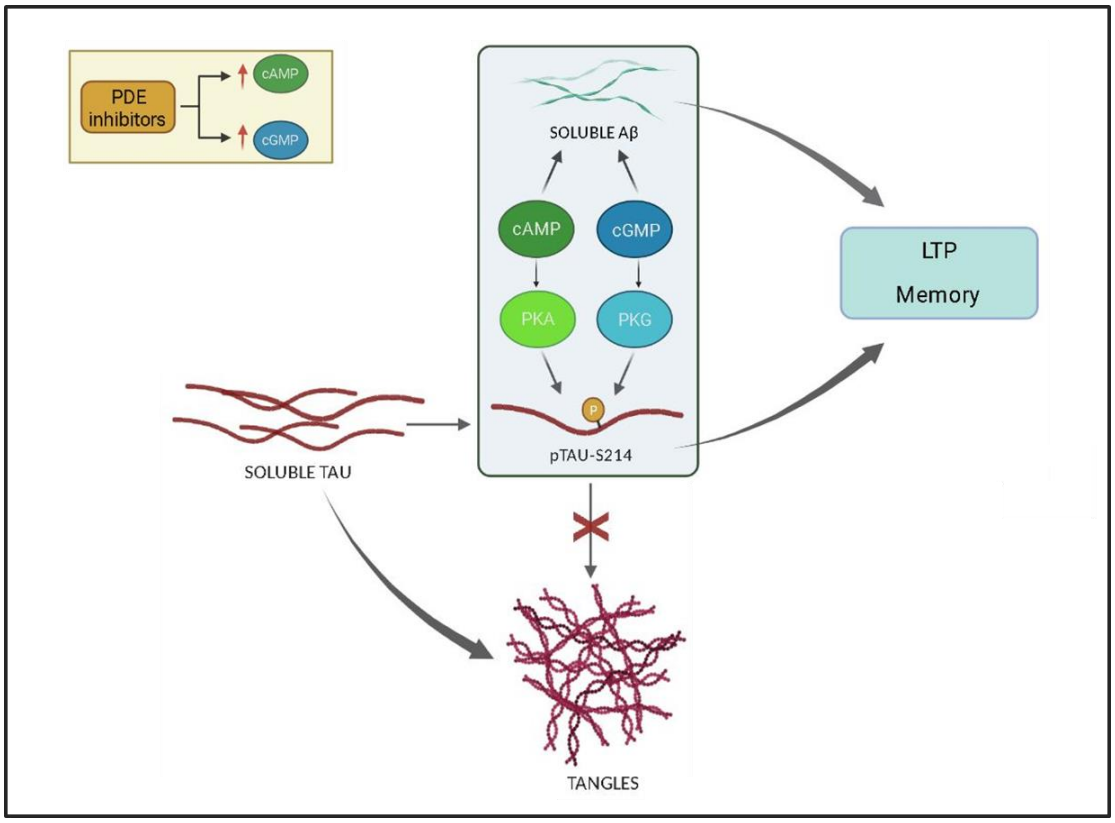


Figure 20: Theoretical model of the effects of cAMP and cGMP on LTP and memory processes. The two cyclic nucleotides, through their respective effectors PKA and PKG, phosphorylate tau at Ser214 and prevent tangles formation. Moreover, cAMP and cGMP favor the physiological production of Aβ peptides. These two events have beneficial effects on LTP and memory formation/consolidation. Therefore, PDE inhibitors, which raise the levels of cyclic nucleotides by inhibiting their degradation, are promising candidates to contrast the cognitive decline associated to Alzheimer's disease and other tauopathies.

7. MATERIALS AND METHODS

7.1. Animals

Sprague–Dawley rats were maintained in the animal facilities of the Department of Pharmacy (DIFAR), University of Genoa (project 02/10/06/2015-OPBA, approved by the local Ethical Committee in accordance with the European (2010/63/EU) and Italian (L.D. n. 26/2014) legislation). C57BL/6 mice were maintained in the animal facilities of Maastricht University (project DEC2013-059, approved by the local Ethical Committee in accordance with the Dutch (Experiments on Animals Act, 2014) and European legislation).

Animals were housed on a regular light/dark cycle (light from 7:00 a.m. to 7:00 p.m.) and kept at constant temperature (22 ± 1°C) and relative humidity (50%) with free access to water and food. In accordance to the 3Rs principle, all efforts were made to minimize animal suffering and to reduce the number of animals to the minimum necessary to produce reliable results.

7.2. Hippocampal slices

This part of the study was conducted in collaboration with Prof. Ernesto Fedele at the Department of Pharmacy (DIFAR, University of Genoa).

Transverse hippocampal slices (250 µm) were obtained from adult male Sprague–Dawley rats (250–300 g) using a Mcllwain tissue chopper. Slices were incubated at 37°C into 2 mL of a physiological solution (125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22mM $NaHCO_3$, 1 mM Na_2HPO_4, 10 mM glucose, pH 7.2–7.4) continuously aerated with 95% O_2 and 5% CO_2, and treated with different concentrations of GEBR-7b (Millipore), forskolin (FSK, Sigma-Aldrich), compound E (CoE, Adipogen) and vardenafil (VDF, Sigma-Aldrich) as indicated. Compounds were dissolved in dimethyl sulfoxide (DMSO) at the concentration of 100 mM (GEBR-7b and FSK), 1 mM (CoE), and 50 mM (VDF), and stored at −20°C until use. Appropriated controls were always run in parallel. At the end of treatments, slices were homogenized in ice-cold RIPA buffer containing 5 mM EDTA, 0.15 M KCl, 25 mM Tris–HCl (pH 7.4), 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulphonyl fluoride, 1% protease inhibitor (Complete Mini, Roche S.p.A.). Homogenates were spun at 10,000 g for

10 minutes at 4°C. Supernatants were centrifuged a second time under the same conditions and then used for immunoblot analyses.

7.3. *In vivo* treatments

This part of the study was conducted in collaboration with Prof. Jos Prickaerts at Maastricht University (The Netherlands).

Adult male C57BL/6 mice were used (25–30 g). VDF was dissolved in a vehicle composed of 98% methyl cellulose tylose solution (0.5%) and 2% Tween80, and intraperitoneally administered to mice (n = 3) in a volume of 4 mL/kg, at a dose of 0.3 mg/kg. Control mice (n = 3) were injected with vehicle only. Dosage and injection volume were based on previous experience with the drug [186, 187]. One hour after injection, mice were sacrificed and brains immediately dissected. Hippocampi were quickly homogenized in ice-cold RIPA buffer and processed as described for hippocampal slices (see above).

7.4. Cell culture treatments

Mouse neuro-2a (N2a) cells were grown in 47% Dulbecco modified Eagle's medium, 47% OptiMEM with 0.1 mM nonessential amino acids, 1% penicillin–streptomycin mixture, and 5% fetal bovine serum. VDF, GEBR-7b, FSK and CoE (dissolved in DMSO and stored at −20°C) were diluted in the culture medium to reach the final concentration.

7.5. Total protein extraction from cell cultures

At the end of treatments, N2a cells were first washed with cold phosphate buffered saline (PBS), then lysed with an ice-cold buffer containing 1 mM EGTA, 50 mM NaF, 2 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM PMSF, 1% protease inhibitors (Complete Mini, Roche S.p.A.) and 1% SDS. Cell scrapers were used to ensure the complete dislodgement of the cells, which were subsequently syringed, using a 25-gauge needle. Cell lysates were then spun at

15,000 g for 10 minutes at 4°C and the supernatant (total protein extracts) stored at -80°C until use.

7.6. Protein quantification

The total protein concentration was determined with bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific). This is a colorimetric method based on the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium. The intensity of the purple-colored reaction obtained from the chelation of two molecules of BCA with one cuprous ion is proportional to the protein concentration. The amount of proteins is then calculated by spectrophotometric analysis at 562 nm, according to the standard curves of albumin prepared on the same microplates.

7.7. Immunoblot analysis

Immunoblot analysis, also called Western blotting (WB), is a biochemical procedure that allows a semi-quantitative evaluation of specific protein expression.

Knowing the total protein concentration of each sample and using the formula:

$$\text{volume} = \frac{\text{mass}}{\text{concentration}}$$

it is possible to evaluate the quantity of each sample that has to be used in order to analyse the same amount of proteins.

In our studies, Laemmli buffer 4X (4% SDS, 0.3% bromophenol blue, 40% glycerol, 100 mM β-mercaptoethanol and 200 mM Tris–HCl buffer, pH 6.8) was diluted to 1X into the protein samples, which were subsequently heated for 5 minutes at 100°C in order to denature the higher order structures. The proteins contained in each sample were then separated by polyacrylamide gel electrophoresis, in the presence of SDS (SDS-PAGE). We used precast gels (Bio-Rad Mini-gels system, 10 x 7.5 x 0.1 cm, 4-20 % polyacrylamide) and sharpmass VII (Euroclone) as a molecular weight marker. Electrophoresis was performed at 140 V with a short pre-run at 70

V. Next, proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane using the Towbin method, with a cold buffer containing 25 mM Tris, 192 mM glycin and 20% methanol. Protein transfer occurred applying a current of 100 V for 60 minutes. Membranes were then incubated for 60 minutes with 5% milk powder in TBS-tween (t/TBS: 200 mM Tris, 1.3 M NaCl (pH 7.5), 0.05% tween 20) in order to saturate possible nonspecific antibody binding sites. Membranes were subsequently incubated for 60 minutes at room temperature (or overnight at 4°C) with a primary antibody specific for the protein of interest. We used the following primary antibodies: monoclonal rabbit anti-tau (phospho S214) (1:1000; Abcam), monoclonal rabbit anti-tau (phospho S202) (1:5000; Abcam), polyclonal rabbit anti-total tau (1:400; Abcam), monoclonal rabbit anti-PKG1 (1:1000; Cell Signaling) and monoclonal mouse anti-β-actin (1:10000; Sigma-Aldrich). At the end of incubations with the primary antibody, membranes were washed with t/TBS and incubated with a secondary anti-rabbit or anti-mouse antibody coupled to horseradish peroxidase (GE Healthcare) for 60 minutes.

Proteins were revealed with an enzyme-linked chemiluminescence detection kit (ECL, GE-Healthcare). Chemiluminescence was visualized by film exposure, and signals were analyzed under non-saturating conditions with an image densitometer (Bio-Rad).

7.8. Aβ peptides evaluation

Aβx-$_{42}$ ELISA kit (Wako Chemicals GmbH) was used to quantify the amount of Aβ_{42} released into culture media from N2a cells and hippocampal slices. At the end of treatments, conditioned media were collected, spun at 1000 g for 10 minutes at 4°C to remove cell debris, and immediately subjected to ELISA.

This assay takes advantage of 96 well-plates coated with the monoclonal antibody BNT77 that binds to the amino acid sequence 11-28 of Aβ. Captured A$\beta_{1\text{-}42}$ is then recognized by another antibody (BC05 HRP-conjugated) that specifically detects the C-terminal portion of A$\beta_{1\text{-}42}$. The peroxidase activity linked to the BC05 antibody is revealed by the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) solution, a specific substrate of HRP. The reaction is stopped by the addition of

sulfuric acid, which produces a yellow colour proportional to the concentration of the antigen-antibody complex ($A\beta_{1-42}$-BC05). The absorbance is then measured at 450 nm using a microplate reader (Bio-Rad iMark™), and the concentration of $A\beta_{1-42}$ is calculated according to the standard curve prepared on the same ELISA plate.

7.9. Evaluation of intracellular cAMP and cGMP

Quantification of intracellular cAMP and cGMP was performed with the DetectX® Direct Cyclic AMP Enzyme Immunoassay and the DetectX® Direct Cyclic GMP Enzyme Immunoassay Kits (Arbor Assay). The test was carried out using a microtiter plate pre-coated with an antibody against mouse IgG. After the samples (and standards) were loaded into the wells, synthetic cAMP (or cGMP) conjugated with peroxidase was added, followed by a mouse IgG against cAMP (or cGMP). In this way, the IgG clings to the coating antibody and binds the cyclic nucleotides. However, since the IgG has a lower affinity for the conjugated nucleotide, compared to that present in the sample, the colorimetric reaction of peroxidase is inversely proportional to the amount of cyclic nucleotides in the sample. Quantitative analyses were performed with a microplate reader (Bio-Rad iMark™) measuring the absorbance at 450 nm.

7.10. RNA interference

Small (or short) interfering RNA (siRNA) is used to induce short-term silencing of protein coding genes. In particular, siRNA is a synthetic RNA duplex whose antisense strand is a perfect reverse complement of the intended target mRNA. Upon transfection into cells, siRNA is loaded into the RNA-induced silencing complex (RISC), the RNA strands are separated, and the antisense strand binds to the target mRNA causing its cleavage and degradation.

In this study, PKG siRNA duplex (directed at the mouse sequence, Accession No. NM_001013833.3) and the control siRNA nontargeting duplex were purchased from Dharmacon. Transfections were performed using Dharmafect at 4 µL/100 nM siRNA, according to the manufacturer's instructions. Silencing efficiency was verified by immunoblotting.

7.11. Kinase assay

This set of experiments was performed in collaboration with Prof. Santina Bruzzone and Prof. Laura Sturla at the Biochemistry Section of DIMES (University of Genoa).

The recombinant human tau used is the 441 amino acid form also known as 2N4R, Tau-F, Tau-4, or Tau441 (UniProt P10636-8, BostonBiochem). The kinase assay was carried out incubating 1 µg of tau with 0.1 or 0.2 µg of purified human recombinant active PKG1alpha (Merck KGaA, Germany) in a kinase buffer containing 25 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 0.5 mM ATP, and 100 µM cGMP. After 60 minutes at 37°C, the reaction was stopped with Laemmli buffer and subjected to SDS-PAGE followed by immunoblotting or by nano-HPLC mass spectrometry.

7.12. Nano-HPLC mass spectrometry

Nano-HPLC mass spectrometry was conducted in collaboration with Prof. Gianluca Damonte and Dr. Annalisa Salis at the Biochemistry Section of DIMES (University of Genoa).

After kinase assay, SDS-PAGE, gel excision and destaining, proteins were reduced, alkylated and digested with trypsin as described by Shevchenko et al. [188]. Nano-HPLC-MS/MS analysis of tryptic peptides was performed on an UltiMate 3000 Nano LC (Thermo Fisher Scientific, Italy). Peptides were first loaded onto a trapping column (Acclaim PepMap 100 C18, Thermo Fisher Scientific) using ACN:H_2O (95:5) + 0.05% trifluoroacetic acid. Peptide separation was carried out at a flow rate of 300 nL/minute on an EASY-Spray column PepMap C18 (Thermo Fisher Scientific) applying a linear gradient of ACN:H_2O (95:5) + 0.08% formic acid, from 4 to 95% in 55 minutes. Analyses were done in the positive ion mode. The MS survey scan was performed in the orbitrap, recording a mass window between 395 and 2000 m/z, with a maximal ion injection time of 100 ms. Experiments were performed in data-dependent acquisition mode, alternating MS and MS/MS. For MS/MS analysis, an isolation window of 2 Da was used. Collision-induced dissociation (CID) was performed with a target value of 5000 ions, a maximal ion

injection time of 50 ms, and a normalized collision energy of 35%. Raw MS/MS spectra were interpreted with Proteome Discoverer (v.1.4, Thermo Fisher Scientific) and peak list files were obtained using SEQUEST algorithm against the UniProt human database. The resulting peptide hits were filtered for a maximum 1% false discovery rate using the percolator tool. The database search parameters were: mass tolerance precursor 20 ppm, mass tolerance fragment CID 0.8 Da, dynamic modification of deamidation (N, Q), oxidation (M) and static modification of alkylation with IAM (C). Phosphorylation of Ser/Thr/Tyr was set as variable modification. PhosphoRS software was used to validate the correct assignment of phosphorylation sites [189]. In any case, the option trypsin with two missed cleavages was selected.

7.13. Detection of tau aggregates

Aggregation of recombinant tau protein (1.3 µg/sample) was carried out with the aid of heparin, as described by Haase et al. [172]. Briefly, at the end of the kinase assay described above, heparin was added, were indicated, at a final concentration of 0.2 µM. After 24 hours at 37°C, reactions were stopped with Laemmli buffer. Samples were heated to 95°C for 5 minutes and subjected to 10% SDS-PAGE followed by Coomassie Brilliant Blue R250 staining and densitometric analysis.

7.14. Thioflavin T-binding assay

This part of the study was conducted in collaboration with Prof. Santina Bruzzone and Prof. Laura Sturla at the Biochemistry Section of DIMES (University of Genoa).
The thioflavin T dye (ThT, Sigma) exhibits a dramatic increase in fluorescence (excitation: 450 nm; emission: 485 nm) when bound to β-sheet-rich structures such as amyloid fibrils or tau aggregates, allowing their detection and quantification [174, 190]. For this reason, at the end of treatments, recombinant tau was transferred to a multiwell plate, labelled with 5 µM ThT, and analysed in time-lapse mode with a Clariostar plus microplate reader (BMG Labtech). Each well contained, where indicated, 1.32 µg of tau, 0.135 µg of PKG, and heparin

(0.18 μM), in a 20 mM Tris–HCl buffer (pH 7.4). As reported in Figure 19, in some samples tau was incubated with heparin for 24 hours before labelling with ThT.

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