



Hypothermia-induced hyperphosphorylation: a new model to study tau kinase inhibitors

SUBJECT AREAS:
TARGET IDENTIFICATION
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Alexis Bretteville^{1,2}, François Marcouiller^{1,2}, Carl Julien^{1,2}, Noura B. El Khoury^{1,2}, Franck R. Petry^{1,2}, Isabelle Poitras^{1,2}, Didier Mougnot^{1,2}, Georges Lévesque^{1,2}, Sébastien S. Hébert^{1,2} & Emmanuel Planel^{1,2}

¹Université Laval, Faculté de médecine, Département de neurosciences et psychiatrie, ²Centre de Recherche du Centre Hospitalier Universitaire de Québec, Axe Neurosciences.

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Correspondence and requests for materials should be addressed to E.P. (emmanuel@planel.org)

Tau hyperphosphorylation is one hallmark of Alzheimer's disease (AD) pathology. Pharmaceutical companies have thus developed kinase inhibitors aiming to reduce tau hyperphosphorylation. One obstacle in screening for tau kinase inhibitors is the low phosphorylation levels of AD-related phospho-epitopes in normal adult mice and cultured cells. We have shown that hypothermia induces tau hyperphosphorylation *in vitro* and *in vivo*. Here, we hypothesized that hypothermia could be used to assess tau kinase inhibitors efficacy. Hypothermia applied to models of biological gradual complexity such as neuronal-like cells, *ex vivo* brain slices and adult non-transgenic mice leads to tau hyperphosphorylation at multiple AD-related phospho-epitopes. We show that Glycogen Synthase Kinase-3 inhibitors LiCl and AR-A014418, as well as roscovitine, a cyclin-dependent kinase 5 inhibitor, decrease hypothermia-induced tau hyperphosphorylation, leading to different tau phosphorylation profiles. Therefore, we propose hypothermia-induced hyperphosphorylation as a reliable, fast, convenient and inexpensive tool to screen for tau kinase inhibitors.

Alzheimer's disease is a neurological disease marked by progressive neuronal loss, as well as memory deficits¹. AD is characterized by two specific histological lesions: amyloid plaques, composed of amyloid- β peptides deposits², and neurofibrillary tangles, composed of hyperphosphorylated and aggregated protein tau^{3,4}. Tau hyperphosphorylation can induce tau aggregation *in vitro*⁵, and decrease its solubility *in vivo*⁶. Tau phosphorylation is a highly regulated process resulting from the balance between kinase and phosphatase activities⁷. During AD development, increased tau phosphorylation is thought to result from a deregulation of these activities⁸. For example, protein phosphatase 2A (PP2A) activity and expression are decreased in AD brains^{9,10}. Tau kinases such as GSK-3 β ^{11,12} and Cdk5¹³ have also been implicated. Many laboratories and pharmaceutical companies have thus focused on the development of specific inhibitors for these kinases. For instance, GSK-3 β inhibitors were shown to reduce tau pathology in a mouse model of neurodegeneration¹⁴, and are currently in clinical trials¹⁵. One of the main roadblocks for drug screening and testing is that normal adult mice and cells in culture have low basal tau phosphorylation levels at many AD-related phospho-epitopes. Previously, we demonstrated that hypothermia induced by either glucose metabolism deregulation¹⁶ or anesthesia¹⁷⁻¹⁹ leads to tau hyperphosphorylation at multiple epitopes in the mouse brain. Here, we tested the applicability of hypothermia as a model to screen for specific kinase inhibitors, based on hypothermia's capability to reliably induce tau hyperphosphorylation. As expected, hypothermic conditions led to rapid tau hyperphosphorylation at major phospho-epitopes deregulated in AD in neuroblastoma cells, metabolically active brain slices, as well as in non-transgenic mice. Treatment in various biological models with known GSK-3 or Cdk5 kinase inhibitors prevented partially or totally hypothermia-induced tau hyperphosphorylation. Based on these observations, we propose hypothermia as a reliable, easy and inexpensive model to screen for and characterize pharmacological modulators of tau phosphorylation.

Results

Anesthesia-induced tau hyperphosphorylation is partially prevented by LiCl administration *in vivo*. We have shown previously that anesthesia-induced hypothermia causes tau hyperphosphorylation *in vivo*¹⁷. In this study, we used ketamine/xylazine anesthesia to induce tau hyperphosphorylation. Adult mice were pre-treated for two



days with LiCl, a well-known GSK-3 inhibitor^{14,20}. On the third day, the mice received a final LiCl injection 75 minutes before anesthesia (Anes). Rectal temperature analysis showed that LiCl injection *per se* induced a significant drop in body temperature after 15 minutes (Figure 1A: 37.9°C Ctl+Veh vs 35.6°C Ctl+LiCl, 37.8°C and Anes+Veh vs 35.8°C Anes+LiCl) and remained constant (~36°C) until anesthesia. Body temperatures of non-treated mice remained unchanged until anesthesia (Figure 1A). Anesthesia induced a progressive and drastic drop in temperature reaching ~26°C after 60 minutes of anesthesia.

As expected, tau phosphorylation significantly increased in anesthetized animals at all phospho-epitopes analyzed (Figure 1B,

C: Ctl+Veh vs Anes+Veh, AT270:~+6x, CP13:~+12x; AT8:~+31x; Tau-1:~+2.5x, AT180:~+6x, MC-6:~+3x and PHF-1:~+4x). Treating anesthetized mice with LiCl, but not vehicle, reduced tau phosphorylation at AT270 (~-22%), AT8 (~-41%), Tau-1 (~+55%) and AT180 (~-53%) phospho-epitopes (Figure 1B, C: Anes+LiCl vs Anes+Veh). Other phospho-epitopes, such as CP13, MC-6 and PHF-1, were also decreased to a lesser extent in LiCl-treated mice but did not reach statistical significance. No significant changes in tau phosphorylation were observed between control groups (Figure 1B, C: Ctl+Veh vs Ctl+LiCl). Likewise, no significant changes were observed in total tau levels in all groups. Notably, GSK-3β serine 9 phosphorylation (pS9),

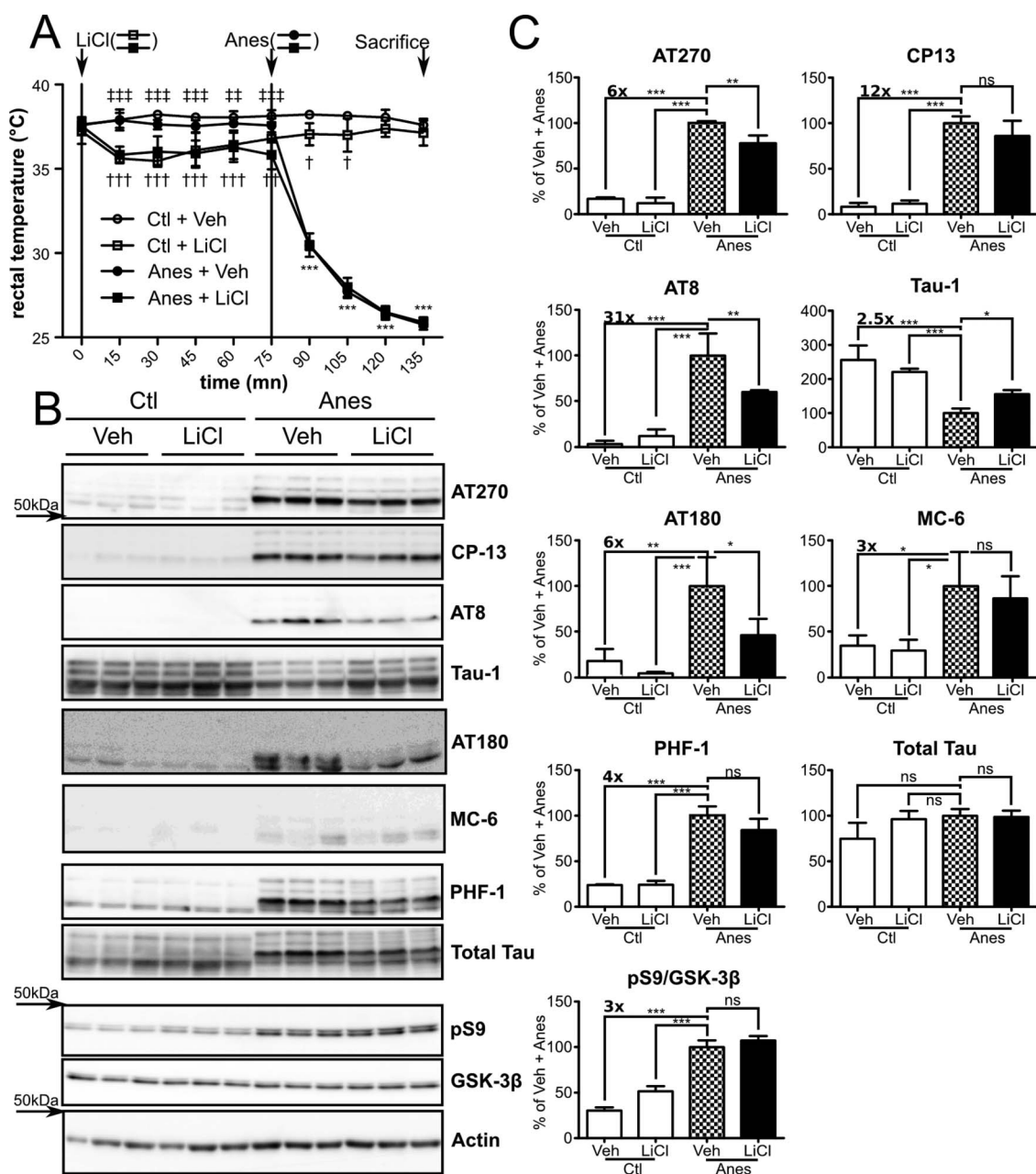


Figure 1 | Anesthesia-induced tau hyperphosphorylation is prevented by LiCl administration *in vivo*. A. Mice rectal temperature curve following LiCl and anesthetic injections. 2-way ANOVA followed by bonferonni's post test was performed. *** $p < 0.001$ Ctl+LiCl vs Anes+LiCl or Ctl+Veh vs Anes+Veh, respectively. ††† $p < 0.001$, †† $p < 0.01$, † $p < 0.05$ Ctl+Veh vs Ctl+LiCl. †††† $p < 0.001$, ††† $p < 0.01$ Anes+Veh vs Anes+LiCl. B. Immunoblots of cortical homogenates extracted protein using using several phospho-tau antibodies (AT270, AT8, CP13, Tau-1, pS262 and PHF-1). Total tau was probed using a pan tau antibody. GSK-3β inhibition was monitored by assessing both GSK-3β pS9 levels and total levels of GSK-3β. Actin probe was used as a loading control. C. Immunoblot quantifications. Ratios of phospho-epitope levels over total tau protein \pm SD are represented as a percentage of vehicle+Anes group condition (checkerboard bar). N=3 per condition.



indicating GSK-3 β inhibition, was significantly increased in the anesthetized groups compared to non-anesthetized mice (Figure 1B, C). A significant increase in GSK-3 β pS9 was observed between control groups (Figure 1B, C: Ctl+Veh vs Ctl+LiCl $p < 0.001$ Bonferroni's post hoc test) but not between anesthetized groups (Anes+Veh vs Anes+LiCl). Taken together, these results demonstrate that anesthesia-induced hypothermia leads to *in vivo* tau hyperphosphorylation that can be attenuated by LiCl administration.

Hypothermia-induced tau hyperphosphorylation is prevented by LiCl treatment in mouse brain slices. As LiCl prevents hypothermia-induced tau hyperphosphorylation *in vivo*, we

intended to transpose these results in an *ex vivo* model. To this end, we performed hypothermia experiments using mouse metabolically active brain slices²¹. After 2h under hypothermia, tau phosphorylation levels were significantly increased at all phospho-epitopes analyzed, including AT270 (~+3x), CP13 (~+5x), AT8 (~+4x), Tau-1 (~-1.5x) and PHF-1 (~+2x) (Figure 2A, B: Ctl 37°C vs Ctl 30°C). On the other hand, slices exposed to hypothermia while treated with LiCl for 2h showed reduced tau phosphorylation levels (CP13:~54%, AT8:~54% and PHF-1:~38% (Figure 2A, B Ctl 30°C vs LiCl 30°C). The same trend was observed on the AT270 and Tau-1 phospho-epitopes even though it did not reach statistical significance. AT180 and MC-6 signals were below the detection

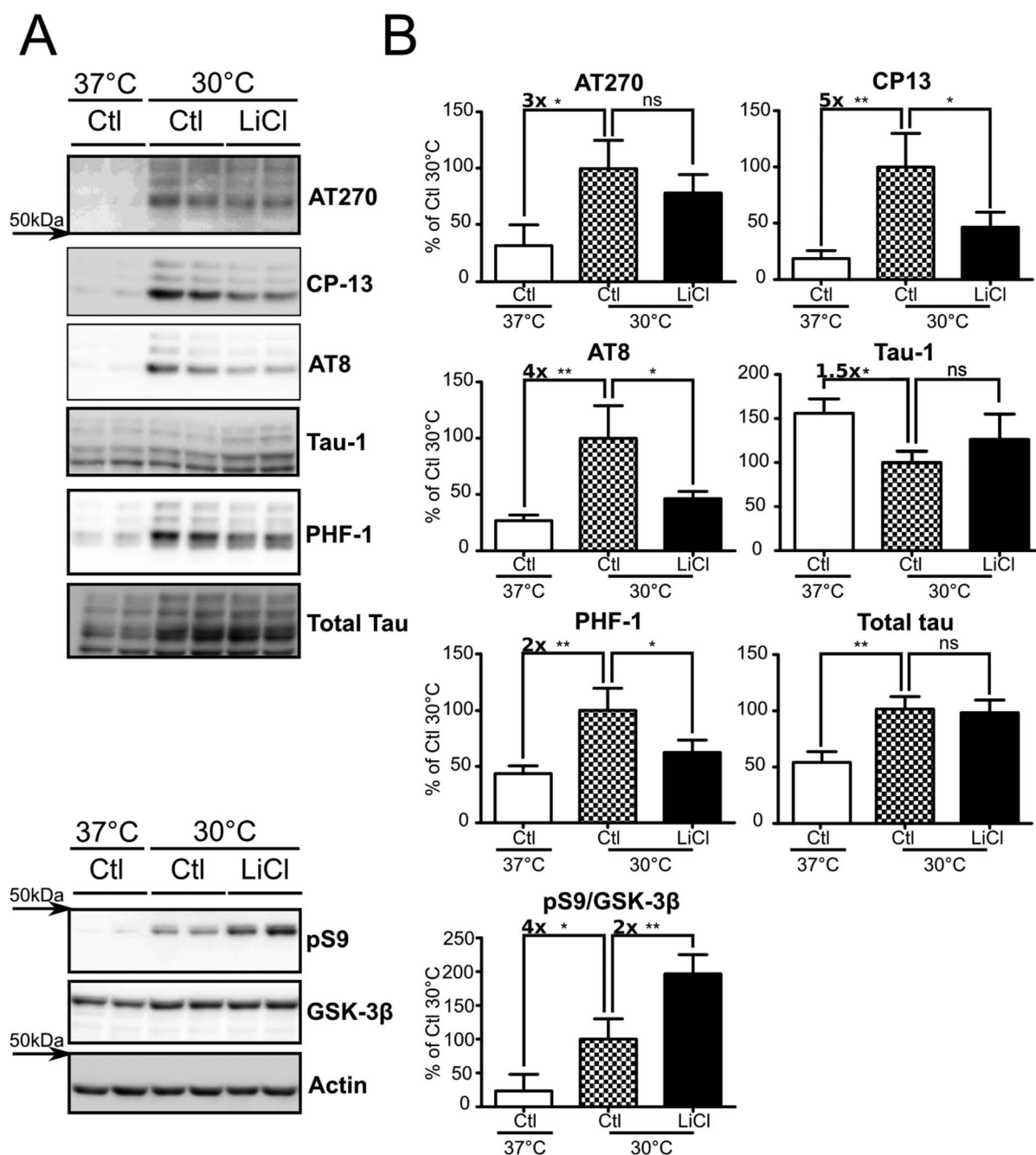


Figure 2 | Hypothermia-induced tau hyperphosphorylation is prevented by LiCl treatment in mouse brain slices. Mouse brain slices were subjected to hypothermia for 2h and treated with either LiCl or medium alone. A. Immunoblots of mouse brain slice proteins using several phospho-tau antibodies (AT270, CP13, AT8 and PHF-1). Total tau was probed using a pan tau antibody. GSK-3 β inhibition was monitored by assessing both GSK-3 β pS9 levels and total GSK-3 β . B. Immunoblot quantifications. Ratios of phospho-epitope levels over total protein levels \pm SD are represented as a percentage of hypothermic non-treated condition Ctl 30°C (Checkerboard bar). N=3 per condition.



threshold (Data not shown). In these experiments, we used an optimized 20 mM LiCl dose (supplementary Figure S1 online), which is consistent with previous findings²². Total tau protein levels were significantly changed by hypothermia but not by LiCl treatment. Hypothermia also induced a ~4-fold GSK-3 β pS9 increase (Figure 2A, B: Ctl 37°C vs Ctl 30°C), while LiCl treatment under hypothermic condition raised GSK-3 β pS9 levels up to ~8-fold (Figure 2A, B). Finally, total GSK-3 β levels were significantly increased (~+20%) with hypothermia. In summary, and as seen *in vivo*, LiCl treatment partially prevented tau hyperphosphorylation induced by hypothermia in *ex vivo* brain slices, through GSK-3 β inhibition.

Hypothermia-induced tau hyperphosphorylation is prevented by LiCl treatment in wild-type SH-SY5Y cells or SH-SY5Y 3R-tau. To further test our experimental paradigm in a cell system more suitable for drug screening, we performed hypothermia experiments in native neuroblastoma SH-SY5Y cells from human origin. Cells exposed to hypothermia (30°C) for 2h showed a significant tau phosphorylation increase at PHF-1 (~+3x) and Tau-1 (~-1.5x) phospho-epitopes (Figure 3 A, B: Ctl 37°C vs Ctl 30°C). Treating cells with LiCl during hypothermia reduced tau phosphorylation to control levels for PHF-1 and Tau-1 phospho-epitopes (Figure 3 A, B: Ctl 30°C vs LiCl 30°C). Total tau levels were not changed in all groups tested. While no increase in GSK-3 β pS9 was observed in hypothermic cells, this epitope was increased (~+50%) in LiCl treated cells. Other tau phospho-epitopes, including, AT8, AT180, CP13 and AT270, were also assessed but were below detection limits, probably because of

low endogenous tau expression and low physiological levels of these phospho-epitopes in these cells.

To overcome these detection limitations, we performed similar experiments in SH-SY5Y cells stably expressing human 3R-tau without any mutation (SH-SY5Y 3R-tau). Here, hypothermic cells exhibited hyperphosphorylation at several phospho-epitopes (AT270:~+2x, CP13:~+4x, AT8:~+6x, Tau-1:~-2x, AT180:~+2x, MC-6:~+15x and PHF-1:~+2.5x (Figure 4 A, B: Ctl 37°C vs Ctl 30°C). As seen previously, LiCl treatment restored tau phosphorylation to control levels at AT270, CP13, AT8, Tau-1, AT180, MC-6 and PHF-1 phospho-epitopes (Figure 4A, B: Ctl 30°C vs LiCl 30°C). Again, total tau protein levels were not affected in these experimental conditions. Finally, while an increase in GSK-3 β pS9 was observed in hypothermic cells with or without LiCl treatment, this effect did not reach statistical significance. In sum, these results show that GSK-3 inhibition by LiCl leads to reduced hypothermia-induced tau hyperphosphorylation in these cells.

Hypothermia-induced tau hyperphosphorylation is differentially prevented by Cdk5 and GSK-3 β inhibitors treatment in SH-SY5Y 3R-tau. To extend our experimental paradigm to other kinase inhibitors, we performed hypothermia experiments using SH-SY5Y 3R-tau cells treated with the GSK-3 β inhibitor AR-A014418 (AR) or the Cdk5 inhibitor roscovitine. Two hours of AR treatment caused a significant decrease of phosphorylation at Tau-1, AT180, MC-6 and PHF-1 phospho-epitopes (+57%, -62%, -95% and -70% respectively) (Figure 5A, B: AR 30°C vs Ctl 30°C). Tau phosphorylation was also decreased to a lesser extent at CP13

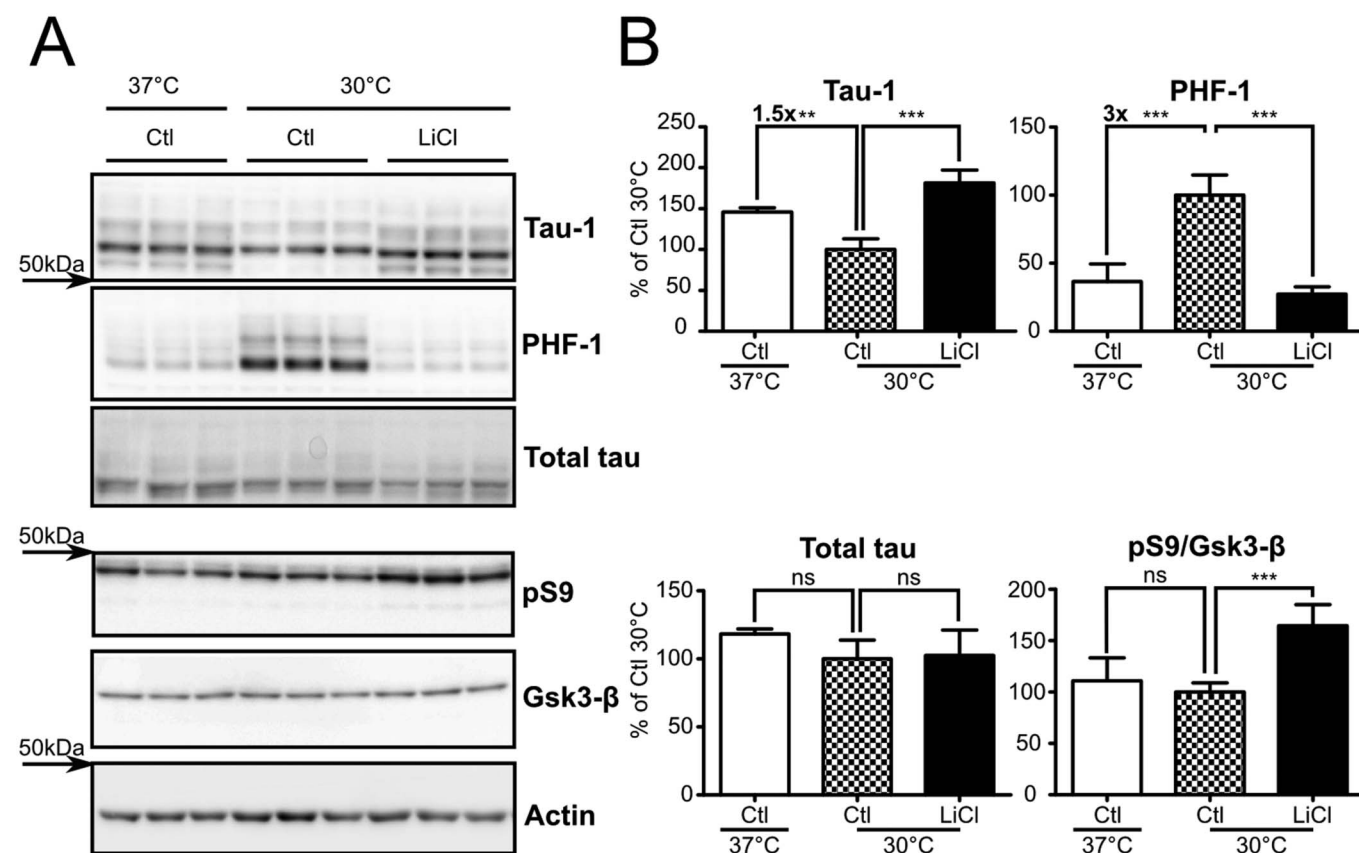


Figure 3 | Hypothermia-induced tau hyperphosphorylation is prevented by LiCl treatment in natives SH-SY5Y cells. Natives SH-SY5Y cells were subjected to 2h of hypothermia and treated with either LiCl or medium alone. A. Immunoblots of cell extracted proteins using phospho-tau antibodies Tau-1 and PHF-1. Total tau was probed using a pan tau antibody. GSK-3 β inhibition was assessed by monitoring both GSK-3 β pS9 levels and total levels. B. Immunoblot quantifications. Ratios of phospho-epitope levels over total protein levels \pm SD are represented as a percentage of hypothermic non-treated condition Ctl 30°C (Checkerboard bar). N=6 per condition.

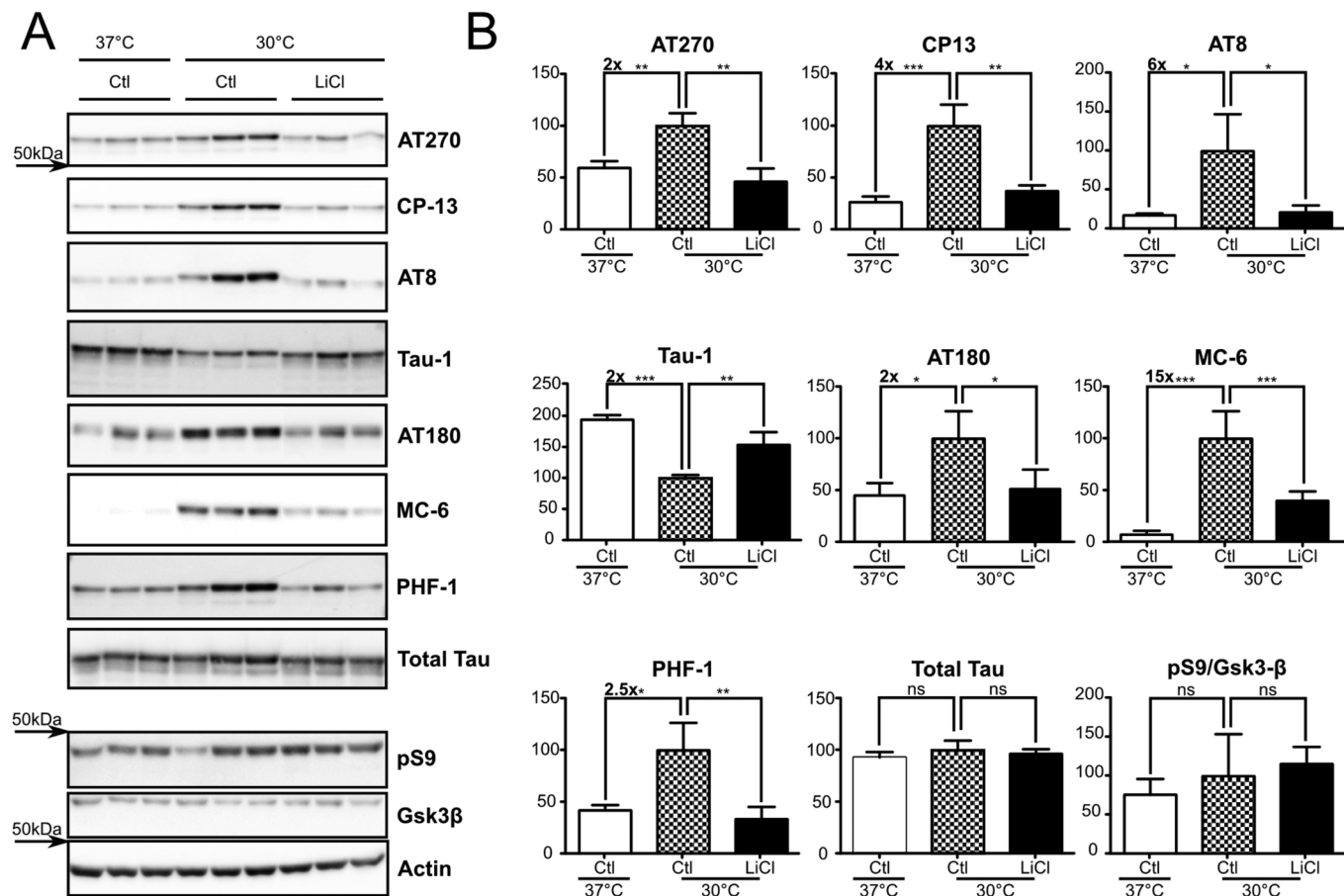


Figure 4 | Hypothermia-induced tau hyperphosphorylation is prevented by LiCl treatment in SH-SY5Y 3R-tau. SH-SY5Y 3R-tau were subjected to hypothermia for 2h and treated with either LiCl or medium alone. **A.** Immunoblots of cell extracted proteins using several phospho-tau antibodies (AT270, CP13, AT8 Tau-1 and PHF-1). Total tau was probed using a pan tau antibody. GSK-3 β inhibition was monitored by assessing both GSK-3 β pS9 levels and total levels. **B.** Immunoblot quantifications. Ratios of phospho-epitope levels over total protein levels \pm SD are represented as a percentage of hypothermic non-treated condition Ctl 30°C (Checkerboard bar). N=3 per condition.

phospho-epitope but did not reach statistical significance. AT270 and AT8 phosphorylation were not decreased by AR treatment with an unexplained slight significant increase of phosphorylation at AT270 phospho-epitope. Cells treated with roscovitine showed significant decreased tau phosphorylation levels at AT180 (−52%) and MC-6 (−92%) phospho-epitopes when compared to untreated conditions (Figure 5A, B: Rscv 30°C vs Ctl 30°C). Notably, roscovitine had no significant effect at AT270, CP13, AT8, Tau-1 and PHF-1 phospho-epitopes. Total tau protein levels remained constant in all experimental conditions. In summary, treatments with Cdk5 inhibitor roscovitine and GSK-3 β inhibitor AR-A014418 leads to different reduced tau phosphorylation patterns reflecting the specificity of inhibitors used.

Discussion

Here, we used hypothermia to induce tau hyperphosphorylation in various biological models ranging from immortal cultured cells, to mice and *ex vivo* metabolically active slices, to test the effects of tau kinase inhibitors. We used the GSK-3 inhibitor LiCl as a proof of concept to demonstrate the feasibility of this approach. As expected, hypothermia induced tau phosphorylation at multiple epitopes in all our models, and treatment with LiCl reduced hypothermia-induced tau hyperphosphorylation. We also extended this study to two other kinase inhibitors, AR-A014418 and roscovitine targeting GSK-3 or Cdk5, respectively, and were able to observe a specific pattern of tau phosphorylation reduction indicative of kinase inhibitor specificity.

All general anesthetics induce hypothermia through the deregulation of both non-shivering and shivering thermogenesis regulation²³. This hypothermia directly results in an *in vivo* increase of AD-related tau phospho-epitopes including AT270 (Thr181), Tau-1 (Ser195/Ser198/Ser199/Ser202), Ser199, CP13 (Ser202), AT8 (Ser202/Thr205), TG-3 (Thr231 conformational), MC-6 (Ser235), Ser262, PHF-1 (Ser396/Ser404) and Ser422^{17,24} at comparable levels to those observed in AD brains¹⁸; reviewed in²⁵. This hypothermia-induced tau hyperphosphorylation is also reversible since we demonstrated earlier that tau phosphorylation levels recover to normal after 1 week of post-anesthesia recovery¹⁹. In the present work, we confirmed these results and showed that ketamine/xylazine led to an hypothermia-induced increase of tau phosphorylation from ~1.5x to ~31x, depending of the phospho-epitope. Among all the phospho-epitopes studied here, Thr231 and Ser262 are accepted to be early tau modifications in neurodegeneration while other tau phosphorylation sites such as Thr181, Ser202/Thr205 and Ser396/Ser404 appear later during disease progression²⁶. Thr231 and Thr181 are used as AD biomarkers^{27,28}, and Ser202/Thr205 is used to stage AD pathology²⁹. Thus, the profiles and levels of tau hyperphosphorylation generated by our hypothermia models are highly relevant to tau pathology observed in AD.

The exact mechanisms underlying tau hyperphosphorylation in AD are not well understood. However, it is commonly accepted that tau hyperphosphorylation results from the imbalance between kinase and phosphatase activities⁸. Among all the phosphatases, PP2A is the major enzyme that dephosphorylates tau³⁰, accounting

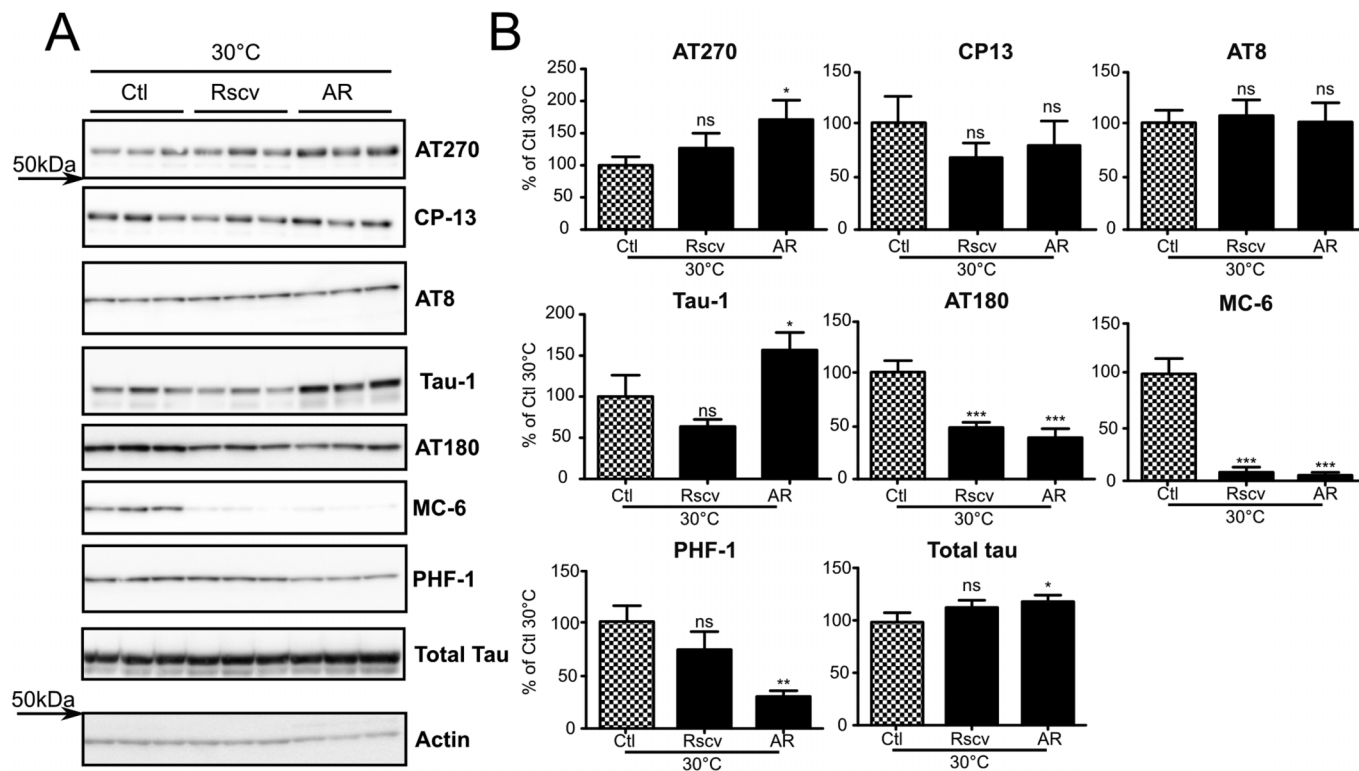


Figure 5 | Hypothermia-induced tau hyperphosphorylation is differentially prevented by Cdk5 and GSK-3 β inhibitor treatment in SH-SY5Y 3R-Tau. SH-SY5Y 3R-tau cells were subjected to hypothermia for 2 h and treated with medium alone or medium containing either Cdk5 inhibitor roscovitine (Rscv) or GSK-3 β inhibitor AR-A014418. **A.** Immunoblots of SH-SY5Y 3R-tau cell extracted proteins using several phospho-tau antibodies (AT8, AT180, MC-6 and PHF-1). Total tau was probed using a pan tau antibody. **B.** Immunoblot quantifications. Ratios of phospho-epitope levels over total protein levels \pm SD are represented as a percentage of hypothermic non-treated condition Ctl 30°C (Checkerboard bar). N=3 per condition.

for more than 70% of the total tau phosphatase activity in the brain, regulating nearly all tau phosphorylation sites³¹. Moreover, its inhibition is associated with AD evolution³². Indeed, both PP2A expression^{10,33} and activity^{9,34} are decreased in AD brains. Tau phosphorylation is exquisitely sensitive to temperature, increasing by 80% for each degree below 37°C, due to exponential decrease in PP2A activity during direct hypothermia¹⁶, or anesthesia-induced hypothermia¹⁷. Therefore, the hypothermia-mediated inhibition of PP2A is a highly relevant model to study modulators of tau phosphorylation targeting tau pathology in AD.

Among tau kinases, GSK-3 β ^{12,35} and Cdk5³⁶ are considered to be the main tau kinases inducing tau hyperphosphorylation in AD. This statement does not conflict with the aforementioned role of PP2A inhibition in tau hyperphosphorylation, because phosphatases merely remove phosphate from tau while kinases are still needed for hyperphosphorylation to take place. In fact, PP2A inhibition is considered to be the functional equivalent of activating all the tau kinases together because PP2A dephosphorylates all known tau epitopes while each kinase is specific for only a set of given sites³⁷. For example, GSK-3 β has been shown *in vitro* and/or *in vivo* to target phosphorylation at sites such as Thr181, Ser202/Thr205, Thr231 and Ser396/Ser404. On the other hand, Cdk5 can phosphorylate about 10 phosphorylation sites *in vitro* including Ser202, Thr231, Ser235, Ser396/Ser404⁷.

In all our biological systems, we observed that LiCl reduced hypothermia-induced tau hyperphosphorylation at almost every epitope analyzed. We also observed that the effects of LiCl was more pronounced in cell culture than in *ex vivo* and *in vivo* models. This could be attributed, in part, to a lower bio-availability of compounds and for the low phosphorylation of some epitopes such as AT180 and MC-6 in *ex vivo* and *in vivo* models compared to cell cultures. Essentially, we observed the same results with another GSK-3 β

inhibitor (AR) at many phospho-epitopes. However, phosphorylation on CP13 (Ser202), AT8 (Ser202/Thr205) and AT270 (Thr181) were not significantly decreased at the dose used. These discrepancies could be explained by both the high specificity of AR to GSK-3 β and the fact that LiCl is also known to inhibit other kinases³⁸. Therefore, the significant decrease of phosphorylation at those phospho-epitopes observed in LiCl treated samples could be the result of a cumulative effect of LiCl on GSK-3 β and other kinases. The use of roscovitine in hypothermic SH-SY5Y 3R-tau, reduced hyperphosphorylation at Thr231 and Ser235 epitopes. However, AT270, (Thr181), CP13 (Ser202), AT8 (Ser202/Thr205), Tau-1 (non-phosphorylated Ser195/Ser198/Ser199/Ser202) and PHF-1 (Ser396/Ser404) epitopes were not significantly decreased. These results could be explained by a lesser activity of Cdk5 at CP13, AT8 and Tau-1 phosphorylation sites⁷ since the activation of Cdk5 in neuroblastoma cells leads to a smaller increase of phosphorylation at Ser202/Thr205 than at Thr231 and Ser235 and does not increase tau phosphorylation at Ser396/Ser404³⁹. Moreover, increased activity of Cdk5 observed in amyloid precursor protein knockout mice does not result in increased tau phosphorylation at Thr181⁴⁰. Therefore, inhibiting Cdk5 by roscovitine in our hypothermia paradigm reduced tau hyperphosphorylation at preferred Cdk5 sites such as Thr231 and Ser235. Altogether, our results show that hypothermia is a suitable model to assess the specificity of tau kinase inhibitors on tau phosphorylation.

One of the main roadblocks for screening and drug testing of tau phosphorylation inhibitors is that immortal cell lines and adult mice show low levels of tau phosphorylation. To override this problem, a large number of both cellular and animal models have been based on the over-expression of mutated human tau to achieve tau hyperphosphorylation⁴¹. However, the use of high expression levels of aggressive tau mutants could lead to pathophysiological mechanisms



Table 1 | Antibodies used and their respective dilutions

| Antibody | Phospho-epitope | Dilution | Provider |
|----------------|-------------------|----------|---------------------------|
| AT100 | pS212/T214 | 1/1000 | Pierce |
| AT180 | pT231 | 1/1000 | Pierce |
| AT270 | pT181 | 1/1000 | Pierce |
| AT8 | pS202/T205 | 1/1000 | Pierce |
| CP13 | pS202 | 1/1000 | Gift of Peter Davies |
| MC-6 | pS235 | 1/1000 | Gift of Peter Davies |
| PHF-1 | pS396/S404 | 1/1000 | Gift of Peter Davies |
| Tau-1 | | 1/10000 | Millipore |
| Tau A0024 | | 1/10000 | Dako Cytomation |
| TG-3 | pT231 | 1/1000 | Gift of Peter Davies |
| GSK-3 β | | 1/1000 | BD biosciences |
| Gsk3 S9 | GSK-3 β pS9 | 1/2000 | Cell signaling Technology |
| Actin- β | | 1/3000 | Sigma |
| pS262 | pS262 | 1/5000 | Life Technologies |

irrelevant to AD, since only non-mutant tau proteins are implicated in the disease. Other studies have induced both hyperphosphorylated⁴² and abnormally phosphorylated tau⁴³ through PP2A inhibition with okadaic acid. However, the high toxicity of okadaic acid, associated to its pro-apoptotic side effects, limits the relevance of such models⁴⁴. Here, we showed that hypothermia led to easily detectable tau phosphorylation levels on non-mutated tau expressed at endogenous levels, which is compatible with tau kinase inhibitors testing.

In conclusion, we have shown in cells, brain slices and mice, that hypothermia is a reliable system to model AD-like tau hyperphosphorylation with endogenous wild-type tau protein levels. Moreover, we demonstrated that hypothermia could be used as a transverse paradigm to evaluate the potency and specificity of tau kinase inhibitors. This is of particular importance as recent evidences implicate pre-fibrillar hyperphosphorylated tau as the toxic species in AD^{45,46}, and therefore fuel a regain of interest in tau kinase inhibitor development and clinical trials¹⁵. Overall, we propose that hypothermia and anesthesia-induced hypothermia represent a fast and useful tool to help in the development and characterization of new tau phosphorylation modulators from cells to animals.

Methods

Kinase Inhibitors. We used Lithium chloride (LiCl) (Sigma-Aldrich, St-Louis, MO, USA), AR-A014418 (Enzo life sciences, Farmingdale, NY, USA), and roscovitine (New England Biolabs, Ipswich, MA, USA).

Animal treatments. 4-month-old C57BL/6 mice of either sex were injected intraperitoneally with either sterile water (control group) or sterile water containing therapeutically relevant dose of 0.6M LiCl for 2 days (once daily at 10 ml/kg), as previously published^{14,47}. On the third day, the mice received a last injection and 75 minutes later, half the mice of each group were anesthetized by injection of ketamine/xylazine (100/10 mg/kg) in sterile water. The other half received a vehicle injection (sterile water). Rectal temperature was monitored every 15 minutes after the first injection until sacrifice, using a rectal probe (Thermalert TH-5; Physitemp, Clifton, NJ, USA). Mice were sacrificed by decapitation 135 minutes after the last LiCl injection (*i.e.* 60 minutes after anesthesia). Brains were removed and dissected on ice, and frozen on dry ice. Tissues were kept at -80°C until processing.

Brain slices. 4-month-old C57BL/6 mice were sacrificed by decapitation. Slices were prepared as previously published⁴⁸. Slices were divided into 2 hemibrain slices and placed into permeable inserts (Transwell Permeable Supports, Corning, Corning, NY, USA). After 30 minutes of recovery, transwells were transferred into new 24-well plates containing pre-oxygenated DMEM or DMEM with 20 mM LiCl. Half the slices and their contra-lateral counterparts were placed in a 5% CO₂ humidified incubator at 30°C for hypothermic conditions or 37°C. After 2 h, the medium was removed and the slices were harvested directly in a modified RIPA buffer (see below). Slice homogenates were kept at -80°C until processing. All animals were handled according to procedures approved by the Comité de Protection des Animaux under the guidelines of the Canadian Council on Animal Care.

Cell cultures. Native SH-SY5Y were purchased from American Type Culture Collection (ATCC #CRL-2266, Manassas, VA, USA) and grown as recommended by the manufacturer. SH-SY5Y stably overexpressing human 3-repeats (SH-SY5Y

3R-tau) tau cells were a kind gift from Dr Luc Buée (Inserm UMR837, Lille, France) and subcultured at 37°C, as previously described⁴⁹. Cells were treated or not with either 20 mM LiCl, or 20 μM AR-A014418 as previously published²², or 20 μM roscovitine⁵⁰ for 2h. For the duration of the treatment, cells were transferred in a 5% CO₂ humidified incubator at 30°C for hypothermic conditions.

Western blots. Samples were prepared as previously described¹⁹. Proteins were quantified, separated on SDS-PAGE gels, blotted onto nitrocellulose membranes and blocked. Membranes were probed with antibodies diluted at the concentration indicated in Table 1. All antibodies were purchased directly from the provider as mentioned in Table 1 except for CP13⁵¹, TG-3 and MC-6⁵², and PHF-1⁵³ incubated with a Horseradish Peroxidase-conjugated secondary antibody (Jackson ImmunoResearch laboratories, West Grove, PA, USA), and revealed by chemiluminescence in a Fujifilm LAS4000 imaging system (Fujifilm Life Science USA, Stamford, CT), as previously described⁵⁴. Of note, contrary to other antibodies, Tau-1 is directed against non-phosphorylated multiple tau epitopes, and therefore the signal decreases when tau is hyperphosphorylated⁵⁵. Immunoblot quantifications were performed by Fujifilm Multigauge software 3.0.

Statistics/Miscellaneous. Statistical analysis was performed with one-way ANOVA followed by the Dunnett's post hoc test. For multiple comparisons, one-way ANOVA followed by the Bonferroni's post hoc test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non significant, except otherwise mentioned. GraphPad Prism 5.0 (GraphPad, La Jolla, CA). LibreOffice suite, The GIMP and Mendeley free softwares were used for data processing, manuscript editing and referencing.

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Author contributions

Conceived and designed the experiments: AB, EP. Performed the experiments: AB, FM, CJ. Analyzed the Data: AB, EP. Contributed reagents/material/analysis tools: DM, GL. Performed revision experiments: NBK, FRP, IP. Wrote the manuscript: AB, DM, GL, SSH, EP. All authors reviewed the manuscript.

Additional information

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