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RESEARCH ARTICLE

D-Pinitol promotes tau dephosphorylation through a cyclin-dependent kinase 5 regulation mechanism: A new potential approach for tauopathies?

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Background and Purpose: Recent evidence links brain insulin resistance with neurodegenerative diseases, where hyperphosphorylated tau protein contributes to neuronal cell death. In the present study, we aimed to evaluate if D-pinitol inositol, which acts as an insulin sensitizer, affects the phosphorylation status of tau protein.

Experimental Approach: We studied the pharmacological effect of D-pinitol on insulin signalling and tau phosphorylation in the hippocampus of Wistar and Zucker rats. To this end, we evaluated by western blotting the Akt pathway and its downstream proteins as being one of the main insulin-mediator pathways. Also, we explored the functional status of additional kinases phosphorylating tau, including PKA, ERK1/2, AMPK and CDK5. We utilized the 3xTg mouse model as a control for tauopathy, since it carries tau mutations that promote phosphorylation and aggregation.

Key Results: Surprisingly, we discovered that oral D-pinitol treatment lowered tau phosphorylation significantly, but not through the expected kinase GSK-3 regulation. An

Abbreviations: DPIN, D-Pinitol; DCI, D-Chiro-inositol; mTOR, mammalian Target of Rapamycin; GSK-3β, Glycogen synthase kinase-3 beta; GS, Glycogen synthase; NFTs, Neurofibrillary Tangles; CDK5, Cyclin-dependent kinase 5; CDK5R1, Cyclin-dependent kinase 5 regulatory subunit 1; MRM, multiple reaction monitoring; RIPA, radioimmunoprecipitation assay buffer lysis buffer; AMPK, AMP-activated protein kinase; PP2A, Protein phosphatase proteins 2A; PP2C, Protein phosphatase proteins2C; PTEN, Tensin homolog deleted on chromosome 10; NFκB, Factor kappa-light-chain-enhancer of activated B cells; STAT3, Signal transducer and activator of transcription 3; IRS-1, Insulin receptor substrate 1.

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extensive search for additional kinases phosphorylating tau revealed that this effect was mediated through a mechanism dependent on the reduction of the activity of the CDK5, affecting both its p35 and p25 subunits. This effect disappeared in leptin-deficient Zucker rats, uncovering that the association of leptin deficiency, obesity, dyslipidaemia and hyperinsulinaemia abrogates D-pinitol actions on tau phosphorylation. The 3xTg mice confirmed D-pinitol effectiveness in a genetic AD-tauopathy.

Conclusion and Implications: The present findings suggest that D-pinitol, by regulating CDK5 activity through a decrease of CDK5R1, is a potential drug for developing treatments for neurological disorders such as tauopathies.

KEYWORDS
Akt, CDK5, D-pinitol, insulin, tau phosphorylation, tauopathy

1 | INTRODUCTION

Inositols are carbohydrates of the family of polyalcohols present both in cellular fluids and as constituents of phospholipids and glycans of plasma membranes. They participate in multiple physiological functions (Best et al., 2010; Saltiel & Cuatrecasas, 1986). Inositols display some **insulin**-like activities, including stimulation of lipogenesis, **glucose transport** and glycogen synthesis (Larner et al., 1979, 2010). They can be synthesized by the body or incorporated in the diet (Saltiel & Cuatrecasas, 1986). Alterations in inositol metabolism play an important role in diseases involving insulin resistance such as diabetes mellitus and (Caputo et al., 2020). D-Pinitol (DPIN) is a naturally occurring inositol, known to act as an insulin sensitizer (Navarro et al., 2020). DPIN is present in several vegetal species, especially found in carob fruit pulp (*Ceratonia siliqua*) in large concentrations, which allows its isolation and industrial production. DPIN is a member of the methylated inositol family (cyclitols). More specifically, DPIN is the 3-*O*-methyl ether of **D-chiro-inositol** (DCI) (Bhat et al., 2009), which is the most studied inositol isomer in biological processes, along with **myo-inositol**, being the major inositol present in mammalian tissues (Figure 1a) (López-Gambero et al., 2020). This compound is soluble in water and slightly soluble in **ethanol**, and it is capable of activating the non-canonical insulin signalling pathway. This natural inositol has been proved to be a potential pharmacological tool for its positive effects in many different diseases: anticancer (Jayasooriya et al., 2015) antidiabetic (Bates et al., 2000; Gao et al., 2015), antioxidant (Sivakumar et al., 2010), hepatoprotective (Choi et al., 2009), immune-suppressor (Kim et al., 2005; Lee et al., 2007), anti-osteoporosis (Liu et al., 2012), antiageing (Hada et al., 2013) and brain insulin-resistance associated disorders, such as Alzheimer's disease (AD) (Lee et al., 2014; López-Gambero et al., 2020; Pitt et al., 2013).

Insulin resistance is described as a poor response of the insulin receptor through the **phosphatidylinositol-3-kinase (PI3K)** / **protein kinase B (Akt)** signalling pathway (Avramoglu et al., 2006; Mehdi et al., 2006). The Akt kinase, also known as protein kinase B (PKB), is a serine (S)/threonine (T)-specific protein kinase involved in the regulation of various signalling downstream pathways including metabolism, angiogenesis, growth, cell proliferation and survival (Kumar et al., 2013). Akt is phosphorylated and therefore activated by PI3K, being an important signalling molecule in the insulin signalling pathway and autophagy through the mammalian **Target of Rapamycin (mTOR)** (Figure 1b). Activation of the insulin pathway suppresses **glycogen synthase kinase-3 beta (GSK-3β)** activity via S9 phosphorylation by Akt (Cross et al., 1995). Consequently, alterations in insulin signalling cause inhibition of Akt, which leads to activation of GSK-3β and deregulation of the different GSK-3β substrates, including activation of the glycogen synthase (GS) and phosphorylation of **tau**. Under normal conditions, tau is constantly phosphorylated and dephosphorylated to regulate the assembly to the microtubule. When this

What is already known

- Brain insulin resistance is linked with neurodegenerative diseases where hyperphosphorylated tau contributes to neuronal loss.
- D-Pinitol, which acts as an insulin sensitizer, may affect the phosphorylation status of tau protein.

What this study adds

- Oral D-pinitol treatment lowered tau phosphorylation significantly, but not through the expected kinase GSK-3 regulation.
- The effect was mediated through a mechanism dependent on the reduction of the CDK5 activity.

What is the clinical significance

- D-Pinitol, by regulating CDK5, is an interesting drug for developing treatments for tauopathies.

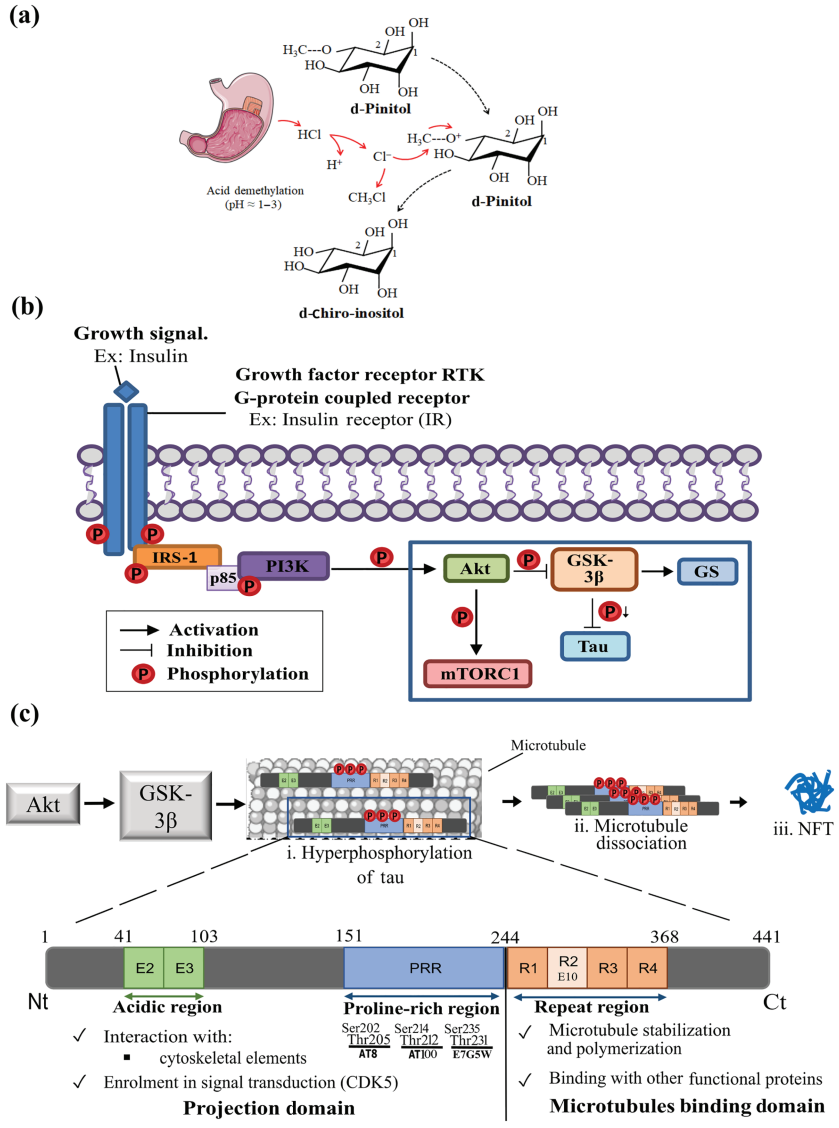

FIGURE 1 (a) Chemical structure of D-pinitol (DPIN, $C_7H_{14}O_6$) and D-chiro-inositol: its 3-*O*-methyl form obtained by acid hydrolysis in the stomach. (b) Schematic diagram depicting the signalling of the protein kinase B (Akt) pathway via insulin receptor substrate 1 (IRS-1). Physiological growth factors, such as insulin, bind to its tyrosine kinase-like receptor (RTK) in the cell membrane. An **insulin-receptor** complex is formed, and this causes a conformational change in the receptor itself. The two intracellular domains phosphorylate each other by modifying their kinase activity and, therefore, the affinity of the two domains to other substrates. The adapter IRS-1 bind now the phosphorylated receptor. The p85 regulatory domain of the Phosphatidylinositol-3-kinase (PI3K) (p85-PI3K) binds the phosphorylated domains of IRS-1, activating PI3K leading to phosphorylated Akt. Then, Akt inhibits by phosphorylation the glycogen synthase kinase-3 beta (GSK-3β). When inactive, GSK-3β is not able to phosphorylate, and this activates the enzyme glycogen synthase (GS). Also, the inhibition of the kinase GSK-3β will contribute to the reduction of phosphorylation on tau protein, decreasing the hyperphosphorylation and therefore the aggregation of tau. Akt also activates by phosphorylation the mammalian target of rapamycin (mTOR) to increase protein synthesis. Additionally, mTOR inhibits autophagy by several mechanisms.
(c) Schematic representation of tau phosphorylation due to the disfunction of the Akt-GSK-3β pathway. In physiological conditions, Akt is phosphorylated and, therefore activated. This is known to result in the inactivation of GSK-3β through its phosphorylation at S9. However, in a pathological condition, such as brain insulin resistance, Akt activates the kinase GSK-3β, which in turn, phosphorylates tau in proline-rich region. When tau protein is hyperphosphorylated (i), it dissociates from the microtubule (ii) and forms insoluble aggregates called neurofibrillary tangles (NFTs) in neurons and glial cells (iii). Both the destabilization of the microtubule and the aggregation of NFTs could lead to cell apoptosis. The domain structure of the longest isoform of human tau is shown below. It differs from other isoforms in the presence or absence of two N-terminal domains (encoded by exons E2 and E3) and one of the repeats in the microtubule binding domain (R2 encoded by exon E10). The projection domain includes an acidic and a proline-rich region, and interacts with cytoskeletal elements and neural plasma membrane. This N-terminal part is also involved in signal transduction pathways by interacting with proteins such as the cyclin-dependent kinase 5 (CDK5). The C-terminal microtubules binding domain, is where tau binds microtubules through repetitive regions, regulating the rate of microtubules polymerization, and also binding with other functional proteins such as protein phosphatase 2A (PP2A). We used the AT8 antibody that recognizes phosphorylated tau in S202 and T205 epitopes; the AT100, recognizing phosphorylated tau in S214 and T212 epitopes; and the E7G5W, recognizing phosphorylated tau in S235 and T231 epitopes.

balance is broken, tau undergoes hyperphosphorylation in the proline-rich region leading to the aggregation of Neurofibrillary Tangles (NFTs) and microtubule fragmentation (Figure 1c). Therefore, studying the deregulation of the Akt pathway is a useful tool for a better understanding of the relationship between metabolic diseases, such as Type 2 diabetes mellitus (T2D), and neurodegenerative disorders.

Recent works have confirmed that insulin is able to modulate the glucose metabolism in the brain (Fernandez et al., 2017; García-Cáceres et al., 2016). Insulin resistance not only occurs in the periphery but also in the brain, where it has recently been linked to the hyperphosphorylation of the protein tau (Boucher et al., 2014), a hallmark for AD. Several studies have indicated that AD is a specific form of diabetes in the brain. Moreover, some authors have even proposed "Type 3 diabetes" for the association of T2D and AD (Akter et al., 2011; De La Monte & Wands, 2008). Because hippocampal damage is a common feature among neurodegenerative diseases, in the present work, we aimed to evaluate the effect of inositol on brain insulin resistance through the Akt signalling pathway in the hippocampi of non-insulin resistant and T2D rats.

The Zucker fatty rats have a genetically homozygous **leptin receptor** mutation, causing **leptin** dysfunction and developing obesity as well as insulin resistance at a young age (Shiota & Printz, 2012). Thus, Zucker rats are the most widely used model for T2D and obesity, and have become an interesting model for studying the close relationship between insulin resistance in the brain and neurodegeneration. The outcome of this study assessed the potential of the inositol DPIN to promote tau dephosphorylation, evaluating insulin-dependent and insulin-Akt-independent pathways in both insulin-resistant and non-insulin-resistant animals. As a control for tauopathy models, the present study addressed DPIN effectiveness in a genetic tauopathy (3xTg mice contain three mutations linked to familial Alzheimer's disease [APP Swedish, MAPT P301L, and PSEN1 M146V] that develop tau hyperphosphorylation and aggregation).

Surprisingly, the actions of inositol were dependent on the cyclin-dependent kinase 5 (**CDK5**) activity modulation by the cyclin-dependent kinase 5 regulatory subunit 1 (CDK5R1), opening alternatives for the treatment of tauopathies. Also, evaluating DPIN effectiveness in a genetic tauopathy allows us to assert that this compound has a pharmacological profile addressing the treatment of tauopathies.

2 | METHODS

2.1 | Animals and ethics statement

Animal experimental procedures were carried out by the European Communities Council Directives 2010/63/EU, Regulation (EC) no. 86/609/ECC (24 November 1986) and Spanish National and Regional Guidelines for Animal Experimentation (Real Decreto 53/2013). Experimental protocols were approved by The Local Ethical Committee for Animal Research of the University of Malaga (CTS-8221, July 2016). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). According to the ARRIVE guidelines (Kilkenny et al., 2010), all efforts were made to minimize animal suffering and reduce the number of animals used per experimental group. Chronic gavage and drinking experiments on Zucker rats (Crl:ZUC-*Lepr*^{fa}; Charles River Laboratories, Barcelona, Spain) were performed on 24 male obese (*fa/fa*) and 24 male lean (+/?) rats for 28 consecutive days (eight animals per treated group), while chronic drinking experiments on Wistar rats (Crl:WI [Han]; Charles River Laboratories, Barcelona, Spain) were performed on 20 male rats for 10 consecutive days (10 animals per treated group). All rats, aged 2 months and weighing 250–300 g, were housed individually during the experiments.

DPIN chronic drinking experiments on mice were performed in the triple-transgenic mice, B6;129-Psen1^{tm1Mpm} Tg (APPSwe, tauP301L)1Lfa/J (named 3xTg) (Oddo et al., 2003) and their wild type (WT) controls MMRRC stock #34830 purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice (eight animals per group) aged 3 months and weighing 24–26 g at the moment of experiments were exposed during 45 consecutive days to DPIN in the water of drinking.

All animals were kept under a standard 12-h light–dark cycle in a room with temperature and humidity control, and water and rat chow pellets were provided ad libitum throughout the studies.

2.2 | Preparation and administration of DPIN

For experiments with Wistar rats, DPIN was dissolved in water for orally daily administration by drinking at a dose of 100 mg·kg^{-1} of body weight (BW) for 10 days. In the case of Zucker rats, the rats had received DPIN by both oral and gavage administration at a dose of 100 mg·kg^{-1} BW for 28 days. We decided to test these two different patterns of administration in order to confirm the persistence of DPIN effects, taking into consideration the low half-life of DPIN when given in a single oral bolus (Navarro et al., 2020). The quantity of DPIN in the drinking water for oral daily dose of 100 mg^{-1} kg^{-1} BW^{-1} was calculated taking into account the standardized daily intake of water for rats, namely 12–15 ml per day as indicated in the "The Laboratory Rat" book (Sharp & Villano, 2013). For this, a solution of 7.41 g L^{-1} was prepared and made available *ad libitum* for all DPIN-treatred groups. During the drinking treatments, the concentration of DPIN in the water was updated, considering the daily increase of BW of the rats and the daily mean drinking volume. All the control groups drank water or were administered with water depending on the case during the treatments. Finalizing the DPIN administration, the animals were anaesthetized with intraperitoneal **sodium pentobarbital** (50 mg·kg^{-1} BW) and sacrificed. Pentobarbital was administered 5 min before rats were killed immediately by decapitation. Brain samples were rapidly removed, and tissues were dissected and immediately frozen at −80°C for late analysis, the whole process taking less than 10 min.

2.3 | Plasma DPIN concentration

Plasma pinitol concentrations were monitored by the Medina Foundation (Parque Tecnológico de Las Ciencias de la Salud, Granada 18,016, Spain), using a specific liquid chromatography–mass spectrometry method. The detection of analytes and internal standards were carried out in multiple reaction monitoring mode (MRM), with electrospray positive ionization. Detection limits were 333 to 20,000 $ng{\cdot}ml^{-1}$ of DPIN. Calculations were performed using an external standard procedure (Navarro et al., 2020).

2.4 | Brain extract

Frozen brains were placed in acrylic mouse brain matrices, and 2-mm-thick slices were cut out using razor blades from the brain matrix. Using the Paxinos and Watson's mouse brain atlas (Paxinos & Franklin, 2012), the hippocampus was dissected out bilaterally. Frozen hippocampus brain samples (17 mg per sample) were homogenized in 1 ml of cold radioimmunoprecipitation assay buffer lysis buffer (RIPA); 50-mM Tris–HCl pH 7.4, 150-mM NaCl, 0.5% NaDOC, 1-mM EDTA, 1% Triton, 0.1% SDS, 1-mM Na3VO4, 1-mM NaF, supplemented with a protease (cOmplete™ Protease Inhibitor Cocktail, Roche, cat. Number: 11836145001) and a phosphatase (Phosphatase Inhibitor Cocktail Set III, Millipore, cat. Number: 524527) inhibitor cocktail. The suspension was incubated for 2 h at 4°C, followed by centrifugation at 12,000 rpm for 15 min at 4°C. The supernatant was transferred to a new clean centrifuge tube, and the Bradford colorimetric method was used to determine the concentration of the total protein. The protein extracts were diluted 1:1 in loading buffer (Ditiotreitol [DTT] 2X) and heated for 5 min at 99°C before being subjected to electrophoresis. Natural product studies are reported in compliance with the recommendations made by the *British Journal of Pharmacology* (Izzo et al., 2020).

2.5 | Western blot analysis

The tissue protein (10–15 μg) was subjected to electrophoresis on 4–12% Criterion XT Precast Bis-Tris gels (Bio-Rad, USA) for 30 min at 80 V and 2 h at 150 V. Proteins were transferred onto a 0.2-μm nitrocellulose membrane (Bio-Rad, USA) for 1 h at 80 V by wet transfer equipment (Bio-Rad, USA). The membrane was washed twice for 5 min in TBST buffer (10-mM Tris–HCl, 150-mM NaCl, 0.1% Tween 20, pH 7.6) and blocked with 2% bovine serum albumin-Tris buffered saline Tween 20 (BSA-TBST) for 1 h at room temperature on a shaker platform. Subsequently, the membrane was incubated with respective primary antibodies (see Table S1 for additional information) overnight at 4°C diluted in 2% BSA-TBST. The following day, the membrane was washed three times for 10 min with TBST. An appropriate HRP conjugated rabbit/mouse secondary antibody (Promega, USA) was diluted 1:10,000 in 2% BSA-TBST and incubated with the membrane for 1 h shaking at room temperature. Finally, the membrane was washed as above and exposed to a chemiluminescent reagent (Santa Cruz, Biotechnology Inc., USA) for 5 min. Stripping/reproving steps were used when necessary. Respective membrane bound protein was then visualized by chemiluminescence (ChemiDoc Imaging System, Bio-Rad, USA). Bands were quantified by densitometric analysis using ImageJ software (http://imagej.nih.gov/ij). Normalization was performed using a reference protein of the same membrane, the γ-adaptin. The results are presented as the ratio between total protein expression and γ-adaptin and the ratio between phosphorylated protein expression and total protein expression (Bass et al., 2017). The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*.

2.6 | Data analysis and statistics

Statistical analysis was conducted in GraphPad Prism, version 9 (GraphPad Software, Inc., USA). Shapiro–Wilk test was used to assess normal distribution of data. Levene's test was used to analyse the assumption of homogeneity of variance. All data are expressed as mean ± SEM. Statistical analysis was undertaken for studies where each group size was of, at least, $n = 5$. One- and two-way analyses of variance (ANOVAs) were assessed, followed by Turkey's post hoc multiple comparisons test. The post hoc tests were conducted only if F in ANOVA achieved a p-value less than 0.05, and there was no statistically significant variance inhomogeneity. The results were considered statistically significant at $P < 0.05$. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology.

2.7 | Materials

The inositol D-pinitol (3-O-methyl-D-chiro-inositol, Caromax®-D-pinitol, DPIN, 98% purity) was provided by Euronutra SL (https://www.euronutra.com/, Málaga, Spain). Doses were selected based on oral pharmacokinetics data from previous research work

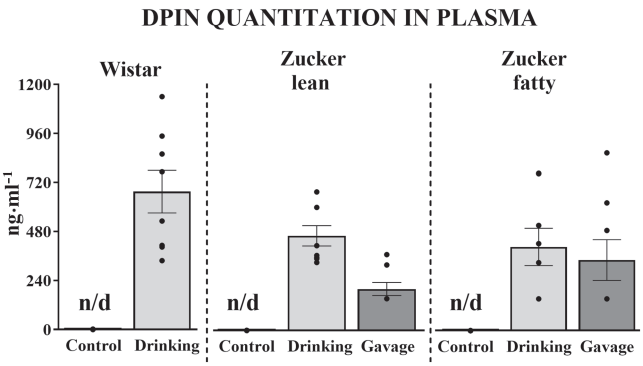

FIGURE 2 Concentration of D-Pinitol (DPIN) in plasma ($ng{\cdot}ml^{-1}$) at the point of sacrifice in both Wistar and Zucker male rats. The values are means ± standard error of the mean (SEM); eight animals per treated group (n/d: no detected).

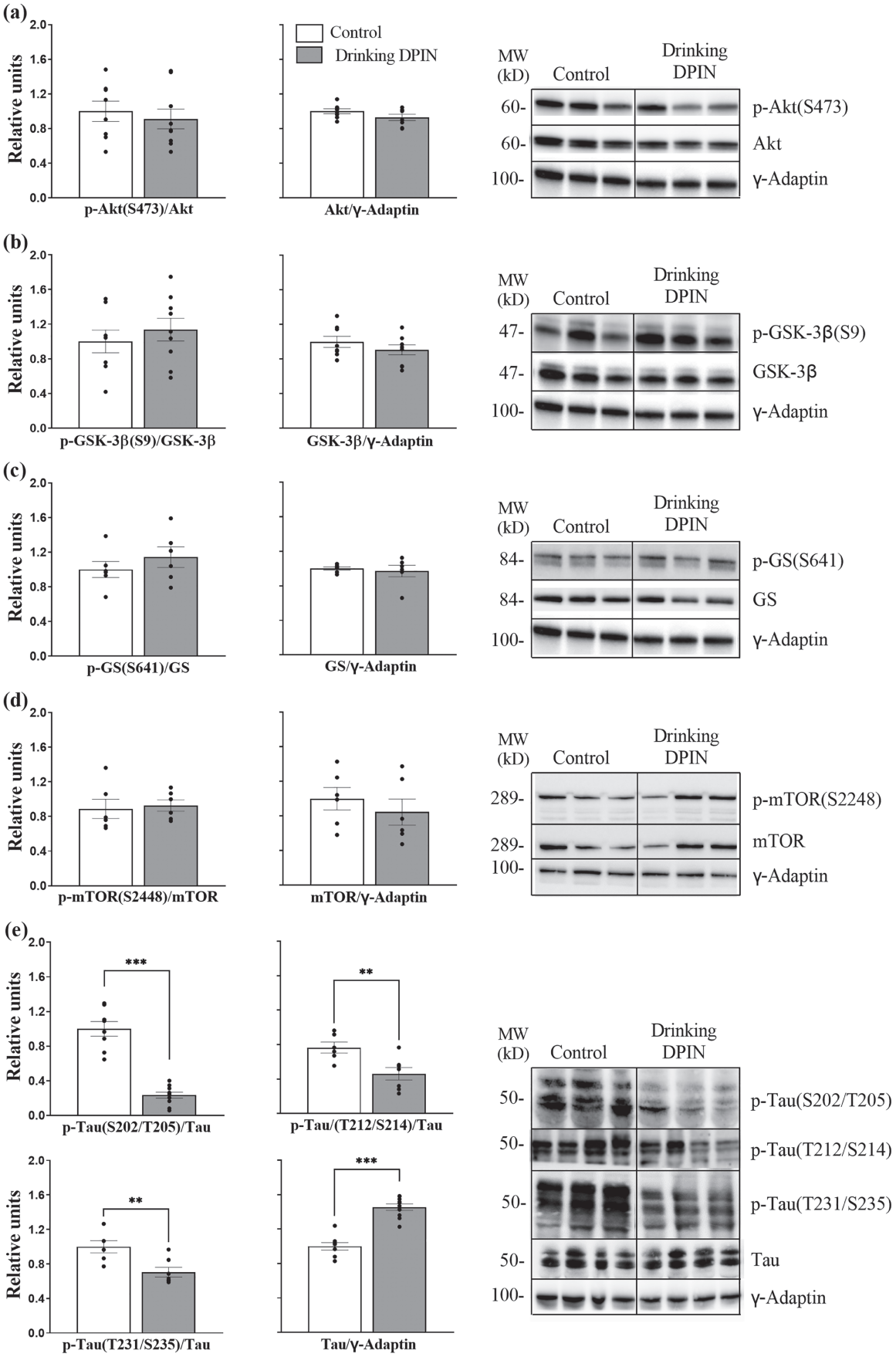

FIGURE 3 Legend on next page.

FIGURE 3 Western blot analysis of the phosphorylation status of the insulin signalling proteins from hippocampus lysates of Wistar rats treated with 100 mg·kg^{-1} of D-pinitol (DPIN). Bar charts show the effect of the DPIN administration on (a) protein kinase B phosphorylation on serine 473 (p-Akt(S476)) and quantity of total Akt; (b) glycogen synthase kinase-3 beta phosphorylation on serine 9 (p-GSK-3β(S9)) and quantity of total GSK-3β; (c) glycogen synthase phosphorylation on serine 641 (p-GS(S641)) and quantity of total GS; (d) mammalian target of rapamycin phosphorylation on serine 2448 (p-mTOR(S2448)) and quantity of total mTOR; and (e) tau [AT8] phosphorylation on serine 202 and threonine 205 (p-Tau(S202/T205)), tau [AT100] phosphorylation on threonine 212 and serine 214 (p-tau(T212/S214)), tau [E7G5W] phosphorylation on threonine 231 and serine 235 (p-Tau(T231/S235)), and quantity of total tau. The blots shown are a representation of all the bands (see Figure S1 for additional information). The corresponding expression of γ-Adaptin is shown as a loading control per lane. Molecular weights (MW) are indicated in kilodaltons (kD). All samples were derived at the same time and processed in parallel. Histograms represent the mean ± SEM, and they have their respective western blot membranes next to six to eight animals per treated group. One-way ANOVA and Tukey's test was performed: (*) $P < 0.05$ vs Control group.

(Navarro et al., 2020). A complete list of primary antibodies, sources, and their corresponding dilutions for protein expression analysis by western blotting are listed in Supplementary Data (Table S1).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

3.1 | Plasma DPIN concentration

First, to confirm that repeated administration of DPIN resulted in active plasma levels, we measured plasma concentrations of DPIN in both Wistar and Zucker rats. As expected, plasma DPIN concentrations were not detected in all Control groups (Figure 2). The levels of DPIN were similar in Drinking groups while they only showed a slightly decreased concentration in Gavage group of Zucker rats. No differences among the administration by Drinking and Gavage was found in the Zucker fatty group. In summary, all experimental groups had a detectable level of DPIN in plasma.

3.2 | Effect of chronic oral administration of DPIN on phosphorylation status of tau protein in the hippocampus of Wistar rats

In order to evaluate the impact of sub chronic administration of DPIN on Akt-dependent signalling, we first treated Wistar rats orally with DPIN in drinking water for 10 days. We aimed to analyse several key proteins of the insulin mediating signalling in the hippocampus. Figure 3a displays the western blot analysis of the phosphorylation state (p-) p-Akt and the levels of total Akt protein, showing that DPIN administration had affected neither its activation by phosphorylation (S473) nor its total expression. Subsequently, we analysed the phosphorylation state and the levels of total GSK-3β, GS, and mTOR as Akt substrates, where both the total level and the phosphorylation state remained unchanged (Figure 3b–d). Surprisingly, we found that the chronic administration of DPIN had a significant effect on tau protein dephosphorylation. The quantity of phospho-tau (p-tau) measured at six different phosphorylation sites (S202/T205, T212/S214, and T231/S235) after compound administration had dropped significantly compared to the Control group, while the levels of total tau protein had significantly increased (Figure 3e). Therefore, while tau was dephosphorylated after the chronic administration of DPIN in a non-diabetic rat model, GSK-3β showed no changes in terms of activation (Figure 3b) or inhibition (Figure S1a).

3.3 | Chronic oral DPIN also reduced tau phosphorylation on Zucker lean rats

We next evaluated the effect of DPIN in an obese model animal of Zucker fatty rats and their lean controls. Figure 4a displays the western blot analysis of the phosphorylation state and the levels of total Akt in the lean controls, showing no differences between groups. While the total amount of GSK-3β was significantly increased after DPIN administration (Figure 4b), no net effect was seen in the Akt and its other substrates, including GS and mTOR (Figure 4c,d). However, tau phosphorylation decreased significantly after DPIN administration both with the drinking and the gavage treatment (Figure 4e). Therefore, as we first saw in the rat Wistar model, the chronic administration of DPIN in Zucker lean rats outcomes both a significant reduction in tau phosphorylation and an increase in the amount of total tau protein, with no changes in GSK-3β activation (Figure 4b) or inhibition (Figure S1b).

3.4 | Obesity-associated with a genetic deficit of leptin signalling precludes DPIN induced tau dephosphorylation in the hippocampus

The profile of the insulin-Akt signalling pathway in the Zucker fatty rats (Figure 5) was different compared with their lean controls (Figure 4). DPIN administration in drinking water resulted in an activation of Akt since the phosphorylation at S473 was significantly increased (Figure 5a). According to the elevated activity of Akt, the

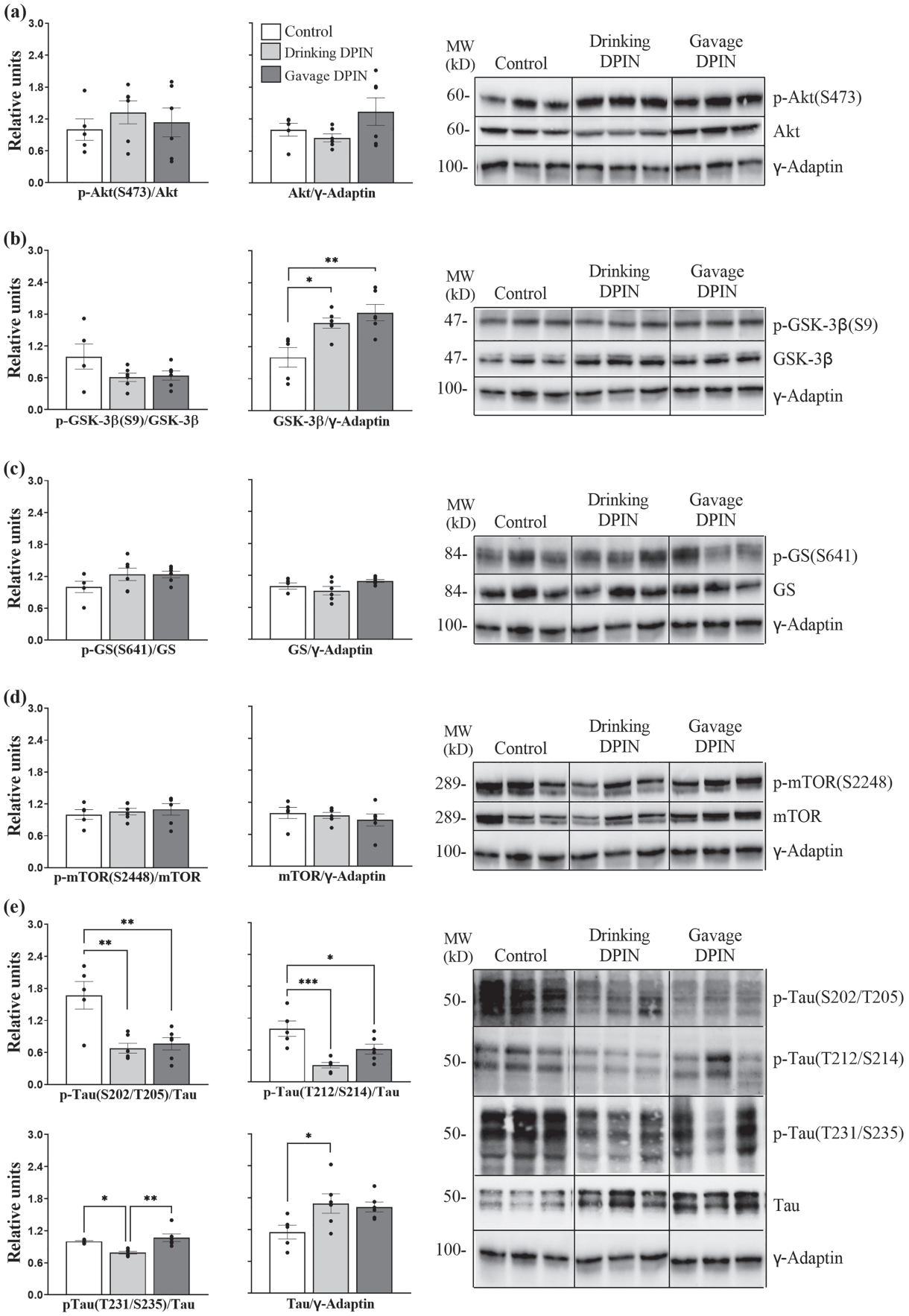

FIGURE 4 Legend on next page.

FIGURE 4 Western blot analysis of the phosphorylation status of the insulin signalling proteins from hippocampus lysates of Zucker lean rats treated with 100 mg·kg^{-1} of D-pinitol (DPIN). Bar charts show the effect of the DPIN administration on (a) protein kinase B phosphorylation on serine 473 (p-Akt(S476)) and quantity of total Akt; (b) glycogen synthase kinase-3 beta phosphorylation on serine 9 (p-GSK-3β(S9)) and quantity of total GSK-3β; (c) glycogen synthase phosphorylation on serine 641 (p-GS(S641)) and quantity of total GS; (d) mammalian target of rapamycin phosphorylation on serine 2448 (p-mTOR(S2448)) and quantity of total mTOR; and (e) tau [AT8] phosphorylation on serine 202 and threonine 205 (p-tau(S202/T205)), tau [AT100] phosphorylation on threonine 212 and serine 214 (p-tau(T212/S214)), tau [E7G5W] phosphorylation on threonine 231 and serine 235 (p-Tau(T231/S235)) and quantity of total tau. The blots shown are a representation of all the bands. The corresponding expression of γ-adaptin is shown as a loading control per lane. Molecular weights (MW) are indicated in kilodaltons (kD). All samples were derived at the same time and processed in parallel. Histograms represent the mean ± SEM, and they have their respective Western blot membranes next to five to six animals per treated group. One-way ANOVA and Tukey's test was performed: (*) $P < 0.05$ vs Control or Drinking DPIN group.

phosphorylation of its substrate GSK-3β (p-GSK3-β) at S9 was also increased after DPIN intake (Figure 5b). Thus, DPIN resulted in a clear inhibition of the GSK-3β kinase activity, since the balance between activating phosphorylation at tyrosine residues was decreased when compared with the inhibitory phosphorylation at serine residues (Figure S1c). When active, GSK-3β phosphorylates and inhibits the enzyme GS. However, even with the inhibition of GSK-3β, there was an increase in GS phosphorylation at S641 inactivating it (Figure 5c). Although not significant, the phosphorylation status (S2448) of mTOR was also slightly increased with DPIN intake (Figure 5d) and therefore activation of this downstream protein. Despite the activation of the Akt cascade proteins after DPIN administration, tau protein phosphorylation status and total level remained unchanged, suggesting that thehyperinsulinaemic/hypertrygliceridaem state derived from leptin signalling deficiency prevents the DPIN from reducing tau phosphorylation (Figure 5e).

3.5 | Cyclin-dependent kinase 5 activator 1-mediated inhibition after DPIN administration as a putative mechanism for tau dephosphorylation

The inhibition of GSK-3β kinase activity is translated into a decrease of tau phosphorylation, as being GSK-3β one of the main tau kinases. Therefore, while DPIN intake did not affect the insulin signalling pathway in the hippocampus, tau protein was highly dephosphorylated. Thus, kinase activation and total expression of Mitogen-activated protein kinase (MAPK)/ERK1/2, AMP-activated protein kinase (AMPK), protein kinase A (PKA), and CDK5 were evaluated to analyse whether those kinases were involved in the above tau dephosphorylation results (see Table S2 for additional information).

As shown in Figure 6a,b, Wistar and Zucker lean rats presented a significant increase in the total amount of PKA with drinking DPIN intake and a decrease in the p25 and p35 subunits of the CDK5R1/CDK5 kinase ratios, suggesting less activation of CDK5. No other changes were observed among the other kinases. On the other hand, Zucker fatty rats (Figure 6c) had a completely different scenario, showing a significant increase in the phosphorylation of **AMPK**, **PKA**, and the total amount of the subunit p25 CDK5R1/CDK5 kinase ratio after DPIN administration. These results were suggestive of a reduction in the activity of the CDK5 kinase in Wistar and Zucker lean rats, leading to less tau phosphorylation. On the contrary, the leptin signalling deficiency in the fatty diabetic rats resulted in activation of the kinases after DPIN administration.

3.6 | An increase in phosphatases activity may be related to tau dephosphorylation in Zucker lean rats

After tau dephosphorylation results on Wistar rats and Zucker lean, we aimed to study whether the action of phosphatases was involved. To this aim, we evaluated the total amount of protein of the serine/threonine protein phosphatase proteins 2A (PP2A) and serine/threonine protein phosphatase proteins 2C (PP2C) and **tensin homolog deleted on chromosome 10 (PTEN).** While there were no changes in the three phosphatases expression (PTEN, PP2A, and PP2C) after DPIN administration in the Wistar rats (Figure 7a), the levels were significantly increased in the Zucker lean rats after DPIN administration by gavage (Figure 7b). On the other hand, in Zucker fatty rats, PTEN and PP2C (Figure 7c) showed a significant decrease in the total amount of protein. Overall, these results suggest a clear activation of phosphatases activity in Zucker lean rats after DPIN administration, an interesting finding when taking into consideration the hippocampal tau dephosphorylation described above.

3.7 | Translational potential of tau dephosphorylation after DPIN administration in the tauopathy 3xTg mice model

Last, we aimed to assess the translational potential of tau dephosphorylation after DPIN administration in an established tauopathy model animal model. To this end, we used the tauopathy mice model 3xTg and a WT group. Interestingly, as we saw previously in the rat models, the phosphorylation site S202/T205 of the AT8 antibody suffered a significant decrease after DPIN was administrated both in the WT and the 3xTg mice (Figure 8a). The AT100 antibody, which recognizes p-tau(T212/S214), shows that the protein was significantly dephosphorylated after DPIN in the 3xTg (Figure 8b), and p-tau (T232/S235) was dephosphorylated in the WT (Figure 8c). The total amount of tau protein was also analysed showing non-variance (Figure 8d).

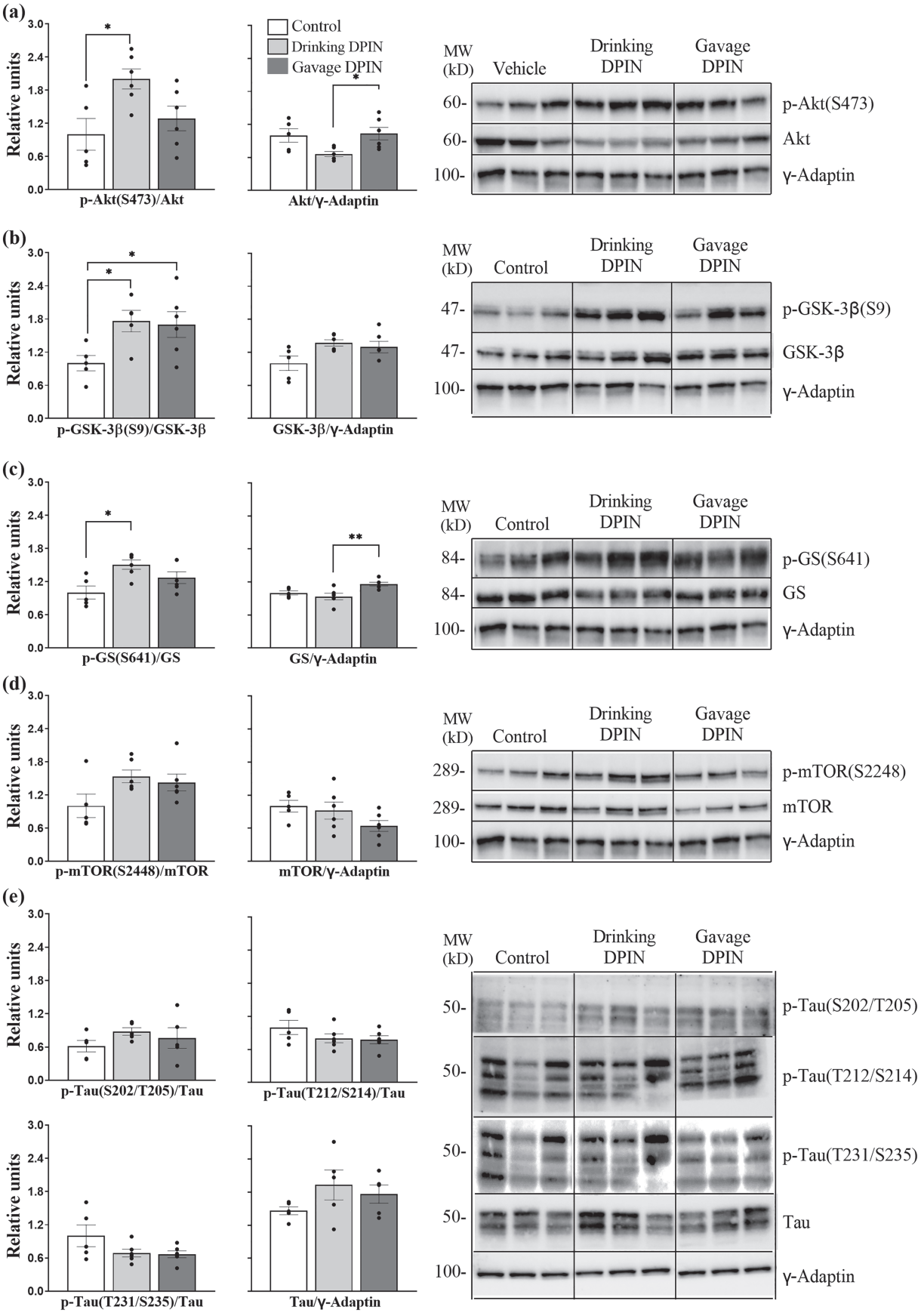

FIGURE 5 Legend on next page.

FIGURE 5 Western blot analysis of the phosphorylation status of the insulin signalling proteins from hippocampus lysates of Zucker fatty rats treated with 100 mg·kg^{-1} of D-pinitol (DPIN). Bar charts show the effect of the DPIN administration on (a) protein kinase B phosphorylation on serine 473 (p-Akt(S476)) and quantity of total Akt; (b) glycogen synthase kinase-3 beta phosphorylation on serine 9 (p-GSK-3β(S9)) and quantity of total GSK-3β; (c) glycogen synthase phosphorylation on serine 641 (p-GS(S641)) and quantity of total GS; (d) mammalian target of rapamycin phosphorylation on serine 2448 (p-mTOR(S2448)) and quantity of total mTOR; and (e) tau [AT8] phosphorylation on serine 202 and threonine 205 (p-tau(S202/T205)), tau [AT100] phosphorylation on threonine 212 and serine 214 (p-tau(T212/S214)), tau [E7G5W] phosphorylation on threonine 231 and serine 235 (p-tau(T231/S235)), and quantity of total tau. The blots shown are a representation of all the bands. The corresponding expression of γ-adaptin is shown as a loading control per lane. Molecular weights (MW) are indicated in kilodaltons (kD). All samples were derived at the same time and processed in parallel. Histograms represent the mean ± SEM, and they have their respective western blot membranes next to five to six animals per treated group. One-way ANOVA and Tukey's test was performed: (*) $P < 0.05$ vs Control or Drinking DPIN group.

4 | DISCUSSION

In this study, we have evaluated the effect of the inositol DPIN on the phosphorylation of tau as a common downstream protein of the insulin signalling Akt-pathway. Deposition of hyperphosphorylated tau is a hallmark of AD and a wider group of neurodegenerative disorders called tauopathies. Apparently, deposition of tau is essential for neurodegeneration in AD, since the absence of tau hyperphosphorylation is sufficient to prevent dementia in familial AD, even when amyloid deposition is widely found in post-mortem brain, as confirmed in a particular lineage of Colombian families (Arboleda-Velasquez et al., 2019). Currently, there is no specific treatment to prevent phosphorylation of tau and its aggregation. Inhibitors of kinases involved in tau phosphorylation, such as GSK-3β or CDK5, have been developed but have failed in Phase II clinical trials since they did not prevent cognitive deterioration nor improved cognition (clinicaltrials.gov: NCT01049399; 12mo 146 subjects, and NCT01110720; 18mo; 313 subjects).

The reduction in the proportion between CDK5 and CDK5R1 (either the membrane-attached p35 or the **calpain**-cleaved p25 isoform) suggests that the regulation of CDK5 expression is affected by DPIN. As expected, since both p35 and p25 are equally affected, neither calpain nor its regulatory protein calpastatin was affected by the treatment (Figure S2). Several microRNAs (Moncini et al., 2017; Sun et al., 2015) and long non-coding RNAs (Spreafico et al., 2018), some of them activated through the nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway, have been identified as regulatory elements controlling the mRNA expression of CDK5R1. Further research will be addressed to identify if this pathway is responsible for the observed reduction in CDK5/CDK5R1-mediated phosphorylation of tau.

Regarding tau protein, there is increasing data showing that insulin regulates tau phosphorylation and heightens the neurofibrillary tangle load (Hong & Lee, 1997; Smet et al., 2005; Theunis et al., 2013). In vivo, mice deficient in insulin receptors in neurons shows an inhibition of PI3K/Akt signalling pathway and an increase in phosphorylation of tau (Schubert et al., 2004). Moreover, obesity-induced peripheral insulin resistance is associated with central insulin resistance that is linked to tau hyperphosphorylation (Špolcová et al., 2014). The pathological effect of insulin resistance on tau mostly has been attributable to the modulation of several downstream pathways, involving tau kinases and phosphatases. Among the diverse modifications of tau, phosphorylation plays a crucial role in regulating the physiological functions of tau. Although dozens of kinases have been described to phosphorylate tau protein (Cavallini et al., 2013), the most studied tau kinase is GSK-3β. This kinase also is closely related to the insulin signalling cascade since it is regulated by Akt. Therefore, alterations in insulin signalling cause inhibition of Akt, which leads to activation of GSK-3β and consequently to greater phosphorylation of tau (Figure 1b). However, our results revealed a clear reduction in tau phosphorylation after inositol intake, in both Wistar and Zucker lean rats, without changes in Akt and its downstream targets including GSK-3β. This finding excludes insulin-sensitizing effects of inositol in the hippocampus as a mechanism for tau dephosphorylation. These data also point to a regional specificity of the actions of DPIN on both tau phosphorylation and brain insulin signalling. Thus, we did not detect changes in the hypothalamus nor in the temporal cortex of the Wistar rats treated with DPIN for 10 days (Figure S3), whereas we did observe a clear activation of PI3K/Akt signalling pathway in the hypothalamus after DPIN administration (Medina-Vera et al., 2021).

Tau protein is a good substrate for protein kinases due to its high of number serine and threonine residues (Arendt et al., 2016) (see Table S2). Most of the kinases related to phosphorylation of tau are part of the proline-directed protein kinase (PDKB) family, which include MAPK (Drewes et al., 1992; Reynolds et al., 1997), GSK-3β (Hanger et al., 1992), AMPK (Domise et al., 2016), CDK5 (Kimura et al., 2014) and PKA (Jicha et al., 1999). We have investigated tau phosphorylation sites with three different antibodies, specifically pSer202/pThr205 (AT8), pThr212/pS214 (AT100) and pThr231/pSer235 (E7G5W). We have decided to investigate these antibodies to search for changes in other kinases, apart from GSK-3β, known to modulate tau phosphorylation in those epitopes, such as PKA, ERK1/2 and CDK5 (Kimura et al., 2014; Stoothoff & Johnson, 2005). Considering that CDK5 is the kinase that is modified by the treatment, we test the phosphorylation levels of some of the specific tau sites that are targeted by CDK5 but not for other kinases. More precisely, pSer235 is a specific phosphorylation site for CDK5 and not for GSK-3β. We added in the analysis the functional status of AMPK as a negative control since this kinase does not phosphorylate tau at S202 or T205 epitopes, but it is regulated through insulin signalling (Domise et al., 2016). Our results showed that in both Wistar rats and Zucker

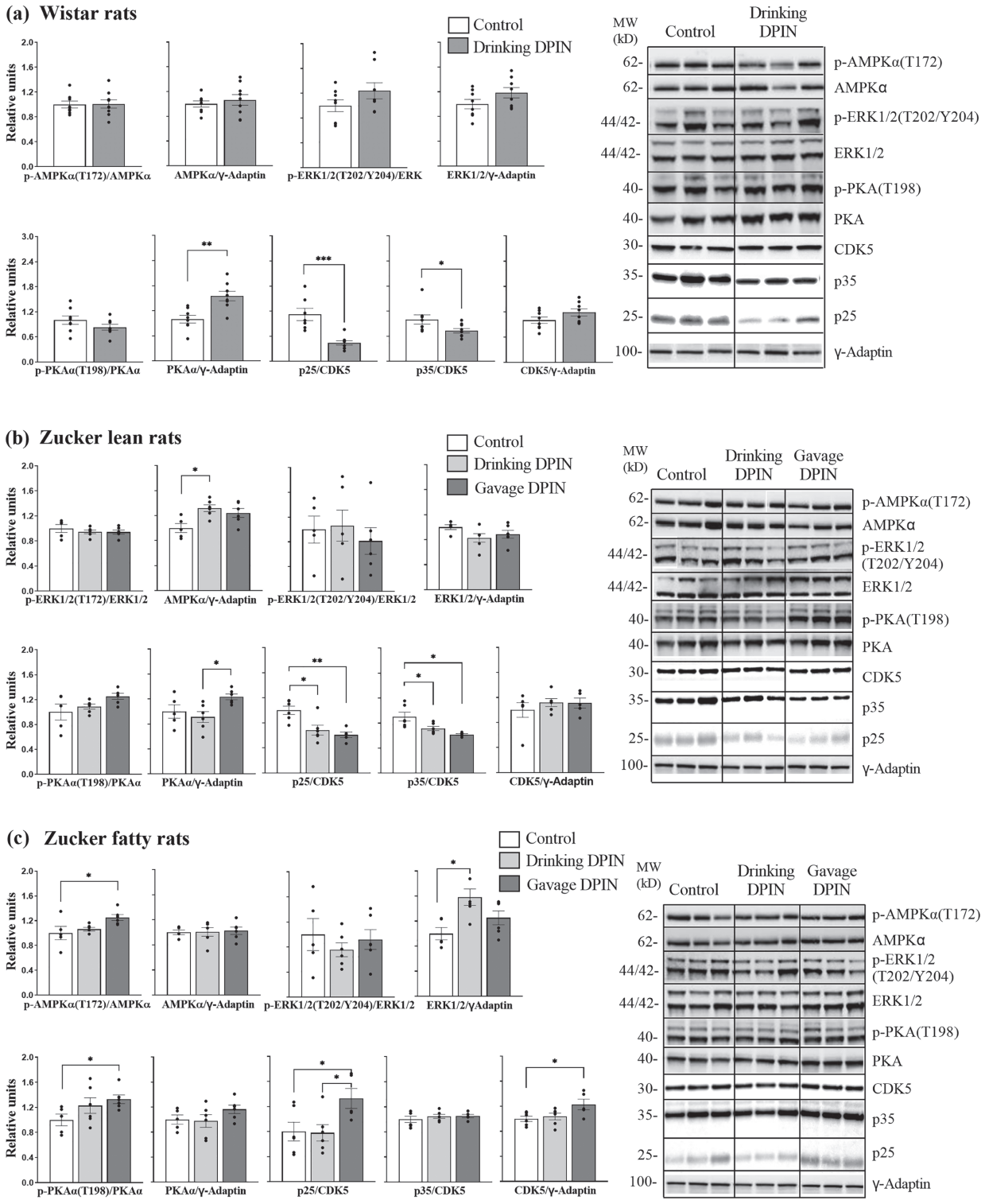

FIGURE 6 Western blot analysis of tau kinases from hippocampus lysates in the hippocampus of Wistar and Zucker rats treated with 100 mg·kg^{-1} of D-pinitol (DPIN). Bar charts show the effect of the DPIN administration on AMP-activated protein kinase alpha phosphorylated on threonine 172 (p-AMPKα(T172)) and total AMPK, mitogen-activated protein kinase phosphorylated on threonine 202 and tyrosine 204 (p-ERK1/2(T202/Y204)) and total (ERK1/2), protein kinase A phosphorylated on tyrosine 198 (p-PKA(T198)) and total PKA, cyclin-dependent kinase 5 activator subunit 1 (CDK5R1)/cyclin-dependent kinase 5 (CDK5) subunits p25 and p35, and the total quantity of CDK5 on (a) Wistar rats; (b) Zucker lean rats; and (c) Zucker fatty rats. The blots shown are a representation of all the bands. The corresponding expression of γ-adaptin is shown as a loading control per lane. Molecular weights (MW) are indicated in kilodaltons (kD). All samples were derived at the same time and processed in parallel. Histograms represent the mean ± SEM, and they have their respective Western blot membranes next to eight animals per treated group in Wistar rats (a), five to six animals per treated group in Zucker rats (b, c). One-way ANOVA and Tukey's test was performed: (*) $P < 0.05$ vs Control or Drinking DPIN group.

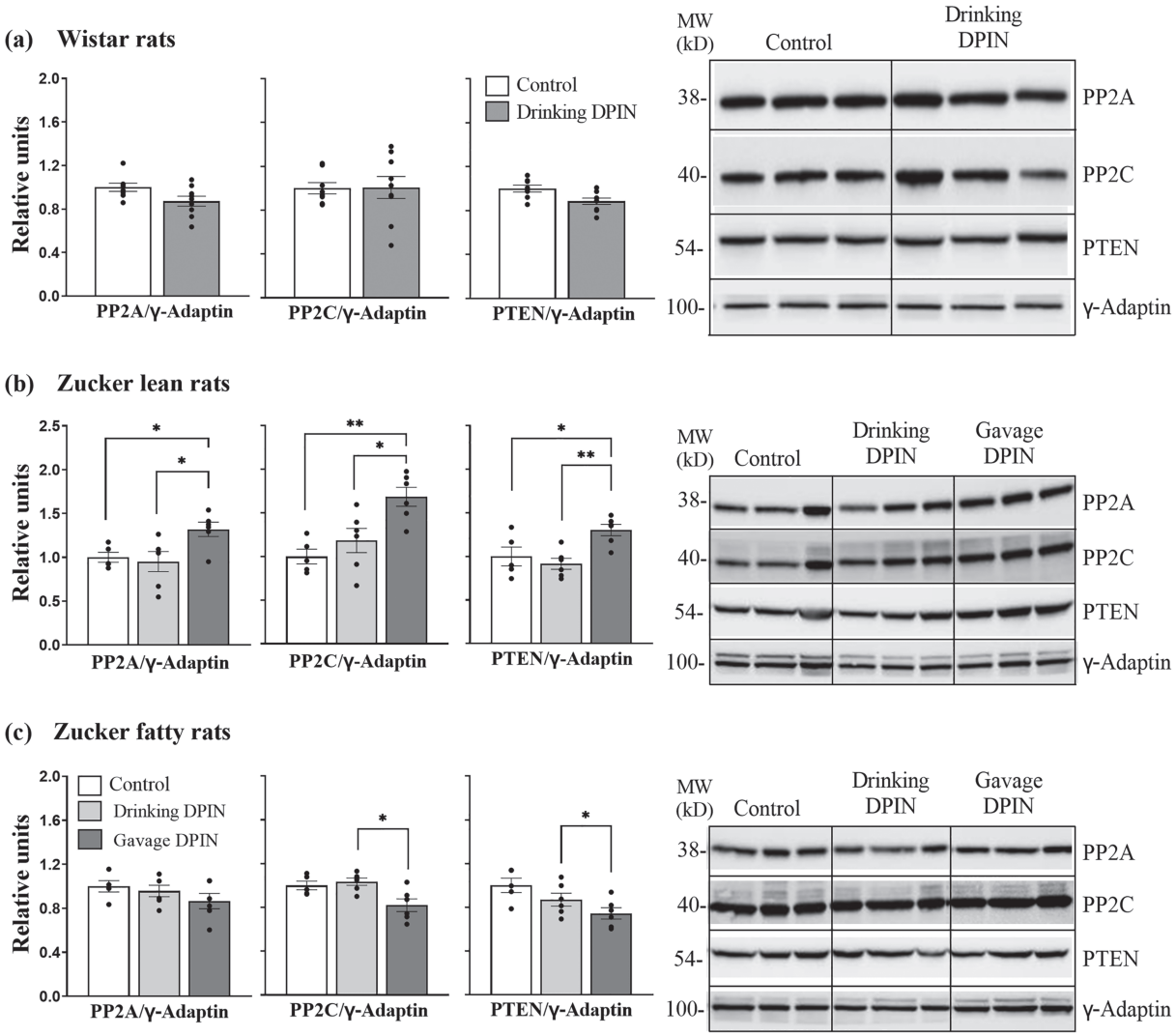

FIGURE 7 Western blot analysis of tau phosphatases from in the hippocampus of Wistar and Zucker rats treated with 100 mg·kg^{-1} of D-pinitol (DPIN). Bar charts show the effect of the DPIN administration on serine/threonine phosphatase proteins 2A (PP2A) and 2C (PP2C) and phosphatase and tensin homologue (PTEN) on (a) Wistar rats, (b) Zucker lean rats, and (c) Zucker fatty rats. The blots shown are a representation of all the bands. The corresponding expression of γ-adaptin is shown as a loading control per lane. Molecular weights (MW) are indicated in kilodaltons (kD). All samples were derived at the same time and processed in parallel. Histograms represent the mean ± SEM, and they have their respective western blot membranes next to eight animals per treated group in Wistar rats (a) and five to six animals per treated group in Zucker rats (b, c). One-way ANOVA and Tukey's test was performed: (*) $P < 0.05$ vs Control or Drinking DPIN group.

lean rats, neither PKA, ERK1, nor AMPK phosphorylation was affected by DPIN. Only drinking DPIN produced a slight increment of total AMPK. However, we did detect changes in CDK5 activity after DPIN treatment, and it was due to alterations in the subunits p25 and p35 of the CDK5R1. CDK5, a small kinase necessary for the correct development of the mammalian central nervous system, is involved in the phosphorylation of tau protein (Kimura et al., 2014). Association of CDK5 with CDK5R1 (p35), its regulatory protein, is required for kinase activation. The accumulation of the truncated fragment of p35 (p25, which is formed by the activity of the protease calpain) occurs in the brains of patients with AD, causing CDK5 deregulation and permanent activation (Patrick et al., 1999). As (Dhavan & Tsai, 2001) mentioned, the p25/CDK5 kinase efficiently phosphorylates tau promoting aggregation. As result of DPIN intake, we found a downregulation of p25/CDK5, which leads to dephosphorylation of tau. Therefore, inositols and, more precisely, DPIN could be used as potential drug candidates to inhibit p25 accumulation and thus permanent CDK5 activation, constituting a potential drug for the treatment of neurological disorders, such as tauopathies.

On the other hand, the scenario in Zucker fatty rats was different. Zucker fatty rats had no changes in the status of tau phosphorylation after DPIN consumption. Interestingly, Zucker fatty rats are leptin-signalling deficient animals that display hyperinsulinaemia, hypertriglyceridemia, and hypercholesterolaemia. All these factors might induce adaptive changes in the brain that precludes the reduction of tau phosphorylation by inositols. The lack of effects on tau

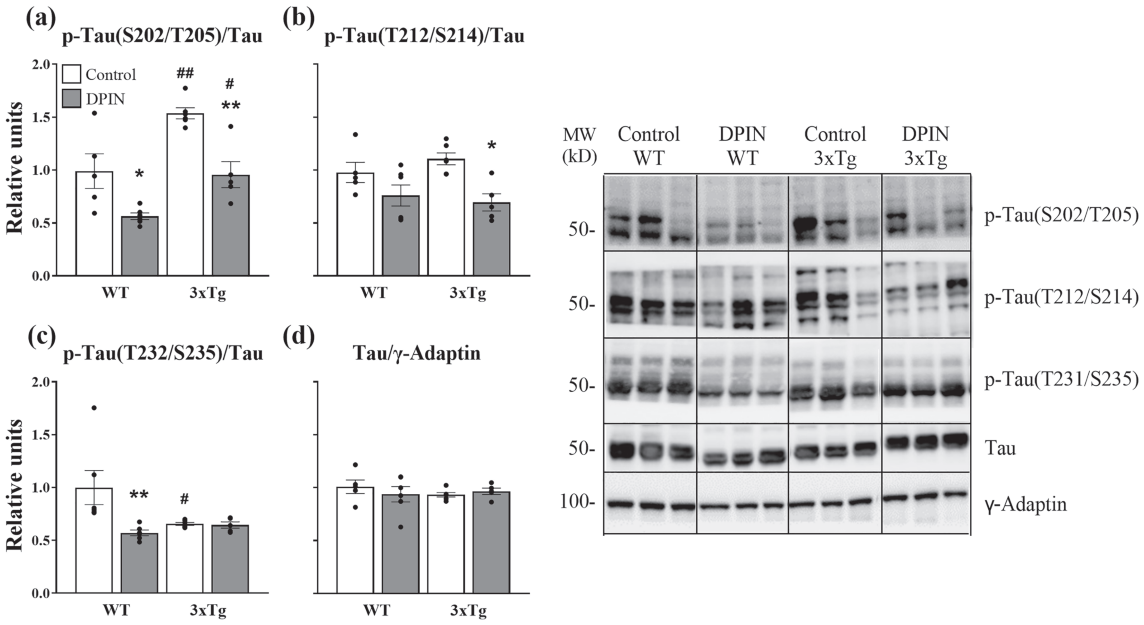

FIGURE 8 Western blot analysis of the phosphorylation status of tau proteins from hippocampus lysates of 3xTg mice treated with 200 mg·kg^{-1} of D-pinitol (DPIN). Bar charts show the effect of the administration on (a) tau [AT8]: phosphorylation on serine 202 and threonine 205 (p-tau(S202,T205)); (b) tau [AT100]: phosphorylation on threonine 212 and serine 214 (p-tau(T212,T214)); (c) tau [E7G5W]: phosphorylation on threonine 232 and serine 235 (p-tau(T232,T235)); and (d) total tau (tau). The corresponding expression of γ-adaptin is shown as a loading control per lane. Molecular weights (MW) are indicated in kilodaltons (kD). All samples were derived at the same time and processed in parallel. Histograms represent the mean ± SEM, and they have their respective western blot membranes next to five to six animals per treated group. Two-way ANOVA and Bonferroni's test was performed:(*) $P < 0.05$ significant differences compared with control group; (#) $P < 0.05$ significant differences compared with WT group.

phosphorylation was associated with opposite changes in CDK5R1/CDK5 when compared with Zucker lean rats. Thus, inositol intake did not reduce p25 but actually increased it. In addition to p25/CDK5, PKA and AMPK kinases were activated by daily gavage administration of DPIN. Thus, the metabolic imbalance present in Zucker fatty rats is sufficient to reverse the actions of DPIN. It is known that leptin signalling reduces tau phosphorylation through different canonical signalling pathways (i.e., through Akt or ERK regulation), thus helping to reduce its aggregation in the hippocampus. In addition, CDK5, through p35 activation, can regulate leptin signalling by targeting the **Signal transducer and activator of transcription 3 (STAT3)**, a key protein in leptin signalling through the **Janus kinase 2 (JAK2)**/STAT3 pathway. Zucker fatty rats have a structural deficit in leptin receptors, so it cannot be activated by leptin. Thus, the potential beneficial effect of leptin over tau phosphorylation is disrupted. In our model, we did not find a reduction of CDK5 dependent phosphorylation, nor a decrease in the CDK5/p35-p25 pathway. We can speculate that the lack of effects of DPIN or the upregulation observed in Zucker fatty on CDK5/p25 is derived from the existence of a compensatory mechanism counteracting the lack of leptin-receptor activation of the JAK2/STAT3 pathway, thus avoiding the beneficial effect on tau phosphorylation derived of the reduction of CDK5 activity. However, under the absence of confirmatory data, this is just a speculative explanation. Further research is needed to unveil mechanisms involved in this particular response of Zucker fatty animals, which might give clues to identify the mechanisms of DPIN action.

In order to have a full view of the actions of DPIN, we also analysed the functional status of three relevant phosphatases regulating the phosphorylation of tau. The balance between kinases and phosphatases may be the key to tau aggregation. PP2A and PP2C and PTEN (Martin et al., 2013) were the phosphatases selected in this study (Stoothoff & Johnson, 2005). Data were different in the three models analysed (Figure 6). While the expression of any phosphatase was altered in Wistar rats, the expression of the three phosphatases was enhanced after gavage administration of DPIN. Strain differences and/or length of inositol administration (10 days in Wistar versus 28 days in Zucker rats) and form of administration (drinking versus gavage) might account for the differences observed. In the case of the Zucker fatty rats, the results are again different. Expression of PP2C and PTEN, but not PP2A, was reduced after gavage administration of DPIN. The changes in phosphatases might account for the phosphorylation status of tau only in Zucker lean and fatty rats, and only after gavage administration, a form of administration that produces a big peak of plasma DPIN (Navarro et al., 2020).

After confirming DPIN effects in tau protein, we evaluated the administration of this compound in a tauopathy model, the 3xTg mice. Because we performed in the rats, six different tau phosphorylation epitopes were analysed, confirming that there is a translation of the

results in this mice model. The 3xTg mice allow us to confirm DPIN effectiveness in a genetic AD-tauopathy and propose it as a preventive compound. Collectively, the present phosphatase data suggest that the CDK5R1/CDK5 alterations are the key element on the reduction phosphorylation of tau produced by inositol, suggesting a potential utility in tauopathies. However, there are important limitations on this subject. There are multiple threonine, serine and tyrosine residues that might be phosphorylated and contribute to tau aggregation. We need to further explore the effects of DPIN on these alternative epitopes. Thus, although the possibility of using inositols as nutritional components for reducing tau hyperphosphorylation is highly promising, further research is needed to evaluate extensively the effectiveness and molecular mechanisms of DPIN in tauopathies and specially in AD.

5 | CONCLUSIONS

Administration of inositol DPIN is capable of reducing phosphorylation of tau in both normal rats and mice and in a genetic model of Alzheimer's disease presenting tau hyperphosphorylation and aggregation. CDK5 inhibition through a marked reduction of both CDK5R1 activator proteins forms (p35 and p25) induced by DPIN administration is proposed as a putative explanation mechanism for the hippocampal tau dephosphorylation described above. Leptin signalling deficiency leading to obesity, dyslipidaemia, and hyperinsulinaemia abrogate DPIN actions on tau phosphorylation. DPIN actions were specific since they did not affect other tau-regulatory proteins, providing a unique pharmacological profile for this natural inositol. Further research is needed to fully address the effectiveness of DPIN treatment on tauopathies, including AD.

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CONFLICT OF INTEREST

Carlos Sanjuan declares he receives salary and has shares in Euronutra Company. The remaining authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

F.R.d.F, J.D. and E.B. were responsible for the conceptualization and data curation. D.M.-V., J.A.N. and J.D. were responsible for the formal analysis. J.D., C.S., P.R. and F.R.d.F. were responsible for the funding acquisition. D.M.-V., J.A.N., J.D., A.J.L.-G., R.T. and E.B. were responsible for the methodology. J.D., J.S., F.R.d.F. and A.G.-A. were responsible for the project administration. F.R.d.F., C.R.-V. and J.D. were responsible for the validation. F.R.d.F., F.J.P., and E.B. were responsible for the supervision. D.M.-V., J.D. and F.R.d.F. were responsible for writing and editing the manuscript. F.J.P., P.R., C.R.-V., J.S., E.B. and F.R.d.F. review the final version of the manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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