The Role of Protein Phosphatase 2A in Alzheimer's disease pathogenesis

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STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provision of the Copyright Act 1968 and any approved embargo.

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I hereby certify that the work embodied in this thesis contains published papers of which I am a joint author. I have included as part of the thesis a written declaration endorsed in writing by my supervisor, attesting to my contribution to the joint publications.

ALEXANDER HOFFMAN

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Abbreviations

3R/4R	**Three Repeat / Four Repeat Tau**	**5HT**	**5-Hydroxy-Triptamine (Serotonin)**
ACh	Acetylcholine	**AD**	Alzheimer's Disease
AICD	Amyloid Intracellular Domain	**AKAP**	A-Kinase Anchoring Protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	**APP**	Amyloid-β Precursor Protein
Aβ	Amyloid-β	**BACE**	B-Amyloid Cleaving Enzyme
BDNF	Brain-Derived Neurotrophic Factor	**BH4**	Tetrahydrobiopterin
CA	Cornu Ammonis	**cAMP**	Cyclic Adenosine Monophosphate
CBD	Corticobasal Degeneration	**CBS**	Cystathionine-β-Synthase
cdk5	Cyclin-dependent protein kinase 5	**CREB**	cAMP-responsive element binding protein
CTF	Carboxy-Terminal Fragment	**D_xR**	Dopamine (1-5) Receptor
ERK	Extracellular Regulated Protein Kinase	**FTD**	Fronto-temporal dementia
GAP	Guanine triphosphate-hydrolysing enzyme Activating protein	**GSK-3β**	Glycogen Synthase Kinase 3β
HA	Hemagglutinin	**Hcy**	Homocysteine
HHcy	Hyper-homocysteinemia	**JNK**	c-Jun N-terminal Kinase
LCMT-1	Leucine Carboxyl-methyltransferase 1	**LTD**	Long-term depression

LTP	Long-term potentiation	**mAChR**	Muscarinic Acetylcholine receptor
MAP2	Microtubule associated protein 2	**mGluR**	Metabotropic glutamate receptor
Mid1	Midline 1 protein	**MT**	Microtubule
MTHFR	5,10- methylene tetrahydrofolate reductase	**NFT**	Neufibrillary Tangle
NGF	Neurotrophic Growth factor	**NMDA**	N-Methyl-D-aspartate
OA	Okadaic Acid	**PDE**	Phospho-diesterase
PHF	Paired Helical Filaments	**Pin1**	Peptidyl- prolyl cis-trans isomerase NIMA-interacting 1
PKA	cAMP-dependent protein kinase A	**PME-1**	Protein Phosphatase Methylesterase 1
PP2A	Protein Phosphatase 2A	**SAH**	S-Adenosyl-Homocysteine
SAM	S-Adenosyl- Methionine	**sAPP**	Secreted Amyloid-β Precursor Protein
SH(x)	Src Homology Domain	**tHcy**	Total Plasma Homocysteine
TIPRL	Target of rapamycin signalling pathway regulator	**TrkA**	Tropomyosin Receptor Kinase A

Thesis Abstract

Sporadic Alzheimer's disease (AD) is the most prevalent form of dementia in Australia and worldwide. Studies on the Familial form of AD have identified many of the molecular participants in the pathophysiology of AD; included in these is the Amyloid-β (Aβ) Precursor Protein (APP), which undergoes proteolytic processing to yield one of the pathological hallmarks of AD, Aβ. Another major hallmark of AD is hyper-phosphorylated, oligomerised Tau protein, which aggregates into Paired Helical Filaments (PHFs), which are then liable to form Neurofibrillary Tangles (NFTs). In this Thesis, we aimed to interrogate potential links between these two major contributors to AD in order to untangle the disease process.

In order to extricate this pathological cascade, we consulted some of the risk factors associated with AD. Among some others, AD risk is heightened by impaired folate metabolism and elevated plasma homocysteine levels; these two conditions may arise through inadequate dietary consumption of folate and associated micronutrients, or genetic impairments in the processing of folate and methyl groups referred to as "One-Carbon Metabolism". A major intracellular signalling mediator which is dependent on the proper cycling of one-carbon groups is the enzymatic family known as Protein Phosphatase 2A (PP2A). Methylated PP2A is widely recognised as the major Tau phosphatase, and deregulated PP2A enzymes have been found to co-exist in AD pathology with hyperphosphorylated Tau and degenerated brain regions most affected in AD. *In vivo* models of One-Carbon metabolism also show that Tau phosphorylation is also significantly elevated in the brain. In this study, we used the same model, mice with genetic deficiencies in the 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme, which were fed a normal folate or folate-deficient diet. We were able to show that the regulation of APP expression and post-translational modification is altered in major substructures on the brain, which helps affirm the link between a risk factor for AD (impaired folate metabolism), existing evidence for this risk in AD showing that PP2A and Tau are dysregulated in this model, and the perturbation of APP regulation.

Since it is evidently a major enzyme deregulated in AD, we endeavoured here to tease apart how PP2A can be precisely controlled by post-translational modification in neurons. The two major post-translational modifications of PP2A are leucine methylation and tyrosine phosphorylation of the catalytic subunit.

Currently, the only known source of the control of PP2A methylation arises from dietary supply of methyl groups, or One-Carbon metabolism, as briefly described above. Some groups have presented evidence suggesting that activation of cAMP signalling in non-neuronal cells affect the activity state of PP2A, while others show similar evidence for changes in PP2A methylation with the initiation of cAMP signalling. We thus deemed it necessary to delineate a more precise understanding of if, and how cAMP signalling affects PP2A methylation in neurons. To do this, we specifically investigated the activity and kinase targeting of cAMP-dependent protein kinase A (PKA) in cultured N2a cells. Indeed, we were able to show that activation of PKA with the cAMP-generating drug Forskolin led to time-sensitive demethylation of PP2A. We also observed that overexpression of the catalytic subunit of PP2A (PP2Ac) reduced the PKA-targeted phosphorylation sites of Tau and the transcription factor CREB, which is heavily involved in learning and memory consolidation. We thereby demonstrate a novel mode of PP2A regulation with direct consequences for both AD pathogenesis and regulation of learning and memory.

PKA-mediated changes in PP2A methylation appeared to have such important consequences, so we also used the MTHFR- and folate-deficient mouse models described above to

investigate a major neural target of PKA and PP2A, CREB. We observed an interesting array of effects of disturbed One-Carbon metabolism on CREB expression and phosphorylation, which is linked to its transcription factor activity state. In the cortex and midbrain regions of mice with genetic deficiency of the MTHFR enzyme, CREB expression was altered, and CREB activity was elevated in the midbrain of these mice. Hence, in this thesis, we demonstrate that disturbed One-Carbon metabolism, which is related to multiple AD risk factors, dysregulates both APP and CREB, both of which are implicated in the AD process. These are linked through previous results from our lab showing that PP2A methylation and Tau phosphorylation are concurrently affected by this model in the same way they are dysregulated in AD pathology.

In contrast to methylation of the catalytic subunit, tyrosine phosphorylation of PP2Ac was first reported to catalytically inactivate the enzyme. Unfortunately, these results have never been corroborated *in vivo* using independent methodologies. Since tyrosine phosphorylated PP2A has been reported to accumulate in close proximity to dystrophic neurites in AD, we deemed it important to substantiate the nature of PP2A tyrosine phosphorylation. If we could confirm that PP2A tyrosine phosphorylation and inactivation occurs in neurons, this may elucidate novel neurotoxic cascades in AD. In two distinct cell lines, we were able to show that the non-receptor tyrosine kinase, Src indeed phosphorylates PP2A, but at two previously unidentified tyrosine sites. We also demonstrate that phosphorylation of one of these sites can impair the Tau phosphatase activity of PP2A in N2a cells.

In the following body of work, we explore the importance of post-translational modifications of PP2A in the context of AD pathogenesis. Using cellular, *in vivo* and *ex vivo* techniques, we use this enzyme to navigate the role of dietary and genetic disturbances in the regulation of major proteins disturbed in AD, specifically Tau, APP and CREB. The novel findings we present provide the foundation for future study in the dysregulation of PP2A in memory disturbances and neurotoxicity in the molecular course of AD.

Chapter 1: Background and Rationale

Statement of originality

I hereby declare that all work enclosed in this thesis, including evaluation of relevant literature and composition of schematic figures was performed independently, with the guidance of my supervisors. Where figures have been adapted, appropriate acknowledgement has been made in the relevant figure legend.

1.1 Alzheimer's disease, a major neurodegenerative disorder

Alzheimer's disease (AD) is the most common form of dementia, and is characterized by an irreversible and progressive loss of neurons and connectivity. AD manifests clinically through a corresponding memory impairment with insidious onset and progressively worsening symptoms. Affected areas include medial temporal regions of the brain – including the amygdala and entorhinal cortex – and substructures of the hippocampus, the subiculum and cornu ammonis field 1 (CA1) [1]. Patients may also present with non-amnestic AD, with symptoms typically manifesting as impairments in language, visuospatial and executive function. This is likely due to atypical AD neuropathology which may spare the hippocampus or predominantly affect the limbic regions [2].

There are two forms of Alzheimer's disease, late onset/sporadic AD, which accounts for the vast majority of cases, and early onset/familial AD. Mutations in the transmembrane Amyloid precursor protein (*APP*) and the Presenilin-1 complex (*PSEN1* [3-5]) have been linked to rare, early onset AD or Familial AD. Sporadic AD has no genetic cause, but several risk factors have been identified. Advanced age is by far the primary risk factor. Other risk factors include: Modifiable lifestyle or behavioural factors (e.g. a lack of physical activity and poor nutrition [6]); a medical history of certain diseases such as hypertension, diabetes, or brain trauma [7-10]; and genetic or age-related physiological changes (e.g. alterations in folate and homocysteine metabolisms (***Fig. 1.1****).* These risk factors, though independent, are not mutually exclusive and can sometimes exist as co-morbidities.

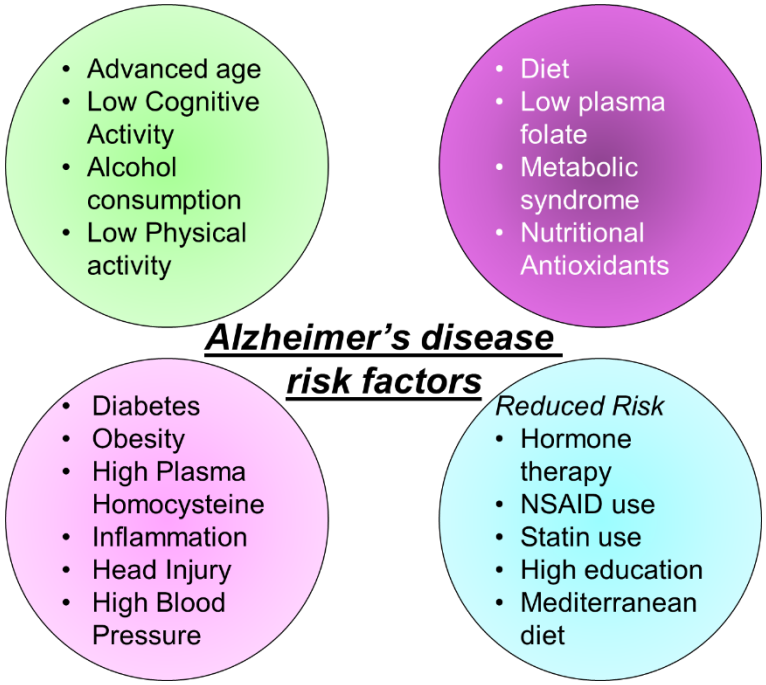


Figure 1.1 *Risk factors for sporadic AD.* While no definite cause of Alzheimer's disease has been determined, several factors have been reported to increase the risk of onset of AD. Of particular relevance to our project is the role of alterations in homocysteine and folate metabolism.

Conversely, the use of certain medications such as non-steroidal anti-inflammatory drugs (NSAIDs), hormone therapy and statins have been linked to a decreased risk of Alzheimer's disease [11-13]. Similarly, lifestyle factors such as the consumption of a Mediterranean diet or high cognitive activity through a high level of education are also associated with reduced risk of AD [6, 14, 15].

The neuropathological hallmarks of AD can be confirmed only by examination of post-mortem brain tissue. First, staining of brain tissue reveals a high density of senile or mature β-Amyloid (Aβ) plaques in AD-affected regions. These dense-core, neuritic plaques are composed of extracellular deposits of Aβ resulting from aberrant processing of APP. The second major neuropathological hallmark of AD is the presence in affected brain regions of intracellular filamentous lesions called neurofibrillary tangles (NFTs). These contain highly phosphorylated Tau proteins [1]. Tau-positive neuropil threads and dystrophic neurites, which are associated with Aβ-containing neuritic plaques, are also a widespread and characteristic lesion of AD. The deregulation of Tau is thought to be a key mediator of Aβ-induced neurotoxic cascades, resulting in synaptic failure, and ultimately neuronal death In AD, there is also decreased brain volume, which is largely attributed to neuron death due to loss of functional connectivity of synapses [16]. This is likely mediated by Tau dysfunction, resulting in cytoskeletal deregulation and perturbed energetics in axonal and dendritic compartments of neurons.

1.2 Proteins implicated in the pathogenesis of Alzheimer's disease

1.2.1 The Amyloid Precursor Protein

1.2.1.1 Regulation and Cellular Function

APP is expressed at high levels in the brain. Of the eight isoforms generated through alternative splicing, the 695 amino acid isoform is the most commonly expressed in neurons, while the 751 and 770 amino acid isoforms are expressed in glia and in peripheral tissue [17]. Expression of APP is upregulated during neuronal differentiation and synaptogenesis, followed by steady-state expression at cellular senescence after full maturation, with minimum turnover [18-20].

APP maturation is differentiated by N-glycosylated or *N-, O-* glycosylated forms. *N-*glycosylated APP, localises mainly to the endoplasmic reticulum after synthesis and is defined as immature APP [21]. Conversely, mature APP is both *N-* and *O-* glycosylated, which is necessary for the cell surface expression [22] and subsequent secretion of APP ([23, 24] ***Fig. 1.2***). Membrane-bound APP is proteolytically processed by either α or β secretase, followed by the γ-secretase complex, with the respective secreted APP fragment (sAPPα or sAPPβ) generated as a result (***Fig. 1.2***). Both membrane localisation and cleavage of APP require its *N-* and *O-* glycosylation [25]. Specific genetic mutations in the secretase components, β-Amyloid Cleaving Enzyme (BACE-1) and PSEN-1 have been causally associated with familial AD (reviewed in [3]). The fragment of APP located within the β and γ secretase cleavage sites form the oligomer- and plaque-forming Aβ fragment.

The α-processing of APP appears to protect against Aβ plaque formation [26] and Glycogen Synthase Kinase-3β (GSK-3β)-mediated Tau phosphorylation [27]. At the synapse, sAPPα stimulates pro-survival signalling cascades, *de novo* protein synthesis in synaptoneurosomes, and the enhancement of threshold frequencies that alter synaptic strength [28-30]. sAPPα promotes neuroprotection via G-protein initiated signalling mediated by Protein Kinase B / Akt [31]. Conversely, fibrillar Aβ may also bind to APP, leading to activation of $G_{\alpha O}$ and subsequent neurotoxicity [32].

Less is understood about the synaptic role of sAPPβ, which is generated by amyloidogenic processing of APP [33]. sAPPβ interacts with Death Receptor 6, leading to axonal degeneration and neuronal death [34]. Recent evidence indicates that this interaction and subsequent axonal pruning may also be required for experience-dependent learning in the somatosensory cortex of mature animals [35].

The APP Intracellular Domain (AICD), formed after γ-secretase cleavage of APP, binds to $G_{\alpha S}$, which stimulates adenylyl cyclase (AC) to synthesise cyclic adenosine monophosphate (cAMP); this second messenger binds to and activates cAMP-dependent protein kinase (PKA) and inhibits GSK-3β [37].

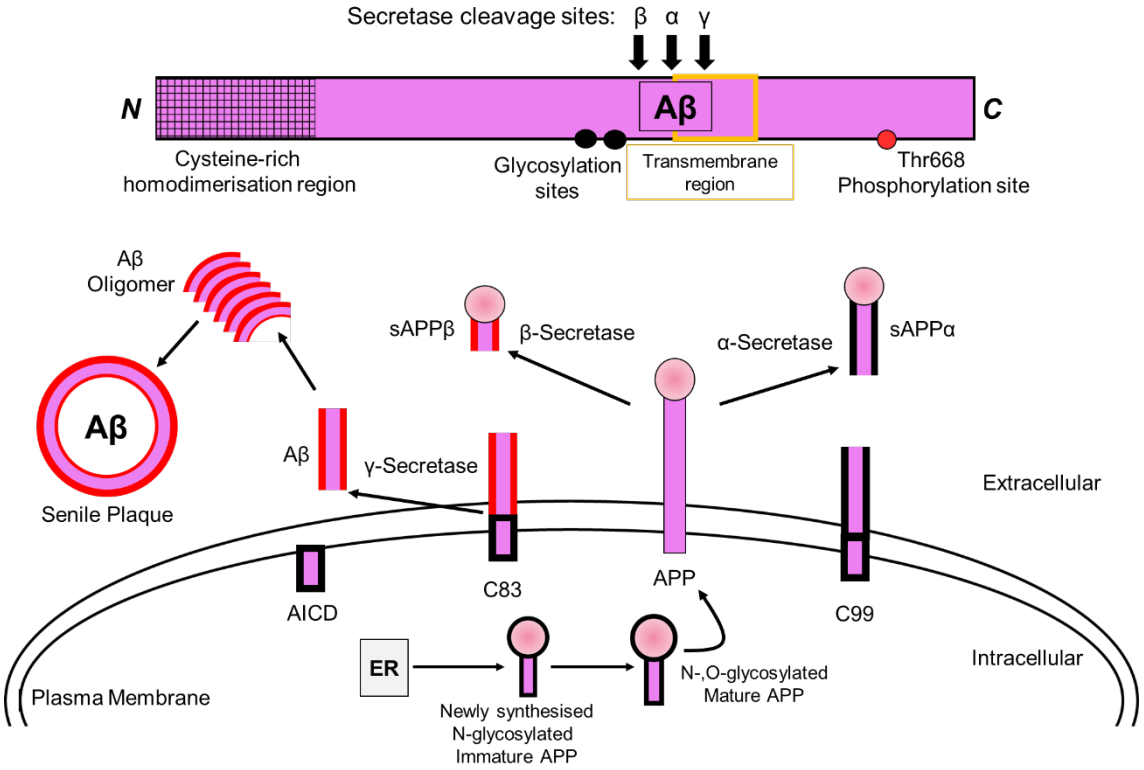


Figure 1.2 Regulation of the Amyloid Precursor Protein. APP has multiple regions that can be post-translationally modified. Newly synthesised APP (immature, *N*-glycosylated APP) translocates to the plasma membrane after *O*-glycosylation. Here, proteolytic processing by α- or β-secretases causes the secretion of sAPPα and sAPPβ, respectively. β-secretase cleavage generates the intracellular C83 peptide fragment. The α-cleavage of APP leads to C99/APP intracellular domain peptide fragments (AICD). Following α- or β-cleavage of APP, γ-secretase cleaves APP in the transmembrane domain, which can lead to Aβ production after β-secretase cleavage of APP. Adapted from Plumber, et. al., 2016 [36].

1.2.1.2 Phosphorylation of APP at Threonine 668 alters protein-protein interactions and subcellular localisation

APP is phosphorylated within its C-terminal intracellular domain– of particular interest at the Threonine 668 residue. Key kinases targeting mature APP in neurons are cyclin-dependent kinase 5 (cdk5) and GSK-3β [38-40]. Other kinases have been shown to phosphorylate immature APP at Thr668, including c-Jun N-terminal Kinase (JNK), under conditions of cellular stress [41, 42]. APP phosphorylation is thought to target the protein to the plasma membrane, including localisation to growth cones and neurite terminals [40, 43]. Neurotrophic growth factor (NGF) stimulates APP phosphorylation and redistribution in immature, developing

neurons, but also impairs APP phosphorylation in mature neurons [44].This suggests that APP-Thr668 phosphorylation has different functions throughout the course of cellular development. The phosphorylation of Thr668 may also serve to stabilise APP at the plasma membrane [45], where it can be processed proteolytically by α- and β-secretase complexes.

Phosphorylation of Thr668 reduces APP binding to Tropomyosin receptor kinase A (TrkA), a high-affinity receptor for NGF in neurons and other tissues. The APP-TrkA interaction has been demonstrated to reduce Aβ generation by facilitating APP transport to the Golgi apparatus [44]. Therefore, phosphorylation of APP at this side contributes to Aβ generation by maintaining APP localisation to the membrane. APP phosphorylation also prevents the neurotoxic caspase-3 and caspase-8 mediated cleavage of APP [46, 47]. Similarly, Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) binds APP-pThr668-Pro669, restoring APP turnover at the plasma membrane, thereby preventing Aβ secretion [45, 48]. Through regulating the interactions of APP with other proteins in the cell, the phosphorylation of Thr668 appears to be a central feature in the regulation of APP.

The γ-secretase C-terminal fragment (CTF) of APP binds to the Fe65 protein, and histone acetyltransferase Tip60 (KAT5 in humans), in complexes known as nuclear spheres (***Fig. 1.3*** [49, 50]). These complexes then translocate to the nucleus, where they regulate gene expression and impair Aβ generation [21, 49]. Thr668 phosphorylation causes a conformational change in the cytoplasmic tail of APP, preventing its interaction with Fe65 interaction and nuclear sphere generation (***Fig. 1.3*** [21, 50]), thereby maintaining the membrane localisation of APP, and facilitating β-secretase cleavage.

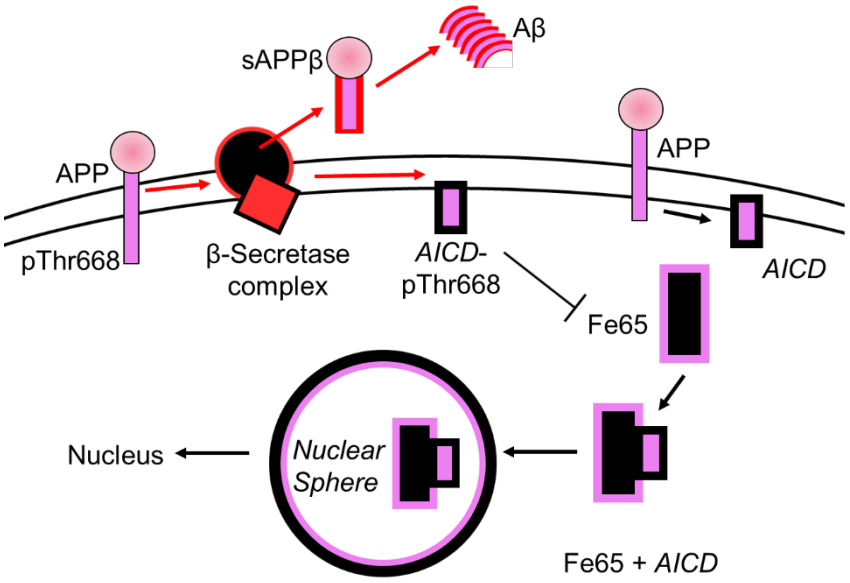


Figure 1.3 APP Thr668 phosphorylation contributes to Aβ formation and intracellular signalling. The phosphorylation of APP can lead to increased β-cleavage and subsequent Aβ secretion by neurons. APP phosphorylation can therefore potentially lead to Aβ deposition. Non-Thr668-phosphorylated APP can associate with Fe65 proteins and translocate to the nucleus to regulate gene transcription in neurons. This interaction is prevented by Thr668 phosphorylation and thereby alters gene expression patterns in neurons.

1.2.1.3 APP phosphorylation has been linked to AD

Given the importance of phosphorylation in regulating protein-protein interactions, subcellular localisation and proteolytic processing of APP, a role in AD seems likely. Indeed, pThr668-APP species have been isolated from AD brain tissue [51]. They are associated with senile plaques and neurofibrillary tangles in AD [52]. Immunohistochemical analyses have also identified pThr668-APP in the dentate gyrus and the entorhinal cortex [53], two areas implicated in the degenerative process of AD. The link between APP phosphorylation, AD markers and neurodegeneration has been confirmed in many cellular and animal models of AD in a majority of published studies [42, 44, 54, 55]. However, it is worth mentioning that some authors, have rather suggested that the phosphorylation of APP at Thr668 is not related to Aβ plaques. This conclusion was based on studies showing that changes in both secreted fragments of APP (sAPPα/β), or insoluble Aβ were undetectable in mice expressing the APP with T668A mutation, preventing its phosphorylation. Moreover, the authors were unable to distinguish any difference in Aβ load in mice bearing the T668A mutation, relative to their WT counterparts [56]. These discrepancies could be explained by another report indicating that both dephospho- (T668A) and phospho-mimicking (T668E) mutations impair APP protein interactions, so that the conformational changes induced by the T668A point mutation are likely responsible for its apparent lack of effect on Aβ generation [21].

1.2.2 The microtubule-associated protein Tau is a key player in AD pathology

1.2.2.1 Structure and Function of Tau

Tau was first identified in neurons as a microtubule-associated protein essential for tubulin polymerisation [57]. There are six Tau isoforms, owing to alternative splicing of N-terminal exons 2, 6 and 10 (0N, 1N, 2N Tau) and the expression of three-repeat (3R) or four-repeat (4R) Tau, referring to the number of C-terminal microtubule binding (MTB) repeat domains (***Fig. 1.4*** [58]). Tau exists as an unfolded protein, with a lack of any substantial secondary structure [59]; however, the microtubule binding repeats form the basis of nuclei for aggregation in AD [60, 61]. The neurofibrillary tangles (NFTs) observed in AD are comprised of Tau aggregates with a characteristic Paired Helical Filament (PHF) structure [62].

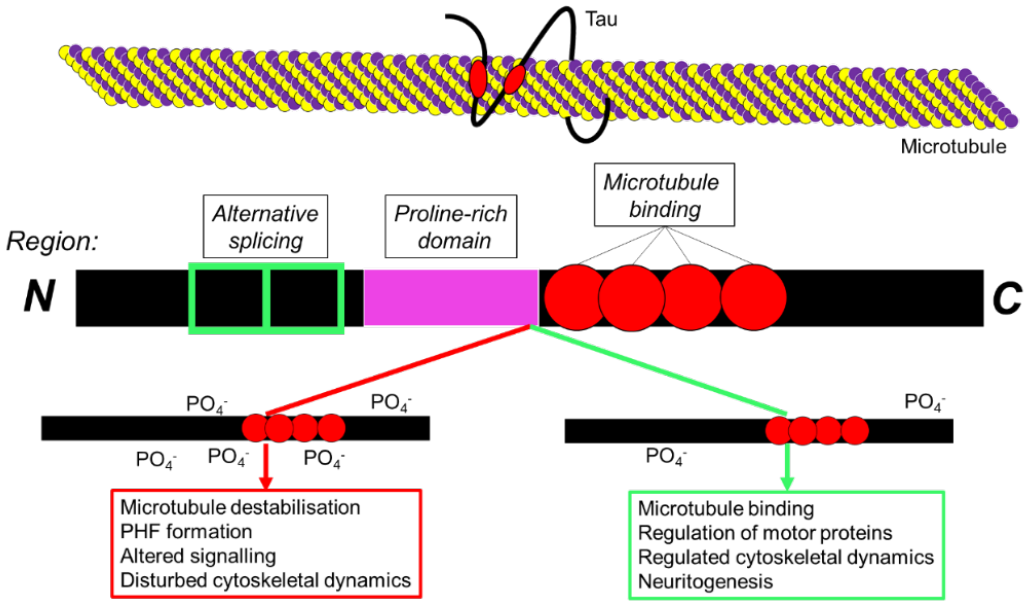


Figure 1.4. Schematic diagram of Tau structure, function and dysfunction. The microtubule-associated protein Tau, encoded by the *MAPT* gene, contains multiple regions implicated in its regulation and function. These include N-terminal alternatively spliced regions in several exons of the *MAPT* gene, proline-rich *PXXP* motifs important for protein-protein interactions, and microtubule binding repeat domains that give rise to juvenile, 3-repeat (3R) Tau, mainly expressed in development or Adult 4-repeat (4R-) Tau (represented). Tau function can also be directed by site-specific phosphorylation. Phosphorylation regulates microtubule binding activity of Tau and the tau-mediated regulation of cytoskeletal dynamics, neuritogenesis and motor proteins involved in axonal transport. Excessive phosphorylation of Tau at specific epitopes can lead to microtubule destabilisation and pTau aggregation, facilitating the formation of characteristic PHFs and NFTs of AD. Adapted from *Goedert, 2004* [63]

The localisation of Tau within neurons is highly dynamic and regulated, and closely linked to its functional specificity. During development, Tau is evenly distributed in the soma and developing neurites, and is primarily localised in the axonal compartment in mature neurons [64, 65]. Notably, 4R Tau is not expressed embryonically or during neurodevelopment, but binds tubulin with a higher affinity [66-68].

Tau is critical to the establishment of neuronal polarity [69], the development and maintenance of axons [70] and neuronal growth and differentiation [71, 72]. Tau participates in the morphology and dynamics of the filamentous actin network known as the lamellipodium, largely by modulating its volume [71] (*for more information, see attached review in Appendix [73]*). Additionally, Tau plays a central role in regulating axonal transport [74, 75]. Neurons have, specialised mechanisms at the Axon Initial Segment to maintain anterograde transport of Tau, which facilitates axonal extension [76]. The retrograde transport of Tau back to the somatodendritic compartment is prevented by a diffusion barrier consisting of intact microtubule networks, and is degraded by site-specific Tau phosphorylation [76]. This is in contrast to the functionally related Microtubule-associated protein 2 (MAP2) protein, which is primarily located in the somatodendritic compartment and rarely observed in the axon [64]. Consequently, Tau is critical in maintaining axonal health. The relatively minor pool of Tau localised to dendritic spines appears to also be highly important in neuron function, through roles in synaptic plasticity and intracellular signalling (discussed below).

1.2.2.2 Phosphorylation of Tau: more than just an agent of disease

Tau distribution and function are essentially informed by site-specific phosphorylation (***Figure 1.3***). What is being increasingly recognised is the importance of Tau in the intracellular signalling process of neurons. While Tau is primarily localised in the axonal compartment [72, 77], the protein-protein interactions and site-directed phosphorylation of Tau in the plasma membrane and microdomains of the plasma membranes in dendrites appear to be equally as important as the canonical functions of Tau.

Adult 4R-Tau has 85 putative sites for phosphorylation -mostly Ser/Thr, and some Tyr residues- many of which have been experimentally validated (***Table 1.1*** [78, 79]). There are several sites of phosphorylation of Tau which are of high significance in the AD process and are mediated by some of the kinases depicted in ***Table 1.1***. These sites of phosphorylation have been well-studied and have been extensively validated (***Table 1.2***). Some of these phospho-epitopes are generated by dual-site phosphorylation, with contribution by more than one kinase, often after priming by one. Some notable phospho-epitopes include PHF-1 and the AT*8* sites (***Table 1.2***).

Kinase	*Target site(s)*	*Functional significance*	*Refs.*
Cyclin-dependent Kinase 5 (cdk5)	Ser199, **Ser202**, **Thr205**, Thr212, Thr231, **Thr235**, **Ser404**	• Primes Tau for GSK-3β phosphorylation. • Promotes Tau aggregation and neurotoxicity. • Regulates Pin1-Tau interaction	[80-83]
p38 MAPK family	Ser199,202,Thr205, Thr231	• p38α/β: Promoted Tau aggregation & neurotoxicity • p38γ: Impairs formation of Tau-Fyn-PSD95 complex • p38γ: Protects against Tau-mediated NMDAR-induced seizures	[84-86]
Cyclic-AMP-dependent protein kinase (PKA)	Thr212, **Ser214**, Thr217, Ser262, Ser404, Ser409, Ser413	• Regulates Tau association to microtubules. • Primes Tau for GSK-3β phosphorylation	[83, 87-89]
Glycogen synthase kinase 3β (GSK-3β)	Thr181, **Ser199**, Ser202, Thr205, Thr212, Ser214,Thr217, **Thr231**, Ser262, **Ser396**, Ser404, Ser409, **Ser413**	• Contributes to AD PHF formation. • Regulates Tau in synaptic plasticity. Modulates Tau association to microtubules.	[83, 90-93]
Abl	Tyr394	• Found in PHFs and foetal brain. • May affect Ser396 phosphorylation by GSK-3β.	[94, 95]
Fyn, Src	**Tyr18**, Tyr197* * *Validated as a Fyn substrate only*	• Found in PHFs and NFTs, and developing neurites. • Facilitates Fyn-Tau binding • Regulation of Fyn-mediated NMDAR excitotoxicity.	[96-99]

Table 1.1 *Notable Tau kinases and their functional importance in neurons.* The major Tau kinases contributing to PHF formation are the proline-directed protein kinases cdk5 and GSK-3β. The other Ser/Thr protein kinase of interest is PKA, whose role in Tau regulation is comparatively unclear. Tyrosine phosphorylation of Tau by Abl and Src-family non-receptor tyrosine kinases (especially Fyn and Src) have garnered a heightened interest recently due to their regulation of non-microtubule binding activities of Tau in neurons. Bold lettering indicates major/primary sites for Tau phosphorylation by respective kinases.

Epitope	*Phosphorylation site*	*Functional consequences*	*Selected References*
AT8	Ser199/202 **+** Thr205	• Microtubule (MT) dissociation of Tau • Implicated in neuro-development • Involved in neurite outgrowth • Increased during mitosis • Structural plasticity of neurons	[100, 101] [102]
AT100	Thr212 **+** Ser214	• MT Dissociation • Nuclear localisation in cellular aging	[103, 104]
AT180	Thr231	• MT Dissociation • Targeting of Pin1 prolyl-isomerase to Tau • Implicated in neuro-development • Structural plasticity of neurons	[105-107]
CP13	Ser202	• Enriched in pre-tangle Tau • Structural plasticity of neurons	[108]
PHF1	Ser396/404	• Highly enriched in Alzheimer PHFs • Essential for synaptic plasticity • Structural plasticity of neurons	[105, 109, 110]

Table 1.2 Common Tau phospho-epitopes and the functional implications of phosphorylation at these sites.

A small fraction of neuronal Tau is phosphorylated on tyrosine residues. Tyr18 phosphorylation is largely mediated by Src-family non-receptor tyrosine kinases (SFKs) Fyn and Src [96, 97, 99]. The association of these kinases with Tau is mediated mainly through their kinase Src-Homology 3 (SH3) domains, which interacts with proline-rich *PXXP* motifs of Tau (***Fig. 1.3***), but also to a smaller extent, through their SH2 domain [111, 112]. Importantly, binding to SH3-Fyn regulates Tau localisation to the plasma membrane. Tau plasma membrane association and interaction with Fyn are both impaired by phosphorylation at the PHF-1 (pSer396/404; ***Table 1.2***) and AT8 (Ser199/202 + Thr205; ***Table 1.2***) phosphoepitopes [113-115]. Interestingly, the phosphorylation at Tyr18 also impairs its interaction with SH3-Fyn and plasma membrane targeting, while enhancing binding to SH2-Fyn [112]. Fyn-tau protein-protein interactions play an important role in modulating excitatory synaptic function, while being independent from the microtubule binding function of Tau [111].

The proline-directed protein kinases (PDPKS) that regulate Tau appear to have similar roles in determining Tau function. GSK-3β, cdk5 and p38α MAP Kinase all contribute to Tau aggregation through phosphorylation of sites such as PHF1, AT8 and AT180 (***Tables 1.2, 1.3*** [85, 109, 116-118]), leading to neurotoxicity. Conversely, recent evidence also shows a protective role of Tau phosphorylation in mouse models of AD, with p38γ phosphorylating Tau at Thr205 and preventing the formation of a Tau-Fyn-PSD95 complex at the post-synaptic

density which mediates excitotoxic stimulation of neurons by Aβ [84]. A major non-PDPK targeting Tau is PKA, which plays a substantial role in determining the binding of Tau to microtubules and interactions with other kinases by phosphorylation [87-89, 119].

1.2.2.3 The role of Tau in neuronal plasticity

Tau distribution in neurons is regulated by a number of mechanisms. For example, Tau mRNA localises to axonal ribosomes via a 3'-untranslated region (UTR) within Tau mRNA and subsequently protein synthesis occurs within the axon [120]. The epitope-specific phosphorylation of Tau (***Table 1.2***) and other cellular mechanisms of partitioning, such as axonal diffusion barriers and proteosomal degradation of Tau, help to restrict its localisation to the axon [76, 121].

Various biological models have helped to establish an essential role for Tau in the initiation and outgrowth of neurite-like processes (the precursors to mature axons and dendrites). Neurite outgrowth is a functional form of neural plasticity and an established model for the study of neurodegenerative mechanisms. In neuroblastoma cells, AT8-phosphorylation of Tau is increased following cAMP-mediated neuritogenesis, and overexpression of Tau enhances neurite outgrowth [102]. AT8 phosphorylation results in microtubule dissociation of Tau, reducing the stability of microtubules, thereby potentially facilitating microtubule reorganisation and extension. Furthermore, in bovine chromaffin cells, insulin-mediated neurite outgrowth involves increased Tau expression [122]. This indicates that both total and phosphorylation levels of Tau are involved in neurite outgrowth.

Long-term changes in synaptic strength and responsiveness have also been linked to Tau function. GSK-3β mediated Tau phosphorylation at Ser-396 is essential for induction of N-methyl-D-aspartate receptor (NMDAR)-mediated down-scaling of synaptic transmission in the hippocampus, also known as long-term depression, LTD, [123]. In demonstrating a role for Tau in LTD, genetic Tau knock-down led to impairments in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-sensitive (AMPA) glutamate receptor internalization. In another recent study, basal synaptic transmission in the mossy fibre tract of the hippocampus was also compromised in Tau knockout mice, as well as transgenic mice expressing a mutant Tau protein that leads to Tau aggregation and mis-sorting in the CA3 region of the hippocampus [124]. Moreover, formation of Tau oligomers was found to induce pre- and postsynaptic structural defects by affecting synaptic vesicle density and synaptic bouton morphology; also altering calcium influx dynamics, resulting in disturbed regulation of synaptic plasticity [124]. Thus, deregulation of Tau in dendrites, despite being a primarily axonal-localised protein, can have far reaching consequences for synapses by promoting their deterioration.

There are other documented forms of neuronal plasticity describing functional and structural changes to neurons associated with characteristic patterns of Tau phosphorylation. Reversible AD-like Tau phosphorylation at multiple epitopes has been observed in diabetic mice [125]. Likewise, a reversible increase in Tau phosphorylation at the AT8, PHF-1, CP13 and AT180 phosphoepitopes (***Table 1.2***) has been observed in hypothermic and anaesthetised mice [126]. Phosphorylation at these sites led to impaired microtubule binding of Tau and cognitive impairment upon mouse arousal. Similarly, in hibernating ground squirrels, highly phosphorylated Tau becomes miss-sorted from its primary axonal localization and redistributed into the neuronal soma and dendrites. This adaptive process leads to changes in neural plasticity, specifically the retraction of mossy fibres from hippocampal CA3 synapses; it is rapidly reversed upon animal arousal [127]. A recent study of Tau in the pyramidal cells of the CA3 hippocampal region of hibernating Hamsters has also given novel insights into its differential physiological roles in apical and basal dendrites [128]. Enrichment of phosphorylated Tau in apical dendrites is correlated with a transient reduction in the size of dendritic spines, but this is not the case in basal dendrites. Unexpectedly, this structural synaptic regression was not correlated with hippocampal memory deficits.

Tau deficiency has previously been associated with behavioural and motor deficits such as hyperactivity, muscle weakness and impairment in fear conditioning tests [80, 129]. However, other evidence suggests Tau reduction prevents seizures, increases inhibitory currents and normalizes excitation/inhibition balance and NMDAR currents in transgenic mouse models of AD overexpressing human APP [130]. Moreover, more recent and advanced genome editing techniques have assisted in a nuanced understanding of the role of Tau in neuronal responses to Aβ insults. For example, the ΔTau74 truncation in which mice express only the projection domain of Tau prevent Aβ-induced excitotoxicity *in vivo* [131]. Similarly, the recent *Tau*$^{\Delta ex1}$ model involves a deletion of the major part of the first exon of the *MAPT* gene and abolishes the expression of Tau also show reduced seizure susceptibility with no impairments in learning and memory formation [132]. Thus, we may conclude that Tau is both important in mediating some forms of neuronal plasticity, and simultaneously facilitating toxic aberrations in neuronal plasticity, including hyperexcitability and excitotoxicity.

1.2.4 Tau pathology and Tauopathies

There are a subset of neuropathologies which involve the toxic accumulation and dysfunction of Tau, known collectively as Tauopathies. These include Pick's disease, Corticobasal Degeneration (CBD) and Frontotemporal dementia (FTD, [133]). Mutations in the *MAPT* gene encoding Tau have been identified in FTD-linked to chromosome 17 (FTD-17), and are attributable to nearly one-third of genetic FTD [134]. Disease-related mutations in Tau lead to energetic deficits at the synapse due to disturbances in cytoskeletal-dependent transport [135,

136] and reduced number of axonal mitochondria [137-139]. In contrast to FTD, genetic Tau mutations have not been found in AD. While there are no known genetic links between Tau and AD, studies of transgenic models bearing FTD-related familial P301L mutation [140-143] continue to provide valuable insights into the role of Tau dysfunction in FTD, but also in AD. Indeed, these genetic models allow us to dissect and understand the direct effects of Tau deregulation on neuronal homeostasis, independently of the effects of Aβ pathology associated with APP models of AD.

In AD, Tau becomes phosphorylated at a rate that is at least three times higher than that under physiological conditions. Current literature suggests that elevated site-directed phosphorylation, rather than gross hyperphosphorylation of Tau, is the driving force behind Tau-mediated microtubule instability [144] and neurodegeneration in AD [145]. For example, phosphorylation at Ser/Thr-Pro sites has been implicated in Tau neurotoxicity in a Drosophila model [146]. Some phosphorylation sites of Tau, such as PHF1 are well-documented to occur in Tau pathology, especially in AD [108, 110], while other sites have different functional implications, such as Thr231, determining Tau as a substrate for Pin1 [82]. Significantly, the mislocalisation, abnormally elevated phosphorylation of Tau, destruction of microtubules and dendritic spines and subsequent neuron dysfunction are hypothesised to be all consequences of Aβ exposure [147].

1.2.5 Neurotoxicity in AD is mediated by Tau

Tau pathology can lead to cognitive impairment and deficits in the structural and functional integrity of neurons. This can occur in models without Aβ pathology [124, 148]. Human studies of sizeable cohorts (n > 2000) have demonstrated that Tau pathology can be observed in individuals up to 20 years before any sign of Aβ pathology becomes visible [149]. Increasing levels of Tau through overexpression sensitises neurons to transport deficits; this leads to a lack of mitochondria at the synapse, resulting in oxidative stress and energy deficits, impairing neurite outgrowth [150].

Tau is required for Aβ-induced deficits in axonal transport [137] that ultimately culminate in disturbing dendritic spine dynamics [151]. Introducing human-derived Aβ into animal models is sufficient to induce LTD and NMDAR-dependent dendritic spine loss [152]. However, the neuronal hyperexcitabiliy and excitotoxicity induced by Aβ pathology in rodent models is abrogated when the animals do not express Tau [130, 153], further indicating that Tau is essential for Aβ-mediated dendritic alterations. These data provide a basis for the hypothesis that Aβ drives Tau dysfunction through synaptic mechanisms.

Primary neuron exposure to toxic oligomeric Aβ also induces a redistribution of Tau to the soma and dendrites, impaired transport of organelles and marked microtubule loss [139]. The

interaction between Fyn and Tau, and the localisation of Fyn at excitatory synapses are essential for the synaptotoxic effects of Aβ. The expression of a truncated form of Tau lacking MBDs or Tau-knock-down models prevent the interaction between Fyn and NMDARs, thereby avoiding Aβ-mediated hyperexcitability *in vivo* [131]. Thus, Tau plays a key role in mediating the neurotoxicity and inducing intracellular signalling pathways that promote neurodegeneration. There is currently strong support for the "Tau axis hypothesis of AD" (outlined in [154]), wherein Aβ and p-Tau converge to produce multiple neurotoxic effects, including deficits in axonal transport, damage to the neuronal cytoskeleton [139], and oxidative stress induced through mitochondrial dysfunction [137].

Since phosphorylation of Tau plays such a critical role in mediating many toxic events observed in AD, the regulation of p-Tau is of particular interest. Like other cellular phosphoproteins, the phosphorylation state of Tau depends on the concerted action of endogenous protein kinases and phosphatases. There are multiple protein kinases (***Table 1.1***) and protein phosphatases regulating Tau [155, 156]. One major Ser/Thr phosphoprotein phosphatase in the brain, protein phosphatase 2A (PP2A), is also the main Tau phosphatase in neurons [157]. Our group has shown that PP2A expression and activity levels decrease in AD-affected brain regions (namely, the cortex and hippocampus) in a manner that correlates with the presence of pTau pathology [158, 159]. Based on the central role of PP2A in modulating pTau levels and its dysfunction in AD [160], we are especially interested in elucidating how exactly PP2A is regulated and how PP2A-Tau functional interactions can change in health and disease.

1.3 A nuanced role for Protein phosphatase 2A in Alzheimer's disease

1.3.1 A brief introduction to the PP2A family

In all cells, the balance of protein phosphorylation is determined by the action of kinases and phosphatases. The major protein Serine and Threonine phosphatase family known as PP2A comprises at least 96 distinct members that collectively contribute to the regulation of most cellular functions [161]. The typical PP2A enzyme exists as a heterotrimer consisting of a scaffolding subunit (A, *PPP2R1A/B* isoforms), a catalytic subunit (PP2Ac, *PPP2CA/B* isoforms) and one of many regulatory B-type (B, B', B" or B'" families) subunits that are encoded by 15 different genes, with 23 regulatory subunits in total identified thus far. This dazzling complexity helps to inform PP2A enzyme substrate specificity, subcellular localisation and differential expression throughout tissues (reviewed in detail in [162]). PP2A is also regulated by a plethora of endogenous and exogenous factors. For instance, inhibitory toxins such as okadaic acid, and endogenously expressed inhibitors such as I_1PP2A and I_2PP2A (also called "SET"), which bind to PP2A and impair its phosphatase activity [163]. Biological "activators" of PP2A have a range of mechanisms and selectivities for PP2A holoenzymes,

and are often classed accordingly. Some major “activators” of PP2A include quinolone, ceramides, memantine and sodium selenate. Those can either act directly on PP2A, indirectly inhibit endogenous PP2A inhibitors, or work in tandem with other activators through different mechanisms (reviewed in [164]). In most cases, though it is worth mentioning that the exact mechanisms underlying the modulation of PP2A activity by endogenous or exogenous activators remain to be fully revealed and validated.

1.3.2 Fine-tuned control of PP2A complexes

In addition to subunit composition, multiple post-translational modifications also modulate PP2A substrate specificity, subcellular compartmentalisation and interaction with a diversity of partners. Catalytic and regulatory-type subunits have been described to be post-translationally modified. Importantly, post-translational modifications such as PP2Ac methylation are important in determining subunit composition and subcellular distribution of PP2A holoenzymes [165].In addition, phosphorylation of certain PP2A subunits has been reported to modulate holoenzyme phosphatase activity, as detailed below.

1.3.2.1 Post-translational modifications in PP2A subunits critically regulate PP2A

PP2A is reversibly methylated (***Fig. 1.5***) at the C-terminal Leucine-309 of the catalytic subunit, by the dedicated Leucine Carboxyl Methyltransferase 1 enzyme (LCMT-1, [166, 167]). Conversely, Protein Phosphatase Methylesterase 1 (PME-1) demethylates PP2Ac. PME-1 also binds to and inactivates PP2A by an independent mechanism [168]. Notably, PP2Ac methylation also critically modulates the binding of the PP2A chaperone protein, α4 and Target of rapamycin signalling pathway regulator (TIPRL), which plays a role in recycling mature PP2A complexes [169].

PP2Ac methylation informs the formation of specific heterotrimeric holoenzymes through regulatory subunit binding. It has been shown to promote B-family (including Bα) subunit recruitment to the core (AC) enzyme (***Fig. 1.5***), while having no significant effect on assembly of holoenzymes containing other regulatory B’-type subunits [170]. Thus, methylation of PP2Ac can significantly influence the biogenesis of (ABαC) holoenzymes and intracellular enzyme composition, thereby affecting PP2A substrate specificity and subcellular localisation [165, 171, 172]. However, the regulation and function of PP2A methylation in neurons is still not well understood. This will be explored in subsequent chapters of this thesis.

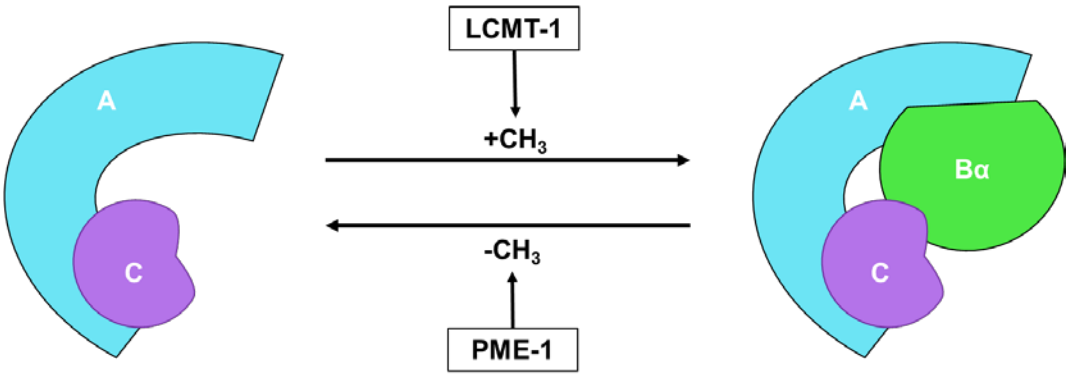


Figure 1.5 PP2A (ABαC) holoenzyme formation. The association of the B-family (especially Bα) regulatory subunits of PP2A with AC dimers is dependent on PP2Ac methylation at the L309 residue by the methyltransferase, LCMT-1. Inactive PP2A can be found in complex with PME-1, which not only demethylates L309 through its function as a methylesterase, but also inactivates PP2Ac through displacement of manganese ions in the catalytic pocket.

Besides methylation, PP2Ac has been reported to be Tyrosine phosphorylated by tyrosine kinases such as viral Src (v-Src), and non-receptor tyrosine kinases, including growth-factor and insulin-mediated signalling, resulting in its inactivation [173, 174]. In these landmark studies, it was noted that this phosphorylation is very transient and labile, based on observations that PP2A is capable of rapid intramolecular auto-dephosphorylation, leading to a restoration of activity. Moreover, tyrosine phosphatases such as Protein Tyrosine Phosphatase 1B (PTP1B) can dephosphorylate tyrosine-phosphorylated PP2Ac. Based on *in vitro* assays with purified PP2Ac, it was proposed that PP2A phosphorylation solely occurs at the Tyr307 (Y307) site. However, it is important to note that this has never been validated *in vivo*, or by using more advanced techniques like mass spectrometry approaches. Of particular, relevance to our project, increased levels of tyrosine phosphorylated PP2Ac have been identified in AD brain tissue, relative to normal controls. In these studies, immunohistochemical and confocal microscopy analyses with commercial pY307-PP2Ac antibodies indicated that tyrosine phosphorylated PP2Ac was associated with pre-tangle and tangle-bearing neurons [175]. Yet, the precise mechanisms of PP2Ac Tyrosine phosphorylation are yet to be fully characterised and will inform part of this Thesis (Chapter 6).

The Thr304 residue of PP2Ac has also been reported to be phosphorylated in vitro by auto-phosphorylation activated protein kinase [176, 177]. Phosphorylation of Thr304 has been linked to PP2A inactivation. It also modulates the association of PP2Ac with B-family members, but not other regulatory subunit types [178, 179]. Yet, the regulation and functional significance of Thr304 phosphorylation remains to be established *in vivo*. Lastly, like many other cellular proteins, PP2Ac is targeted for degradation by ubiquitination. It has been proposed that the targeting of PP2Ac by the E3 ubiquitin ligase Midline 1 (Mid1) determines the phosphorylation of many microtubule-associated proteins by influencing the levels of PP2A associated with the microtubule network [180]. The α4 subunit provides a scaffold for

the association between Mid1 and PP2Ac at microtubules [181]. Mid1 mono-ubiquitinates α4 prior to a change in α4 conformation, which leads to Mid1-mediated PP2Ac ubiquitination, inactivation and degradation [182]. This represents one example of the fine-tuned control of PP2Ac levels and activity in cells.

1.3.2.2 Modifications in A and B-type subunits can also influence PP2A regulation

B'-subunit family members have also been reported to be phosphorylated at several sites. Of relevance to the CNS, activation of cAMP/PKA signalling promotes the phosphorylation of the B56δ subunit at Ser566; this promotes PP2A activation and subsequent dephosphorylation of DARP-32 at Thr75, which coordinates the efficacy of dopaminergic neurotransmission in striatal neurons [183]. Likewise, PKC phosphorylates the B'δ subunit at Ser566, and the resulting activation leads to the Ser40 dephosphorylation and inactivation of tyrosine hydroxylase, a key enzyme in the synthesis of catecholamines [184]. Phosphorylation of B'δ at Ser573 [185] and phosphorylation of B'α at Ser41 [186] that display opposite effects on PP2A catalytic activity have also been reported in non-neuronal models.

It is likely that similar phosphorylation of B' subunits occurs in the neuronal context and is relevant to the regulation of other established neuronal functions of (AB'C) holoenzyme. For instance, in Drosophila, PP2A-B' homologs are essential for presynaptic functioning, vesicle density and active zone formation, regulated by GSK-3β [187]. B' complexes are critical in distal axon, synaptic dense-core vesicle and Ca^{2+} channel organisation via association with the F-actin binding protein Liprin-α1, which itself is essential for active zone assembly [188]. Lastly, the scaffolding (A) subunit of PP2A is also Ser/Thr phosphorylated. Ser303, Thr268 and Ser314 have all been identified as sites for PP2A-A phosphorylation *in vivo*, which may modulate the localisation and subunit interaction dynamics of PP2A heterotrimers [189]. Yet, much remains to be learned on the regulatory function of PP2A subunit phosphorylation.

1.3.2.3 Differential distribution of PP2A holoenzymes modulate their function

Another major mode of PP2A regulation lies in the holoenzyme subcellular compartmentalisation. Post-translational modifications of PP2A subunits can influence the enzyme spatial distribution and thus its ability to interact with, and dephosphorylate substrates. PP2Ac methylation promotes the accumulation of (ABαC) in lipid rafts and the subsequent dephosphorylation of Tau in the plasma membrane [165]. Demethylated pools of PP2Ac, conversely, are concentrated to non-raft domains of the plasma membrane. Additionally, the A subunit is palmitoylated, which targets PP2A complexes to the plasma membrane, thereby contributing to functional roles of PP2A in this compartment [190].

1.3.3 PP2A is an important enzyme in neuron homeostasis

While a primary focus of PP2A in neurons and AD has typically centred on its role as a Tau phosphatase, PP2A also plays other important functional roles, for example in the synaptic compartment. The viral (SV40) *small t* antigen binds to the A and C subunits of PP2A in a similar way to B-type subunits [191], rendering the enzyme inactive. Mice with inducible *small t* expression in forebrain and hippocampal regions are entirely deficient in NMDAR-mediated LTD induction [192]. Meanwhile PP2A dephosphorylates and modulates the function of multiple receptor subunits that contribute to excitatory transmission [193, 194]. In addition to receptor subunits, PP2A can also dephosphorylate and regulate the permeability of neuronal Ca^{2+} channels [195, 196].

The role of PP2A in neuronal plasticity is not limited to regulation of membrane-bound proteins like ion channels and receptors. Modulation of several Actin-regulating proteins in dendritic spines is also PP2A-dependent and determines the morphology and synaptic function of the spines [197]. PP2A also dephosphorylates tubulin and associates with microtubules [198]. This is in addition to the binding to and dephosphorylation of microtubule-associated proteins such as Tau and MAP2 [199], and the regulation of dynamic microtubule instability [198, 200] by PP2A. There are extensive roles played by PP2A in different aspects of cytoskeletal regulation in neurons – we have reviewed this area in depth and attached the published article in the appendix of this thesis.

1.3.3.1 PP2A plays a critical role in the regulation of the neuronal cytoskeleton – <u>See attached review [73] for in-depth discussion</u>

The microtubule, F-actin and neurofilament networks play a critical role in neuronal cell morphogenesis, polarity and synaptic plasticity. Significantly, the assembly/disassembly and stability of these cytoskeletal networks is crucially modulated by protein phosphorylation and dephosphorylation events. In the attached review, we examined the role of PP2A (and other phosphoprotein phosphatases PP1 and Calcineurin) in the homeostasis of the neuronal cytoskeleton. There is strong evidence that these enzymes interact with and dephosphorylate a variety of cytoskeletal proteins, resulting in major regulation of neuronal cytoskeletal dynamics. Conversely, we also discussed how multi-protein cytoskeletal scaffolds can also influence the regulation of PP2A, PP1 and Calcineurin, with important implications for neuronal signalling and homeostasis. Deregulation of these scaffolds and PP2A are associated with many neurological diseases. The article was part of a Special Issue entitled “Cytoskeleton-dependent regulation of neuronal network formation” in *Molecular and Cellular Neuroscience*.

1.3.3.2 PP2A is the major Tau phosphatase and Tau regulator in the brain

PP2A represents about ~71% of all Tau phosphatase activity in neurons [157]. The most potent PP2A species in the dephosphorylation of Tau is the ABαC heterotrimer [201, 202], which binds to the MTB-domain of Tau. Therefore, PP2A-Tau binding does not occur at the microtubule. Likewise, PP2A only dephosphorylates Tubulin-unbound Tau [73]. The proline-rich region (*PXXP* motifs, ***Fig. 1.3***) just upstream of the MTB repeat domains is critical for PP2A binding of Tau, and as such, PP2A-ABαC has a much higher affinity for 4R-Tau, compared to foetal and juvenile 3R-Tau [199, 201]. Of note, FTD-17 mutations in Tau (such as the P301L substitution in the *PXXP* domain) in the PP2A-ABαC binding region impair PP2A-Tau association by as much as 70% [140], while phosphorylation-mimicking glutamate clusters in the N-terminal proline rich region of Tau also inhibit PP2A binding [203]. Similarly, phosphorylation of Tau at Thr231 also inhibits PP2A binding so that pThr231-Tau is poorly dephosphorylated by PP2A [199, 204]. Importantly, PP2A plays a greater role in the regulation of Tau phosphorylation than the Tau kinases GSK-3β and cdk5, since simultaneous phosphatase and kinase inhibition results in Tau hyperphosphorylation in the mouse hippocampus [205].

1.4 PP2Ac Methylation: Classical and new modes of regulation

1.4.1 One-carbon metabolism is the typical regulator of PP2A methylation

The activity of LCMT-1, and therefore the methylation state of PP2A are dependent on cellular availability of methyl donors. The universal methyl donor for LCMT-1 and all other methyltransferases is S-adenosylmethionine (SAM). Supply of SAM is dependent on a complex pathway of biochemical reactions described as “one-carbon metabolism” (***Fig. 1.6***). This cycle ultimately transforms dietary folate into the methyl donor SAM. Briefly, dietary folate is first converted to 5-Methylfolate (5-MTHF) via a series of metabolic reactions, including the conversion of 5,10-MTHF to 5-MTHF by 5,10-Methylenetetrahydrofolate reductase (MTHFR). The methyl group of 5-MTHF is then used in the remethylation of homocysteine (Hcy) to methionine by methionine synthase, for which dietary vitamin B12 is a cofactor. Methionine is converted by methionine adenosyltransferase to SAM which donates the methyl group to methyltransferases, leading to functional methylation of proteins, lipids or DNA [167, 206-208]. This reaction results in the generation of S-Adenosyl-homocysteine (SAH), which is then hydrolysed to Hcy. Hcy can re-enter this transmethylation cycle. Alternatively, Hcy may also be converted to cystathionine and cysteine via Cystathionine-β-Synthase (CBS), via the transsulfuration pathway [209].

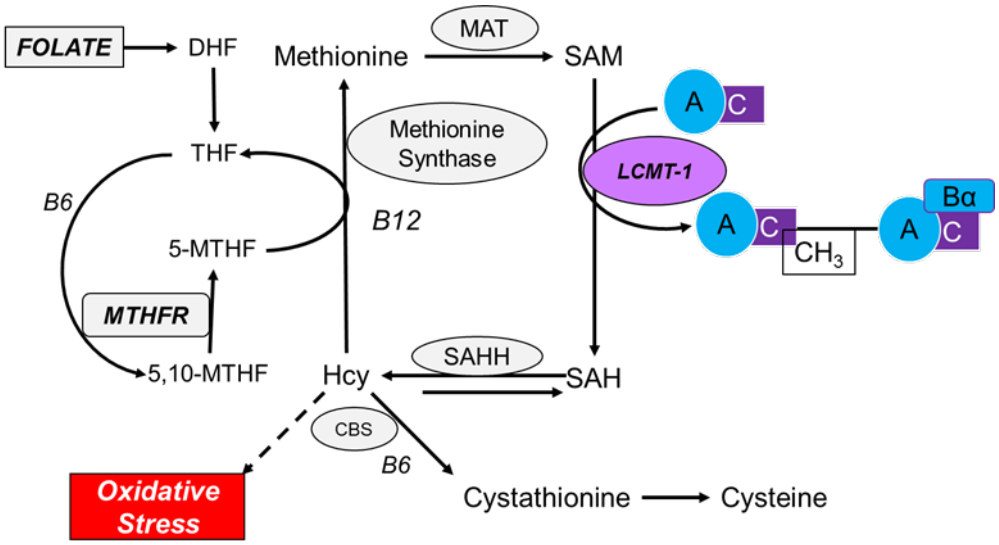


Figure 1.6: One-carbon metabolism controls LCMT-1 activity and PP2Ac methylation. In this cycle, methionine is metabolised into the universal methyl donor S-adenosylmethionine (SAM) by methionine adenosyl-transferase (MAT). After methyl group transfer to methyltransferases like LCMT-1, the metabolite S-adenosylhomocysteine (SAH) is produced and hydrolysed to homocysteine by SAH-hydrolase (SAHH). Homocysteine can be metabolised into cystathionine and then cysteine through a transsulfuration pathway involving cystathionine β synthase (CBS) and requiring vitamin B6 as cofactor. Alternatively, homocysteine can be converted to methionine by methionine synthase in a reaction requiring 5-methyltetrahydrofolate (5-MTHF) and vitamin B12. Significantly, the generation of the active folate metabolite, 5-MTHF, is under the control of the 5, 10-methylene tetrahydrofolate reductase (MTHFR) enzyme.

1.4.2 Altered one-carbon metabolism: A risk factor for Alzheimer's disease

Several epidemiological studies have linked disturbances in folate and Hcy metabolism with brain atrophy, cognitive decline and increased risk for AD [210-212]. Elevated plasma Hcy levels nearly double the risk of developing AD in the elderly [213, 214]. Recent meta-analyses further support a causal link between elevated plasma total Hcy (tHcy) and AD [215] and a link between common polymorphisms in the *MTHFR* gene critical in one-carbon cycling in cells, and increased risk for AD [216].

Elevated plasma total Hcy (tHcy) was initially noted in clinical cases of AD [217] just prior to this being confirmed in post-mortem histopathological investigation [218]. Subsequent larger-scale studies in cohorts of people already diagnosed with the disease have also identified plasma tHcy as a strong risk factor for AD [214]. Long-term, prospective studies retrospectively assessing the risk of AD development found that women with mid-life elevations in tHcy were more prone to the development of dementia [219]. Additionally, neuroimaging studies have indicated that elevated Hcy worsens cerebral white matter lesions in patients with AD [220], and that poor vitamin B12 status is associated with intensified cerebral white matter lesions [221]. Common polymorphisms in *MTHFR*, elevated plasma tHcy and reduced plasma folate levels have each been independently associated with increased AD risk [222]. Recent meta-analyses further support a causal role for the *MTHFR* C677T polymorphism in AD by elevating circulating tHcy [215, 216]. A recent consortium statement from experts also suggests that elevated plasma tHcy is a modifiable risk factor for development of cognitive decline dementia and AD [223].

Clinical trials aiming to alleviate AD symptoms with B-vitamin supplementation have yielded mixed results, likely owing to the complex heterogeneity of the disease and the swathe of risk factors involved in AD (***Fig. 1.1***), and in some instances, arguably poor trial design. Properly conducted double-blinded randomised trials in 800+ participants have unarguably demonstrated that long-term folic acid supplementation enhances cognitive function as assessed by the diagnostic mini-mental state examination, MMSE [224]. However, it was reported that B-vitamin supplementation (folate, B9 and B12) is not an effective treatment in AD patients wherein plasma tHcy levels are not elevated [225]. This suggests that using supplementation with B-vitamins, including folate, to slow or reverse symptoms of AD may be ineffective unless an individual is already B-vitamin deficient. Other findings suggest that while B-vitamin supplementation could assist the slowing of grey matter atrophy in AD [226], the patient must start with healthy ω-3 fatty acid levels, otherwise whole brain atrophy is unaffected [227]. This reiterates the importance of interacting risk factors of AD, including dietary, genetic and metabolic risk factors (***Fig. 1.1***). In addition, it is worth mentioning that some of the published negative B-vitamin trials may be flawed due to poor design, for instance

those with small cohorts, are non-randomised non-blinded and by not adequately taking into account vitamin absorption and genetic heterogeneity of participants [223]. Another confounding factor in clinical trials assessing effects of plasma tHcy and the onset of AD are measurements of cognitive activity; otherwise meaningful studies have been limited by a lack of baseline measurements of cognition in placebo groups [228]. Other essential criteria identified by the above consortium of experts include baseline levels of B-vitamins and plasma tHcy, an absence of dementia at the start of the trial, and the trial must have a clinically meaningful duration to measure losses in brain volume [223]. A similar confounding factor in such studies includes the effect of common *MTHFR* polymorphisms which affect folic acid absorption; un-metabolised folic acid does not enter the brain [229], and could even be detrimental [230, 231]. It thus remains to be seen whether further trials with active B-vitamin compounds can mitigate or prevent conversion to dementia.

1.4.3 Disturbances in one-carbon metabolism affect neuronal function and homeostasis by several mechanisms

First, disturbances in one-carbon metabolism (illustrated in ***Fig. 1.6*** and ***Table 1.3***) exert damaging effects in the brain through a deficit in methyl group supply essential for neuronal homeostasis, including those necessary for protein and epigenetic modifications. Hereditary malabsorption of folate is linked to retardation in neural development [232]. *MTHFR* gene polymorphisms that lead to decreased MTHFR activity are common in humans – up to ~50% of some ethnic populations possess at least one C677T allele. Meanwhile, severe MTHFR deficiency due to the TT genotype, which affects ~12% of populations, leads to hyperhomocysteinaemia (HHcy) and decreased methionine synthase activity (***Fig. 1.5***). This is associated with neurological abnormalities [233]. Mouse models of severe MTHFR deficiency show a number of behavioural and neural biochemical deficits including HHcy and altered methylation potential, as well as increased mortality rates [234]. They have a loss of hippocampal volume, increased hippocampal apoptosis, disturbances in neurotransmitter metabolism and reduced cognition and memory [235]. MTHFR deficient mouse models also show reductions in cerebellar volume, with subsequent disturbances in spatial memory [235]. This suggests that the brain is particularly sensitive to disturbances in one-carbon metabolism, and that methyl group supply is essential for neuron function.

Treatment of Neuro-2A (N2a) neuroblastoma cells with exogenous Hcy also blocks the extension of neurites [236]. Exposure to Hcy leads to apoptotic cell death of Purkinje fibres cultured *in vitro*, following reduced neurite length and arborisation [237]. Hippocampal and Purkinje cell death may also be exacerbated by aberrant synaptic function in animal models wherein one-carbon metabolism is disturbed. Many changes in synaptic function induced by alterations in one-carbon metabolism are linked to glutamate receptors. Animals with severe

MTHFR deficiency exhibit a range of neurological abnormalities including seizures that are typical of glutamate hyperexcitability [234]. Conversely, excess 5-MTHF stimulates kainate-type glutamate receptors and lowers seizure threshold in offspring [238]. Notably, Hcy and its metabolite, L-Homocysteic acid, bind to glutamate and glycine binding sites of NDMARs [239], suggesting a direct mechanism for the hyperexcitability observed in these models. Direct treatment of *ex vivo* slices with Hcy bi-directionally alters the activity-dependent increases in synaptic strength underlying cellular memory and long-term potentiation (LTP), in a concentration-dependent manner [240]. *In vivo*, chronic Hcy exposure enhances reversal learning performance of mice in the Morris Water Maze [241], suggesting an enhancement of LTD induction that is consistent with overall prolonged NDMAR activation.

Hcy is also readily oxidised and its metabolites have been linked to oxidative stress and neuronal damage [242]. Aside from the transmethylation pathway catalysed by methionine synthase, illustrated in ***Fig 1.6***, Hcy can be metabolised by CBS in the transsulfuration pathway, which leads to the formation of glutathione (GSH), a major cellular antioxidant [243]. Importantly, methionine synthase activity is impaired by the presence of free radicals, while the opposite is true for CBS [244, 245]. Therefore, transsulfuration of Hcy to the antioxidant GSH is also sensitive to redox changes in the brain [246]; this pathway is a major redox buffer system in mammalian cells. It has also been demonstrated that, in addition to elevated Hcy, there is also a pronounced loss of GSH in the AD brain [247]. This imbalance may contribute to apoptotic cell death in the hippocampus and can be mediated by intracellular Ca^{2+} elevation and caspase activation [248].

Similarly, folate deprivation also induces neuronal impairment. Dietary folate deficiency impairs vesicular transport, synaptic plasticity and neurite outgrowth and increases sensitivity to apoptosis [249]. Significantly, active folate also possesses antioxidant properties [250], and folate deficiency has been associated with oxidative stress through its ability to increase Hcy [222, 251, 252], or by independent mechanisms such as the scavenging of free radicals [253-256]. Thus, perturbed one-carbon metabolism leads not only to neurotoxicity, but also induces severe changes in synaptic function before cell death ultimately occurs.

Type	Experimental Paradigm	Neurological Indications	Selected Refs.
In vivo dietary interventions	Methionine-enriched (Including High-Methionine/Low-Folate)	• ↑ pThr668-APP • ↑$A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$ secretion • ↑Tau expression and Alzheimer-like phosphorylation	[257, 258]
	"B-vitamin" (B6, B12 and Folate) deficient	• ↑PS1, BACE1 expression and Tau phosphorylation in TgCRND8 (dual APP mutations) and wild-type mice. GSK-3β activation and impaired PP2A activity. Rescued by SAM supplementation. • ↑$A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$, sAPPα/β • Elevated Homocysteine, Impaired DNA repair and sensitisation to Aβ • Acceleration of Aβ formation in Tg2576 (APP-Swedish mutation). Higher GSK-3β activity.	[259-261]
	pseudo-MTHFR deficiency	• Induced by high-folate/methionine diets. Short-term memory impairments	[231]
In vivo genetic disturbances of enzymes involved in one-carbon metabolism	Cystathionine-β-Synthase deficient	• ↑pThr668-APP, ↑ Alzheimer-like pTau, ↓PP2Ac methylation. • Exacerbated $A\beta_{40}$, $A\beta_{42}$ deposition in female APP / PS1 transgenic mouse model of AD	[258, 262]
	5,10-methylenetetrahydrofolate reductase deficient	• ↑ Alzheimer-like pTau and ↓PP2Ac methylation in Cortex, Hippocampus, Midbrain and Cerebellum • Impaired motor function, long-term and short-term memory. Reduced whole brain and hippocampal volume, reduced thickness of CA1 and CA3 regions of the hippocampus. Deregulated Choline signalling systems. • Reduced volume of Cerebellum and cerebral Cortex, with enlarged lateral ventricles	[235, 263, 264]
	Methionine synthase deficient	• Altered Choline metabolism and neurotransmission. Impaired learning and memory.	[265]

Table 1.3 In vivo models and consequences of disturbed one-carbon metabolism. This table describes some key models utilised *in vivo* which induce deficiencies in one-carbon metabolism by dietary or genetic means. Key neurological consequences relevant to AD are outlined.

1.4.3.1 One carbon metabolism is tightly linked to neurotransmission

Not only do alterations in folate metabolism in the brain affect responses to neurotransmission, but these aberrations impair the synthesis of multiple neurotransmitters – SAM donates a critical methyl group to tetrahydrobiopterin (BH_4), which is an essential cofactor for phenylalanine, tryptophan and tyrosine hydroxylases, the rate limiting enzymes in catecholamine (adrenaline, noradrenaline and dopamine) and tryptamine (serotonin and melatonin) synthesis [266].

Additionally, cholinergic signalling in the brain is also tightly linked to folate metabolism. Acetylcholine (ACh) is a highly important neurotransmitter in the CNS [267]. Its precursor, choline, can be converted to betaine [268]. In turn, betaine serves as a methyl donor, allowing for Hcy remethylation in the absence of SAM [269]. However, it is worth noting that this pathway only occurs in the peripheral tissue (for example, the liver), but not in the CNS, due to the absence of betaine Hcy methyltransferase in the brain. Brain choline efflux facilitates peripheral betaine conversion and is inhibited by N-methyl-nicotinamide, which is formed by SAM-dependent methylation [270]. In conditions of dietary folate or genetic MTHFR deficiencies, peripheral tissues have a reduced SAM:SAH ratio. As a compensatory mechanism, brain choline efflux is increased to restore the balance between methyl donors and metabolites [271]. Reduced ACh supply has been linked *in vivo* to lowered dietary folate, MTHFR deficiency and impaired cognition in an SAM-dependent manner [267].

1.4.4 Disturbed one-carbon metabolism induces deregulation of Tau and APP, at least in part by altering PP2A methylation

Since LCMT-1 activity is dependent on SAM supply (***Fig. 1.6***), the methylation of PP2Ac is also contingent on continuous SAM supply. Our group has demonstrated in murine models that severe MTHFR deficiency and low dietary folate can synergise to downregulate PP2Ac methylation and Bα expression with a concomitant increase in pTau formation in the cortex, hippocampus and cerebellum [263]. Importantly, PP2Ac methylation and LCMT-1 expression are reduced in affected brain regions from autopsy AD brain tissue [158, 159]. *In vivo* injection of Hcy also induces Tau phosphorylation in the hippocampus by impairing PP2A methylation [272, 273].

The phosphorylation of APP at Thr668 has implications for neuronal cell biology and the pathogenesis of AD. APP is yet another protein which is subject to regulation by PP2A via dephosphorylation. PP2A regulates APP directly by dephosphorylation, and indirectly by regulating kinases which target APP [274, 275]. The incubation of neuronal cells with the broad-spectrum phosphatase inhibitor Okadaic Acid (OA) has been used as a basic model to identify potential functions of PP1 and PP2A [42, 276]. OA treatment of N2a cells not only

leads to APP Thr668 phosphorylation, but also an increase in β and γ-secretase activities, with eventual Aβ generation [276, 277].The inhibition of PP2A promotes JNK activation and APP phosphorylation at Thr668 [42, 274, 278].

Despite the limitations of using OA as an experimental model to specifically inhibit PP2A, other evidence indicates that PP2A regulates the phosphorylation of APP in neurons. Our group has shown that impaired PP2Ac methylation results in increased phosphorylation of APP at Thr668 and concomitant generation of sAPPβ, with a parallel loss of the neuroprotective sAPPα [258] in both cells and *in vivo*. These effects appear to be a product of the reduced methylation potential in N2a cells and *in vivo* from an altered SAM:SAH ratio and Hcy exposure [258]. The loss of methyl-PP2Ac has functional implications for the development of AD, however we will also explore the consequences of impaired one-carbon metabolism on the phosphorylation of APP and the related mechanisms in Chapter 5, using the MTHFR-deficient mouse model used in previous studies and in Chapter 4 (***Table 1.3***, [263]).

1.5 PP2A methylation: a potential target of cAMP/PKA signalling

1.5.1 Neuronal cAMP/PKA signalling: overview

The signalling cascade affected by the generation of cAMP is a particularly important one in neurons. Activation of adenylate cyclase is responsible for the formation of cAMP following local spikes in Ca^{2+} concentration, or ligand binding to G-protein coupled receptors (GPCRs, ***Fig. 1.7***). The binding of cAMP to one of two regulatory subunits on the tetrameric cAMP-dependent protein kinase (PKA), leads to the release of two PKA catalytic subunits which phosphorylate Ser/Thr residues within [Arg-Arg-X-Ser*/Thr*-X] motifs where X is a hydrophobic amino acid [279]. Within the cell, cAMP availability and binding to PKA is determined by temporal diffusion gradients, degradation of cAMP by phosphodiesterases and cellular localisation and distribution of PKA, determined by A-kinase anchoring proteins (AKAPs, reviewed in [280]). There is also a pool of PKA and adenylate cyclase localised to the nucleus, indicating that PKA signalling in this compartment has a high level of significance [281]. This complex spatiotemporal regulation contributes to cAMP/PKA signal specificity.

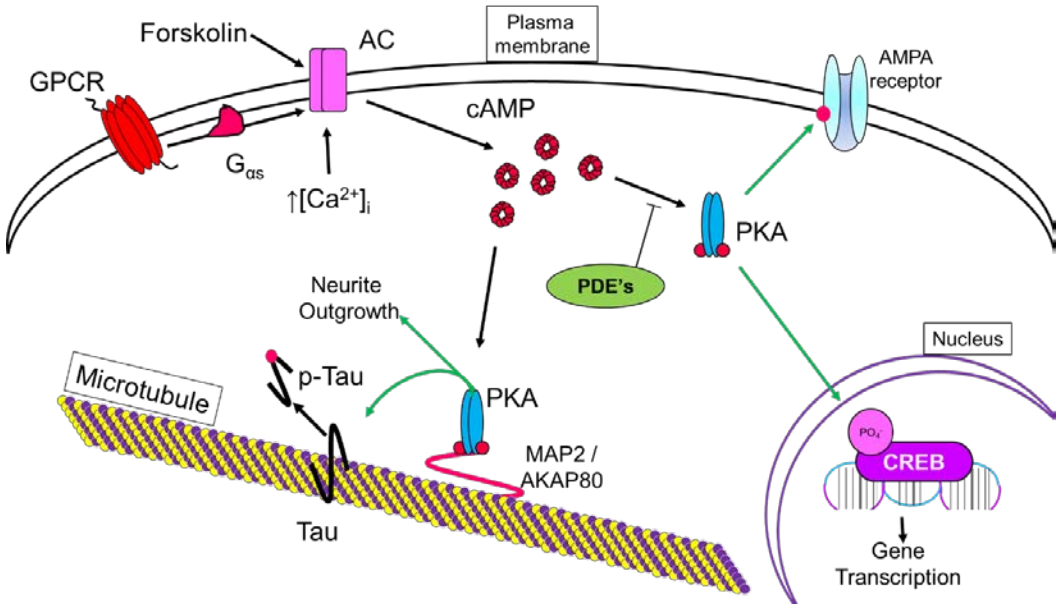


Figure 1.7: Neuronal cAMP/PKA signalling. Adenylate cyclase (AC) becomes activated following stimulation of metabotropic neuronal receptors, or elevation of intracellular calcium (which can occur in response to neuronal calcium influx from ionotropic glutamate receptors or signal-dependent release of calcium from the endoplasmic reticulum). Calmodulin plays an important role in modulating the activation of adenylate cyclase by calcium. Adenylate cyclase is also directly activated by drugs like Forskolin. Activation of the enzyme stimulates the synthesis of cAMP. Subsequently, cAMP binds to the regulatory subunits of PKA, causing release and activity of two catalytic subunits. PKA activity is therefore dependent on available pools of cAMP, degraded by phosphodiesterases (PDEs). Active PKA directly phosphorylates glutamate receptors, stabilising them at the plasma membrane, which contributes to LTP. PKA phosphorylates and activates the nuclear transcription factor CREB, which promotes consolidation of LTP by promoting gene transcription of proteins involved in synaptic plasticity. PKA is targeted to the microtubule network by microtubule-associated protein 2 (MAP2) and phosphorylates Tau at Ser214, which modulates the binding of Tau to MTs. PKA signalling is also involved in neurite outgrowth and adult neurogenesis in the hippocampus.

1.5.1.1 CREB: An important transcription factor targeted by cAMP/PKA signalling

The nuclear target of PKA signalling, the cAMP-Responsive Element Binding protein (CREB), plays an important role in regulating the expression of multiple proteins involved in key neuronal functions (see below). CREB targets regions of DNA known as cAMP-responsive elements (CREs) that regulate gene transcription. CREB is activated by PKA-mediated phosphorylation at the Ser133 site; thus, this phosphorylation is often used as a readout for CREB activity [282, 283]. PKA also phosphorylates and activates Ser/Thr kinases in neurons which target CREB, such as mitogen-activated protein kinase (MAPK) and calcium-calmodulin activated protein kinase (CaMK) family members [284, 285]. CREB phosphorylation induces association with CREB-Binding Protein (CBP) [286] and CREB-regulated transcriptional coactivators (CRTCs), leading to gene transcription via a basic leucine zipper region within CREB binding to CREs [287].

1.5.1.2 cAMP/PKA signalling: an important regulator of the cytoskeleton

The cAMP-PKA signalling pathway regulates multiple aspects of neuronal functionality. PKA signalling critically modulates neurite outgrowth and differentiation and regulation of cytoarchitecture (***Figure 1.7***). In multiple models of neuronal differentiation, the PKA signalling pathway is essential for initiating and extending neurite outgrowth [288-291]. The generation of cAMP and its subsequent binding to PKA leads to the release of the PKA catalytic subunit, resulting in the phosphorylation and activation of CREB. Active CREB facilitates the transcription of multiple cytoskeletal elements contributing to neurite extension [289, 290]. In pheochromocytoma (PC12) cells, PKA also targets the Rho-family Guanine triphosphate hydrolase (GTPase) Rac1, leading to actin binding and filament assembly to facilitate axonal differentiation [289]. PKA signalling is capable of responding to extracellular cues such as Ca^{2+} to more subtly regulate cytoskeletal dynamics. In filopodia, cAMP can determine the response to these extracellular cues in a graded manner, leading to responsive attraction of the growth cone, induced by dynamic reorganisation of the actin cytoskeleton induced by PKA [292-295].

1.5.1.3 cAMP/PKA signalling: A central mediator of synaptic plasticity, learning and memory

PKA plays an essential role in the molecular mechanisms underpinning and the consolidation of learning and memory in the hippocampus, referred to as LTP [296-298]. Actin-mediated internalisation of AMPA receptors in hippocampal neurons is mediated by PKA in an activity-dependent manner [299]. PKA also directly phosphorylates the AMPA receptor subunits GluA1 and GluR1, in plasma membrane, thereby facilitating LTP [298, 300]. Importantly, PKA is targeted to the plasma membrane in a complex with AMPA receptors by AKAP5 [301], suggesting that PKA targeting dynamics and excitatory receptor function are tightly linked.

Aside from directly modulating glutamate receptor function at the synapse to mediate LTP induction, cAMP-PKA signalling also consolidates synaptic plasticity in a long-term manner. Late-LTP (L-LTP) refers to the protein transcription-dependent consolidation of activity-dependent strengthening of synapses, and is dependent on CREB function [302-306]. Protein levels of GluA1 have been reported to be reduced in mice expressing dominant-negative CREB (S133A mutation), suggesting this as one mode of memory consolidation by CREB [307]. Neuronal CREB activity has also been linked to dendritic spine density [308, 309], which is likely mediated by its ability to impact the expression of cytoskeletal proteins.

Consolidation of glutamate-induced synaptic plasticity may also occur through the stimulation of the dopamine (type 1 and 5, also referred to D1 and D5) and β-adrenergic receptors. Activation of these GPCRs results in cAMP generation and subsequent targeting of glutamate receptors by PKA. The AMPAR subunit GluA1 is phosphorylated by PKA in response to D1 receptor stimulation, circumventing memory deficits *in vivo* [310], while stimulation of D1 and

D5 receptors also enhances the magnitude of LTP [311, 312]. Similarly, D1 receptor activation leads to NMDAR-GluN1 subunit phosphorylation [313], which modulates Ca^{2+} permeability and therefore NMDAR excitability [314]. The stimulation of β-adrenergic receptors also leads to PKA-mediated increase in the amplitude of excitatory post-synaptic potentials via GluN1 [315]. Both dopamine and adrenaline–type receptors enhance NMDAR Ca^{2+} permeability through GluN2B phosphorylation, mediated by PKA [316].

1.5.1.4 CREB is also implicated in neuronal cell survival

Notably, several studies support an important function for CREB in mediating cell survival in multiple regions of the brain [317, 318]. CREB is responsive to growth factors, such as NGF [318]. It is also responsible for the transcription of other growth factors, such as brain-derived neurotrophic factor (BDNF) in developing cortical neurons [319]. A role for CREB in neurogenesis, survival and neuron maturation in the sub-ventricular zone of the brain has been demonstrated through expression of a dominant-negative S133A CREB mutant [320]. Others have found that expression of this non-functional mutant in the hippocampus leads to increased cell death, and impaired expression of a group of genes known as Activity-Regulated Inhibitors of Cell Death [321]. Overexpression of wild-type CREB in the same study resulted in increased BDNF expression and cell survival.

1.5.2 Alterations in cAMP/PKA signalling in AD promote neuronal dysfunction

The cAMP/PKA signalling pathway is affected in AD and in the normal ageing process. Some evidence suggests that Aβ-mediated impairment of mitochondria leads to inhibition of CREB activity subsequent synaptic degeneration [322]. PKA-mediated regulation of CREB is impaired in the AD hippocampus [323, 324]. PKA has also been identified as a Tau kinase (***Table 1.1***). Phosphorylation at the Ser214 residue by PKA makes Tau a more favourable substrate for GSK-3β, another key kinase in AD pathogenesis [88, 89], thereby facilitating Tau aggregation [325]. The cAMP pathway has also been implicated in the alternative splicing of Tau at exon 10, which is implicated in PHF-Tau pathology [326, 327].

As such, this pathway has been proposed as a therapeutic target in AD. Indeed, the phosphodiesterase type-4 (PDE-4) inhibitor Rolipram, which increases the available pool of cAMP in cells can reverse the effects of Aβ toxicity in rodent models of AD [328-330]. However, lower PDE4 activity in the aged prefrontal cortex of primates, induces the miss-localisation and phosphorylation of Tau at Ser214 in dendritic spines, which is associated with heightened risk for neurodegeneration [331]. Additionally, Rolipram treatment in wild-type mice enhances Tau phosphorylation without affecting CREB activity [332], which could as well promote neurotoxicity. If translated to healthy human subjects, there would be little benefit of Rolipram in improving learning and memory. This indicates that perhaps the broad-spectrum targeting

of cAMP-PKA signalling may be ineffective in the treatment of AD. Rather, it might be more beneficial to target specific signalling molecules like PKA or PKA substrates.

1.5.3 cAMP/ PKA signalling can alter PP2A methylation

There is some preliminary evidence for a role of PKA signalling in regulating PP2A, and more specifically, PP2Ac methylation. In non-neuronal cells, pharmacological activation of cAMP/PKA signalling enhances PP2A methylation. Models employed include *in vivo* experiments in kidney and myoblast cell cultures [333]. Early studies in *Xenopus* oocytes also indicate increased PP2A methylation as a consequence of adenylate cyclase activation [334]. Critically, PP2A has also been demonstrated to be the phosphatase responsible for nuclear dephosphorylation of PKA-targeted CREB in a range of systems [335-337].

The potential link between neuronal cAMP/PKA signalling and PP2A methylation has important implications for AD. As described above, PP2Ac methylation is important for B-subunit binding and formation the ABαC holoenzymes that primarily mediate dephosphorylation of Tau. Furthermore, the regulation of PP2Ac methylation contributes to the sensitivity of excitatory neurons to exogenous Aβ *in vivo* [338]. Considering the role of PKA signalling in cell survival, Tau phosphorylation, and synaptic function, the discovery of any potential cross-talk in the regulation of PP2A and PKA would be important for the understanding of neuronal homeostasis and neurodegenerative processes underlying AD. To that end, Chapter 3 of this thesis will explore the connection between PKA signalling and PP2Ac methylation state. We will also investigate if PP2A enzymes can modulate cAMP-PKA mediated targeting of CREB, Tau phosphorylation and the stimulation of neurite outgrowth.

1.5.4 cAMP/PKA signalling could be disturbed by impaired one-carbon metabolism

Animal models inducing disturbances in one-carbon metabolism typically induce deficits in learning and memory, as well as in domains of brain function like motor learning and performance (***Table 1.3***, [241, 339, 340]). It is therefore interesting that Hcy exposure has been linked to hippocampal deficits *in vivo* [241], and alterations in cellular CREB activity [239]. Other *in vivo* investigations have demonstrated that deficiencies in methyl donors lead to impaired CREB activity in the cerebellum [341]. A loss of cholinergic neurons in models of impaired one-carbon metabolism also occurs *in vivo* [340, 342]. Since alterations in one-carbon metabolism are a risk factor for AD, and CREB becomes down-regulated in AD, there could be a cross-talk between metabolic and signalling pathways that regulate CREB and AD markers. Critically, nuclear CREB has been reported to be dephosphorylated by PP2A [335-337], and both CREB and PP2A become impaired in AD [158, 159, 323]. Thus, we propose in Chapter 5 to investigate how cellular and *in vivo* alterations of folate metabolism affect PP2A and CREB activity.

1.6 Deregulation of Src-mediated PP2Ac Tyrosine phosphorylation could also be linked to neurodegenerative processes in AD

While alterations in one-carbon metabolism or specific signalling pathways could contribute to the pathological process of AD through impairment of PP2A methylation, this key tau phosphatase is also regulated by tyrosine phosphorylation. In this context, it has been reported that PP2A tyrosine phosphorylation is also enhanced in AD, resulting in inhibition of PP2A activity [175]. Src kinase has been identified as a primary tyrosine kinase mediating PP2A tyrosine phosphorylation. The function of Src-family kinases is also closely linked with Tau in neurons [97, 111, 131]. Since the deregulation of Src kinase also occurs in AD [343], one aim of our thesis project will be to revisit the functional relevance of Src-mediated tyrosine phosphorylation of PP2A.

1.6.1 Structure and regulation of Src-family kinases

Src-family kinases are the most common non-receptor type tyrosine kinases. Aside from Src, they include Fyn, Yes, Fgr, Lck, Lyn, Hck and Blk [344]. SFKs are activated in response to extracellular stimuli, such as growth factor binding to respective receptors; they play a diversity of regulatory roles in cells including differentiation, survival, endocytosis and adhesion [345]. The most prominent Src family kinases in the brain appear to be Src and Fyn [346]. All Src family kinases share “Src Homology” (SH) Domains, which help to determine kinase functions. In Src family kinases, there are three of these domains, SH1, SH2 and SH3 (***Fig. 1.8***).

When inactive, the C-terminal Tyr527 residue of SFKs is phosphorylated, facilitating an auto-inhibitory loop within the SH1/kinase domain, which allows the 527 residue of a SFK to bind to its own SH2 domain, and induces SH2-SH3 binding, thereby “locking” itself ([344, 346], ***Fig 1.8***). Dephosphorylation of Tyr527 by protein tyrosine phosphatases (PTPs), such as PTPα causes for a conformational change in SFKs, which enables Tyr416 phosphorylation within the SH1 domain to occur, allowing kinase activity [347]. Substrate recognition is facilitated by the SH2 (phosphotyrosine binding) and SH3 (Proline rich / PXXP binding) domains, while the SH3 domain also regulates cytoskeletal association and substrate targeting of SFKs via proline binding. The N-terminus of SFKs contains a unique domain of ~55 amino acids, that can either be myristoylated at a conserved Gly2 site, or palmitoylated at cysteine residues in all SFKs except for Src and Blk [344]. Myristoylation and palmitoylation are needed for membrane association of SFKs, while the dephosphorylation of Tyr527 requires SFKs to be myristoylated [348].

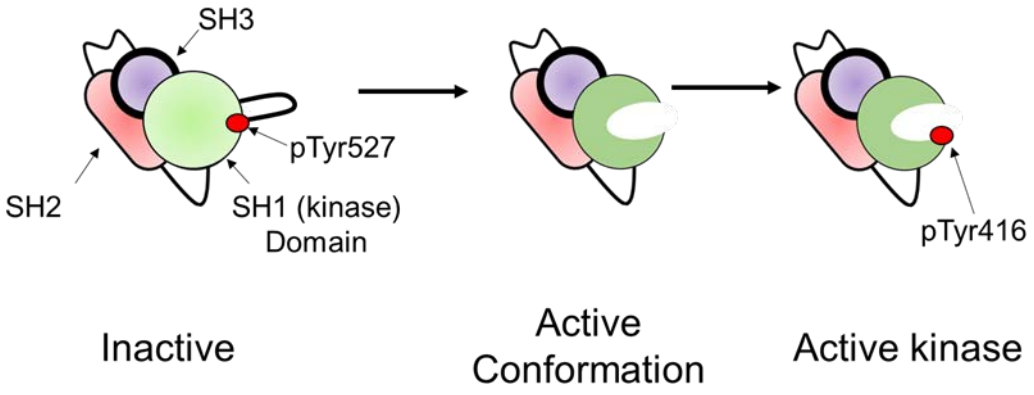


Figure 1.8: Src family kinase structure. Src family kinases consist of three highly conserved regions, SH3 domains that bind proline-rich regions of interacting proteins, SH2 phosphotyrosine binding domains and the kinase domains, SH1. Src is subject to auto inhibitory Tyr527 phosphorylation; upon dephosphorylation, Tyr416 phosphorylation is facilitated, enabling kinase activity.

1.6.2 Functional significance of Src in neuronal homeostasis

The cellular Src kinase (also called c-Src or simply Src) plays a critical role in mediating responses to extracellular stimuli such as neurotransmitters [349, 350] or growth factors [351]. It can also integrate signals from neurotransmitters to activate growth factor signalling [352]. Src modulates intracellular responses of neurons to extracellular stimuli through regulation of endocytic mechanisms [353, 354]. Accordingly, the neuronal axonal and microtubule cytoskeletal networks are regulated by Src [355, 356].

Src has an integral role of the kinase in several distinct neuronal populations, for instance, Src activity affects serotonergic cells (i.e. those that respond to serotonin, 5-HT) in the frontal cortex. In these cells, Src is found in close proximity to serotonin receptors 5-$HT_{1\text{-}7}$R in complex with the scaffolding protein β-Arrestin. The association of β-Arrestin complexes with 5-HT_2 receptors can in turn potentiate responses to GPCRs [350, 357]. The β-Arrestin Src complex can also regulates GPCRs, via endocytic mechanisms. Src interacts via its SH1 and SH3 domains with the β-Arrestins proline-rich domain to form complexes with GPCRs and facilitate their internalisation [358]. Src regulates neuron excitability in response to GPCR ligands such as Pituitary Adenylyl Cyclase Activating Peptide (PACAP) by facilitating the formation of β-Arrestin receptor complexes [354]. Src/β-Arrestin protein-protein interactions facilitate the 5-HT_2R response to serotonin in the frontal cortex by forming a complex with the kinase Akt. This leads to activation of Akt, which elicits a behavioural response [350]. Stimulation of 5-HT_{1A}R or 5-HT_4R induces Src-mediated activation of Erk [349, 352] via transactivation of the platelet-derived growth factor β receptor (PDGFβ-R) in SH-SY-5Y cells [352]. Conversely, Src also modulates cellular responses to 5-HT_{1A}R by inhibitory tyrosine phosphorylation, thus dampening the effects of 5-HT receptor signalling *in vivo* [359].

Glutamatergic cell function and response to excitation are also mediated by Src activity. The NDMAR subunit GluN3A is less sensitive to Mn^{2+} ions and is less Ca^{2+} permeable than other NMDAR subunits, and can be tyrosine phosphorylated by Src, leading to NMDAR internalisation [353]. Src thus participates in the removal of less excitable glutamate receptors in neurons. Initial observations suggested that only Fyn-, but not Src- or Yes- knockout mice have severely impaired LTP and long-term memory formed in the CA1 region of the hippocampus [360]. However, more recent evidence indicates that Src tyrosine kinase activity is both necessary and sufficient for LTP induction in CA1 neurons [361]. One underlying mechanism may be the LTP-induced phosphorylation of Tyr1070 on NMDAR-GluN2B, stabilising their surface expression at the plasma membrane, thereby allowing neurons to be more responsive to excitatory stimuli [362]. This represents both a response to glutamatergic inputs, and a modulatory response of glutamate receptors in neurons. Src family kinases also enhance glutamate receptor sensitivity through dopamine receptor signalling. NMDAR-NR2B subunits have impaired phosphorylation following neuronal treatment with the SFK inhibitor “PP2”, thereby blocking LTP induction in the hippocampus [312]. Conversely, Src also participates in LTD through AMPA receptor internalisation, with Src-dependent phosphorylation of the GluR2 subunit needed for this process [363].

Src is also an important regulator of neurite outgrowth. In response to stimulation of muscarinic Acetylcholine receptors (mAChRs), Src promotes Erk and CREB activation in primary hippocampal neurons, thereby inducing neurite extension [364]. Additionally, mAChR activation leads to Src-dependent transactivation of fibroblastic growth factor receptors, resulting in neurite formation in primary hippocampal neurons and primary axon extension in adult hippocampal cells [365]. Src is highly concentrated in growth cones [366], assisting in axon guidance [367]. Moreover, *in vitro*, Src directly phosphorylates p190-RhoGTPase activating protein (GAP) [368], an adhesion molecule critical for neuromorphogenesis [369]. Transgenic mice deficient in Src and Fyn have a complete lack of Rho phosphorylation, with subsequent deficits in axon guidance and outgrowth [370]. Beyond Rho-GAP activation through tyrosine phosphorylation, the cellular distribution and protein-protein interactions of Src also regulate neurite-related processes. Overexpression of constitutively active Src in Aplysia models leads to cortactin association, resulting in increased density, stability and elongation of filopodia [355]. Meanwhile, lipid-raft associated Src shows higher tyrosine kinase activity and leads to neurite outgrowth, and axonal elongation in cortical neurons [371].

1.6.3 Src becomes deregulated in Alzheimer's disease and plays a role in the disease process

1.6.3.1 Src has a unique relationship with Tau

Tau associates with SFKs *in vivo*, including Src [111, 114]. Enhanced Tau phosphorylation or FTD-17 mutations to the *MAPT* gene increases its association with the SH3 domains of Src. The resulting conformational changes lead to increased Src kinase activity [114, 115, 356], demonstrating a role for Tau as a signalling protein. Through the association with Src, Tau can modulate the growth-factor induced remodelling of the cytoskeleton [356]. Conversely, Src (and Fyn) are capable of phosphorylating Tau at the N-terminal Tyr18 residue [96, 97, 111]. The complete functional implications of this are not entirely understood. However, it regulates the association of Tau to the plasma membrane, and potentially the association of Tau with Src family kinases such as Fyn [112, 372]. These results suggest that Tau and SFKs can regulate each other, suggesting potentially complex feedback mechanisms.

1.6.3.2 Src regulates APP subcellular distribution and proteolytic processing

The secretion of sAPPα has been linked to Src-induced activation of α-secretase following 5-HT_4 receptor activation in neurons [373]. This is thought to occur via the regulation of Munc18-interacting (MINT) adaptor proteins, which can determine the subcellular targeting of APP in neurons [374]. Indeed, Src phosphorylates MINT1 at multiple tyrosine residues to allow differential targeting of APP to the Golgi apparatus or the plasma membrane [375]. Additionally, Src-mediated phosphorylation of MINT2 facilitates the endocytic internalisation of APP [376]. Similarly, muscarinic receptor activation also results in sAPPα formation in a Src-dependent manner, and involves Erk activation [377].

1.6.3.3 Src dysfunction has been linked to AD

The SFK Fyn has been hypothesised to play a central role in AD by mediating toxic functions of Aβ and Tau [131, 378]. While most AD studies have so far focused on Fyn of AD has surrounded [131, 378] there is accumulating evidence that deregulation of Src participates in the disease process.

Recent data have demonstrated Src dysfunction in a transgenic mouse model of AD with mutations in *APP/PSEN1/MAPT* genes (3xTg) [379]. This is associated with impaired phosphorylation of NMDAR-GluN2B subunits, and deregulation of actin cytoskeletal dynamics in dendritic spines. These results imply that AD-like changes in neurons undermine Src-mediated regulation of excitatory synapses. This can lead to the synaptic dysfunction typically seen in experimental models of AD. Corroborating these observations, Src kinase activity is progressively impaired in AD patients with increasing Braak stage [343]- a measure of the

severity of pTau pathology. Thus, the disease process may progressively induce a complete loss of Src function.

Like Fyn, Src may also mediate neurotoxicity in AD. In *APP/PSEN1* mouse models of AD pathology, Aβ exposure induces reactive gliosis in a Src dependent manner [380]. In neurons, Src-dependent activation of Rac1 promotes the γ-secretase cleavage of APP and subsequent Aβ secretion [381]. Fyn-Tau SH3 interactions have also been implicated in targeting protein complexes in NMDAR-containing post-synaptic densities, which mediate excitotoxic responses to Aβ *in vivo* [131]. Recent evidence suggests that Tyr18 phosphorylation of Tau, which is also mediated by Src, is important for NMDAR-dependent toxicity in primary neuronal cultures [98]. The precise nature and cause of Src dysfunction in AD, and what other consequences it may have are still somewhat unclear.

1.6.4 A potential role of Src in the deregulation of PP2A in AD

1.6.4.1 Src has been reported to inactivate PP2A via Tyr307 phosphorylation

The first interaction between PP2A and Src was first reported more than two decades ago when expression of the viral Src (v-Src) oncogene, which is constitutively active, was shown to phosphorylate PP2Ac at the Tyr307 residue *in vitro*; this phosphorylation was associated with inhibition of PP2Ac catalytic activity [173]. The phosphorylation of PP2Ac was also reported to occur in v-Src-transformed fibroblasts [174]. It was similarly observed after epidermal growth factor and insulin stimulation, catalysed by Src and the related SFK, Lck [173].

Tyr307-phosphorylation of PP2Ac has been proposed to be an important mode of PP2A regulation in multiple cell and tissue types, and has been used in numerous studies as a readout of PP2A inactivation. Over the years, PP2Ac-Tyr307 phosphorylation has been associated with functions as diverse as axon recovery in the CNS [382] and sperm motility [383]. More significantly, it has been suggested to play a role in many major diseases including several cancer types [384, 385]. Of particular relevance to our project, deregulation of PP2Ac tyrosine phosphorylation has been implicated in AD pathology [175].

Yet, it is worth mentioning that the PP2Ac Tyr307 phosphorylation site has never been found or validated in vivo using mass spectrometry since the initial in vitro experiments performed by Chen et. al. [173, 174]. Rather, the majority of results reporting changes in PP2A-pTyr307 have relied on the use of commercially available antibodies directed towards this site. Significantly, our group and our collaborators have recently found these “anti-pY307-PP2Ac” antibodies are rather unreliable due to their non-specifity, with their blanket use proving to be highly problematic in delineating PP2A function [386]. This discovery raises serious questions

about the validity of functional PP2A phosphorylation studies that have relied on these antibodies.

1.6.4.2 A possible link between Src and PP2A in AD

Another way by which Src-dependent signalling could contribute to AD pathogenesis may be through the reported ability of this kinase to directly phosphorylate and inactivate PP2A. Indeed, Src-mediated PP2A deregulation may participate in the neuronal response to various "stress" or toxic stimuli. For example, it has been reported that in cerebral ischemia [387], and in Aβ-treated cortical neurons, Src-mediated phosphorylation of PP2Ac leads to activation of $Erk_{1/2}$ [388], resulting in PHF-1 phosphorylation of Tau. Metabotropic glutamate receptor-dependent activation of Src also leads to PP2Ac phosphorylation and inactivation, and subsequent Erk activation in striatal neurons [389].

Post-mortem studies in human have suggested that Src may promote indirect Tau phosphorylation at Ser/Thr sites by impairing PP2A activity via tyrosine phosphorylation [390]. In agreement with this hypothesis, enhanced PP2A phosphorylation at Tyr307 has been reported to occur with neurofibrillary pathology in AD [175]. However, the proven transient and labile essence of PP2A tyrosine phosphorylation [174], combined with the demonstration that all commercial anti-pY307 antibodies are flawed, raises some serious doubts on the conclusions drawn from these studies performed in autopsy tissue. Thus, while there is strong support for a role of Src in PP2A regulation, the modalities of this regulation remain to be elucidated. In Chapter 6, we will strive to re-evaluate the role of Src in PP2Ac phosphorylation using different cell models.

It is also interesting that conversely, PP2A can dephosphorylate and inhibit Src activity in vitro [391, 392] so that downregulation of PP2A in AD could also in theory promote Src deregulation. Altogether, these observations are consistent with the concept that deregulation of Src-PP2A functional interactions could contribute to p-Tau pathology, albeit precise causes and underlying mechanisms are still unclear.

We also propose that the regulation of PP2A (and therefore the Ser/Thr phosphorylation of Tau) is more complex than has previously been suggested. If we can validate that the phosphorylation of PP2Ac at tyrosine residues impairs phosphatase activity, the effects on Tau phosphorylation would be twofold in AD pathogenesis. The reduced expression of PP2A-Bα [158], and impaired methylation of PP2Ac [159] in AD means that regular PP2A-Tau binding dynamics are disturbed, which would also result in enhanced SFK-Tau protein-protein interactions [97]. Indeed, PP2A and SFK enzymes bind Tau in the same regions, and therefore compete for binding. Moreover, AD-like phosphorylation inhibits the association of Tau with PP2A, thereby preventing dephosphorylation of p-Tau at Ser/Thr sites. At the same time,

detachment of PP2A and Tau phosphorylation enhance the binding of SFKs to p-Tau, and thereby the tyrosine phosphorylation of Tau [199]. Thus, decreased PP2A methylation *and* Src-mediated PP2A phosphorylation may both drive the accumulation of Tau species phosphorylated at Ser/Thr and Tyr sites, as observed in the AD brain. Thus, to gain some better understanding of Tau regulation, it is important to delineate the precise role of Src-mediated PP2A phosphorylation.

1.7 Hypotheses and Aims

It is widely recognised that Tau and Aβ play central roles in the onset of AD. However, what is less understood is how Tau and APP are regulated in neurons. Current evidence strongly supports an essential role for PP2A in Tau regulation [155, 160], with a growing body of literature also implicating PP2A in the regulation of APP in neurons [258, 393, 394]. Recent *in vivo* studies from our group also indicate that methylation of PP2Ac protects against the neurotoxic effects and memory impairment induced by Aβ [338]. PP2Ac methylation depends directly on the proper functioning of one-carbon metabolism. This suggests a compelling link between one-carbon metabolism, APP phosphorylation, Aβ formation, Tau phosphorylation and neurodegenerative processes in AD.

What is currently unknown, however, is how PP2Ac methylation is otherwise regulated. One-carbon metabolic supply of methyl groups is undoubtedly critical for PP2Ac methylation, but we believe that other signalling pathways could also modulate PP2Ac methylation. These effects could be direct and/or cross-talk with the biochemical pathways that are part of the one-carbon cycle. In other cell types, cAMP signalling has been suggested to alter PP2Ac methylation and cellular distribution [333, 334]. *Thus, we aim to interrogate the role of cAMP/PKA signalling in the regulation of neuronal PP2Ac methylation, and investigate the functional effects on selected PKA and PP2A substrates. We hypothesise that PKA activation will modulate the methylation and compartmentalisation of PP2Ac to regulate their shared substrates* ***(Fig. 1.9)***.

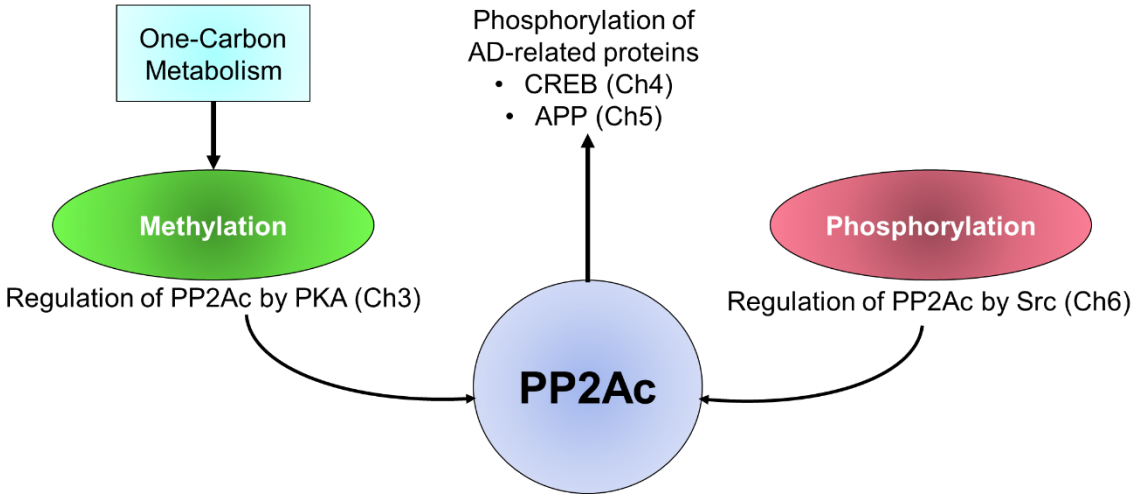


Figure 1.9 Regulation of PP2Ac in Alzheimer's disease. In this thesis, we aim to clarify the precise mechanisms of PP2Ac regulation by post-translational modification and their functional consequences for AD. Our fist aim (Chapter 3) will be to provide evidence of a novel link between cAMP/PKA signalling and neuronal PP2Ac methylation. Our second aim is to investigate how the disturbed methylation of PP2Ac in cells and *in vivo* models of MTHFR and folate deficiencies affects CREB and APP phosphorylation and expression (Chapters 4 and 5), since those are both functionally relevant substrates of PP2A in AD. Our final aim is to re-evaluate and clarify the role of Src in the regulation of PP2A by tyrosine phosphorylation of the catalytic subunit (Chapter 6).

Furthermore, PP2A regulates several important proteins in neurons involved in AD. Our group has repeatedly demonstrated a role for PP2A in Tau dephosphorylation *in vitro, in vivo* and in Alzheimer's disease [158, 159, 395, 396], while also linking Tau phosphorylation with disturbed PP2Ac methylation in a model of disturbed one-carbon metabolism *in vivo* [263]. Other observations from our group also suggest that disturbances in methyl-group supply may result in disturbed APP processing and deregulated phosphorylation of Thr668, caused by demethylated PP2Ac [258].

Additionally, recent evidence has suggested a link between disturbed one-carbon metabolism *in vivo* and the deregulation of a number of important proteins in the brain, including CREB [341, 397]. There is also evidence implicating PP2A in the regulation of CREB, mainly through the dephosphorylation of this transcription factor [335, 337].

Since PP2A methylation affects the phosphorylation of APP and CREB, and becomes impaired in mice with genetic MTFHR and dietary folate deficiencies, these disturbances may also manifest in this model. *Therefore, we propose to assess the effect of altered one-carbon metabolism on important PP2A substrates in the brain, CREB and APP. We hypothesise that the phosphorylation and possibly the expression of these proteins may be deregulated in MTHFR and folate deficient mouse models (**Fig. 1.9**).*

Lastly, Src-mediated phosphorylation of PP2Ac at Tyr307 and subsequent inhibition of PP2A catalytic activity was first described *in vitro* over 25 years ago [173, 174]. Since then, reports have suggested that Src leads to increased Tau phosphorylation through PP2Ac inhibition [387, 389, 390, 398]. Despite these studies, and others indicating that PP2Ac-pTyr307 is increased in AD [175], it remains to be validated that PP2A is indeed phosphorylated at this site. Since there is a complex interplay between the binding of SFKs and PP2A to Tau, this represents a pressing issue. *Thus, we propose to clarify the nature of Src-mediated tyrosine phosphorylation of PP2A, and assess its functional consequences for Tau phosphorylation. We will also investigate the effects of these functional interactions on neuritogenesis, which becomes impaired in AD. We hypothesise that Src targets PP2Ac for phosphorylation at residues other than Tyr307, leading to deregulation of Tau and neurite outgrowth.*

Chapter 2: Materials and Methods

Acknowledgement of Collaboration

I hereby acknowledge that the work enclosed in this thesis was performed in collaboration with other researchers.

The methodology in the following chapter (Chapter 2) was compiled by myself following instruction and technical advice from my academic supervisors (A/Prof. Estelle Sontag, Dr. Jean-Marie Sontag and Prof. Alan Brichta).

The acute preparation of mouse brains was conceptualised and implemented by myself. This was done with invaluable advice and optimisation from the laboratory of Prof. Alan Brichta and Dr. Rebecca Lim, including Hannah Drury and Dr. Lauren Poppi. The tissue was generously donated by members of the same laboratory, the laboratory of A/Prof. Pradeep Tanwar, particularly Dr. Manish Kumar Jhamb, Arnab Ghosh and Shafiq Syed, and the laboratory of A/Prof. Doug Smith.

The MTHFR deficient mice from which we analysed brain tissue were cared for and sacrificed by our collaborators Drs. Teodoro Bottiglieri and Brandi Wasek (Center of Metabolomics, Institute of Metabolic Disease, Baylor Scott & White Research Institute, Dallas, TX, USA). The specialised metabolite analysis of these tissues was performed by Dr. Brandi Wasek.

2.0 Reagents

2.0.1 Chemicals

Unless indicated, all reagents were purchased from Sigma.

2.0.2 Plasmids

In this thesis, COS-7 and N2a cells were transfected with plasmids listed in ***Table 2.1*** below. Listed cDNA constructs are all mouse genes.

Encoded protein	Plasmid
HA-PP2Ac^{WT}	pcDNA3.1 HA-wild-type PP2AC
HA-PP2Ac$^{L309\Delta}$	pcDNA3.1 HA-PP2AC-L309Δ
Src^{CA}	pRc/CMV Src-Y529F
HA$_3$-PP2Ac$^{\#1}$	pcDNA5/TO HA$_3$-PP2AC-tyrosine phosphomutant #1 (Y → F)
HA$_3$-PP2Ac$^{\#2}$	pcDNA5/TO HA$_3$-PP2AC-tyrosine phosphomutant #2 (Y → F)
HA$_3$-PP2Ac$^{\#3}$	pcDNA5/TO HA$_3$-PP2AC-tyrosine phosphomutant #3 (Y → F)
HA-PP2Ac$^{\#3}$	pcDNA5/TO HA-PP2AC-tyrosine phosphomutant #3 (Y → F)
Myc-PME-1	pBABE-Myc-wild-type PME-1

Table 2.1 Plasmids used in this Thesis

2.0.3 Antibodies

Antigen	Origin species	MW of target (kDa)	Source	Catalogue number
Actin	Mouse	42	Merck Millipore	MAB1501
APP (total)	Rabbit	116	Merck Millipore	MABN2287
Phospho-APP (Thr668)	Rabbit	116	Cell Signaling Technology, Fisher Scientific	2451 AF2508
PP2A Bα subunit	Mouse, monoclonal	55	Merck Millipore	05-592
PP2A total catalytic subunit	Mouse	36	BD Biosciences	610556
Demethylated PP2A catalytic subunit	Mouse	36	Upstate biotechnology	05-421
Methylated PP2A catalytic subunit	Mouse, monoclonal	36	Dr. E. Ogris	†
CREB (total)	Rabbit	42	Cell Signaling Technology	9197
Phospho-CREB (Ser133)	Rabbit	42	Cell Signaling Technology	91985
Phospho-CREB (Ser133)	Goat	42	Santa Cruz biotechnology	sc-7978 (*discontinued*)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Mouse	36	Merck Millipore	AB2302
GSK-3β	Mouse	46	Cell Signaling Technology	9832S
Phospho-GSK-3β	Rabbit	46	Cell Signaling Technology	9323S
Hemagluttinin (HA)	Mouse	Range	Covance	MMS-10R
LSD1	Rabbit	>100	Merck Millipore	ABE365
Na^+/K^+ ATPase	Mouse	116	Abcam	ab254025
PKA Substrates	Rabbit	Range	Cell Signaling	9624S
PME-1	Mouse, monoclonal	42	Dr. E. Ogris	†
Src	Mouse	59	Merck Millipore	05-184
Phospho-Src (Tyr416)	Rabbit	50-59	Cell Signaling Technology	9643S
Tau, total	Rabbit	50-75	Rpeptide	T-1308-1
Phospho-Tau (CP13)	Mouse	50-75	Dr. P. Davies	†
Phospho-Tau (PHF-1)	Mouse	50-75	Dr. P. Davies	†
Phospho-Tau (Ser214)	Rabbit	50-75	Cell Signaling Technology	77348S
Tubulin	Rabbit	55	Merck Millipore	ABT170
β-III Tubulin	Rabbit	55	AbCam	ab18207
Phospho-Tyrosine	Rabbit	Range	Cell Signaling Technology	8954S

Table 2.2 Primary Antibodies used in this Thesis

† : Denotes primary antibodies obtained from Dr. E. Orgris or Dr. P. Davis which are not commercially available

2.1 Cell culture and transfection

Mouse neuroblastoma (Neuro-2a cells, N2a, American Type Culture Collection, ATCC) were maintained in RPMI 1640 culture medium (Thermo Fisher Scientific, Mulgrave, Vic.) containing 25mM Hepes (pH 7.4), 10% foetal bovine serum (FBS, Bovogen biologicals, Essendon, Vic) and 10µg/ml gentamicin (Thermo Fisher Scientific, Mulgrave, Vic). COS-7 cells (ATCC; a gift from Dr. Rick Thorne, University of Newcastle) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Mulgrave, Vic), and supplemented as above. Cells were transiently transfected using Metafectene Pro reagent, following manufacturer's instructions (Biontex Laboratories, Munich, Germany). Cells were transfected with plasmids expressing the hemagglutinin (HA) tagged wild-type PP2Ac (WT-PP2Ac), the methyl-deficient L309Δ PP2Ac (L309Δ-PP2Ac), three PP2Ac de-phospho mutants (Y→F amino acid substitutions) labelled as PP2Ac #1, PP2Ac #2 and PP2Ac #3, as well as constitutively catalytic active Src (Y527F mutation, Src^{CA}). Cells mock-transfected with empty vectors (EV) were used as controls and referred to accordingly as "control cells" or "EV". They behaved in the same way as non-transfected cells in our experiments.

2.2 Cell treatment

2.2.1 Activation and inhibition of cAMP/PKA signalling

N2a cells were grown to confluence in 100mm dishes (Corning, NY, USA) in RPMI 1640 medium containing normal folate and 10% Foetal Bovine Serum. Cells were serum-starved 16-18h prior to experiments by replacing the normal culture medium with RPMI 1640 medium containing 0.5% FBS. When indicated, cells were incubated in the same medium in the presence of 50µM Forskolin for 0 to 60 minutes as indicated in Results. In other experiments, cells were incubated for 30 minutes at 37°C with the cAMP analogue 8-Br-cAMP (50µM) in the same medium. Cells were also treated with the cAMP/PKA inhibitors H89 (30µM) and KT5720 (10µM) in RPMI1640 containing 0.1% FBS for 45 minutes prior to incubation with Forskolin for 15 minutes. Lastly, to inhibit endogenous PME-1, cells were incubated for 3h with 20µM AMZ-30 for 3 hours in RPMI 1640 medium containing 0.5% FBS.

2.2.2 Folate deficiency

To study the effects of folate depletion, cells were washed once with room temperature PBS, then incubated for up to four hours in RPMI 1640 cell culture medium without folic acid (ThermoFisher Scientific) in the presence of 5% FBS (Bovogen), prior to harvesting.

2.2.3 SAH-Hydrolase inhibition and SAM supplementation

When indicated, prior to harvesting, N2a cells were starved overnight in RPMI medium containing 0.1% FBS in the presence of 100μM of either S-Adenosyl Methionine (SAM) or SAH-hydrolase inhibitor 3-deazaadenosine (3DA), or vehicle.

2.2.4 Pervanadate treatment of N2a and COS-7 cells

Under sterile and dark conditions, sodium orthovanadate (Na_3VO_4, 250mM) was added to 30% H_2O_2 and PBS to a final concentration volume of 2.5ml. This reaction was left in dark conditions at room temperature to yield a 100x (10mM) solution of Pervanadate (PV). This 100x solution was added to cell culture medium (DMEM) at a dilution of 1:100 for a final concentration of 100μM. Serum starved cells were incubated with PV for 15 minutes at 37°C.

2.3 Preparation of cellular homogenates

2.3.1 *Total cell extracts*

Following treatment, cells were rinsed with room temperature PBS, then harvested in ice-cold extraction buffer E [25mM Tris pH7.4, 150mM NaCl, 1% Nonidet-P40, Complete Mini™ protease and phoSTOP™ phosphatase inhibitor cocktails (Roche, Dee Why , NSW, Australia), 5mM PMSF, 1mM DTT, 0.5μM okadaic acid (Merck Millipore, Bayswater, VIC, Australia)] according to our previous methods [199]. Total cell extracts were sonicated on ice then centrifuged for 10 minutes at 13,000x*g*. Supernatants were collected and stored at -80°C until further analysis.

2.3.2 *Subcellular fractionation*

2.3.2.1 Nuclear purification of proteins

Nuclei were isolated from confluent 100mm dishes according to a published protocol [399]. Briefly, cells were harvested in 500μl of RSB buffer (10mM Tris pH 7.4, 10mM NaCl, 3mM $MgCl_2$, with cOmplete Mini ™ protease and phoSTOP ™ phosphatase inhibitor cocktails, 0.5μM okadaic acid, 5mM PMSF), and centrifuged at 960x*g* for 1 minute. The pellet was resuspended in Buffer RSB-G40 (RSB + 10% glycerol and 0.25% NP-40) with 1mM DTT, before centrifugation at 10,000x*g* for 1 minute. The supernatant (non-nuclear fraction) was collected and stored at -80°C for future analysis.

The pellet was rinsed once in RSB-G40 and once in RSB-G (RSB + 10% glycerol). The final pellet was resuspended in 100μl RSB-G40 buffer and homogenised. The sample was centrifuged for 5 minutes at 12, 000 x *g.* The supernatant (nuclear fraction) was stored at -80°C for subsequent analysis. The nuclear marker Lysine-specific demethylase 1 (*See 2.8 Gel Electrophoresis and Western Blotting*) was used as a protein loading control in this compartment. In other experiments, nuclei were prepared as described in section 2.3.2.2.

2.3.2.2 *Isolation of membrane fractions*

Subcellular fractionation of cells was performed according to our previously published methods [165]. Briefly, cultured cells were washed in PBS, then incubated for 5 minutes at room temperature in a hypotonic sucrose buffer solution S [10mM Tris pH7.4, 250mM Sucrose, 1mM EDTA, cOmplete Mini^{TM} protease and phoSTOP^{TM} phosphatase inhibitor cocktails, 1mM DTT, 0.5μM okadaic acid, 5mM PMSF]. Cells were then harvested in the same ice-cold sucrose buffer and incubated on ice for 15 minutes. Cells were homogenised using a glass-Teflon Dounce homogeniser (DWK Life Sciences, New Jersey, USA). Aliquots of total cell homogenates were kept for future analysis, and remaining cell homogenates were first centrifuged for 10 minutes at 800 x *g* to pellet cell nuclei. The pellet was rinsed one time in buffer S and re-centrifuged for 10 minutes at 800 x *g*. The supernatant was discarded and the pellet (nuclear fraction P1) was resuspended in buffer E and sonicated.

The first post-nuclei supernatant was centrifuged for 1h at 100,000 x *g* to separate cell membranes (pellet) from the cytosolic cell fraction S2 (supernatant). Pelleted membrane fractions were resuspended in buffer E, sonicated and centrifuged for 5 min at 12,000x*g* to remove insoluble material. The supernatant (Membrane fraction P2) was collected.

All cell fractions were stored at -80ºC until further analysis. Membrane enrichment was verified using Western blotting with antibodies directed against Na^+/K^+-ATPase (see 2.8: *Gel Electrophoresis and Western blotting*, below)

2.3.4 *Separation of cellular detergent-soluble and –insoluble pools*

Cells were rinsed in PBS and collected in ice-cold E buffer, as described in section 2.3.1. Aliquots of total cell extracts were kept for further analyses. The remaining cell homogenates were centrifuged for 45 minutes at 26,330*xg*. The supernatant (detergent-soluble fraction) was collected and stored at -80ºC. The pellet (detergent-insoluble fraction) was resuspended by sonication in buffer E prior to storage at -80ºC.

2.4 Immunoprecipitation

2.4.1 Immunoprecipitation of PP2Ac^{WT} and PP2Ac phosphorylation-incompetent mutants

Cultured N2a or COS-7 extracts were homogenised in ice-cold buffer E, without DTT. Extracts were homogenised on ice using a microtube pestle (Eppendorf). Homogenates were then centrifuged at 13,000*xg* for 10 minutes. A small aliquot of the total protein extract was stored at -80ºC for future analysis by Western blotting. The remaining homogenates were mixed with 40μl anti-HA antibody-tagged magnetic beads (Cell Signaling, Genesearch, Arundel, Qld) and incubated at 4ºC overnight. Immunoprecipitates were washed five times in the same harvesting buffer. After the last wash, LDS-based SDS-PAGE loading buffer was added directly to the beads and samples analysed by NU-PAGE as described in section 2.8.

2.4.2 PKA phosphorylation assay

Growing COS-7 cells were transfected with the pBabe Puro Myc-PME-1 plasmid. Cells were harvested 36h post-transfection in ice-cold modified buffer E, containing 0.5% sodium deoxycholate, but without DTT. Cells were homogenised with a motorised pestle (Eppendorf). Extracts were then centrifuged for 5 minutes at 13,000 *x g* at 4°C. The remaining protein extract was added to a 20µl aliquot of anti-Myc antibody-tagged magnetic bead suspension (Cell Signaling, Genesearch, Arundel, Qld). This bead slurry was left to incubate overnight at 4°C on a rotating mixer (Select Bioproducts, Edison, NJ, USA). The next day, the cell extract was removed and the beads were washed five times in 500µl of the same E buffer used in the protein extraction. After the fifth wash, the beads were resuspended in a PKA reaction buffer, containing recombinant mouse catalytic subunit of cAMP-dependent Protein Kinase A (α-isoform, New England Biolabs).The phosphorylation reaction was started by adding 30µl PKA reaction mix containing 50mM DTT, 0.01% Brij-35, 0.2mM ATP and 1,250 units of recombinant PKA-cα. The suspension was incubated for 30 minutes at 30°C. The reaction was stopped with the addition of 15µl gel loading buffer. The sample was then heated for 10 minutes at 72°C, before separation by NU-PAGE and Western blot analysis.

2.5 *Analysis of neurite outgrowth in N2a cells by confocal microscopy*

Growing N2a cells were seeded on poly-L-Lysine coated coverslips at a density of $1.5x10^4$ cells/mL in RPMI cell culture medium. The next day, cells were incubated overnight in starvation medium (RPMI 1640 medium with 0.5% FBS) to initiate the process of differentiation. As indicated in results, cells were incubated in the same medium with either Forskolin, *Rp*-cAMPs, 8-Br-cAMP, KT5720, H89 or PP2 (Merck Millipore, Bayswater, VIC, Australia) for 24h to 48h.

To investigate neurite outgrowth, cells were fixed and permeabilized for 5min in ice-cold methanol [236]. After washing with PBS cells were incubated in a PBS plus 5% BSA solution (blocking step). Cells were then incubated for 1h in PBS+3% BSA with a primary antibody directed against β-III Tubulin (Abcam). Cells were washed and the secondary Alexa Fluor 488 labelled antibody (Life Technologies, Mulgrave, VIC, Australia) was added for 1hr. After washing in PBS, coverslips were mounted with Fluoromount (Fisher Scientific) and examined on an Olympus FV1000 CLSM confocal microscope using a 60× objective.

Captured images (*z*-stacks) were transferred to Adobe Photoshop/Illustrator CS5 for printing (Adobe Systems). Images were processed using ImageJ software (National Institute of Health, USA), and neurites were measured using the ImageJ Plugin application Neurite Tracer [400].

2.6 *Preparation of acute cortical slices from mouse brain*

3-6 month old male C57BL6 mice were anaesthetized with ketamine (100mg kg^{-1} I.P.) and decapitated. Brains were rapidly removed and placed in ice-cold sucrose substituted artificial cerebrospinal fluid (sACSF; 250mM sucrose, 25mM $NaHCO_3$, 10mM glucose, 2.5mM KCl, 1mM NaH_2PO_4, 1mM $MgCl_2$ and 2.5mM $CaCl_2$ [401]). Coronal slices (350µm) were sectioned using a vibrating microtome (Leica VT-1200S, Heidelberg, Germany) and then transferred to an incubation chamber containing oxygenated ACSF (118mM NaCl substituted for sucrose [401]). Slices were equilibrated for 1h at room temperature (22-24ºC) prior to macro dissection of the cortex or hippocampus (**Fig. 2.1**). After isolation, cortical slices were placed for 0-60min at room temperature into fresh, carbogenated ACSF containing either 50µM Forskolin, 50µM Forskolin plus 2.5µM Rolipram, or vehicle.

Total homogenates were prepared from slices the cortex, in 500µl buffer E. Following sonication on ice, samples were centrifuged at 13,000x*g* to remove insoluble material. The supernatant was stored at -80ºC until further analysis by Western blot.

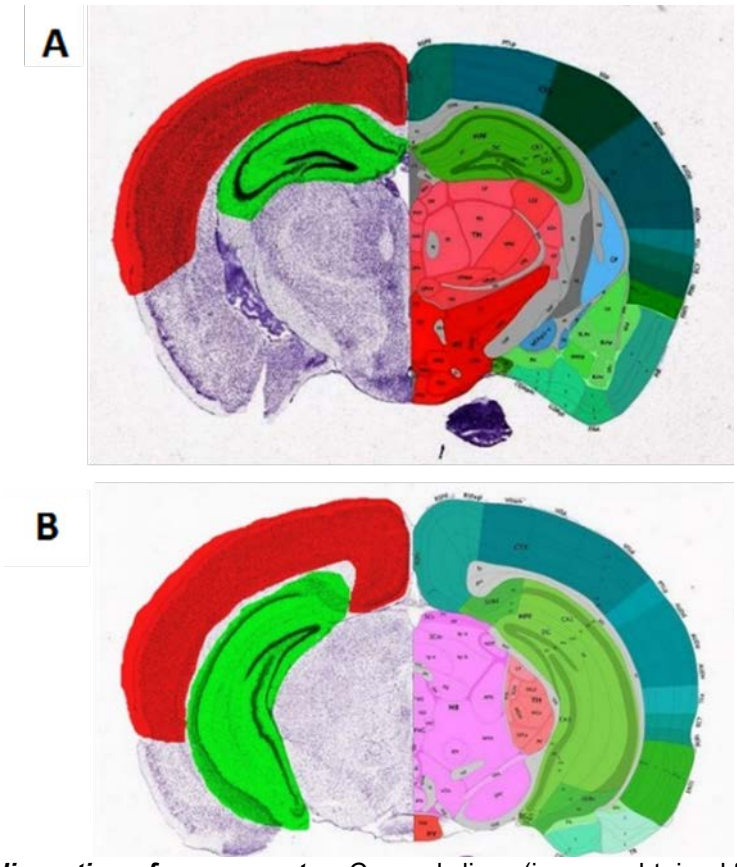


Figure 2.1 Macrodissection of mouse cortex. Coronal slices (images obtained from *Mouse Brain Atlas [402]* at depths of ***A.*** ~-2.3mm and ***B.*** ~-3.3mm, relative to Bregma. Highlighted in left hemisphere of each slice, in red, are examples of cortical regions that were dissected from coronal slices

2.7 MTHFR-deficient mouse models and dietary interventions

Brain tissue homogenates were obtained from previous studies performed in wild-type (WT) *MTHFR*$^{+/+}$, heterozygous (HET) *MTHFR*$^{+/-}$ and homozygous (NULL) *MTHFR*$^{-/-}$ female mice [263]. Briefly, 5-week old WT, HET and NULL mice fed on a normal chow diet containing 6.7mg/kg folate (Harlan Teklad, Madison WI) were sacrificed by CO_2 asphyxiation and different brain regions were collected and flash frozen (***Fig 2.2.A***). Additionally, 16-month-old WT and HET mice were fed for 6 months with either the above normal-folate containing control diet (CD) or a low folate diet (LF; Harlan Teklad, Madison, WI) with only 0.2 mg/kg folate, prior to sacrifice (***Fig. 2.2.B***).

Figure 2.2 Mouse models of MTHFR- and folate- deficiencies. A. Wild-type (WT) *MTHFR*$^{+/+}$, heterozygous (HET) *MTHFR*$^{+/-}$ and homozygous (NULL) *MTHFR*$^{-/-}$ female mice were fed a normal folate diet for 5 weeks before being sacrificed. Cortex, midbrain and cerebellar regions were collected and analysed for expression and phosphorylation of CREB or APP. Due to limitations in available tissue, the only region we were able to use from NULL mice in the analysis of CREB was the cortex. ***B***. Aged (16-month-old) wild-type mice were fed a normal- or low-folate containing diet and sacrificed after 6 months on the diet (final age of ~22 months). The cortex, hippocampus (Chapter 5 only) midbrain and cerebellar regions were collected and analysed for expression and phosphorylation of APP and CREB. Mice rearing, housing and sacrifice were performed in the laboratory of our collaborator Dr. T. Bottiglieri, (Baylor University, Dallas, TX, USA).

The CD and LF diets supplied to aged mice, also contained succinylsulfathiazole (10 mg/kg) to inhibit gastrointestinal bacterial growth and prevent absorption of folate from this source [263, 403]. Plasma tHcy was determined by high pressure liquid chromatography with fluorescence detection [404] and plasma folate was quantified with a Simultrac-SNB RIA kit (MP Biomedicals). Total homogenates were prepared from the cortex, cerebellum or midbrain mouse brain regions at a ratio of 1 g tissue/10 ml buffer E. Following sonication on ice, samples were centrifuged at 13,000x*g* to remove insoluble material. The supernatant was flash frozen and stored at -80^{o}C.

2.8 Gel electrophoresis and Western blotting

Protein extracts (~50µg) of cell and mouse brain homogenates were separated on NU-PAGE Bis-Tris 4%-12% gradient mini or midi gels (Thermo Fisher Scientific). Precision Plus™ Protein Standards (BIO-RAD) were used as molecular weight standards. Proteins were transferred overnight onto nitrocellulose membranes in a tris-glycine-methanol transfer buffer. Western blotting was performed according to the manufacturer's instructions (LI-COR Biosciences, Millennium Science, VIC, Australia). Briefly, membranes were incubated for 1h in a Bovine Serum albumin (BSA)-PBS based blocking buffer (Odyssey blocking buffer, LI-COR Biosciences, Millennium Science, VIC, Australia) prior to sequential incubation with the indicated primary antibody (***Table 2.1***) and a species-specific Infrared IRDye®-labelled secondary antibody (LI-COR) for 1h each, in the same blocking buffer containing 0.1% Tween20.

Western blotting was performed using the Odyssey™ Infrared imaging system (LI-COR Biosciences). Band intensity was determined by two separate investigators using Image Studio Lite version 5.0 Software (LI-COR Biosciences) to accurately quantify protein levels.

2.9 ***Statistics***

Pairwise comparisons were analysed using Student's T-test. Where appropriate, in animal-based experiments, data was analysed using one-way ANOVA with Dunnett's test. Differences where $p < 0.05$ were considered statistically significant. Data is presented as mean ± standard deviation (SD) or standard error of the mean (SEM), as indicated.

Chapter 3: Regulation of PP2Ac methylation by cAMP/PKA signalling

Acknowledgement of Collaboration

I hereby acknowledge that the work enclosed in this thesis was performed in collaboration with other researchers.

The work in the following chapter (Chapter 3) was primarily performed by myself (Alexander Hoffman), though I received assistance in some experiments from fellow lab members (Goce Taleski) and my supervisors (Dr. Jean-Marie Sontag and A/Prof. Estelle Sontag).

This practical assistance was in addition to invaluable technical advice from my supervisors (Dr. Jean-Marie Sontag, Prof. Alan Brichta and A/Prof. Estelle Sontag).

All figures and results presented in this chapter were obtained and composed by me (Alexander Hoffman). In addition, the methodology for the mouse cortical slices was conceptualised, optimised and implemented by me with the generous technical expertise and assistance of Prof. Alan Brichta, Dr. Rebecca Lim, Hannah Drury and Dr. Lauren Poppi.

Mouse brain tissue was generously donated by the laboratories of Prof. Alan Brichta, Dr. Rebecca Lim, A/Prof. Doug Smith and A/Prof. Pradeep Tanwar

3.0 Rationale and Significance

PP2A is responsible for the regulation of many key neuronal functions, including synaptic plasticity, neuronal differentiation, cell cycle progression and survival [338] [73, 200, 236, 338, 405]. Functional PP2A heterotrimers are formed by the association of specific A, B, and C subunits which determine PP2A enzyme substrate specificity [Chapter 1,406]. One mechanism that determines subunit composition is the C-terminal methylation of the catalytic subunit at the leucine 309 residue. Methylation at this site allows B-family regulatory subunit binding to the core AC dimer and the formation of PP2A-ABαC enzymes. Of particular interest to our studies is the link between methylation and PP2A-ABαC biogenesis, since this PP2A isoform is the primary Tau phosphatase in neurons [157].

By controlling the supply of the methyl donor, SAM, the one-carbon metabolic cycle, is currently the only mechanism demonstrated to influence neuronal LCMT-1 activity, and therefore PP2Ac methylation in cells and *in vivo* (Refer to Chapter 1). Interestingly, it has been suggested that cAMP/PKA signalling is able to modulate PP2A methylation and activity in a number of non-neuronal cell types. For example, induction of cAMP signalling in *Xenopus* oocytes leads to PP2Ac methylation [334], though a precise understanding of how this occurs is still unknown. Others have shown that elevation of cAMP increases PP2A activity in kidney cells; the authors proposed that this effect may occur as a result of increased methylation of PP2Ac [333]. Furthermore, use of the adenylate cyclase activator Forskolin is associated with activation of PP2A enzymes [407]. Together, these data indicate that there may be a link between the generation of cAMP and PP2Ac methylation in neurons, as observed in other cell types.

In this chapter, we wanted to investigate if, and how cAMP signalling could affect PP2Ac methylation in neurons. We observed that cAMP generation in N2a cells led to a time-dependent demethylation of PP2Ac, and a redistribution of methylated PP2Ac and PP2A-ABαC complexes. We also observed that PP2Ac methylation can affect the phosphorylation state of two downstream targets of cAMP signalling, the transcription factor CREB, and the microtubule-associated protein Tau. These observations support a role for the cAMP-regulated methylation of PP2Ac in neuronal processes such as synaptic plasticity, differentiation and survival, especially as these functions are mediated by PP2A, CREB and Tau.

3.1 Introduction

The cAMP/PKA signalling pathway is critical for proper neuron function and becomes deregulated in AD. Ligand binding to G-protein coupled receptors or elevated intracellular Ca^{2+} result in the activation of adenylate cyclase, leading to cAMP formation and PKA activation. cAMP binding to two regulatory subunits of tetrameric PKA induces the release of the two catalytic subunits that phosphorylate PKA substrates. Phosphodiesterases are responsible for the degradation and cycling of cAMP, and subsequent inactivation of PKA. There are many identified phosphodiesterase isoforms with different expression patterns throughout tissues, including in different brain regions [408]. PKA is localised and tethered to various intracellular compartments by A-Kinase Anchoring proteins (AKAPs) that control the subcellular targeting of the cAMP/PKA pathway. The regulation of PKA signalling in neurons has been reviewed in the introduction of this thesis and has been extensively described in the literature [409, 410].

Synaptic transmission in neurons is dependent on cAMP/PKA signalling. Excitatory AMPA receptor (AMPAR)-mediated synaptic transmission leads to rapid generation of cAMP by adenylate cyclase in the postsynaptic neuron [411]. PKA activation promotes AMPAR stabilisation at the synapse, contributing to post-synaptic plasticity [300, 316]. It induces actin-mediated internalisation of AMPARs in hippocampal neurons upon LTP induction [299]. In addition to these functions at dendritic spines, cAMP/PKA signalling activity is also localised to other subcellular compartments, notably the nucleus. The major nuclear target of cAMP/PKA signalling is the transcription factor CREB. CREB targets regulatory regions of DNA known as cAMP-responsive elements (CREs) and is activated by PKA-mediated phosphorylation at the Ser133 site. CREB is also targeted by a handful of other kinases, including CaMKIV and MAPK, which are also regulated by PKA [282, 283]. CREB phosphorylation induces the association of CBP with CREB [286] or of CREB-regulated transcriptional coactivators (CRTCs) with CREB. Binding of these proteins to CREB leads to gene transcription [287].

CREB is responsible for regulating the transcription of cytoskeletal regulatory proteins, either by inducing their expression, for example that of Doublecortin [412], or by suppressing the transcription of others, like p250GAP and Tau [413, 414]. PKA- mediated activation of CREB is essential for late phase (protein transcription-dependent) LTP [307, 415], and other aspects of neurotransmission and synaptic function. For instance, it affects the expression of the brain-specific growth factors, NGF and BDNF, as well as tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine [307, 319, 416, 417].

The cAMP/PKA/CREB signalling axis is highly important for the formation and consolidation of LTP. Given the importance of PKA-CREB activity in memory formation, it is logically a major

signalling pathway impaired in AD, a disease characterised by significant memory loss. This conclusion is supported by studies showing reduced CREB phosphorylation and activity in the hippocampus of AD-affected patients [323], and experimental *in vivo* models exposed to exogenous AD-like Aβ concentrations [418]. Furthermore, treatment with the phosphodiesterase inhibitor Rolipram improves hippocampal-dependent memory formation in mouse models of AD [328-330]. Similarly, increasing the expression of CREB in AD mouse models also alleviates memory deficits [419]. Conversely, PKA-mediated phosphorylation of Tau at Ser214 enhances the association of Tau with GSK-3β [325], a key Tau kinase implicated in AD pathology [88, 103]. Phosphorylation of Tau at Ser214 also facilitates the dissociation of Tau from microtubules [87]; this is thought to be a key event in AD, by promoting Tau aggregation. It also causes subpopulations of neurons to be more vulnerable to degeneration in the prefrontal cortex of primates [331]. Thus, activation of PKA could have a positive (enhancing CREB activity) or a negative (enhancing AD-like p-Tau pathology) impact in neurons. While the cAMP/PKA/CREB signalling pathway undoubtedly plays a role in AD, the exact nature of this role remains to be further examined.

Another subset of regulatory enzymes critical to neuronal homeostasis includes PP2A [161]. As mentioned above, the typical PP2A enzyme exists as a heterotrimer of a scaffolding A subunit (two isoforms), a catalytic C subunit (two isoforms) and one of many regulatory B-type subunits (B, B', B" or B'" families). In addition to subunit composition, the catalytic subunit is also post-translationally modified by methylation. PP2Ac methylation promotes the recruitment of Bα to the AC enzyme and stabilisation of PP2A-ABαC holoenzymes (***Figs. 1.5*, *1.6*** [170]). In contrast, it does not significantly affect binding of B'-, B"- or B''''/Striatin regulatory subunits [420] to the core enzyme. Thus, potential alterations in PP2A methylation ultimately result in a loss of PP2A-ABαC, and changes in intracellular PP2A subunit composition. This is highly relevant to our thesis project, since PP2A-ABαC enzymes are the primary Tau Ser/Thr phosphatase in neurons [155, 157], and have been shown to be down-regulated in AD autopsy brain tissue in tandem with p-Tau pathology [158, 159]. PP2A-ABαC enzymes also regulate other aspects of Tau function, including its microtubule-binding and stabilising activities [201, 395].

PP2Ac methylation can be reversed by Protein Phosphatase Methylesterase 1 (PME-1). PME-1 has been suggested to play multiple roles in the regulation of PP2A in cells. Aside from PP2A demethylation, PME-1 can also bind to and inactivate PP2A by displacement of the core Mn^{2+} ions in the catalytic subunit [168]. PME-1 also functions as a chaperone for newly produced, demethylated PP2Ac, which is important to prevent ubiquitination of PP2A and subsequent proteasomal degradation [421].

Little is currently known about the regulation of PP2Ac methylation in neurons, aside from the contribution of the one-carbon metabolic cycle. Like all methylation reactions, PP2Ac methylation by LCMT-1 is dependent on the available cellular pool of SAM [236, 403] and is impaired via feedback inhibition of LCMT-1 by S-Adenosyl Homocysteine (SAH).

Studies in non-neuronal cells suggests that the cAMP signalling pathway can regulate PP2Ac methylation [333, 334]. Similarly, PP2A regulates CREB via dephosphorylation in multiple cell types, including hippocampal neurons [335-337]. In this chapter, our goal was to first examine how the activation of cAMP/PKA signalling affects PP2Ac methylation in N2a cells and in *ex vivo* cortical slices prepared from mouse brain tissue. Furthermore, we aimed to investigate how this signalling module affects the regulation of CREB phosphorylation and other targets common to both PP2A and PKA, such as Tau.

We found that activation of cAMP/PKA signalling leads to rapid changes in PP2Ac methylation and redistribution of PP2Ac in neuronal cells. We also observed a complex interplay between cAMP/PKA and PP2A-ABαC signalling pathways, which affected their function in the regulation of CREB, Tau and neurite outgrowth.

3.2 Results

3.2.1. PP2Ac methylation and methylation-dependent PP2A subcellular distribution are altered by cAMP/PKA signalling

3.2.1.1 Activation of cAMP/PKA signaling leads to time-dependent demethylation of PP2Ac

To investigate whether PKA signaling can affect PP2Ac methylation, we treated serum-starved N2a cells for 0 to 60 minutes with the widely used adenylate cyclase activator, Forskolin. Activation of PKA by Forskolin led to a significant ~20% to ~60% increase in demethylated PP2Ac at the 15, 20 and 30 minute time points (***Fig. 3.1 A-B***). After one hour, the Forskolin-mediated increase in PP2Ac demethylation was no longer observed and amounts of demethylated PP2Ac returned to basal levels.

In order to verify if the demethylation of PP2Ac following Forskolin exposure was mediated by PKA, we pre-incubated a subset of N2a cells for 45 minutes with either 30μM H89 (a pharmacological inhibitor of Forskolin) or vehicle prior to stimulation with 50μM Forskolin for 20 minutes. As a control, other subsets of cells were incubated in parallel for 0-30 minutes with vehicle + 50μM Forskolin (***Fig. 3.1***). Notably, H89+Forskolin-incubated N2a cells showed significantly less (approximately ~20%) PP2Ac demethylation, compared to untreated, serum starved N2a control cells (***Fig. 3.1 C-D***). In addition, there was ~50% less demethylated PP2Ac in H89+Forskolin-treated N2a cells, relative to N2a cells incubated with vehicle +50μM Forskolin (***Fig. 3.1 C-D***). This provided evidence that the effects we observed on PP2Ac demethylation were more specifically initiated by activation of cAMP-PKA signaling.

Peak effects of Forskolin were also recapitulated by incubating N2a cells with 2.5μM of the phosphodiesterase inhibitor, Rolipram (***Fig. 3.1 E-F***). Indeed, demethylated PP2Ac levels were increased by ~70% in Rolipram-treated cells compared to vehicle-treated controls. These findings suggest that activation of cAMP signaling promote a time- and PKA-dependent demethylation of PP2Ac in N2a cells.

To confirm these data obtained in cultured cells, we next examined the effects of cAMP/PKA activators in acute mouse cortical slice preparations. As seen in N2a cells, we observed a significant increase in PP2Ac demethylation after incubation of mouse cortical slices at room temperature with 50μM Forskolin for 15 minutes (***Fig. 3.2.A***). Likewise, when we incubated cortical slices with 50μM Forskolin combined with 2.5μM Rolipram (to prevent the degradation of cAMP by phosphodiesterases), we observed a significant increase in demethylated PP2Ac after 15 minutes. This effect was preserved after 60 minutes of incubation with a combination of Rolipram and Forskolin (***Fig. 3.2.B***). These data confirm that sustained elevation of cAMP can induce marked demethylation of PP2A in neuronal cells.

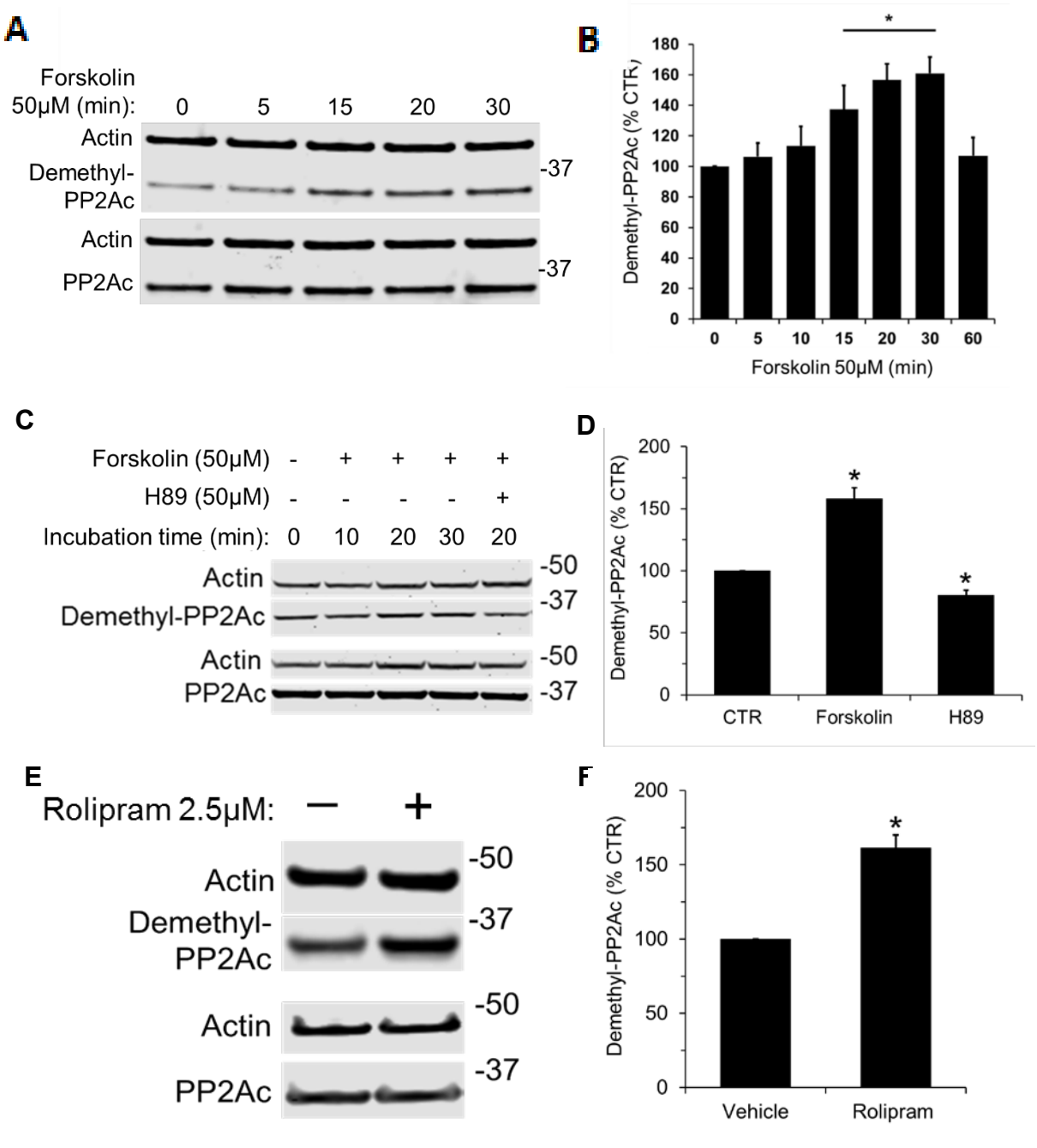


Figure 3.1 Pharmacological activation of PKA promotes PP2Ac demethylation. (**A**) Serum-starved N2a cells were incubated for the indicated times with 50μM Forskolin or vehicle, prior to harvesting, Duplicate aliquots of total cell homogenates were analysed by Western blot for the relative expression of demethylated and total PP2Ac using validated antibodies. Actin was used to normalise each blot for protein loading. Representative Western blots are shown. (**B**) Demethylated PP2A levels were quantified after densitometric analysis of blots and normalisation for total PP2Ac expression levels. Values shown represent mean ± S.D. (n = 5; *p<0.05). (**C**) Subsets of serum-starved N2a cells were pre-incubated with either 30μM H89 or vehicle for 45 minutes prior to stimulation with 50μM Forskolin for the indicated time. (**D**) Subsets of serum-starved N2a cells were pre-incubated with 30μM H89 or vehicle for 45 minutes prior to incubation with Forskolin or vehicle for 20 minutes. Demethylated PP2Ac levels were quantified after densitometric analysis of blots and normalisation for total PP2Ac expression levels in untreated (Vehicle), vehicle + Forskolin-incubated (Forskolin), and H89+Forskolin (H89)-treated N2a cells. Values shown represent mean ± S.D. (n = 5; p <0.05). (**E**) N2a cells were incubated for 45 minutes with 2.5μM Rolipram or vehicle. Cells were harvested and duplicate aliquots (~25μg proteins) of total cell homogenates were analysed by Western blot for the relative expression of demethylated PP2Ac as described in (A). (F) Demethylated PP2A levels were quantified after densitometric analysis of blots and normalisation for total PP2Ac expression levels, as in (D) and (F). Data presented are mean ± S.D. (n = 5; p < 0.05).

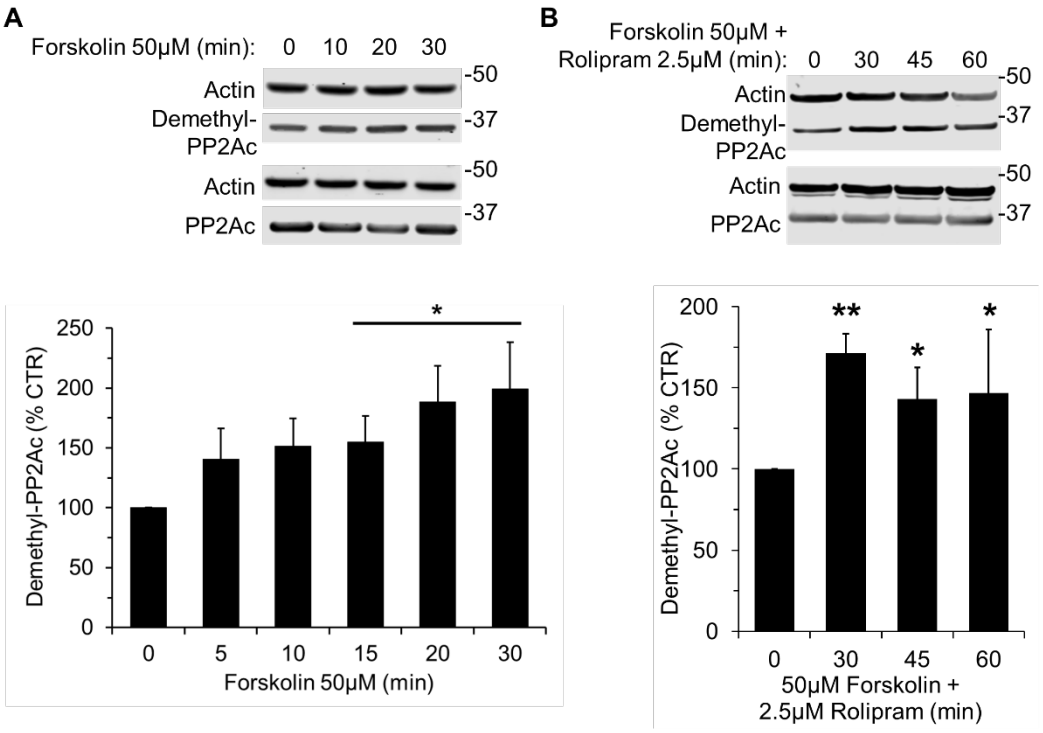


Figure 3.2 PKA activation induces PP2Ac demethylation in acute preparations of mouse cortex. Brain cortical tissue from 4- to 6 month-old female mice was obtained after sacrifice and sectioned into 350μm-thick coronal slices in an ice-cold sucrose-supplemented artificial CSF. Prior to homogenisation, isolated slices of mouse cortex were incubated with either 50μM Forskolin or vehicle for 0-30 minutes (**A**), or a combination of either 50μM Forskolin + 2.5μM Rolipram, or vehicle for 0-60 minutes (**B**). Duplicate aliquots of homogenates (~15μl protein extract per well) were analysed by Western blot for the relative expression of demethylated and total PP2Ac using validated antibodies. Actin was used to normalise each blot for protein loading. (**A**) Representative Western blots of experiments performed with Forskolin (upper panel). Demethylated PP2A levels were quantified after densitometric analysis of blots and normalisation for total PP2Ac expression (lower panel). Values shown represent mean ± S.D. (n = 5, *p<0.05, Forskolin versus vehicle-treated controls). (**B**) Representative Western blot (upper panel) for experiments performed with Forskolin + Rolipram and quantification of demethylated PP2A levels (lower) after densitometric analysis of blots as described in (**A**). Values shown represent mean ± S.E.M. (n = 3, *p<0.05, **p<0.01, Forskolin + Rolipram-treated slices, versus vehicle-treated controls).

3.2.1.2 PP2Ac methylation status varies in different cellular compartments

By affecting PP2A subunit composition, PP2A methylation state is also an important determinant of the subcellular distribution of PP2A enzymes [165, 172, 405]. Demethylated PP2A enzymes are found in complex with PME-1 in the cytoplasm, but are typically enriched in the nucleus of COS-7 and HeLa cells [172, 422]. LCMT-1 and methylated PP2A enzymes are abundant in the cytoplasm, but also present in smaller quantities in the cell nucleus. Pools of LCMT-1 and methylated PP2A-ABαC enzymes are also found in cholesterol- and glycosphingolipid-rich lipid rafts [165]. PP2A methylation levels have been reported to fluctuate during the cell cycle in non-neuronal cells, and PP2Ac methylation is essential for the metaphase-anaphase transition in the cell cycle [405].

Thus, we next assessed the spatial distribution profile of methylated and demethylated PP2A enzymes in our N2a cell model. To that end, we comparatively analysed by Western blot the expression levels of methylated and demethylated PP2Ac in homogenates of total, nuclear, cytosolic and membrane fractions prepared from unstimulated, serum starved N2a cells. We found that the vast majority of endogenous PP2Ac is methylated, with only 17± 5.3% of total endogenous PP2Ac in demethylated form in total homogenates from these cells (n = 5). This is in agreement with previous reports showing that the bulk of cellular PP2A is in a methylated form in 3T3 fibroblasts and in mouse brain [338]. Furthermore, membrane-associated PP2A was predominantly methylated (***Fig. 3.3***), in agreement with our earlier findings [165]. As expected from earlier studies in non-neuronal cells [172], the nuclear fraction was the most enriched in demethylated PP2Ac (***Fig 3.3***).

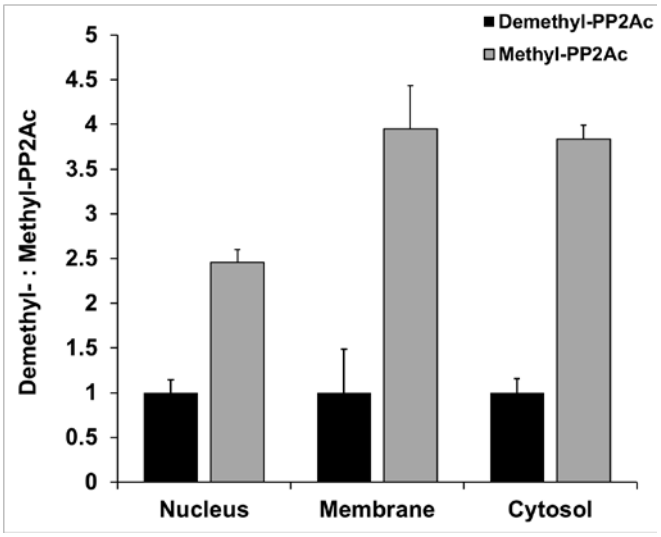


Figure 3.3 Subcellular distribution of demethylated and methylated PP2Ac. Unstimulated N2a cells were separated by subcellular fractionation (*see Chapter 2: Materials and methods*) into nuclear, membrane and cytosolic compartments. 50µg of protein from each purified cellular fraction were then analysed by Western blot to assess the distribution of demethylated PP2Ac using compartment-specific protein loading controls (*see Materials and methods*). The graph presented here shows mean values ± S.D. for the proportion of methylated- (grey bars) relative to demethylated- PP2Ac (black bars) in each compartment, (n = 5 for nucleus, n = 6 for membrane, n = 3 for cytosol)

3.2.1.3 cAMP/PKA signalling leads to nuclear enrichment of demethylated PP2A and PME-1

PKA signalling is subject to a highly organised spatiotemporal compartmentalisation, since the functional effects and targets of PKA activation differ in the plasma membrane, cytosol, cytoskeleton and nucleus [281]. Likewise, methylated and demethylated PP2A pools are also regulated in a compartment-specific manner [165, Fig. 3.3]. We have demonstrated that incubation of N2a cells with Forskolin induces an overall increase in endogenous PP2Ac demethylation (***Figs. 3.1 & 3.2***) and the cellular compartment that is most enriched in demethylated PP2Ac is the nucleus [Fig. 3.3, 172]. Thus, we next examined whether Forskolin stimulation correlates with the specific nuclear enrichment of demethylated PP2Ac. N2a cells were incubated for 0-30 minutes with 50μM Forskolin and nuclear proteins were isolated. After 15 minutes of incubation with Forskolin, we observed a significant increase in the nuclear-associated pools of demethylated PP2Ac and PME-1, compared to vehicle-treated controls (***Fig. 3.4***).

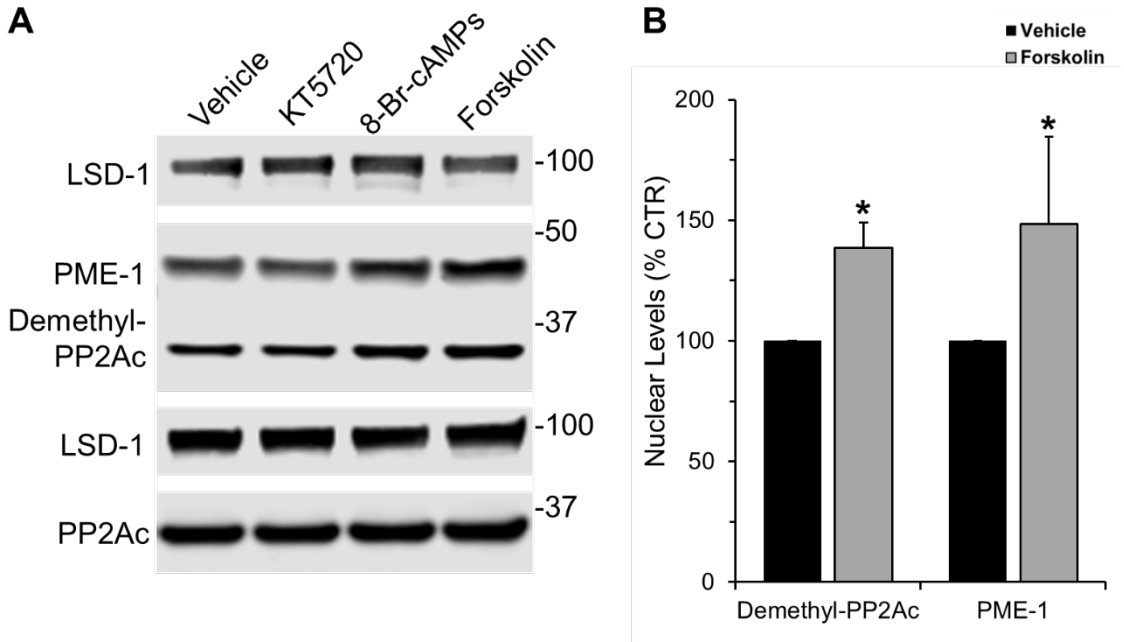


Figure 3.4 Forskolin enhances the nuclear distribution of demethylated PP2Ac and PME-1. N2a cells were starved for 16h in low-serum containing medium and incubated with either 1μM KT5720, 100μM 8-Br-cAMPs, 50μM Forskolin, or vehicle. Total cell homogenates were prepared and nuclear fractions purified as described in *Materials and methods*. Duplicate aliquots of nuclear fractions were analysed by Western blot for the expression of demethylated or total PP2Ac, and PME-1 using validated antibodies. Actin or LSD-1 were used to control for protein loading; LSD-1 was also used to assess the purity of nuclear fractions. (**A**) Representative Western blots. (**B**) Demethylated PP2A and PME-1 levels were quantified after densitometric analysis of blots and normalisation as described in previous figures. Demethylated PP2Ac and PME-1 levels in vehicle- (Black bars) and Forskolin-treated cells (15 minutes, Grey bars) are represented. Values shown represent mean ± S.E.M. (n = 4, *p<0.05: Forskolin-, versus vehicle-treated cells).

This is in agreement with previous observations showing a close association of PME-1 and demethylated PP2Ac in the nucleus [172]. Additionally, this effect was also observed when N2a cells were incubated with the cAMP analogue, 8-Bromo-cAMP. Nuclear PP2A demethylation was significantly inhibited when cells were incubated in the presence of the PKA inhibitor, KT5720 (***Fig. 3.4***), clearly indicating that this is a PKA-dependent signalling event in N2a cells.

3.2.1.4.: PKA-induced PP2A holoenzyme redistribution in cell compartments

We have shown in our results so far that PKA-dependent demethylation of PP2A is associated with its nuclear redistribution in N2a cells. Since the methylation status of PP2Ac can also determine the subcellular compartmentalisation of PP2A-ABαC holoenzymes [165], we also examined whether PKA signalling induces their redistribution. Following incubation with 50μM Forskolin, a significant loss of total PP2Ac and Bα was observed at the plasma membrane (***Fig. 3.5***). Importantly, the plasma membrane is highly enriched in methylated PP2Ac-ABαC enzymes and the Bα subunit is always found associated with the A and C subunits *in vivo*; thus, the loss of Bα reflects a loss of the PP2A-ABαC holoenzyme [258, 405, 406, 423].

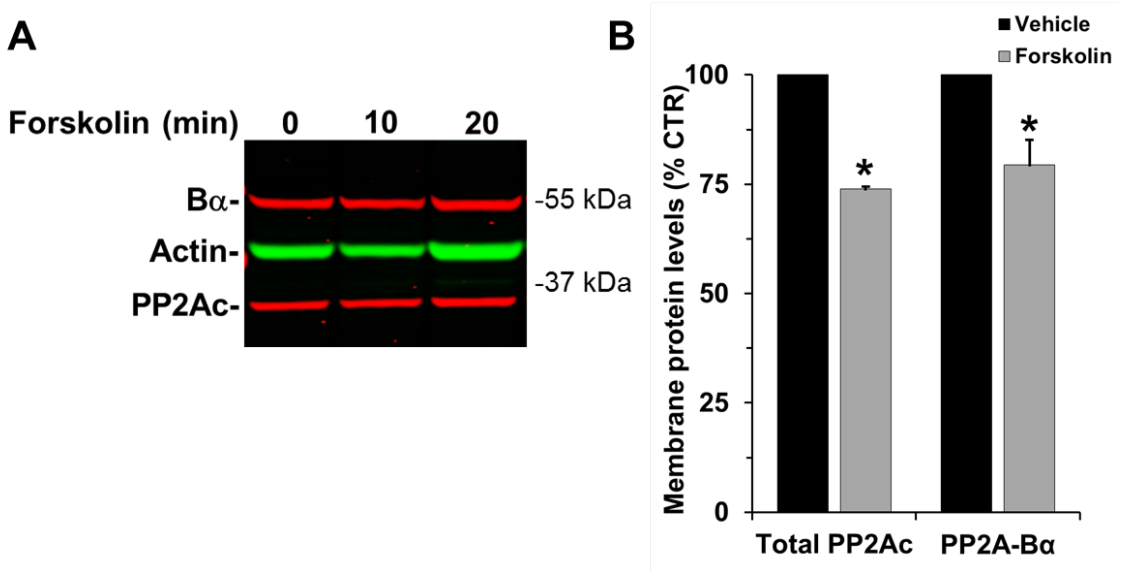


Figure 3.5 Incubation of N2a cells with Forskolin induces a loss of membrane-associated PP2A-ABαC. Serum-starved N2a cells were incubated with 50μM Forskolin or vehicle for 0- 20 minutes prior to harvesting. Membrane fractions were then isolated as described in *Materials and methods*. Relative expression of PP2Ac and PP2A-Bα were analysed by Western blot using validated antibodies, following separation of proteins by SDS-PAGE. Aliquots were loaded by volume, with each lane containing 25μl of membrane isolates. Actin was used to normalise each blot for protein loading (**A**). Representative Western blot. (**B**) PP2Ac and PP2A-Bα levels were quantified after densitometric analysis of blots. PP2Ac and PP2A-Bα expression levels in membrane-enriched N2a compartments after treatment for 20 minutes with Forskolin (Grey bars) or vehicle (Black bars). Values shown represent mean ± S.E.M ($n = 4$, * $p < 0.05$, Forskolin-treated cells compared to vehicle-treated controls)

We also analysed purified nuclear fractions for potential changes in PP2A subunit levels. As observed in purified membrane fractions, there was an increase in nuclear PP2Ac and Bα subunits levels in N2a cells exposed to 50μM Forskolin (***Fig. 3.6***). This increase was observed 5 minutes after drug stimulation, and persisted for 10-15 minutes. These results, together with those shown in ***Fig. 3.5***, indicate that following activation of PKA signalling in N2a cells, there is a translocation of PP2A-ABαC complexes. At the same time, rapid, localised activation of pre-existing pools of nuclear PKA [281] could mediate the more rapid (< 5 minutes) nuclear redistribution of PP2A subunits as a result of changes in PP2Ac methylation.

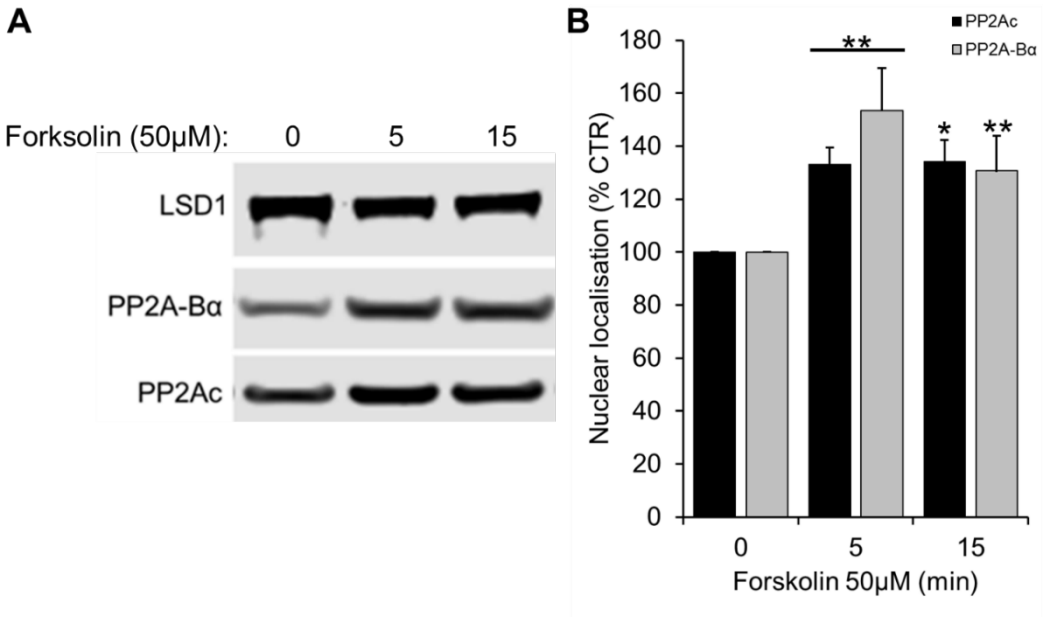


Figure 3.6 Stimulation of cAMP/PKA signaling by Forskolin leads to nuclear enrichment of PP2Ac and Bα subunits. Serum-starved N2a cells were incubated for 0-15 minutes in 50μM Forskolin, harvested and separated into subcellular compartments, including nuclear-enriched fractions.Nuclear-enriched fractions were analysed for relative expression of PP2Ac and PP2A-Bα using validated antibodies. LSD1 was used to normalise for protein loading and to verify nuclear enrichment (**A**) Representative Western blots. (**B**) Nuclear PP2Ac (Black bars) and PP2A-Bα (Grey bars) expression levels were quantified after densitometric analysis of blots. Values shown represent mean ± S.D. (*n* = 5, ***p< 0.01, *p<0.05*, Forskolin compared to vehicle treated cells)

3.2.1.5: Activation of cAMP/PKA signalling does not lead to PME-1 phosphorylation

Considering our observations that PKA activation induces PP2Ac demethylation (***Figs. 3.1, 3.2***), and that there is a nuclear enrichment of both PME-1 and demethylated PP2Ac, we wanted to determine whether PKA can directly regulate PME-1 activity. In this context, there is some emerging evidence that PME-1 activity can be modulated by phosphorylation. PME-1 phosphorylation at a non-identified Ser/Thr residue by Salt-Inducible Kinase-1 has been reported in Opossum Kidney cells, and is associated with inhibition of the methylesterase [424]. However, phosphorylation of PME-1 that causes increased methylesterase activity has not yet been described in any cell type.

In order to investigate whether PME-1 is phosphorylated by PKA, we used Myc-conjugated magnetic beads to immunoprecipitate Myc-tagged PME-1 (Myc-PME-1) from total homogenates of transiently transfected COS-7 cells. For this particular experiment, we chose to use COS-7 rather than N2a cells since these widely-used cells have a better transfection efficiency and express higher levels of recombinant proteins. Serum-starved cells expressing Myc-PME-1 were incubated with 50μM Forskolin or vehicle, prior to immunoprecipitation and extensive washing. Immunoprecipitates were then analysed by Western blot. Additionally, we analysed total homogenates of COS-7 cells to verify the overall level of phosphorylation of cellular proteins driven by PKA activation.

As expected, in total homogenates, activation of cAMP/PKA signalling resulted in a significant increase in the amounts of proteins labelled with a well-validated antibody recognising PKA-specific RRXS*/T*-phosphomotifs, relative to unstimulated cells (***Fig. 3.7***). This confirmed that many endogenous proteins became phosphorylated by PKA activation. In contrast, we did not observe any noteworthy PKA-induced phosphorylation of PME-1 in our Myc-PME-1 co-immunoprecipitates, suggesting that PME-1 is not a prevalent PKA substrate. Likewise, parallel *in vitro* kinase assays performed by Dr. Jean-Marie Sontag showed that PME-1 was not phosphorylated by recombinant PKA. PKA-mediated phosphorylation of PME-1 is therefore not the anticipated mechanism of regulation of PP2Ac methylation by PKA, which is likely complex and will require further investigation.

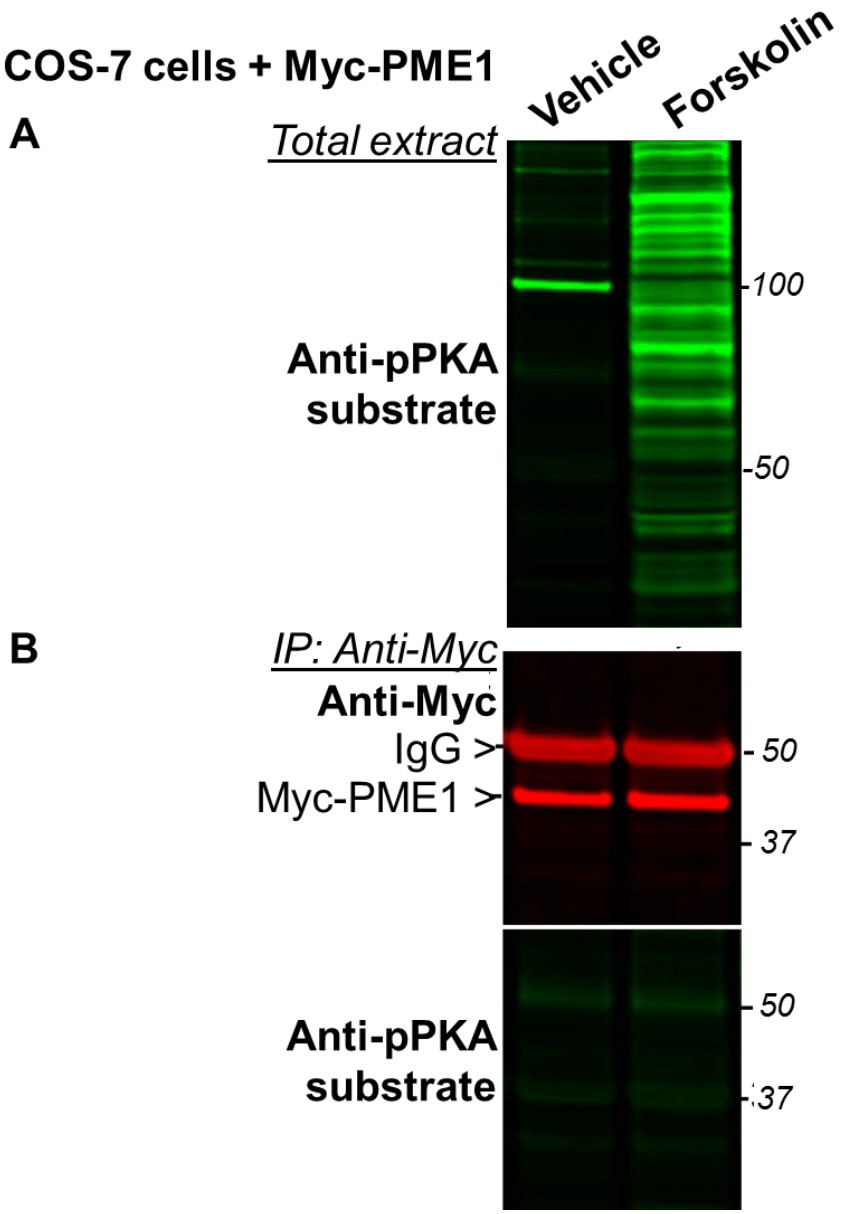


Figure 3.7 Activation of cAMP/PKA signalling does not result in phosphorylation of PME-1* in COS-7 cells**. Myc-PME-1 was immunoprecipitated from total homogenates of COS-7 cells transiently expressing Myc-PME-1 that were incubated with either 50μM Forskolin or vehicle. ***A. Western blot of total COS-7 homogenates probed with a validated antibody recognising phosphorylated PKA substrates (pPKA substrate, [RRXS*/T*]) to verify efficacy of Forskolin in PKA activation in COS-7 cells. ***B***. Western blot analysis of immunoprecipiated Myc-PME-1 with anti-Myc and anti-pPKA substrate antibodies.

3.2.2 Functional effects of PKA-mediated regulation of PP2Ac demethylation

3.2.2.1 PP2Ac methylation modulates CREB activity in N2a cells

We next focused our attention on assessing the functional significance of PKA-mediated PP2A demethylation. Of particular interest, CREB is a major target for phosphorylation in the PKA signaling pathway. Furthermore, PP2A may target CREB for dephosphorylation in the hippocampus [337]. We thus assessed whether there is a link between PKA-induced PP2Ac demethylation and CREB phosphorylation. Firstly, we re-analysed the N2a cell homogenates for PKA-induced changes in CREB phosphorylation (see ***Fig. 3.2***). We primarily focused on 0-15 minute time points, since stimulation of PKA for 5-15 minutes correlated with the greatest increase in PP2Ac demethylation in the nucleus, where CREB is localized (***Fig. 3.4***). There was a ~2 to ~5-fold increase in CREB phosphorylation at Ser133 in Forskolin-treated N2a cells, relative to vehicle-treated controls (***Fig. 3.8.A***). These results indicate that in N2a cells, PKA-dependent CREB phosphorylation at Ser133, and its subsequent activation occur concomitantly with PP2Ac demethylation.

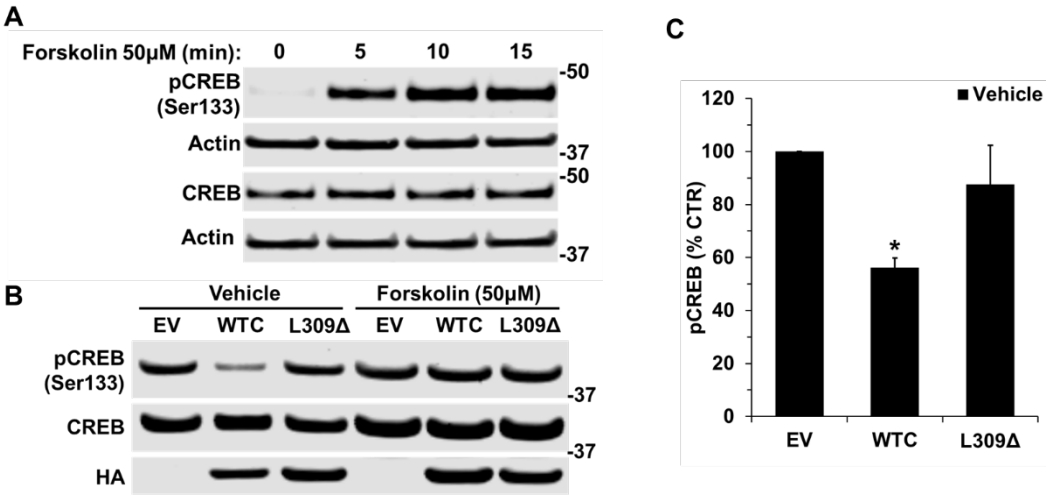


Figure 3.8: PP2Ac^{WT}-induced CREB dephosphorylation is methylation-dependent Serum-starved N2a cells were incubated for 0 to 15 minutes with 50μM Forskolin, prior to harvesting and homogenisation. N2a cells were also transfected with plasmids encoding either, HA-PP2Ac^{WT} (WTC) HA-PP2Ac$^{L309\Delta}$ (L309Δ), or the empty vector (EV), then incubated for 20 minutes with 50μM Forskolin or vehicle. Duplicates aliquots of N2a homogenates were analysed by Western blot for the relative expression of CREB phosphorylated at Ser133 (pCREB) and total CREB using validated antibodies. Actin was used to normalise each blot for protein loading. (**A**). Representative Western blot for pCREB and total CREB in N2a cells incubated for 0-15 minutes with Forskolin. (**B**) Representative Western blot for pCREB and CREB from N2a cells transfected with EV, PP2Ac^{WT}, or PP2Ac$^{L309\Delta}$, incubated with vehicle or Forskolin. (**C**) pCREB levels were quantified after densitometric analysis of blots and normalisation for total CREB expression levels in vehicle-treated cells. Values shown represent mean ± S.D., compared to vehicle-treated, N2a cells transfected with EV plasmid (n = 4; *p<0.05).

We next compared the effects of expressing HA-tagged wild-type PP2A catalytic subunit ($PP2Ac^{WT}$) and the methylation-incompetent L309Δ mutant ($HA\text{-}PP2Ac^{L309\Delta}$) in N2a cells. Of note, ectopic PP2Ac cannot be expressed at high levels in transfected or infected cells due to autoregulation of cellular PP2Ac levels [200, 425]. Indeed, transfection with a plasmid encoding $HA\text{-}PP2Ac^{WT}$ leads only to a ~30% enhancement of total cellular PP2Ac expression. Moreover, expressed PP2Ac subunits replace some of the endogenous protein due to this regulation; they typically represent ~30-38% of total PP2Ac in transfected N2a cells [258]. Of note, expressed $HA\text{-}PP2Ac^{WT}$ is methylated to similar levels as endogenous $PP2Ac^{WT}$, so that enhanced $PP2Ac^{WT}$ expression correlates with increased PP2A methylation and activity levels in unstimulated N2a cells expressing $PP2Ac^{WT}$. In contrast, the basal levels of demethylated PP2Ac are increased by ~35-40% in N2a cells expressing $PP2Ac^{L309\Delta}$, due to the partial replacement of endogenous PP2Ac (which is primarily in a methylated form) by the methylation-incompetent $PP2Ac^{L309\Delta}$ mutant [258]. The enhanced PP2A demethylation in N2a cells expressing $PP2A^{L309\Delta}$ is accompanied by a concomitant loss of cellular Bα protein levels, likely due to defects in PP2A-ABαC biogenesis and stabilization, and the notorious instability of free, monomeric PP2A subunits [406].

The expression of ectopic HA-tagged $PP2Ac^{WT}$, which increases total cellular PP2A activity, was associated with a decrease in the basal phosphorylation state of endogenous CREB at Ser133 in unstimulated N2a cells, compared to EV-transfected cells (***Fig. 3.8 B and C***). This is in agreement with the published role of PP2A as a CREB phosphatase [335, 337]. However, enhanced dephosphorylation of CREB was not observed in cells expressing the methyl-incompetent $PP2Ac^{L309\Delta}$ mutant (***Fig. 3.8 B and C***), suggesting that CREB dephosphorylation by PP2A is dependent on methylation of the phosphatase.

CREB phosphorylation levels were enhanced in all Forskolin-treated, transfected N2a cells (***Fig. 3.8.B***). These data suggest that Forskolin induces tandem demethylation of PP2Ac and phosphorylation of CREB. Significantly, this would mean that PKA can mediate CREB phosphorylation, either directly via its role as a CREB kinase, or indirectly, by circumventing PP2A-mediated dephosphorylation of CREB via demethylation of PP2Ac.

3.2.2.2: PME-1 inhibition alters CREB phosphorylation

As described above, PKA plays a role in the demethylation of PP2Ac (***Fig 3.1***), and the redistribution of demethylated PP2Ac and PME-1 enzymes to the nucleus in N2a cells (***Fig. 3.4***). We also show that CREB dephosphorylation in vehicle-treated N2a cells is also influenced by PP2A methylation state (***Fig. 3.8***). Therefore, our next aim was to determine if PME-1 inhibition could affect the phosphorylation of CREB, since it is the sole enzyme that mediates PP2Ac demethylation [426]. Serum-starved N2a cells were incubated for 3 hours with the previously validated PME-1 inhibitor AMZ-30 [427]. This led to a significant reduction of pCREB levels (***Fig. 3.9***), suggesting that PME-1-induced PP2A demethylation and/or inactivation [171, 426] can impair PP2A-dependent dephosphorylation of CREB in N2a cells.

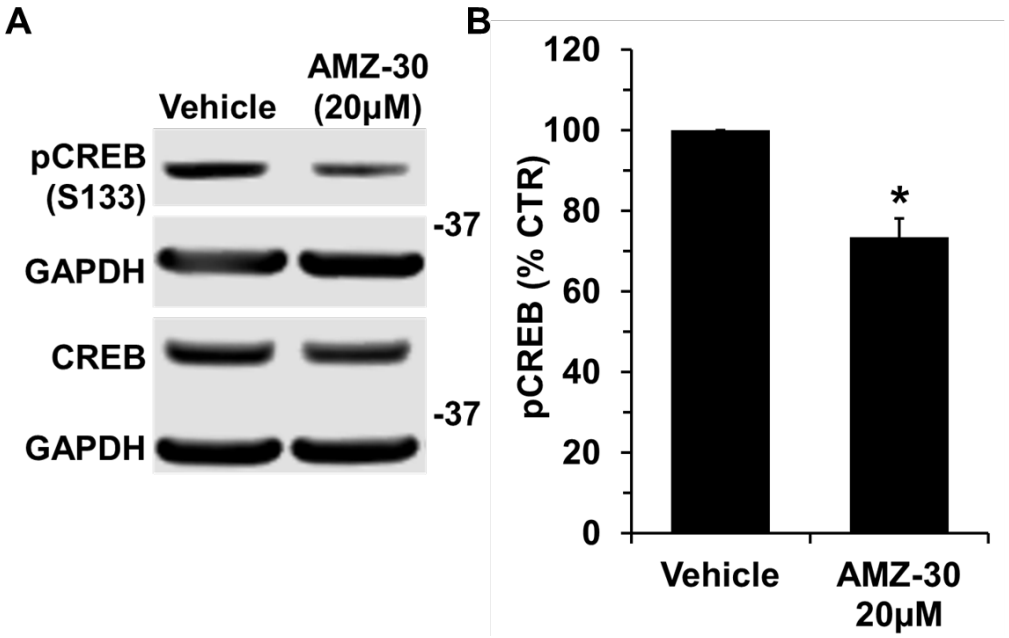


Figure 3.9: PME-1 inhibition impairs CREB phosphorylation. N2a cells were incubated for 3 hours with either the PME-1 inhibitor AMZ-30 (20μM), or vehicle for 3h, prior to harvesting. Total N2a cell homogenates were analysed in duplicate by Western blot for pCREB and total CREB. Western blots were re-probed with anti-GAPDH antibodies to control for protein loading (**A**) Representative Western blot. (**B**) Quantified pCREB levels after densitometric analysis of blots and normalisation for total CREB expression. Values shown represent mean ± S.E.M. (n = 4, * p < 0.05).

3.2.2.3 PKA-dependent Tau phosphorylation is impaired by PP2Ac overexpression

Our group has previously demonstrated that enhanced PP2Ac methylation leads to reduced Tau phosphorylation in N2a cells and *in vivo* [263, 396, 403]. Furthermore, PP2A demethylation correlates with increased Tau phosphorylation in AD [159]. Two of the phosphorylation sites targeted by PP2A-ABαC, the levels of which are dependent on PP2Ac methylation, include the PKA-targeted Ser214 residue and the phospho-epitope PHF-1, which is abundant in NFTs in late-stage AD [258]. Indeed our lab has previously shown that only very low levels of Tau phosphorylated at the Ser214 and PHF-1 epitopes can be detected in unstimulated, vehicle-treated N2a cells [258], in agreement with numerous studies showing that neuronal Tau is primarily in a dephosphorylated state under normal, basal conditions. Moreover, in these studies, expression of HA-PP2Ac$^{L309\Delta}$ enhanced the baseline phosphorylation of Tau at these sites, while immunoreactivity with anti-pSer214 or PHF-1 antibodies was further decreased in HA-PP2Ac^{WT}-expressing N2a cells [258].

To determine how the phosphorylation of Tau at these sites is more specifically affected by the PKA-induced demethylation of PP2Ac, I performed Western blot analyses of duplicate homogenates from Forskolin-treated N2a cells that had been transfected with plasmids encoding either PP2Ac^{WT}, PP2Ac$^{L309\Delta}$, or the corresponding EV. As expected incubation of EV-transfected controls with Forskolin promoted the phosphorylation of Tau at Ser214, a PKA-sensitive site, as evidenced by strong immunoreactivity with specific anti-pSer214 antibodies in stimulated versus unstimulated cells (***Fig. 3.10. A, B***). However, ectopic expression of PP2Ac^{WT} counteracted the effects of Forskolin and promoted the dephosphorylation of Tau at this site (***Fig. 3.10 A, B***). In contrast to PP2Ac^{WT}, expression of PP2Ac$^{L309\Delta}$ failed to induce the dephosphorylation of Tau at the pSer214 phospho-epitope. Indeed, following incubation with 50μM Forskolin, PP2Ac$^{L309\Delta}$-expressing cells had similar levels of pSer214-Tau, compared to EV-transfected N2a cells (***Fig. 3.10 A, B***).

Phosphorylation of Tau at the PHF-1 (pSer396/404) phospho-epitope has also been reported to be enhanced by Forskolin *in vivo* [88]. Accordingly, there was a strong immunoreactivity with the PHF-1 antibody in Forskolin-stimulated N2a controls transfected with EV, relative to unstimulated controls (***Fig. 3.10 A, C***). While expression of PP2Ac^{WT} promotes the dephosphorylation of Tau at the PHF-1 epitope in unstimulated N2a cells [258], Forskolin-induced Tau phosphorylation at PHF-1 was augmented by PP2Ac^{WT} expression (***Fig. 3.10 A, C***). This stimulatory effect is likely mediated by the demethylation of expressed PP2A^{WT}, since expression of PP2Ac$^{L309\Delta}$, which enhances cellular demethylation also stimulated the Forskolin-mediated phosphorylation of Tau at PHF-1. These results suggest that while cAMP/PKA activation can induce the demethylation of PP2Ac, there are different consequences for site-directed Tau phosphorylation.

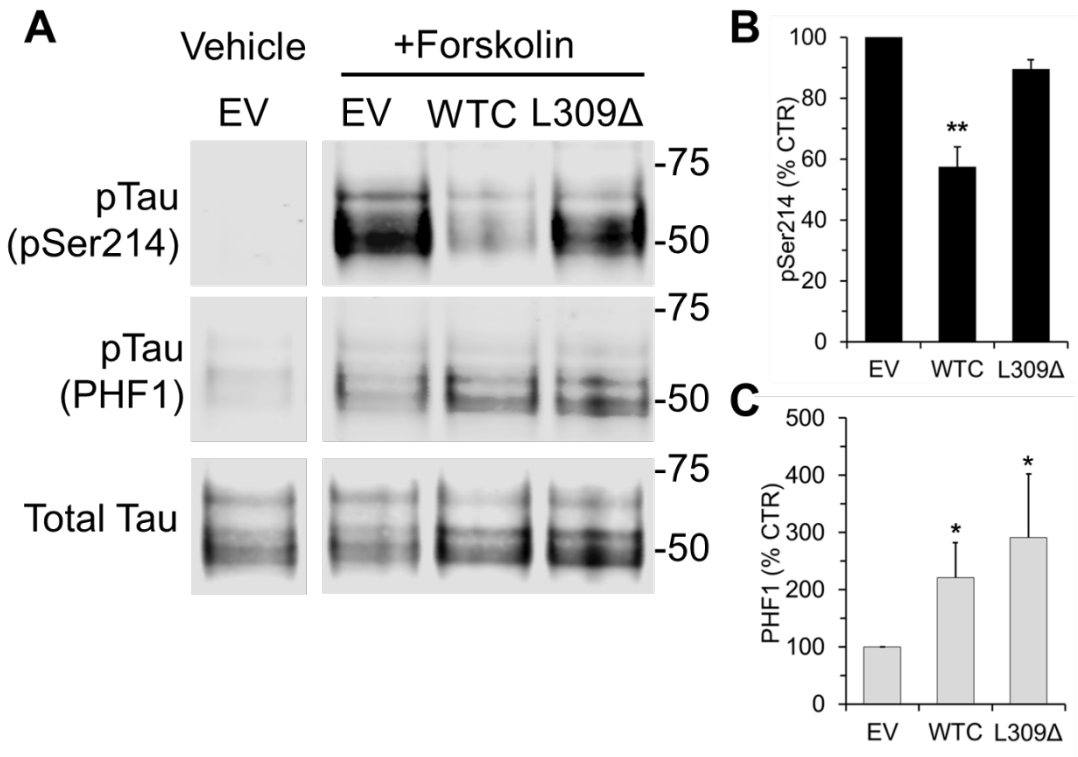


Figure 3.10 Effects of modulating PP2A methylation on cAMP/PKA-dependent Tau phosphorylation. N2a cells homogenates described in ***Figure 3.8*** were also analysed by Western blot for relative expression of Tau phosphorylated at Ser214 (pSer214) or the PHF-1 epitope, using validated antibodies. (**A**) Representative Western blot for pSer214 and total Tau (**B**) pSer214-Tau levels were quantified in Forskolin-treated cells after densitometric analysis of blots and normalisation for total Tau. Values represent mean ± S.E.M. (n = 4, **p<0.01). (**C**) PHF-1 levels were quantified in Forskolin-treated cells after densitometric analysis of blots and normalisation for total Tau. Values represent mean ± S.E.M. (n = 4, *p<0.05).

3.2.2.4 PP2Ac methylation mediates cAMP-dependent N2a differentiation

It is well-established that cAMP/PKA signalling contributes to neurite outgrowth [288, 291, 428-430]. Previous data from our group also strongly implicates PP2Ac methylation in the process of neurite extension [236]. In order to examine whether or not the regulation of neuritogenesis by PP2Ac methylation and cAMP/PKA signalling occur independently, we differentiated N2a cells in low-serum media, as previously reported [236]. To induce cAMP-mediated neurite outgrowth, we incubated N2a cells in the presence of 20μM Forskolin for 24h; as expected from earlier studies, cell differentiation was significantly enhanced in Forskolin-treated, compared to vehicle-treated cells (***Fig. 3.11***). In contrast, neuritogenesis was inhibited when cells were incubated with R_p-cAMPs, a potent and specific competitive inhibitor of the activation of cAMP-dependent protein kinases (***Fig. 3.11***), thus confirming the role of cAMP signalling in N2a cell differentiation.

We next incubated N2a cells stably expressing HA-PP2Ac^{WT} in differentiation medium containing 20μM Forskolin. The average neurite length and number of neurite-bearing cells was significantly increased in these cells, compared to controls under the same experimental conditions (***Fig. 3.11, B & C***). These data suggest that PP2Ac^{WT} contributes to and enhances cAMP/PKA-dependent neurite outgrowth. To test whether or not PP2Ac methylation plays a role in this process, we also incubated N2a cells stably expressing PP2Ac$^{L309\Delta}$ in the same media for 24h. These cells showed a substantially impaired capacity to differentiate, in stark contrast to control and PP2Ac^{WT} expressing N2a cells (***Fig 3.11 B & C***). These results suggest that cAMP/PKA-induced neurite outgrowth depends on the integrity of PP2A methylation in N2a cells.

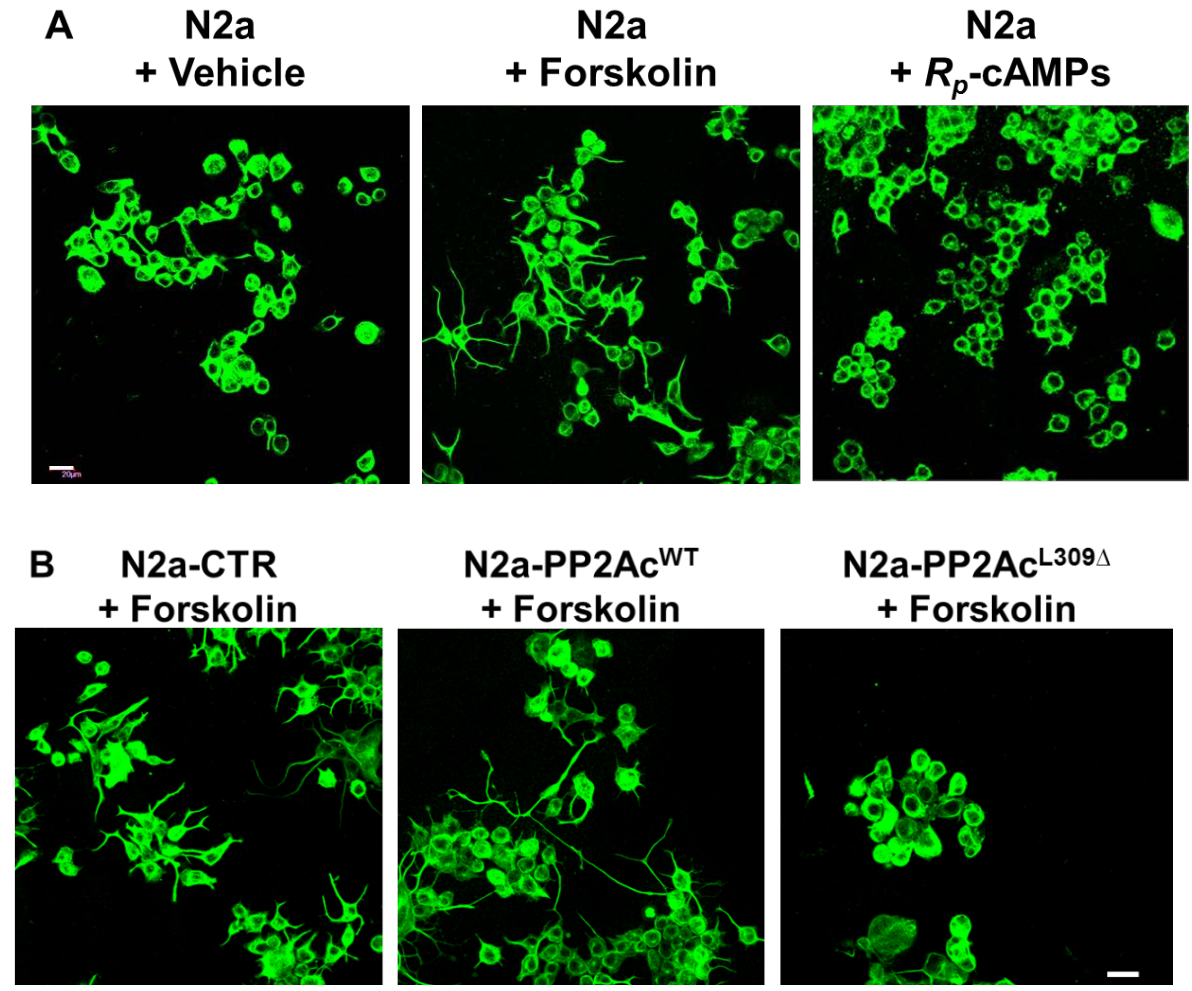


Figure 3.11 PP2Ac methylation mediates Forskolin-induced N2a neurite outgrowth. Wild-type, or stably-expressing, $PP2Ac^{WT}$ and $PP2Ac^{L309\Delta}$ N2a cells were differentiated in low-serum medium and incubated for 24 hours with either 20µM Forskolin, 20µM Rp-cAMPs or vehicle. After differentiation, cells were fixed and stained for βIII-Tubulin. (***A***) Representative immunofluorescent staining of N2a cells incubated with either vehicle, Forskolin or Rp-cAMPs. White scale bars = 20µm (***B***) Representative immunofluorescent staining of control wild-type N2a cells, and $PP2Ac^{WT}$- or $PP2Ac^{-L309\Delta}$-expressing N2a cells, after incubation with 20µM Forskolin for 24h. White scale bars = 20µm. (***C***) Cells were counted and neurites measured to assess average neurite length and proportion of neurite-bearing cells. Data presented as mean values ± S.D. (* $p < 0.01$).

3.3 Discussion

Methyl-group supply via one-carbon metabolism is well-established as a major determinant of PP2A methylation. However, in this chapter we sought to come to a more refined understanding of how this post-translational modification occurs. Our first aim was to assess if PP2A methylation could be altered in an alternative model to that of one-carbon metabolic supply. Our second aim was to assess the functional consequences of PKA-mediated PP2A demethylation in neurons. Here, we present evidence of a novel mechanism for the modulation of PP2Ac methylation involving cAMP/PKA signalling and the redistribution of PP2A holoenzymes between cell compartments, which also holds functional significance for the regulation of CREB, Tau and neurite outgrowth.

3.3.1 PP2Ac demethylation is induced by Forskolin

Our results indicate that cAMP generation and subsequent activation of PKA lead to a time-dependent demethylation of PP2Ac in N2a cells and acute mouse cortical slice preparations. We observed a consistent effect on the demethylation of PP2Ac in both of these models using Forskolin alone and in combination with Rolipram (***Figs. 3.2*** and ***3.3***). Other groups have previously suggested that, in rodent kidney or amphibian ovaries, enhancing cAMP availability with Forskolin leads to increased PP2Ac methylation [333, 334]. Similarly, other nebulous claims implicating increased “PP2A activity” following Forskolin exposure in non-neuronal models also differ from the results we present here [431, 432]. The contrast between our data showing that cAMP/PKA activity leads to PP2Ac demethylation, and other data from various cell types suggests that there may be a unique cross-talk between PP2A and PKA in neurons. This would align with other reports of indirect cross-talk between PKA and PP2A. As an example, PKA and PP2A respectively phosphorylate and dephosphorylate the same AMPAR subunit residues and neuronal L-type calcium channel (Cav1.2) residues, both of which are important for modulating neuron excitability [194-196, 433]. Aside from these indirect interactions and opposing functions, there is little evidence in neurons with which we can compare these novel findings. This may be potentially a significant cellular signalling event, since PP2A and PKA share many substrates at the plasma membrane which are important for neuron function, such as the GluA1-AMPAR subunit [301, 434].

Since PP2Ac methylation affects important functions of PP2A, such as subcellular distribution and substrate targeting of the holoenzyme, we then decided to focus on these functional effects of cAMP/PKA-mediated demethylation of PP2Ac.

3.3.2 Forskolin leads to redistributed PP2A enzymes

In this chapter, we observed that in addition to demethylation of PP2Ac, cAMP/PKA signalling induced the redistribution of PP2A. We investigated the effects of cAMP/PKA signalling on

membrane-associated PP2Ac and Bα, since this compartment is enriched in methylated PP2A-ABαC complexes [165], and PKA signalling can also be localised here [280, 409]. Following incubation of N2a cells with 50μM Forskolin, we saw a reduced pool of total PP2Ac, demethylated PP2Ac and PP2A-Bα in the plasma membrane (***Fig. 3.5***). This effect was blocked by the PKA inhibitor H89. PKA signalling at the plasma membrane has not only been demonstrated before, but has been recorded at high levels, localised to lipid rafts [435], signalling microdomains at the plasma membrane which also contain high concentrations of methylated PP2Ac and PP2A-ABαC enzymes [165]. PKA can localise here via anchoring by AKAPs, but also myristoylation of the catalytic subunit (PKA-C), which is thought to be the primary facilitator of substrate phosphorylation at the plasma membrane [436].

In contrast, activation of cAMP/PKA signalling with Forskolin in N2a cells also led to enhanced PP2Ac, Bα, PME-1 and demethylated PP2Ac content in the nuclear-enriched fraction (***Figs. 3.4*** & ***3.6***). This re-distribution of PP2A enzymes was abrogated by co-treatment with the PKA inhibitor KT5720. Notably, PME-1 and demethyated -PP2Ac are primarily localised to the nucleus in basal conditions [172], while there is also a pool of nuclear-associated adenylate cyclase which can induce rapid activation of PKA in this compartment [281]. Further studies would ideally investigate, and compare the dynamics of PKA activation in neurons with the redistribution of PP2A enzymes in response to this.

PME-1 can demethylate PP2A *and* inactivate the enzyme through displacement of the critical manganese ions in the active site of PP2Ac [171], as well as acting as a chaperone for latent, or inactive PP2A enzymes [421]. Moreover, PP2A inactivation and holoenzyme disassembly has recently been shown to be dependent on PP2Ac demethylation [169]. The proteins found to be mediating this are TIPRL (TOR pathway signalling regulator), and another major PP2A latency chaperone, α4. Recent findings have demonstrated a role for phosphorylation of PME-1 in modulating its activity state [424]. We therefore hypothesised that a mechanism involving either the methylesterase or chaperone functions of PME-1 which may underlie the PKA-induced PP2A demethylation and redistribution which we observed in our present studies. After immunoprecpitiating PME-1 from Forskolin-treated COS-7 cells, we found that PKA does not phosphorylate PME-1 (***Fig. 3.7***). We therefore concluded that an alternative mode of PP2Ac demethylation and redistribution, induced by PKA exists and remains to be investigated in future studies. Such alternative mechanisms may involve a PKA-dependent regulation of other aforementioned proteins or chaperones of PP2A, such as α4.

3.3.3 Functional significance of PKA-induced PP2Ac demethylation

a. CREB phosphorylation

CREB is the major substrate and target of cAMP/PKA in the nucleus, and plays highly important, neuron-specific functions [302]. PP2A has been shown to dephosphorylate PKA-phosphorylated CREB [335, 337], though the specific holoenzyme responsible for this has not been described. Incubation of N2a cells and sections of mouse brain with Forskolin and Rolipram led to a reduced proportion of demethylated PP2Ac (***Figs. 3.1, 3.2***).Changes in the pool of methylated PP2Ac affects the subunit composition of PP2A complexes within cells, particularly the formation of PP2A-ABαC [437]. These holoenzymes are responsible for many processes within neurons, including adaptations in excitability to external stimuli [338]. These previous findings, along with our current data demonstrating that Forskolin also causes an increase in nuclear PP2A-Bα (***Fig. 3.6***), led us to develop the hypothesis that PKA-induced demethylation of PP2A is closely linked to the phosphorylation of CREB.

To determine the effect of PP2A methylation state on CREB, we ectopically expressed $PP2Ac^{WT}$ and $PP2Ac^{L309\Delta}$ in N2a cells treated with vehicle or Forskolin. In vehicle-treated cells, expression of $PP2Ac^{WT}$ induced a significant loss of pCREB, compared to EV-transfected cells (***Fig. 3.8***). This relative loss in CREB phosphorylation was not recapitulated when N2a cells were transfected with plasmids encoding $PP2Ac^{L309\Delta}$ (***Fig. 3.8***). We thus concluded that the methylation state of PP2Ac under basal conditions can determine the phosphorylation (and therefore the activity state) of a major PKA substrate in neurons, CREB. In contrast, we could not observe a similar effect in $PP2Ac^{WT}$-transfected N2a cells incubated with 50μM Forskolin (***Fig. 3.8***), suggesting that PKA-induced PP2A demethylation may also contribute indirectly to CREB phosphorylation, in addition to direct phosphorylation by PKA. Interestingly, Forskolin-treated N2a cells transfected with plasmids encoding $PP2Ac^{WT}$ and $PP2Ac^{L309\Delta}$ had similar levels of pCREB, compared to vehicle-treated, EV-transfected cells, supporting PKA-induced PP2A demethylation as a mechanism of enhancing CREB activity in neurons.

In supporting experiments, we found that AMZ-30, an irreversible PME-1 inhibitor which prevents PP2Ac demethylation [427], induces a decrease in CREB phosphorylation (***Fig. 3.9***). This suggests that, while direct phosphorylation of PME-1 is not the mechanism underlying the PKA-dependent demethylation of PP2A, the demethylation of PP2A by PME-1 is still an important event in the PP2A-mediated dephosphorylation of CREB.

In our current experiments, we have been able to significantly alter the phosphorylation of CREB via the manipulation of PP2A methylation, without directly changing the activity of PKA. Given our results demonstrating that PKA activation induces both the demethylation of PP2A and the phosphorylation of CREB, it would then appear that CREB, as a downstream target

of PKA signalling is subject to nascent regulation by PP2A. The temporal dynamics of PKA-targeting of CREB have been typically described in the order of tens of minutes [438, 439], although this has recently been shown to vary between brain regions; for example, CREB phosphorylation in mouse striatal dopaminergic neurons is much more responsive to extracellular stimuli, compared to that in mouse neocortex [439, 440]. Additionally, Forskolin-induced, PKA-mediated CREB phosphorylation has been reported to occur after 17 minutes in mouse brain slices [441], compared to cultured cells, in which nuclear adenylate cyclase can activate nuclear PKA much faster [281]. This is notable, since we observed an extremely similar time-course for PKA-mediated PP2A demethylation in neuronal cells. It is unclear whether or not this is linked to the temporal dynamics of PKA-mediated CREB phosphorylation in neurons. Future experiments should be undertaken in order to delineate differences between direct PKA-mediated CREB phosphorylation, and indirect PKA-induced impairments in PP2A-mediated CREB dephosphorylation, and what the intracellular signalling dynamics of these processes are.

b. Tau phosphorylation direct and indirect mechanisms

Elevated site-directed Tau phosphorylation is a major event in the cellular pathological processes in AD [58, Chapter 1]. The major proportion of Tau dephosphorylation is mediated by PP2A, as compared to other major phosphoprotein phosphatases, PP1 and Calcineurin [157]. PP2A-Bα expression is impaired in Alzheimer's disease, along with reduced methylation of PP2Ac and a concomitant exacerbation of phosho-Tau pathology [158, 159]. There are several key phospho-epitopes of Tau targeted by methyl-PP2Ac, including PHF-1 and Ser214 [258]. The Ser214 site is phosphorylated by PKA *in vivo* and primes Tau for subsequent phosphorylation by Glycogen Synthase Kinase 3β (GSK-3β), another major Tau kinase [88, 89, 103]. The importance of the phosphorylation of Tau at Ser214 is not completely elucidated, however it appears to be critical in negatively regulating the association of Tau to microtubules [87] and interactions with kinases such as GSK-3β [325]. Expression of a Tau-Ser214Ala mutation in primary hippocampal neurons impairs neurite extension [442], but may promote initial neurite formation, suggesting that the impaired microtubule binding capacity of pSer214-Tau may play a role in the dynamic process of neurite outgrowth. Furthermore, activation of PKA by Forskolin incubation leads to Tau phosphorylation at PHF-1 [88, 443].

In our experimental conditions, we did not detect pSer214-Tau in vehicle-treated N2a cells (***Fig. 3.10***). However, following incubation of N2a cells with Forskolin, we were able to detect marked pSer214-Tau (***Fig. 3.10***). Transfection of plasmids encoding $PP2Ac^{WT}$ in N2a cells prior to incubation with Forskolin impaired the phosphorylation of Tau at this epitope by ~45%, compared to EV-transfected cells, which was not observed following the expression of the methylation-incompetent $PP2Ac^{L309\Delta}$ (***Fig. 3.10***). These data suggest that, while Forskolin

induces Tau phosphorylation, the expression of $PP2Ac^{WT}$ abrogates this effect. Furthermore, PP2A-mediated dephosphorylation at this site is significantly affected by the methylation of PP2A, since Forskolin-treated N2a cells expressing $PP2Ac^{L309\Delta}$ had similar levels of pSer214-Tau to EV-transfected cells. This is in support of previous findings presented by our group, identifying that PP2A-ABαC dephosphorylated Tau at this site [258], and evidence presented by other groups demonstrating that Tau phosphorylation is mostly dependent on PP2A activity levels [205]. Levels of pSer214-Tau were mostly preserved in $PP2Ac^{L309\Delta}$-transfected cells following incubation with Forskolin (***Fig. 3.10***). However, ectopic $PP2Ac^{L309\Delta}$ expression did not induce Tau phosphorylation at Ser214 in vehicle-treated N2a cells. This supports a conclusion in which Tau phosphorylation is mostly regulated by PP2A, but can be acutely regulated by PKA-induced demethylation of PP2A.

Interestingly, we observed a much higher level of endogenous Tau phosphorylation at PHF-1 (Ser396 & 404), compared to Ser214 (***Fig. 3.10***). The PHF-1 epitope has been linked with p-Tau pathology in AD [105, 110], but may also contribute to other functions of Tau, such as the induction of LTD [123, 444], and association with the plasma membrane [113], which is also determined by PP2A methylation [165]. PHF-1 phosphorylation has been suggested to be enhanced with PKA-mediated Ser214 phosphorylation [88], though our findings did not support this, as we observed overall similar levels of PHF-1 immunoreactivity between Forskolin-treated cells and vehicle-treated cells.

Oddly, some previous data from other groups have indicated that incubation of primary hippocampal neurons [443], or treatment *in vivo* [445] with Forskolin leads to concurrent increases in PP2A activity and Tau phosphorylation at both Ser214 and PHF-1 epitopes. These results appear counterintuitive, since elevated PP2A activity would be incompatible with increased Tau phosphorylation [157]. Furthermore, impairments in PP2Ac methylation have been repeatedly reported to enhance PHF-1 phosphorylation [396, 446, 447], which is what our present data confirms, as Forskolin led to both impaired PP2A methylation and a relative increase in PHF-1 phosphorylation in $PP2Ac^{WT}$-transfected cells.

Interestingly, it has been suggested that cAMP/PKA signalling is able to modulate PP2A methylation and activity in a number of cell types. Some have suggested that treatment of primary neurons [8] or exposure in vivo [9] with Forskolin leads to a simultaneous increase in Tau phosphorylation at the Ser214 and PHF-1 epitopes, and in PP2A activity. Since PP2A is a major Tau phosphatase, whose activity generally results in Tau dephosphorylation, these results are somewhat curious. A possible explanation is that enhanced methylation causes PP2A-mediated dephosphorylation of GSK-3β at the inhibitory Ser9 residue, which causes enhanced Tau phosphorylation. This cross-talk mechanism of Tau phosphorylation has been described in other models *in vivo* [422, 448, 449].

The results we present in this chapter represent a novel mechanism of cAMP/PKA-mediated PP2Ac demethylation, and also show that cAMP/PKA signalling can alter the phosphorylation of Tau both directly and indirectly, through the demethylation of PP2A. What requires further investigation, however, is the conditions that lead to a differential phosphorylation between pSer214-Tau and PHF-1 phosphorylated Tau, since they have previously been suggested to be linked [88], and a more clear mechanism of how PP2Ac methylation contributes to this.

c. Neurite Outgrowth

Models of neurite-like extensions and branching in cultured cells are a useful model for the assessment of conditions required for neuronal plasticity and structural integrity. Our group has previously demonstrated that PP2Ac methylation is essential for optimal neurite outgrowth [236], a conclusion we also reached in the appraisal of our current results (***Fig. 3.11***). PKA signalling is also an essential component of neurite outgrowth [291, 429, 430, 450]. We were able to show that incubation with Forskolin led to demethylation of PP2A (***Figs. 3.1, 3.2***), and that PKA-dependent demethylation of PP2A significantly affected the phosphorylation of CREB and Tau (***Figs. 3.8, 3.10***). Importantly, both CREB and Tau also play important roles in neuron differentiation and neurite outgrowth [71, 102, 290, 291, 412]. Thus, we hypothesised that cAMP/PKA-induced changes in PP2Ac methylation may mediate neurite outgrowth in N2a cells.

We successfully induced N2a cell differentiation in serum-depleted growth medium (0.5% FBS) with 20μM Forskolin (***Fig. 3.11***). Conversely, we prevented the formation of processes from N2a by incubation in the same differentiation medium with the addition of the inhibitory cAMP analogue Rp-cAMPs (20μM). Interestingly, Forskolin-induced neurite outgrowth was significantly enhanced in N2a cells stably overexpressing $PP2Ac^{WT}$ (***Fig. 3.11B***). Meanwhile, stable expression of the methylation-incompetent $PP2Ac^{L309\Delta}$ inhibited the effects of Forskolin on the proportion of differentiating N2a cells (***Fig. 3.11***). These data suggest that PKA-induced neuritogenesis is mediated by PP2A which is methylation-*competent*.

Previous work suggests that the impairment of neurite outgrowth in $PP2Ac^{L309\Delta}$-expressing N2a cells is due to impaired binding of the Bα regulatory subunit. In several cell types, mutations to the carboxyl-tail of PP2Ac have been shown to disturb binding of regulatory subunits [178, 423]. Furthermore, the L309Δ mutation annihilates both Bα and B'α binding to PP2Ac in N2a cells, and cannot bind to microtubules [451]. We also showed that $PP2Ac^{WT}$ expression reduced Forskolin-mediated Tau phosphorylation at Ser214, but $PP2Ac^{L309\Delta}$ expression did not (***Fig. 3.10***). Notably, phosphorylation of Tau at Ser214 has been linked to dissociation of the protein from microtubules [87], and PP2A does not bind microtubule-associated Tau [199, 395]. In light of these previous studies, our results suggest that Forskolin-

induced phosphorylation of Tau at Ser214, and demethylation of PP2A may be linked and serve to regulate the association of Tau at the microtubule network, which is essential for neurite outgrowth [70, 71]. Our present data compliments previous work in that we are able to show that these interactions between PP2A-ABαC, Tau and the microtubule network are essential to neurite outgrowth, since $PP2Ac^{L309\Delta}$ expression prevents neurite outgrowth despite incubation of N2a cells with Forskolin, which itself is sufficient in inducing this cellular process.

Another result of incubation of N2a cells with Forskolin that we observed was the redistribution of PP2A-ABαC complexes off the plasma membrane. This may also be significant, since both the localisation to the plasma membrane and the induction of neurite outgrowth are enhanced by PP2Ac methylation [165, 236] and are altered by incubation with Forskolin (***Figs 3.5, 3.11***). Additionally, expression of $PP2Ac^{WT}$ in tandem with Forskolin incubation did not reduce PHF-1 Tau phosphorylation in the same way that pSer214-Tau was reduced in the same conditions (***Fig. 3.10***). Significantly, PHF-1 phosphorylation impairs Tau association with the plasma membrane [113], which has been suggested to play a role in the development of neuronal polarity and association with growth cones, in a microtubule-binding-independent manner [69, 372, 452]. Altogether, our results taken with current available evidence indicate that PKA may induce the dissociation of PP2A and Tau from the plasma membrane, facilitating Tau association with the microtubule network, thereby inducing neurite outgrowth. These processes are linked in our results through the PKA-dependent demethylation and redistribution of PP2A, though precise mechanism deserve more attention in future studies.

3.3.4 Significance for the study of Alzheimer's disease

Investigations of cAMP signalling in the contexts of aging and disease have helped shed light on to what possible and probable roles this pathway plays in the brain. In human hippocampi, CREB expression and activity is lowered with age [323], whilst CREB activity is impaired in the parietal and frontal cortices, and the hypothalamus *in vivo* [453, 454]. Furthermore, in the senescence-accelerated mouse-prone 8 model of accelerated aging, CREB activity in the hippocampus is impaired [455]. The cAMP/PKA/CREB pathway is implicated in AD not only through impairments of learning and memory, but also via direct impairment by Aβ and other factors central to AD pathogenic cascades. BACE1 and Aβ both lead to a lowered pool of cAMP in brain lysates and hippocampi [456, 457]. Studies linking Aβ and Aβ-regulating enzymes involved with AD pathogenesis support the experimental utility of cAMP-elevating drugs such as Rolipram in preclinical models, which demonstrate an amelioration of cognitive impairments induced by Aβ [328-330]. Importantly, our group and collaborators have recently demonstrated that the sensitivity of neurons to Aβ exposure is dependent on the regulation of PP2Ac methylation [338].

Our present results indicate that the relationship between PKA signalling and the regulation of PP2Ac requires further attention in the study of AD. Here, we show that a major Tau kinase, PKA, not only directly mediates acute phosphorylation of Tau, but also facilitates indirect Tau phosphorylation through PP2Ac demethylation. Models of aging *in vivo* show that cAMP/PKA signalling leads to elevated Tau phosphorylation and a higher risk for neuronal degeneration [331]. The proposed mechanism was an inactivation of dendritic PDE4, leading to accumulation of pS214-Tau and gradual loss of spines and subsequently neurons. It is important to note that the main target of Rolipram in the prospective treatment of AD are PDEs [328, 330]. Recent evidence also shows that long-term, low concentrations of Forskolin leads to an increase in PP2Ac expression and activity, possibly, in response to elevated short-term Tau phosphorylation [443].

Thus, there is likely a complex interaction between cAMP/PKA signalling and PP2A in neurons, which, as we have demonstrated in this chapter, has functional effects for the neuronal regulation of PKA-driven CREB and Tau phosphorylation, and cAMP/PKA-induced neurite outgrowth. These observations have significant implications for the study of Alzheimer's disease, learning and memory and neuronal survival, since these are all directly affected by CREB, Tau, neurite outgrowth and PP2Ac methylation.

3.4 Concluding remarks

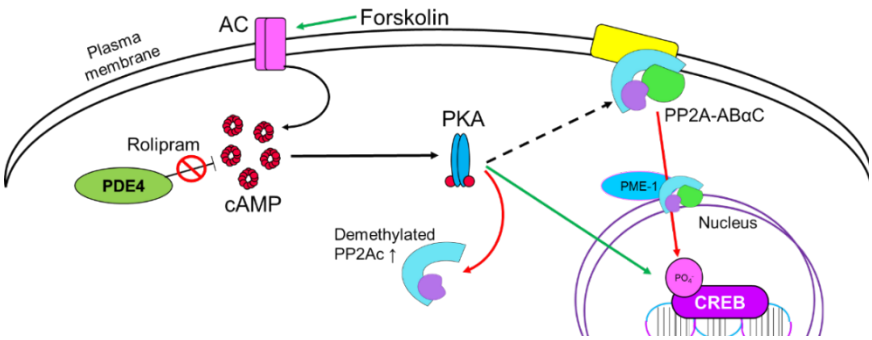
Little is understood about the regulation of PP2Ac methylation aside from the influence of one-carbon metabolism. While one-carbon metabolic inputs are important in the AD process (see chapters 4 and 5), there should conceivably be more than one mode of regulating such an important post-translational modification to an enzyme which is evolutionarily conserved from yeast through to humans [458]. Here, we investigated a cAMP-mediated, time-dependent demethylation of PP2Ac in N2a cells, acute cortical slices from mouse brain (***Fig. 3.3***). This effect was reproduced with the phosphodiesterase inhibitor Rolipram and the cAMP analogue 8-Bromo-cAMP, and eliminated with two inhibitors of PKA with distinct mechanisms of action, H89 and KT5720. *The novel mechanism of PP2Ac demethylation by cAMP/PKA signalling was found to have four major functional implications:*

1. *A PKA-induced, time-dependent redistribution of PP2A-ABαC complexes from the plasma membrane to the nucleus (**Fig. 3.12.A**)*
2. *A PKA-induced, PP2Ac-mediated regulation of CREB phosphorylation (**Fig. 3.12.A**)*
3. *Regulation of Ser214 Tau phosphorylation by the PKA-induced alteration of PP2Ac methylation (**Fig. 3.12.B**)*

4. *That PP2Ac methylation state mediates cAMP-induced neurite outgrowth (**Fig. 3.12.B**)*

These results represent one novel mode of PP2Ac regulation. The more characterised mechanism of PP2Ac methylation is through one-carbon metabolism and availability of methyl-groups within the cell. Our group has previously used a murine model of genetic *mthfr* and dietary folate deficiency to demonstrate that one-carbon metabolism affects PP2Ac methylation and Tau phosphorylation *in vivo* [263]. Since we have demonstrated a novel mode of regulating the same events (PP2Ac methylation), we then focussed on investigating whether or not these pathways intersect (Chapter 4). We additionally have investigated the effects of this model on the other major molecular aspect of AD, the Amyloid Precursor Protein (APP, Chapter 5).

A.



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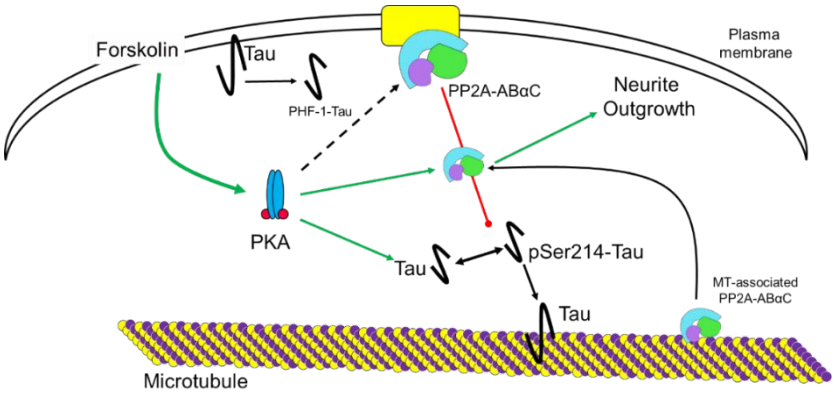

Figure 3.12 PP2Ac regulation by PKA in neurons. Schematic diagram shows that cAMP can be generated via Forskolin-induced adenylyl cyclase (AC) activation, phosphodiesterase (PDE) inhibition or G-Protein Couped Receptor (GPCR) activation. (**A**) Subsequent PKA activation leads to demethylation of PP2Ac, redistribution of PP2A-ABαC from the plasma membrane and movement of PP2Ac, PP2A-Bα and PME-1 into the nucleus, which we have shown in this chapter regulates CREB phosphorylation in a PP2Ac-methylation-dependent manner. (**B**) Overexpression of the methylation-incompetent $PP2Ac^{L309\Delta}$ led to altered PKA targeting of Tau. $PP2Ac^{L309\Delta}$ overexpression also impaired PKA-induced neurite outgrowth. This may be mediated by the redistribution of PKA and/or PP2A-ABαC to and from microtubules through competitive binding or redistribution from the plasma membrane.

Chapter 4: One-carbon metabolism alters CREB regulation in cells and *in vivo*

Acknowledgement of Collaboration

I hereby acknowledge that the work enclosed in the following chapter of this thesis was performed in collaboration with other researchers.

The work in the following chapter (Chapter 4) was primarily performed by myself (Alexander Hoffman), though I received assistance in some experiments from fellow lab members (Goce Taleski) and my supervisors (Dr. Jean-Marie Sontag and A/Prof. Estelle Sontag).

The tissue from MTHFR-deficient mice was donated from our collaborator Dr. Teodoro Bottiglieri and Dr. Brandi Wasek (Baylor, Dallas TX).

All figures and results, including data analysis presented in this chapter were obtained and composed by me (Alexander Hoffman), with input and feedback from my supervisors A/Prof. Estelle Sontag and Dr. Jean-Marie Sontag.

4.0 Rationale and significance

Disturbances in one-carbon metabolism are a risk factor for a range of cognitive and neurodegenerative impairments and diseases, including AD [216, 218, 459, 460]. Proper supply of folate and methyl-groups are essential for cellular, particularly neuronal, function and survival. The cAMP-responsive element binding protein (CREB) is a transcription factor associated with responses to extracellular stimuli, neurite outgrowth, cell survival and learning and memory [302, 305, 318, 461]. CREB activity is also impaired in the AD process [323], and has garnered interest as a potential therapeutic target to restore cognitive deficits observed in AD. Drugs like Rolipram, which target cAMP/PKA signalling, and subsequently, enhance the activity of CREB, have been shown to alleviate Aβ-induced cell death and impairments in learning and memory *in vivo* [328, 329].

In the previous chapter, we showed that cAMP-PKA signalling can regulate PP2Ac methylation. We also observed that this mechanism can influence CREB phosphorylation. In this chapter, we wanted to assess if the one-carbon metabolic cycle can regulate CREB. For this purpose, we studied CREB phosphorylation levels in N2a cells incubated with molecules that can stimulate or inhibit the methylation cycle (***Fig. 4.1***). Additionally, we also explored CREB regulation in different brain regions from wild-type and 5,10-methylenetetrahydrofolate reductase (MTHFR)-deficient mice fed diets with or without folate.

Previous data from our group has shown that these mice also have impaired methylation of PP2Ac and that there was an associated increase in Tau phosphorylation [263]. *We hypothesise that MTHFR and/or folate deficiency in mice will alter the regulation of CREB* (**Fig. 4.1**)

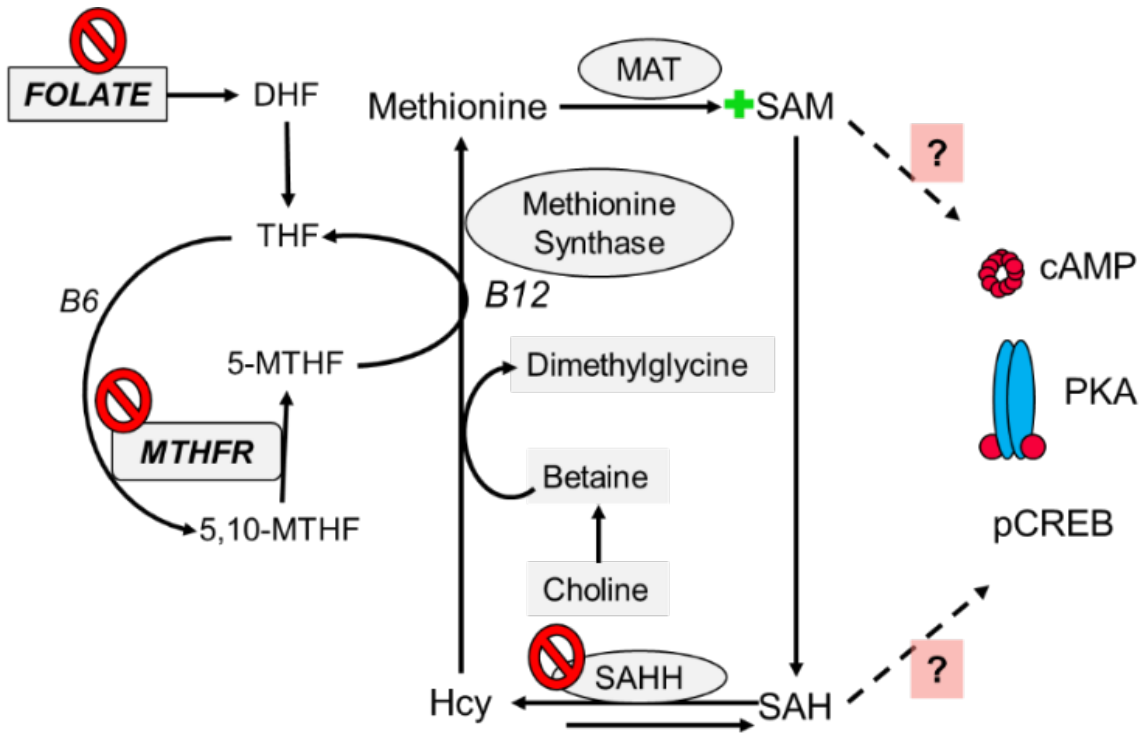

Fig 4.1 Illustration of one carbon metabolic cycle. Methyl groups in cells originate from dietary folates which are converted to 5-MTHF from 5,10-MTHF by Methylenetetrahydrofolate reductase (MTHFR). 5-MTHF donates a methyl group to facilitate the re-methylation of homocysteine (Hcy) to Methionine. This amino acid is then converted to the universal methyl donor S-adenosyl-methionine (SAM). After donation of the methyl group, the metabolite S-adenosyl-homocysteine (SAH) is formed. SAH hydrolysed into homocysteine. Aside from 5-MTHF, Choline may be converted to betaine which can aid in the re-methylation of Hcy. This pathway does not exist in neurons, but only in the periphery. Abbreviations; 5,10-MTHF (5,10-methylenetetrahydrofolate), DHF (dihydrofolate), MAT (methionine adenosyl-transferase), SAHH (S-Adenosyl-Homocysteine Hydrolase). Red circles indicate enzymatic reactions in enzymes targeted by molecules and mutations for the purpose of this study; these include folate deficiency in N2a cells and *in vivo*, MTHFR deficiency *in vivo* and inhibition of SAHH by 3-Deazaadenosine (3DA) in N2a cells. Green "+" next to SAM indicates that N2a cells were incubated in medium supplemented with this molecule.

4.1 Introduction: One-carbon metabolism impairs PP2Ac methylation and may affect CREB

Impaired folate metabolism leads to increases in total plasma homocysteine (tHcy), which can contribute to delayed development, neurological impairment, motor dysfunction, gait abnormalities, seizures, and thrombotic events [459]. Active folate (methylfolate, 5-MTHF) is needed for the re-methylation of Hcy to Methionine. This step is essential for the generation of the methyl donor S-adenosyl-methionine (SAM). SAM acts as a cofactor for dedicated methyltransferases involved in protein, DNA and lipid methylation reactions [462]. These reactions are critical for an array of biosynthetic pathways, including neurotransmitters, membrane lipids and nucleic acids. The rate-limiting enzyme in the conversion of folate to 5-MTHF is 5,10-methylenetetrahydrofolate reductase (MTHFR). There is a relatively high prevalence of functional genetic *Mthfr* polymorphisms in the general population, with the common human *Mthfr* 677C→T gene polymorphism occurring in 30% to 40% of people in Western countries [463]. This polymorphism is associated with mild enzymatic deficiency of MTHFR, and is the most frequent inborn cause of hyperhomocysteinemia (hHcy) [464]. The occurrence of MTHFR-677TT genotype is less common. It presents at a rate of ~10% [463], and is associated with significantly reduced enzyme activity and has a much more severe phenotype, leading to conditions such as spina bifida and developmental delays [463].

Mouse models with severe MTHFR deficiency show a number of behavioural and biochemical deficits in multiple regions of the brain including hHcy and altered methylation potential. There are significant reductions in hippocampal volume, increased hippocampal apoptosis, disturbances in neurotransmitter metabolism occur, along with impaired cognition and memory in these models [3].

In neurons, LTP and LTD underpin the molecular mechanisms of learning and memory [465]. Many signalling pathways are involved in LTP induction, but the cAMP-dependent protein kinase (PKA) is one which is essential to this process [296, 297, 466]. It is a critical pathway for plasticity of neurons, cell survival, neurotransmitter synthesis, transport and regulation of neurotransmission. PKA stabilises synaptic expression of AMPA-receptors (AMPARs) and enhances their conductance by phosphorylation of the GluR1 subunit *in vivo* [298, 300]. The cAMP-Responsive Element Binding protein, CREB can be activated through phosphorylation at the Serine 133 site, leading to binding and transcription of regions of DNA known as cAMP-Responsive Elements [467]. PKA-dependent activation of CREB is essential for an array of neuronal functions including LTP [307], expression of NMDARs [468], and neuronal survival through neurotrophic signalling [318].

Alterations in LTP induction are typically observed in models of disturbed one-carbon metabolism. Direct exposure to Hcy *in vivo*, or dietary interventions are associated with impaired LTP induction. [240, 273, 469, 470]. These changes are connected to short-term (consolidation of synaptic strength by post-translational modifications to receptor subunits [471, 472]) and long-term effects (at the transcriptional and translational levels [296]). There is some evidence that Hcy could alter CREB function in liver cell models [473, 474], while maternal disturbances in one-carbon metabolism lead to changes in CREB expression in their offspring [397]. Therefore, there may be a link between disturbed one-carbon metabolism and the regulation of CREB.

In this chapter, we are investigating if one-carbon metabolism can regulate CREB function. Since perturbations in one-carbon metabolism has been shown in numerous models to induce cognitive and structural deficits, and CREB is a critical mediator of both of these processes, we suggest that cAMP-PKA signalling (i.e. CREB activity) may be partly controlled by one-carbon metabolic inputs. For this purpose, we have used validated genetic models of mild and severe *Mthfr* deficiency [263], as well as dietary folate deficiency to determine the effects on CREB protein expression and phosphorylation in different brain regions.

4.2 Results

4.2.1 Altered methyl-group supply in N2a cells leads to changes in CREB phosphorylation

In order to assess how one-carbon metabolism may alter CREB phosphorylation, we incubated serum-starved N2a cells with the methyl-donor SAM or the SAH-hydrolase inhibitor 3DA (***Fig. 4.2***). In vehicle-treated cells, a small amount of CREB remained phosphorylated at Ser133 (~10-15% of total CREB levels). Overnight incubation with 3DA significantly reduced pCREB levels by ~50%, compared to vehicle-treated cells. Conversely, supplementation of N2a cells with 100μM SAM overnight led to a consistent increase in CREB phosphorylation by ~40% from basal levels (***Fig. 4.2***). These results suggest that CREB phosphorylation may depend on the availability of methyl donors in N2a cells.

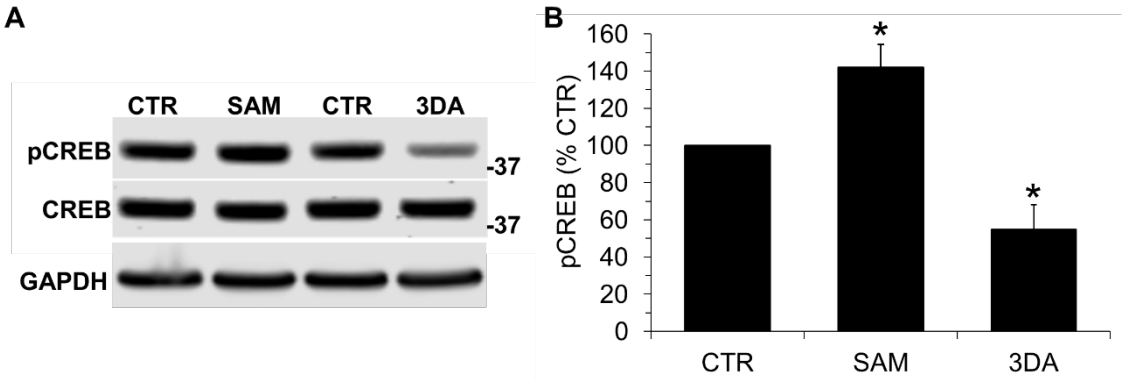

Figure 4.2 CREB phosphorylation depends on methyl-group availability. N2a cells were incubated overnight with Vehicle compound, S-Adenosyl-Methionine (SAM) or 3DA. Cells were harvested the next day. Homogenised extracts were analysed by Western blot for relative phosphorylation of CREB at Ser133 (pCREB) and total CREB validated antibodies. Protein loading was normalised using GAPDH in each blot. **A**. Representative Western blots. **B**. Phosphorylated CREB levels were quantified after densitometric analysis of blots and normalisation of total CREB expression levels. Values shown represent mean ± S.D. (*p<0.05 n = 5).

Folate a necessary co-factor for the remethylation of homocysteine to methionine, which is the primary source of SAM. Thus, we next wanted to see if changes in folate status can also affect CREB phosphorylation. Changing N2a cell culture medium from normal-folate containing RPMI to folate deficient (FD) RPMI led to a slight decrease (~20% reduction) in pCREB levels after 2h of culture and a significant decrease in pCREB levels (~30% ± 4, p < 0.05) after 4 hours of folate deprivation (***Fig 4.3***). Prolonged culture of cells in FD media led to the detachment of N2a cells and loss of viability, which is why the maximum time point of 4 hours was selected. These data supported previous evidence indicating that folate deficiency impairs cognitive processes such as learning and memory, likely because CREB mediates protein-transcription dependent LTP [339, 466, 475]. These new results confirmed that

disturbances in one-carbon metabolism in N2a cells can alter CREB phosphorylation in neuronal cells (***Figs. 4.2 & 4.3***).

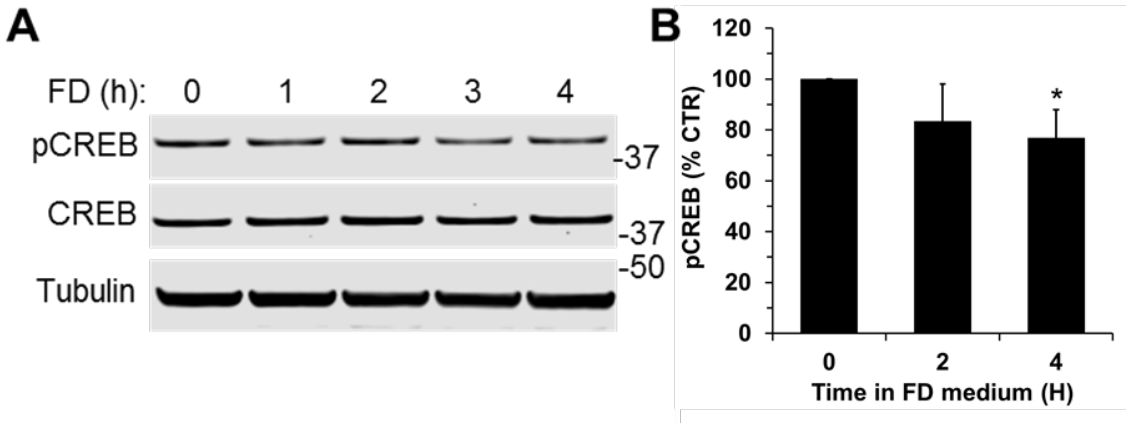

Figure 4.3: Folate deficiency impairs CREB phosphorylation in N2a cells. Serum-starved N2a cells were incubated in folate-deficient (FD) cell culture media for 0-4 hours prior to harvesting. N2a cell homogenates were analysed by Western blotting for relative expression levels of pCREB, followed by Western blotting for total CREB expression levels. Blots were also probed with anti-Tubulin antibodies to control for protein loading. ***A.*** Representative Western blot for pCREB and total CREB. ***B***. Phosphorylated CREB levels were quantified following densitometric analysis of Western lots and normalisation of total CREB levels. Values shown are representative of mean ± S.D. (*n = 4, * p < 0.05 versus normal folate control)*

Since we were able to demonstrate in N2a cells that CREB phosphorylation is modifiable through alterations in one-carbon metabolism, we then hypothesised that CREB phosphorylation and/or expression may be regulated by one-carbon metabolism in the brain.

4.2.2 MTHFR deficiency in vivo *has brain region specific effects on CREB expression and activity*

In addition to our experiments in N2a cells, we wanted to further investigate the effects of disturbed one-carbon metabolism *in vivo.* We used a previously validated model of MTHFR deficiency, using 6-week-old WT mice, or mice HET or NULL for the expression of MTHFR. HET mice are used to model the expression of the MTHFR-C677T allele in humans, while NULL mice model the TT genotype. The effects of the MTHFR-C677T polymorphism are discussed in Chapter 1. Briefly, presence of one MTHFR-C677T allele leads to a ~35% loss of MTHFR activity in human, while the MTHFR-677TT genotype can cause a ~65% loss of MTHFR activity, depending on ethnic background in humans [463].

We placed 16-month-old WT and HET mice on a low-folate (LF) diet for 6 months (*see Chapter 2: Materials and Methods*). First, plasma metabolites were analysed to verify the effect of both genotype and diet on the levels of tHcy and methylfolate (5-MTHF). In 6-week old mice, we found that tHcy levels were mildly, but significantly (~1.6-fold) increased in HET mice, compared to WT littermates (***Fig. 4.4.A***). Conversely, in NULL mice, we saw a substantially larger increase in tHcy (~10-fold), compared to aged-matched WT mice (***Fig. 4.4.A***).

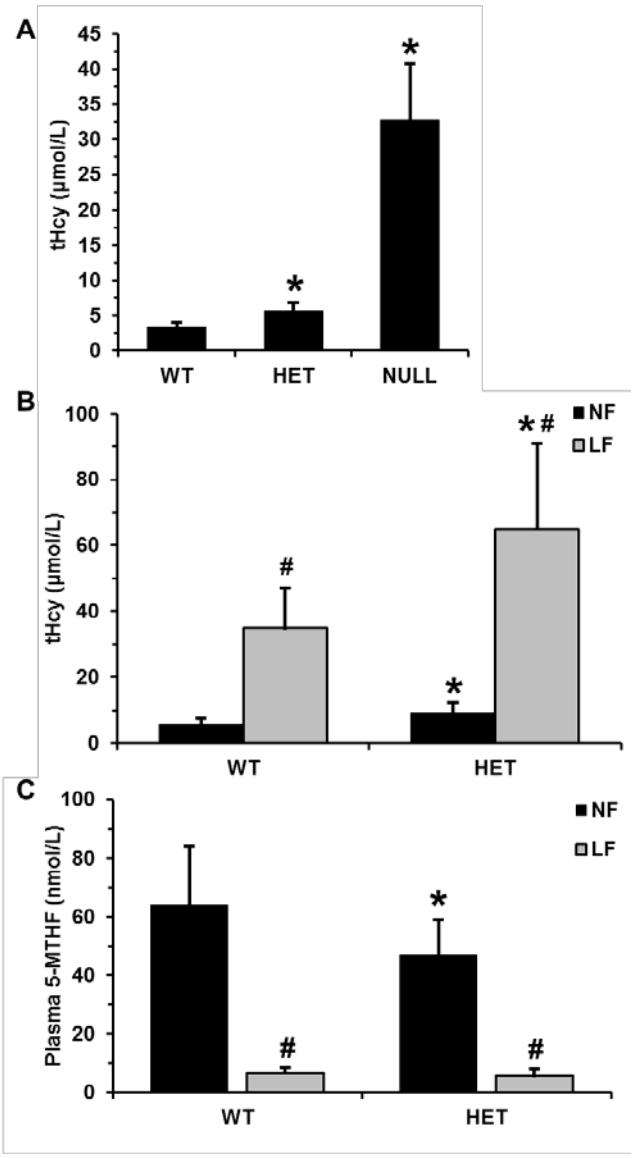

Figure 4.4 Plasma metabolite analysis in MTHFR- and folate-deficient mice. Plasma obtained from 6-week-old (**A**) and 22-month-old (**B, C**) WT, HET or NULL mice was analysed for tHcy and 5-MTHF levels. **A.** High pressure liquid chromatography was used for the determination of tHcy in 6-week-old WT, HET or NULL mice. **B.** Levels of tHcy in 22-month-old WT and HET mice fed a Normal Folate- (NF) or Low Folate-containing (LF) diet for 6 months. **C.** 5-MTHF levels in 22-month-old mice fed NF or LF diets for 6 months determined by radioimmunoassay. Data presented as Mean ± S.D. (*n* = 8-10 per group, * $p < 0.05$, HET or NULL mice, relative to WT in groups fed the same diet, $^{\#}$ $p < 0.05$, LF versus NF diets within the same genotype groups.

We also assessed tHcy and 5-MTHF levels in WT and HET animals fed normal-folate (NF) or LF diets. In both WT and HET mice, LF diets significantly increased tHcy and decreased plasma 5-MTHF levels, compared to animals of the same respective genotype fed a NF-containing diet (***Fig. 4.4***). Furthermore, we also observed that HET mice fed a NF diet continued to show increased plasma levels of tHcy and decreased 5-MTHF, compared to WT on the same diet (***Fig. 4.4***). Notably, in this chapter, we recorded the effects of LF diets in WT

mice only, as available brain tissue was exhausted in experiments for previous studies, and in Chapter 5 [263, 476].

After analysis of plasma metabolites, we then assessed homogenates from different brain regions of these mice for the phosphorylation and expression of CREB. An initial study done by our group has already shown there is a significant decrease in PP2Ac methylation in the cortex, hippocampus, midbrain and cerebellum of these mice [263]. These animals also exhibit cognitive impairment and loss of hippocampal volume [235]. Since CREB contributes to processes of learning and memory, and is necessary for cellular survival, we hypothesised that these animals would display disturbances in the phosphorylation and/or expression levels of CREB in one or more regions of the brain. In the cortex of 6-week-old WT, HET, or NULL mice fed a normal folate diet, we observed a significant increase in the expression of CREB in both HET (~26% ± 6) and NULL (42% ± 7) animals compared to WT controls (***Fig. 4.5***). We also observed a decreasing trend in CREB phosphorylation in the cortex of mice with severe MTHFR deficiency, compared to WT and HET mice, although this decrease was not statistically significant.

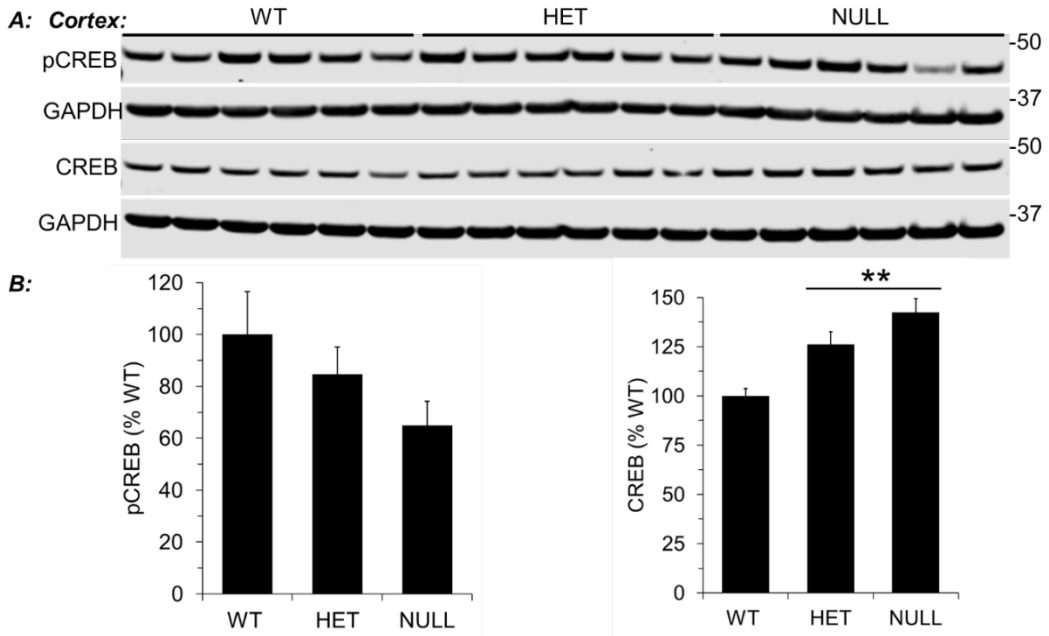

Figure 4.5 CREB expression is increased in the Cortex of young MTHFR deficient mice. Homogenates obtained from cortices of 5-week old WT, HET and NULL mice fed a normal folate-containing diet were analysed in duplicate for expression levels of CREB and CREB phosphorylated at Ser133 (pCREB). GAPDH was used as a protein loading control. **A.** Representative Western blot. **B.** Relative pCREB phosphorylation and CREB expression levels following densitometric analysis including normalisation for Protein loading and total CREB expression. Values shown are representative of mean ± S.D. (n = 6 mice per group, ** $p < 0.01$, HET and NULL compared to WT).

Because of previously mentioned studies using the same tissue extracts (see [263] and Chapter 5), we had insufficient homogenate amount to properly analyse changes in CREB in

the midbrain and cerebellum of NULL animals. Despite these limitations, we are able to present data from the cerebellum and midbrain from wild-type mice and mice with moderate MTHFR deficiency (HET). In contrast to the cortex, we observed a significant reduction in the expression of CREB in the midbrain of HET mice, compared to WT (***Fig. 4.5***). Interestingly, we also observed a significant (~2.5 fold) increase in pCREB in the midbrain of heterozygous mice, compared to wild-type animals (***Fig. 4.5***). The contrast between our results in the cortex and midbrain of MTHFR$^{+/-}$ animals is interesting, as they support previous observations suggesting that different regions of the brain show varying sensitivities to disturbances in one-carbon metabolism [263].

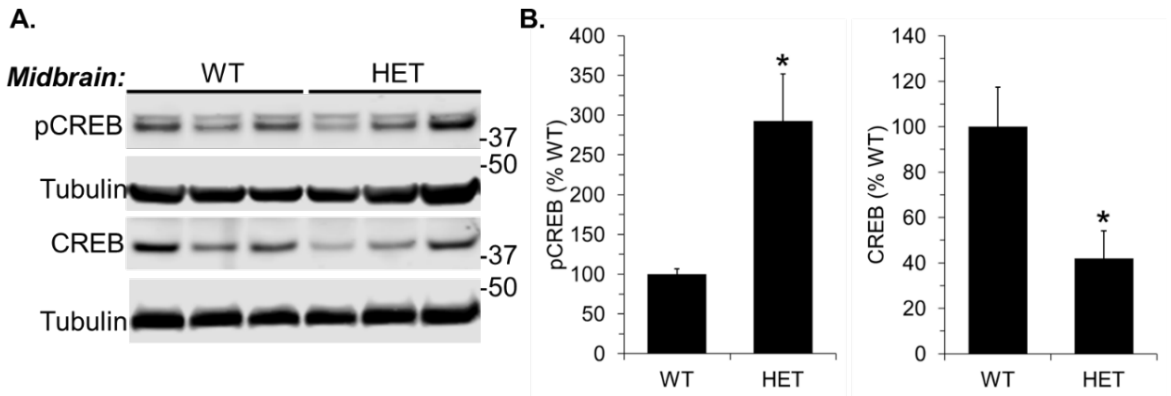

Figure 4.6 CREB expression and phosphorylation is deregulated in the midbrain of young mice with moderate MTHFR deficiency Homogenates obtained from midbrains of 5-week old WT and HET mice fed a normal folate-containing diet were analysed in duplicate for expression levels of CREB and CREB phosphorylated at Ser133 (pCREB). Tubulin was used as a protein loading control. **A.** Representative Western blot. **B.** Relative pCREB phosphorylation and CREB expression levels following densitometric analysis including normalisation for protein loading and total CREB expression. Values shown are representative of mean ± S.D. (*n* = mice 6 per group, * $p < 0.05$, compared to WT).

Another region suggested to be sensitive to changes in one-carbon metabolism is the cerebellum [475, 477], particularly in early stages of development. Unexpectedly, we found no significant difference in the phosphorylation or expression of CREB in the cerebellum of 5-week old HET mice compared to WT (***Fig 4.7***). This may be, in part, explained by the differential roles played by CREB and synaptic-dependent processes in different brain regions.

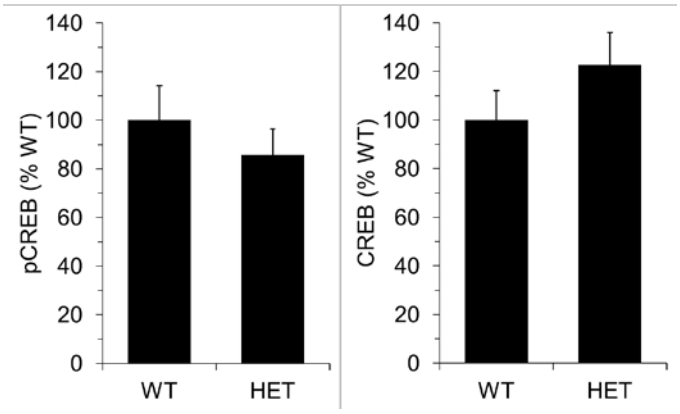

Figure 4.7: MTHFR deficiency and CREB in the cerebellum. Homogenates obtained from cerebellar regions of 5-week old WT and HET mice fed a normal folate-containing diet were analysed in duplicate for expression levels of CREB and CREB phosphorylated at Ser133 (pCREB). Tubulin was used as a protein loading control. Homogenates were run on SDS-PAGE and Western blots for pCREB, CREB (used to normalise pCREB levels) and Tubulin (to normalise for protein loading. Were performed Data presented here is representative analysis of densitometry of described Western blots. Data represented as mean ± S.D. (*n* = 6 mice per group)

4.3.3 Dietary supply of folate affects the phosphorylation and expression of CREB in the midbrain of aged mice

Previous data from our group suggest the effects of genetic *Mthfr* deficiency may be recapitulated by dietary folate deficiency in aged mice [263]. 22-month old WT mice were fed a normal-folate-containing diet (NF) or a low-folate diet (LF) for 6 months. Brain homogenates were again analysed by Western blotting. We found a significant loss of CREB expression in mice fed a LF diet compared to those on a NF in the midbrain (44% ± 7, ***Fig. 4.8.A***). When normalised for total CREB expression, we found that pCREB was significantly increased, by over 2.5-fold (***Fig. 4.8.A***). These data are similar to those observed in young mice with moderate MTHFR deficiency fed a normal diet (***Fig. 4.6***). In contrast to our results in the midbrain, we saw no effect of LF diets in the cortex or cerebellum (***Fig. 4.8.B, C***). Because of limited tissue availability, we were unable to investigate the effect of aging on CREB phosphorylation and expression. However, impaired CREB activity with advancing age *in vivo* has been described by others in the past [455, 478].

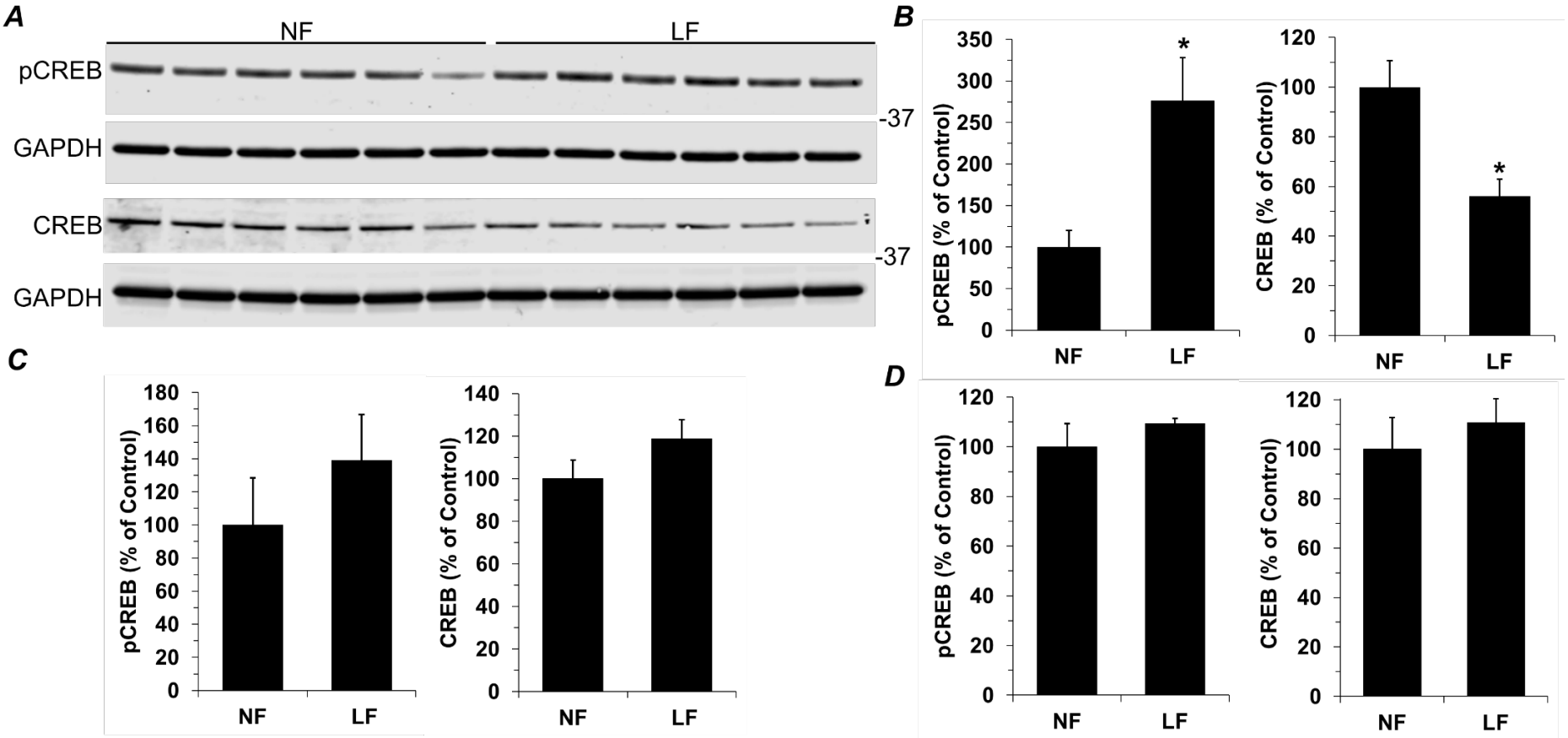

Figure 4.8: Feeding aged mice a low-folate diet leads to deregulation CREB in the midbrain. 22-month-old WT mice were fed a normal folate (normal folate-containing, NF) or a low-folate diet (LF). Homogenates of the cortex, midbrain and cerebellum were analysed by Western blotting for relative expression levels of phosphorylated CREB (pCREB), total CREB and GAPDH for protein loading control. ***A.*** Representative Western blots for midbrain region, showing expression levels of pCREB and CREB. Blots were re-probed with primary antibodies directed against GAPDH to control for protein loading. **B.** Relative pCREB and total CREB levels determined by densitometric analysis of Western blots. **C.** Relative expression levels of pCREB and total CREB after densitometric analysis of cortical homogenates from 22-month-old mice fed NF or LF diets **D.** Relative expression levels of pCREB and total CREB after densitometric analysis of homogenates from the cerebellum of 22-month-old WT mice fed NF or LF diets. Data presented as mean ± S.D. (n = 6 mice per group, * $p < 0.05$)

4.3 Discussion

In this work, we present data demonstrating a relationship between methyl group supply in neuronal cells through the one-carbon metabolic cycle and the phosphorylation and expression of CREB. We found treatment with the methyl donor SAM to N2a cells led to an increase in CREB phosphorylation at Serine 133 (pCREB), which is necessary for CREB-induced gene transcription. Similarly, inhibition of SAH-Hydrolase with 3DA, as well as folate deprivation led to a decrease in pCREB in the same cells. We also demonstrated *in vivo*, that CREB expression and phosphorylation is differentially regulated between brain regions in young mice with mild and severe MTHFR deficiency and aged mice fed a normal or folate-deficient diet. Our findings may have important ramifications for the study of Alzheimer's disease, given that impaired one-carbon metabolism, including low dietary folate and increased tHcy [213, 218] represent independent risk factors for AD. Importantly, CREB function and PP2A methylation are also impaired in AD [158, 159, 323]. Others have demonstrated a role for PP2A in the regulation of CREB [335, 337], while we have shown that CREB phosphorylation is tightly modulated by PP2Ac methylation (chapter 3). Our data strongly suggest that, in neurons, one-carbon metabolism and PP2A methylation in neurons contribute to the regulation of CREB by modulating the phosphorylation state of CREB (***Fig. 4.9***). The regulation of CREB by one-carbon metabolism and by PP2A methylation may be independent, but not mutually exclusive modes.

4.3.1 Methyl-supply in N2a cells is associated with CREB activity

Our data suggests that SAM contributes to CREB activity in neuronal cells, possibly through cAMP-PKA signalling. Conversely, decreasing cellular SAM levels through 3DA treatment of N2a cells led to impaired CREB phosphorylation (***Fig. 4.2***). Indeed, SAH-hydrolase inhibition with 3DA blocks Hcy formation, therefore impairing the restoration of an endogenous SAM pool by Hcy re-methylation (***Fig 4.9*** [479]). Interestingly, a study on the effect of SAM on cAMP and Calmodulin-dependent kinase activity places SAM at the crossroads between one-carbon metabolism and PKA [480]. Their data suggests that SAM may facilitate cAMP binding to the PKA regulatory subunit, which would also boost enzymatic activity (***Fig. 4.9***).

Further evidence of convergence of these pathways is that cAMP can directly bind to SAH-hydrolase at the adenosine binding site. This leads to increased SAH-hydrolase activity *in vitro*. We suggest that SAM supply regulates cAMP-PKA signalling in neurons, thereby affecting CREB and PP2A function, which may have consequences for the regulation of learning and memory, and perhaps other neuron functions (***Fig 4.9***). PP2A is also regulated by this one-carbon supply of SAM, via LCMT-1-mediated methylation.

Our results in N2a cells suggest a functional link between the regulation of CREB and one-carbon cycling. Previous studies in differentiated neurons indicate that both folate and SAM availability contribute to electrophysiological firing patterns and synaptic activity [481]. Since CREB activity is increased following excitatory activation of synapses [307] our results suggest that methyl group supply is critical for CREB function in response to extracellular stimuli. Here, we found an important role for folate in the regulation of CREB in N2a cells and in the midbrain of aged mice.

There is limited available data on the effect of folate deficiency on CREB activity in neurons. In intestinal epithelial cells, however, reduced folate uptake coincides with reduced PKA activity, which is upstream of CREB [482]. Similarly, CREB expression and activity are impaired in cancer cell lines with impaired Reduced Folate Carrier (RFC) protein expression levels [483]. In this study, treatment with Forskolin led to increased CREB activity and expression and subsequent restoration of RFC expression. In agreement with this, our N2a cell results show a significant loss of CREB phosphorylation following 4 hours of folate deficiency (***Fig. 4.2***), which is consistent with our data from SAM/3DA treated N2a cells. Evidence from others indicates that maternal imbalance of critical micronutrients (B12 and folate) leads to impaired transcription of CREB mRNA as well as that of the neuroprotective BDNF [397], which is also subject to CREB-dependent transcription [319, 484].

Follow-up studies would ideally focus on identifying the molecular connection between one-carbon metabolism and cAMP modulation. We would want to identify what is the mechanism of SAM-dependent enhancement of cAMP-PKA binding and assess if folate supply and methylation play direct roles in PKA activity or subcellular localisation. We suggest that this interplay may be extremely complex, but essential in maintaining neuron function. Changes in that interaction could be part of the mechanisms involved in AD onset, especially since low folate status and elevated homocysteine have been identified as risk factors in the disease.

4.4.2 MTHFR status changes CREB expression in the cortex of young mice

Genetic deficiency of *Mthfr* and dietary deficiency of folate, choline or methionine causes memory impairments in animal models [235, 475, 485]. These animals show deficits in learning and memory through a loss of cholinergic metabolites [235] and altered DNA methylation of the AMPAR subunit GluR1 [485]. These could partly explain some of the deficits in hippocampal-dependent learning and memory, which is mediated by CREB [307]. Although we did not have access to hippocampal tissue in these animals, we present evidence in other regions suggesting that altered regulation of folate/one-carbon metabolism may affect CREB.

In the cortex of young HET and NULL mice, we observed a significant increase in CREB expression (***Fig. 4.5***). Similarly, others have demonstrated a reduction in CREB mRNA in the

cortex of rat pups following prenatal vitamin B12 deprivation and elevated folate supplementation [397], which could also result in lowered CREB expression. We did not see any significant differences in CREB phosphorylation in the cortex between genotypes. CREB function in the cortex has been linked to adaptation of dendritic spines in response to environmental stimuli [486], and MTHFR-deficiency *in vivo* has been suggested to prevent spatial memory formation [235, 487]. Future work should focus on a link between MTHFR status and behavioural abnormalities arising from disturbed regulation of CREB.

4.4.3 MTHFR deficiency and folate-deficient diet similarly alter midbrain regulation of CREB

We found that MTHFR deficient young mice fed a normal diet and wild-type mice fed a folate deficient diet both show a significant increase in pCREB and decrease in CREB expression (***Figs. 4.6*** and ***4.8.A***), compared to WT and normal folate diet controls, respectively. These results differ from what we observed in other brain regions of the same animals. This implies that regulation of CREB expression and activity is more or less sensitive to alterations in one-carbon metabolism in different brain regions. Our group has already shown that PP2Ac methylation is impaired in the midbrain of these mice [263]. We have also shown, in chapter 3, that PP2Ac methylation modulates pCREB in N2a cells (see ***Fig. 4.9***). Therefore, the increased CREB phosphorylation in the midbrain could be mediated by a loss of PP2A methylation.

Given the abundance of behavioural and structural deficits observed in MTHFR and folate-deficient mice [235, 487], it is important to emphasise that the consequences of CREB phosphorylation are dependent on the stimulus. For example, while CREB activation is associated with cellular survival in some models [317, 318], there are other examples where increased CREB phosphorylation leads to toxicity in the midbrain following nicotine exposure [488]. Therefore, the increase in CREB phosphorylation in the midbrain of HET mice compared to WT, and of old WT mice fed a LF diet compared to a normal diet may be another example of CREB phosphorylation increasing in response to a toxic stimulus.

An important feature of the midbrain is the high presence of dopaminergic (DA) neurons. Our group has made a link between acute exposure of these cells to high levels of L-DOPA and changes in one-carbon metabolism. Treatment of neurons with L-DOPA led to a significant decrease of methylated PP2Ac, and a significant increase in pTau [396]. These changes induced by L-DOPA were enhanced by folate deficiency. It was suggested to be mediated by an increased workload of catechol-*O*-methyltransferase (COMT), thereby unbalancing SAM: SAH ratio. We saw varying intensities of changes in CREB expression and phosphorylation in MTHFR-HET mice compared to WT, and in mice fed a LF diet compared to those fed NF-containing diets, depending on the brain regions we investigated. Together, these results suggest that the midbrain may be a region of the brain which is especially sensitive to

disturbances in methyl-group supply, especially in terms of CREB phosphorylation and expression. It is also possible that pTau and pCREB are elevated by the same mechanism, namely impaired PP2Ac methylation, especially since disturbances in PP2A methylation, Tau phosphorylation and CREB activity all occur in AD [158, 159, 323]. We suggest that impaired PP2A-dependent dephosphorylation of CREB may underlie the changes in CREB activity we observe in the midbrain induced by perturbed one-carbon metabolism.

4.4 Concluding remarks and significance for Alzheimer's disease and interactions with PP2A

A potential limitation of our results is the effect of aging on CREB regulation. CREB expression and phosphorylation are reduced with the aging process [455, 478, 489], which we were unable to assess here due to the limited amount of available brain tissue. Our results indicate an interesting and complex heterogeneity in the interaction between *Mthfr* genotypes and the expression and activity of CREB, which reflect the existing polymorphisms and their phenotypes in humans [463, 464, 490-492]. Our results show a complex picture of CREB expression and activity, even when comparing different brain regions from WT mice. This is in agreement with many observations showing the complex and varied expression patterns and different functions of CREB between brain regions.

In the hippocampus, where CREB function has been most extensively studied, CREB plays an essential role in long term memory formation and LTP [283, 328]. The extracellular-dependent activity-induced phosphorylation of CREB induces gene transcription of proteins such as AMPA-type glutamate receptor subunits, which enhances the responsiveness of neurons to excitation [302-304, 466]. Importantly, in aging, AD, and MTHFR deficiency in mice, LTP and memory-dependent behavioural outcomes are impaired [235, 328, 487, 493-497]. However, in other areas of the brain, CREB phosphorylation is increased with advanced age in the prefrontal cortex [454], an area responsible for the accession of working memory. The inhibition of cAMP signalling in the prefrontal cortex appears to improve working memory performance [498]. Models of aging report higher levels of pCREB following training in passive avoidance tests, which may be due to a change in CREB function, or may be a compensatory mechanism [455]. Meanwhile, chronic enhancement of CREB activity appears to impair spatial memory recall [499]. This suggests that CREB phosphorylation may have divergent functions between brain regions, in addition to being dependent on the nature of the upstream stimulus. It is important to validate our results in a similar model, taking into account the effect of aging, and observing possible region-dependent behavioural outcomes.

MTHFR deficiency leads to problems with working memory in mice [487] and in humans, for example in schizophrenic patients carrying the *Mthfr* C667T allele [500]. It appears that there may be a connection between one-carbon metabolism, CREB regulation and changes in

behavioural performance, but the mechanisms involved remain unclear. Some possible explanation may be that components of one-carbon metabolism like SAM may affect CREB function through modulation of cAMP-PKA dynamics [501], and that methylation-dependent PP2A enzyme formation can impair CREB dephosphorylation (chapter 3, ***Fig. 4.9***). In this chapter we provide a unique insight into how the impairments in memory induced by one-carbon metabolism may be mediated.

Elevated homocysteine and low folate levels represent independent risk factors for the development of AD [210, 213, 218, 502], and CREB function is impaired in the AD brain and models of the disease [323, 328, 329, 419]. Our results provide a link between these risk factors and impaired CREB function in the brain which may be important in the study of AD. Similar to previous investigations surrounding folate supply and one-carbon metabolism, in our model we are unable to delineate if the effects we observed are due the toxic effects of elevated homocysteine accumulation or a deficit in methylation reactions due to impaired folate supply, or an interaction between the two. In conclusion, we suggest that the regulation of CREB in different brain regions is affected by one-carbon supply and may influence the development of AD through novel mechanisms. Taken with our results from chapter 3, we suggest that CREB phosphorylation is dependent on both PKA kinase activity, which can be enhanced by SAM-mediated cAMP binding to PKA (***Fig. 4.9***), and also the phosphatase activity of PP2A, which is dependent on cellular SAM availability. Furthermore, PP2A methylation is also affected by PKA activity in neurons. Future studies will work to properly uncover the details of this intracellular signalling cycle.

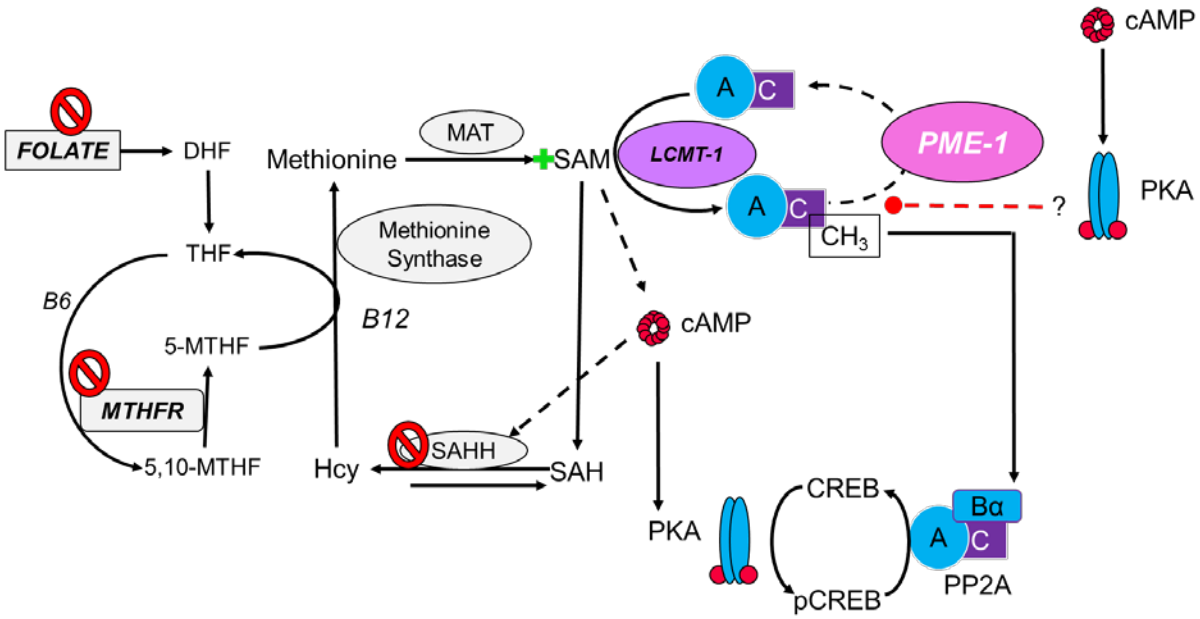

Figure 4.9: One-Carbon metabolism and the regulation of CREB. Schematic diagram of proposed mechanism of interaction between one-carbon metabolism, PP2A and cAMP-PKA signalling in neuronal-type cells based on our experimental results. SAM supply enhances pCREB (refer to dashed arrow in figure). Incubation in N2a cells in medium without folate (red crossed circle) or inhibition of SAHH impairs the phosphorylation of CREB (pCREB). This indicates that availability of methyl groups are necessary for proper CREB function. *In vivo*, however we found regional differences in the effects of genetic MTHFR deficiency in young animals and dietary folate deficiency in aged animals, suggesting that regulation of CREB by one-carbon metabolism is brain-region dependent and may change based on cell function in any particular brain region.

Chapter 5 – Methylenetetrahydrofolate reductase (MTHFR) deficiency deregulates regional brain Amyloid-β precursor protein

Acknowledgement of Collaboration

I hereby acknowledge that the work enclosed in this thesis was performed in collaboration with other researchers.

The work in the following chapter (Chapter 5) was primarily performed by myself (Alexander Hoffman), and fellow lab member Goce Taleski and my supervisors (Dr. Jean-Marie Sontag and A/Prof. Estelle Sontag).

This practical assistance was in addition to invaluable technical advice from my supervisors (Dr. Jean-Marie Sontag, Prof. Alan Brichta and A/Prof. Estelle Sontag).

The brain tissue utilized in this study was obtained by our collaborator Dr. Teodoro Bottiglieri (Center of Metabolomics, Institute of Metabolic Disease, Baylor Scott & White Research Institute, Dallas, TX, USA). The analyses of the metabolites relevant to the results herein were performed by Dr. Brandi Wasek, of the same laboratory.

This chapter comprises a significant portion of an original research article first published in the Journal of Alzheimer's disease, vol. 64, no. 1, pp. 223-237, 8th June 2018, of which I am the first author. Many figures herein may be subject to copyright by the publisher.

5.0 Rationale and significance

One-carbon metabolism is important for PP2A methylation. As previously indicated, PP2A methylation drives holoenzyme assembly and protein targeting. Our group has shown that the PP2A-ABαC isoform regulates Tau phosphorylation [201]. There are also significant decreases in PP2A-ABαC and methylated PP2A in brain from AD patients that correlate with increased levels of pTau [158, 159]. Similarly, mice homozygous and heterozygous for *Mthfr* mimicking the C677T polymorphism also show an increase in pTau, in conditions where animals were fed a normal or low-folate diet [263]. We therefore suggested that PP2A-ABαC is a key enzyme that is deregulated in AD. Loss of expression and changes in PP2Ac methylation due to *Mthfr* polymorphisms or limitations in folate supply from diets could be one mechanism responsible for the accumulation of pTau in neurons. Indeed, it is well-recognized that folate absorption is impaired with aging [503], therefore, we have suggested a strong link between one-carbon metabolism, PP2A methylation and ABαC formation, and Tau phosphorylation.

PP2A can also regulate APP phosphorylation, in a methylation dependent manner [258]. The phosphorylation of APP at the C-terminal Thr668 site has been suggested to contribute to Aβ formation [21, 51], and has been found to co-localize with senile plaques and neurofibrillary tangles of AD [52]. APP phosphorylation is reported to alter subcellular localization, protein-protein interactions and trafficking of the protein. We have previously identified a link between pTau and pAPP which is also observed in AD, with that link being disturbed by one-carbon metabolism and PP2Ac methylation. We therefore hypothesized that MTHFR and folate deficiencies could contribute to APP phosphorylation. We have used the same mouse models as the previous chapter, and hope to further demonstrate a mechanistic role for PP2A methylation and one-carbon metabolism in the regulation of APP, a key protein deregulated in the AD process.

5.1 Introduction

The metabolically active form of folate (5-methyltetrahydrofolate, 5-MTHF) is a critical cofactor in the supply of methyl-groups in cells. One-carbon metabolic networks integrate nutrient input to regulate multiple cellular processes essential for neuronal homeostasis. Folate-dependent single-carbon reactions support several critical biosynthetic pathways, including methylation of proteins, lipids and DNA [462]. Unsurprisingly, disturbances in folate metabolism are associated with the onset of an array of neurological disorders. Several epidemiological studies have linked alterations in one-carbon metabolism with an increased risk for cognitive decline, brain atrophy and AD [459]. Likewise, low folate status and hyperhomocysteinemia are independent risk factors for AD [213, 214].

Low folate status also leads to increased levels of tHcy, which exerts neurotoxic effects via a range of mechanisms. The most common genetic cause of hyperhomocysteinemia in humans is due to polymorphisms in the *MTHFR* gene encoding MTHFR [464]. The common human *MTHFR* C677T polymorphism is associated with increased risk for late-onset AD [216, 222, 460]. Molecular mechanisms by which altered MTHFR activity may contribute to AD pathogenesis are not fully understood.

The abnormal accumulation of oligomers of Aβ peptides derived from β- and γ-secretase cleavage of the Amyloid-β precursor protein (APP) is believed to trigger the neurodegenerative cascade in AD [504]. Aβ production and neurodegeneration are promoted by APP phosphorylation at the Threonine 668 (Thr668) site in several models *in vivo* [21, 51, 53, 55, 275, 505-507] Interestingly, hyperhomocysteinemia induced either by genetic cystathionine-β-synthase *(Cbs)* deficiency [258, 262], homocysteine injection [508], or dietary B-vitamin deficiency [259, 509] increases pThr668-APP or Aβ generation in APP rodent models of AD. This supports a role for the deregulation of homocysteine metabolism in APP phosphorylation at Thr668.

Our group has also provided evidence indicating that MTHFR and folate deficiency *in vivo* lead to elevated Tau phosphorylation and impaired PP2Ac methylation [263]. Impaired PP2Ac methylation is associated with pTau in AD brain tissue [158], and pAPP in other mouse models of disturbed one-carbon metabolism, and promotes the amyloidogenic cleavage of APP in N2a cells [258]. We hypothesized that MTHFR-deficiency *in vivo* leads to disturbances in the regulation of APP expression and phosphorylation, which are typical to the pathological processes seen in AD patients and experimental AD models.

5.2 Results

5.2.1 MTHFR deficiency is associated with APP-Thr668 phosphorylation in the cortex, hippocampus and midbrain of young mice

To test the hypothesis that genetic alterations in MTHFR activity can affect the regulation of APP, we first analysed tissue homogenates from different brain regions obtained from young transgenic *Mthfr* knockout mice fed a normal folate-containing diet (NF diet). Mice with experimentally compromised expression of MTHFR have been shown to model the effects of mild and severe MTHFR deficiency, respectively. They mimic the biochemical and other deficits observed in human populations with the common MTHFR C677T polymorphism [234, 235, 510, 511]. In the plasma and brain tissue of MTHFR-deficient mice, 5-MTHF levels are decreased, which is associated with elevated tHcy and subsequent disturbances in the methylation cycle [263]. According to previously published data from our group, the 5-week-old HET mice used in this study had significant (~1.6-fold increase), but mildly elevated tHcy levels over basal levels measured in their WT littermates [263]. Compared to aged-matched WT mice, NULL mice were hyperhomocysteinemic, showing a ~10-fold increase in tHcy levels.

Here, we have used brain tissue samples from the same WT, HET and NULL mice for the expression of MTHFR that were described in Chapter 4 of this thesis and previous studies by our group [263]. APP phosphorylation at Thr668 was significantly enhanced in the cortex, hippocampus and midbrain of HET and NULL mice, compared to WT animals (***Fig. 5.1***). There was no change in APP phosphorylation in the cerebellum of mice either HET or NULL mice, compared to WT mice (***Fig. 5.1***). These data support our hypothesis that genetic MTHFR deficiency modelling the C677T polymorphism can lead to increased APP phosphorylation associated with AD [52].

5.2.2 Aged mice have elevated pThr668-APP in the hippocampus with mild MTHFR deficiency

The MTHFR C677T polymorphism is a risk factor for the onset of sporadic AD, an age-related disorder [216, 460]. Therefore, we also sought to assess APP phosphorylation state in 22-month-old -WT and -HET animals fed a normal folate-containing diet. Importantly, NULL mice could not be assessed for changes in APP as they experience premature mortality [234, 235]. Decreased MTFHR activity led to reductions in plasma total folate levels relative to WT controls, as expected ($p < 0.01$). We also noted that plasma tHcy levels were further elevated ($p < 0.01$) in 22-month-old WT and HET mice, compared to their respective 5-week-old littermates, indicating an effect of aging on metabolite accumulation.

As observed in young animals (***Fig. 5.1***), aged HET mice had no change in pThr668-APP levels in the cerebellum (***Fig. 5.2***). Similarly, there was a significant increase in the phosphorylation of APP in the hippocampus of aged HET mice, relative to WT controls (***Fig. 5.2***). Additionally, there was no change in APP phosphorylation in the cortex or midbrain of aged mice with mild MTHFR deficiency fed a normal-folate-containing diet. These results demonstrate that MTHFR deficiency leads to an increase in hippocampal APP phosphorylation in aged mice.

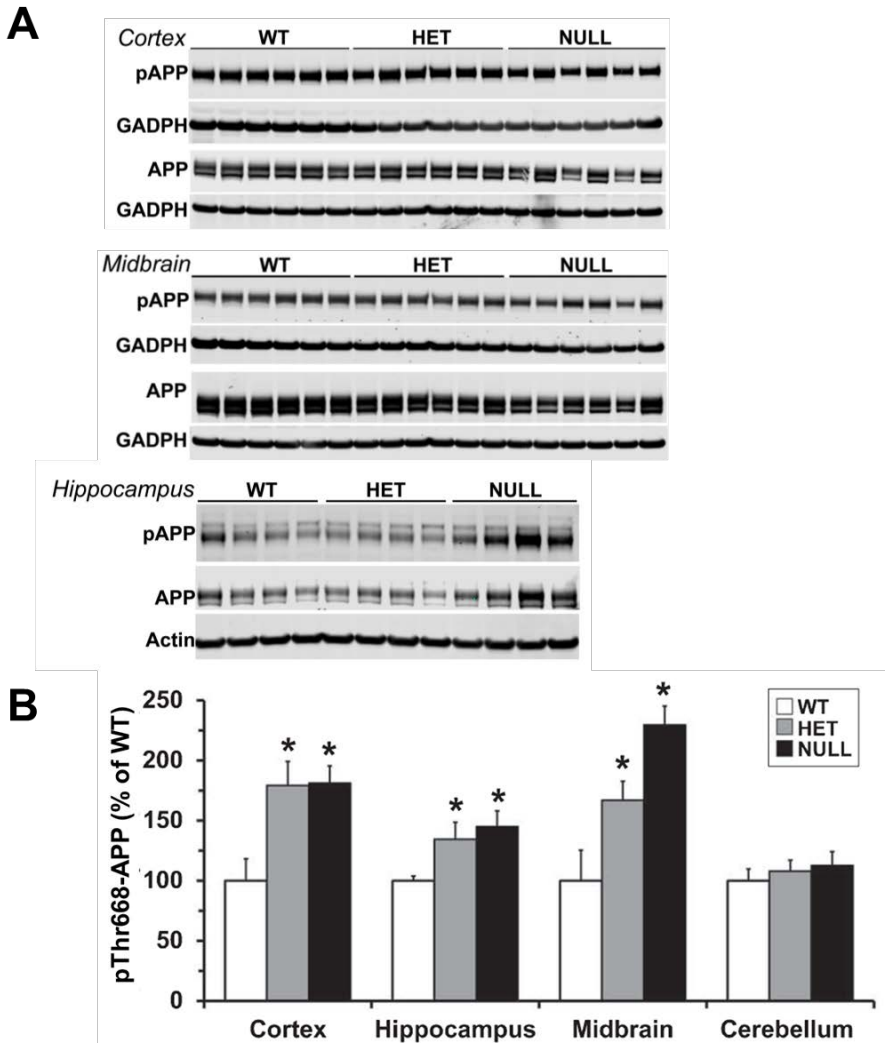

Figure 5.1 MTHFR deficiency leads to increased APP phosphorylation in the cortex, hippocampus and midbrain of young mice. Regional homogenates were prepared from 5-week old WT, HET or -NULL mouse models of MTHFR deficiency. Duplicate aliquots of homogenates were separated by NU-PAGE and analysed for APP expression and phosphorylation (pAPP) by Western blot. GAPDH or Actin were used to normalize each blot for protein loading. **A.** Representative Western blots. **B.** Relative pThr668-APP levels were quantified following densitometric analysis of Western blots and normalization for total APP levels. Values represent mean ± S.D. (n = 6 mice/group, * $p < 0.05$ for HET or NULL vs. WT control mice).

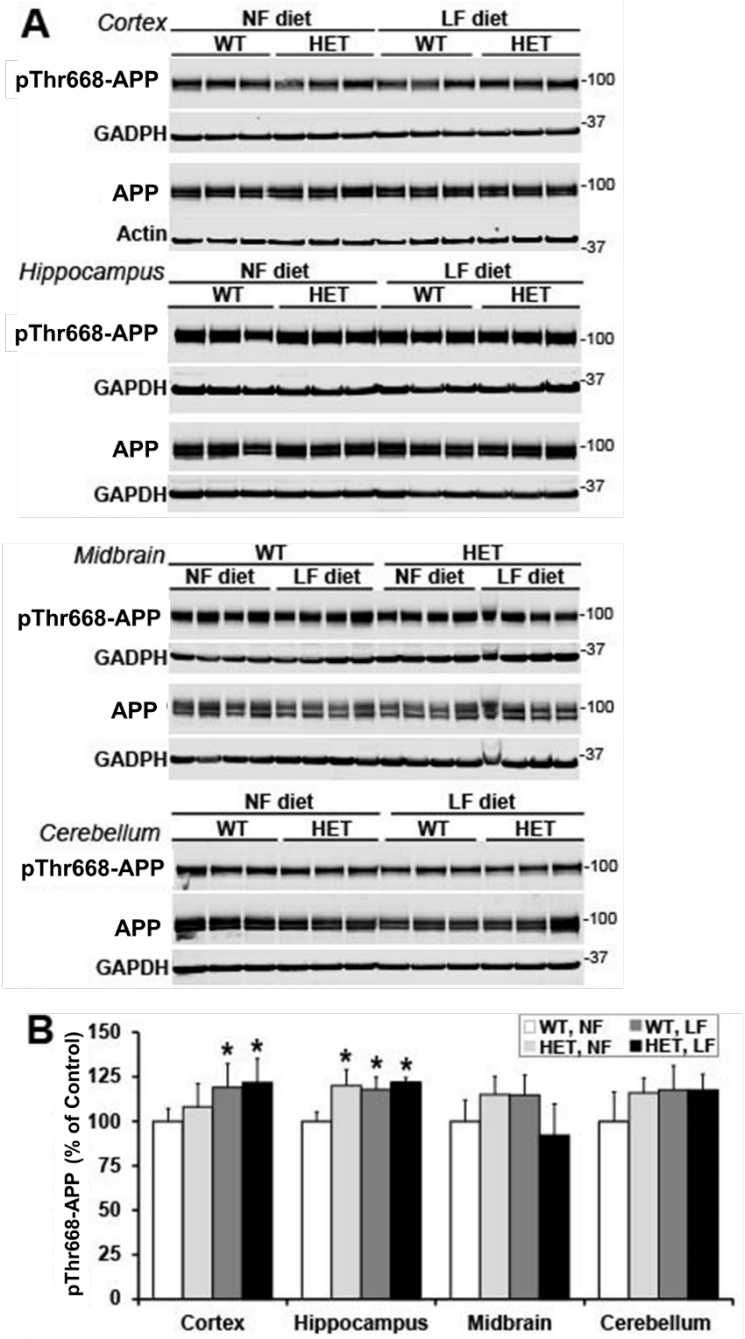

Figure 5.2 Effect of MTHFR status and dietary folate on APP phosphorylation in old mice. Homogenates from cortical, hippocampal, midbrain and cerebellar brain regions of 22-month-old WT or HET mice, fed a normal folate-containing (NF) or low folate (LF) diet were analysed for APP phosphorylation levels by Western blotting, as described in ***Fig. 5.1.***. **A.** Representative Western blots. **B.** Relative pThr668-APP levels were quantified following densitometric analysis of the blots and normalization for total APP levels. Values represent mean ± S.D. (n = 8 mice/group, * $p < 0.05$ relative to WT mice fed a normal folate containing diet).

5.2.3 Dietary folate deficiency leads to elevated pThr668-APP in the cortex and the hippocampus of old mice

The functional consequences of MTHFR C677T polymorphisms can be exacerbated by inadequate dietary folate intake [464]. Considering this, we found it important to assess the effect of dietary folate deficiency in WT and HET animals on APP expression and phosphorylation. For this purpose, we analysed pThr668-APP by Western blot from parallel cohorts of WT and HET mice fed either a normal folate-containing (NF) or low-folate diet (LF, refer to Chapters 2 and 4 for a description of these models).

Dietary folate deficiency induced an increase in pThr668-APP in the cortex of both WT and HET mice, compared to mice of the same genotype fed NF diets (***Fig. 5.2***). Likewise, pThr668-APP levels were elevated in the hippocampal homogenates from both WT and HET mice fed the LF diet, compared to WT controls on the NF diet. In the hippocampus, the functional effect of dietary folate deficiency was comparable to that of mild MTHFR deficiency in young (***Fig. 5.1***) and old (***Fig. 5.2***) HET mice fed a NF diet. However, there was no significant change in APP phosphorylation in the midbrain or cerebellum of WT or HET mice fed the LF diet, compared to mice fed the NF diet (***Fig. 5.2***). These data indicate that dietary folate or genetic MTHFR deficiencies can significantly increase the phosphorylation of APP at Thr668 in the cortex and hippocampus of aged mice.

5.2.4 MTHFR and folate status alter APP expression in the cortex, midbrain and hippocampus

Since the increased Thr668 phosphorylation we observed may be due to increased expression of APP, we also verified APP protein levels in brain homogenate obtained from the young and old mice analysed above. In young animals (***Fig. 5.3.A***), we observed a significant decrease in APP expression in the cortex of NULL mice, as well as in the midbrain of HET and NULL mice. Conversely, we saw a significant increase in the expression of APP in the hippocampus of NULL mice (***Fig. 5.3.A***). There was no change in the cerebellum of either HET or NULL mice fed a normal diet in APP expression, compared to WT mice. Similarly, the LF diet did not induce any change in APP expression in the cerebellum (***Fig. 5.3***). These results indicate that a brain-region specific regulation of APP expression exists in response to genetic MTHFR deficiency.

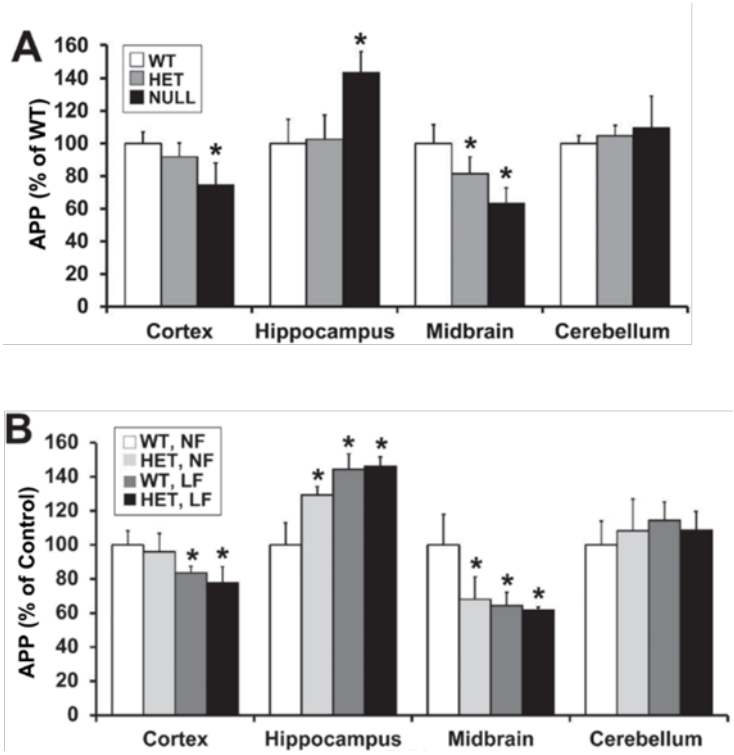

Figure 5.3 MTHFR and folate deficiencies induce brain-region specific alterations in APP expression. Homogenates from young mice (***Fig. 5.1***) and old mice (***Fig. 5.2***) were analysed by Western blotting for total APP expression levels, after normalization from protein loading. **A.** Relative APP expression in brain regions taken from young WT, HET and NULL mice fed a NF-containing diet. Values represent mean ± S.D. (n = 6 mice/group, * $p < 0.05$ for HET or NULL vs. WT control mice). **B.** Relative APP expression in brain regions obtained from old WT and HET mice fed NF and LF diets. Values represent mean ± S.D. (n = 8 mice/group, * $p < 0.05$ relative to WT mice fed a normal-folate containing diet).

In the cortex, midbrain and hippocampus of aged mice, we observed changes in APP expression with diet and *Mthfr* genotype. WT and HET mice on a LF diet had significantly lowered APP expression in the cortex, compared to those on a normal-folate containing diet (***Fig. 5.3.B***). There was also lower APP expression in the midbrain of 22-month-old HET mice fed a NF diet. In the hippocampus, however, HET mice fed a normal diet, and both WT and HET mice fed a LF diet all showed elevated expression of APP at 22 months of age, compared to WT mice on a NF diet (***Fig. 5.3.B***). These results emphasize the role of dietary folate in the brain and support our hypothesis that APP expression is affected in a brain-region-specific manner in response to impairments in one-carbon metabolism.

5.2.5 Reduced inhibitory GSK-3β Ser9 phosphorylation parallels increased pThr668-APP in MTHFR and folate deficient mice

Finally, we were interested to see what the potential mechanisms behind disturbed pThr668 phosphorylation of APP may be. In this context, we and others have previously reported that dietary folate deficiency and hyperhomocysteinemia can independently promote the demethylation of PP2Ac [258, 272, 403, 448]. Importantly, the demethylation of PP2Ac is sufficient to promote APP phosphorylation at Thr668 in cultured cells and *in vivo* [258, 338]. Our group has previously demonstrated that PP2Ac is demethylated in the same tissues analysed here [263].

Besides affecting PP2A methylation, impaired folate and homocysteine metabolism might also enhance the activity of protein kinases targeting APP. We hypothesized that one such kinase could be Glycogen Synthase Kinase-3β (GSK-3β). Aberrant activation of GSK-3β has been linked to hyperphosphorylation of APP at Thr668 [55] and AD-related neurodegenerative processes [512]. Thus, we used homogenates from the cortex of young MTHFR- WT, HET and NULL mice, and the cortex and hippocampus taken from 22-month-old WT and HET mice fed NF or LF diets to assess the phosphorylation of GSK-3β at Ser9. We looked at these regions since they showed the most pronounced changes in APP phosphorylation at Thr668, a site targeted by GSK-3β [54, 55]. Importantly, Ser9 phosphorylation inhibits GSK-3β kinase activity. Western blot analysis of cortical homogenates from young mice showed that phosphorylation of GSK-3β at Ser9 was significantly decreased in response to MTHFR deficiency (***Fig. 5.4.A***), suggesting that increased GSK-3β contributes to the enhanced phosphorylation of APP in these animals. We also assessed the effect of dietary folate deficiency on GSK-3β phosphorylation. Folate deficiency was associated with a significant reduction in pSer9-GSK3β in the cortex and the hippocampus of aged mice (***Fig. 5.4.B***). These results, and others previously generated from our lab [263], indicate that impaired PP2Ac methylation and enhanced GSK-3β activity contribute to increased APP phosphorylation in response to disturbances in one-carbon metabolism in the cortex and hippocampus.

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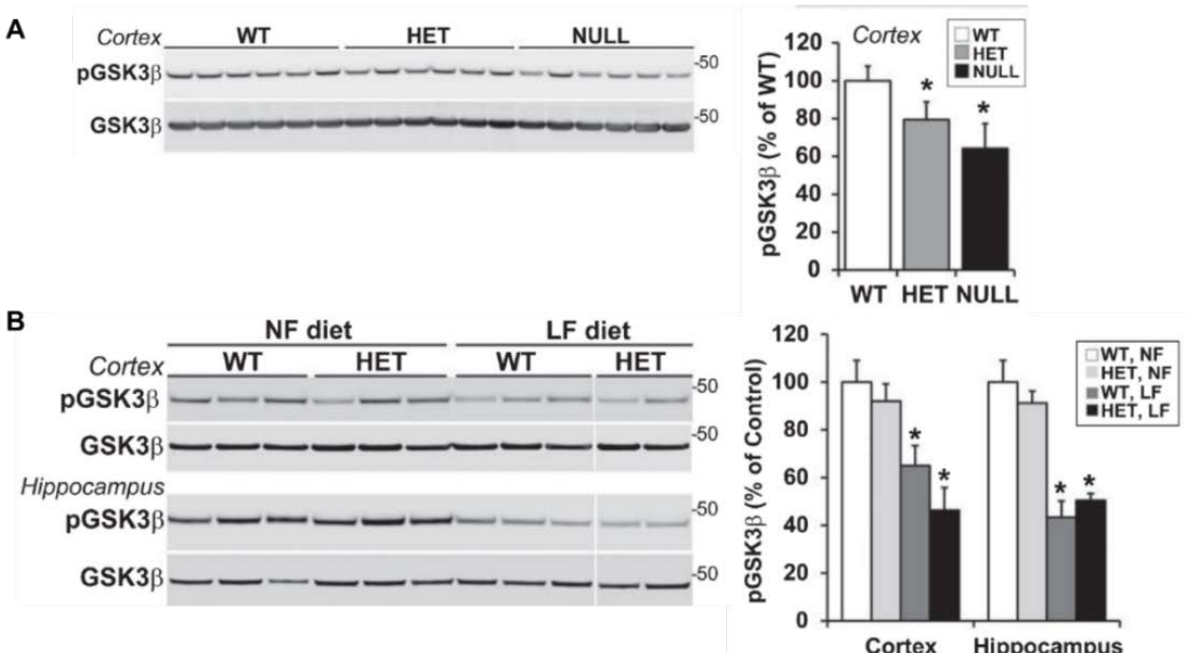

Figure 5.4 Effect of MTHFR and folate status on GSK-3β activity in young and old mice. Homogenates from young mice (***Fig. 5.1***) and old mice (***Fig. 5.2***) were analysed by Western blotting for inactive GSK-3β (pGSK-3β) total GSK-3β and by re-probing the same blots used in previous figures, where GAPDH or Actin were used as protein loading controls. **A.** Representative Western blots and densitometric analyses of pGSK-3β levels in WT, HET, and NULL mice fed a NF diet. Values represent mean ± S.D. (n = 6, p < 0.05, compared to WT). **B**. Representative blots and analysis of pGSK-3β levels in old WT and HET mice fed NF and LF diets. Values represent mean ± S.D. (n = 8 p < 0.05, compared to WT mice fed NF diet).

5.3 Discussion

There is substantial evidence that disturbances in folate and homocysteine metabolism are important risk factors for the onset of sporadic AD [210, 213-215, 459]. It is also well recognized that abnormally enhanced production of Aβ contributes to the neurodegenerative process in AD. However, much remains to be elucidated on how risk factors for AD, including disturbed one-carbon metabolism, affect the molecular determinants of the disease. In particular, there is limited direct evidence linking the phosphorylation and processing of APP with dietary folate deficiency and impairments in MTHFR function.

Previous work from our laboratory has demonstrated a link between one-carbon metabolism, altered proteolytic cleavage and phosphorylation of APP at Thr668 in N2a cells and CBS-deficient mice [258]. There have been several other studies into the regulation of Aβ production in regards to the one-carbon metabolic cycle, though few exist to demonstrate a role for this cycle in direct regulation of APP protein expression and phosphorylation. Indeed, disturbances in folate metabolism can lead to compromised cellular methylation reactions [462], which cause a variety of changes in intracellular signalling, through disrupted targeting of protein, DNA or lipid methyltransferases. In previous work, we showed that one such protein with disturbed methylation and altered function was PP2A, which targets APP for phosphorylation [258, 476]. PP2A has similarly been shown to regulate the activity of GSK-3β through dephosphorylation of the pSer9 site [422, 449, 513]. In this chapter, we wanted to demonstrate how disturbed methylation dynamics *in vivo* can affect these two proteins which play well-established roles in AD pathogenesis [3, 33, 51, 512, 514]. Specifically, we show that genetic-induced MTHFR deficiency can alter APP expression and/or phosphorylation at Thr668 in a brain region- and age-dependent manner. In these studies, we used well-characterized *Mthfr* knockout mice. This model is widely used to recapitulate the effects of MTHFR deficiency, which is associated with the common human C677T polymorphism, a risk factor for sporadic AD [216, 222, 460].

5.3.1 One-carbon metabolism has been linked to AD through various mechanisms

Disturbances in one-carbon metabolism have been identified as risk factors for the onset of sporadic AD. Epidemiologic data indicates on a large scale that multiple aspects of one-carbon metabolism contribute to these risks. These include elevated tHcy, suboptimal intake and impaired absorption of dietary folate. Meta-analyses observing the effects of elevated tHcy in humans, with cohorts ranging from 2,500 to 15,000+ have identified strong links between elevated tHcy (plasma concentrations above 10-12μmol/L) and a positive risk of developing non-familial AD [6, 214, 515]. Particularly relevant to our investigations is the evidence indicating that elevated tHcy arising from the *Mthfr*-C677T polymorphism heightens AD risk [215, 460]. Similarly, low-folate and B-vitamin containing diets and the risk for developing AD

have also been linked through multiple meta-analyses [222, 459] and population-based longitudinal studies [516]. Importantly, many of the rigorous investigations linking AD with tHcy levels control for dietary folate, and vice-versa. Therefore, it is important to note that, while low folate-containing diets and elevated levels of tHcy often occur simultaneously, they represent independent risk factors for AD [213, 502]. This supports the notion that low dietary folate and elevated tHcy increase AD risk through overlapping, but also specific mechanisms.

Perturbations in the one-carbon metabolic cycle are associated with increased Aβ load in animal models [260, 262, 508, 517]. In humans, factors that impair cellular methylation potential are associated with higher rates of cognitive decline [518], and grey matter volume in AD patients [212]. Likewise, diet-induced HHcy has repeatedly been shown to exacerbate cognitive and neuropathological symptoms [260]. High-methionine diets exacerbate Aβ formation [257]; the elevated production of Aβ induced by enhanced pThr668-APP and γ-secretase function is reversible through folate and B-vitamin supplementation [508]. tHcy levels have also been tied to enhanced secretion of Aβ via impaired DNA methylation of the promoter regions of BACE and PS1. This leads to higher expression of β-secretase complex enzymes and amyloidogenic processing of APP [259, 517]. Similarly, folate-deficient diets have been linked to higher PS1 and BACE expression, due to the same mechanism [517]. Folate deficiency impairs DNA methylation in a SAM-dependent manner, underlining the importance of methyl-donor supply in the brain.

Notably, the MTHFR-deficient mouse model that we have used in this thesis causes characteristic increases in tHcy and compromised cellular methylation potential [263, 510]; it closely models the effects of C677T polymorphism which occurs in humans. The HET mice used here model the CT genotype in humans, and NULL mice model severe genetic deficiency of MTHFR (TT genotype). This polymorphism heightens the risk for the development of AD and other psychopathologies [216, 519]. While the precise mechanisms surrounding low folate, elevated homocysteine and AD risk are still somewhat unknown, what is clear is that genetic MTHFR deficiency does recapitulate several conditions of AD *in vivo*. This includes behavioural defects and hippocampal atrophy [235], increased PP2Ac demethylation and Tau phosphorylation [263], and altered regulation of APP expression and phosphorylation, according to the data we have presented in this chapter. This contributes to a growing body of work linking AD with models of impaired one-carbon metabolism through shared molecular, cellular and neuropathological disturbances as described above.

5.3.2 APP phosphorylation is sensitive to MTHFR deficiency in a brain-region- dependent manner

We found that MTHFR deficiency in young mice was sufficient to markedly enhance APP phosphorylation at Thr668 in AD susceptible brain regions such as the hippocampus and the

cortex (***Fig. 5.1***). The cerebellum, a region typically spared from severe neurodegeneration in AD, showed no change in APP phosphorylation. Conversely, in aged HET mice fed a LF-diet, we observed an increase in pThr668-APP in the hippocampus only (***Fig. 5.2***). Due to an insufficient amount of tissue available to us, we were unable to determine whether or not the normal ageing process was responsible for the observed increase in APP phosphorylation levels in the cortex and midbrain of WT mice. If it was the case, this would account for a lack of an effect in these regions in aged mice, since MTHFR deficiency enhanced APP phosphorylation in young animals.

Previous work from our group has demonstrated that disturbances in one-carbon metabolism leads to phosphorylation and amyloidogenic processing of APP [258]. Here, pThr668-APP and levels of sAPPβ, the secreted form of APP resulting from β-secretase cleavage, were significantly reduced by the treatment of N2a cells with the methyl donor, SAM. Furthermore, in HHcy mice, APP phosphorylation was also significantly enhanced. As discussed above, the MTHFR-deficient mice we studied here also had significantly higher tHcy, compared to WT mice (Chapter 4). Our current results are thus consistent with those previously linking methyl-group supply with the regulation of APP in cultured neurons. Other studies *in vivo* have connected hyperhomocysteinemia with APP phosphorylation at Thr668 and an increase in the severity of amyloidogenesis [259, 262, 508, 509]. Furthermore, in the hippocampus, exposure to Aβ oligomers induces neuronal death by excitotoxic stimulation of glutamate receptors [131], which is the same mechanism by which Hcy and its derivatives are hypothesized to exert toxic effects in the brain [520, 521]. Our present data demonstrating enhanced pThr668-APP induced by genetic MTHFR deficiency is in agreement with these studies suggesting that elevations in Hcy promotes Aβ generation *in vivo*.

5.3.3 Genetic MTHFR deficiency and dietary folate deficiency interact to alter APP phosphorylation

Disturbed methylation potential *in vivo* has far-reaching consequences for cellular homeostasis. Importantly, we found that LF diets enhanced the phosphorylation of APP at Thr668 in the cortex and hippocampus of aged WT and HET mice, compared to WT mice fed a NF diet (***Fig. 5.2***). This meant that LF diets were able to induce increases in pThr668-APP levels in these regions whereas genetic MTHFR deficiencies did not. Since AD is an age-related disorder, and Aβ deposition worsens with age [522], these results provide an important insight into how AD risk factors interact and develop with worsening age.

Our results demonstrating that the hippocampus was more susceptible to changes in APP phosphorylation are in agreement with observations suggesting that the hippocampus is especially sensitive to MTHFR- and folate deficiencies [235, 263]. The hippocampus is also the brain region which is most severely affected in AD [2, 523] and is especially vulnerable to early-onset Aβ-mediated functional impairments in AD transgenic models [524]. Accordingly,

these findings taken with previous data from our group showing that MTHFR combined with low LF induces AD-like changes in the brain [263] support the notion that methyl-group supply is essential for neuronal homeostasis. Importantly, the effect of MTHFR deficiency was not augmented in HET mice fed a LF diet, as we expected. This likely reflects the effect of prolonged feeding of mice with this diet, which induced a maximal and similar decrease in plasma folate levels in both genotypes (see Chapter 4 for plasma folate analysis). In our conditions, we observed a ~10-fold reduction in 5-MTHF (64±20 nmol/L in NF versus 6.8±1.6 nmol/L in LF) in WT mice on this diet, while HET mice showed a similarly significant loss of plasma 5-MTHF after 6 months on the LF diet (47±12nmol/L compared to 5.8±2.1 nmol/L).

Interestingly, there are some reports showing that total folate levels remain unchanged in the brain of MTHFR deficient mice [511]. However, cerebral 5-MTHF levels are decreased, and non-methylfolate species are increased in the brain of these animals [511]. This altered biological dispersal of folate derivatives affects the flux of one-carbon units between methylation reactions and nucleotide synthesis [511]. Thus, MTHFR deficiency induces defects in cellular methylation potential in mice [510], the effect of which is conceivably compounded by a loss of 5-MTHF in the mice fed a LF diet.

A possible cause for the preferential loss of 5-MTHF in the brain of animals with deficiencies in methyl-group supply such as that of MTHFR or folate may partly originate in the periphery. The conversion of Hcy to Methionine is dependent on the methionine synthase-mediated transmethylation of 5-MTHF, with vitamin B12 required as a cofactor [245, 525]. The alternative pathway for Hcy remethylation is the donation of a methyl group from the metabolite of Choline, Betaine, mediated by Betaine-Homocysteine Methyltransferase (BHMT) [269, 526]. Importantly, this pathway does not exist within the brain, since BHMT is not expressed in this organ [527]. Therefore, restrictions in 5-MTHF supply induced by genetic MTHFR deficiency or LF diets could lead to neuronal efflux of choline to the periphery as a compensatory mechanism, further compounding the loss of methyl-donors for Hcy remethylation in the brain. A further consequence of this includes a reduction in the neurotransmitter, acetylcholine, which has been previously described in MTHFR deficient mice [235]. Methionine synthase deficiency has also been shown to lead to cognitive impairment in a matter which involves disturbances in choline metabolism [265], suggesting an intimate link between disturbances in one-carbon metabolism and the regulation of neuronal choline.

Another important role of folate and its derivatives include antioxidant activity. While loss of 5-MTHF and elevations in tHcy should be considered independent biological events, they often occur in tandem [528]. Besides inducing damage through direct excitotoxic stimulation, Hcy can cause damage to neurons through oxidative stress [529]. Conversely, 5-MTHF shows

potent *anti*oxidant activity through the scavenging off free radicals and by preventing the oxidation of BH_4, which reduces intracellular superoxide generation via the coupling of Nitric oxide synthase as a cofactor [256]. The metabolite analysis of the mice we used here shows the loss of 5-MTHF and elevation of tHcy in response to LF diets (*Chapter 4*), which indicates a substantial imbalance in 5-MTHF as an antioxidant and tHcy as a source of oxidative stress [256].

Furthermore, folic acid protects against glutamate-induced excitoxicity [530], which is caused by Hcy, occurring through the same mechanism by which glutamate exerts this toxicity [521, 531]. Restrictions in normal one-carbon metabolic cycling can thereby induce APP phosphorylation through PP2A [476] and GSK-3β. For instance, oxidative stress and excitoxicity can enhance GSK-3β activity, mediating Thr668 phosphorylation of APP and, ultimately, neurodegeneration [55, 512]. Similar mechanisms may underlie the enhanced GSK-3β activity and APP phosphorylation in MTHFR deficient mice, and those fed LF diets, which we report here (***Fig. 5.4***). Similarly, our group has previously found that PP2A methylation is impaired in the cortex and hippocampus of MTHFR deficient mice and in mice fed LF diets [263]. Since PP2A is a major APP-Thr668 phosphatase, and PP2A demethylation is sufficient to promote the accumulation of p-Thr668 APP in cells and *in vivo* [258, 338], it is likely that impairment of PP2A methylation is a major contributor to the increased phosphorylation of APP observed in our studies.

Importantly, the sensitivity of hippocampal neurons to Aβ has been shown to critically depend on the methylation state of PP2A *in vivo* [338], which is dependent on methyl-group supply. Furthermore, recent evidence has shown that dietary supplementation of betaine abrogates neuronal impairments induced by Aβ peptide injection [532]. Betaine supplementation also prevents Aβ-induced oxidative stress, which is mediated in the brain by nitric oxide synthase [533]. It is unclear whether the positive effects in this study were directly induced by betaine itself, or a reduced antioxidant and Hcy-remethylation load of 5-MTHF that would arise as a consequence of increased intracellular Betaine.

Altogether, reduced neuronal 5-MTHF supply could thereby result in neuronal dysfunction through a number of mechanisms: 1 – a positive feedback cycle of neuronal choline efflux and 5-MTHF depletion, leading to cognitive impairments induced by reduced neuronal acetylcholine levels. 2 –Neuronal damage and death induced by Hcy-induced oxidative stress and excitotoxicity, which is not reversed by the antioxidant function of folate 3 – Elevated phosphorylation of APP and Tau as a result of impaired PP2A methylation and activity and increased GSK-3β activity, leading to neurotoxicity and disturbances in synaptic plasticity.

5.3.4 Changes in APP expression due to one-carbon metabolic impairments vary by brain region

Despite being well-validated as the precursor to Aβ species in AD, APP also exerts important functions in neuronal and synaptic plasticity and maintenance [534]. MTHFR and folate deficiencies induce impairments in learning and memory [235, 475, 500], which may be linked to APP through synaptic dysfunction, since MTHFR polymorphisms and dietary folate deficiency are risk factors for AD [216, 218, 460, 502]. Our present results show that genetic- or diet-induced folate deficiency affect total APP expression in our mouse models, in a complex, brain region specific manner.

In the hippocampus, genetic MTHFR and dietary folate deficiencies were associated with upregulation of APP protein levels, in both young and old mice (***Fig. 5.3***). However, in the cortex, APP expression was impaired in MTHFR deficient mice, compared to WT mice, and in mice fed a LF diet, compared to a NF-containing diet (***Fig. 5.3***). Similarly, APP expression was also significantly reduced in the midbrain of young HET and NULL mice, compared to WT, and in mice fed a LF diet (***Fig. 5.3***).

There are a number of regulatory factors that control APP gene expression in response to external stimuli, including growth factors, stress and inflammation [535]. While there have been few studies linking one-carbon supply and APP expression in humans, animal and cellular models have provided some insights into possible mechanisms of altered APP expression. In mice expressing familial AD-related mutations in APP and PS1 and fed folate-deficient diets, there is a limited expression profile of several miRNA, which caused elevated gene expression of APP and BACE1 [536]. The increased expression of these genes leads to augmentation of the Aβ pathology observed in this model, as well as in N2a cells used in the same study. Conversely, N2a cells overexpressing APP have reduced APP expression following folic acid supplementation, which is mediated by increased DNA-methyltransferase expression and activity [537]. Additionally, alterations in SAM availability or SAH levels are also able to modulate transcription of PS1 and BACE1 genes, which exacerbate Aβ pathology [259, 517]. There is further evidence demonstrating that supplementation of folate in APP/PS1 mice leads to impaired hippocampal expression of APP through changes in methylation potential and enhanced APP promoter methylation [538]. Altogether, previous evidence from other groups suggests that restrictions in methyl-donor availability could lead to enhanced expression of APP, which we observe in the hippocampus, a region especially sensitive to these restrictions. Importantly, this does not account for the differences we observe between the hippocampus, cortex and midbrain, suggesting a differential regional regulation of APP expression and sensitivity to disturbances in one-carbon metabolism.

Since APP is required for neuronal homeostasis and synapse formation [539], a loss of cortical APP could contribute to neurodevelopmental delays and cognitive deficits in human with severe MTHFR deficiency [459, 462, 464]. Disturbances in folate metabolism lead to the elevation on tHcy and impairment of PP2A methylation [160, 459], which may be linked to the APP dysfunction and changes in APP expression that we observe. Interestingly, the accumulation of APP has been reported to occur at sites of axonal injury in the brain and the upregulation of APP is considered a marker of central nervous system axonal injury [540]. Deregulated APP expression may contribute to neuronal dysfunction and/or degeneration in states of folate deficiency and hyperhomocysteinemia, as well as contributing to AD pathogenesis.

Since only AD models are able to generate detectable levels of Aβ *in vivo*, we were unable to ascertain the precise degree to which Aβ production is affected by MTHFR status in our investigations. Future studies examining the effect of MTHFR and/or folate deficiency in mouse models of amyloidogenesis would yield important outcomes.

5.4 Concluding remarks

In this chapter, our data describe one-carbon metabolic cycle control of APP expression and phosphorylation in MTHFR deficient mouse models fed either a normal or folate deficient diet. We show that these effects are specific to the brain regions we investigated. This model has already been shown to induce pathological processes similar to those observed in AD. Specifically, MTHFR-deficient mice have impaired hippocampal-dependent behavioural outcomes, in line with the observed hippocampal atrophy [235]. The animals we analysed here also have impaired PP2A methylation and enhanced Tau phosphorylation [263]. Additionally, PP2A has been previously identified as the phosphatase regulating APP phosphorylation at Thr668 [258], and has been proposed to regulate GSK-3β function [449, 513] (***Fig 5.5***). These findings, taken in conjunction with our results in chapter three and chapter four, suggest that compromised one-carbon metabolism and AD pathology are linked by PP2A function (***Fig 5.5***). Past work by our lab has demonstrated that PP2Ac methylation is impaired in MTHFR-deficient mice and mice fed FD diets [263]. Our results in chapter 3 show that PP2Ac methylation is modified by cAMP-PKA signalling and contributes to the regulation of the phosphorylation of the transcription factor CREB (***Fig. 5.5***).

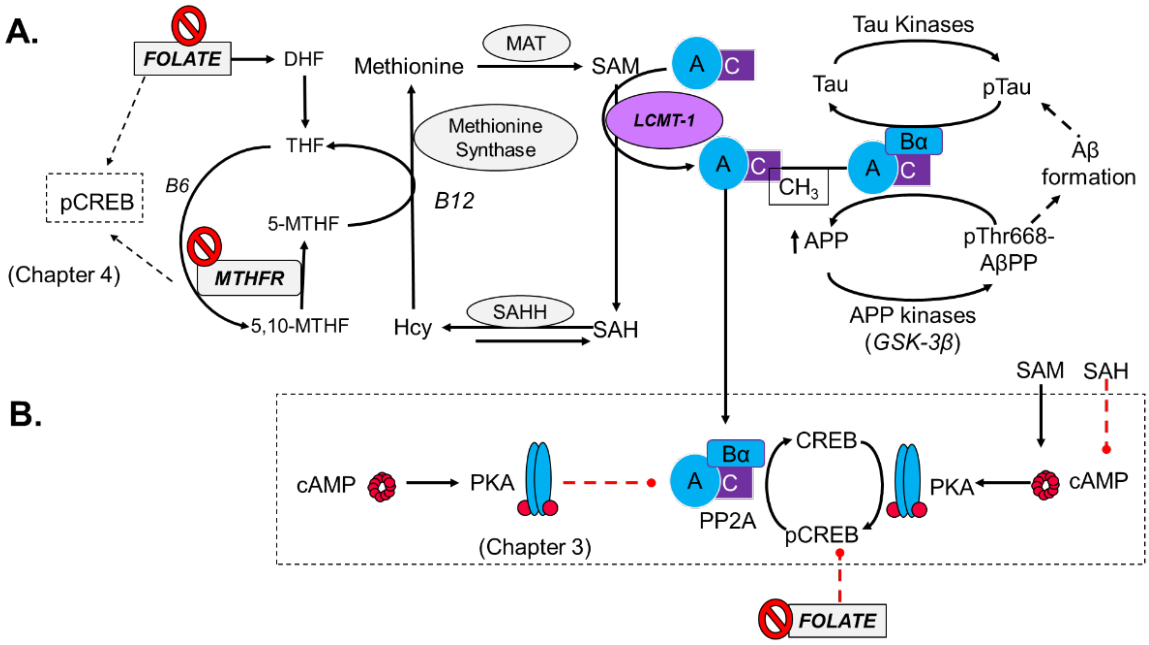

Figure 5.5 One-Carbon metabolism and the regulation of APP by PP2A. Schematic diagram of the interaction between one-carbon metabolism and APP phosphorylation *in vivo*. **A.** The regulation and cycling of one-carbon groups in cells is shown here. Dietary folate deficiency and genetic MTHFR deficiency (Red circles) both lead to PP2A demethylation and APP phosphorylation and expression (this chapter), and changes in CREB phosphorylation and expression (Chapter 4). **B.** PP2A methylation is also regulated by cAMP/PKA signalling (Chapter 3). Together, our findings suggest that altered PP2A methylation is a likely contributor to the deregulation of Tau, APP and CREB.

In chapter four, we demonstrated that CREB phosphorylation and expression are also modified by disturbances in one-carbon metabolism in N2a cells and in MTHFR-HET and NULL mice, and in WT mice fed a FD diet (***Fig. 5.5***). Therefore, the changes we have documented that affect PP2Ac methylation, Tau phosphorylation, CREB phosphorylation and expression, and APP phosphorylation and expression, may partly explain the neurological abnormalities observed in mouse models of disturbed one-carbon metabolism. Furthermore, folate deficiency and hyperhomocysteinaemia are both risk factors for the development of AD [217, 218, 261, 516], and loss of PP2Ac methylation and PP2A-ABαC enzymes occur concomitantly with pTau pathology in AD [158, 159]. These results indicate that similar molecular events occur in both disturbed one-carbon metabolism *in vivo* as those occurring in AD pathology. We emphasize the importance of the investigation of APP regulation by one-carbon metabolism in the context of an AD model to further explore these mechanisms in future studies. Meanwhile, we also conclude that APP regulation is multifaceted *in vivo*, but that PP2A methylation is central to this regulation, which itself is altered by the AD risk factors, dietary folate and elevated homocysteine.

Chapter 6: PP2Ac regulation by tyrosine phosphorylation

Acknowledgement of Collaboration:

I hereby acknowledge that the work enclosed in the following chapter of this thesis was performed in collaboration with other researchers.

The work in the following chapter (Chapter 4) was primarily performed by myself (Alexander Hoffman), though I received assistance in some experiments from fellow lab members (Goce Taleski) and my supervisors (Dr. Jean-Marie Sontag and A/Prof. Estelle Sontag).

We received additional help in the form of expertise from our international collaborators Drs. Brian Wadzinski and Egon Ogris, and their respective laboratories. Their contributions are acknowledged throughout.

The figures and results, including data analysis presented in this chapter were obtained and composed by me (Alexander Hoffman), with invaluable input and feedback from my supervisors A/Prof. Estelle Sontag and Dr. Jean-Marie Sontag.

6.0 Rationale and Significance

In previous chapters of this thesis, we have focused our interest on the regulation of PP2Ac methylation. In this chapter, we are now looking at another major post-translational modification of PP2Ac - the tyrosine phosphorylation of the catalytic subunit.

Initial *in vitro* data from Chen, *et. al.* using recombinant purified PP2Ac indicated tyrosine phosphorylation occurs at the Tyr307 site and leads to the inhibition of PP2A catalytic activity [173]. Tyr307 phosphorylation was reported to be mediated by RTKs and non-RTK, such as Src. Indeed, this phosphorylation event was initially observed in viral-transformed fibroblasts expressing a constitutively active mutant form of Src (v-Src) [173, 174].

However, Tyr307 phosphorylation of PP2A holoenzymes has never been confirmed *in vivo* by mass spectrometry. Notably, most studies investigating its functional role in cells have relied on the use of commercial anti-phospho-Tyr307-PP2Ac antibodies. Unfortunately, these antibodies have recently been shown to be quite unreliable in their use, because of a lack of specificity [386]. Furthermore, preliminary data from a collaborative effort involving our group, Dr. B. Wadzinski (*Vanderbilt University, U.S.A*.) and Dr. E. Ogris (*University of Vienna, Austria*) suggest that Tyr307 may not be the primary site for PP2A tyrosine phosphorylation.

Of particular relevance to our project, enhanced tyrosine phosphorylation of PP2A has been reported to occur in AD, in close proximity to dystrophic neurites; the authors suggested that increased Tyr307 phosphorylation-induced PP2A inactivation could lead to aberrant Tau phosphorylation and accumulation in AD-affected brain regions [175]. Likewise, *in vivo* studies using anti-phospho-Tyr307-PP2Ac antibodies have also indicated that PP2A tyrosine phosphorylation leads to aberrant Ser/Thr phosphorylation of Tau in a Src-dependent manner [391, 541]. Together, these observations support a hypothesis in which increased Src-mediated PP2Ac phosphorylation results in p-Tau pathology in AD, and contributes to the neurodegenerative process. However, other data do not support a role for enhanced Src activity in AD, since reduced Src activity is observed in some mouse models of AD [379], and in AD brain tissue with higher Braak staging [343].

In light of these observations, it is difficult to confirm if there is an active Src → PP2A phosphorylation → Tau neurotoxic pathway. In this chapter, we thus aimed to thoroughly investigate the role of Src-mediated PP2Ac tyrosine phosphorylation, in order to provide some clarity on this ambiguous triad. We also examined the functional consequences of Src-mediated PP2Ac phosphorylation in neurons and how this contributes to neuronal homeostasis and neuropathological processes in AD.

6.1 Introduction

Aside from methylation, the other well-documented post translational modification of PP2Ac is its phosphorylation. The tyrosine phosphorylation of PP2Ac was first reported to occur in response to viral Src (v-Src) expression and growth factor signalling *in vitro* and in fibroblasts [173, 174]. Since then Src-mediated phosphorylation of PP2Ac has been widely reported in a variety of cell types [387, 398]. Similarly, the tyrosine phosphorylation – and reported inactivation – of PP2A at the Tyr307 site has been widely described in both neuronal and non-neuronal cell lines [542, 543].

Pathological processes in AD are likely to be substantially affected by deregulation of Src. There may be at least two mechanisms by which Src dysfunction can lead to increased Tau Ser/Thr phosphorylation. The first mechanism may involve Src-mediated PP2Ac tyrosine phosphorylation, leading to inhibition of its catalytic activity, and consequently impairment of Tau dephosphorylation. Indeed, several studies have reported that PP2A phosphorylation in neurons has functional consequences for intracellular signalling pathways involving Src and Tau. In models of cerebral ischemia, there is a Src-dependent increase in $Erk_{1/2}$ activity facilitated by phosphorylation of PP2A at Tyr307 [387]. In Aβ-treated cortical neurons, Src was found to induce the tyrosine phosphorylation of PP2Ac, resulting in a consequent activation of $Erk_{1/2}$, and phosphorylation of Tau at the PHF-1 epitope [388]. Glutamate receptor-dependent activation of Src also promotes PP2Ac tyrosine phosphorylation and inactivation, and subsequent $Erk_{1/2}$ activation in striatal neurons [389]. Studies of human cases of Parkinsonism-Dementia of Guam, an environmental toxin-induced Tauopathy, also suggest that Src phosphorylates PP2Ac at Tyr307, culminating in Tau Ser/Thr phosphorylation [390]. Indeed, phosphorylation of PP2Ac at Tyr307 has been observed in neurofibrillary pathology in AD [175].

The second mechanism by which Src may affect Tau Ser/Thr phosphorylation involves the highly regulated interaction between Tau and Src. This interaction can indirectly affect PP2A-Tau protein-protein binding dynamics that modulate the ability of PP2A to dephosphorylate Tau. Indeed, Tau can bind to Src via the Src Homology 3 (SH3) domain, which enhances Src activity due to conformational changes in the kinase [356]. The association of Tau and Src is impaired by phosphorylation at Ser/Thr sites, or by expression of Tau mutants with phospho-mimicking, Ser/Thr → Glu amino acid substitutions in the proline-rich regions that associate with Src-SH3 [111, 114]. Significantly, the Src-family kinase member, Fyn, competes with PP2A for Tau binding [199]. Since Src and Fyn associate with the same domains in Tau [97, 111, 115], a similar PP2A/Src competition for Tau binding could, in theory, also occur with Src, though this has not yet been investigated. In this chapter, our first aim was to examine Src-

mediated phosphorylation of PP2Ac, and investigate its functional consequences for the phosphorylation of Tau at chosen Ser/Thr epitopes.

Additionally, PP2A, Tau and Src are all essential for neurite outgrowth. We have demonstrated in previous work [236], and in this thesis (Chapter 3) that PP2A is critically required for the extension and development of neurite-like processes. The expression and Ser/Thr phosphorylation of Tau is also important for development and maturation of neurites [544-546]. Src-dependent activation of Rho-GAP also mediates neurite outgrowth induced by Erk and cAMP/PKA signalling pathways [547, 548]. The neuron-enriched splice variant, N1-Src, has also recently been identified as absolutely necessary in primary neurogenesis *in vivo* [549]. Thus, in this chapter, we also aimed to demonstrate a relationship between Src, PP2A and neurite outgrowth.

Significantly, the majority the data published on Src-dependent PP2Ac phosphorylation relied on the widely used commercial anti-phospho-Tyr307 antibodies. It has been extensively reported that the detection of phospho-Tyr307 positive PP2Ac in cells and *in vivo* can be used as a readout of the presence of inactive PP2A holoenzymes. Therefore, any observed changes in signalling involving PP2A was attributed to its inactivation through phosphorylation at this site. However, data from our collaboration with Drs. Wadzinski and Ogris recently called into question the validity of this model of PP2A-site specific tyrosine phosphorylation, due to the flaws in the commercial anti-phospho-Tyr307--PP2Ac antibodies used [386]. In addition, the results of *Chen and colleagues* [173, 174], have not been successfully reproduced by other laboratories, nor has any research group provided independent evidence of PP2A phosphorylation at Tyr307 by mass spectrometry.

As such, the final part of this thesis has three hypotheses:

1. *The non-receptor tyrosine kinase Src phosphorylates PP2Ac, but this occurs at novel sites, rather than the previously suggested Tyr307 site*
2. *Src activity leads to Ser/Thr phosphorylation of Tau by inhibition of PP2A by phosphorylation at these novel sites*
3. *Src activity is important for PP2A-dependent neurite outgrowth*

The primary aim of this work is to validate *demonstrate a* direct role for Src in the regulation of PP2A, with direct functional outcomes relevant to both normal neuronal homeostasis and pathogenic processes in Alzheimer's disease.

6.2 Results

6.2.1 Validation of PP2Ac Tyrosine phosphorylation by Src

6.2.1.1 Expression of constitutively active mutants of the tyrosine kinase, Src

To address the functional role of Src kinase in PP2Ac regulation, a plasmid encoding a constitutively active c-Src mutant with a Y→F mutation in the auto-regulatory C-terminal 527 residue (Src^{CA}) was first generated and verified by sequencing, as detailed in Materials & methods *(Chapter 2)*. COS-7 and N2a cells were then transiently transfected with plasmids encoding Src^{CA}. The expression and activity of transfected Src^{CA} was verified by Western blotting of total cell extracts, using anti-phospho-Tyr416 (p-Tyr416) Src and anti-total Src antibodies in N2a and COS-7 cells (***Fig. 6.1***). As expected, total Src increased substantially upon transfection, and was immunoreactive with the anti- p-Tyr416 Src antibody recognizing active Src kinase in both N2a and COS-7 cell lines.

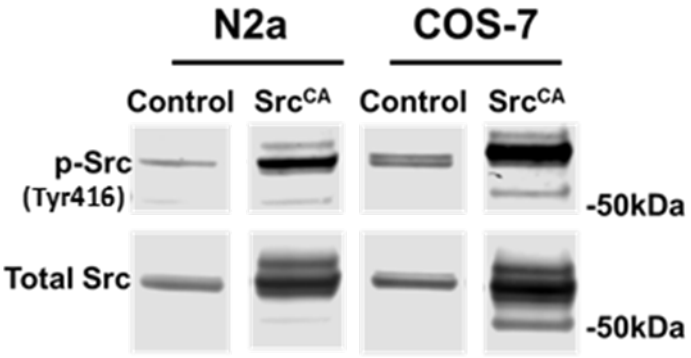

Figure 6.1: ***Ectopic expression of Src^{CA} mutant in N2a and COS-7 cell lines.*** N2a and COS-7 cells were transfected with constitutively active Src (Src^{CA}). Protein aliquots from total cell homogenates were prepared and analysed by Western blot for transfected Src^{CA}. Src activity was assessed by probing for phosphorylation of the Tyr416 residue in the Src-kinase domain. Blots were then re-probed with anti-Src primary antibody to detect total (endogenous and transfected Src^{CA}) Src. As expected, Src^{CA}-transfected N2a and COS-7 cells showed an increase in the expression of total Src protein and kinase-active Src.

6.2.1.2 PP2Ac Tyrosine residues are substrates of Src

The successful overexpression of Src^{CA} allowed us to investigate whether or not we could observe Src-induced PP2Ac tyrosine phosphorylation in N2a and COS-7 cells. COS-7 cells were co-transfected with plasmids encoding HA-tagged wild-type PP2Ac ($PP2Ac^{WT}$) together with Src^{CA} or empty vector (EV). Transfected cells were treated with either the phosphotyrosine phosphatase (PTP) inhibitor, Pervanadate (PV, *see chapter 2, Materials and Methods*) or vehicle, prior to cell lysis and immunoprecipitation with anti-HA-conjugated magnetic beads. HA-PP2Ac immuno-precipitates and total cellular lysates were analysed by

Western blot with a commercial, well-validated pan-phospho-Tyr directed primary antibody (***Fig. 6.2***).

As expected, the overexpression of Src^{CA} and treatment of cells with PV were each sufficient to significantly increase the tyrosine phosphorylation of endogenous proteins, compared to EV-transfected or vehicle-treated cells, respectively (***Fig. 6.2.A***, shown are COS-7 cell lysates). Incubation with PV further synergized with transfected Src^{CA} to increase the overall tyrosine phosphorylation of proteins in COS-7 (***Fig. 6.2.A***).

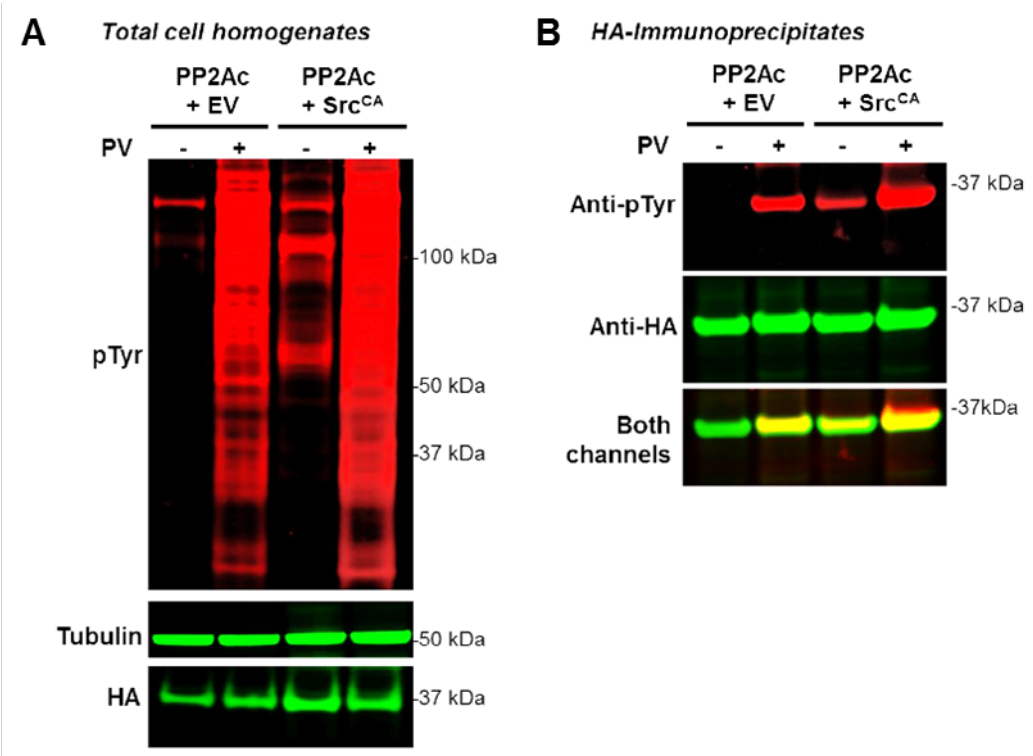

Figure 6.2 PP2Ac Tyrosine phosphorylation is enhanced by Src^{CA} overexpression and Pervanadate treatment. Representative Western blots of (**A**) total cell homogenates and (**B**) HA-immunoprecipitates prepared from COS-7 cells expressing HA-tagged PP2Ac that were co-transfected with plasmids encoding either Src^{CA} or an empty vector (EV). Cells were also incubated with pervanadate (+) or vehicle (-) for 15 min prior to harvesting. Anti-pan-phospho-tyrosine antibodies (pTyr) were used to assess tyrosine phosphorylation of proteins in response to Src^{CA} and PV. Anti-Tubulin antibodies were used to control for protein loading. Similar results were observed in 4 separate experiments.

We next analysed HA-tagged PP2Ac immunoprecipitates prepared from N2a and COS-7 cells for potential tyrosine phosphorylation (***Fig. 6.2.B***, shown are immunoprecipitates from COS-7 cells). Firstly, we noted that tyrosine phosphorylation of PP2A was not detectable in HA-PP2Ac precipitates prepared from EV-expressing, vehicle treated controls. In contrast pTyr immunoreactivity was clearly visible in HA-PP2Ac immunoprecipitates from PV-treated or Src^{CA} overexpressing COS-7 cells (***Fig. 6.2.B***). Moreover, PV further enhanced the tyrosine phosphorylation of PP2A in Src^{CA} overexpressing COS-7 cells (***Fig. 6.2.B***). Similar results were obtained in experiments performed in N2a cells (n = 4).

6.2.1.3 Is PP2Ac phosphorylated at Tyr307?

Since we observed that expression of active Src or treatment of cells with PV can induce PP2Ac tyrosine phosphorylation, we next wanted to verify whether or not this occurred at the previously described Tyr307 site [173, 174]. In order to do this, we first overexpressed Src^{CA} in a number of cell types, including N2a, MEF, U-87, HEK293, and 3T3 cells, and analysed cell homogenates with anti-phospho-Tyr and anti-phospho-Tyr-307-PP2Ac antibodies. As a control, we also took a duplicate aliquot from Src^{CA}-expressing cell homogenates incubated with PV, and treated it with alkaline phosphatase (AP), which is known to induce broad-spectrum dephosphorylation of all endogenous proteins. Analysis of cell extracts showed that, as expected, there was a significant increase in overall tyrosine phosphorylation of proteins in Src^{CA}-overexpressing and PV-treated COS-7 cells (***Fig. 6.3***). Moreover, AP treatment caused blanket protein dephosphorylation, as evidenced by the disappearance of the signal (***Fig. 6.3***). In stark contrast, there was no change in the immunoreactivity of two antibodies directed towards pTyr307-PP2Ac following either overexpression of Src^{CA}, PV treatment, or AP incubation (***Fig. 6.3***). These antibodies also strongly cross-reacted with non-phosphorylated PP2Ac in control cells. The two antibodies that we used include rabbit "anti-PP2Ac-pY307", clone E155, available through Abcam, and goat "anti-PP2Ac-pY307", purchased from Santa Cruz biotechnology, which have been widely used in over 100 peer-reviewed publications. Together, our present findings indicate that these antibodies do not specifically recognise phosphorylated tyrosine residues in PP2Ac.

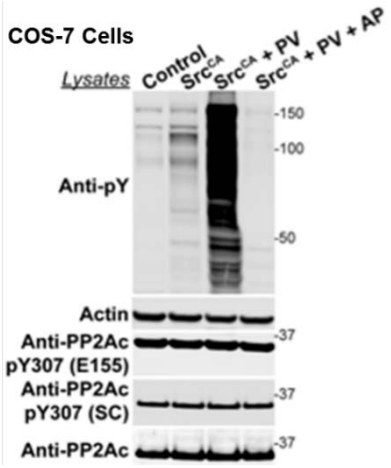

Figure 6.3 Widely utilised commercial pTyr307-PP2Ac antibodies do not specifically recognise tyrosine phosphorylated PP2A. COS-7 cells were transfected with a plasmid encoding Src^{CA} or the empty vector alone (EV) then incubated with pervanadate (PV) or vehicle. A duplicate aliquot of the lysate from Src^{CA}-transfected and PV-treated cells was incubated with alkaline phosphatase (AP; 1 unit/µg protein) to induce protein dephosphorylation. Representative Western blots from total cell lysates are shown. Anti-pan-phospho-tyrosine antibodies were used to assess total tyrosine phosphorylation in each condition. Two common anti-pTyr307-PP2Ac antibodies, "anti-pY307 clone E155" (Abcam) and "anti-pY307" (Santa Cruz) showed no change in immunoreactivity, despite Src^{CA} overexpression, or treatment with PV or AP. Actin was used as protein loading control. Representative blots from 4 separate experiments are shown.

Since the use of anti-PP2Ac-Tyr307 antibodies appeared to be inherently problematic, to validate the existence of the pTyr307 site, we then investigated whether Src^{CA} or PV can indeed induce the tyrosine phosphorylation of PP2Ac at this site using alternative methods. First, we comparatively analysed PP2A tyrosine phosphorylation in COS-7 cells overexpressing HA-tagged $PP2Ac^{WT}$ or the phospho-incompetent $PP2Ac^{Y307F}$ mutant, together with Src^{CA} or the EV. Again, a subset of cells was incubated with PV to maximally induce and preserve protein tyrosine phosphorylation or vehicle. As expected, both Src^{CA} overexpression and PV treatment induced tyrosine phosphorylation of the endogenous protein pool of COS-7 cells (***Fig. 6.4.A***).

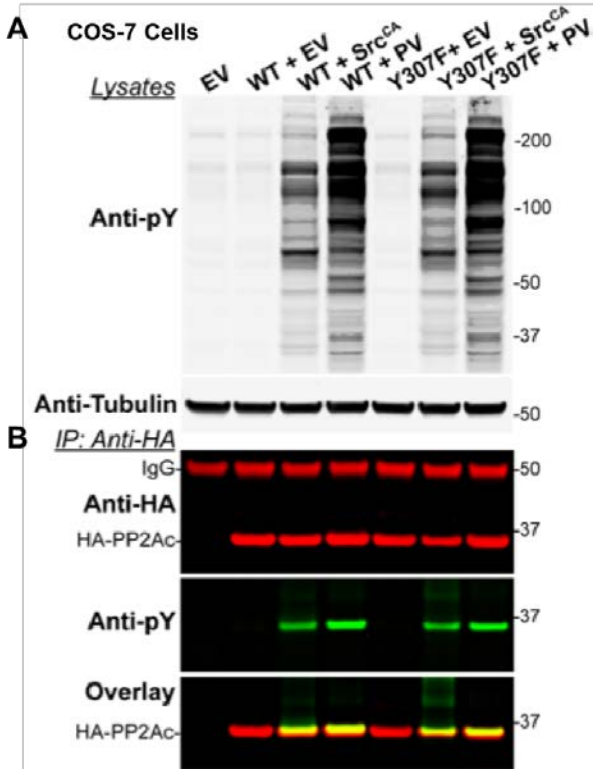

Fig. 6.4 PP2A is not significantly phosphorylated at Tyr307 in response to Src^{CA} or PV. Representative Western blots from (**A**) total cell lysates and (**B**) HA-immunoprecipitates prepared from mock-transfected COS-7 cells (EV) or COS-7 cells transfected with plasmids encoding HA-$PP2Ac^{WT}$ (WT), HA-$PP2Ac^{Y307F}$ (Y307F), together with Src^{CA} or empty vector (EV). Subsets of transfected cells were also treated with pervanadate (PV) or vehicle. Blots were probed with anti-phospho-tyrosine to assess tyrosine phosphorylation levels of both the endogenous pool of proteins (**A**) and HA-PP2Ac immunoprecipitates (**B**). Representative blots are shown from 4 separate experiments.

We were unable to detect tyrosine-phosphorylated PP2Ac in the absence of Src^{CA} or PV in HA-PP2Ac immunnoprecipitates prepared from cells (***Fig. 6.4.B***). Furthermore, in HA-$PP2Ac^{Y307F}$-expressing cells, Src^{CA} overexpression and PV treatment were still able to induce the phosphorylation of PP2Ac at levels comparable to those seen in HA-$PP2Ac^{WT}$-expressing cells (***Fig. 6.4.B***), indicating that PP2Ac phosphorylation occurs at sites other than Tyr307.

Importantly, these results were reproduced in experiments in multiple cell lines, including N2a, MEF, U-87, HEK293, and 3T3 cells, performed by several investigators in our groups and in laboratories of our collaborators, Drs. Wadzinski and Ogris. In support of these results, we were unable to detect phosphorylated Tyr307 in six different phospho-proteomic analyses of HA-PP2Ac immunoprecipitates performed in collaboration with Drs. Wadzinksi and Ogris. Likewise, a careful review of up-to-date protein modification databases (PhosphoSitePlus, PhosphoNET) also reveals a number of discovery-mode mass spectrometry investigations that have failed to identify pTyr307 in a variety of cells. Thus, although it has been reported that PP2Ac is solely phosphorylated on Tyr307 [174], our findings suggest that Tyr307 is not a major, primary tyrosine phosphorylation site in PP2Ac.

6.2.1.4 Expression of novel phosphorylation-incompetent mutants of PP2Ac

Our collaborative phospho-proteomic data analyses rather suggest the existence of novel Src-dependent PP2Ac tyrosine phosphorylation sites. Due to the sensitive nature of the intellectual property in question, and at the explicit request of our collaborators, we have elected not to reveal the precise nature of these tyrosine residues. To validate and understand the functional role of these newly identified phosphotyrosine sites, phosphorylation-incompetent PP2Ac mutants (Y→F mutations) were constructed in our laboratory, and verified by sequencing. These mutants (HA-tagged PP2Ac$^{\#1}$, PP2Ac$^{\#2}$, and PP2Ac$^{\#3}$) were expressed via transient transfection of COS-7 cells, and their expression verified by Western blot using antibodies raised against HA (***Fig 6.5***).

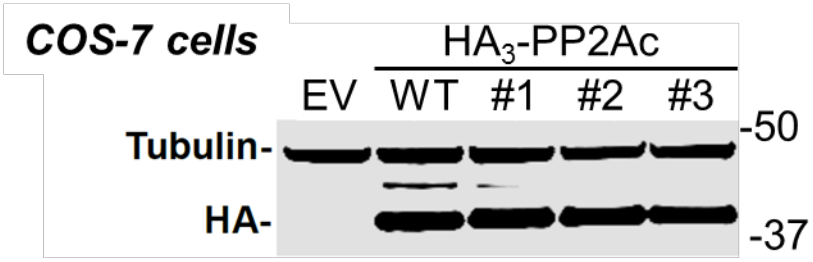

Figure 6.5 Expression of PP2Ac Tyrosine phosphorylation-incompetent mutants in COS-7 cells. PP2Ac Y→F mutants were transiently transfected in COS-7 cells. Cells were then homogenised and 30µg proteins from total protein extracts separated by NU-PAGE. Shown is a representative Western blot demonstrating HA_3-tagged PP2Ac expression in COS-7 cells 48h post-transfection. Blotting with anti-Tubulin antibodies was used to control for protein loading.

6.2.1.5 Mutation of selected PP2Ac Tyrosine sites inhibits PP2Ac phosphorylation

To validate putative Src-targeted tyrosine phosphorylation sites on PP2Ac, Src^{CA} was co-expressed together with HA_3-tagged PP2Ac^{WT} and three candidate phospho-incompetent (Y → F) PP2Ac mutants. Expression of Src^{CA} was validated by Western blot of total COS-7 cell lysates. Western blot analysis of HA-PP2Ac immune complexes isolated from pervanadate-

treated cells revealed that tyrosine phosphorylation of PP2Ac$^{\#1}$ and PP2Ac$^{\#2}$ mutants were decreased relative to PP2Ac^{WT}, and was almost absent in PP2Ac$^{\#3}$. Mutants were verified using anti-HA antibodies (***Fig. 6.6***). While HA$_3$-PP2Ac^{WT} showed substantial immunoreactivity to anti-pY antibodies when co-expressed with Src^{CA}, each of the Y → F HA$_3$-PP2Ac mutants tested had reduced levels of tyrosine phosphorylation, with PP2Ac$^{\#3}$ being the most resistant to Src^{CA}-dependent phosphorylation.

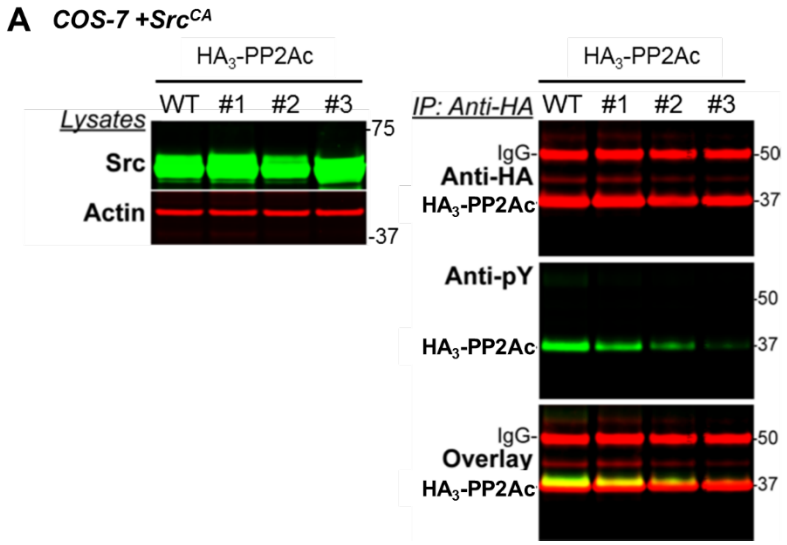

Figure 6.6 HA$_3$-PP2Ac$^{\#3}$mutant is resistant to Src^{CA}-dependent tyrosine phosphorylation. Total cell lysates and HA-immunoprecipitates were prepared from COS-7 cells transiently expressing either HA$_3$-PP2Ac^{WT} or HA$_3$-PP2Ac$^{\#1}$, HA$_3$-PP2Ac$^{\#2}$ or HA$_3$-PP2Ac$^{\#3}$, together with EV or Src^{CA}. Representative Western blots of total cell lysates and immunoprecipitates are shown. Src^{CA} expression in COS-7 cells was validated using anti-Src antibody; anti-Actin antibody was used to control for protein loading. The immunoprecipitation and phosphorylation of HA-PP2Ac^{WT} and PP2Ac mutants was analysed by probing blots with anti-HA and anti-pan-phospho-Tyrosine (Anti-pY) antibodies. Blots representative of *n* = 4 experiments.

We found similar results in PV-treated COS-7 cells (***Fig. 6.7***). As expected, analysis of HA$_3$-PP2Ac immunoprecipitates confirmed that PV induced a significant tyrosine phosphorylation of PP2Ac^{WT}. In contrast, the tyrosine phosphorylation of PP2Ac$^{\#1}$ and PP2Ac $^{\#2}$ were decreased relative to WT, and almost absent in PP2Ac$^{\#3}$. Comparable results were obtained in several experiments undertaken in N2a cells. Since the PP2Ac mutant showing the most resistance to tyrosine phosphorylation by Src and PV was PP2Ac#3, it was preferentially chosen for further functional experiments.

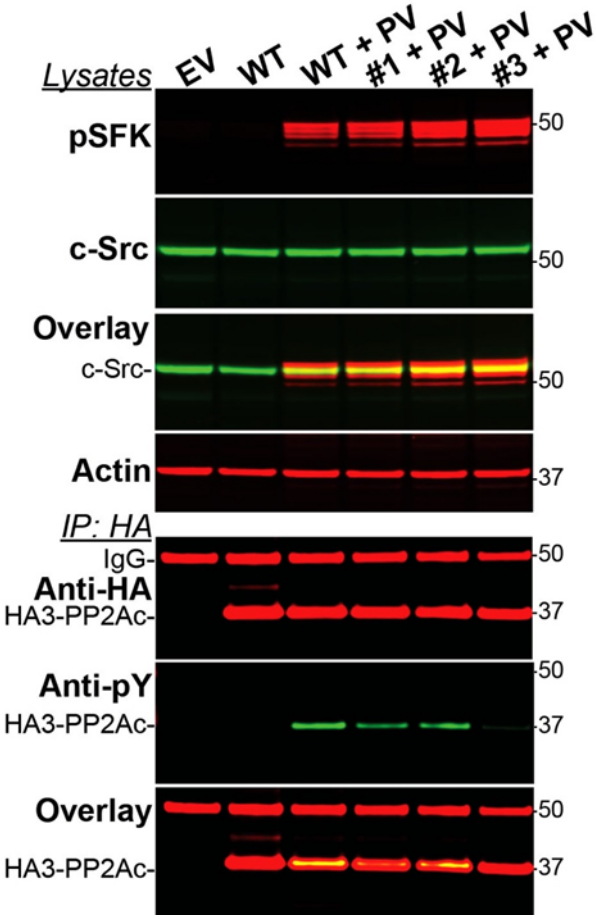

Figure 6.7 The HA_3-$PP2Ac^{\#3}$ mutant is resistant to PV-dependent tyrosine phosphorylation. COS-7 cells were co-transfected with plasmids encoding HA_3-$PP2Ac^{WT}$ or HA_3-$PP2Ac^{\#3}$ together with Src^{CA} or EV. Cells were incubated with Pervanadate (PV) or vehicle, prior to immunoprecipitation with anti-HA magnetic beads. Immunoprecipitates were analysed by Western blot using anti-pY and anti-HA antibodies to determine the tyrosine phosphorylation state of PP2Ac. Shown are representative blots from *n* = 3 experiments.

6.2.2 Functional role of PP2Ac tyrosine phosphorylation in N2a cells

6.2.2.1 Serine phosphorylation of Tau is enhanced by Src^{CA} expression

Because of the potential link between Src, PP2A and Tau phosphorylation, we next examined whether changes in Src-induced PP2Ac phosphorylation can indeed affect PP2A- dependent Tau dephosphorylation. We examined whether the phosphorylation of endogenous Tau at the Ser202 (identified by the CP13 antibody) and Ser396/404 (identified by the PHF-1 antibody) residues in N2a cells could be affected by the tyrosine phosphorylation of PP2Ac. We transiently transfected N2a cells with plasmids encoding either Src^{CA} or EV, together with plasmids encoding $PP2Ac^{WT}$, $PP2Ac^{\#3}$ or EV (***Fig. 6.8***), and assessed Tau phosphorylation by Western blot.

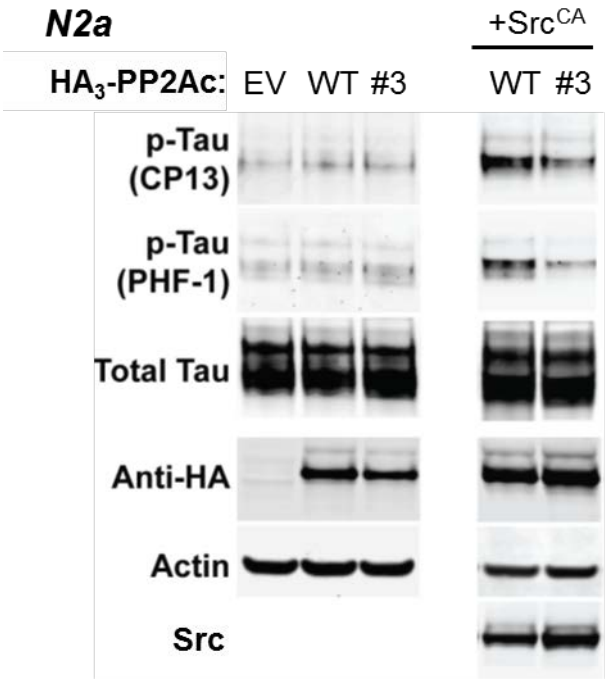

Figure 6.8 $PP2Ac^{\#3}$ blocks Src-mediated Serine/Threonine Tau phosphorylation. Total cell homogenates were prepared from N2a cells transfected with EV or HA_3-$PP2Ac^{WT}$ or HA_3-$PP2Ac^{\#3}$ ± Src^{CA}. Cell lysates were analysed by Western blotting for endogenous pTau using CP13 (pSer202) and PHF-1 (pSer396/Ser404) antibodies. Blots were probed with anti-HA antibodies to verify expression of HA_3-$PP2Ac^{WT}$ and HA_3-$PP2Ac^{\#3}$, while anti-Tubulin antibodies were used as protein loading controls. Blots shown are representative of *n* = 3 experiments.

Both of these phospho-Tau epitopes have been linked to structural plasticity of neurons *in vivo,* and in Tau dysfunction in AD [108]. The phospho-Tau CP13 epitope is typically observed in pre-tangle p-Tau pathology [550], while the phospho-Tau PHF-1 epitope is frequently observed in AD p-Tau tangle pathology. Both antibodies are highly specific, and having been validated *in vivo* [108]. We observed that Tau is dephosphorylated at these epitopes in unstimulated N2a cells expressing $PP2Ac^{WT}$, as has been shown in our previous work ([258], ***Fig. 6.8***). Tau is dephosphorylated to similar levels in $PP2Ac^{\#3}$-expressing N2a cells, indicating that this mutant, like $PP2Ac^{WT}$, is catalytically active towards p-Tau.

Expression of Src^{CA} in $PP2Ac^{WT}$-overexpressing N2a cells enhances CP13 phosphorylation by ~3.5-fold and PHF-1 phosphorylation by 2-fold (***Fig. 6.8***). However, Src^{CA}-mediated Tau phosphorylation was completely abolished in cells co-expressing $PP2Ac^{\#3}$ (n = 4; p <0.01). Together, these data indicate that Src-dependent phosphorylation of PP2Ac (at one or possibly multiple sites) can impair the ability of PP2A to dephosphorylate Tau.

6.2.2.2 PP2Ac –mediated neurite outgrowth is blocked by SFK inhibition

Besides regulating Tau, studies have shown that Src also plays a very significant role in neuritogenesis [366, 371]. We have demonstrated the importance of PP2A in the regulation of neurite outgrowth in N2a cells, as outlined in Chapter 3 of our thesis. To further investigate the functional interaction of Src and PP2A, we then examined whether Src can affect PP2Ac-induced neurite outgrowth in N2a cells. Cells stably overexpressing $PP2Ac^{WT}$ were differentiated for ~20h by serum starvation in the presence of the specific SFK inhibitor, PP2, or vehicle. Cells were then fixed and stained with anti-β-III tubulin primary antibody in order to assess neurite outgrowth by confocal microscopy (***Fig. 6.9***). In agreement with earlier studies [236, 551, 552], N2a-$PP2Ac^{WT}$ cells generated neurites (processes ≥ one cell diameter) at a rate of ~26% upon incubation in differentiation medium containing vehicle. However, there was a dramatic inhibition of neurite outgrowth in N2a-$PP2Ac^{WT}$ cells incubated in the differentiation medium containing PP2, with only ~5.5% of these cells growing neurites (***Fig. 6.9***).

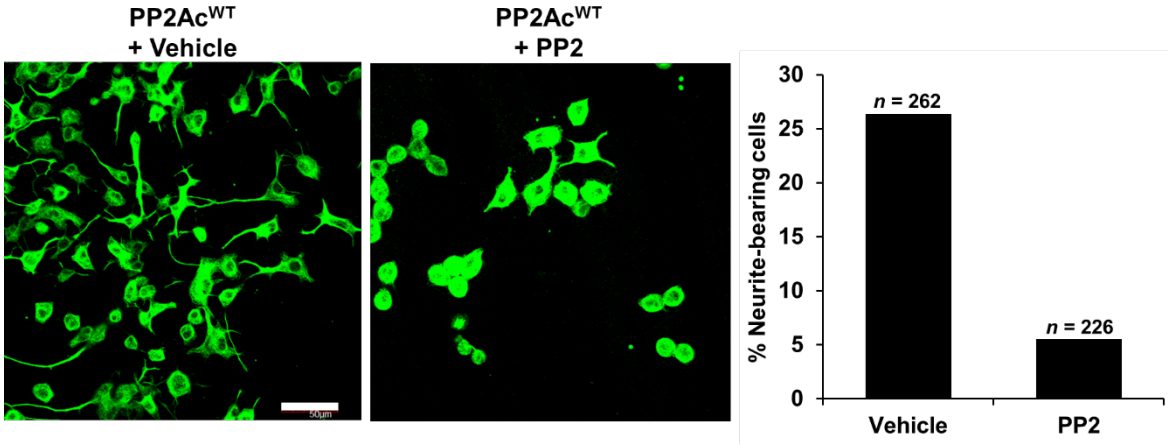

Figure 6.9: Src Family kinase inhibition blocks PP2Ac-dependent neurite outgrowth. Immunofluorescent staining for β-III tubulin in N2a cells stably expressing $PP2Ac^{WT}$. Cells were cultured in low-serum differentiation medium with the Src-family kinase inhibitor PP2, or vehicle. The proportion of cells bearing neurites longer or equal to the diameter of the cell body was determined for each condition, and data presented graphically (n = 262 for vehicle- incubated control cells; n = 226 for PP2-treated cells). White scale bar, 50μm length.

Together, these findings suggest that Src-mediated PP2A tyrosine phosphorylation could be an important contributor to the role of PP2A in the regulation of both Tau and neurite outgrowth.

6.3 Discussion

6.3.1 Significance of PP2A phosphorylation

Tyrosine phosphorylation of the 307 residue of PP2Ac was first hypothesised to catalytically inactivate the phosphatase [173]. The tyrosine phosphorylation of PP2Ac has since been suggested to play an important role in a number of intracellular signalling pathways. For instance, it has been linked to insulin receptor-induced metabolic signalling [176], for which PP2A inactivation is a prerequisite [553]. PP2Ac tyrosine phosphorylation and inactivation has also been implicated in restriction of axonal transport in neurons [554]. A major signalling pathway affected by PP2Ac inactivation is the Ras-Raf-Erk, or MAP kinase signalling pathway [555], involved in the differentiation of neurons following extracellular mitogens, including insulin. It was one of the first signalling pathways reported to cause PP2Ac-Tyr307 phosphorylation [174]. Previous work has demonstrated that PP2Ac regulates this pathway bi-modally, meaning that PP2A impairs Ras1 activity, but can activate Raf signalling downstream in some types of CNS cells [556].

Several studies suggest that PP2Ac phosphorylation is increased in AD [175]. PP2Ac phosphorylation has been proposed to lead to Tau hyperphosphorylation in multiple animal models of neurodegeneration through inactivation of Tau phosphatase activity and through a concomitant increase in MAP kinase signalling [387, 389, 555]. Notably in these reports, Src was responsible for MAP kinase activity and the tyrosine phosphorylation of PP2Ac. However, as noted earlier, all these studies exclusively relied on the use of flawed anti--pTyr307-PP2Ac) antibodies, which prompted us to re-examine the role of Src as a PP2A tyrosine kinase.

In this chapter, we were able to demonstrate that Src can phosphorylate PP2A at novel sites other than Tyr307, which has functional implications for PP2A regulation, Tau phosphorylation and neuronal cell differentiation.

6.3.2 Src phosphorylates PP2Ac at novel tyrosine sites

To re-examine the regulation of PP2A by tyrosine phosphorylation, we used PV, a PTP inhibitor that induces an irreversible oxidation of the cysteine residues in PTPs, resulting in enzymatic inhibition [557]. This leads to activation of many endogenous tyrosine kinases (including receptor and non-receptor) that are usually maintained in an inactivated state by PTPs. In addition, broad inhibition of PTPs by PV helps to preserve the phosphorylation of tyrosine residues in target proteins. This is important in the context of studying PP2Ac tyrosine phosphorylation, since it has been shown that this phosphorylation event is extremely transient and labile, and phospho-PP2Ac can be quickly dephosphorylated by autodephosphorylation and/or PTPs [173]. We also expressed a constitutively active mutant of Src (Y527F mutation, Src^{CA}, ***Fig. 6.1***) for specifically studying the Src-PP2A relationship. We chose to express this

mutant, rather than wild-type Src, since PP2A has been reported to bind to and inhibit Src activity *in vitro* [392], which could have confounded the interpretation of our results.

Stimulation of tyrosine phosphorylation with Src^{CA} and/or PV induced a substantial increase in the tyrosine phosphorylation state of a plethora of proteins in total homogenates, compared to vehicle-treated cells (***Fig. 6.1***). Likewise, there was a synergistic effect of PV and Src^{CA} in the tyrosine phosphorylation of immunoprecipitated PP2Ac (***Fig. 6.2***). These findings suggest that besides Src, other tyrosine kinases are involved in the tyrosine phosphorylation of PP2A, and that inhibition of PTPs helps to preserve Src^{CA}-mediated tyrosine phosphorylation of PP2Ac.

Once we were able to validate that Src really phosphorylates PP2Ac, it was important to assess whether this indeed occurred on the Tyr307 residue identified as the *sole* phosphorylation site in PP2Ac [173]. Our group and collaborators have recently raised reservations about the commercially available anti-pTyr307-PP2Ac antibodies [386], and the lack of independent verification of results presented by *Chen* and colleagues [173, 174]. We therefore attempted to detect changes in phosphorylation of PP2Ac using the two most common "anti-pTyr307-PP2Ac" antibodies following incubation of lysates taken from cells overexpressing Src^{CA} ± PV ± alkaline phosphatase (***Fig. 6.3***). As expected, incubation of PV-treated cell homogenates with AP induced dephosphorylation of all proteins, as demonstrated by Western blot with an anti-pan-phosphotyrosine antibody (***Fig. 6.3***). Yet, anti-pTyr307-PP2Ac antibodies remained immunoreactive towards PP2Ac in the same AP-treated extracts, indicating that these antibodies strongly cross-react with dephosphorylated PP2Ac.

We were unable to show that Src phosphorylated PP2Ac at Tyr307 by using commonly-used antibodies (***Fig. 6.3***). This led to our interpretation that these antibodies cross-react with dephosphorylated PP2A, and/or that other kinases potentially phosphorylate PP2A at Tyr307. We next immunoprecipitated HA-$PP2Ac^{WT}$ and HA-tagged $PP2Ac^{Y307F}$ mutant from cells co-transfected with Src^{CA} or EV, and treated with PV or vehicle, and compared their tyrosine phosphorylation levels by blotting with anti-pan-phosphotyrosine antibodies (***Fig. 6.4.B***). In HA-$PP2Ac^{WT}$–expressing cells, the presence of Src^{CA} was necessary to observe tyrosine phosphorylation of PP2A, which was enhanced by incubation with PV. Of note, the absence of detectable PP2A tyrosine phosphorylation under basal, unstimulated conditions suggests that PP2A tyrosine phosphorylation only occurs with significant activation of tyrosine kinases, and is likely transient and labile. Interestingly, when normalising for the amounts of immunoprecipitated material, there were similar levels of PP2Ac tyrosine phosphorylation in HA-$PP2Ac^{Y307F}$ immunoprecipitates (***Fig. 6.4.B***). These data further demonstrate that Tyr307 is neither the major, nor sole tyrosine phosphorylation site as originally published [173]. Indeed, our findings rather suggest that PP2Ac tyrosine phosphorylation primarily occurs at

non-Tyr307 sites. However, we cannot exclude the possibility that PP2A can become phosphorylated at Tyr307 in other cells lines and under other experimental conditions, but this remains to be experimentally validated using mass spectrometry and specific antibodies.

In order to further examine the nature of PP2Ac phosphorylation, we generated three unique HA_3-tagged tyrosine phospho-incompetent (Y → F) mutants and their HA_3-tagged $PP2Ac^{WT}$ counterpart, and expressed each of these together with Src^{CA} in N2a and COS-7 cells (***Fig. 6.5***). Accordingly, we found that mutants $PP2Ac^{\#1}$, $PP2Ac^{\#2}$ and $PP2Ac^{\#3}$ all impaired PP2Ac phosphorylation with increasing degrees of efficacy, with $PP2Ac^{\#3}$ being the least tyrosine-phosphorylated (***Fig. 6.6.A***). Furthermore, $PP2Ac^{\#3,}$ and to a lesser extent, $PP2Ac^{\#1}$ and $PP2Ac^{\#2}$, were also resistant to PV-mediated tyrosine phosphorylation (***Fig. 6.7***).

Taken together, these data indicate that the regulation of the tyrosine phosphorylation of PP2Ac is not only poorly understood and mis-characterised, but also complex and may even be functionally diverse, depending on which site becomes phosphorylated. The two sites that represent the minor portion of PP2Ac tyrosine phosphorylation remain to be studied in more detail. However, the $PP2Ac^{\#3}$ tyrosine-phospho-incompetent mutant represents a substantial portion of PP2Ac tyrosine phosphorylation induced by Src and PTP inhibition in COS-7 and N2a cells, so we chose to focus on this mutant to investigate some functional consequences of PP2A phosphorylation at this site.

6.3.3 Src-mediated PP2Ac phosphorylation enhances Tau phosphorylation

The major substrate of PP2A in neurons that we are particularly interested in is the microtubule-associated protein, Tau. We hypothesised that if Src-mediated phosphorylation of PP2Ac impairs the phosphatase activity of PP2A, as has been suggested by others previously [173, 174], the phosphorylation of Tau would be increased. When we expressed Src^{CA} in N2a cells to induce tyrosine phosphorylation of PP2Ac, we observed a marked increase in Tau phosphorylation at serine residues of Tau (***Fig. 6.7***), CP13 (pSer202, [108]) and PHF-1 (pSer396+404, [105, 109, 110]), which are involved in neuronal plasticity and early- and late-stage AD p-Tau pathology, respectively [108]. Co-expression of Src^{CA} and the $PP2Ac^{\#3}$ Y→F mutant did not lead to a similar increase in Tau phosphorylation to that seen in $PP2Ac^{WT}$-expressing N2a cells (***Fig. 6.8***). These findings suggest that: (1) The tyrosine residue altered in the $PP2Ac^{\#3}$ mutant is targeted by Src for phosphorylation, (2) the Y→F PP2Ac mutant retains catalytic activity in N2a cells towards pTau (***Fig. 6.8.B***) and (3) phosphorylation at this site inhibits the Tau phosphatase activity of PP2A. However, we cannot exclude the possibility that the $PP2Ac^{\#3}$ mutant could also interfere with Src-mediated Tau phosphorylation by other indirect mechanisms than preventing inhibition of the catalytic activity of PP2A. For instance, the mutant could affect assembly of Src/Tau, PP2A/Src or PP2A/Tau

protein-protein complexes, and/or PP2A subunit composition and distribution. Future studies will be required to test those hypotheses.

Observations surrounding changes in PP2Ac phosphorylation in neuropathology are signified by either Tyr307 phosphorylation using the commercially available antibodies described above, or make use of pan-phospho-tyrosine antibodies in identifying changes in PP2A regulation.

PP2Ac-Tyr307 phosphorylation has been reported to be markedly increased in AD autopsy brain tissue in tandem with neurofibrillary pathology [175]. Unfortunately, those studies relied on poorly characterised anti-pTyr307-PP2Ac antibodies, so most of these observations should be considered critically (refer to [386]). Moreover, detecting high levels of phosphorylated PP2A in human post-mortem brain tissue- with typically elevated post-mortem intervals- is quite surprising, since PP2A tyrosine phosphorylation is normally very transient and labile, and requires special processing and buffers to be preserved [173].

Src has been previously suggested to contribute to Tau Ser/Thr phosphorylation in neurons through PP2A phosphorylation at Tyr307 and subsequent inhibition of its catalytic subunit [390, 541]. In one report, Tyr307 phosphorylation of PP2Ac corresponded with phosphorylation of Tau at the 12E8 epitope (pSer262/356 and Ser199), Thr205, Thr212 and Ser396 residues [390]. These sites are typically associated with microtubule dissociation of Tau, and structural plasticity of neurons and neurodevelopment, in which Tau appears to play an important regulatory role [100, 102].

In neuroblastoma cells, PP2Ac phosphorylated on Tyr307 residues led to Tau phosphorylation at Ser199/202 + Thr205 (AT8), Thr212 + Ser214 (AT100) and Thr217, concurrent with detection of active Src [391]. The increases in pTau observed with PP2Ac-Tyr307 phosphorylation was abrogated with the SFK inhibitor PP2 [390]. Similarly, in rat brain and N2a cells, Src-dependent phosphorylation of Tyr307-PP2Ac was reported to cause Ser396/404, Ser214 and Thr205 phosphorylation of Tau [541]. Again, all of these studies relied on anti-pTyr307-PP2Ac antibodies so it is not clear whether Src-mediated Tau phosphorylation was actually mediated by Src-dependent tyrosine phosphorylation of PP2A. Nevertheless, our group has demonstrated that most of these phospho-epitopes of Tau are substrates of PP2A [258]. This is in addition to the dephosphorylation of Ser199 and Ser396/404, which we demonstrate to be impaired by Src^{CA} dependent phosphorylation of PP2Ac at one of three newly identified tyrosine residues targeted by Src; indeed, this phosphorylation does not occur in cells over-expressing a novel phosphorylation-resistant Y→F PP2Ac mutant (***Fig 6.8***). Interestingly, in another earlier study relying on the use of pan-pTyr antibodies, it was shown that stimulation of neuronal mGluR5 can induce the tyrosine phosphorylation of PP2Ac in a Src-dependent manner [389].

Together, these observations support our hypothesis that Src-mediated tyrosine phosphorylation of PP2Ac leads to enhanced phosphorylation of Tau. However, underlying mechanisms are likely to be complex, and the respective effects of individual or combined tyrosine phosphorylation site(s) on PP2A regulation remain to be clarified.

6.3.4 Inhibition of Src-family kinases disrupts PP2Ac-dependent neurite outgrowth

PP2A activity, Tau expression and phosphorylation, and Src activity are all important for extension of neurite-like processes in neurons [71, 236, 371, 545]. The remodelling of the cytoskeleton is essential for process formation. Dynamic regulation of microtubules and neurite outgrowth are major functions of Tau [70, 545, 558]. Importantly, proteins which regulate cytoskeletal dynamics are targeted by Src [366, 370, 371]. Since our findings indicate that Src regulates Tau Ser phosphorylation through PP2Ac tyrosine phosphorylation, and since PP2A, Tau and Src are key determinants of neuritogenesis, we hypothesised that Src-mediated phosphorylation of PP2Ac may also play a functional role in neurite outgrowth.

In agreement with earlier work from our lab [236], we have demonstrated that overexpression of $PP2Ac^{WT}$ is sufficient to induce the extension of neurites in N2a cells (***Fig. 6.9***). Using the SFK inhibitor, PP2, we observed a substantial impairment of neuritogenesis, in cells overexpressing $PP2Ac^{WT}$. This suggests that PP2A-directed neurite outgrowth is dependent on Src-family kinases. This likely occurs through phosphorylation at one or more of the novel tyrosine sites on PP2Ac which we report to be targeted by Src^{CA} in N2a cells. Critically, phosphorylation of Tau at CP13 and PHF-1 epitopes mediate dissociation of Tau from microtubules, allowing for neuronal structural plasticity [105, 108-110], i.e. the dynamic outgrowth of neurite-like processes. Whether or not the mechanism by which SFK mediates PP2A-dependent neurite outgrowth involves Tau phosphorylation events remains to be tested experimentally.

6.4 Concluding remarks

In this chapter, our overall goal was to re-evaluate and better delineate a role for the non-receptor tyrosine kinase Src in the regulation of PP2Ac. We show that Src indeed does phosphorylate PP2Ac in several neuronal and non-neuronal cell lines, in agreement with previous reports [173, 174]. However, in contrast to earlier studies, we demonstrate that *Src does not phosphorylate PP2Ac at Tyr307, but rather at multiple tyrosine residues.* This novel finding has functional significance for many fields within medical science, since PP2A is a central signalling molecule that becomes deregulated in various diseases besides AD, including cancer [162, 559]. Furthermore, we demonstrate that, by phosphorylating PP2A, Src can regulate the phosphorylation of Tau at Ser residues involved in the AD pathological

process. We also provide the first evidence that SFK is essential for PP2A-induced neurite outgrowth, suggesting a vital role of both of these enzymes in neuroplasticity.

In conclusion, we have identified a novel mode of regulation of PP2A, with direct implications for the regulation of neuronal homeostasis – including Tau, neuritogenesis and signalling (***Fig. 6.10***) – and the pathogenesis of AD.

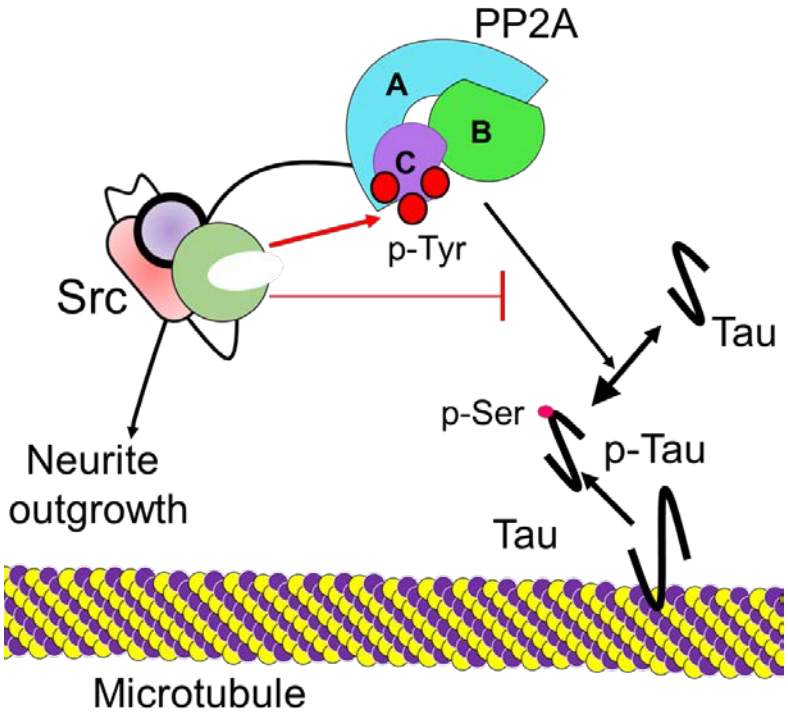

Figure 6.10 Schematic representation of Src-mediated regulation of PP2A by phosphorylation. The phosphorylation of PP2Ac at specific tyrosine residues results in the accumulation of Tau phosphorylated at Serine residues associated with pathological accumulation of Tau. Additionally, we have shown that PP2Ac-induced neurite outgrowth depends on Src activity.

Chapter 7: Discussion & Significance

7.1 Rationale for this study

Although the symptoms, staging and onset of AD have gradually been well-characterised over the past century, the full molecular course of the pathology is still unclear. The risk factors for AD are currently categorised behaviourally, medically, metabolically and nutritionally (refer to Chapter 1). The knowledge surrounding risk factors of AD is continuously becoming more refined. Furthermore, the molecular pathological hallmarks associated with brain atrophy have been described for some time now; Aβ plaque deposition, arising from aberrant processing of the Amyloid Precursor Protein, and neurofibrillary tangles, occurring as a result of intracellular paired helical filaments of the microtubule-associated protein Tau, are found in the AD brain. These hallmarks often manifest with a predictable and characteristic spread, implying a causative role in the disease process [1].

Some of the pathological events which contribute to the cognitive decline in AD have been characterised, or at least identified. Exposure of glutamatergic excitatory synapses to Aβ oligomers leads to hyper excitability of neurons, triggering intracellular signalling cascades which ultimately result in neurotoxicity [131, 153, 433]. This excitotoxic activation of neurons induced by Aβ promotes synapse loss, which functions as a precursor to much of the neuron death seen in the AD process [16, 560, 561], and appears to be mediated by deregulation of Tau [130, 153]. Notably, several risk factors for AD, especially elevated tHcy and low dietary folate are also capable of inducing neuron dysfunction through similar mechanisms. Elevated Hcy in particular, can cause aberrant activation of glutamate receptors in a comparable way to Aβ, resulting in excitotoxic cell death [521, 562], as well as enhanced Tau phosphorylation [263, 272]. Importantly, risk factors for AD like folate deficiency and increased tHcy are not only independent risk factors for the disease, but also synergise to cause further deficits such as cognitive impairment, damage to cerebral white matter and reduced hippocampal volume [217, 218, 518]. While AD is certainly a complex disease with many risk factors, what remains to be uncovered is how these risk factors precisely relate to the molecular pathology of this major neurodegenerative disorder.

One such link between risk factors for AD and molecular dysfunction is likely the family of enzymes known as PP2A. The formation of a key neuronal phosphatase, PP2A-ABαC, is dependent on methyl-group supply to cells [406, 423, 437]. As previously discussed at length throughout this thesis, methyl-group supply to cells (*i.e. one-carbon metabolism*) is dependent on dietary folate availability, and is impaired by elevated levels of Hcy [209, 525]. Thus, PP2A could contribute to the pathological processes observed in AD. Indeed, PP2A is the major Tau phosphatase in the brain [157], and helps to determine the affinity of Tau for microtubules through dephosphorylation of Tau in a site-specific manner [201, 395]. Furthermore, PP2A methylation and the expression of the heterotrimeric isoform of PP2A which dephosphorylates

Tau, PP2A-ABαC, is down-regulated in AD [158, 159, 563]. Importantly, these deficiencies in PP2A observed in AD directly correspond to Tau phosphorylation, Tau pathology and neurodegeneration. Furthermore, our group has previously connected disturbed one-carbon metabolism with demethylated PP2Ac, phosphorylated Tau and APP, and the formation of Aβ in cells and *in vivo* [165, 263], thereby linking a prominent risk factor for AD onset and the molecular regulation of Tau and the APP. While recognising that PP2A, as an important enzyme in the AD pathological process, is important in the study of the disease, a full understanding of PP2A regulation in neurons is yet to be achieved.

Current knowledge regarding the post-translational modifications of PP2A is incomplete. It is recognised that methylation of PP2A is mediated by LCMT-1 [168], and that PME-1 demethylates and binds to inactive PP2A [426]. Aside from these two enzymes, it is currently not known how else PP2A methylation and demethylation can be precisely modulated in response to extracellular stimuli. PP2A is also purportedly inactivated by phosphorylation at the Tyr307 residue [173, 174], which has been difficult to completely validate independently [386]. If tyrosine phosphorylation does indeed contribute to PP2A inactivation and Tau phosphorylation in AD pathology, as has been suggested [175], then it is important to verify the nature of this phosphorylation experimentally, using methods which are not dependent on poorly-validated antibodies directed against “pY307-PP2A”.

In this thesis, we aimed to delineate how dysregulation of PP2A contributes to the AD process, and indeed how PP2A is regulated in physiological conditions. We also wanted to verify if proteins affected in the disease process, APP and CREB, are affected by disturbed one-carbon metabolism (specifically genetic MTHFR and dietary folate deficiencies), since this is a demonstrated risk factor for AD, and dysregulates neuronal PP2A and Tau *in vivo*. Finally, we aimed to clarify the nature of PP2A-Tyr phosphorylation in a variety of cell types, and uncover how this affects the phosphorylation of Tau by PP2A.

7.2 Novel regulation of PP2Ac methylation

Since PP2A plays a central role as the major Tau Ser/Thr phosphatase in the brain, and the methylation of PP2A is impaired in the degenerative process of AD [157-159], investigating how this is regulated was our primary objective. Importantly, PP2A methylation modulates excitotoxic responses and sensitivity of hippocampal neurons to Aβ exposure [338]. This places PP2A methylation well within the suggested molecular pathways underlying AD pathology. Currently, understanding of PP2Ac methylation is limited, in that the proposed mechanisms follow a relatively straightforward sequence involving LCMT-1, PP2Ac, and demethylation by PME-1 (refer to Chapter 1 for more details). As with any methyltransferase,

LCMT-1 activity is determined by cellular SAM supply, which is dependent on dietary folate supply and subject to feedback inhibition by SAH [167, 403, 406].

Various data in non-neuronal cells indicates that cAMP signalling can lead to increased methylation or activity of PP2A. In both *Xenopus* oocytes and rodent ventricular myocytes, elevation of cAMP levels purportedly result in PP2A methylation [334, 564]. Similarly, in rodent kidney cells, activation of adenylyl cyclase is suggested to activate the phosphatase [333]. It is unclear in this particular study however, if cAMP led to increased PP2A activity due to modifications in PP2Ac methylation status, or by other mechanisms, including phosphorylation of regulatory subunits such as PP2A-B56δ [183]. Importantly, PKA, a kinase dependent on cAMP levels, can phosphorylate PP2A- B56δ, which causes elevated phosphatase activity towards certain substrates.

PKA is an important enzyme in the brain, as it targets many substrates essential for normal neuron functions. Some typical roles of PKA include promoting cell survival and synaptic plasticity in acute ways, which are dependent on post-translational modification of existing proteins such as glutamate receptor subunits [301, 315, 316]. Other functions of PKA involve long-term regulation of cellular elements, which involve gene transcription and *de novo* protein synthesis, via phosphorylation and activation of the transcription factor CREB [296, 318, 486]. Notably, CREB plays major functions in neurons, including the consolidation of hippocampal-based memory, and cellular survival [307, 317, 318, 415, 466]. PKA signalling, specifically PKA-dependent CREB activation, is impaired in AD [323]. A number of research groups have suggested potential therapeutic value for the treatment of AD in the PDE inhibitor Rolipram, since it is capable of eliminating Aβ-induced impairments in CREB activity and associated cognitive function *in vivo* [328-330]. Since CREB function and PP2A methylation are both impaired in AD [159, 323], we believed it was important to determine the effect of cAMP/PKA signalling on PP2A methylation and function. Hence, we hypothesised that, cAMP/PKA signalling could alter PP2Ac methylation, and that this cascade may be linked to CREB phosphorylation.

7.2.1 PKA activation causes PP2A demethylation in neuronal cells

We started by incubating N2a cells with pharmacological agents designed to alter PKA activity. Incubation of N2a cells with the cAMP-elevating drug Forskolin at a concentration of 50μM led to a time-dependent increase in the demethylation of PP2A, which was prevented by prior exposure of N2a cells with the PKA inhibitor, H89 (***Fig. 3.1***). Similarly, incubation of both N2a cells and sections of mouse cortex with Rolipram augmented the Forskolin-induced demethylation of PP2A (***Figs. 3.1, 3.2***). This is in contrast to previous results in other cell types suggesting that cAMP leads to PP2A methylation [334, 564]. Methylation of PP2Ac can

substantially determine the subcellular distribution of the enzyme [165], and PKA signalling is a tightly compartmentalised series of events [409]. This may partly explain why cAMP led to PP2A demethylation in our experiments, compared to that in other cell types, as the structure and function of neurons differ greatly from that of myocytes and oocytes.

Subsequently, we attempted to determine the spatial dynamics of PKA-induced demethylation of PP2Ac. In order to achieve this, we isolated nuclear and membrane fractions from N2a cells incubated with Forskolin or vehicle. We found that in vehicle-treated N2a cells, most PP2Ac is methylated, with methyl-PP2Ac being distributed in the cytoplasm, membrane and nuclear compartments (***Fig 3.4***). However, demethyl-PP2Ac was enriched in the nuclear compartment and least enriched in the membrane. These observations confirmed previous reports from our own group and others [165, 172].

Since previous reports from *Longin* and colleagues suggest that PME-1 localised demethyl-PP2Ac to the nucleus [172], we attempted to verify whether or not this was occurring in N2a cells incubated with Forskolin. We observed a significant increase in both demethyl-PP2Ac and PME-1 in the nucleus after a 12-minute incubation of N2a cells with Forskolin (***Fig. 3.4***), suggesting that PKA activation led to the demethylation of PP2Ac by PME-1, which may then chaperone the inactive phosphatase to translocate to the nucleus. We also incubated N2a cells with the PKA inhibitor compound KT58720, and the PKA-activating cAMP analogue 8-Br-cAMPs, to determine whether the effects we observed were PKA-dependent. Indeed, incubation of N2a cells with KT5720 prior to 15-minute incubation with Forskolin abrogated the effects on PP2A demethylation (***Fig. 3.4***). We observed a similar effect of 8-Br-cAMPs to Forskolin in N2a cells, suggesting that the mechanism underlying the nuclear enrichment of demethyl-PP2A enzymes was PKA-dependent.

We next considered the possibility that PME-1 could be a substrate of PKA. In this model, PKA-induced PME-1 Ser/Thr phosphorylation would activate PME-1, thereby mediating the demethylation of PP2A and the redistribution of both PME-1 and demethyl-PP2A enzymes to the nucleus. However, the data we obtained showed that PME-1 was not significantly phosphorylated following activation of cAMP/PKA signalling (***Fig. 3.7***), ruling out this model. These findings suggest that another mechanism underlies PKA-induced enrichment of demethylated PP2Ac in the nucleus. It will need to be interrogated in more details in future experiments.

We then aimed to determine if there was any alteration in the membrane distribution of PP2A. After isolating membrane-associated proteins from N2a cells, we observed a loss of total PP2Ac and PP2A-Bα from this subcellular compartment (***Fig. 3.5***). This suggests that Forskolin induced a loss of PP2A-ABαC holoenzymes from the membrane [165]. Similarly, we

observed an enrichment of both total PP2Ac and Bα in the nucleus under the same conditions (***Fig. 3.6***), suggesting a PKA-dependent mechanism of redistribution of PP2A- ABαC holoenzymes. Importantly, recycling of inactive PP2A enzymes is a methylation-sensitive process [169], and PME-1 is bound to inactive PP2Ac [172, 426]. We were unable to determine whether or not PP2A-Bα could be associated with such complexes. Thus, our results suggest that PKA-induced demethylation and inactivation of PP2A would facilitate phosphorylation of PKA substrates, and thereby prevent immediate dephosphorylation by PP2A. These two enzymes have multiple shared substrates which are important in neuron function, including those involved in signal transduction. One such target includes the transcription factor CREB, which, as described above is central to processes of learning and memory and is impaired in AD.

7.2.2 PKA-induced PP2A demethylation affects the phosphorylation of CREB and Tau

Importantly, previous reports suggesting a role for PP2A-mediated CREB dephosphorylation and inactivation do not specify the subunit composition or methylation state of PP2A. To assist in our characterisation of a possible regulation of CREB by PP2A, we overexpressed wild-type PP2Ac and the methylation-incompetent mutant $PP2Ac^{L309\Delta}$. This allowed us to interrogate a precise role of PP2A demethylation at L309 in relation to CREB phosphorylation in Forskolin or vehicle-treated N2a cells. We observed a significant decrease in phosphorylated CREB in $PP2Ac^{WT}$-transfected N2a cells incubated with vehicle (***Fig. 3.8***). In contrast, $PP2Ac^{L309\Delta}$-transfected cells, showed no observable change in pCREB (***Fig. 3.8***), compared to EV-transfected cells.

When N2a cells were incubated with Forskolin, CREB phosphorylation was induced after 5 minutes and sustained for at least 15 minutes (***Fig. 3.8***). This was the period of time where there was maximal demethylation of PP2Ac. Interestingly, we did not observe any significant effect on pCREB in either $PP2Ac^{WT}$- or $PP2A^{L309\Delta}$- transfected N2a cells that were incubated with Forskolin (***Fig. 3.8***). Together, these data suggest that, as previously reported, CREB is phosphorylated by PKA and leads to activation and gene transcription [302], while we provide evidence that only methylated PP2A can dephosphorylate CREB. Furthermore, PKA-induced PP2A demethylation or ectopic expression of $PP2A^{L309\Delta}$ led to diminished CREB dephosphorylation. Our results also suggest that PKA-dependent PP2A demethylation and redistribution are dependent on PME-1, since we also observed that pharmacological inhibition of PME-1 with AMZ-30 led to CREB dephosphorylation (***Fig. 3.9***), independently of direct PKA activation.

Another important mutual substrate of PP2A and PKA in neurons is Tau. Since Tau has important functions in neuronal homeostasis and plasticity, and Tau dysregulation is linked to

AD, we wanted to evaluate the role of PKA-induced PP2A demethylation in Tau phosphorylation. PP2A is the phosphatase responsible for the major proportion of Tau dephosphorylation [157], and PP2A-ABαC is the species which most effectively mediates this dephosphorylation and the microtubule affinity of Tau [201, 395]. PP2Ac methylation and Bα expression are impaired in AD, and concomitantly occur with phospho-Tau pathology [158, 159]. Similarly, PKA-mediated phosphorylation of Tau at Ser214 also regulates the affinity of Tau for microtubules and interactions with other binding partners of Tau, including kinases such as GSK-3β [88, 89, 325, 565]. The AD-related phosphoepitope of Tau, PHF-1 is targeted by PP2A for dephosphorylation, while PHF-1 levels are enhanced by PKA activity, mediated via GSK-3β [83, 88, 403]. Phosphorylation at the PHF-1 epitope has been associated with late-stage phospho-Tau pathology, microtubule dissociation of Tau, reduced association of Tau with the plasma membrane and long-term depression of synaptic plasticity [108, 110, 113, 123]. Thus, we decided to interrogate the effects of PKA-induced changes in PP2A methylation in relation to these Tau phospho-epitopes, as they appear to be closely linked to both PP2A methylation and PKA activity.

Previous data from our lab have demonstrated that in unstimulated N2a cells, $PP2A^{L309\Delta}$ expression leads to heightened cellular PP2A demethylation and a concomitant increase in phosphorylation at PHF-1 and at the PKA-targeted pSer214 site [258]. Therefore, we assessed the effect of Forskolin incubation in tandem with $PP2Ac^{WT}$ and $PP2Ac^{L309\Delta}$ expression on Tau phosphorylation. In $PP2Ac^{WT}$-overexpressing cells, pSer214-Tau was significantly decreased, compared to EV-transfected cells (***Fig. 3.10***). This suggests that while PKA can demethylate endogenous pools of PP2A, the presence of additional, ectopic $PP2Ac^{WT}$ is sufficient to overcome this effect by enhancing total cellular PP2A Tau phosphatase activity. There was no difference in pSer214-Tau levels in $PP2Ac^{L309\Delta}$-transfected cells, compared to EV-transfected cells (***Fig. 3.10***), suggesting that PKA activation induced maximal phosphorylation of Tau, while $PP2Ac^{L309\Delta}$ failed to dephosphorylate pSer214-Tau.

Other groups have reported that incubation with Forskolin enhances PHF-1 phosphorylation *in vivo* [445]. Since Forskolin induced PP2A demethylation, we concluded that PHF-1 was another site that we should investigate in terms of the PKA-PP2A interaction. We found that in EV-transfected cells, Forskolin did indeed boost PHF-1 signal detected by Western blot (***Fig. 3.10***). Surprisingly, we also observed that PHF-1 phosphorylation was augmented by $PP2Ac^{WT}$ expression. The effects of PKA-mediated PP2A demethylation on pCREB and pTau are outlined in ***Table 7.1***. Unlike Ser214, the PHF-1 epitope is primarily targeted, by GSK-3β, rather than PKA [116]; moreover, PKA-mediated phosphorylation of Tau enhances targeting of Tau by GSK-3β [88]. Critically, PP2A has been reported to activate GSK-3β by

dephosphorylation of the inhibitory Ser9 site [422, 449]. Thus, we propose that in $PP2Ac^{WT}$-tranfected cells, a combination of Tau phosphorylation by PKA at Ser214 and the increased presence of PP2A synergise to enhance the activity of GSK-3β towards Tau. This conclusion is consistent with previous reports linking PP2A and GSK-3β-dependent Tau phosphorylation [422, 448, 513].

	pCREB Vehicle	pCREB Forskolin	pSer214 Vehicle	pSer214 Forskolin	PHF-1 Vehicle	PHF-1 Forskolin
$PP2Ac^{WT}$ VS. EV	↓	—	Not detectable	↓	↓	↑
$PP2Ac^{L309\Delta}$ VS. EV	—	—	Not detectable	—	—	—

Table 7.1 Effects of PP2Ac expression and Forskolin on CREB and Tau phosphorylation. Summarised results from chapter 3, indicating significant changes in phosphorylation of CREB or Tau. "↑" represents a significant increase, "↓" represents a significant decrease and "—" represents no significant change, compared to empty-vector (EV) transfected cells.

We report in this thesis that the PKA activity induces demethylation of PP2Ac at L309, and $PP2Ac^{WT}$ overexpression in N2a cells leads to significant changes in the phosphorylation of CREB and Tau, compared to EV-transfected cells, where $PP2Ac^{L309\Delta}$ expression does not. These data suggest that dynamic methylation/demethylation reactions are necessary in the balance of Tau phosphorylation and CREB phosphorylation/activity in neurons. The deletion of the C-terminal Leucine 309 residue of PP2Ac ($PP2Ac^{L309\Delta}$) also disturbs the binding of several regulatory subunits, most notably the PP2A-Bα subunit [451]; in turn, decreased association of Bα with the core enzyme inhibits the binding of PP2A to both Tau and microtubules. Thus, the dynamics of PP2A-CREB regulatory interactions and dephosphorylation likely also depend on PP2A-Bα, which is supported by its translocation to the nucleus following PKA activation with Forskolin (***Fig. 3.6***).

7.2.3 PP2A mediates PKA-initiated neurite outgrowth

Importantly, Tau and CREB are both involved in neurite outgrowth through the regulation of microtubule dynamics and transcription of various components of the neuronal architecture, respectively [70, 102, 291, 412, 544, 566]. We have demonstrated that both PKA and PP2A are responsible for the phosphorylation and dephosphorylation of Tau and CREB. Therefore, we turned our focus to how PKA signalling and PP2A methylation interact through the

assessment of neurite outgrowth. In order to achieve this, we induced the differentiation of N2a cells stably overexpressing $PP2Ac^{WT}$ and $PP2Ac^{L309\Delta}$ by incubating them with Forskolin. Previous results from our group and others indicate that PP2A methylation is an essential component in neurite outgrowth [236, 552]. We were able to successfully confirm these findings by showing that expression of $PP2Ac^{WT}$ significantly enhanced neurite outgrowth in both vehicle and Forskolin-treated N2a cells (***Fig. 3.11***). In contrast, $PP2Ac^{L309\Delta}$-expressing N2a cells had a severely impaired capacity to initiate neurite extension, despite the presence of Forskolin.

Linking PP2A methylation with PKA activity in neurite outgrowth has several functional consequences in the understanding of neuron differentiation. Activation of PKA by cAMP has been centrally tied to the initiation of neuronal differentiation [288], with mechanisms including inhibition of the GTPase RhoA via phosphorylation [567], thereby interfering with changes in actin dynamics that are responsible for neurite retraction. CREB is also involved in differentiation, in response to changes in neuronal cell adhesion molecules [290]. Thus, cAMP/PKA signalling serves to maintain the integrity of the actin cytoskeleton. Interestingly, the dephosphorylation of the actin-binding protein cofilin promotes the disassembly of F-actin: in non-neuronal models PP2A mediates alterations in the actin cytoskeleton through its ability to dephosphorylate cofilin [568-570]. Therefore, since RhoA is directly upstream of cofilin in the regulation of F-actin formation, PKA-mediated PP2A demethylation may serve as both a direct and an indirect controlling factor in actin dynamics.

Tau has previously been recognised as a microtubule binding protein with important roles in neuronal differentiation [571, 572]. The phosphorylation of Tau at Ser214 has been shown to affect its microtubule binding activity [87, 565], while PHF-1 phosphorylation affects Tau localisation to the plasma membrane and its ability to bind organelles while associated with the microtubule network [113, 573]. Our group has also demonstrated in previous work that PP2Ac methylation is critical for neurite outgrowth [236]. Here, we observed differential changes in the patterns of Tau phosphorylation with PP2Ac methylation competency and Forskolin treatment. Perhaps the effect of Tau phosphorylation here may underlie the impaired neurite outgrowth induced by $PP2Ac^{L309\Delta}$. Future investigations are needed to fully delineate the regulatory relationship between PKA and PP2A, and the contribution of Tau phosphorylation in neurite outgrowth.

7.2.4 Concluding remarks: PP2A and PKA – competitors or collaborators?

The evidence presented in this thesis linking PP2A and PKA signalling directly affects our understanding of the molecular pathology of AD. PP2Ac methylation is impaired in AD-affected brain regions, concomitantly with increased Tau phosphorylation [159]. PKA-mediated Tau

phosphorylation is associated with degeneration of vulnerable neurons in aged primates [331], and PKA-phosphorylated CREB is impaired in AD [323]. On the surface, it would appear as though PKA and PP2A compete for phosphorylation and dephosphorylation of their shared substrates – notable examples include CREB [335, 337], Tau [258, 331], voltage-gated calcium channels [195, 196] and glutamate receptor subunits [433]. Our current results show that PKA-mediated demethylation of PP2Ac facilitates phosphorylation of Tau and CREB in N2a cells, and therefore potentially holds important functions in the brain which mediate AD-related cellular dysfunction. This indicates that PKA directs the regulation of PP2A methylation in neurons, thereby controlling PP2A function to this extent. Notwithstanding the potential validity of this conclusion in light of current knowledge regarding the function of these enzymes in neurons, we suggest that perhaps, instead, PKA demethylates PP2A to provide a cohesive intracellular cell signalling cascade that regulates CREB, Tau and Neurite outgrowth. We look to future experiments to test this emergent hypothesis.

In that respect, our group has recently demonstrated that impaired methylation of PP2Ac by genetic MTHFR and dietary folate deficiencies lead to elevated Tau phosphorylation in a number of regions [263]. Since we have presented evidence linking cAMP/PKA signalling with PP2A methylation and Tau phosphorylation, we then hypothesised that the regulation of CREB may also be aberrantly affected in these animals. This provided the basis for Chapter 4 of this thesis.

7.3 Disturbed one-carbon metabolism and the regulation of CREB and APP

7.3.1 Methyl-group supply and CREB phosphorylation in N2a cells

One of our key observations from Chapter 3 is that PP2Ac methylation is important for the dephosphorylation of CREB. Our group has also recently observed impaired methylation of PP2Ac and increased Tau phosphorylation in the brains of MTHFR- and folate-deficient mice [263]. These animals show increased levels of circulating and neural Hcy, impaired learning and memory [267, 339, 487] and hippocampal atrophy [235]. Importantly, elevated tHcy and low plasma folate are risk factors for the onset of AD [213, 217, 218]. CREB activity is also impaired in AD [323, 418], a disease characterised by memory loss and cell death, which are both affected by CREB function. Therefore, we hypothesised that CREB function may be affected by *in vivo* disturbances of one-carbon metabolism such as MTHFR and folate deficiencies. As previously reported, PP2A regulates CREB phosphorylation [335, 337], which likely occurs in a methylation-dependent manner (Chapter 3). Thus, this provided evidence-based grounding for our hypothesis that MTHFR and folate deficiencies may affect CREB function.

First, we conducted experiments in N2a cells to verify any possible effect on CREB phosphorylation following alterations in one-carbon metabolism. Supplementation of culture media with the methyl donor SAM enhanced CREB phosphorylation (***Fig. 4.3***). Conversely, incubation with the SAH-hydrolase inhibitor 3DA, which generates a lowered SAM: SAH ratio and impaired methylation potential in cells led to significantly impaired CREB phosphorylation. Furthermore, restriction of folate in cell culture medium also impaired CREB phosphorylation (***Fig. 4.5***). These results were unexpected, since folate deficiency induces PP2Ac demethylation [258, 403], while PP2A demethylation enhanced pCREB (Chapter 3). These findings suggest that there are also PP2A-independent mechanisms underlying SAM-mediated CREB phosphorylation. Indeed, others have reported in rat brain, that the supply of SAM can enhance cAMP binding to PKA regulatory subunits, thus facilitating enzymatic activation of PKA [480]. Both folate deprivation and 3DA-mediated SAH-hydrolase inhibition restrict cellular SAM and methyl-group supply, which would contribute to impaired CREB phosphorylation.

7.3.2 In vivo disturbances of one-carbon metabolism can alter CREB regulation

Since we observed a significant effect on CREB phosphorylation following alterations in one-carbon metabolism in our cultured cells, we nextaimed to determine whether genetic-induced MTHFR deficiency or dietary folate deficiency in young and old mice have a synergistic effect on the regulation of CREB. In the cortex of young heterozyogous (HET) or homozygous (NULL) *Mthfr* mice, we observed no change in CREB phophorylation, but a significant increase in CREB expression, compared to wild-type animals (***Fig. 4.5***). Conversely, CREB phosphorylation was significantly increased in the midbrain of HET mice (***Fig. 4.6***), emphasising the diversity in responses of brain regions to perturbed MTHFR activity. Furthermore, this increase in CREB phosphorylation supports a model in which methylated PP2Ac dephosphorylates CREB. Importantly, our group has previously demonstrated that these same animals have significantly impaired PP2Ac methylation in the midbrain [263]. While CREB phosphorylation is typically associated with CREB activity [282, 335], it is important to note that the significantly impaired expression of CREB may translate to a net reduction in overall CREB-dependent transcriptional activity. These results suggest that in young animals with moderate and severe MTHFR deficiency, CREB may be deregulated through both altered expression and phosphorylation levels.

AD risk is increased with advanced age. CREB is not only dysregulated in humans with AD [323], but also in mouse models of accelerated aging [455]. However, MTHFR deficient mice do not age well, experiencing a higher rate of premature mortality [235, 510]. Therefore, to investigate severe disturbances in one-carbon metabolism in aged animals, we chose to utilise dietary interventions through the restriction of folate. Low-folate diets typically result in

elevated tHcy and reduced cellular methylation potential [209], in a manner similar to that induced by MTHFR deficiency. Therefore, we fed 16-month old wild-type mice either a NF or LF diet and compared the effects on the expression and phosphorylation of CREB. We observed a significant increase in CREB phosphorylation, and a significant reduction in CREB expression in the midbrain of aged mice fed the LF diet (***Fig. 4.8***). This is consistent with our observations in young MTHFR deficient animals, suggesting a possible shared mechanism. These observations were also consistent with those suggesting that MTHFR [487, 574] and folate [249, 475, 487, 575] deficiencies can lead to memory deficits and impaired cellular survival, especially in midbrain regions. Our present data indicate that impaired cellular survival and memory formation arising as consequence of impaired one-carbon metabolism may be due to disturbed CREB function. It this will be interesting in the future to assess the precise mechanisms underlying CREB impairment in the midbrain following disturbed one-carbon metabolism. Future studies will also ideally investigate a potential role for PP2A, since these mice have impaired PP2Ac methylation [263], which is important in the regulation of CREB in N2a cells (Chapter 3).

7.3.3 APP phosphorylation and expression are regulated by one-carbon metabolism in vivo

Elevated tHcy resulting from imbalances in one-carbon metabolism [6, 515] have been associated with an increased risk for AD. The *Mthfr*-C677T polymorphism, the most common inborn cause of HHcy [491], also increases the risk for AD [215, 460], as do low B-vitamin / folate diets [222, 459]. The formation of characteristic Aβ plaques in the disease has been linked with enhanced β- and γ-secretase mediated proteolytic processing of APP, which is also promoted by occur phosphorylation of APP at Thr668 [51, 52]. Critically, folate and vitamin B12 supplementation mitigate APP phosphorylation and γ-secretase induced Aβ formation *in vivo* [508].

Thus, there may be a link between aberrant one-carbon metabolism and the molecular processes underlying AD pathogenesis. We were especially interested in assessing whether changes in folate availability- either via genetic or dietary interventions- could affect APP phosphorylation and expression. To that end, we used the same *Mthfr*-deficient mouse model described in Chapter 4. Briefly, we analysed homogenates taken from the cortex, hippocampus, midbrain and cerebellum of WT, HET or NULL mice at 6 weeks or 22-months of age. Young mice were fed a NF diet only; 16-month-old mice were fed for 6 months either a NF or LF diet, prior to sacrifice.

We found that genetically induced MTHFR deficiency significantly affected the expression and phosphorylation of APP. MTHFR deficiency induced an increase in pAPP in brain regions which are quickest to degenerate in models of AD, namely the hippocampus and the cortex

(***Fig. 5.1,*** [523, 576]). An age-dependent increase in pAPP may have also masked this effect in the cortex and midbrain of WT mice fed a LF diet, compared to WT mice on a NF diet. This may provide some explanation as to why we saw little to no effect of genotype in these regions (***Fig. 5.2***). Our results support previous observations that suggest that the hippocampus is particularly sensitive to disturbances in one-carbon metabolism [235, 263], as there was a significant increase in APP phosphorylation at Thr668, which accumulates in AD and in transgenic models of AD [52].

In addition to altered APP phosphorylation, we also observed changes in the protein expression levels of APP. Interestingly, these changes varied by region. In the cortex and midbrain of young NULL mice, we observed a significantly reduced APP expression in tandem with increased APP phosphorylation, compared to wild-type animals. This was in contrast to the hippocampus, where we saw significant increases in both APP expression and phosphorylation compared to wild-type mice. These changes were generally similar in aged WT mice on a LF diet or in HET mice; they are summarised in ***Table 7.2***. The differences in the expression levels of APP between the cortex/midbrain and the hippocampus may indicate that APP may be differentially regulated between these regions.

	Young HET		Young NULL		Old, WT LFD		Old, HET NFD		Old, HET LFD	
	pAPP	**tAPP**	**pAPP**	**tAPP**	**pAPP**	**tAPP**	**pAPP**	**tAPP**	**pAPP**	**tAPP**
Cortex	↑	—	↑	↓	—	—	↑	—	↑	↓
Hippocampus	↑	—	↑	↑	—	↑	↑	↑	↑	↑
Midbrain	↑	↓	↑	↓	—	↓	—	↓	—	↓
Cerebellum	—	—	—	—	—	—	—	—	—	—

Table 7.2 Changes in APP regulation as a consequence of disturbed one-carbon metabolism. Summarised results from chapter 5, indicating significant changes in total APP expression (tAPP) or phosphorylation at Thr668 (pAPP) in Young HET/NULL mice compared to wild-type/aged mice fed a low-folate diet (LFD) or a normal-folate containing diet (NFD). "↑" represents significant increases, "↓" represents significant decreases and "—" represents no significant change.

The changes in pAPP we observed in our mice are likely mediated by altered PP2A methylation. Indeed, our group has previously demonstrated that *in vitro* (N2a cells), and *in vivo* (CBS deficient mice), impaired one-carbon metabolism and reduced cellular methylation potential result in diminished PP2A methylation and concomitantly increased pTau and pThr668-APP levels. Moreover, the accumulation of demethylated PP2A – induced either by the downregulation of LCMT-1 or overexpression of PME-1 – is sufficient to promote APP

phosphorylation at Thr668 in N2a cells [258] and *in vivo* [338]. In the same *Mthfr* animals analysed here for pAPP, there is also impaired PP2Ac methylation and a parallel increase in Tau phosphorylation [263], suggesting a causal link between impaired PP2A methylation and enhanced phosphorylation of Tau and APP. Notably, these alterations mirror the molecular pathology of AD.

Furthermore, we have identified a kinase that likely contributes to the increased phosphorylation of Tau and APP in the context of disturbed one-carbon metabolism, Glycogen Synthase Kinase 3β. We found significantly reduced inhibitory phosphorylation of GSK-3β in tandem with increased Tau and APP phosphorylation, and impaired PP2A methylation (***Fig. 5.4***). GSK-3β, a major Tau kinase [103, 116], has been shown by others to phosphorylate APP [577]. It becomes activated following neuronal exposure to Aβ, leading to Tau phosphorylation [54].

In conclusion, our findings provide further mechanistic insights and evidence for a strong link between altered folate metabolism, deregulation of key signalling molecules (i.e. PP2A and GSK-3β), and the deregulation of two major hallmarks of AD, Tau and APP. Our experimental data strongly support a role genetic- or diet- induced for impairment of one-carbon metabolism as a risk factor for AD.

7.3.4 Potential links between PP2A, CREB and APP

Impairments in both PP2A methylation and CREB phosphorylation have been observed in AD [158, 159, 323]. Increased APP phosphorylation at Thr668 has also been found to be associated with AD brain lesions [51]. Activation of PKA signaling, including the activation of CREB, has been reported to ameliorate learning and memory defecits in rodent models of AD [328, 329]. We have demonstrated that PP2A methylation state can alter the phosphorylation of Tau and CREB, depending on PKA activity (Chapter 3). In Chapters 4 and 5, we also demonstrated that modulating methyl group supply *in vitro* and *in vivo* alter how CREB and APP are expressed and phosphorylated. We suggest that the changes in APP and CREB are likely related to the concomitant dowregulation of PP2Ac methylation [258, 263] occuring in these models (***Fig 7.1***), with significant implications for AD pathological processes.

The progression of AD is characterised by a typical pattern of synapse, neuron and grey matter loss, concomitantly with depositions of aggregated pTau and Aβ [1]. Interestingly, the MTHFR-deficient mouse model that we used is characterised by a loss of hippocampal volume, as well as learning and memory defecits [235]. CREB is essential for the molecular processes underlying learning and memory [466], as well as neuronal survival [318]; it becomes functionally impaired pathological concentrations of Aβ *in vivo* [418]. Additionally, our group has previously demonstrated that neuronal vulnerability to excitotoxic stimulation by Aβ is

dependent on PP2A methylation [338]. Here, we propose that the the downregulation of PP2Ac methylation links Aβ formation and dysregulation of CREB through aberrant phosphorylation of APP at Thr668, and that impaired PP2A methylation and APP phosphorylation act synergistically to affect CREB function in AD. In addition to the role played by enhanced PP2A demethylation in the aberrant phosphorylation of Tau in AD [158, 159]. Our findings thus provide new potential mechanisms of AD pathogenesis.

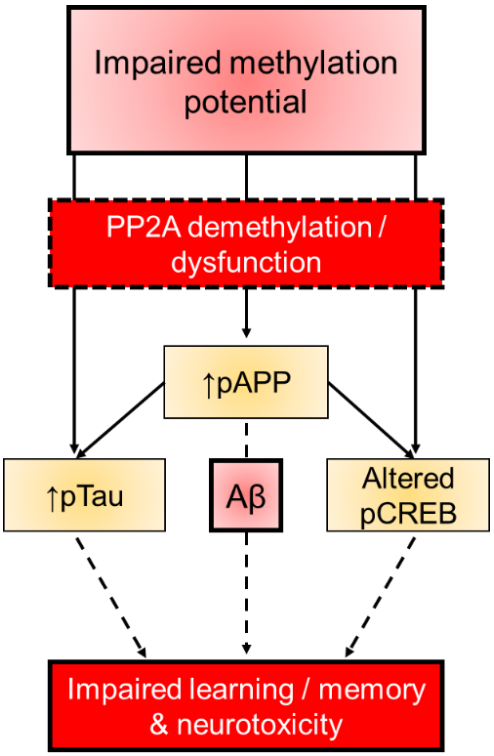

Figure 7.1 Disturbed one-carbon metabolism induces changes in Tau, APP and CREB via PP2Ac demethylation. In Chapters 4 and 5 of this thesis, we used *in vitro* and *in vivo* models of disturbed one-carbon metabolism which lead to impaired cellular methylation potential. Previous observations by our lab implicate demethyl-PP2Ac-mediated increases in pTau *in vivo*. Here we provide evidence that similar perturbations lead to disturbances in the regulation of APP and CREB. These can subsequently lead to neurotoxicity and impairments in learning and memory typically observed in AD and in models of AD.

7.4 Novel insights into PP2Ac tyrosine phosphorylation

In addition to methylation, we also aimed to better characterise the tyrosine phosphorylation of PP2Ac. The tyrosine phosphorylation of PP2Ac was first reported *in vitro* and later shown to occur in vSrc-transformed fibroblasts and following activation of growth factor signalling [173, 174]. Src-mediated phosphorylation – and reported "inactivation" – of PP2Ac has since been widely reported in a variety of cell types [387, 398, 542, 543]. In chapter 6, our primary aim was to characterise the mechanism of PP2Ac regulation by tyrosine phosphorylation induced by Src, with a secondary goal to investigate how Src, via PP2A, could affect Tau in neuronal cells.

Src activity in neurons can regulate Tau in several ways. Tau can bind to Src at the SH3 domains, inducing conformational changes in Src, thereby modulating its kinase activity [356]. Tau and Src associate in a phosphorylation-dependent manner; AD-like phosphorylation of Tau or phospho-mimicking, glutamate mutations on Tau at one or more sites in proline-rich regions impair this SH3 interaction [111, 114]. The other mechanism of Src-mediated Tau regulation may occur through the tyrosine phosphorylation of PP2Ac, which could then induce the accumulation of Ser/Thr phosphorylated Tau.

7.4.1 Src phosphorylates PP2Ac at multiple, novel tyrosine residues

Initial work on the Src-induced tyrosine phosphorylation of PP2Ac in fibroblasts suggested that it specifically occurred only on the Tyr307 residue [173, 174]. However, these conclusions have not been validated by mass spectrometry. To further investigate this important regulatory process, we used the PTP inhibitor, PV, and expression of a constitutively active Src mutant (Y529F, Src^{CA}) to promote PP2Ac tyrosine phosphorylation in N2a and COS-7 cells. We confirmed that both PV and Src^{CA} induced the phosphorylation of PP2A in these cells by Western blot analysis of HA-$PP2Ac^{WT}$ immunoprecipitates with pan-pY antibodies (***Fig 6.2***). Not only could we induce PP2Ac tyrosine phosphorylation in these conditions, but PV treatment of Src^{CA} expressing cells led to a synergistic increase in pY immunoreactivity.

In collaboration with Dr. Brian Wadzinski (*Vanderbilt University*), we generated three unique Y → F point mutations on PP2Ac at three potential PP2Ac tyrosine phosphorylation sites (denoted by HA-$PP2Ac^{\#1\ /\ \#2\ /\ \#3}$). We verified the expression of these mutants by Western blotting (***Fig. 6.3***). All three mutants showed decreased phosphorylation compared to $PP2Ac^{WT}$ upon Western blot analysis (***Fig. 6.4***). The greatest effect was observed in $PP2Ac^{\#3}$, while the most modest was seen in $PP2Ac^{\#1}$. Compared to $PP2Ac^{WT}$ controls, $PP2Ac^{\#3}$ induced a ~5-fold decrease in tyrosine phosphorylation in HA-immunoprecipitates when co-expressed with Src^{CA}, while this mutant abrogated most of the tyrosine phosphorylation induced by PV (***Fig. 6.4***). These data supported our hypothesis that PP2Ac is phosphorylated at multiple sites by Src, and that Tyr307 is not a sole site of PP2Ac phosphorylation in cells.

These findings suggest a direct kinase-substrate relationship between Src and PP2Ac. However, we cannot exclude the possibility that other tyrosine kinases could also target PP2A for phosphorylation at these tyrosine sites, as well as potentially other tyrosine residues which were not investigated here.

7.4.2 Implications of PP2Ac Tyrosine phosphorylation: pTau and neurite outgrowth

PP2Ac tyrosine phosphorylation has been reported to occur in tandem with neurofibrillary tangle deposition in AD [175]. Our second aim in Chapter 6 was to determine the effect of Src-dependent phosphorylation of PP2Ac using Tau as a validated substrate of PP2A in N2a cells. Tau represents a particularly attractive substrate of PP2A to investigate in this respect, given the central role of the functional PP2A-Tau relationship in neurons and its disruption in AD. We hypothesised that Src-induced PP2A phosphorylation results in accumulation of Ser/Thr-phosphorylated Tau, since Src-mediated PP2A phosphorylation has been reported to inactivate PP2A [174].

We investigated the effects of Src^{CA}-dependent PP2A phosphorylation on Tau at two unique phosphoepitopes. These included CP13, which has been identified to play a role in Tau pathology in AD, before NFTs start to form, and PHF-1, which is a site that becomes phosphorylated as a precursory event to PHF formation and NFT development [108]. When we co-expressed Src^{CA} or empty vector (EV; as a control), with $PP2Ac^{WT}$ or $PP2Ac^{\#3}$ in N2a cells, we observed different patterns of Tau phosphorylation. Co-transfection of N2a cells with Src^{CA} in tandem with $PP2Ac^{WT}$ led to significant increases in Tau phosphorylation at CP13 and PHF-1, compared to N2a cells transfected with $PP2Ac^{WT}$ and EV (***Fig. 6.8***). However, in N2a cells transfected with Src^{CA} and HA-$PP2Ac^{\#3}$, we did not observe any change in Tau phosphorylation at these pSer-epitopes (***Fig. 6.8***). These results demonstrate that Src-mediated phosphorylation of PP2Ac induces Tau Ser/Thr phosphorylation at AD-related epitopes. However, underlying molecular mechanisms remain to be elucidated, since our group and collaborators were unable to confirm that Src-mediated PP2A tyrosine phosphorylation indeed inhibits PP2A catalytic activity *in vitro*, as initially published [173, 174]. Moreover, it is possible that distinct tyrosine phosphorylation sites could induce differential effects on PP2A activity, protein-protein interactions, distribution or scaffolding functions, all of which may converge to promote Tau phosphorylation. Other results in different models also suggest that one kinase responsible for the increase in Src-mediated Tau phosphorylation may be Erk [387]. This remains to be tested in our model.

Since Src, PP2A and Tau are all involved in the differentiation of neurons, we hypothesised that Src can also affect PP2A-dependent neurite outgrowth. To test this hypothesis, we grew N2a cells stably overexpressing $PP2Ac^{WT}$. These differentiate significantly more than wild-

type N2a cells (chapter 3, [236]). We then treated N2a-PP2Ac^{WT} cells with the SFK inhibitor, PP2, or vehicle. PP2 treatment entirely abrogated PP2Ac^{WT}-induced neurite outgrowth in N2a cells (***Fig. 6.9***). While PP2Ac function is essential in neurite outgrowth, our results suggest that PP2Ac mediates SFK -dependent neurite outgrowth in N2a cells.

Src is highly concentrated in growth cones [366], assisting in axon guidance [367]. Overexpression of constitutively active Src in *Aplysia* models leads to cortactin association, resulting in increased density, stability and elongation of filopodia [355]. Conversely, Src and Fyn-deficient mice are deficient in the phosphorylation of Rho, a second-messenger which plays essential roles in neuronal morphology [370]. These animals also show deficits in axon guidance and outgrowth, indicating that SFKs play absolutely essential roles in neuronal morphology and differentiation. Similarly, Src phosphorylates p190-RhoGAP *in vitro*, indicating that this interaction is direct [369]. As discussed along with our previous observations regarding cAMP/PKA signalling in neuritogenesis, Rho signalling facilitates F-actin stabilisation in neurons. Meanwhile, PP2A activates cofilin, leading to actin remodelling; it has extensive roles in regulating cytoskeletal dynamics during neurite outgrowth (reviewed in [73]). Thus, a likely interplay in this system lies in the interactions between PP2A, Src and Tau. The precise pathways underlying SFK/PP2A dependent neuritogenesis remain to be elucidated.

7.5 Conclusions and perspectives:

In this thesis, we have provided evidence underlining the importance of PP2Ac post-translational modifications in normal neuronal function and in AD, as illustrated in ***Fig. 7.2***.

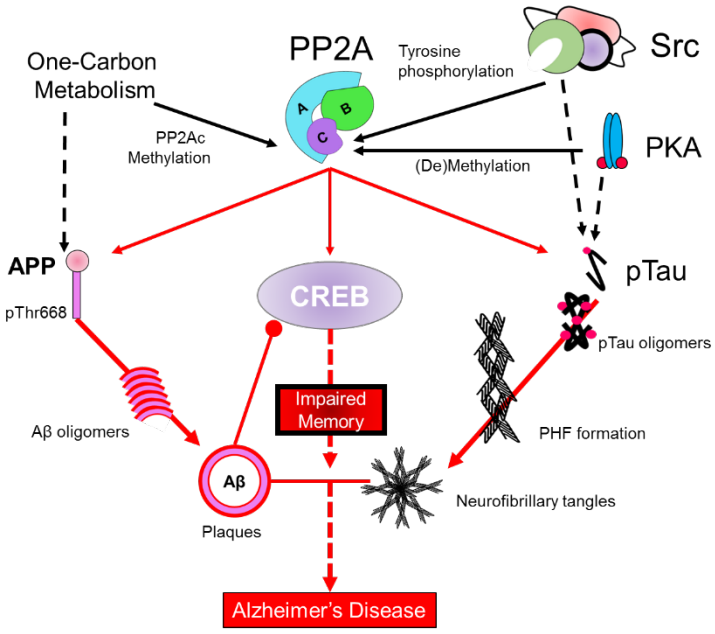

Figure 7.2 Involvement of PP2A in normal neuronal homeostasis and in AD pathogenesis. In this thesis, we have provided evidence further implicating dysregulation of PP2A in AD in novel ways. We have shown that PP2Ac demethylation can be induced by PKA, and that Src mediates novel tyrosine phosphorylation of PP2A. We present evidence indicating that disturbed one-carbon metabolism *in vivo* alters the phosphorylation of APP, which has been shown to promote Aβ formation, through a mechanism involving PP2A and GSK-3β. We have also observed one-carbon metabolism-dependent changes in the regulation of CREB in N2a cells and *in vivo*, which provides some molecular explanation for the link between impaired one-carbon metabolism and deficits in learning and memory.

Firstly, we demonstrate a role for the activation of PKA in the regulation of PP2Ac demethylation and distribution in neuronal cells, which in turn directly affects the phosphorylation and function of Tau and CREB, modulating neurite outgrowth (*Chapter 3*). We also built on earlier observations by showing that one-carbon metabolism not only regulates the methylation and Tau phosphatase activity of PP2A, but can also modulate the expression and/or phosphorylation of CREB and APP in both N2a cells and *in vivo* (*Chapters 4 and 5*). We also demonstrate *in vivo* that genetic- or diet- induced alterations in folate status can promote the dysregulation GSK-3β, which has a well-documented role in the phosphorylation of Tau and neuron dysfunction in AD. Finally, we provide novel insights in the regulation of PP2Ac by tyrosine phosphorylation induced by Src. We demonstrate that Src-dependent PP2A tyrosine phosphorylation leads to Tau phosphorylation at the AD-related

CP13 and PHF-1 sites. Additionally, we demonstrate a direct link between Src and PP2A in modulating neurite outgrowth.

References

1. Braak, H. and E. Braak, *Neuropathological stageing of Alzheimer-related changes.* Acta Neuropathologica, 1991. **82**(4): p. 239-259.
2. Murray, M.E., et al., *Neuropathologically defined subtypes of Alzheimer's disease with distinct clinical characteristics: a retrospective study.* Lancet Neurol, 2011. **10**(9): p. 785-96.
3. Selkoe, D.J., *Alzheimer's Disease: Genes, Proteins, and Therapy*. Vol. 81. 2001. 741-766.
4. Saura, C.A., et al., *Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration.* Neuron, 2004. **42**(1): p. 23-36.
5. Benitez, B.A., et al., *The PSEN1, p.E318G variant increases the risk of Alzheimer's disease in APOE-epsilon4 carriers.* PLoS Genet, 2013. **9**(8): p. e1003685.
6. Beydoun, M.A., et al., *Epidemiologic studies of modifiable factors associated with cognition and dementia: systematic review and meta-analysis.* BMC Public Health, 2014. **14**: p. 643.
7. Ott, A., et al., *Diabetes mellitus and the risk of dementia: The Rotterdam Study.* Neurology, 1999. **53**(9): p. 1937-42.
8. Watson, G.S. and S. Craft, *The role of insulin resistance in the pathogenesis of Alzheimer's disease: implications for treatment.* CNS Drugs, 2003. **17**(1): p. 27-45.
9. Steen, E., et al., *Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes?* J Alzheimers Dis, 2005. **7**(1): p. 63-80.
10. Stozicka, Z., N. Zilka, and M. Novak, *Risk and protective factors for sporadic Alzheimer's disease.* Acta Virol, 2007. **51**(4): p. 205-22.
11. Zhang, C., et al., *NSAID Exposure and Risk of Alzheimer's Disease: An Updated Meta-Analysis From Cohort Studies.* Frontiers in Aging Neuroscience, 2018. **10**(83).
12. McGuinness, B. and P. Passmore, *Can statins prevent or help treat Alzheimer's disease?* J Alzheimers Dis, 2010. **20**(3): p. 925-33.
13. Resnick, S.M. and V.W. Henderson, *Hormone therapy and risk of alzheimer disease: A critical time.* JAMA, 2002. **288**(17): p. 2170-2172.
14. Vassallo, N. and C. Scerri, *Mediterranean diet and dementia of the Alzheimer type.* Curr Aging Sci, 2013. **6**(2): p. 150-62.
15. Singh, B., et al., *Association of mediterranean diet with mild cognitive impairment and Alzheimer's disease: a systematic review and meta-analysis.* J Alzheimers Dis, 2014. **39**(2): p. 271-82.
16. Selkoe, D.J., *Alzheimer's disease is a synaptic failure.* Science, 2002. **298**(5594): p. 789-91.
17. Bayer, T.A., et al., *It all sticks together--the APP-related family of proteins and Alzheimer's disease.* Mol Psychiatry, 1999. **4**(6): p. 524-8.
18. Hung, A.Y., et al., *Increased expression of beta-amyloid precursor protein during neuronal differentiation is not accompanied by secretory cleavage.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9439-43.
19. Loffler, J. and G. Huber, *Beta-amyloid precursor protein isoforms in various rat brain regions and during brain development.* J Neurochem, 1992. **59**(4): p. 1316-24.
20. Moya, K.L., et al., *The amyloid precursor protein is developmentally regulated and correlated with synaptogenesis.* Dev Biol, 1994. **161**(2): p. 597-603.
21. Ando, K., et al., *Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of beta-amyloid.* J Biol Chem, 2001. **276**(43): p. 40353-61.

22. Leem, J.Y., et al., *A role for presenilin 1 in regulating the delivery of amyloid precursor protein to the cell surface.* Neurobiol Dis, 2002. **11**(1): p. 64-82.
23. Pahlsson, P. and S.L. Spitalnik, *The role of glycosylation in synthesis and secretion of beta-amyloid precursor protein by Chinese hamster ovary cells.* Arch Biochem Biophys, 1996. **331**(2): p. 177-86.
24. Galbete, J.L., et al., *Cholesterol decreases secretion of the secreted form of amyloid precursor protein by interfering with glycosylation in the protein secretory pathway.* Biochem J, 2000. **348 Pt 2**: p. 307-13.
25. Tomita, S., Y. Kirino, and T. Suzuki, *Cleavage of Alzheimer's amyloid precursor protein (APP) by secretases occurs after O-glycosylation of APP in the protein secretory pathway. Identification of intracellular compartments in which APP cleavage occurs without using toxic agents that interfere with protein metabolism.* J Biol Chem, 1998. **273**(11): p. 6277-84.
26. Obregon, D., et al., *Soluble amyloid precursor protein-alpha modulates beta-secretase activity and amyloid-beta generation.* Nat Commun, 2012. **3**: p. 777.
27. Deng, J., et al., *Soluble amyloid precursor protein alpha inhibits tau phosphorylation through modulation of GSK3beta signaling pathway.* J Neurochem, 2015. **135**(3): p. 630-7.
28. Claasen, A.M., et al., *Secreted amyloid precursor protein-alpha upregulates synaptic protein synthesis by a protein kinase G-dependent mechanism.* Neurosci Lett, 2009. **460**(1): p. 92-6.
29. Taylor, C.J., et al., *Endogenous secreted amyloid precursor protein-alpha regulates hippocampal NMDA receptor function, long-term potentiation and spatial memory.* Neurobiol Dis, 2008. **31**(2): p. 250-60.
30. Ishida, A., et al., *Secreted form of beta-amyloid precursor protein shifts the frequency dependency for induction of LTD, and enhances LTP in hippocampal slices.* Neuroreport, 1997. **8**(9-10): p. 2133-7.
31. Milosch, N., et al., *Holo-APP and G-protein-mediated signaling are required for sAPPalpha-induced activation of the Akt survival pathway.* Cell Death Dis, 2014. **5**: p. e1391.
32. Sola Vigo, F., et al., *Amyloid-beta precursor protein mediates neuronal toxicity of amyloid beta through Go protein activation.* Neurobiol Aging, 2009. **30**(9): p. 1379-92.
33. Vassar, R., *beta-Secretase, APP and Abeta in Alzheimer's disease.* Subcell Biochem, 2005. **38**: p. 79-103.
34. Nikolaev, A., et al., *APP binds DR6 to trigger axon pruning and neuron death via distinct caspases.* Nature, 2009. **457**(7232): p. 981-9.
35. Marik, S.A., et al., *Physiological role for amyloid precursor protein in adult experience-dependent plasticity.* Proceedings of the National Academy of Sciences, 2016. **113**(28): p. 7912-7917.
36. Plummer, S., et al., *The Neuroprotective Properties of the Amyloid Precursor Protein Following Traumatic Brain Injury.* Aging Dis, 2016. **7**(2): p. 163-79.
37. Deyts, C., et al., *Novel GalphaS-protein signaling associated with membrane-tethered amyloid precursor protein intracellular domain.* J Neurosci, 2012. **32**(5): p. 1714-29.
38. Aplin, A.E., et al., *In vitro phosphorylation of the cytoplasmic domain of the amyloid precursor protein by glycogen synthase kinase-3beta.* J Neurochem, 1996. **67**(2): p. 699-707.
39. Oishi, M., et al., *The cytoplasmic domain of Alzheimer's amyloid precursor protein is phosphorylated at Thr654, Ser655, and Thr668 in adult rat brain and cultured cells.* Mol Med, 1997. **3**(2): p. 111-23.
40. Iijima, K., et al., *Neuron-specific phosphorylation of Alzheimer's beta-amyloid precursor protein by cyclin-dependent kinase 5.* J Neurochem, 2000. **75**(3): p. 1085-91.
41. Quiroz-Baez, R., E. Rojas, and C. Arias, *Oxidative stress promotes JNK-dependent amyloidogenic processing of normally expressed human APP by differential*

modification of alpha-, beta- and gamma-secretase expression. Neurochem Int, 2009. **55**(7): p. 662-70.
42. Ahn, J.H., et al., *c-Jun N-terminal Kinase (JNK) induces phosphorylation of amyloid precursor protein (APP) at Thr668, in okadaic acid-induced neurodegeneration.* BMB Rep, 2016. **49**(7): p. 376-81.
43. Ando, K., et al., *Role of phosphorylation of Alzheimer's amyloid precursor protein during neuronal differentiation.* J Neurosci, 1999. **19**(11): p. 4421-7.
44. Triaca, V., et al., *NGF controls APP cleavage by downregulating APP phosphorylation at Thr668: relevance for Alzheimer's disease.* Aging Cell, 2016. **15**(4): p. 661-72.
45. Ma, S.L., et al., *Prolyl isomerase Pin1 promotes amyloid precursor protein (APP) turnover by inhibiting glycogen synthase kinase-3beta (GSK3beta) activity: novel mechanism for Pin1 to protect against Alzheimer disease.* J Biol Chem, 2012. **287**(10): p. 6969-73.
46. Gervais, F.G., et al., *Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation.* Cell, 1999. **97**(3): p. 395-406.
47. Taru, H., K. Yoshikawa, and T. Suzuki, *Suppression of the caspase cleavage of beta-amyloid precursor protein by its cytoplasmic phosphorylation.* FEBS Lett, 2004. **567**(2-3): p. 248-52.
48. Pastorino, L., et al., *The prolyl isomerase Pin1 regulates amyloid precursor protein processing and amyloid-beta production.* Nature, 2006. **440**(7083): p. 528-34.
49. Cao, X. and T.C. Sudhof, *A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60.* Science, 2001. **293**(5527): p. 115-20.
50. Bukhari, H., et al., *Membrane tethering of APP c-terminal fragments is a prerequisite for T668 phosphorylation preventing nuclear sphere generation.* Cell Signal, 2016. **28**(11): p. 1725-34.
51. Lee, M.-S., et al., *APP processing is regulated by cytoplasmic phosphorylation.* The Journal of Cell Biology, 2003. **163**(1): p. 83-95.
52. Shin, R.W., et al., *Amyloid precursor protein cytoplasmic domain with phospho-Thr668 accumulates in Alzheimer's disease and its transgenic models: a role to mediate interaction of Abeta and tau.* Acta Neuropathol, 2007. **113**(6): p. 627-36.
53. Chang, K.A., et al., *Phosphorylation of amyloid precursor protein (APP) at Thr668 regulates the nuclear translocation of the APP intracellular domain and induces neurodegeneration.* Mol Cell Biol, 2006. **26**(11): p. 4327-38.
54. Oliveira, J.M., et al., *Amyloid-beta Modulates Both AbetaPP and Tau Phosphorylation.* J Alzheimers Dis, 2015. **45**(2): p. 495-507.
55. Kirouac, L., et al., *Activation of Ras-ERK Signaling and GSK-3 by Amyloid Precursor Protein and Amyloid Beta Facilitates Neurodegeneration in Alzheimer's Disease.* eNeuro, 2017. **4**(2).
56. Sano, Y., et al., *Physiological mouse brain Abeta levels are not related to the phosphorylation state of threonine-668 of Alzheimer's APP.* PLoS One, 2006. **1**: p. e51.
57. Witman, G.B., et al., *Tubulin requires tau for growth onto microtubule initiating sites.* Proc Natl Acad Sci U S A, 1976. **73**(11): p. 4070-4.
58. Mandelkow, E.M. and E. Mandelkow, *Biochemistry and cell biology of tau protein in neurofibrillary degeneration.* Cold Spring Harb Perspect Med, 2012. **2**(7): p. a006247.
59. Schweers, O., et al., *Structural studies of tau protein and Alzheimer paired helical filaments show no evidence for beta-structure.* J Biol Chem, 1994. **269**(39): p. 24290-7.
60. von Bergen, M., et al., *Tau aggregation is driven by a transition from random coil to beta sheet structure.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2005. **1739**(2): p. 158-166.
61. Jeganathan, S., et al., *The Natively Unfolded Character of Tau and Its Aggregation to Alzheimer-like Paired Helical Filaments.* Biochemistry, 2008. **47**(40): p. 10526-10539.

62. Kosik, K.S., C.L. Joachim, and D.J. Selkoe, *Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease.* Proc Natl Acad Sci U S A, 1986. **83**(11): p. 4044-8.
63. Goedert, M., *Tau protein and neurodegeneration.* Semin Cell Dev Biol, 2004. **15**(1): p. 45-9.
64. Binder, L.I., A. Frankfurter, and L.I. Rebhun, *Differential localization of MAP-2 and tau in mammalian neurons in situ.* Ann N Y Acad Sci, 1986. **466**: p. 145-66.
65. Papasozomenos, S.C. and L.I. Binder, *Phosphorylation determines two distinct species of Tau in the central nervous system.* Cell Motil Cytoskeleton, 1987. **8**(3): p. 210-26.
66. Bullmann, T., et al., *Pattern of tau isoforms expression during development in vivo.* Int J Dev Neurosci, 2009. **27**(6): p. 591-7.
67. Goedert, M., et al., *Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain.* EMBO J, 1989. **8**(2): p. 393-9.
68. Goode, B. and S. Feinstein, *Identification of a novel microtubule binding and assembly domain in the developmentally regulated inter-repeat region of tau.* The Journal of Cell Biology, 1994. **124**(5): p. 769-782.
69. Kempf, M., et al., *Tau binds to the distal axon early in development of polarity in a microtubule- and microfilament-dependent manner.* J Neurosci, 1996. **16**(18): p. 5583-92.
70. Dawson, H.N., et al., *Loss of tau elicits axonal degeneration in a mouse model of Alzheimer's disease.* Neuroscience, 2010. **169**(1): p. 516-31.
71. Liu, C.W., G. Lee, and D.G. Jay, *Tau is required for neurite outgrowth and growth cone motility of chick sensory neurons.* Cell Motil Cytoskeleton, 1999. **43**(3): p. 232-42.
72. Mandell, J.W. and G.A. Banker, *A spatial gradient of tau protein phosphorylation in nascent axons.* J Neurosci, 1996. **16**(18): p. 5727-40.
73. Hoffman, A., G. Taleski, and E. Sontag, *The protein serine/threonine phosphatases PP2A, PP1 and calcineurin: A triple threat in the regulation of the neuronal cytoskeleton.* Mol Cell Neurosci, 2017.
74. Dixit, R., et al., *Differential regulation of dynein and kinesin motor proteins by tau.* Science, 2008. **319**(5866): p. 1086-9.
75. Götz, J., et al., *Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform.* The EMBO Journal, 1995. **14**(7): p. 1304-1313.
76. Li, X., et al., *Novel diffusion barrier for axonal retention of Tau in neurons and its failure in neurodegeneration.* EMBO J, 2011. **30**(23): p. 4825-37.
77. Caceres, A. and K.S. Kosik, *Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons.* Nature, 1990. **343**(6257): p. 461-3.
78. Martin, L., X. Latypova, and F. Terro, *Post-translational modifications of tau protein: implications for Alzheimer's disease.* Neurochem Int, 2011. **58**(4): p. 458-71.
79. Morris, M., et al., *Tau post-translational modifications in wild-type and human amyloid precursor protein transgenic mice.* Nat Neurosci, 2015. **18**(8): p. 1183-9.
80. Kimura, T., K. Ishiguro, and S. Hisanaga, *Physiological and pathological phosphorylation of tau by Cdk5.* Front Mol Neurosci, 2014. **7**: p. 65.
81. Castro-Alvarez, J.F., et al., *Long- and short-term CDK5 knockdown prevents spatial memory dysfunction and tau pathology of triple transgenic Alzheimer's mice.* Front Aging Neurosci, 2014. **6**: p. 243.
82. Kimura, T., et al., *Isomerase Pin1 stimulates dephosphorylation of tau protein at cyclin-dependent kinase (Cdk5)-dependent Alzheimer phosphorylation sites.* J Biol Chem, 2013. **288**(11): p. 7968-77.
83. Wang, J.-Z., I. Grundke-Iqbal, and K. Iqbal, *Kinases and phosphatases and tau sites involved in Alzheimer neurofibrillary degeneration.* The European journal of neuroscience, 2007. **25**(1): p. 59-68.

84. Ittner, A., et al., *Site-specific phosphorylation of tau inhibits amyloid-beta toxicity in Alzheimer's mice.* Science, 2016. **354**(6314): p. 904-908.
85. Maphis, N., et al., *Selective suppression of the alpha isoform of p38 MAPK rescues late-stage tau pathology.* Alzheimers Res Ther, 2016. **8**(1): p. 54.
86. Lauretti, E. and D. Pratico, *Glucose deprivation increases tau phosphorylation via P38 mitogen-activated protein kinase.* Aging Cell, 2015. **14**(6): p. 1067-74.
87. Schneider, A., et al., *Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments.* Biochemistry, 1999. **38**(12): p. 3549-58.
88. Liu, S.J., et al., *Tau becomes a more favorable substrate for GSK-3 when it is prephosphorylated by PKA in rat brain.* J Biol Chem, 2004. **279**(48): p. 50078-88.
89. Liu, F., et al., *PKA modulates GSK-3beta- and cdk5-catalyzed phosphorylation of tau in site- and kinase-specific manners.* FEBS Lett, 2006. **580**(26): p. 6269-74.
90. Cho, J.-H. and G.V.W. Johnson, *Primed phosphorylation of tau at Thr231 by glycogen synthase kinase 3β (GSK3β) plays a critical role in regulating tau's ability to bind and stabilize microtubules.* Journal of Neurochemistry, 2004. **88**(2): p. 349-358.
91. Ahmed, T., et al., *Rescue of impaired late-phase long-term depression in a tau transgenic mouse model.* Neurobiol Aging, 2015. **36**(2): p. 730-9.
92. Niewiadomska, G., M. Baksalerska-Pazera, and G. Riedel, *Altered cellular distribution of phospho-tau proteins coincides with impaired retrograde axonal transport in neurons of aged rats.* Ann N Y Acad Sci, 2005. **1048**: p. 287-95.
93. Lin, Y.T., et al., *The binding and phosphorylation of Thr231 is critical for Tau's hyperphosphorylation and functional regulation by glycogen synthase kinase 3beta.* J Neurochem, 2007. **103**(2): p. 802-13.
94. Derkinderen, P., et al., *Tyrosine 394 is phosphorylated in Alzheimer's paired helical filament tau and in fetal tau with c-Abl as the candidate tyrosine kinase.* J Neurosci, 2005. **25**(28): p. 6584-93.
95. Chukwu, J.E., et al., *Tau Antibody Structure Reveals a Molecular Switch Defining a Pathological Conformation of the Tau Protein.* Sci Rep, 2018. **8**(1): p. 6209.
96. Lee, G., et al., *Phosphorylation of tau by fyn: implications for Alzheimer's disease.* J Neurosci, 2004. **24**(9): p. 2304-12.
97. Lee, G., *Tau and src family tyrosine kinases.* Biochim Biophys Acta, 2005. **1739**(2-3): p. 323-30.
98. Miyamoto, T., et al., *Phosphorylation of tau at Y18, but not tau-fyn binding, is required for tau to modulate NMDA receptor-dependent excitotoxicity in primary neuronal culture.* Molecular Neurodegeneration, 2017. **12**: p. 41.
99. Scales, T.M., et al., *Tyrosine phosphorylation of tau by the SRC family kinases lck and fyn.* Mol Neurodegener, 2011. **6**: p. 12.
100. Illenberger, S., et al., *The endogenous and cell cycle-dependent phosphorylation of tau protein in living cells: implications for Alzheimer's disease.* Mol Biol Cell, 1998. **9**(6): p. 1495-512.
101. Preuss, U. and E.M. Mandelkow, *Mitotic phosphorylation of tau protein in neuronal cell lines resembles phosphorylation in Alzheimer's disease.* Eur J Cell Biol, 1998. **76**(3): p. 176-84.
102. Yoshizaki, C., M. Tsukane, and T. Yamauchi, *Overexpression of tau leads to the stimulation of neurite outgrowth, the activation of caspase 3 activity, and accumulation and phosphorylation of tau in neuroblastoma cells on cAMP treatment.* Neurosci Res, 2004. **49**(4): p. 363-71.
103. Zheng-Fischhofer, Q., et al., *Sequential phosphorylation of Tau by glycogen synthase kinase-3beta and protein kinase A at Thr212 and Ser214 generates the Alzheimer-specific epitope of antibody AT100 and requires a paired-helical-filament-like conformation.* Eur J Biochem, 1998. **252**(3): p. 542-52.
104. Gil, L., et al., *Aging dependent effect of nuclear tau.* Brain Res, 2017. **1677**: p. 129-137.

105. Goedert, M., et al., *Epitope mapping of monoclonal antibodies to the paired helical filaments of Alzheimer's disease: identification of phosphorylation sites in tau protein.* Biochem J, 1994. **301 (Pt 3)**: p. 871-7.
106. Milenkovic, I., et al., *The physiological phosphorylation of tau is critically changed in fetal brains of individuals with Down syndrome.* Neuropathol Appl Neurobiol, 2018. **44**(3): p. 314-327.
107. Nakamura, K., et al., *Proline Isomer-Specific Antibodies Reveal the Early Pathogenic Tau Conformation in Alzheimer's Disease.* Cell, 2012. **149**(1): p. 232-244.
108. Petry, F.R., et al., *Specificity of anti-tau antibodies when analyzing mice models of Alzheimer's disease: problems and solutions.* PLoS One, 2014. **9**(5): p. e94251.
109. Lovestone, S., et al., *Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells.* Curr Biol, 1994. **4**(12): p. 1077-86.
110. Otvos, L., Jr., et al., *Monoclonal antibody PHF-1 recognizes tau protein phosphorylated at serine residues 396 and 404.* J Neurosci Res, 1994. **39**(6): p. 669-73.
111. Lee, G., et al., *Tau interacts with src-family non-receptor tyrosine kinases.* J Cell Sci, 1998. **111 (Pt 21)**: p. 3167-77.
112. Usardi, A., et al., *Tyrosine phosphorylation of tau regulates its interactions with Fyn SH2 domains, but not SH3 domains, altering the cellular localization of tau.* FEBS J, 2011. **278**(16): p. 2927-37.
113. Pooler, A.M., et al., *Dynamic association of tau with neuronal membranes is regulated by phosphorylation.* Neurobiol Aging, 2012. **33**(2): p. 431 e27-38.
114. Reynolds, C.H., et al., *Phosphorylation regulates tau interactions with Src homology 3 domains of phosphatidylinositol 3-kinase, phospholipase Cgamma1, Grb2, and Src family kinases.* J Biol Chem, 2008. **283**(26): p. 18177-86.
115. Bhaskar, K., S.H. Yen, and G. Lee, *Disease-related modifications in tau affect the interaction between Fyn and Tau.* J Biol Chem, 2005. **280**(42): p. 35119-25.
116. Hanger, D.P., et al., *Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase.* Neurosci Lett, 1992. **147**(1): p. 58-62.
117. Li, X., et al., *Activation of glycogen synthase kinase-3 induces Alzheimer-like tau hyperphosphorylation in rat hippocampus slices in culture.* J Neural Transm, 2006. **113**(1): p. 93-102.
118. Plattner, F., M. Angelo, and K.P. Giese, *The roles of cyclin-dependent kinase 5 and glycogen synthase kinase 3 in tau hyperphosphorylation.* J Biol Chem, 2006. **281**(35): p. 25457-65.
119. Deng, S.S., et al., *Protein kinase A rescues microtubule affinity-regulating kinase 2-induced microtubule instability and neurite disruption by phosphorylating serine 409.* J Biol Chem, 2015. **290**(5): p. 3149-60.
120. Aronov, S., et al., *Axonal tau mRNA localization coincides with tau protein in living neuronal cells and depends on axonal targeting signal.* J Neurosci, 2001. **21**(17): p. 6577-87.
121. Zempel, H. and E. Mandelkow, *Lost after translation: missorting of Tau protein and consequences for Alzheimer disease.* Trends Neurosci, 2014. **37**(12): p. 721-32.
122. Nemoto, T., et al., *Insulin-induced neurite-like process outgrowth: acceleration of tau protein synthesis via a phosphoinositide 3-kinase~mammalian target of rapamycin pathway.* Neurochem Int, 2011. **59**(6): p. 880-8.
123. Regan, P., et al., *Tau phosphorylation at serine 396 residue is required for hippocampal LTD.* J Neurosci, 2015. **35**(12): p. 4804-12.
124. Decker, J.M., et al., *Pro-aggregant Tau impairs mossy fiber plasticity due to structural changes and Ca(++) dysregulation.* Acta Neuropathol Commun, 2015. **3**: p. 23.
125. El Khoury, N.B., et al., *Insulin dysfunction and Tau pathology.* Front Cell Neurosci, 2014. **8**: p. 22.

126. Whittington, R.A., et al., *Anesthesia and tau pathology.* Prog Neuropsychopharmacol Biol Psychiatry, 2013. **47**: p. 147-55.
127. Arendt, T., et al., *Reversible paired helical filament-like phosphorylation of tau is an adaptive process associated with neuronal plasticity in hibernating animals.* J Neurosci, 2003. **23**(18): p. 6972-81.
128. Bullmann, T., et al., *Tau phosphorylation-associated spine regression does not impair hippocampal-dependent memory in hibernating golden hamsters.* Hippocampus, 2016. **26**(3): p. 301-18.
129. Ikegami, S., A. Harada, and N. Hirokawa, *Muscle weakness, hyperactivity, and impairment in fear conditioning in tau-deficient mice.* Neurosci Lett, 2000. **279**(3): p. 129-32.
130. Roberson, E.D., et al., *Amyloid-beta/Fyn-induced synaptic, network, and cognitive impairments depend on tau levels in multiple mouse models of Alzheimer's disease.* J Neurosci, 2011. **31**(2): p. 700-11.
131. Ittner, L.M., et al., *Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models.* Cell, 2010. **142**(3): p. 387-97.
132. Tan, D.C.S., et al., *Generation of a New Tau Knockout (tauDeltaex1) Line Using CRISPR/Cas9 Genome Editing in Mice.* J Alzheimers Dis, 2018. **62**(2): p. 571-578.
133. Lee, G. and C.J. Leugers, *Tau and tauopathies.* Prog Mol Biol Transl Sci, 2012. **107**: p. 263-93.
134. van Swieten, J. and M.G. Spillantini, *Hereditary frontotemporal dementia caused by Tau gene mutations.* Brain Pathol, 2007. **17**(1): p. 63-73.
135. LaPointe, N.E., et al., *The amino terminus of tau inhibits kinesin-dependent axonal transport: implications for filament toxicity.* J Neurosci Res, 2009. **87**(2): p. 440-51.
136. Kanaan, N.M., et al., *Pathogenic forms of tau inhibit kinesin-dependent axonal transport through a mechanism involving activation of axonal phosphotransferases.* J Neurosci, 2011. **31**(27): p. 9858-68.
137. Vossel, K.A., et al., *Tau Reduction Prevents Aβ-Induced Defects in Axonal Transport.* Science, 2010. **330**(6001): p. 198-198.
138. Shahpasand, K., et al., *Regulation of mitochondrial transport and inter-microtubule spacing by tau phosphorylation at the sites hyperphosphorylated in Alzheimer's disease.* J Neurosci, 2012. **32**(7): p. 2430-41.
139. Zempel, H. and E.M. Mandelkow, *Linking amyloid-beta and tau: amyloid-beta induced synaptic dysfunction via local wreckage of the neuronal cytoskeleton.* Neurodegener Dis, 2012. **10**(1-4): p. 64-72.
140. Goedert, M., et al., *Reduced binding of protein phosphatase 2A to tau protein with frontotemporal dementia and parkinsonism linked to chromosome 17 mutations.* J Neurochem, 2000. **75**(5): p. 2155-62.
141. Lewis, J., et al., *Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein.* Nat Genet, 2000. **25**(4): p. 402-5.
142. Bhaskar, K., et al., *Tyrosine phosphorylation of tau accompanies disease progression in transgenic mouse models of tauopathy.* Neuropathol Appl Neurobiol, 2010. **36**(6): p. 462-77.
143. Xia, D., C. Li, and J. Gotz, *Pseudophosphorylation of Tau at distinct epitopes or the presence of the P301L mutation targets the microtubule-associated protein Tau to dendritic spines.* Biochim Biophys Acta, 2015. **1852**(5): p. 913-24.
144. Drewes, G., et al., *Microtubule-associated protein/microtubule affinity-regulating kinase (p110mark). A novel protein kinase that regulates tau-microtubule interactions and dynamic instability by phosphorylation at the Alzheimer-specific site serine 262.* J Biol Chem, 1995. **270**(13): p. 7679-88.
145. Gendron, T.F. and L. Petrucelli, *The role of tau in neurodegeneration.* Mol Neurodegener, 2009. **4**: p. 13.
146. Steinhilb, M.L., et al., *S/P and T/P phosphorylation is critical for tau neurotoxicity in Drosophila.* J Neurosci Res, 2007. **85**(6): p. 1271-8.

147. Zempel, H., et al., *Aβ Oligomers Cause Localized Ca2+ Elevation, Missorting of Endogenous Tau into Dendrites, Tau Phosphorylation, and Destruction of Microtubules and Spines.* The Journal of Neuroscience, 2010. **30**(36): p. 11938-11950.
148. van Eersel, J., et al., *Early-onset axonal pathology in a novel P301S-Tau transgenic mouse model of frontotemporal lobar degeneration.* Neuropathol Appl Neurobiol, 2015. **41**(7): p. 906-25.
149. Braak, H., et al., *Stages of the pathologic process in Alzheimer disease: age categories from 1 to 100 years.* J Neuropathol Exp Neurol, 2011. **70**(11): p. 960-9.
150. Stamer, K., et al., *Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress.* J Cell Biol, 2002. **156**(6): p. 1051-63.
151. Thies, E. and E.M. Mandelkow, *Missorting of tau in neurons causes degeneration of synapses that can be rescued by the kinase MARK2/Par-1.* J Neurosci, 2007. **27**(11): p. 2896-907.
152. Shankar, G.M., et al., *Amyloid-[beta] protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory.* Nat Med, 2008. **14**(8): p. 837-842.
153. Roberson, E.D., et al., *Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model.* Science, 2007. **316**(5825): p. 750-4.
154. Ittner, L.M. and J. Gotz, *Amyloid-beta and tau--a toxic pas de deux in Alzheimer's disease.* Nat Rev Neurosci, 2011. **12**(2): p. 65-72.
155. Martin, L., et al., *Tau protein phosphatases in Alzheimer's disease: the leading role of PP2A.* Ageing Res Rev, 2013. **12**(1): p. 39-49.
156. Martin, L., et al., *Tau protein kinases: involvement in Alzheimer's disease.* Ageing Res Rev, 2013. **12**(1): p. 289-309.
157. Liu, F., et al., *Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation.* Eur J Neurosci, 2005. **22**(8): p. 1942-50.
158. Sontag, E., et al., *Altered expression levels of the protein phosphatase 2A ABalphaC enzyme are associated with Alzheimer disease pathology.* J Neuropathol Exp Neurol, 2004. **63**(4): p. 287-301.
159. Sontag, E., et al., *Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis.* J Neuropathol Exp Neurol, 2004. **63**(10): p. 1080-91.
160. Sontag, J.M. and E. Sontag, *Protein phosphatase 2A dysfunction in Alzheimer's disease.* Front Mol Neurosci, 2014. **7**: p. 16.
161. Virshup, D.M. and S. Shenolikar, *From promiscuity to precision: protein phosphatases get a makeover.* Mol Cell, 2009. **33**(5): p. 537-45.
162. Sontag, E., *Protein phosphatase 2A: the Trojan Horse of cellular signaling.* Cellular Signalling, 2001. **13**(1): p. 7-16.
163. Arnaud, L., et al., *Mechanism of inhibition of PP2A activity and abnormal hyperphosphorylation of tau by I2(PP2A)/SET.* FEBS Lett, 2011. **585**(17): p. 2653-9.
164. Voronkov, M., S.P. Braithwaite, and J.B. Stock, *Phosphoprotein phosphatase 2A: a novel druggable target for Alzheimer's disease.* Future Medicinal Chemistry, 2011. **3**(7): p. 821-833.
165. Sontag, J.M., V. Nunbhakdi-Craig, and E. Sontag, *Leucine carboxyl methyltransferase 1 (LCMT1)-dependent methylation regulates the association of protein phosphatase 2A and Tau protein with plasma membrane microdomains in neuroblastoma cells.* J Biol Chem, 2013. **288**(38): p. 27396-405.
166. De Baere, I., et al., *Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue.* Biochemistry, 1999. **38**(50): p. 16539-47.
167. Lee, J. and J. Stock, *Protein phosphatase 2A catalytic subunit is methyl-esterified at its carboxyl terminus by a novel methyltransferase.* J Biol Chem, 1993. **268**(26): p. 19192-5.
168. Lee, J., et al., *A specific protein carboxyl methylesterase that demethylates phosphoprotein phosphatase 2A in bovine brain.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 6043-7.

169. Wu, C.G., et al., *Methylation-regulated decommissioning of multimeric PP2A complexes.* Nat Commun, 2017. **8**(1): p. 2272.
170. MacKay, K.B., et al., *Circumventing embryonic lethality with Lcmt1 deficiency: generation of hypomorphic Lcmt1 mice with reduced protein phosphatase 2A methyltransferase expression and defects in insulin signaling.* PLoS One, 2013. **8**(6): p. e65967.
171. Xing, Y., et al., *Structural mechanism of demethylation and inactivation of protein phosphatase 2A.* Cell, 2008. **133**(1): p. 154-63.
172. Longin, S., et al., *Spatial control of protein phosphatase 2A (de)methylation.* Exp Cell Res, 2008. **314**(1): p. 68-81.
173. Chen, J., B.L. Martin, and D.L. Brautigan, *Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation.* Science, 1992. **257**(5074): p. 1261-4.
174. Chen, J., S. Parsons, and D.L. Brautigan, *Tyrosine phosphorylation of protein phosphatase 2A in response to growth stimulation and v-src transformation of fibroblasts.* J Biol Chem, 1994. **269**(11): p. 7957-62.
175. Liu, R., et al., *Phosphorylated PP2A (tyrosine 307) is associated with Alzheimer neurofibrillary pathology.* J Cell Mol Med, 2008. **12**(1): p. 241-57.
176. Guo, H. and Z. Damuni, *Autophosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A.* Proc Natl Acad Sci U S A, 1993. **90**(6): p. 2500-4.
177. Damuni, Z., H. Xiong, and M. Li, *Autophosphorylation-activated protein kinase inactivates the protein tyrosine phosphatase activity of protein phosphatase 2A.* FEBS Lett, 1994. **352**(3): p. 311-4.
178. Ogris, E., D.M. Gibson, and D.C. Pallas, *Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular B subunit but not polyomavirus middle tumor antigen.* Oncogene, 1997. **15**(8): p. 911-7.
179. Longin, S., et al., *Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic Subunit.* J Biol Chem, 2007. **282**(37): p. 26971-80.
180. Trockenbacher, A., et al., *MID1, mutated in Opitz syndrome, encodes an ubiquitin ligase that targets phosphatase 2A for degradation.* Nat Genet, 2001. **29**(3): p. 287-94.
181. McConnell, J.L., et al., *Alpha4 is a ubiquitin-binding protein that regulates protein serine/threonine phosphatase 2A ubiquitination.* Biochemistry, 2010. **49**(8): p. 1713-8.
182. Watkins, G.R., et al., *Monoubiquitination promotes calpain cleavage of the protein phosphatase 2A (PP2A) regulatory subunit alpha4, altering PP2A stability and microtubule-associated protein phosphorylation.* J Biol Chem, 2012. **287**(29): p. 24207-15.
183. Ahn, J.H., et al., *Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56delta subunit.* Proc Natl Acad Sci U S A, 2007. **104**(8): p. 2979-84.
184. Ahn, J.H., et al., *Protein kinase C-dependent dephosphorylation of tyrosine hydroxylase requires the B56delta heterotrimeric form of protein phosphatase 2A.* PLoS One, 2011. **6**(10): p. e26292.
185. Ranieri, A., et al., *beta-Adrenergic regulation of cardiac type 2A protein phosphatase through phosphorylation of regulatory subunit B56delta at S573.* J Mol Cell Cardiol, 2018. **115**: p. 20-31.
186. Kirchhefer, U., et al., *Protein phosphatase 2A is regulated by protein kinase Calpha (PKCalpha)-dependent phosphorylation of its targeting subunit B56alpha at Ser41.* J Biol Chem, 2014. **289**(1): p. 163-76.
187. Viquez, N.M., et al., *PP2A and GSK-3beta act antagonistically to regulate active zone development.* J Neurosci, 2009. **29**(37): p. 11484-94.
188. Chia, P.H., M.R. Patel, and K. Shen, *NAB-1 instructs synapse assembly by linking adhesion molecules and F-actin to active zone proteins.* Nat Neurosci, 2012. **15**(2): p. 234-42.

189. Kotlo, K., et al., *PR65A Phosphorylation Regulates PP2A Complex Signaling.* PLoS ONE, 2014. **9**(1): p. e85000.
190. Martin, B.R., et al., *Global profiling of dynamic protein palmitoylation.* Nat Methods, 2011. **9**(1): p. 84-9.
191. Sontag, E., et al., *The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation.* Cell, 1993. **75**(5): p. 887-97.
192. Nicholls, R.E., et al., *Transgenic mice lacking NMDAR-dependent LTD exhibit deficits in behavioral flexibility.* Neuron, 2008. **58**(1): p. 104-17.
193. Chan, S.F. and N.J. Sucher, *An NMDA receptor signaling complex with protein phosphatase 2A.* J Neurosci, 2001. **21**(20): p. 7985-92.
194. Toda, A.M. and R.L. Huganir, *Regulation of AMPA receptor phosphorylation by the neuropeptide PACAP38.* Proc Natl Acad Sci U S A, 2015. **112**(21): p. 6712-7.
195. Davare, M.A., M.C. Horne, and J.W. Hell, *Protein phosphatase 2A is associated with class C L-type calcium channels (Cav1.2) and antagonizes channel phosphorylation by cAMP-dependent protein kinase.* J Biol Chem, 2000. **275**(50): p. 39710-7.
196. Hall, D.D., et al., *Binding of protein phosphatase 2A to the L-type calcium channel Cav1.2 next to Ser1928, its main PKA site, is critical for Ser1928 dephosphorylation.* Biochemistry, 2006. **45**(10): p. 3448-59.
197. Chen, Y.K., et al., *CTTNBP2, but not CTTNBP2NL, regulates dendritic spinogenesis and synaptic distribution of the striatin-PP2A complex.* Mol Biol Cell, 2012. **23**(22): p. 4383-92.
198. Hiraga, A. and S. Tamura, *Protein phosphatase 2A is associated in an inactive state with microtubules through 2A1-specific interaction with tubulin.* Biochem J, 2000. **346 Pt 2**: p. 433-9.
199. Sontag, J.M., et al., *The protein phosphatase PP2A/Balpha binds to the microtubule-associated proteins Tau and MAP2 at a motif also recognized by the kinase Fyn: implications for tauopathies.* J Biol Chem, 2012. **287**(18): p. 14984-93.
200. Sontag, E., et al., *A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle.* The Journal of Cell Biology, 1995. **128**(6): p. 1131-44.
201. Sontag, E., et al., *Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies.* J Biol Chem, 1999. **274**(36): p. 25490-8.
202. Xu, Y., et al., *Structure of a protein phosphatase 2A holoenzyme: insights into B55-mediated Tau dephosphorylation.* Mol Cell, 2008. **31**(6): p. 873-85.
203. Eidenmuller, J., et al., *Phosphorylation-mimicking glutamate clusters in the proline-rich region are sufficient to simulate the functional deficiencies of hyperphosphorylated tau protein.* Biochem J, 2001. **357**(Pt 3): p. 759-67.
204. Landrieu, I., et al., *Molecular implication of PP2A and Pin1 in the Alzheimer's disease specific hyperphosphorylation of Tau.* PLoS One, 2011. **6**(6): p. e21521.
205. Planel, E., et al., *Inhibition of protein phosphatase 2A overrides tau protein kinase I/glycogen synthase kinase 3 beta and cyclin-dependent kinase 5 inhibition and results in tau hyperphosphorylation in the hippocampus of starved mouse.* J Biol Chem, 2001. **276**(36): p. 34298-306.
206. Hirata, F., et al., *Phospholipid methylation: a possible mechanism of signal transduction across biomembranes.* Prog Clin Biol Res, 1981. **63**: p. 383-8.
207. Fuks, F., *[DNA methyltransferases: from chromatin remodeling to cancer].* Med Sci (Paris), 2003. **19**(4): p. 477-80.
208. Ghosh, S.K., et al., *Enzymic methylation of myelin basic protein in myelin.* Biochem J, 1991. **275 (Pt 2)**: p. 381-7.
209. Tchantchou, F., *Homocysteine metabolism and various consequences of folate deficiency.* J Alzheimers Dis, 2006. **9**(4): p. 421-7.

210. Shen, L. and H.F. Ji, *Associations between Homocysteine, Folic Acid, Vitamin B12 and Alzheimer's Disease: Insights from Meta-Analyses.* J Alzheimers Dis, 2015. **46**(3): p. 777-90.
211. Reynolds, E.H., *The neurology of folic acid deficiency.* Handb Clin Neurol, 2014. **120**: p. 927-43.
212. Madsen, S.K., et al., *Higher homocysteine associated with thinner cortical gray matter in 803 participants from the Alzheimer's Disease Neuroimaging Initiative.* Neurobiol Aging, 2015. **36 Suppl 1**: p. S203-10.
213. Ravaglia, G., et al., *Homocysteine and folate as risk factors for dementia and Alzheimer disease.* Am J Clin Nutr, 2005. **82**(3): p. 636-43.
214. Seshadri, S., et al., *Plasma homocysteine as a risk factor for dementia and Alzheimer's disease.* N Engl J Med, 2002. **346**(7): p. 476-83.
215. Hu, Q., et al., *Homocysteine and Alzheimer's Disease: Evidence for a Causal Link from Mendelian Randomization.* J Alzheimers Dis, 2016.
216. Rai, V., *Methylenetetrahydrofolate Reductase (MTHFR) C677T Polymorphism and Alzheimer Disease Risk: a Meta-Analysis.* Mol Neurobiol, 2016.
217. McCaddon, A., et al., *Total serum homocysteine in senile dementia of Alzheimer type.* Int J Geriatr Psychiatry, 1998. **13**(4): p. 235-9.
218. Clarke, R., et al., *Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease.* Arch Neurol, 1998. **55**(11): p. 1449-55.
219. Zylberstein, D.E., et al., *Midlife homocysteine and late-life dementia in women. A prospective population study.* Neurobiol Aging, 2011. **32**(3): p. 380-6.
220. Hogervorst, E., et al., *Plasma homocysteine levels, cerebrovascular risk factors, and cerebral white matter changes (leukoaraiosis) in patients with Alzheimer disease.* Arch Neurol, 2002. **59**(5): p. 787-93.
221. de Lau, L.M., et al., *Plasma vitamin B12 status and cerebral white-matter lesions.* J Neurol Neurosurg Psychiatry, 2009. **80**(2): p. 149-57.
222. Coppede, F., et al., *Folate, homocysteine, vitamin B12, and polymorphisms of genes participating in one-carbon metabolism in late-onset Alzheimer's disease patients and healthy controls.* Antioxid Redox Signal, 2012. **17**(2): p. 195-204.
223. Smith, A.D., et al., *Homocysteine and Dementia: An International Consensus Statement.* J Alzheimers Dis, 2018. **62**(2): p. 561-570.
224. Durga, J., et al., *Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomised, double blind, controlled trial.* Lancet, 2007. **369**(9557): p. 208-16.
225. Aisen, P.S., et al., *High-dose B vitamin supplementation and cognitive decline in Alzheimer disease: a randomized controlled trial.* Jama, 2008. **300**(15): p. 1774-83.
226. Douaud, G., et al., *Preventing Alzheimer's disease-related gray matter atrophy by B-vitamin treatment.* Proc Natl Acad Sci U S A, 2013. **110**(23): p. 9523-8.
227. Jerneren, F., et al., *Brain atrophy in cognitively impaired elderly: the importance of long-chain omega-3 fatty acids and B vitamin status in a randomized controlled trial.* Am J Clin Nutr, 2015. **102**(1): p. 215-21.
228. Clarke, R., et al., *Effects of homocysteine lowering with B vitamins on cognitive aging: meta-analysis of 11 trials with cognitive data on 22,000 individuals.* Am J Clin Nutr, 2014. **100**(2): p. 657-66.
229. Mitchell, E.S., N. Conus, and J. Kaput, *B vitamin polymorphisms and behavior: evidence of associations with neurodevelopment, depression, schizophrenia, bipolar disorder and cognitive decline.* Neurosci Biobehav Rev, 2014. **47**: p. 307-20.
230. Christensen, K.E., et al., *High folic acid consumption leads to pseudo-MTHFR deficiency, altered lipid metabolism, and liver injury in mice.* Am J Clin Nutr, 2015. **101**(3): p. 646-58.
231. Bahous, R.H., et al., *High dietary folate in pregnant mice leads to pseudo-MTHFR deficiency and altered methyl metabolism, with embryonic growth delay and short-term memory impairment in offspring.* Hum Mol Genet, 2017. **26**(5): p. 888-900.

232. Geller, J., et al., *Hereditary folate malabsorption: family report and review of the literature.* Medicine (Baltimore), 2002. **81**(1): p. 51-68.
233. Watkins, D. and D.S. Rosenblatt, *Update and new concepts in vitamin responsive disorders of folate transport and metabolism.* J Inherit Metab Dis, 2012. **35**(4): p. 665-70.
234. Lawrance, A.K., et al., *Complete deficiency of methylenetetrahydrofolate reductase in mice is associated with impaired retinal function and variable mortality, hematological profiles, and reproductive outcomes.* J Inherit Metab Dis, 2011. **34**(1): p. 147-57.
235. Jadavji, N.M., et al., *Severe methylenetetrahydrofolate reductase deficiency in mice results in behavioral anomalies with morphological and biochemical changes in hippocampus.* Mol Genet Metab, 2012. **106**(2): p. 149-59.
236. Sontag, J.M., et al., *Regulation of protein phosphatase 2A methylation by LCMT1 and PME-1 plays a critical role in differentiation of neuroblastoma cells.* J Neurochem, 2010. **115**(6): p. 1455-65.
237. Oldreive, C.E. and G.H. Doherty, *Neurotoxic effects of homocysteine on cerebellar Purkinje neurons in vitro.* Neurosci Lett, 2007. **413**(1): p. 52-7.
238. Girotto, F., et al., *High dose folic acid supplementation of rats alters synaptic transmission and seizure susceptibility in offspring.* Sci Rep, 2013. **3**: p. 1465.
239. Poddar, R. and S. Paul, *Homocysteine–NMDA receptor-mediated activation of extracellular signal-regulated kinase leads to neuronal cell death.* Journal of neurochemistry, 2009. **110**(3): p. 1095-1106.
240. Christie, L.A., G. Riedel, and B. Platt, *Bi-directional alterations of LTP after acute homocysteine exposure.* Behav Brain Res, 2009. **205**(2): p. 559-63.
241. Algaidi, S.A., et al., *Long-term homocysteine exposure induces alterations in spatial learning, hippocampal signalling and synaptic plasticity.* Exp Neurol, 2006. **197**(1): p. 8-21.
242. Pierozan, P., et al., *Synergistic Toxicity of the Neurometabolites Quinolinic Acid and Homocysteine in Cortical Neurons and Astrocytes: Implications in Alzheimer's Disease.* Neurotox Res, 2018. **34**(1): p. 147-163.
243. Vitvitsky, V., et al., *A functional transsulfuration pathway in the brain links to glutathione homeostasis.* J Biol Chem, 2006. **281**(47): p. 35785-93.
244. Taoka, S., et al., *Evidence for heme-mediated redox regulation of human cystathionine beta-synthase activity.* J Biol Chem, 1998. **273**(39): p. 25179-84.
245. Chen, Z., S. Chakraborty, and R. Banerjee, *Demonstration that mammalian methionine synthases are predominantly cobalamin-loaded.* J Biol Chem, 1995. **270**(33): p. 19246-9.
246. Mosharov, E., M.R. Cranford, and R. Banerjee, *The Quantitatively Important Relationship between Homocysteine Metabolism and Glutathione Synthesis by the Transsulfuration Pathway and Its Regulation by Redox Changes.* Biochemistry, 2000. **39**(42): p. 13005-13011.
247. Saharan, S. and P.K. Mandal, *The emerging role of glutathione in Alzheimer's disease.* J Alzheimers Dis, 2014. **40**(3): p. 519-29.
248. Ovey, I.S. and M. Naziroglu, *Homocysteine and cytosolic GSH depletion induce apoptosis and oxidative toxicity through cytosolic calcium overload in the hippocampus of aged mice: involvement of TRPM2 and TRPV1 channels.* Neuroscience, 2015. **284**: p. 225-33.
249. Akchiche, N., et al., *Homocysteinylation of neuronal proteins contributes to folate deficiency-associated alterations of differentiation, vesicular transport, and plasticity in hippocampal neuronal cells.* FASEB J, 2012. **26**(10): p. 3980-92.
250. Rezk, B.M., et al., *Tetrahydrofolate and 5-methyltetrahydrofolate are folates with high antioxidant activity. Identification of the antioxidant pharmacophore.* FEBS Lett, 2003. **555**(3): p. 601-5.
251. Huang, R.F., et al., *Folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers.* J Nutr, 2001. **131**(1): p. 33-8.

252. Coppola, A., et al., *Reduced in vivo oxidative stress following 5-methyltetrahydrofolate supplementation in patients with early-onset thrombosis and 677TT methylenetetrahydrofolate reductase genotype.* Br J Haematol, 2005. **131**(1): p. 100-8.
253. Joshi, R., et al., *Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity.* Free Radic Biol Med, 2001. **30**(12): p. 1390-9.
254. Nakano, E., J.A. Higgins, and H.J. Powers, *Folate protects against oxidative modification of human LDL.* Br J Nutr, 2001. **86**(6): p. 637-9.
255. Lai, K.G., et al., *Novel roles of folic acid as redox regulator: Modulation of reactive oxygen species sinker protein expression and maintenance of mitochondrial redox homeostasis on hepatocellular carcinoma.* Tumour Biol, 2017. **39**(6): p. 1010428317702649.
256. Stanhewicz, A.E. and W.L. Kenney, *Role of folic acid in nitric oxide bioavailability and vascular endothelial function.* Nutr Rev, 2017. **75**(1): p. 61-70.
257. McCampbell, A., et al., *Induction of Alzheimer's-like changes in brain of mice expressing mutant APP fed excess methionine.* J Neurochem, 2011. **116**(1): p. 82-92.
258. Sontag, E., et al., *Protein phosphatase 2A methyltransferase links homocysteine metabolism with tau and amyloid precursor protein regulation.* J Neurosci, 2007. **27**(11): p. 2751-9.
259. Fuso, A., et al., *B-vitamin deprivation induces hyperhomocysteinemia and brain S-adenosylhomocysteine, depletes brain S-adenosylmethionine, and enhances PS1 and BACE expression and amyloid-β deposition in mice.* Molecular and Cellular Neuroscience, 2008. **37**(4): p. 731-746.
260. Zhuo, J.M. and D. Pratico, *Acceleration of brain amyloidosis in an Alzheimer's disease mouse model by a folate, vitamin B6 and B12-deficient diet.* Exp Gerontol, 2010. **45**(3): p. 195-201.
261. Kruman, II, et al., *Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease.* J Neurosci, 2002. **22**(5): p. 1752-62.
262. Pacheco-Quinto, J., et al., *Hyperhomocysteinemic Alzheimer's mouse model of amyloidosis shows increased brain amyloid beta peptide levels.* Neurobiol Dis, 2006. **22**(3): p. 651-6.
263. Sontag, J.M., et al., *Altered protein phosphatase 2A methylation and Tau phosphorylation in the young and aged brain of methylenetetrahydrofolate reductase (MTHFR) deficient mice.* Front Aging Neurosci, 2014. **6**: p. 214.
264. Chen, Z., et al., *Postnatal cerebellar defects in mice deficient in methylenetetrahydrofolate reductase.* Int J Dev Neurosci, 2005. **23**(5): p. 465-74.
265. Jadavji, N.M., et al., *Mouse model for deficiency of methionine synthase reductase exhibits short-term memory impairment and disturbances in brain choline metabolism.* Biochem J, 2014. **461**(2): p. 205-12.
266. Bottiglieri, T., *S-Adenosyl-L-methionine (SAMe): from the bench to the bedside--molecular basis of a pleiotrophic molecule.* Am J Clin Nutr, 2002. **76**(5): p. 1151S-7S.
267. Chan, A., et al., *Dietary and genetic compromise in folate availability reduces acetylcholine, cognitive performance and increases aggression: Critical role of S-adenosyl methionine.* The Journal of Nutrition Health and Aging, 2008. **12**(4): p. 252-261.
268. Galletti, P., et al., *Transport and metabolism of double-labelled CDPcholine in mammalian tissues.* Biochem Pharmacol, 1985. **34**(23): p. 4121-30.
269. Zeisel, S.H., *Choline: needed for normal development of memory.* J Am Coll Nutr, 2000. **19**(5 Suppl): p. 528S-531S.
270. Williams, A.C. and D.B. Ramsden, *Nicotinamide homeostasis: a xenobiotic pathway that is key to development and degenerative diseases.* Med Hypotheses, 2005. **65**(2): p. 353-62.

271. Kohlmeier, M., et al., *Genetic variation of folate-mediated one-carbon transfer pathway predicts susceptibility to choline deficiency in humans.* Proc Natl Acad Sci U S A, 2005. **102**(44): p. 16025-30.
272. Zhang, C.E., et al., *Homocysteine induces tau phosphorylation by inactivating protein phosphatase 2A in rat hippocampus.* Neurobiol Aging, 2008. **29**(11): p. 1654-65.
273. Chai, G.S., et al., *Betaine attenuates Alzheimer-like pathological changes and memory deficits induced by homocysteine.* J Neurochem, 2013. **124**(3): p. 388-96.
274. Kins, S., et al., *Activation of the ERK and JNK signaling pathways caused by neuron-specific inhibition of PP2A in transgenic mice.* Am J Pathol, 2003. **163**(3): p. 833-43.
275. Park, S.S., et al., *Asp664 cleavage of amyloid precursor protein induces tau phosphorylation by decreasing protein phosphatase 2A activity.* J Neurochem, 2012. **123**(5): p. 856-65.
276. Yoon, S.Y., et al., *BACE inhibitor reduces APP-beta-C-terminal fragment accumulation in axonal swellings of okadaic acid-induced neurodegeneration.* Neurobiol Dis, 2006. **22**(2): p. 435-44.
277. Zhang, J.W., et al., *Mechanism of protein phosphatase-2Aregulating phosphorylation of amyloid precursor proteosome and Abeta generation.* Bratisl Lek Listy, 2015. **116**(3): p. 184-90.
278. Liu, C., et al., *Resveratrol prevents cadmium activation of Erk1/2 and JNK pathways from neuronal cell death via protein phosphatases 2A and 5.* J Neurochem, 2015. **135**(3): p. 466-78.
279. Johnson, D.A., et al., *Dynamics of cAMP-dependent protein kinase.* Chem Rev, 2001. **101**(8): p. 2243-70.
280. Tasken, K. and E.M. Aandahl, *Localized effects of cAMP mediated by distinct routes of protein kinase A.* Physiol Rev, 2004. **84**(1): p. 137-67.
281. Sample, V., et al., *Regulation of nuclear PKA revealed by spatiotemporal manipulation of cyclic AMP.* Nat Chem Biol, 2012. **8**(4): p. 375-82.
282. Ofir, R., et al., *CREB represses transcription of fos promoter: role of phosphorylation.* Gene Expr, 1991. **1**(1): p. 55-60.
283. Impey, S., et al., *Cross Talk between ERK and PKA Is Required for Ca2+ Stimulation of CREB-Dependent Transcription and ERK Nuclear Translocation.* Neuron, 1998. **21**(4): p. 869-883.
284. Ahn, S., D.D. Ginty, and D.J. Linden, *A late phase of cerebellar long-term depression requires activation of CaMKIV and CREB.* Neuron, 1999. **23**(3): p. 559-68.
285. Sun, C., et al., *Mechanisms for luteinizing hormone induction of growth hormone gene transcription in fish model: crosstalk of the cAMP/PKA pathway with MAPK-and PI3K-dependent cascades.* Mol Cell Endocrinol, 2014. **382**(2): p. 835-50.
286. Radhakrishnan, I., et al., *Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions.* Cell, 1997. **91**(6): p. 741-52.
287. Heinrich, A., et al., *Lithium enhances CRTC oligomer formation and the interaction between the CREB coactivators CRTC and CBP--implications for CREB-dependent gene transcription.* Cell Signal, 2013. **25**(1): p. 113-25.
288. Aglah, C., T. Gordon, and E.I. Posse de Chaves, *cAMP promotes neurite outgrowth and extension through protein kinase A but independently of Erk activation in cultured rat motoneurons.* Neuropharmacology, 2008. **55**(1): p. 8-17.
289. Goto, A., et al., *Phosphorylation of STEF/Tiam2 by protein kinase A is critical for Rac1 activation and neurite outgrowth in dibutyryl cAMP-treated PC12D cells.* Mol Biol Cell, 2011. **22**(10): p. 1780-90.
290. Jessen, U., et al., *The transcription factors CREB and c-Fos play key roles in NCAM-mediated neuritogenesis in PC12-E2 cells.* J Neurochem, 2001. **79**(6): p. 1149-60.
291. Wan, G., et al., *Cyclic AMP signalling through PKA but not Epac is essential for neurturin-induced biphasic ERK1/2 activation and neurite outgrowths through GFRalpha2 isoforms.* Cell Signal, 2011. **23**(11): p. 1727-37.

292. Munck, S., et al., *Spatiotemporal properties of cytoplasmic cyclic AMP gradients can alter the turning behaviour of neuronal growth cones.* Eur J Neurosci, 2004. **19**(4): p. 791-7.
293. Nicol, X., K.P. Hong, and N.C. Spitzer, *Spatial and temporal second messenger codes for growth cone turning.* Proc Natl Acad Sci U S A, 2011. **108**(33): p. 13776-81.
294. Tojima, T., et al., *Second messengers and membrane trafficking direct and organize growth cone steering.* Nat Rev Neurosci, 2011. **12**(4): p. 191-203.
295. Han, J., et al., *Spatial targeting of type II protein kinase A to filopodia mediates the regulation of growth cone guidance by cAMP.* J Cell Biol, 2007. **176**(1): p. 101-11.
296. Abel, T., et al., *Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory.* Cell, 1997. **88**(5): p. 615-26.
297. Schafe, G.E. and J.E. LeDoux, *Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala.* J Neurosci, 2000. **20**(18): p. RC96.
298. Lee, H.K., et al., *Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity.* Nature, 2000. **405**(6789): p. 955-9.
299. Loebrich, S., et al., *Regulation of glutamate receptor internalization by the spine cytoskeleton is mediated by its PKA-dependent association with CPG2.* Proc Natl Acad Sci U S A, 2013. **110**(47): p. E4548-56.
300. Banke, T.G., et al., *Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase.* J Neurosci, 2000. **20**(1): p. 89-102.
301. Diering, G.H., A.S. Gustina, and R.L. Huganir, *PKA-GluA1 coupling via AKAP5 controls AMPA receptor phosphorylation and cell-surface targeting during bidirectional homeostatic plasticity.* Neuron, 2014. **84**(4): p. 790-805.
302. Bernabeu, R., et al., *Involvement of hippocampal cAMP/cAMP-dependent protein kinase signaling pathways in a late memory consolidation phase of aversively motivated learning in rats.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 7041-6.
303. Bourtchuladze, R., et al., *Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein.* Cell, 1994. **79**(1): p. 59-68.
304. Deisseroth, K., H. Bito, and R.W. Tsien, *Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity.* Neuron, 1996. **16**(1): p. 89-101.
305. Martin, K.C., et al., *Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage.* Cell, 1997. **91**(7): p. 927-38.
306. Stevens, C.F., *CREB and memory consolidation.* Neuron, 1994. **13**(4): p. 769-70.
307. Middei, S., et al., *CREB is necessary for synaptic maintenance and learning-induced changes of the AMPA receptor GluA1 subunit.* Hippocampus, 2013. **23**(6): p. 488-99.
308. Sargin, D., et al., *CREB regulates spine density of lateral amygdala neurons: implications for memory allocation.* Front Behav Neurosci, 2013. **7**: p. 209.
309. Serita, T., H. Fukushima, and S. Kida, *Constitutive activation of CREB in mice enhances temporal association learning and increases hippocampal CA1 neuronal spine density and complexity.* Sci Rep, 2017. **7**: p. 42528.
310. Hao, J.R., et al., *L-Stepholidine rescues memory deficit and synaptic plasticity in models of Alzheimer's disease via activating dopamine D1 receptor/PKA signaling pathway.* Cell Death Dis, 2015. **6**: p. e1965.
311. Otmakhova, N.A. and J.E. Lisman, *D1/D5 dopamine receptor activation increases the magnitude of early long-term potentiation at CA1 hippocampal synapses.* J Neurosci, 1996. **16**(23): p. 7478-86.
312. Stramiello, M. and J.J. Wagner, *D1/5 receptor-mediated enhancement of LTP requires PKA, Src family kinases, and NR2B-containing NMDARs.* Neuropharmacology, 2008. **55**(5): p. 871-7.
313. Snyder, G.L., et al., *A dopamine/D1 receptor/protein kinase A/dopamine- and cAMP-regulated phosphoprotein (Mr 32 kDa)/protein phosphatase-1 pathway regulates dephosphorylation of the NMDA receptor.* J Neurosci, 1998. **18**(24): p. 10297-303.

314. Skeberdis, V.A., et al., *Protein kinase A regulates calcium permeability of NMDA receptors.* Nat Neurosci, 2006. **9**(4): p. 501-10.
315. Raman, I.M., G. Tong, and C.E. Jahr, *Beta-adrenergic regulation of synaptic NMDA receptors by cAMP-dependent protein kinase.* Neuron, 1996. **16**(2): p. 415-21.
316. Murphy, J.A., et al., *Phosphorylation of Ser1166 on GluN2B by PKA is critical to synaptic NMDA receptor function and Ca2+ signaling in spines.* J Neurosci, 2014. **34**(3): p. 869-79.
317. Monti, B., L. Marri, and A. Contestabile, *NMDA receptor-dependent CREB activation in survival of cerebellar granule cells during in vivo and in vitro development.* Eur J Neurosci, 2002. **16**(8): p. 1490-8.
318. Riccio, A., et al., *Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons.* Science, 1999. **286**(5448): p. 2358-61.
319. Fukuchi, M., et al., *Excitatory GABA induces BDNF transcription via CRTC1 and phosphorylated CREB-related pathways in immature cortical cells.* J Neurochem, 2014. **131**(2): p. 134-46.
320. Herold, S., et al., *CREB signalling regulates early survival, neuronal gene expression and morphological development in adult subventricular zone neurogenesis.* Mol Cell Neurosci, 2011. **46**(1): p. 79-88.
321. Tan, Y.W., et al., *Increasing levels of wild-type CREB up-regulates several activity-regulated inhibitor of death (AID) genes and promotes neuronal survival.* BMC Neurosci, 2012. **13**: p. 48.
322. Du, H., et al., *Cyclophilin D deficiency rescues Abeta-impaired PKA/CREB signaling and alleviates synaptic degeneration.* Biochim Biophys Acta, 2014. **1842**(12 Pt A): p. 2517-27.
323. Yamamoto-Sasaki, M., et al., *Impaired phosphorylation of cyclic AMP response element binding protein in the hippocampus of dementia of the Alzheimer type.* Brain Res, 1999. **824**(2): p. 300-3.
324. Liang, Z., et al., *Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain.* J Neurochem, 2007. **103**(6): p. 2462-70.
325. Sadik, G., et al., *Phosphorylation of tau at Ser214 mediates its interaction with 14-3-3 protein: implications for the mechanism of tau aggregation.* J Neurochem, 2009. **108**(1): p. 33-43.
326. Shi, J., et al., *Cyclic AMP-dependent protein kinase regulates the alternative splicing of tau exon 10: a mechanism involved in tau pathology of Alzheimer disease.* J Biol Chem, 2011. **286**(16): p. 14639-48.
327. Ksiezak-Reding, H., B. Shafit-Zagardo, and S.H. Yen, *Differential expression of exons 10 and 11 in normal tau and tau associated with paired helical filaments.* J Neurosci Res, 1995. **41**(5): p. 583-93.
328. Vitolo, O.V., et al., *Amyloid beta -peptide inhibition of the PKA/CREB pathway and long-term potentiation: reversibility by drugs that enhance cAMP signaling.* Proc Natl Acad Sci U S A, 2002. **99**(20): p. 13217-21.
329. Gong, B., et al., *Persistent improvement in synaptic and cognitive functions in an Alzheimer mouse model after rolipram treatment.* The Journal of Clinical Investigation, 2004. **114**(11): p. 1624-1634.
330. Cheng, Y.F., et al., *Inhibition of phosphodiesterase-4 reverses memory deficits produced by Abeta25-35 or Abeta1-40 peptide in rats.* Psychopharmacology (Berl), 2010. **212**(2): p. 181-91.
331. Carlyle, B.C., et al., *cAMP-PKA phosphorylation of tau confers risk for degeneration in aging association cortex.* Proc Natl Acad Sci U S A, 2014. **111**(13): p. 5036-41.
332. Giralt, A., et al., *Increased PKA signaling disrupts recognition memory and spatial memory: role in Huntington's disease.* Hum Mol Genet, 2011. **20**(21): p. 4232-47.
333. Feschenko, M.S., et al., *A novel cAMP-stimulated pathway in protein phosphatase 2A activation.* J Pharmacol Exp Ther, 2002. **302**(1): p. 111-8.

334. Floer, M. and J. Stock, *Carboxyl methylation of protein phosphatase 2A from Xenopus eggs is stimulated by cAMP and inhibited by okadaic acid.* Biochem Biophys Res Commun, 1994. **198**(1): p. 372-9.
335. Wadzinski, B.E., et al., *Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation.* Mol Cell Biol, 1993. **13**(5): p. 2822-34.
336. Zakany, R., et al., *Protein phosphatase 2A is involved in the regulation of protein kinase A signaling pathway during in vitro chondrogenesis.* Exp Cell Res, 2002. **275**(1): p. 1-8.
337. Mauna, J.C., et al., *Protein phosphatases 1 and 2A are both required for long-term depression and associated dephosphorylation of cAMP response element binding protein in hippocampal area CA1 in vivo.* Hippocampus, 2011. **21**(10): p. 1093-104.
338. Nicholls, R.E., et al., *PP2A methylation controls sensitivity and resistance to beta-amyloid-induced cognitive and electrophysiological impairments.* Proc Natl Acad Sci U S A, 2016.
339. Troen, A.M., et al., *Cognitive impairment in folate-deficient rats corresponds to depleted brain phosphatidylcholine and is prevented by dietary methionine without lowering plasma homocysteine.* J Nutr, 2008. **138**(12): p. 2502-9.
340. Pirchl, M., C. Ullrich, and C. Humpel, *Differential effects of short- and long-term hyperhomocysteinaemia on cholinergic neurons, spatial memory and microbleedings in vivo in rats.* Eur J Neurosci, 2010. **32**(9): p. 1516-27.
341. El Hajj Chehadeh, S., et al., *Early methyl donor deficiency alters cAMP signaling pathway and neurosteroidogenesis in the cerebellum of female rat pups.* Am J Physiol Endocrinol Metab, 2014. **307**(11): p. E1009-19.
342. Ehrlich, D. and C. Humpel, *Chronic vascular risk factors (cholesterol, homocysteine, ethanol) impair spatial memory, decline cholinergic neurons and induce blood-brain barrier leakage in rats in vivo.* J Neurol Sci, 2012. **322**(1-2): p. 92-5.
343. Rosenberger, A.F., et al., *Protein Kinase Activity Decreases with Higher Braak Stages of Alzheimer's Disease Pathology.* J Alzheimers Dis, 2015. **49**(4): p. 927-43.
344. Okada, M., *Regulation of the SRC family kinases by Csk.* Int J Biol Sci, 2012. **8**(10): p. 1385-97.
345. Thomas, S.M. and J.S. Brugge, *Cellular functions regulated by Src family kinases.* Annu Rev Cell Dev Biol, 1997. **13**: p. 513-609.
346. Brown, M.T. and J.A. Cooper, *Regulation, substrates and functions of src.* Biochim Biophys Acta, 1996. **1287**(2-3): p. 121-49.
347. Zheng, X.M., Y. Wang, and C.J. Pallen, *Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase.* Nature, 1992. **359**(6393): p. 336-9.
348. Bagrodia, S., S.J. Taylor, and D. Shalloway, *Myristylation is required for Tyr-527 dephosphorylation and activation of pp60c-src in mitosis.* Mol Cell Biol, 1993. **13**(3): p. 1464-70.
349. Barthet, G., et al., *5-hydroxytryptamine 4 receptor activation of the extracellular signal-regulated kinase pathway depends on Src activation but not on G protein or beta-arrestin signaling.* Mol Biol Cell, 2007. **18**(6): p. 1979-91.
350. Schmid, C.L. and L.M. Bohn, *Serotonin, but not N-methyltryptamines, activates the serotonin 2A receptor via a ss-arrestin2/Src/Akt signaling complex in vivo.* J Neurosci, 2010. **30**(40): p. 13513-24.
351. Matsuoka, H. and M. Inoue, *Src mediates endocytosis of TWIK-related acid-sensitive K+ 1 channels in PC12 cells in response to nerve growth factor.* Am J Physiol Cell Physiol, 2015. **309**(4): p. C251-63.
352. Kruk, J.S., et al., *5-HT(1A) receptors transactivate the platelet-derived growth factor receptor type beta in neuronal cells.* Cell Signal, 2013. **25**(1): p. 133-43.
353. Chowdhury, D., et al., *Tyrosine phosphorylation regulates the endocytosis and surface expression of GluN3A-containing NMDA receptors.* J Neurosci, 2013. **33**(9): p. 4151-64.

354. Tompkins, J.D., et al., *Src family kinase inhibitors blunt PACAP-induced PAC1 receptor endocytosis, phosphorylation of ERK, and the increase in cardiac neuron excitability.* Am J Physiol Cell Physiol, 2018. **314**(2): p. C233-c241.
355. He, Y., et al., *Src and cortactin promote lamellipodia protrusion and filopodia formation and stability in growth cones.* Mol Biol Cell, 2015. **26**(18): p. 3229-44.
356. Sharma, V.M., et al., *Tau impacts on growth-factor-stimulated actin remodeling.* J Cell Sci, 2007. **120**(Pt 5): p. 748-57.
357. Gainetdinov, R.R., et al., *Desensitization of G protein-coupled receptors and neuronal functions.* Annu Rev Neurosci, 2004. **27**: p. 107-44.
358. Miller, W.E., et al., *beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis.* J Biol Chem, 2000. **275**(15): p. 11312-9.
359. Goodfellow, N.M., et al., *Mice with compromised 5-HTT function lack phosphotyrosine-mediated inhibitory control over prefrontal 5-HT responses.* J Neurosci, 2014. **34**(17): p. 6107-11.
360. Grant, S.G., et al., *Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice.* Science, 1992. **258**(5090): p. 1903-10.
361. Lu, Y.M., et al., *Src activation in the induction of long-term potentiation in CA1 hippocampal neurons.* Science, 1998. **279**(5355): p. 1363-7.
362. Lu, W., et al., *Phosphorylation of Tyrosine 1070 at the GluN2B Subunit Is Regulated by Synaptic Activity and Critical for Surface Expression of N-Methyl-D-aspartate (NMDA) Receptors.* J Biol Chem, 2015. **290**(38): p. 22945-54.
363. Ahmadian, G., et al., *Tyrosine phosphorylation of GluR2 is required for insulin-stimulated AMPA receptor endocytosis and LTD.* EMBO J, 2004. **23**(5): p. 1040-50.
364. Zhao, W.Q., D.L. Alkon, and W. Ma, *c-Src protein tyrosine kinase activity is required for muscarinic receptor-mediated DNA synthesis and neurogenesis via ERK1/2 and c-AMP-responsive element-binding protein signaling in neural precursor cells.* J Neurosci Res, 2003. **72**(3): p. 334-42.
365. Di Liberto, V., et al., *Existence of muscarinic acetylcholine receptor (mAChR) and fibroblast growth factor receptor (FGFR) heteroreceptor complexes and their enhancement of neurite outgrowth in neural hippocampal cultures.* Biochim Biophys Acta, 2017. **1861**(2): p. 235-245.
366. Maness, P.F., et al., *c-src gene product in developing rat brain is enriched in nerve growth cone membranes.* Proc Natl Acad Sci U S A, 1988. **85**(14): p. 5001-5.
367. Morse, W.R., et al., *p59fyn and pp60c-src modulate axonal guidance in the developing mouse olfactory pathway.* J Neurobiol, 1998. **36**(1): p. 53-63.
368. Roof, R.W., et al., *Phosphotyrosine (p-Tyr)-dependent and -independent mechanisms of p190 RhoGAP-p120 RasGAP interaction: Tyr 1105 of p190, a substrate for c-Src, is the sole p-Tyr mediator of complex formation.* Mol Cell Biol, 1998. **18**(12): p. 7052-63.
369. Brouns, M.R., et al., *The adhesion signaling molecule p190 RhoGAP is required for morphogenetic processes in neural development.* Development, 2000. **127**(22): p. 4891-903.
370. Brouns, M.R., S.F. Matheson, and J. Settleman, *p190 RhoGAP is the principal Src substrate in brain and regulates axon outgrowth, guidance and fasciculation.* Nat Cell Biol, 2001. **3**(4): p. 361-7.
371. Zhao, H., et al., *Neurite outgrowth is dependent on the association of c-Src and lipid rafts.* Neurochem Res, 2009. **34**(12): p. 2197-205.
372. Brandt, R., J. Leger, and G. Lee, *Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain.* J Cell Biol, 1995. **131**(5): p. 1327-40.
373. Pimenova, A.A., et al., *Regulation of amyloid precursor protein processing by serotonin signaling.* PLoS One, 2014. **9**(1): p. e87014.

374. Sullivan, S.E., et al., *Mint proteins are required for synaptic activity-dependent amyloid precursor protein (APP) trafficking and amyloid beta generation.* J Biol Chem, 2014. **289**(22): p. 15374-83.
375. Dunning, C.J., et al., *Multisite tyrosine phosphorylation of the N-terminus of Mint1/X11alpha by Src kinase regulates the trafficking of amyloid precursor protein.* J Neurochem, 2016. **137**(4): p. 518-27.
376. Chaufty, J., S.E. Sullivan, and A. Ho, *Intracellular amyloid precursor protein sorting and amyloid-beta secretion are regulated by Src-mediated phosphorylation of Mint2.* J Neurosci, 2012. **32**(28): p. 9613-25.
377. Canet-Aviles, R.M., et al., *Muscarine enhances soluble amyloid precursor protein secretion in human neuroblastoma SH-SY5Y by a pathway dependent on protein kinase C(alpha), src-tyrosine kinase and extracellular signal-regulated kinase but not phospholipase C.* Brain Res Mol Brain Res, 2002. **102**(1-2): p. 62-72.
378. Nygaard, H.B., C.H. van Dyck, and S.M. Strittmatter, *Fyn kinase inhibition as a novel therapy for Alzheimer's disease.* Alzheimers Res Ther, 2014. **6**(1): p. 8.
379. Mota, S.I., et al., *Impaired Src signaling and post-synaptic actin polymerization in Alzheimer's disease mice hippocampus - Linking NMDA receptors and the reelin pathway.* Exp Neurol, 2014. **261C**: p. 698-709.
380. Dhawan, G. and C.K. Combs, *Inhibition of Src kinase activity attenuates amyloid associated microgliosis in a murine model of Alzheimer's disease.* J Neuroinflammation, 2012. **9**: p. 117.
381. Gianni, D., et al., *Platelet-derived growth factor induces the beta-gamma-secretase-mediated cleavage of Alzheimer's amyloid precursor protein through a Src-Rac-dependent pathway.* J Biol Chem, 2003. **278**(11): p. 9290-7.
382. Cheng, P., et al., *Protein phosphatase 2A (PP2A) activation promotes axonal growth and recovery in the CNS.* J Neurol Sci, 2015. **359**(1-2): p. 48-56.
383. Dudiki, T., et al., *Changes in Carboxy Methylation and Tyrosine Phosphorylation of Protein Phosphatase PP2A Are Associated with Epididymal Sperm Maturation and Motility.* PLoS One, 2015. **10**(11): p. e0141961.
384. Neviani, P., et al., *The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein.* Cancer Cell, 2005. **8**(5): p. 355-68.
385. Wong, L.L., et al., *Tyrosine phosphorylation of PP2A is regulated by HER-2 signalling and correlates with breast cancer progression.* Int J Oncol, 2009. **34**(5): p. 1291-301.
386. Ogris, E., et al., *Specificity of research antibodies: "trust is good, validation is better".* Hum Pathol, 2018. **72**: p. 199-201.
387. Hu, X., et al., *Src kinase up-regulates the ERK cascade through inactivation of protein phosphatase 2A following cerebral ischemia.* BMC Neurosci, 2009. **10**: p. 74.
388. Williamson, R., et al., *Rapid tyrosine phosphorylation of neuronal proteins including tau and focal adhesion kinase in response to amyloid-beta peptide exposure: involvement of Src family protein kinases.* J Neurosci, 2002. **22**(1): p. 10-20.
389. Mao, L., et al., *Role of protein phosphatase 2A in mGluR5-regulated MEK/ERK phosphorylation in neurons.* J Biol Chem, 2005. **280**(13): p. 12602-10.
390. Arif, M., et al., *Tau pathology involves protein phosphatase 2A in Parkinsonism-dementia of Guam.* Proc Natl Acad Sci U S A, 2014. **111**(3): p. 1144-9.
391. Yang, C.C., et al., *Morroniside-Induced PP2A Activation Antagonizes Tau Hyperphosphorylation in a Cellular Model of Neurodegeneration.* J Alzheimers Dis, 2016. **51**(1): p. 33-44.
392. Yokoyama, N. and W.T. Miller, *Inhibition of Src by direct interaction with protein phosphatase 2A.* FEBS Lett, 2001. **505**(3): p. 460-4.
393. Kamat, P.K., S. Rai, and C. Nath, *Okadaic acid induced neurotoxicity: an emerging tool to study Alzheimer's disease pathology.* Neurotoxicology, 2013. **37**: p. 163-72.
394. Kamat, P.K., et al., *Molecular and cellular mechanism of okadaic acid (OKA)-induced neurotoxicity: a novel tool for Alzheimer's disease therapeutic application.* Mol Neurobiol, 2014. **50**(3): p. 852-65.

395. Sontag, E., et al., *Regulation of the phosphorylation state and microtubule-binding activity of Tau by protein phosphatase 2A.* Neuron, 1996. **17**(6): p. 1201-7.
396. Bottiglieri, T., et al., *Acute administration of L-DOPA induces changes in methylation metabolites, reduced protein phosphatase 2A methylation, and hyperphosphorylation of Tau protein in mouse brain.* J Neurosci, 2012. **32**(27): p. 9173-81.
397. Sable, P., et al., *Maternal micronutrient imbalance alters gene expression of BDNF, NGF, TrkB and CREB in the offspring brain at an adult age.* Int J Dev Neurosci, 2014. **34**: p. 24-32.
398. Barisic, S., et al., *Tyrosine phosphatase inhibition triggers sustained canonical serine-dependent NFkappaB activation via Src-dependent blockade of PP2A.* Biochem Pharmacol, 2010. **80**(4): p. 439-47.
399. Michaelson, D., et al., *Rac1 accumulates in the nucleus during the G2 phase of the cell cycle and promotes cell division.* J Cell Biol, 2008. **181**(3): p. 485-96.
400. Longair, M.H., D.A. Baker, and J.D. Armstrong, *Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes.* Bioinformatics, 2011. **27**(17): p. 2453-2454.
401. de Oliveira, R.B., et al., *Ketamine anesthesia helps preserve neuronal viability.* J Neurosci Methods, 2010. **189**(2): p. 230-2.
402. Science., A.I.f.B. *Allen Developing Mouse Brain Atlas* 2015 [cited 2016; Available from: http://developingmouse.brain-map.org.
403. Sontag, J.M., et al., *Folate deficiency induces in vitro and mouse brain region-specific downregulation of leucine carboxyl methyltransferase-1 and protein phosphatase 2A B(alpha) subunit expression that correlate with enhanced tau phosphorylation.* J Neurosci, 2008. **28**(45): p. 11477-87.
404. Ubbink, J.B., W.J. Hayward Vermaak, and S. Bissbort, *Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum.* J Chromatogr, 1991. **565**(1-2): p. 441-6.
405. Lee, J.A. and D.C. Pallas, *Leucine carboxyl methyltransferase-1 is necessary for normal progression through mitosis in mammalian cells.* J Biol Chem, 2007. **282**(42): p. 30974-84.
406. Tolstykh, T., et al., *Carboxyl methylation regulates phosphoprotein phosphatase 2A by controlling the association of regulatory B subunits.* Embo j, 2000. **19**(21): p. 5682-91.
407. Cristobal, I., et al., *PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect.* Leukemia, 2011. **25**(4): p. 606-14.
408. Kelly, M.P., et al., *Select 3',5'-cyclic nucleotide phosphodiesterases exhibit altered expression in the aged rodent brain.* Cell Signal, 2014. **26**(2): p. 383-97.
409. Dell'Acqua, M.L., et al., *Regulation of neuronal PKA signaling through AKAP targeting dynamics.* Eur J Cell Biol, 2006. **85**(7): p. 627-33.
410. Antoni, F.A., *New paradigms in cAMP signalling.* Mol Cell Endocrinol, 2012. **353**(1-2): p. 3-9.
411. Vanhoose, A.M., M.D. Ritchie, and D.G. Winder, *Regulation of cAMP levels in area CA1 of hippocampus by Gi/o-coupled receptors is stimulus dependent in mice.* Neurosci Lett, 2004. **370**(1): p. 80-3.
412. Shmueli, O., et al., *DCX in PC12 cells: CREB-mediated transcription and neurite outgrowth.* Hum Mol Genet, 2001. **10**(10): p. 1061-70.
413. Dhar, M., et al., *Leptin induces hippocampal synaptogenesis via CREB-regulated microRNA-132 suppression of p250GAP.* Mol Endocrinol, 2014. **28**(7): p. 1073-87.
414. Liu, H., et al., *PKA-CREB Signaling Suppresses Tau Transcription.* J Alzheimers Dis, 2015. **46**(1): p. 239-48.
415. Middei, S., et al., *CREB selectively controls learning-induced structural remodeling of neurons.* Learn Mem, 2012. **19**(8): p. 330-6.
416. Ghee, M., et al., *AP-1, CREB and CBP transcription factors differentially regulate the tyrosine hydroxylase gene.* Brain Res Mol Brain Res, 1998. **55**(1): p. 101-14.

417. Boyarskikh, U.A., et al., *Downregulation of serotonergic gene expression in the Raphe nuclei of the midbrain under chronic social defeat stress in male mice.* Mol Neurobiol, 2013. **48**(1): p. 13-21.
418. Tong, L., et al., *Beta -amyloid-(1-42) impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival Is not compromised.* J Biol Chem, 2001. **276**(20): p. 17301-6.
419. Yiu, A.P., A.J. Rashid, and S.A. Josselyn, *Increasing CREB Function in the CA1 Region of Dorsal Hippocampus Rescues the Spatial Memory Deficits in a Mouse Model of Alzheimer's Disease.* Neuropsychopharmacology, 2011. **36**: p. 2169.
420. Janssens, V., S. Longin, and J. Goris, *PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail).* Trends in Biochemical Sciences, 2008. **33**(3): p. 113-121.
421. Yabe, R., et al., *Protein Phosphatase Methyl-Esterase PME-1 Protects Protein Phosphatase 2A from Ubiquitin/Proteasome Degradation.* PLoS ONE, 2015. **10**(12): p. e0145226.
422. Wang, Y., et al., *Cross talk between PI3K-AKT-GSK-3beta and PP2A pathways determines tau hyperphosphorylation.* Neurobiol Aging, 2015. **36**(1): p. 188-200.
423. Yu, X.X., et al., *Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen.* Mol Biol Cell, 2001. **12**(1): p. 185-99.
424. Sjostrom, M., et al., *SIK1 is part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 16922-7.
425. Ruediger, R., et al., *Constant expression and activity of protein phosphatase 2A in synchronized cells.* Mol Cell Biol, 1991. **11**(8): p. 4282-5.
426. Ogris, E., et al., *A protein phosphatase methylesterase (PME-1) is one of several novel proteins stably associating with two inactive mutants of protein phosphatase 2A.* Journal of Biological Chemistry, 1999. **274**(20): p. 14382-91.
427. Bachovchin, D.A., et al., *Discovery and optimization of sulfonyl acrylonitriles as selective, covalent inhibitors of protein phosphatase methylesterase-1.* J Med Chem, 2011. **54**(14): p. 5229-36.
428. Ma'ayan, A., et al., *Neuro2A differentiation by Galphai/o pathway.* Sci Signal, 2009. **2**(54): p. cm1.
429. Pale, T., E.B. Frisch, and A.D. McClellan, *Cyclic AMP stimulates neurite outgrowth of lamprey reticulospinal neurons without substantially altering their biophysical properties.* Neuroscience, 2013. **245**: p. 74-89.
430. Sepe, M., et al., *Proteolytic control of neurite outgrowth inhibitor NOGO-A by the cAMP/PKA pathway.* Proc Natl Acad Sci U S A, 2014. **111**(44): p. 15729-34.
431. Moon, E.Y. and A. Lerner, *PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A.* Blood, 2003. **101**(10): p. 4122-30.
432. Hsiung, S.C., et al., *Inhibition of 5-HT1A receptor-dependent cell survival by cAMP/protein kinase A: role of protein phosphatase 2A and Bax.* J Neurosci Res, 2008. **86**(10): p. 2326-38.
433. Wang, D., et al., *Binding of amyloid beta peptide to beta2 adrenergic receptor induces PKA-dependent AMPA receptor hyperactivity.* Faseb j, 2010. **24**(9): p. 3511-21.
434. Stockwell, J., et al., *Protein phosphatase role in adenosine A1 receptor-induced AMPA receptor trafficking and rat hippocampal neuronal damage in hypoxia/reperfusion injury.* Neuropharmacology, 2016. **102**: p. 254-65.
435. Depry, C., M.D. Allen, and J. Zhang, *Visualization of PKA activity in plasma membrane microdomains.* Mol Biosyst, 2011. **7**(1): p. 52-8.
436. Tillo, S.E., et al., *Liberated PKA Catalytic Subunits Associate with the Membrane via Myristoylation to Preferentially Phosphorylate Membrane Substrates.* Cell Rep, 2017. **19**(3): p. 617-629.

437. Bryant, J.C., R.S. Westphal, and B.E. Wadzinski, *Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit.* Biochem J, 1999. **339 (Pt 2)**: p. 241-6.
438. Hagiwara, M., et al., *Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A.* Molecular and Cellular Biology, 1993. **13**(8): p. 4852-4859.
439. Hu, E., et al., *VIP, CRF, and PACAP act at distinct receptors to elicit different cAMP/PKA dynamics in the neocortex.* Cereb Cortex, 2011. **21**(3): p. 708-18.
440. Yapo, C., et al., *Switch-like PKA responses in the nucleus of striatal neurons.* J Cell Sci, 2018. **131**(14).
441. Gervasi, N., et al., *Dynamics of protein kinase A signaling at the membrane, in the cytosol, and in the nucleus of neurons in mouse brain slices.* J Neurosci, 2007. **27**(11): p. 2744-50.
442. Yang, Y.C., C.H. Lin, and E.H. Lee, *Serum- and glucocorticoid-inducible kinase 1 (SGK1) increases neurite formation through microtubule depolymerization by SGK1 and by SGK1 phosphorylation of tau.* Mol Cell Biol, 2006. **26**(22): p. 8357-70.
443. Wang, H.H., et al., *Forskolin Induces Hyperphosphorylation of Tau Accompanied by Cell Cycle Reactivation in Primary Hippocampal Neurons.* Mol Neurobiol, 2018. **55**(1): p. 696-706.
444. Kimura, T., et al., *Microtubule-associated protein tau is essential for long-term depression in the hippocampus.* Philos Trans R Soc Lond B Biol Sci, 2014. **369**(1633): p. 20130144.
445. Tian, Q., et al., *Biphasic effects of forskolin on tau phosphorylation and spatial memory in rats.* J Alzheimers Dis, 2009. **17**(3): p. 631-42.
446. Sun, L., et al., *Inhibition of protein phosphatase 2A- and protein phosphatase 1-induced tau hyperphosphorylation and impairment of spatial memory retention in rats.* Neuroscience, 2003. **118**(4): p. 1175-82.
447. Zhou, X.W., et al., *Tau hyperphosphorylation correlates with reduced methylation of protein phosphatase 2A.* Neurobiol Dis, 2008. **31**(3): p. 386-94.
448. Nicolia, V., et al., *B vitamin deficiency promotes tau phosphorylation through regulation of GSK3beta and PP2A.* J Alzheimers Dis, 2010. **19**(3): p. 895-907.
449. Chu, D., et al., *GSK-3beta is Dephosphorylated by PP2A in a Leu309 Methylation-Independent Manner.* J Alzheimers Dis, 2015.
450. Leung, Y.M., et al., *Voltage-gated K+ channels play a role in cAMP-stimulated neuritogenesis in mouse neuroblastoma N2A cells.* J Cell Physiol, 2011. **226**(4): p. 1090-8.
451. Nunbhakdi-Craig, V., et al., *Expression of protein phosphatase 2A mutants and silencing of the regulatory Bα subunit induce a selective loss of acetylated and detyrosinated microtubules.* Journal of Neurochemistry, 2007. **101**(4): p. 959-971.
452. Pooler, A.M. and D.P. Hanger, *Functional implications of the association of tau with the plasma membrane.* Biochem Soc Trans, 2010. **38**(4): p. 1012-5.
453. Titus, D.J., et al., *Age-dependent alterations in cAMP signaling contribute to synaptic plasticity deficits following traumatic brain injury.* Neuroscience, 2013. **231**: p. 182-194.
454. Ramos, B.P., et al., *Dysregulation of protein kinase a signaling in the aged prefrontal cortex: new strategy for treating age-related cognitive decline.* Neuron, 2003. **40**(4): p. 835-45.
455. Tomobe, K., Y. Okuma, and Y. Nomura, *Impairment of CREB phosphorylation in the hippocampal CA1 region of the senescence-accelerated mouse (SAM) P8.* Brain Res, 2007. **1141**: p. 214-7.
456. Chen, Y., et al., *Alzheimer's β-Secretase (BACE1) Regulates the cAMP/PKA/CREB Pathway Independently of β-Amyloid.* The Journal of Neuroscience, 2012. **32**(33): p. 11390-11395.
457. Zhang, C., et al., *RNA interference-mediated knockdown of long-form phosphodiesterase-4D (PDE4D) enzyme reverses amyloid-beta42-induced memory deficits in mice.* J Alzheimers Dis, 2014. **38**(2): p. 269-80.

458. Favre, B., et al., *The catalytic subunit of protein phosphatase 2A is carboxyl-methylated in vivo.* J Biol Chem, 1994. **269**(23): p. 16311-7.
459. Smith, A.D. and H. Refsum, *Homocysteine, B Vitamins, and Cognitive Impairment.* Annu Rev Nutr, 2016. **36**: p. 211-39.
460. Stoccoro, A., et al., *The Methylenetetrahydrofolate Reductase C677T Polymorphism and Risk for Late-Onset Alzheimer's disease: Further Evidence in an Italian Multicenter Study.* J Alzheimers Dis, 2017. **56**(4): p. 1451-1457.
461. Bartsch, D., et al., *CREB1 encodes a nuclear activator, a repressor, and a cytoplasmic modulator that form a regulatory unit critical for long-term facilitation.* Cell, 1998. **95**(2): p. 211-23.
462. Bottiglieri, T., *Folate, vitamin B(1)(2), and S-adenosylmethionine.* Psychiatr Clin North Am, 2013. **36**(1): p. 1-13.
463. Botto, L.D. and Q. Yang, *5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review.* Am J Epidemiol, 2000. **151**(9): p. 862-77.
464. Leclerc, D. and R. Rozen, *[Molecular genetics of MTHFR: polymorphisms are not all benign].* Med Sci (Paris), 2007. **23**(3): p. 297-302.
465. Nabavi, S., et al., *Engineering a memory with LTD and LTP.* Nature, 2014.
466. Kida, S., *A Functional Role for CREB as a Positive Regulator of Memory Formation and LTP.* Exp Neurobiol, 2012. **21**(4): p. 136-40.
467. Naqvi, S., K.J. Martin, and J.S. Arthur, *CREB phosphorylation at Ser133 regulates transcription via distinct mechanisms downstream of cAMP and MAPK signalling.* Biochem J, 2014. **458**(3): p. 469-79.
468. Lau, G.C., et al., *Up-regulation of NMDAR1 subunit gene expression in cortical neurons via a PKA-dependent pathway.* J Neurochem, 2004. **88**(3): p. 564-75.
469. Viggiano, A., et al., *Methionine-enriched diet decreases hippocampal antioxidant defences and impairs spontaneous behaviour and long-term potentiation in rats.* Brain Res, 2012. **1471**: p. 66-74.
470. Christie, L.A., et al., *Enhanced hippocampal long-term potentiation in rats after chronic exposure to homocysteine.* Neurosci Lett, 2005. **373**(2): p. 119-24.
471. Buard, I., et al., *CaMKII "autonomy" is required for initiating but not for maintaining neuronal long-term information storage.* J Neurosci, 2010. **30**(24): p. 8214-20.
472. Kaeser-Woo, Y.J., et al., *Synaptotagmin-12 phosphorylation by cAMP-dependent protein kinase is essential for hippocampal mossy fiber LTP.* J Neurosci, 2013. **33**(23): p. 9769-80.
473. Woo, C.W.H., et al., *Hyperhomocysteinemia induces hepatic cholesterol biosynthesis and lipid accumulation via activation of transcription factors*. Vol. 288. 2005. E1002-E1010.
474. Woo, C.W., Y.L. Siow, and K. O, *Homocysteine activates cAMP-response element binding protein in HepG2 through cAMP/PKA signaling pathway.* Arterioscler Thromb Vasc Biol, 2006. **26**(5): p. 1043-50.
475. Berrocal-Zaragoza, M.I., et al., *Folate deficiency in rat pups during weaning causes learning and memory deficits.* Br J Nutr, 2014. **112**(8): p. 1323-32.
476. Hoffman, A., et al., *Methylenetetrahydrofolate Reductase Deficiency Deregulates Regional Brain Amyloid-beta Protein Precursor Expression and Phosphorylation Levels.* J Alzheimers Dis, 2018. **64**(1): p. 223-237.
477. Partearroyo, T., et al., *Low and high dietary folic acid levels perturb postnatal cerebellar morphology in growing rats.* Br J Nutr, 2016. **115**(11): p. 1967-77.
478. Kudo, K., et al., *Age-related disturbance of memory and CREB phosphorylation in CA1 area of hippocampus of rats.* Brain Res, 2005. **1054**(1): p. 30-7.
479. Chiang, P.K. and G.L. Cantoni, *Perturbation of biochemical transmethylations by 3-deazaadenosine in vivo.* Biochem Pharmacol, 1979. **28**(12): p. 1897-902.
480. Zanotti, S., et al., *Modifications in brain cAMP- and calcium/calmodulin-dependent protein kinases induced by treatment with S-adenosylmethionine.* Neuropharmacology, 1998. **37**(8): p. 1081-9.

481. Serra, M., et al., *Folate and S-adenosylmethionine modulate synaptic activity in cultured cortical neurons: acute differential impact on normal and apolipoprotein-deficient mice.* Phys Biol, 2008. **5**(4): p. 044002.
482. Hamid, A. and J. Kaur, *Role of signaling pathways in the regulation of folate transport in ethanol-fed rats.* J Nutr Biochem, 2009. **20**(4): p. 291-7.
483. Rothem, L., M. Stark, and Y.G. Assaraf, *Impaired CREB-1 phosphorylation in antifolate-resistant cell lines with down-regulation of the reduced folate carrier gene.* Mol Pharmacol, 2004. **66**(6): p. 1536-43.
484. Zheng, F., et al., *Regulation of brain-derived neurotrophic factor expression in neurons.* International Journal of Physiology, Pathophysiology and Pharmacology, 2012. **4**(4): p. 188-200.
485. Tomizawa, H., et al., *Methyl-donor deficiency in adolescence affects memory and epigenetic status in the mouse hippocampus.* Genes Brain Behav, 2015. **14**(3): p. 301-9.
486. Pignataro, A., et al., *CREB Regulates Experience-Dependent Spine Formation and Enlargement in Mouse Barrel Cortex.* Neural Plast, 2015. **2015**: p. 651469.
487. Jadavji, N.M., et al., *MTHFR deficiency or reduced intake of folate or choline in pregnant mice results in impaired short-term memory and increased apoptosis in the hippocampus of wild-type offspring.* Neuroscience, 2015. **300**: p. 1-9.
488. Ciani, E., et al., *Neurochemical correlates of nicotine neurotoxicity on rat habenulo-interpeduncular cholinergic neurons.* Neurotoxicology, 2005. **26**(3): p. 467-74.
489. Matsumoto, A., *Age-dependent changes in phosphorylated cAMP response element-binding protein immunoreactivity in motoneurons of the spinal nucleus of the bulbocavernosus of male rats.* Neurosci Lett, 2000. **279**(2): p. 117-20.
490. Frosst, P., et al., *A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase.* Nat Genet, 1995. **10**(1): p. 111-3.
491. Goyette, P., et al., *Severe and mild mutations in cis for the methylenetetrahydrofolate reductase (MTHFR) gene, and description of five novel mutations in MTHFR.* Am J Hum Genet, 1996. **59**(6): p. 1268-75.
492. Jacques, P.F., et al., *Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations.* Circulation, 1996. **93**(1): p. 7-9.
493. Tozzi, A., et al., *Region- and age-dependent reductions of hippocampal long-term potentiation and NMDA to AMPA ratio in a genetic model of Alzheimer's disease.* Neurobiol Aging, 2015. **36**(1): p. 123-33.
494. Drulis-Fajdasz, D., et al., *Involvement of cellular metabolism in age-related LTP modifications in rat hippocampal slices.* Oncotarget, 2015. **6**(16): p. 14065-81.
495. Gooney, M., et al., *BDNF-induced LTP in dentate gyrus is impaired with age: analysis of changes in cell signaling events.* Neurobiol Aging, 2004. **25**(10): p. 1323-31.
496. Wang, H.W., et al., *Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus.* Brain Res, 2002. **924**(2): p. 133-40.
497. Wang, Q.W., M.J. Rowan, and R. Anwyl, *Inhibition of LTP by beta-amyloid is prevented by activation of beta2 adrenoceptors and stimulation of the cAMP/PKA signalling pathway.* Neurobiol Aging, 2009. **30**(10): p. 1608-13.
498. Ramos, B.P., et al., *Alpha2A-adrenoceptor stimulation improves prefrontal cortical regulation of behavior through inhibition of cAMP signaling in aging animals.* Learn Mem, 2006. **13**(6): p. 770-6.
499. Viosca, J., et al., *Chronic enhancement of CREB activity in the hippocampus interferes with the retrieval of spatial information.* Learn Mem, 2009. **16**(3): p. 198-209.
500. Kontis, D., et al., *COMT and MTHFR polymorphisms interaction on cognition in schizophrenia: an exploratory study.* Neurosci Lett, 2013. **537**: p. 17-22.
501. Gobejishvili, L., et al., *S-adenosylmethionine decreases lipopolysaccharide-induced phosphodiesterase 4B2 and attenuates tumor necrosis factor expression via cAMP/protein kinase A pathway.* J Pharmacol Exp Ther, 2011. **337**(2): p. 433-43.

502. Kageyama, M., M. Hiraoka, and Y. Kagawa, *Relationship between genetic polymorphism, serum folate and homocysteine in Alzheimer's disease.* Asia Pac J Public Health, 2008. **20 Suppl**: p. 111-7.
503. Hughes, C.F., et al., *Vitamin B12 and ageing: current issues and interaction with folate.* Ann Clin Biochem, 2013. **50**(Pt 4): p. 315-29.
504. Nisbet, R.M., et al., *Tau aggregation and its interplay with amyloid-beta.* Acta Neuropathol, 2015. **129**(2): p. 207-20.
505. Vingtdeux, V., et al., *Phosphorylation of amyloid precursor carboxy-terminal fragments enhances their processing by a gamma-secretase-dependent mechanism.* Neurobiol Dis, 2005. **20**(2): p. 625-37.
506. Mazzitelli, S., et al., *The loss of c-Jun N-terminal protein kinase activity prevents the amyloidogenic cleavage of amyloid precursor protein and the formation of amyloid plaques in vivo.* J Neurosci, 2011. **31**(47): p. 16969-76.
507. Lombino, F., et al., *An intracellular threonine of amyloid-beta precursor protein mediates synaptic plasticity deficits and memory loss.* PLoS One, 2013. **8**(2): p. e57120.
508. Zhang, C.-E., et al., *Hyperhomocysteinemia Increases β-Amyloid by Enhancing Expression of γ-Secretase and Phosphorylation of Amyloid Precursor Protein in Rat Brain.* The American Journal of Pathology, 2009. **174**(4): p. 1481-1491.
509. Li, M.H., et al., *Disturbance of endogenous hydrogen sulfide generation and endoplasmic reticulum stress in hippocampus are involved in homocysteine-induced defect in learning and memory of rats.* Behav Brain Res, 2014. **262**: p. 35-41.
510. Chen, Z., et al., *Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition.* Hum Mol Genet, 2001. **10**(5): p. 433-43.
511. Ghandour, H., et al., *Mice deficient in methylenetetrahydrofolate reductase exhibit tissue-specific distribution of folates.* J Nutr, 2004. **134**(11): p. 2975-8.
512. Hooper, C., R. Killick, and S. Lovestone, *The GSK3 hypothesis of Alzheimer's disease.* J Neurochem, 2008. **104**(6): p. 1433-9.
513. Qian, W., et al., *PP2A regulates tau phosphorylation directly and also indirectly via activating GSK-3beta.* J Alzheimers Dis, 2010. **19**(4): p. 1221-9.
514. Dewachter, I., et al., *GSK3beta, a centre-staged kinase in neuropsychiatric disorders, modulates long term memory by inhibitory phosphorylation at serine-9.* Neurobiol Dis, 2009. **35**(2): p. 193-200.
515. Van Dam, F. and W.A. Van Gool, *Hyperhomocysteinemia and Alzheimer's disease: A systematic review.* Archives of Gerontology and Geriatrics, 2009. **48**(3): p. 425-430.
516. Wang, H.X., et al., *Vitamin B(12) and folate in relation to the development of Alzheimer's disease.* Neurology, 2001. **56**(9): p. 1188-94.
517. Fuso, A., et al., *S-adenosylmethionine/homocysteine cycle alterations modify DNA methylation status with consequent deregulation of PS1 and BACE and beta-amyloid production.* Mol Cell Neurosci, 2005. **28**(1): p. 195-204.
518. Feng, L., et al., *Associations between elevated homocysteine, cognitive impairment, and reduced white matter volume in healthy old adults.* Am J Geriatr Psychiatry, 2013. **21**(2): p. 164-72.
519. Gilbody, S., S. Lewis, and T. Lightfoot, *Methylenetetrahydrofolate reductase (MTHFR) genetic polymorphisms and psychiatric disorders: a HuGE review.* Am J Epidemiol, 2007. **165**(1): p. 1-13.
520. Lipton, S.A., et al., *Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor.* Proc Natl Acad Sci U S A, 1997. **94**(11): p. 5923-8.
521. Flott-Rahmel, B., et al., *Homocysteic and homocysteine sulphinic acid exhibit excitotoxicity in organotypic cultures from rat brain.* Eur J Pediatr, 1998. **157 Suppl 2**: p. S112-7.
522. Braak, H., et al., *Age, neurofibrillary changes, A beta-amyloid and the onset of Alzheimer's disease.* Neurosci Lett, 1996. **210**(2): p. 87-90.

523. Schönheit, B., R. Zarski, and T.G. Ohm, *Spatial and temporal relationships between plaques and tangles in Alzheimer-pathology.* Neurobiology of Aging, 2004. **25**(6): p. 697-711.
524. Busche, M.A., et al., *Critical role of soluble amyloid-beta for early hippocampal hyperactivity in a mouse model of Alzheimer's disease.* Proc Natl Acad Sci U S A, 2012. **109**(22): p. 8740-5.
525. Reynolds, E., *Vitamin B12, folic acid, and the nervous system.* Lancet Neurol, 2006. **5**(11): p. 949-60.
526. Zeisel, S., *Choline, Other Methyl-Donors and Epigenetics.* Nutrients, 2017. **9**(5).
527. Sunden, S.L., et al., *Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene.* Arch Biochem Biophys, 1997. **345**(1): p. 171-4.
528. Cascalheira, J.F., et al., *Serum homocysteine: interplay with other circulating and genetic factors in association to Alzheimer's type dementia.* Clin Biochem, 2009. **42**(9): p. 783-90.
529. Ho, P.I., et al., *Folate deprivation induces neurodegeneration: roles of oxidative stress and increased homocysteine.* Neurobiol Dis, 2003. **14**(1): p. 32-42.
530. Budni, J., et al., *Folic Acid Protects Against Glutamate-Induced Excitotoxicity in Hippocampal Slices Through a Mechanism that Implicates Inhibition of GSK-3beta and iNOS.* Mol Neurobiol, 2018. **55**(2): p. 1580-1589.
531. Martin, P.M., et al., *The sigma receptor ligand (+)-pentazocine prevents apoptotic retinal ganglion cell death induced in vitro by homocysteine and glutamate.* Brain Res Mol Brain Res, 2004. **123**(1-2): p. 66-75.
532. Ibi, D., et al., *Involvement of GAT2/BGT-1 in the preventive effects of betaine on cognitive impairment and brain oxidative stress in amyloid beta peptide-injected mice.* Eur J Pharmacol, 2019. **842**: p. 57-63.
533. McCarthy, R.C., et al., *Characterization of a novel adult murine immortalized microglial cell line and its activation by amyloid-beta.* Journal of neuroinflammation, 2016. **13**(1): p. 21.
534. Hoe, H.S., H.K. Lee, and D.T. Pak, *The upside of APP at synapses.* CNS Neurosci Ther, 2012. **18**(1): p. 47-56.
535. Chen, Y., et al., *Intracerebroventricular streptozotocin exacerbates Alzheimer-like changes of 3xTg-AD mice.* Mol Neurobiol, 2014. **49**(1): p. 547-62.
536. Liu, H., et al., *Folic acid deficiency enhances abeta accumulation in APP/PS1 mice brain and decreases amyloid-associated miRNAs expression.* J Nutr Biochem, 2015. **26**(12): p. 1502-8.
537. Li, W., et al., *Folic Acid Inhibits Amyloid beta-Peptide Production through Modulating DNA Methyltransferase Activity in N2a-APP Cells.* Int J Mol Sci, 2015. **16**(10): p. 25002-13.
538. Liu, H., et al., *Folic acid attenuates the effects of amyloid beta oligomers on DNA methylation in neuronal cells.* Eur J Nutr, 2016. **55**(5): p. 1849-62.
539. Muresan, V. and Z. Ladescu Muresan, *Amyloid-beta precursor protein: Multiple fragments, numerous transport routes and mechanisms.* Exp Cell Res, 2015. **334**(1): p. 45-53.
540. Sherriff, F.E., L.R. Bridges, and S. Sivaloganathan, *Early detection of axonal injury after human head trauma using immunocytochemistry for beta-amyloid precursor protein.* Acta Neuropathol, 1994. **87**(1): p. 55-62.
541. Xiong, Y., et al., *Zinc induces protein phosphatase 2A inactivation and tau hyperphosphorylation through Src dependent PP2A (tyrosine 307) phosphorylation.* Neurobiol Aging, 2013. **34**(3): p. 745-56.
542. Roberts, K.G., et al., *Essential requirement for PP2A inhibition by the oncogenic receptor c-KIT suggests PP2A reactivation as a strategy to treat c-KIT+ cancers.* Cancer Res, 2010. **70**(13): p. 5438-47.

543. Wong, L.L., et al., *Silencing of the PP2A catalytic subunit causes HER-2/neu positive breast cancer cells to undergo apoptosis.* Experimental Cell Research, 2010. **316**(20): p. 3387-96.
544. Drubin, D.G., et al., *Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors.* J Cell Biol, 1985. **101**(5 Pt 1): p. 1799-807.
545. Biernat, J. and E.M. Mandelkow, *The development of cell processes induced by tau protein requires phosphorylation of serine 262 and 356 in the repeat domain and is inhibited by phosphorylation in the proline-rich domains.* Mol Biol Cell, 1999. **10**(3): p. 727-40.
546. Dawson, H.N., et al., *Inhibition of neuronal maturation in primary hippocampal neurons from tau deficient mice.* J Cell Sci, 2001. **114**(Pt 6): p. 1179-87.
547. Schmid, R.-S., W.M. Pruitt, and P.F. Maness, *A MAP Kinase-Signaling Pathway Mediates Neurite Outgrowth on L1 and Requires Src-Dependent Endocytosis.* The Journal of Neuroscience, 2000. **20**(11): p. 4177-4188.
548. Jeon, C.Y., et al., *Control of neurite outgrowth by RhoA inactivation.* J Neurochem, 2012. **120**(5): p. 684-98.
549. Lewis, P.A., et al., *N1-Src Kinase Is Required for Primary Neurogenesis in Xenopus tropicalis.* J Neurosci, 2017. **37**(35): p. 8477-8485.
550. Espinoza, M., et al., *Differential incorporation of tau isoforms in Alzheimer's disease.* J Alzheimers Dis, 2008. **14**(1): p. 1-16.
551. Strack, S., *Overexpression of the protein phosphatase 2A regulatory subunit Bgamma promotes neuronal differentiation by activating the MAP kinase (MAPK) cascade.* Journal of Biological Chemistry, 2002. **277**(44): p. 41525-32.
552. Tanabe, A., et al., *MARCKS dephosphorylation is involved in bradykinin-induced neurite outgrowth in neuroblastoma SH-SY5Y cells.* J Cell Physiol, 2012. **227**(2): p. 618-29.
553. Ugi, S., et al., *Protein phosphatase 2A forms a molecular complex with Shc and regulates Shc tyrosine phosphorylation and downstream mitogenic signaling.* Mol Cell Biol, 2002. **22**(7): p. 2375-87.
554. Liu, X., et al., *Berberine attenuates axonal transport impairment and axonopathy induced by Calyculin A in N2a cells.* PLoS One, 2014. **9**(4): p. e93974.
555. Dalton, G.D. and A.C. Howlett, *Cannabinoid CB1 receptors transactivate multiple receptor tyrosine kinases and regulate serine/threonine kinases to activate ERK in neuronal cells.* Br J Pharmacol, 2012. **165**(8): p. 2497-511.
556. Wassarman, D.A., et al., *Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in Drosophila.* Genes Dev, 1996. **10**(3): p. 272-8.
557. Huyer, G., et al., *Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate.* J Biol Chem, 1997. **272**(2): p. 843-51.
558. Leugers, C.J. and G. Lee, *Tau potentiates nerve growth factor-induced mitogen-activated protein kinase signaling and neurite initiation without a requirement for microtubule binding.* J Biol Chem, 2010. **285**(25): p. 19125-34.
559. Sontag, J.M. and E. Sontag, *Regulation of cell adhesion by PP2A and SV40 small tumor antigen: an important link to cell transformation.* Cell Mol Life Sci, 2006. **63**(24): p. 2979-91.
560. Ingelsson, M., et al., *Early Abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain.* Neurology, 2004. **62**(6): p. 925-31.
561. Jacobsen, J.S., et al., *Early-onset behavioral and synaptic deficits in a mouse model of Alzheimer's disease.* Proc Natl Acad Sci U S A, 2006. **103**(13): p. 5161-6.
562. Loureiro, S.O., et al., *Homocysteine activates calcium-mediated cell signaling mechanisms targeting the cytoskeleton in rat hippocampus.* Int J Dev Neurosci, 2008. **26**(5): p. 447-55.
563. Vogelsberg-Ragaglia, V., et al., *PP2A mRNA expression is quantitatively decreased in Alzheimer's disease hippocampus.* Exp Neurol, 2001. **168**(2): p. 402-12.

564. Longman, M.R., et al., *Regulation of PP2AC carboxylmethylation and cellular localisation by inhibitory class G-protein coupled receptors in cardiomyocytes.* PLoS One, 2014. **9**(1): p. e86234.
565. Jicha, G.A., et al., *cAMP-dependent protein kinase phosphorylations on tau in Alzheimer's disease.* J Neurosci, 1999. **19**(17): p. 7486-94.
566. Cheng, H.C., H.M. Shih, and Y. Chern, *Essential role of cAMP-response element-binding protein activation by A2A adenosine receptors in rescuing the nerve growth factor-induced neurite outgrowth impaired by blockage of the MAPK cascade.* J Biol Chem, 2002. **277**(37): p. 33930-42.
567. Leemhuis, J., et al., *The protein kinase A inhibitor H89 acts on cell morphology by inhibiting Rho kinase.* J Pharmacol Exp Ther, 2002. **300**(3): p. 1000-7.
568. Kilian, P., et al., *Angiotensin II type 2 receptor stimulation increases the rate of NG108–15 cell migration via actin depolymerization.* Endocrinology, 2008. **149**(6): p. 2923-2933.
569. Oleinik, N.V., N.I. Krupenko, and S.A. Krupenko, *ALDH1L1 inhibits cell motility via dephosphorylation of cofilin by PP1 and PP2A.* Oncogene, 2010. **29**(47): p. 6233-44.
570. Ambach, A., et al., *The serine phosphatases PP1 and PP2A associate with and activate the actin-binding protein cofilin in human T lymphocytes.* Eur J Immunol, 2000. **30**(12): p. 3422-31.
571. Hanemaaijer, R. and I. Ginzburg, *Involvement of mature tau isoforms in the stabilization of neurites in PC12 cells.* J Neurosci Res, 1991. **30**(1): p. 163-71.
572. Maccioni, R.B. and V. Cambiazo, *Role of microtubule-associated proteins in the control of microtubule assembly.* Physiol Rev, 1995. **75**(4): p. 835-64.
573. Farah, C.A., et al., *Tau interacts with Golgi membranes and mediates their association with microtubules.* Cell Motil Cytoskeleton, 2006. **63**(11): p. 710-24.
574. Roffman, J.L., et al., *MTHFR 677C>T effects on anterior cingulate structure and function during response monitoring in schizophrenia: a preliminary study.* Brain Imaging Behav, 2011. **5**(1): p. 65-75.
575. Duan, W., et al., *Dietary folate deficiency and elevated homocysteine levels endanger dopaminergic neurons in models of Parkinson's disease.* J Neurochem, 2002. **80**(1): p. 101-10.
576. Simon, A.M., et al., *Overexpression of wild-type human APP in mice causes cognitive deficits and pathological features unrelated to Abeta levels.* Neurobiol Dis, 2009. **33**(3): p. 369-78.
577. Acevedo, K.M., et al., *Phosphorylation of amyloid precursor protein at threonine 668 is essential for its copper-responsive trafficking in SH-SY5Y neuroblastoma cells.* J Biol Chem, 2014. **289**(16): p. 11007-19.

Appendix

The following review article "***The protein serine/threonine phosphatases PP2A, PP1 and calcineurin: A triple threat in the regulation of the neuronal cytoskeleton***" was first published in *Molecular and Cellular Neuroscience 84 (2017) 119–131.* I appear as first author, and there were invaluable contributions made by members of the Sontag lab, Goce Taleski and Estelle Sontag.

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The protein serine/threonine phosphatases PP2A, PP1 and calcineurin: A triple threat in the regulation of the neuronal cytoskeleton

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ABSTRACT

The microtubule, F-actin and neurofilament networks play a critical role in neuronal cell morphogenesis, polarity and synaptic plasticity. Significantly, the assembly/disassembly and stability of these cytoskeletal networks is crucially modulated by protein phosphorylation and dephosphorylation events. Herein, we aim to more closely examine the role played by three major neuronal Ser/Thr protein phosphatases, PP2A, PP1 and calcineurin, in the homeostasis of the neuronal cytoskeleton. There is strong evidence that these enzymes interact with and dephosphorylate a variety of cytoskeletal proteins, resulting in major regulation of neuronal cytoskeletal dynamics. Conversely, we also discuss how multi-protein cytoskeletal scaffolds can also influence the regulation of these phosphatases, with important implications for neuronal signalling and homeostasis. Not surprisingly, deregulation of these cytoskeletal scaffolds and phosphatase dysfunction are associated with many neurological diseases.

Contents

Abbreviations: AD, Alzheimer disease; CaM, calmodulin; CNA/B, calcineurin A/B subunit; Glu-MTs, detyrosinated microtubules; GSK3β, glycogen synthase kinase 3β; MAP, microtubule-associated protein; MARCKS, myristoylated alanine-rich C-kinase substrate; MT, microtubule; MTCL1, microtubule crosslinking factor 1; NF, neurofilament; NMDA, N-Methyl-D-Aspartate; PKA, protein kinase A; PP1c, protein phosphatase 1 catalytic subunit; PP2Ac, protein phosphatase 2A catalytic subunit; ROCK, Rho-associated protein kinase; SH3, Src Homology 3 domain; Tyr-MTs, tyrosinated microtubules.

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1. Introduction

The cytoskeleton plays a critical role in the establishment of the neuronal cell polar morphology, which is essential for regulated synaptic plasticity. Alterations in the phosphorylation state of cytoskeletal proteins in response to various extracellular signals occurs *via* changes in the relative activities of protein kinases and phosphatases. It is a major regulatory mechanism ensuring homeostasis of the neuronal cytoskeleton. The majority of neuronal cell phospho-Serine/Threonine dephosphorylation is mediated by the phosphoprotein phosphatases (PPPs) type 1 (PP1 or PPP1), 2 (PP2A or PPP2) and 3 (PPP3, also called PP2B or calcineurin). These ubiquitously expressed enzymes, which contain a highly conserved catalytic domain (Fig. 1A), regulate nearly all cellular processes while displaying differences in substrate recognition (Reviewed in Virshup and Shenolikar, 2009). As illustrated in Fig. 1B, a key feature of PP2A, PP1 and calcineurin, is that they are

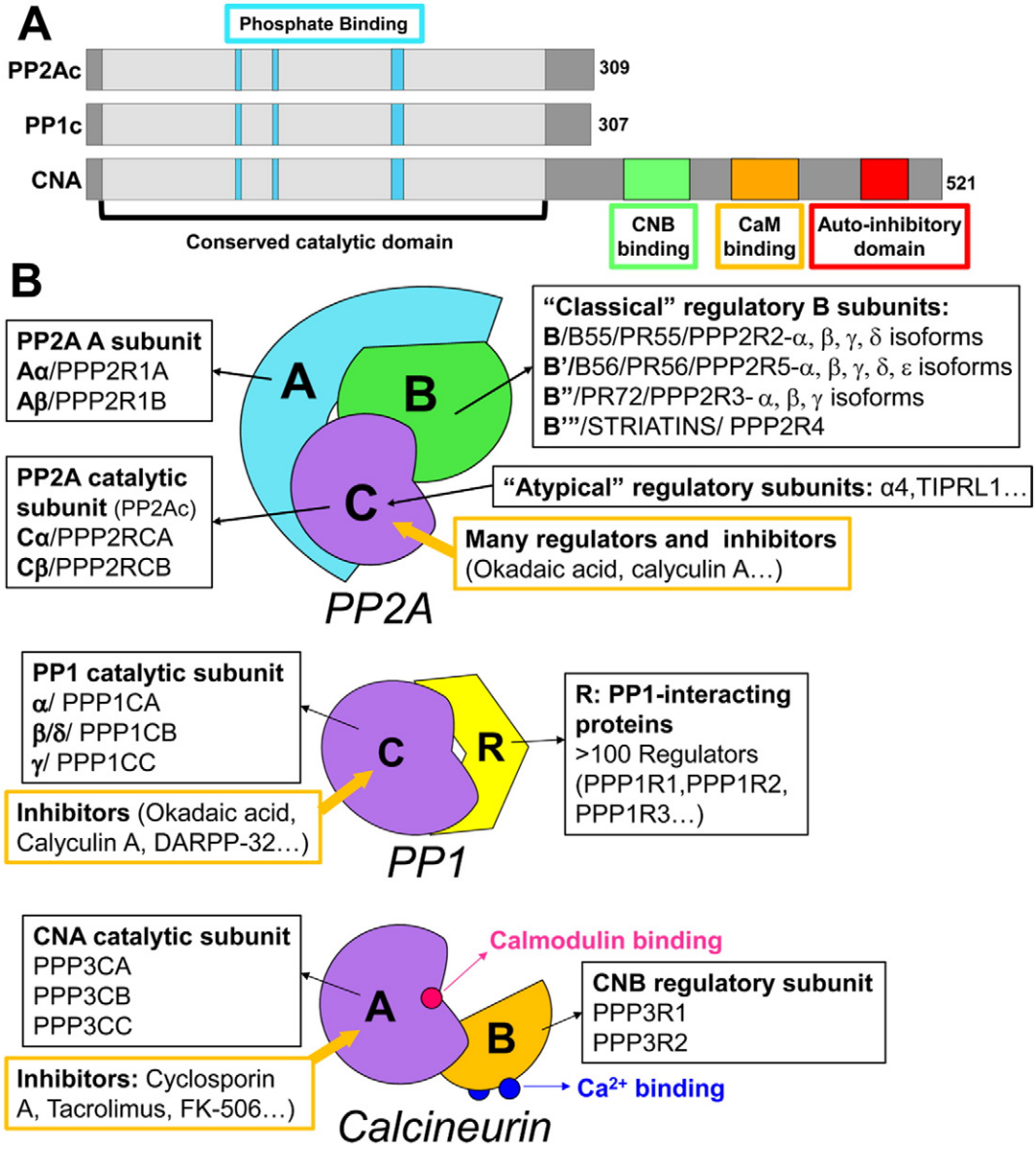

Fig. 1. Schematic structure of PP2A, PP1 and calcineurin enzymes. **A**. These phosphatases all share a ~ 30 kDa conserved catalytic domain. The calcineurin A subunit contains additional B subunit- and calmodulin (CaM)-binding domains, and an auto-inhibitory domain. **B**. Subunit interactions play a critical role in the regulation of these enzymes, which are targeted by many natural inhibitors. For details, see text.

multimeric enzymes. Notably, crystal structure analyses of these phosphatases have shown that binding of diverse regulators to the catalytic subunit provides a specific substrate recognition platform, thereby explaining the substrate specificity displayed by distinct oligomeric holoenzymes (Cho and Xu, 2007; Egloff et al., 1997; Griffith et al., 1995; Kelker et al., 2009; Maynes et al., 2004; Terrak et al., 2004; Xu et al., 2008; Xu et al., 2006). In this review, we discuss how these major phosphatases have truly emerged as critical regulators of the neuronal microtubule (MT), neurofilament and actin cytoskeleton.

1.1. PP2A

Protein phosphatase 2A (PP2A) is an essential enzyme that represents up to 1% of total cellular proteins (Lin et al., 1998; Ruediger et al., 1991). The "PP2A" family of enzymes (Fig. 1B) comprises at least 96 distinct members that exert non redundant cellular functions. The typical mammalian PP2A enzyme exists as a heterotrimer of a scaffolding A subunit (PPP2R1 A/B isoforms), a catalytic C subunit (PP2Ac or PPP2C A/B isoforms) and one of 23 regulatory B-type subunits belonging to one of 4 distinct families: B (PPP2R), B' (PPP2R5), B" (PPP2R3) or B"'/striatins (PPP2R4); B, B' and B" families each comprise several isoforms (named A, B, C…or α, β, γ…). Other atypical regulatory subunits, such as α4, can also associate with PP2Ac. The dazzling complexity of regulatory subunits, which are differentially expressed in different tissues and cells, informs PP2A enzyme biogenesis, substrate specificity, stability and subcellular localisation (Reviewed in Sents et al., 2012; Sontag, 2001). For instance, structural differences affecting the binding to the scaffolding A and catalytic C subunits have been found to underlie the contrasting functions of the B and B′ families of regulatory subunits (Cho and Xu, 2007; Xu et al., 2008; Xu et al., 2006).

PP2A activity is also regulated by many cellular regulators and natural inhibitors, such as okadaic acid (OA). However, it is worth mentioning that these commonly used inhibitors not only inhibit all PP2A isoforms, but also other Ser/Thr phosphatases, including PP1 and other PPP family members, at the concentrations needed to completely abrogate cellular PP2A activity. This has several limitations in the interpretation of studies performed with these inhibitors (Swingle et al., 2007), in particular in neurons wherein PP2A and PP1 enzymes are very abundant.

1.2. PP1

Functional PP1 enzymes (Fig. 1B) classically consist of a complex between the catalytic "C" subunit (PP1c or PPP1C; four isoforms) and one or more regulators (R or PPP1R). PP1-binding domains allow a single PP1c subunit to recruit more than one regulator, using what is described as a "molecular-lego strategy". There is especially an incredible diversity of PP1-interacting proteins in the brain. The nearly 200 vertebrate PP1-interacting proteins identified so far show preferential docking to individual PP1 isoforms, thereby supporting PP1 functional specificity and diversity (Reviewed in Heroes et al., 2013). Several structural studies have given insights into how regulatory interactions control the access of substrate to the catalytic site (Egloff et al., 1997; Kelker et al., 2009; Terrak et al., 2004). PP1 is also inhibited by many toxins, such as OA and tautomycin, as well as unique endogenous protein inhibitors (Kelker et al., 2009; Shi, 2009).

1.3. Calcineurin

Calcineurin was first identified as the principal target of the immunosuppressive drugs, Cyclosporin A and FK506/Tacrolimus, the binding of which physically prevents the recruitment of macromolecular substrates to the active site (Griffith et al., 1995). Interestingly, while calcineurin and PP1 share 40% sequence identity, crystal structure data of a chimeric PP1-Calcineurin mutant have contributed to explain how PP1 and calcineurin vastly differ in their sensitivity to inhibitory toxins such as OA and microcystin-LR, due to a divergent amino acid sequence in the β12–β33 loop of PP1c (Maynes et al., 2004).

Calcineurin is a calcium- and calmodulin-dependent enzyme found in many cell types, but is especially enriched in neuronal soma and processes (Ferreira et al., 1993). Notably, it is the most abundant calmodulin-binding protein in adult brain (Klee et al., 1979), where it plays an important role in memory and plasticity (Mansuy, 2003). Calcineurin comprises a catalytic "A or CNA" subunit (PPP3C A/B/C isoforms), which interacts with calmodulin in a calcium-dependent fashion, and a regulatory "B or CNB" subunit (PPP3R 1/2 isoforms), which contains four calcium-binding domains (Fig. 1B). The subunits act in concert to regulate calcineurin activity. Calcineurin isoforms are also compartmentalised in cells through interaction with various anchoring proteins, thereby determining substrate specificity (Dodge and Scott, 2003).

2. Pools of PP2A, PP1 and calcineurin are associated with the neuronal microtubule cytoskeleton

Neuronal MTs exist as a mixture of different populations of α- and β-tubulin isotypes, with αβ-tubulin heterodimers being the building blocks of MTs (Barra et al., 1988). Studies from the past decades have established that many Ser/Thr phosphatases are associated with the neuronal MT cytoskeleton, either directly, or indirectly through MT-associated proteins (MAPs). The regulated MT attachment of these major enzymes allows exquisite spatial modulation of MAPs phosphorylation and MT assembly and stability, as described below.

2.1. Selected PP2A enzymes are associated in an inactive state with the microtubule cytoskeleton

Pools of regulatory Bα (or PPP2R2A)-containing PP2A holoenzymes (PP2A/Bα) directly bind to purified MTs *in vitro* and in neuronal cells (Hiraga and Tamura, 2000; Merrick et al., 1997; Sontag et al., 1995; Sontag et al., 1999). Notably, the targeting of PP2A to MTs involves in part a direct association of PP2Ac with polymerised, but not monomeric tubulin (Fig. 2). Yet, binding of regulatory subunits to PP2Ac greatly modulates the strength of this association, with Bα-, and to a lesser extent Bβ (or PPP2R2B)-containing PP2A isoforms having the highest affinity for MTs (Hiraga and Tamura, 2000; Sontag et al., 1999). Although the exact domain of interaction of PP2A with neuronal MTs remains to be determined, PP2A associates with MTs *via* a domain distinct from that of tau and MAP2, two major MAPs. Other studies have confirmed that both PP2A/Bα and PP2A/Bβ co-sediment with MTs, but have suggested a potential recruitment of these enzymes to MTs by a yet unidentified scaffolding protein (Price et al., 1999).

The binding of PP2A to MTs has a dramatic inhibitory effect on its catalytic activity, so that MT-bound PP2A is maintained in an inactive state, relative to MT-unbound, cytosolic PP2A (Hiraga and Tamura, 2000; Sontag et al., 1999). The sequestration of inactive PP2A on MTs could serve to preserve basal levels of Ser/Thr phosphorylation of major MAPs, such as MAP2 and tau, at the MT level. Those MAPs are the targets of many Ser/Thr kinases under normal physiological conditions (Reviewed in Martin et al., 2013b; Sanchez et al., 2000). While PP2A is a major tau and MAP2 phosphatase, it can only dephosphorylate these phospho-MAPs when they are not bound to MTs (Sontag et al., 1999; Sontag et al., 2012).

Interestingly, PP2A was reported to be the only phosphatase mediating dephosphorylation of the neuronal phosphorylated βIII tubulin isoform, which inhibits MAP2-stimulated MT assembly (Khan and Luduena, 1996). PP2A preferentially dephosphorylates unassembled tubulin (Sontag et al., 1999). While PP2A can influence MT assembly and dynamics (see also below), the observation that tubulin assembly decreases PP2A activity *in vitro* suggests that cellular PP2A activity can also be modulated by MT dynamics. Thus, MT depolymerisation results in a substantial increase in active cytosolic PP2A pools available for

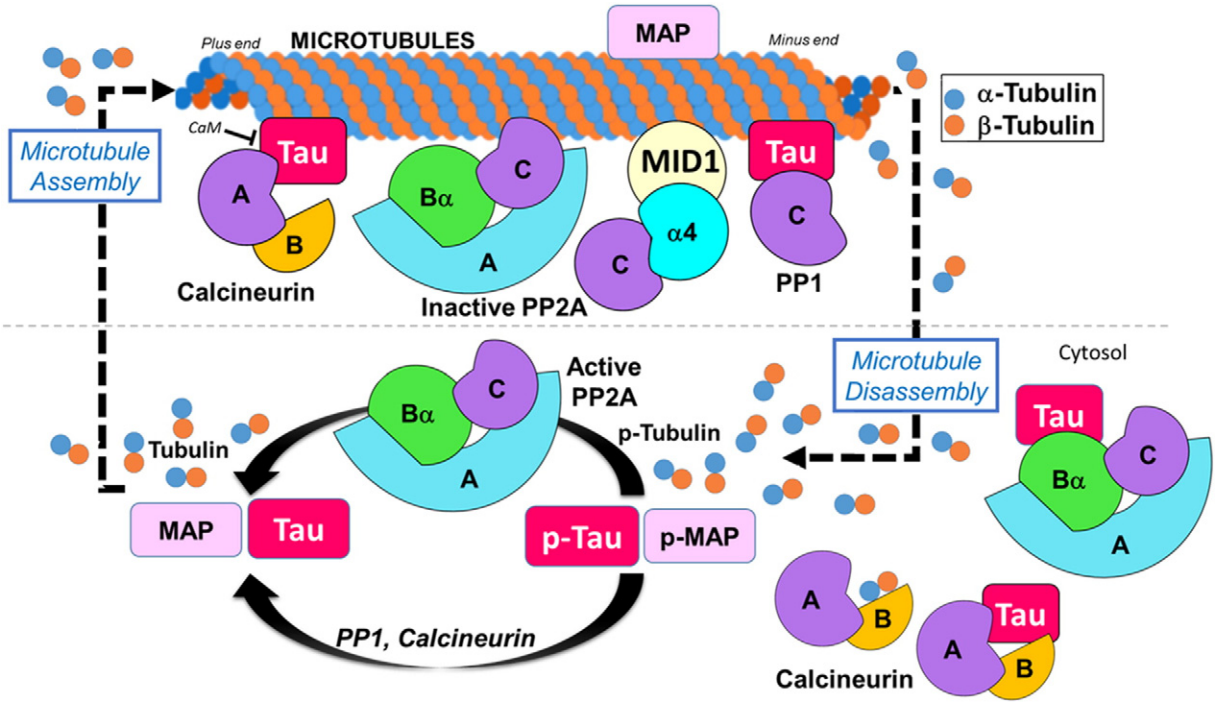

Fig. 2. The regulated binding of PP2A, PP1 and calcineurin to neuronal microtubules influences the spatial dephosphorylation of tubulin and MAPs. PP2A/Bα and α4/PP2Ac enzymes are sequestered in an inactive state on MTs. MT depolymerisation releases PP2A (and MAPs), resulting in enhanced dephosphorylation of cytosolic tubulin, tau and other MAPs like MAP2 and MAP1B. Dephosphorylation is likely promoted by the direct association of cytosolic PP2A/Bα with tau, MAP2 (not shown) and unassembled tubulin. In contrast to PP2A, both calcineurin and PP1 are anchored to MTs by tau; they have the potential to dephosphorylate both cytosolic and MT-bound MAPs. The calcineurin CNB subunit also binds to tubulin *in vitro*. For details, see text.

dephosphorylating tau (Sontag et al., 1999), MAP2 (Fan et al., 2002), and other cellular phosphoproteins (Fig. 2).

In addition to "classical" PP2A/Bα holoenzymes, other less abundant PP2A oligomeric species are also found associated with MTs. Indeed, the atypical PP2A regulatory subunit, α4, which binds to PP2Ac, is tethered to MTs through formation of a complex with the E3 ubiquitin ligase, MID1 (Schweiger and Schneider, 2003; Short et al., 2002). Under conditions of cellular stress, cytosolic α4 normally serves to sequester and stabilize PP2Ac in an inactive form until it can be incorporated into active PP2A holoenzymes (Kong et al., 2009). At the MT level, MID1-mediated monoubiquitination can trigger calpain-dependent cleavage of α4, resulting in altered stability and targeting of PP2Ac for proteosomal degradation; in turn, the cellular loss of PP2A can induce changes in the phosphorylation state of tau, a preferred PP2A substrate (Watkins et al., 2012). Notably, increased α4 cleavage and destabilization of MTs in AD could contribute to the pathological increase in tau phosphorylation observed in this disorder; conversely, mutations in MID1 that decrease α4 cleavage are associated with MAP hypophosphorylation in the developmental disorder, Opitz syndrome (Watkins et al., 2012).

Altogether, these findings suggest close structural and functional interrelationships between PP2A, MTs and MAPs.

2.2. PP1 and calcineurin also interact with the microtubule cytoskeleton

There is much less information regarding the association of other Ser/Thr phosphatases with MTs. The PP1c β isoform was found to co-localise with neuronal MTs, but its association with β-tubulin is not direct (Strack et al., 1999). Indeed, in contrast to PP2A, tau could serve to anchor PP1c to MTs (Liao et al., 1998), which likely contributes to the MT-regulatory function of PP1 (Fig. 2). Others have failed, however, to identify PP1 in MT co-sedimentation experiments in neuronal cells (Merrick et al., 1997).

Distribution studies have shown that calcineurin is present in neuronal growth cones, and cell soma and processes, where it is associated with the neuronal cytoskeleton (Ferreira et al., 1993). *In vitro*, the CNB subunit can bind to bovine brain α/β-tubulin, and calcineurin dephosphorylates tubulin phosphorylated by calcium-, calmodulin-dependent protein kinase (Goto et al., 1985; Li and Handschumacher, 2002). Calcineurin is responsible for alterations in the MT cytoskeleton during changes in synaptic function (Silverman-Gavrila et al., 2013). It also binds to tau and co-localises with tau on MTs (Yu et al., 2008) (Fig. 2). Calcineurin is especially well positioned to mediate interactions between MT and actin cytoskeletal systems during neuritogenesis. Specific inhibition of calcineurin prevents axonal elongation, affects tau phosphorylation state and interferes with the establishment of cell polarity (Ferreira et al., 1993).

3. PP2A, PP1 and calcineurin also directly bind to selected MAPs in a regulated manner

3.1. PP2A/Bα isoforms can directly bind to the major neuronal MAPs, tau and MAP2

Besides interacting with MTs, we have reported that PP2A can directly bind to tau and MAP2 (Sontag et al., 1996; Sontag et al., 1999; Sontag et al., 2012), which share many structural and functional similarities (Lewis et al., 1988). The high molecular weight MAP2 isoform is a major neuronal MAP present in neuronal cell bodies and enriched in dendrites. It regulates MT and actin cytoskeletal organization, dendritic morphogenesis and organelle trafficking (Reviewed in Farah and Leclerc, 2008). In contrast, tau is a primary axonal MAP that plays a role in regulating neuronal MT dynamics, promoting axonal outgrowth and regulating axonal trafficking (Reviewed in Wang and Mandelkow, 2016). PP2A/Bα holoenzymes co-purify with and co-sediment with MAP2 and tau isolated from bovine grey matter and white matter, respectively (Sontag et al., 2012). However, neither MAP can serve as a scaffolding protein for PP2A on MTs, since PP2A still associates well

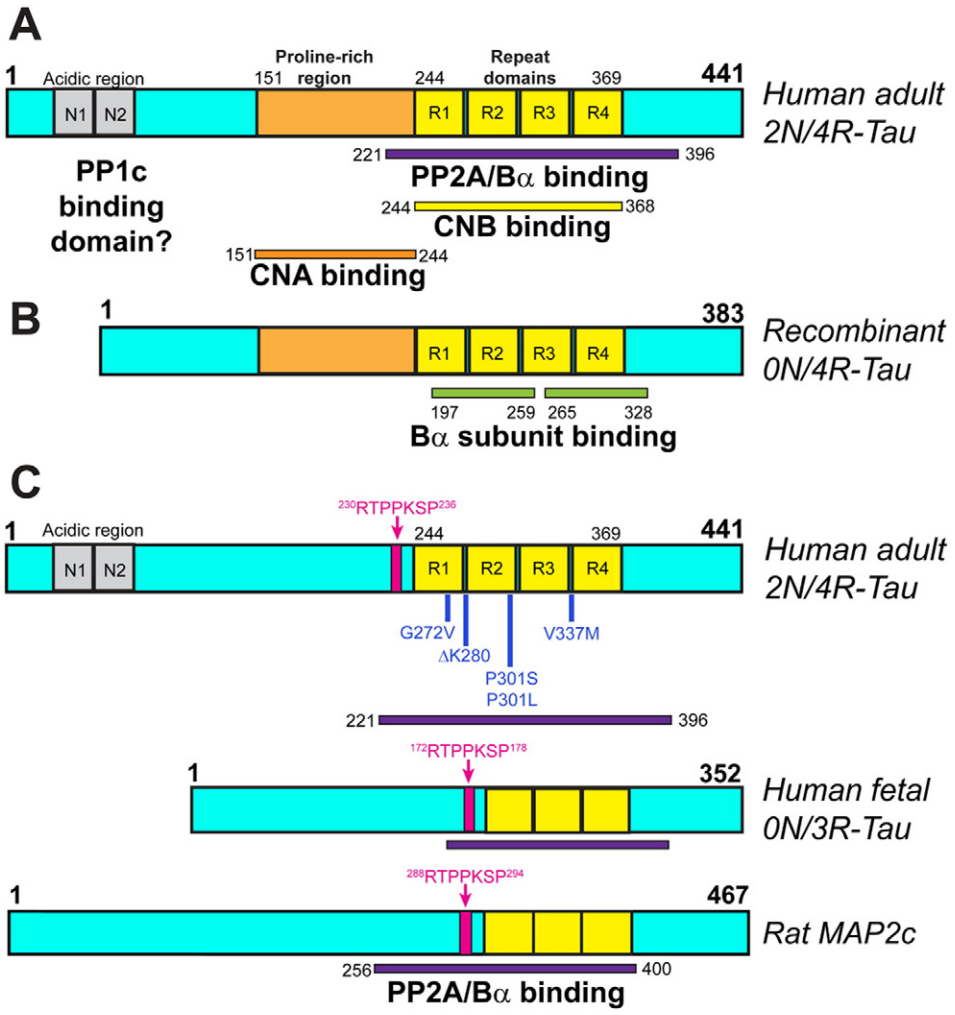

Fig. 3. PP2A, PP1 and calcineurin interact with tau. **A**. The PP2A/Bα trimeric enzyme interacts with a domain encompassing the MT-binding repeats and upstream proline-rich region of the longest adult tau 2N/4R–Tau isoform containing 2 N-terminal inserts (2N) and four MT-binding repeats (4R). Calcineurin CNA and CNB subunits independently bind to these two separate regions of tau. PP1 binds to tau *via* an unknown motif. **B**. Two peptides within the MT-binding domain mediate the direct interaction of a recombinant tau isoform with PP2A regulatory Bα subunit. **C**. Frontotemporal dementia-associated tau mutations (blue) inhibit the interaction of PP2A with tau. The conserved RTPPKSP motif present in all tau and MAP2 isoforms is critical for binding to PP2A. Represented here are the shortest MAP2c and fetal tau isoforms containing only three MT-binding repeats. For details, see text.

with MTs after their cleavage by subtilisin, which impairs tau and MAP2 binding (Sontag et al., 1999; Sontag et al., 2012).

Studies using purified proteins have shown that PP2A binds to a domain encompassing the MT-binding repeats and upstream proline-rich region of both tau and MAP2 (Fig. 3A); these domains are shared by all known isoforms of tau and MAP2. Interaction of PP2A with these MAPs is isoform-dependent, with PP2A/Bα holoenzymes displaying the highest affinity (Sontag et al., 1996; Sontag et al., 1999; Sontag et al., 2012). Structural studies have provided further proof for the preferential interaction of the Bα subunit with tau. Binding studies with recombinant proteins have specifically identified two non-overlapping basic peptide sequences within the MT-binding domain of tau that promote the interaction of tau with the acidic groove located in the center of the seven-bladed β propeller of Bα (Xu et al., 2008) (Fig. 3B). *In vitro* binding assays with purified native and recombinant proteins have shown that soluble MAP2 and tau can compete for binding to PP2A/Bα, suggesting the existence of a common binding domain (Sontag et al., 2012). Significantly, conserved SH3-binding PXXP proline-rich motifs in adult and fetal MAP2 and tau isoforms, are also required for optimal PP2A-MAP2 and PP2A-Tau interactions (Fig. 3C); more specifically, the RTPPKSP sequence in tau and MAP2 is critical for PP2A binding (Sontag et al., 2012).

PP2A/Bα binds more tightly to adult tau isoforms containing four MT-binding repeats (4R-Tau), relative to tau isoforms containing three MT-binding repeats (3R-Tau), including fetal tau (Sontag et al., 1999; Sontag et al., 2012). Notably, kinases like Fyn that bind to SH3-binding PXXP proline-rich motifs in MAP2 and tau, can inhibit the interaction of PP2A/Bα to both the shortest fetal tau and juvenile MAP2c isoforms *in vitro* (Sontag et al., 2012). Moreover, disease-associated tau phosphorylation in the proline-rich region (Eidenmuller et al., 2000; Eidenmuller et al., 2001) and frontotemporal dementia-associated tau mutations (Goedert et al., 2000) inhibit the interaction of tau with PP2A (Fig. 3C), while increasing its affinity for Fyn (Bhaskar et al., 2005). Thus, changes in PP2A-tau interactions have the potential to affect tau phosphorylation and function by changing levels of the kinases associated with tau. Notably, the ^{230}RpTPPKSP^{236} phosphopeptide containing the phospho-Thr231 residue markedly inhibits PP2A-tau interactions (Sontag et al., 2012). Phosphorylation of tau at Thr231 decreases its affinity for PP2A, and could explain why tau phosphorylated at this site is poorly dephosphorylated by PP2A/Bα (Landrieu et al., 2011). Importantly, the pseudo-phosphorylation of several Ser/Thr sites in the proline-rich region (residues 172–251), including Thr231 (Sengupta et al., 1998), can induce the displacement of tau from MTs and its redistribution to the cytosol. It is also associated with marked

tau functional deficits, since it impairs tau's ability to promote MT nucleation and process formation (Eidenmuller et al., 2000; Eidenmuller et al., 2001). Likewise, phosphorylation of the RTPPKSP motif in MAP2c at the corresponding Thr residue, which occurs during development (Zamora-Leon et al., 2001), inhibits its ability to associate with PP2A/Bα (Sontag et al., 2012).

3.2. PP1 and calcineurin also directly bind to tau

Like PP2A, both PP1 and calcineurin can directly form a complex with tau. However, the domain responsible for the interaction of tau with PP1 (Liao et al., 1998) has not yet been mapped. It has been reported that calcineurin co-localises with tau, and both CNA and CNB subunits bind to tau (Fig. 2 and 3A). Interestingly, this calcineurin-tau protein interaction is inhibited by calmodulin and participates in the regulation of the dephosphorylation of tau by the phosphatase (Yu et al., 2008). Using truncation mutants, the authors found that the CNA subunit interacts with the proline-rich domain of tau, and this interaction involves the calmodulin-binding domain; however, CNA and tau do not compete significantly for binding to CNB. In contrast, CNB interacts with the MT-binding repeats of tau (Fig. 3A), so that tau cannot anchor CNB to MTs, as observed with PP2A. Thus, it is likely that only the CNA subunit mediates binding of calcineurin to MT-associated tau, while both CNA and CNB subunits have the potential to interact with MT-unbound tau. It remains to be established whether the CNB subunit, which binds to tubulin *in vitro* (Li and Handschumacher, 2002), directly anchors calcineurin to assembled MTs in a tau-independent manner.

In any case, in contrast to PP2A enzymes that can only dephosphorylate MT-unbound tau, the reported association of both PP1 and calcineurin with MT-associated tau may provide a regulatory mechanism for the control of tau phosphorylation state at the MT level.

Together, these observations strongly support an important role for abundant neuronal MAPs as localised scaffolds for major regulatory signalling molecules. Conversely, the spatial compartmentalization of active/inactive PP2A, PP1 and calcineurin, which are differentially regulated by distinct signal transduction pathways, can allow for complex modulation of MAP-MT interactions and MT assembly.

4. PP2A, PP1 and calcineurin dephosphorylate MAPs, thereby affecting microtubule assembly

Like most cellular proteins, MAPs are regulated by dynamic and reversible phosphorylation events, wherein PP2A, PP1 and calcineurin play a central regulatory role. In turn, changes in MAPs phosphorylation state influence cytoskeletal dynamics, as well as other cellular processes, which are important for morphogenesis and synaptic plasticity in the developing and mature brain.

4.1. Tau is dephosphorylated by PP2A, PP1 and calcineurin

The study of tau phosphorylation has been a major focus of attention due to its central role in the pathogenesis of tauopathies, such as Alzheimer disease (AD) (Reviewed in Spillantini and Goedert, 2013). Indeed, abnormal, excessive phosphorylation of tau has been linked to cytoskeletal pathology, synaptotoxicity, impairment in learning and memory, and eventually neuronal cell death (Reviewed in Wang and Mandelkow, 2016). Each of the major PPPs have been identified as tau phosphatases (Goedert et al., 1992; Gong et al., 1994a; Gong et al., 1994b; Sontag et al., 1996), but PP2A is by far the most significant, contributing to ~71% of total brain tau phosphatase activity (Liu et al., 2005). Biochemical and structural studies have demonstrated that PP2A/Bα isoform is the primary neuronal PP2A isoform that mediates tau dephosphorylation at many phosphoepitopes (Reviewed in Sontag and Sontag, 2014). The direct PP2A tau phosphatase activity is intimately linked to its ability to associate with tau (Sontag et al., 1996). Yet, PP2A can also indirectly regulate tau phosphorylation state by acting on tau kinases, such as glycogen synthase kinase 3β (GSK3β) (Wang et al., 2015). Besides PP2A/Bα, other PP2A isoforms can also dephosphorylate tau, albeit to a lesser degree (Sontag et al., 1996). They likely modulate tau phosphorylation through indirect mechanisms, *e.g.* by regulating the activity of tau kinases like PP2A/PPP2R5D enzymes (Louis et al., 2011; Yu et al., 2014), or by affecting PP2A stability like the α4-PP2Ac complex (Watkins et al., 2012).

Specific inhibition of PP2A/Bα is associated with enhanced tau phosphorylation, and subsequent inability of tau to bind to and stabilize MTs (Sontag et al., 1996). Deregulation of the MT cytoskeleton could in turn contribute to the inhibition of neurite outgrowth observed following silencing of PP2A/Bα (Sontag et al., 2010). Of note, many physiologically relevant metabolic alterations, such as starvation (Planel et al., 2001), hypothermia (El Khoury et al., 2016), diabetes (Papon et al., 2013), estrogen deficiency (Zhou et al., 2008) and disturbances of folate and homocysteine metabolism (Sontag et al., 2007; Sontag et al., 2008; Sontag et al., 2014; Yoon et al., 2007; Zhang et al., 2008) can inhibit PP2A, resulting in increased tau phosphorylation in many animal models. In addition, specific inhibition of PP2A worsens phospho-tau pathology in transgenic mice (Deters et al., 2009). There is also a significant down-regulation of neuronal PP2A/Bα expression levels (Sontag et al., 2004) and PP2A activity (Gong et al., 1995; Gong et al., 1993; Sontag et al., 2004) in AD, which correlate with the accumulation of phospho-tau pathology, cementing the critical role played by this phosphatase in tau regulation at multiple epitopes. Many mechanisms have been proposed to lead to PP2A dysfunction in AD (Reviewed in Sontag and Sontag, 2014).

In contrast to PP2A, PP1 only dephosphorylates few tau phosphoepitopes, and accounts for ~11% of total brain tau phosphatase activity (Liu et al., 2005). However, some of these sites are preferentially dephosphorylated by PP1 (Rahman et al., 2005). PP1 has the potential to dephosphorylate MT-bound tau, which could increase the affinity of tau for MTs. Conversely, axonal PP1 can be activated by pathological filamentous forms of tau, resulting in inhibition of anterograde, kinesin-based fast axonal transport (Kanaan et al., 2011).

Calcineurin can dephosphorylate tau (Gong et al., 1994c; Goto et al., 1985) at few sites (Wei et al., 2002), but its activity represents only ~7% of total brain tau phosphatase activity (Liu et al., 2005). Interestingly, the tau phosphatase activity of both calcineurin and PP2A is regulated during brain development (Mawal-Dewan et al., 1994). Deregulation of calcineurin contributes to abnormal tau hyperphosphorylation in mouse models of Huntington's disease, an autosomal dominant neurodegenerative disorder (Gratuze et al., 2015). In contrast, partial calpain cleavage of calcineurin has been associated with its activation in AD, but is insufficient to counteract tau hyperphosphorylation in this disorder (Qian et al., 2011).

4.2. MAP2 is also differentially dephosphorylated by PP2A, PP1 and calcineurin

Like tau, MAP2 isoforms are also phosphorylated at numerous sites by a variety of cellular Ser/Thr kinases, and dephosphorylated by Ser/Thr phosphatases, resulting in modulation of MT organization and stability (Sanchez et al., 2000). The protein kinase A (PKA)-phosphorylated Thr-220 phosphosite of the juvenile MAP2c isoform is dephosphorylated *in vitro* by CNA > PP2Ac > PP1c; this dephosphorylation regulates nucleation of MTs and the MT binding activity of MAP2 (Alexa et al., 2002). Calcineurin may also be involved in dephosphorylation of MAP2 following NMDA receptor activation (Halpain and Greengard, 1990). However, other studies have shown that inhibition of PP2A, but not of calcineurin, induces phosphorylation of MAP2 at multiple sites in rat brain, impairing its MT binding activity (Gong et al., 2000). Likewise, phosphorylated sites within the C-terminal proline-rich region of adult MAP2 are preferentially dephosphorylated *in vitro* by PP2A and PP1, but not calcineurin (Sanchez et al., 1996). MAP2 is also the major MAP dephosphorylated by PP2A in bovine grey matter. As observed

with tau, PP2A/Bα binds to and dephosphorylates MAP2 more efficiently than PP2A dimers and PP2Ac (Sontag et al., 2012).

In agreement with the hypothesis that MAP2 cannot anchor PP2A to MTs and can only bind to cytosolic PP2A (Sontag et al., 2012), MAP2 appears to sequestrate PP2A into the somatic compartment of neuronal cells. There, it acts as a scaffold for the phosphatase, as well as for kinases such as PKA (Dell'Acqua et al., 2006). Distribution studies also indicate that PP2A most likely associates with and dephosphorylates MAP2 in neuronal cell bodies rather than in dendrites (Sontag et al., 2012). Indeed, other phosphatases, such as PP1 α and γ1 isoforms, which are specifically concentrated at dendrites and regulate synaptic plasticity (Ouimet et al., 1995), are better positioned than PP2A/Bα to dephosphorylate dendritic MAP2. Nevertheless, detachment of PP2A from MTs leads to activation of PP2A–mediated dephosphorylation of MAP2. This results in inhibition of dendrite outgrowth (Fan et al., 2002), suggesting that PP2A does not need to be associated with dendrites *per se* to regulate MAP2 function in dendritic morphogenesis.

4.3. MAP1B is also a target of Ser/Thr phosphatases

Like tau and MAP2, MAP1B undergoes phosphorylation-dependent regulation during neurodevelopment. MAP1B can be phosphorylated at multiple sites that are conventionally classified into two modes of phosphorylation, which not only influence its MT binding, but also impact neuronal morphology and maturation. Mode I phosphorylation is mediated by proline-directed protein kinases and is limited to growing axons; dephosphorylation of these sites occurs upon neuron maturation (Gordon-Weeks et al., 1993; Riederer et al., 1993; Ulloa et al., 1993b). Mode II phosphorylation of MAP1B is catalysed by casein kinase II and affects MT binding and neurite outgrowth (Ulloa et al., 1993a). While PP2A can dephosphorylate both modes of MAP1B phosphorylation, PP1 dephosphorylates mode II and calcineurin mode I sites only (Gong et al., 2000; Ortega-Gutierrez et al., 2008; Ulloa et al., 1993b). Inhibition of PP2A, and to a lesser extent of calcineurin, enhances MAP1B phosphorylation and inhibit its MT binding activity (Gong et al., 2000).

Together, these studies show that complex protein-protein and enzyme-substrate interactions govern the regulation of major neuronal MAPs, as determined by a delicate balance between phosphatase and kinase expression levels and activity. In turn, this significantly impacts MT assembly.

5. PP2A, PP1 and calcineurin and the regulation of microtubule dynamics

5.1. Inhibition of PP2A and PP1 selectively affect stable microtubules

MTs are the molecular machinery involved in the establishment of neuronal polarity and cellular transport, and exhibit a dynamically regulated structure. The most characteristic MT behavior encompasses alternating phases between slow, plus-end growth ("rescue"), rapid depolymerisation ("catastrophe"), and pausing, referred to as dynamic instability. There is strong evidence that PP2A and PP1 can modulate MT dynamics. Stimulation of cells with OA induces a two-fold increase in the frequency of catastrophe transitions and dramatically eliminates rescue transitions (Gliksman et al., 1992; Shelden and Wadsworth, 1996). OA also reduces the density of MTs in the vicinity of MT-organising centres, and induces a three-fold increase in net gain and loss of polymers at MT plus ends (Shelden and Wadsworth, 1996).

Interestingly, a specific subset of MTs comprised of detyrosinated α-tubulin (Glu-MTs) exhibits long turnover rates and increased resistance to the MT-depolymerising drug, nocodazole (Gundersen and Bulinski, 1988; Khawaja et al., 1988). Like Glu-MTs, MTs comprised of acetylated α-tubulin are selectively stabilized, in particular under depolymerizing conditions (Piperno et al., 1987). In contrast, the more dynamic or labile MTs are composed of tyrosinated α-tubulin (Tyr-MTs) (Gundersen and Bulinski, 1988). Significantly, studies in neuronal and nonneuronal cell types have demonstrated that stable acetylated and Glu-MTs, rather than Tyr-MTs, are especially vulnerable to OA- or calyculin-A-induced MT depolymerisation (Gurland and Gundersen, 1993; Merrick et al., 1997; Yoon et al., 2008). Of note, in these studies, these inhibitors did not affect tubulin synthesis and turnover, and did not up-regulate any MT severing activity under the experimental conditions utilised. Short incubation of cultured neuronal NT2N cells with low concentrations of OA that preferentially inhibit PP2A, but not PP1, was sufficient to lead to a significant loss of Glu-MTs. However, the loss of stable MTs was further exacerbated when cells were treated with OA and/or calyculin-A for a prolonged time, or using higher drug concentrations, which result in concomitant PP1 inhibition (Gurland and Gundersen, 1993; Merrick et al., 1997; Yoon et al., 2008). Thus, it has been proposed that PP2A is preferentially involved in the initial step of MT depolymerisation (Merrick et al., 1997). Accordingly, specific deregulation of cellular PP2A activity and subunit composition, and silencing of the regulatory Bα subunit are sufficient to induce a selective breakdown of acetylated and Glu-MTs; they also enhance the sensitivity of MTs to nocodazole-induced MT depolymerization in both neuronal and nonneuronal cells (Nunbhakdi-Craig et al., 2007). These results unambiguously implicate PP2A in the regulation of MT stability. More specifically, PP2A/Bα holoenzymes, which co-purify and are associated with neuronal MTs, are well positioned to directly influence MT dynamic instability (Hiraga and Tamura, 2000; Sontag et al., 1995).

The pool of Glu-tubulin is generated through enzymatic removal of the COOH-terminal tyrosine residue on the tubulin α-chain by an ill-defined tubulin carboxypeptidase. Conversely, a tubulin tyrosine ligase mediates re-addition of the tyrosine residue on α-tubulin (Barra et al., 1988). There is ample evidence that these evolutionary conserved cycles of tubulin tyrosination/detyrosination play a critical physiological role in most eukaryotic cell types; in particular, they are essential for neuronal organization and neurite extension (Erck et al., 2005). In this context, it is interesting that, besides markedly reducing Glu-tubulin levels, PP2A inhibitors (Merrick et al., 1997; Yoon et al., 2008) and specific deregulation of PP2A (Nunbhakdi-Craig et al., 2007) also increased Tyr-tubulin levels. Of note, a significant depletion of tubulin carboxypeptidase activity has been found in cells treated with inhibitors of PP2A/PP1, but not other Ser/Thr and Tyr phosphatase inhibitors (Contin et al., 2003). Such changes could underlie the changes in tubulin tyrosinated state observed in many studies using PP2A/PP1 inhibitory drugs.

In agreement with the critical role of MTs in neuronal homeostasis, OA-mediated loss of Glu-MTs in NT2N neuronal cells correlates with rapid degeneration of axons, which are enriched in stable MTs (Merrick et al., 1997). MTs accumulate intensely around soma and proximal neurites in OA-treated neurons, likely as a result of impairment of MT retrograde transport to distal neurites. Indeed, OA induces the calpain-dependent cleavage of dynein and dynactin, which are components of the dynein-motor complex. In turn, MT transport deficits promote neurite retraction (Yoon et al., 2008). Breakdown of the MT cytoskeleton following inhibition of PP1 also induces retraction of growth cones in NGF-differentiated PC12 cells (Reber and Bouron, 1995).

Altogether, these findings delineate an important functional role for PP2A and PP1 activities in the regulation of MT stability, while emphasizing that the stable and dynamic subsets of MTs are differentially regulated by protein phosphorylation-dependent mechanisms.

5.2. Indirect regulation of MT dynamics via the regulation of MAPs

As mentioned earlier, dynamic changes in MT organization are also indirectly influenced by MAPs. Both tau and MAP2 modulate MT dynamics (Gamblin et al., 1996; Panda et al., 2003). Recently, structural studies have revealed that tau binds at the interface between tubulin α/β heterodimers (Kadavath et al., 2015). Since stable MTs and tau are both enriched in the axonal compartment, another mechanism by

which phosphatase inhibitors such as OA could affect MT dynamics could involve their ability to deregulate tau phosphorylation. Indeed, treatment of neuronal cells with OA and specific targeting and inhibition of PP2A both induce the phosphorylation of tau at many epitopes, and concomitant detachment of tau from MTs (Merrick et al., 1997; Sontag et al., 1996). PP2A inhibition also inhibits the formation of nocodazole-resistant, tau-mediated MT bundles (Sontag et al., 1996), indicating both tau-dependent and-independent roles of PP2A in regulating MT dynamics.

Recent studies strongly suggest that the phosphorylation of tau at key epitopes, rather than a quantitative "blanket increase" in tau phosphorylation, can differentially influence its association with MTs (Reviewed in Hanger et al., 2009; Wang and Mandelkow, 2016). Indeed, the phosphorylation of the conserved KXGS motif in both Tau and MAP2 regulates their MT-binding capacity (Drewes et al., 1995; Ozer and Halpain, 2000). Phosphorylation of Ser262 reduces the affinity of tau for MTs, rendering MTs more dynamically unstable (Drewes et al., 1995). Likewise, tau phosphorylation at Ser214 (Schneider et al., 1999) and Thr231 (Sengupta et al., 1998) in the region flanking the MTBD of tau, promotes its detachment from MTs. By contrast, tau-MT interactions are not significantly affected by phosphorylation of other sites. Notably, the activity of many of the tau/MAP2 protein kinases that phosphorylate these key MT-binding regulatory sites can be upregulated by PP2A/PP1 inhibition, which also prevents tau/MAP2 dephosphorylation (Reviewed in Martin et al., 2013b; Sontag and Sontag, 2014). Moreover, binding of particular protein kinases could also modulate tau/MAP2 phosphorylation, in turn altering MT dynamics. For instance, the tyrosine kinase Fyn, which binds to proline-rich motifs in Tau and MAP2, inhibits the interaction of these MAPs with PP2A/Bα (Sontag et al., 2012). Not surprisingly, alterations in tau phosphorylation due to an imbalance between protein kinases' and phosphatases' activities (Reviewed in Martin et al., 2013a; Martin et al., 2013b) has been linked to neuronal MT disassembly and defects in kinesin-dependent axonal cargo transport, ultimately leading to synapse loss (Reviewed in Wang and Mandelkow, 2016). Interestingly, it has also been proposed that the abnormal hyperphosphorylated tau in AD could promote MT disassembly by sequestering normal tau, as well as MAP2 and MAP1, into filamentous aggregates (Alonso et al., 1996; Alonso et al., 1994; Alonso et al., 1997).

Besides regulating tau and MAP2, both PP2A and PP1 have also been involved in the dephosphorylation of doublecortin, an important MAP involved in MT stabilization and neuronal cell migration (Schaar et al., 2004; Shmueli et al., 2006). In particular, the PP1-targeting protein, spinophilin/Neurabin II, promotes PP1γ1-dependent dephosphorylation of doublecortin, resulting in localised MT bundling, an event critical for axonal growth (Bielas et al., 2007). Notably, mutations in doublecortin alter MT dynamics and cause X-linked lissencephaly ("smooth brain"), a neuronal migration disorder associated with epilepsy and mental retardation in humans.

Lastly, MT crosslinking factor 1 (MTCL1), a cytoskeletal crosslinking protein that interacts with both F-actin and MTs, has recently been identified as a protein targeted by PP2A in epithelial cells. More specifically, PP2A PPP2R5E (B′ε) regulatory subunit can bind to and stabilize MTCL1, thereby contributing to MT organization (Hyodo et al., 2016). Interestingly, MTCL1 has also a function in the developing brain, where it is essential for axonal outgrowth and dendritic arborisations; yet, it remains to be established whether MTCL1 is also regulated by PP2A in neurons.

In conclusion, major Ser/Thr phosphatases are targeted, either directly or indirectly, to localised cytoskeletal protein scaffolds, where they modulate the phosphorylation state of MT and MAPS *via* multimodal and intricate mechanisms. There is strong evidence that deregulation of these phosphatases and disruption of these multi-protein scaffolds has severe consequences on MT cytoskeletal organization and dynamics, resulting in disturbances in neuronal plasticity and homeostasis. On the other side, MTs could also be viewed as reservoirs of intracellular phosphatases. Together with MAPs, they may act as platforms to sequester major phosphatases and kinases in discrete neuronal compartments, *e.g.* cell body, dendrite or axon, allowing for exquisite control of localised signal transduction and integration.

6. Regulation of neurofilaments by PP2A, PP1 and calcineurin

6.1. PP2A, PP1 and calcineurin co-purify with and/or dephosphorylate neurofilament proteins

Neurofilaments (NFs) are another predominant constituent of the cytoskeletal scaffolding underlying the axonal architecture; they make up more than 60% of total protein in the axoplasm (Brown and Lasek, 1993). NFs act as essential determinants in radial growth of myelinated axons, which optimises conduction velocity (Hoffman et al., 1987; Sakaguchi et al., 1993). Furthermore, NFs support neuronal polarisation through their elastic fibrous properties (Wagner et al., 2007). NFs exist as heteropolymers and assembles from five intermediate filament proteins, namely NF heavy (NF-H), medium (NF-M), and light (NF-L) polypeptides, along with α-internexin in the central nervous system (Yuan et al., 2006), or peripherin, mostly expressed by neurons of the peripheral nervous system (Beaulieu et al., 1999). The amino-terminal rod domain promotes intermolecular interactions between NF subunits, thereby playing an important role in their polymerization into coiled-coil structures and ultimately, NF protofibrils. Uniquely, N-terminal domains of NF proteins harbour a MT polymerization inhibitory motif that binds unassembled tubulin. These flexible NF-MT interactions can influence MT polymerization and the markedly asymmetric shape of neurons (Bocquet et al., 2009). Thus, NF-NF and NF-MT interactions are integral to the formation of the dynamic axonal lattice.

Of particular interest, the C-terminal tail of NF-M and NF-H harbour highly phosphorylated Lys-Ser-Pro repeats, so that NFs, and especially NFH, are the most phosphorylated proteins in the nervous system. NF phosphorylation influences filament calibre, inter-filament interaction, NF-MT interactions, plasticity, and stability (Pant and Veeranna, 1995). In the axon, phosphorylation of NF-M and NF-H creates linearly aligned, spaced lattices of NFs, whereas dephosphorylation favours collapsed meshes in the soma and dendrites (Pant and Veeranna, 1995). In the normal neuron, NF-H, NF-M, and NF-L are phosphorylated to different degrees along the NF length, likely due to the complex regulatory interplay between protein kinases' and phosphatases' activities. PP1 catalytic subunit, and PP2A A, Bα and C subunits have been isolated in NF fractions purified from white matter (Saito et al., 1995; Strack et al., 1997) (Fig. 4). They interact with NFs independently of their activities. PP2A and PP1 contribute ~60% and ~10–20%, respectively, of total NF subunit phosphatase activity *in vitro*. Calcineurin has also been involved in dephosphorylation of NFs *in vitro* (Eyer and Leterrier, 1988), in depolarised neuronal cultures (Mata et al., 1997), and in the rat brain (Tanaka et al., 1993).

6.2. Functional significance of NF (de)phosphorylation in neuronal homeostasis

PP2A-mediated dephosphorylation of NF-L, which is assembly incompetent, induces its assembly into filamentous forms (Saito et al., 1995). Thus, by increasing the phosphorylation of NF triplet proteins and inducing disassembly of NFs, inhibition of PP2A may also contribute to axonal degeneration in OA-treated cells.

During their transport along axons, newly synthesised NF-M and NF-L specifically undergo a significant turnover of phosphate group. This dynamic modification may represent a fundamental mechanism to coordinate interactions of NFs with other proteins during their axonal transport, and their incorporation into the stationary cytoskeleton along axons (Nixon and Lewis, 1986). Significantly, this process is attributed to C-terminal domain dephosphorylation (Fig. 4). Inhibition of PP2A/PP1 by OA stimulates a rapid *de novo* accumulation of

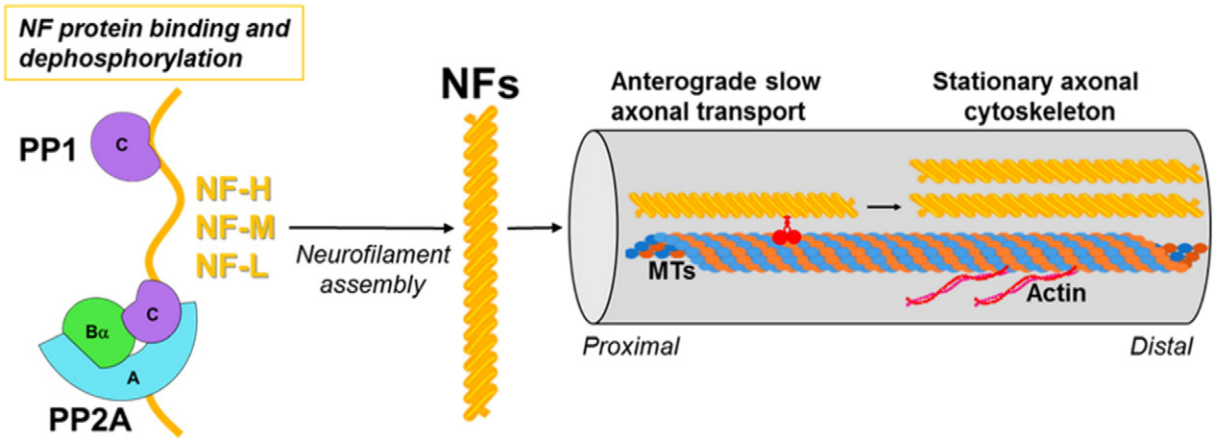

Fig. 4. PP2A- and PP1-dependent regulation of neurofilament architecture. Neurofilaments (NFs) are intermediate filaments that abundantly occupy axons and constitute the fundamental axonal cytoskeletal framework. Both PP2A and PP1 can interact with and dephosphorylate NF proteins, and play an essential role in the regulation of anterograde transport of somatic NFs along axons, which is required for incorporation of NFs into the stationary cytoskeletal lattice supporting axonal calibre and function. Inhibition of PP2A/PP1 activity promotes the accumulation of phosphorylated NFs in the soma and proximal axon by hindering axonal transport. For details, see text.

phospho-NFs in somas and proximal axons *in situ*, and decreases transport rate (Jung and Shea, 1999). Likewise, other studies report aberrant somatic phosphorylation of NF proteins in response to several PP2A/PP1 inhibitors (OA, microcystin LR and fostriecin), which impedes anterograde transport of NF-H into axons of primary cortical neurons (Rudrabhatla et al., 2009; Wang et al., 2001). Activation of PP1 triggers the translocation of NFs from the soma into axonal neurites, while direct PP1 inhibition promotes the accumulation of phospho-NFs and NF bundles in cell bodies (Lee et al., 2014).

Under normal physiological conditions, PP2A activity is high in neuronal cell bodies and phosphorylation of NF occurs selectively in axonal compartments (Rudrabhatla et al., 2009). Moreover, NF dynamics play a critical role in the developing and mature brain, and decreased PP2A activity has been shown to underlie age-related NF hyperphosphorylation (Veeranna et al., 2011). Interestingly, decreased NF and tau protein levels are found in mossy fibers of calcineurin α knockout mice, which could contribute to their deficits in learning and memory (Kayyali et al., 1997).

6.3. Functional significance in disease

Cytoskeletal phosphorylation status is also often altered in neurological disease. For instance, hyperphosphorylated NF proteins are present in AD-affected brain regions (Wang et al., 2001), wherein PP2A/Bα holoenzymes are known to be down-regulated (Sontag et al., 2004). Furthermore, phosphorylated NFs abnormally accumulate in motor neurons from patients with amyotropic lateral sclerosis (Munoz et al., 1988), and this is causally linked to neuronal cell death in transgenic mouse models of the disease (Williamson et al., 1998). Abnormal up-regulation of PP2A and PP1 mediating excessive dephosphorylation of NF proteins has also been observed in a mouse model of Krabbe disease, a genetic demyelinating syndrome characterised by a deficiency of large-calibre axons (Cantuti-Castelvetri et al., 2012). This is not surprising considering that PP2A is a key regulator of cellular signal transduction pathways and affiliated kinases. For instance, PP2A can positively regulate GSK3β activity (Wang et al., 2015). This kinase can induce NF bundling in somas and is essential for facilitating NF-NF interactions fostering the incorporation of NFs into the stationary phase (Lee et al., 2014). In contrast to PP2A activity, GSK3β also inhibits axonal transport of NFs and mediates NF accumulation within somas (Lee et al., 2014).

Together, these findings support a direct and indirect role for PP2A- and PP1-mediated dephosphorylation of NFs in the regulation of axonal calibre and NF dynamics.

7. Regulation of the neuronal actin cytoskeleton by PP2A, PP1 and Calcineurin

Actin filaments are another important constituent of the neuronal cytoskeleton. Actin within cells exists as globular (G-Actin) or filamentous (F-Actin) forms. Actin polymerization is tightly regulated and mediates a vast array of processes that are critical for neuronal morphogenesis and plasticity (Spence and Soderling, 2015). Actin polymerization can be regulated either by uncapping filament barbed ends, severing filaments, or *de novo* nucleation. It is well established that many actin regulatory proteins are regulated by Ser/Thr phosphorylation/dephosphorylation, and several studies in nonneuronal cells support an important role for PP2A, PP1 and calcineurin in the regulation of actin dynamics (see examples below). However, the specific function of these enzymes in the regulation of the neuronal actin cytoskeleton remains somewhat sketchy. Yet, it is likely that many actin regulatory proteins modulated by these phosphatases in nonneuronal cells could be targeted as well in neurons.

Actin dynamics in the dendritic spine -as well as in many other cellular systems- are largely regulated by Rho-family GTPases, including RhoA, Rac-1 and Cdc42 (Spence and Soderling, 2015). The Rho-GTPases regulate the downstream formation of specific actin structures, such as stress fibers, ruffles, lamellipodia and filopodia. Because of their central role in signal transduction, a great deal of interest has focused on the identification of downstream pathways that link these G proteins to the actin cytoskeleton (Hall, 1998). For instance, RhoA stimulates ROCK activity, subsequently phosphorylating and activating LIM domain kinase (LIMK); the loss of LIMK alters spine morphology and enhances long term potentiation (Meng et al., 2002). The critical target of LIMK is cofilin, an F-actin severing protein, the phosphorylation of which inhibits its F-actin disassembly activity (Yang et al., 1998). Notably, calcium-dependent calcineurin activation has been linked to enhanced cofilin dephosphorylation, contributing to synaptic dysfunction and impaired long term potentiation (Tu et al., 2014). PP1 and PP2A have also been reported to bind to and dephosphorylate cofilin in nonneuronal cell types, leading to its activation (Ambach et al., 2000; Oleinik et al., 2010). PP2A is likely involved in migration of neuronal cells *via* cofilin-dependent actin depolymerisation (Kilian et al., 2008). Stimulus-dependent PP1- and calcineurin-mediated cofilin dephosphorylation have been implicated in PC12 cell differentiation (Meberg et al., 1998).

The Rho/ROCK pathway is also important for neurite morphogenesis. One of the downstream effector of the Rho/ROCK pathway is the myristoylated, alanine-rich C kinase substrate protein (MARCKS),

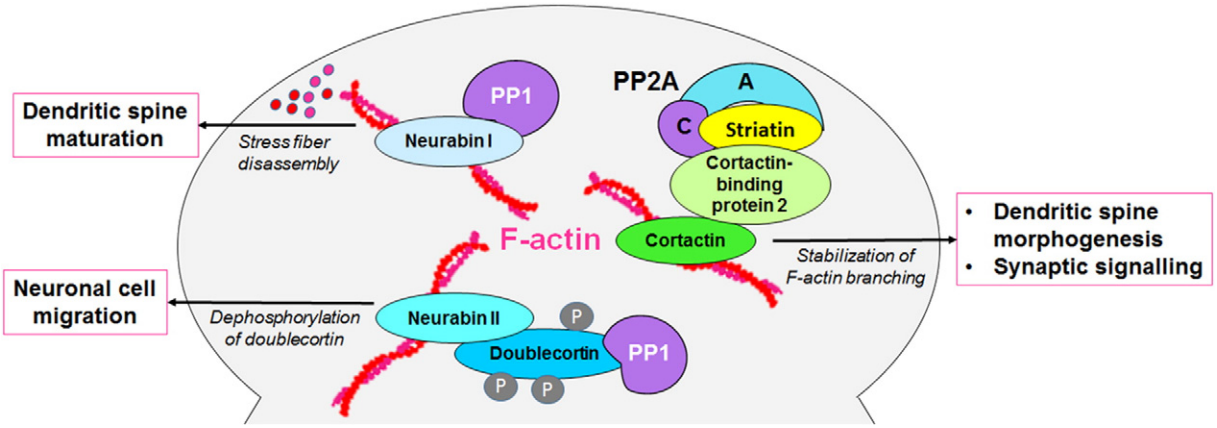

Fig. 5. Targeting of PP2A and PP1 to the actin cytoskeleton. Selected PP2A and PP1 enzymes have been found to be recruited to the F-actin network by specific actin-binding proteins in dendritic spines. This leads to actin rearrangements that modulate synaptic function and spine morphogenesis. Neuronal PP2A, PP1 and calcineurin also directly regulate F-actin disassembly by interacting with and/or dephosphorylating F-actin severing factors; they also function in Rho GTPase-mediated signalling that critically regulates F-actin assembly and branching (not shown). For details, see text.

which plays a key role in F-actin assembly, neurite outgrowth and dendrite branching. Notably, PP2A can dephosphorylate MARCKS to promote bradykinin-induced neurite outgrowth (Tanabe et al., 2012). Specific deregulation of PP2A is also associated with alterations in the actin cytoskeletal network in differentiated N2a cells (Sontag et al., 2010). In *Xenopus*, calcineurin acts on the growth cone actin cytoskeleton; Ca^{2+} waves lead to calcineurin-mediated dephosphorylation of the actin polymerization factor, GAP43, thereby promoting F-actin instability and slowing neurite extension (Lautermilch and Spitzer, 2000). Furthermore, the Rho/ROCK pathway is involved in the regulation of profilin, which facilitates linear F-actin formation and the inhibition of axon growth in neurons (Fujita and Yamashita, 2014). OA and knockdown of PP1c increase profilin phosphorylation in nonneuronal cells (Shao and Diamond, 2012). While not yet demonstrated, PP1/PP2A phosphatases may similarly regulate profilin in neurons.

F-actin assembly in neurons is also modulated by Rac1 and Cdc42, which link extracellular signals and actin nucleation through pathways involving the Wiskott-Aldrich Syndrome Protein (WASP) family of proteins, and the Arp2/3 complex, the actin nucleation machinery. These regulatory mechanisms are essential for branched F-actin formation, spine maintenance and functional and morphological alterations to spines, contributing to plasticity (Kim et al., 2013). Recent findings in mouse sensory neurons indicate a role for PP2A in interacting with and dephosphorylating Dock6, a guanine nucleotide exchange factor that activates Rac1 and Cdc42, thereby critically modulating axon outgrowth during neuronal development (Miyamoto et al., 2013).

Notably, some selected phosphatases are also targeted to the actin cytoskeleton through their docking to selective actin-binding proteins (Fig. 5). For instance, PP1 is anchored to F-actin-rich clusters in the postsynaptic density through its binding to Neurabin I, a neuron-specific actin-binding protein enriched in dendritic spines. By controlling actin rearrangement, this protein complex plays a critical role in modulating the morphology and maturation of spines; its disruption significantly affects synaptic transmission and hippocampal plasticity (Hu et al., 2006). Moreover, the PP1-Neurabin I complex promotes the disassembly of actin stress fibers in mouse hippocampal neurons (Oliver et al., 2002). The actin-binding protein, Neurabin II, also serves to target PP1 and doublecortin to F-actin; this promotes dephosphorylation of doublecortin, a key regulator of neuronal cell migration during development (Shmueli et al., 2006). Another example is the brain cortactin-binding protein 2, which targets striatin-containing PP2A isoforms (Fig. 1B) to actin stress fibers in dendritic spines (Chen et al., 2012). Cortactin-binding protein 2, which stably resides at dendritic spines, interacts with cortactin, a factor that stabilizes F-actin branching. The cortactin/cortactin-binding protein 2/PP2A protein complex is thought to regulate dendritic spine morphogenesis and synaptic signalling (Chen et al., 2012).

Lastly, many studies have implicated interactions between the actin and MT cytoskeleton in neurite outgrowth and axon guidance. In this context, the regulation of tau and MAP2 by Ser/Thr phosphatases could also indirectly influence actin remodelling. For instance, tau can indirectly impact actin dynamics through its signalling function (Sharma et al., 2007). MAP2c has been involved in the reorganization of lamellipodia during differentiation (Dehmelt et al., 2003), and in F-actin binding and bundling (Roger et al., 2004). The interaction of MAP2 with both MTs and F-actin is critical for neurite initiation, during which networks of MTs and F-actin are reorganized in a coordinated manner (Dehmelt and Halpain, 2005). Many other MAPs, like doublecortin and MTCL1, can bind both MTs and F-actin, and are regulated by PP2A and/or PP1.

In conclusion, PP2A, PP1 and calcineurin have clearly emerged as key regulators of neuronal MT, actin and NF dynamics. However, much remains to be learnt on their precise function in the regulation of the neuronal cytoskeletal architecture and its function in synaptic plasticity.

Conflicts of interest

The authors declare no conflicts of interests.

Acknowledgments

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References

Alexa, A., Schmidt, G., Tompa, P., Ogueta, S., Vazquez, J., Kulcsar, P., Kovacs, J., Dombradi, V., Friedrich, P., 2002. The phosphorylation state of threonine-220, a uniquely phosphatase-sensitive protein kinase A site in microtubule-associated protein MAP2c, regulates microtubule binding and stability. Biochemistry 41, 12427–12435.

Alonso, A.C., Zaidi, T., Grundke-Iqbal, I., Iqbal, K., 1994. Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. Proc. Natl. Acad. Sci. U. S. A. 91, 5562–5566.

Alonso, A.C., Grundke-Iqbal, I., Iqbal, K., 1996. Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. Nat. Med. 2, 783–787.

Alonso, A.D., Grundke-Iqbal, I., Barra, H.S., Iqbal, K., 1997. Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. Proc. Natl. Acad. Sci. U. S. A. 94, 298–303.

Ambach, A., Saunus, J., Konstandin, M., Wesselborg, S., Meuer, S.C., Samstag, Y., 2000. The serine phosphatases PP1 and PP2A associate with and activate the actin-binding protein cofilin in human T lymphocytes. Eur. J. Immunol. 30, 3422–3431.

Barra, H.S., Arce, C.A., Argarana, C.E., 1988. Posttranslational tyrosination/detyrosination of tubulin. Mol. Neurobiol. 2, 133–153.

Beaulieu, J.M., Robertson, J., Julien, J.P., 1999. Interactions between peripherin and neurofilaments in cultured cells: disruption of peripherin assembly by the NF-M and NF-H subunits. Biochem. Cell Biol. 77, 41–45.

Bhaskar, K., Yen, S.H., Lee, G., 2005. Disease-related modifications in tau affect the interaction between Fyn and Tau. J. Biol. Chem. 280, 35119–35125.

Bielas, S.L., Serneo, F.F., Chechlacz, M., Deerinck, T.J., Perkins, G.A., Allen, P.B., Ellisman, M.H., Gleeson, J.G., 2007. Spinophilin facilitates dephosphorylation of doublecortin by PP1 to mediate microtubule bundling at the axonal wrist. Cell 129, 579–591.

Bocquet, A., Berges, R., Frank, R., Robert, P., Peterson, A.C., Eyer, J., 2009. Neurofilaments bind tubulin and modulate its polymerization. J. Neurosci. Off. J. Soc. Neurosci. 29, 11043–11054.

Brown, A., Lasek, R.J., 1993. Neurofilaments move apart freely when released from the circumferential constraint of the axonal plasma membrane. Cell Motil. Cytoskeleton 26, 313–324.

Cantuti-Castelvetri, L., Zhu, H., Givogri, M.I., Chidavaenzi, R.L., Lopez-Rosas, A., Bongarzone, E.R., 2012. Psychosine induces the dephosphorylation of neurofilaments by deregulation of PP1 and PP2A phosphatases. Neurobiol. Dis. 46, 325–335.

Chen, Y.K., Chen, C.Y., Hu, H.T., Hsueh, Y.P., 2012. CTTNBP2, but not CTTNBP2NL, regulates dendritic spinogenesis and synaptic distribution of the striatin-PP2A complex. Mol. Biol. Cell 23, 4383–4392.

Cho, U.S., Xu, W., 2007. Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. Nature 445, 53–57.

Contin, M.A., Purro, S.A., Bisig, C.G., Barra, H.S., Arce, C.A., 2003. Inhibitors of protein phosphatase 1 and 2A decrease the level of tubulin carboxypeptidase activity associated with microtubules. Eur. J. Biochem. 270, 4921–4929.

Dehmelt, L., Halpain, S., 2005. The MAP2/Tau family of microtubule-associated proteins. Genome Biol. 6, 204.

Dehmelt, L., Smart, F.M., Ozer, R.S., Halpain, S., 2003. The role of microtubule-associated protein 2c in the reorganization of microtubules and lamellipodia during neurite initiation. J. Neurosci. 23, 9479–9490.

Dell'Acqua, M.L., Smith, K.E., Gorski, J.A., Horne, E.A., Gibson, E.S., Gomez, L.L., 2006. Regulation of neuronal PKA signaling through AKAP targeting dynamics. Eur. J. Cell Biol. 85, 627–633.

Deters, N., Ittner, L.M., Gotz, J., 2009. Substrate-specific reduction of PP2A activity exaggerates tau pathology. Biochem. Biophys. Res. Commun. 379, 400–405.

Dodge, K.L., Scott, J.D., 2003. Calcineurin anchoring and cell signaling. Biochem. Biophys. Res. Commun. 311, 1111–1115.

Drewes, G., Trinczek, B., Illenberger, S., Biernat, J., Schmitt-Ulms, G., Meyer, H.E., Mandelkow, E.M., Mandelkow, E., 1995. Microtubule-associated protein/microtubule affinity-regulating kinase (p110mark). A novel protein kinase that regulates tau-microtubule interactions and dynamic instability by phosphorylation at the Alzheimer-specific site serine 262. J. Biol. Chem. 270, 7679–7688.

Egloff, M.P., Johnson, D.F., Moorhead, G., Cohen, P.T., Cohen, P., Barford, D., 1997. Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. EMBO J. 16, 1876–1887.

Eidenmuller, J., Fath, T., Hellwig, A., Reed, J., Sontag, E., Brandt, R., 2000. Structural and functional implications of tau hyperphosphorylation: information from phosphorylation-mimicking mutated tau proteins. Biochemistry 39, 13166–13175.

Eidenmuller, J., Fath, T., Maas, T., Pool, M., Sontag, E., Brandt, R., 2001. Phosphorylation-mimicking glutamate clusters in the proline-rich region are sufficient to simulate the functional deficiencies of hyperphosphorylated tau protein. Biochem. J. 357, 759–767.

El Khoury, N.B., Gratuze, M., Petry, F., Papon, M.A., Julien, C., Marcouiller, F., Morin, F., Nicholls, S.B., Calon, F., Hebert, S.S., Marette, A., Planel, E., 2016. Hypothermia mediates age-dependent increase of tau phosphorylation in db/db mice. Neurobiol. Dis. 88, 55–65.

Erck, C., Peris, L., Andrieux, A., Meissirel, C., Gruber, A.D., Vernet, M., Schweitzer, A., Saoudi, Y., Pointu, H., Bosc, C., Salin, P.A., Job, D., Wehland, J., 2005. A vital role of tubulin-tyrosine-ligase for neuronal organization. Proc. Natl. Acad. Sci. U. S. A. 102, 7853–7858.

Eyer, J., Leterrier, J.F., 1988. Influence of the phosphorylation state of neurofilament proteins on the interactions between purified filaments in vitro. Biochem. J. 252, 655–660.

Fan, Q.W., Yu, W., Gong, J.S., Zou, K., Sawamura, N., Senda, T., Yanagisawa, K., Michikawa, M., 2002. Cholesterol-dependent modulation of dendrite outgrowth and microtubule stability in cultured neurons. J. Neurochem. 80, 178–190.

Farah, C.A., Leclerc, N., 2008. HMWMAP2: new perspectives on a pathway to dendritic identity. Cell Motil. Cytoskeleton 65, 515–527.

Ferreira, A., Kincaid, R., Kosik, K.S., 1993. Calcineurin is associated with the cytoskeleton of cultured neurons and has a role in the acquisition of polarity. Mol. Biol. Cell 4, 1225–1238.

Fujita, Y., Yamashita, T., 2014. Axon growth inhibition by RhoA/ROCK in the central nervous system. Front. Neurosci. 8, 338.

Gamblin, T.C., Nachmanoff, K., Halpain, S., Williams Jr., R.C., 1996. Recombinant microtubule-associated protein 2c reduces the dynamic instability of individual microtubules. Biochemistry 35, 12576–12586.

Gliksman, N.R., Parsons, S.F., Salmon, E.D., 1992. Okadaic acid induces interphase to mitotic-like microtubule dynamic instability by inactivating rescue. J. Cell Biol. 119, 1271–1276.

Goedert, M., Cohen, E.S., Jakes, R., Cohen, P., 1992. p42 MAP kinase phosphorylation sites in microtubule-associated protein tau are dephosphorylated by protein phosphatase 2A1. Implications for Alzheimer's disease [corrected]. FEBS Lett. 312, 95–99.

Goedert, M., Satumtira, S., Jakes, R., Smith, M.J., Kamibayashi, C., White, C.L., Sontag, E., 2000. Reduced binding of protein phosphatase 2A to tau protein with frontotemporal dementia and parkinsonism linked to chromosome 17 mutations. J. Neurochem. 75, 2155–2162.

Gong, C.X., Singh, T.J., Grundke-Iqbal, I., Iqbal, K., 1993. Phosphoprotein phosphatase activities in Alzheimer disease brain. J. Neurochem. 61, 921–927.

Gong, C.X., Grundke-Iqbal, I., Damuni, Z., Iqbal, K., 1994a. Dephosphorylation of microtubule-associated protein tau by protein phosphatase-1 and -2C and its implication in Alzheimer disease. FEBS Lett. 341, 94–98.

Gong, C.X., Grundke-Iqbal, I., Iqbal, K., 1994b. Dephosphorylation of Alzheimer's disease abnormally phosphorylated tau by protein phosphatase-2A. Neuroscience 61, 765–772.

Gong, C.X., Singh, T.J., Grundke-Iqbal, I., Iqbal, K., 1994c. Alzheimer's disease abnormally phosphorylated tau is dephosphorylated by protein phosphatase-2B (calcineurin). J. Neurochem. 62, 803–806.

Gong, C.X., Shaikh, S., Wang, J.Z., Zaidi, T., Grundke-Iqbal, I., Iqbal, K., 1995. Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. J. Neurochem. 65, 732–738.

Gong, C.X., Wegiel, J., Lidsky, T., Zuck, L., Avila, J., Wisniewski, H.M., Grundke-Iqbal, I., Iqbal, K., 2000. Regulation of phosphorylation of neuronal microtubule-associated proteins MAP1b and MAP2 by protein phosphatase-2A and -2B in rat brain. Brain Res. 853, 299–309.

Gordon-Weeks, P.R., Mansfield, S.G., Alberto, C., Johnstone, M., Moya, F., 1993. A phosphorylation epitope on MAP 1B that is transiently expressed in growing axons in the developing rat nervous system. Eur. J. Neurosci. 5, 1302–1311.

Goto, S., Yamamoto, H., Fukunaga, K., Iwasa, T., Matsukado, Y., Miyamoto, E., 1985. Dephosphorylation of microtubule-associated protein 2, tau factor, and tubulin by calcineurin. J. Neurochem. 45, 276–283.

Gratuze, M., Noel, A., Julien, C., Cisbani, G., Milot-Rousseau, P., Morin, F., Dickler, M., Goupil, C., Bezeau, F., Poitras, I., Bissonnette, S., Whittington, R.A., Hebert, S.S., Cicchetti, F., Parker, J.A., Samadi, P., Planel, E., 2015. Tau hyperphosphorylation and deregulation of calcineurin in mouse models of Huntington's disease. Hum. Mol. Genet. 24, 86–99.

Griffith, J.P., Kim, J.L., Kim, E.E., Sintchak, M.D., Thomson, J.A., Fitzgibbon, M.J., Fleming, M.A., Caron, P.R., Hsiao, K., Navia, M.A., 1995. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. Cell 82, 507–522.

Gundersen, G.G., Bulinski, J.C., 1988. Selective stabilization of microtubules oriented toward the direction of cell migration. Proc. Natl. Acad. Sci. U. S. A. 85, 5946–5950.

Gurland, G., Gundersen, G.G., 1993. Protein phosphatase inhibitors induce the selective breakdown of stable microtubules in fibroblasts and epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 90, 8827–8831.

Hall, A., 1998. Rho GTPases and the actin cytoskeleton. Science 279, 509–514.

Halpain, S., Greengard, P., 1990. Activation of NMDA receptors induces rapid dephosphorylation of the cytoskeletal protein MAP2. Neuron 5, 237–246.

Hanger, D.P., Anderton, B.H., Noble, W., 2009. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. Trends Mol. Med. 15, 112–119.

Heroes, E., Lesage, B., Gornemann, J., Beullens, M., Van Meervelt, L., Bollen, M., 2013. The PP1 binding code: a molecular-lego strategy that governs specificity. FEBS J. 280, 584–595.

Hiraga, A., Tamura, S., 2000. Protein phosphatase 2A is associated in an inactive state with microtubules through 2A1-specific interaction with tubulin. Biochem. J. 346 (Pt 2), 433–439.

Hoffman, P.N., Cleveland, D.W., Griffin, J.W., Landes, P.W., Cowan, N.J., Price, D.L., 1987. Neurofilament gene expression: a major determinant of axonal caliber. Proc. Natl. Acad. Sci. U. S. A. 84, 3472–3476.

Hu, X.D., Huang, Q., Roadcap, D.W., Shenolikar, S.S., Xia, H., 2006. Actin-associated neurabin-protein phosphatase-1 complex regulates hippocampal plasticity. J. Neurochem. 98, 1841–1851.

Hyodo, T., Ito, S., Asano-Inami, E., Chen, D., Senga, T., 2016. A regulatory subunit of protein phosphatase 2A, PPP2R5E, regulates the abundance of microtubule crosslinking factor 1 (MTCL1). FEBS J.

Jung, C., Shea, T.B., 1999. Regulation of neurofilament axonal transport by phosphorylation in optic axons in situ. Cell Motil. Cytoskeleton 42, 230–240.

Kadavath, H., Hofele, R.V., Biernat, J., Kumar, S., Tepper, K., Urlaub, H., Mandelkow, E., Zweckstetter, M., 2015. Tau stabilizes microtubules by binding at the interface between tubulin heterodimers. Proc. Natl. Acad. Sci. U. S. A. 112, 7501–7506.

Kanaan, N.M., Morfini, G.A., LaPointe, N.E., Pigino, G.F., Patterson, K.R., Song, Y., Andreadis, A., Fu, Y., Brady, S.T., Binder, L.I., 2011. Pathogenic forms of tau inhibit kinesin-dependent axonal transport through a mechanism involving activation of axonal phosphotransferases. J. Neurosci. 31, 9858–9868.

Kayyali, U.S., Zhang, W., Yee, A.G., Seidman, J.G., Potter, H., 1997. Cytoskeletal changes in the brains of mice lacking calcineurin A alpha. J. Neurochem. 68, 1668–1678.

Kelker, M.S., Page, R., Peti, W., 2009. Crystal structures of protein phosphatase-1 bound to nodularin-R and tautomycin: a novel scaffold for structure-based drug design of serine/threonine phosphatase inhibitors. J. Mol. Biol. 385, 11–21.

Khan, I.A., Luduena, R.F., 1996. Phosphorylation of beta III-tubulin. Biochemistry 35, 3704–3711.

Khawaja, S., Gundersen, G.G., Bulinski, J.C., 1988. Enhanced stability of microtubules enriched in detyrosinated tubulin is not a direct function of detyrosination level. J. Cell Biol. 106, 141–149.

Kilian, P., Campbell, S., Bilodeau, L., Guimond, M.O., Roberge, C., Gallo-Payet, N., Payet, M.D., 2008. Angiotensin II AT2 receptor stimulation increases the rate of NG108-15 cell migration via actin depolymerization. Endocrinology.

Kim, I.H., Racz, B., Wang, H., Burianek, L., Weinberg, R., Yasuda, R., Wetsel, W.C., Soderling, S.H., 2013. Disruption of Arp2/3 results in asymmetric structural plasticity of dendritic spines and progressive synaptic and behavioral abnormalities. J. Neurosci. Off. J. Soc. Neurosci. 33, 6081–6092.

Klee, C.B., Crouch, T.H., Krinks, M.H., 1979. Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc. Natl. Acad. Sci. U. S. A. 76, 6270–6273.

Kong, M., Ditsworth, D., Lindsten, T., Thompson, C.B., 2009. alpha4 is an essential regulator of PP2A phosphatase activity. Mol. Cell 36, 51–60.
Landrieu, I., Smet-Nocca, C., Amniai, L., Louis, J.V., Wieruszeski, J.M., Goris, J., Janssens, V., Lippens, G., 2011. Molecular implication of PP2A and Pin1 in the Alzheimer's disease specific hyperphosphorylation of Tau. PLoS One 6, e21521.
Lautermilch, N.J., Spitzer, N.C., 2000. Regulation of calcineurin by growth cone calcium waves controls neurite extension. J. Neurosci. Off. J. Soc. Neurosci. 20, 315–325.
Lee, S., Pant, H.C., Shea, T.B., 2014. Divergent and convergent roles for kinases and phosphatases in neurofilament dynamics. J. Cell Sci. 127, 4064–4077.
Lewis, S.A., Wang, D.H., Cowan, N.J., 1988. Microtubule-associated protein MAP2 shares a microtubule binding motif with tau protein. Science 242, 936–939.
Li, W., Handschumacher, R.E., 2002. Identification of two calcineurin B-binding proteins: tubulin and heat shock protein 60. Biochim. Biophys. Acta 1599, 72–81.
Liao, H., Li, Y., Brautigan, D.L., Gundersen, G.G., 1998. Protein phosphatase 1 is targeted to microtubules by the microtubule-associated protein tau. J. Biol. Chem. 273, 21901–21908.
Lin, X.H., Walter, J., Scheidtmann, K., Ohst, K., Newport, J., Walter, G., 1998. Protein phosphatase 2A is required for the initiation of chromosomal DNA replication. Proc. Natl. Acad. Sci. U. S. A. 95, 14693–14698.
Liu, F., Grundke-Iqbal, I., Iqbal, K., Gong, C.X., 2005. Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. Eur. J. Neurosci. 22, 1942–1950.
Louis, J.V., Martens, E., Borghgraef, P., Lambrecht, C., Sents, W., Longin, S., Zwaenepoel, K., Pijnenborg, R., Landrieu, I., Lippens, G., Ledermann, B., Gotz, J., Van Leuven, F., Goris, J., Janssens, V., 2011. Mice lacking phosphatase PP2A subunit PR61/B'delta (Ppp2r5d) develop spatially restricted tauopathy by deregulation of CDK5 and GSK3beta. Proc. Natl. Acad. Sci. U. S. A. 108, 6957–6962.
Mansuy, I.M., 2003. Calcineurin in memory and bidirectional plasticity. Biochem. Biophys. Res. Commun. 311, 1195–1208.
Martin, L., Latypova, X., Wilson, C.M., Magnaudeix, A., Perrin, M.L., Terro, F., 2013a. Tau protein phosphatases in Alzheimer's disease: the leading role of PP2A. Ageing Res. Rev. 12, 39–49.
Martin, L., Latypova, X., Wilson, C.M., Magnaudeix, A., Perrin, M.L., Yardin, C., Terro, F., 2013b. Tau protein kinases: involvement in Alzheimer's disease. Ageing Res. Rev. 12, 289–309.
Mata, M., Honegger, P., Fink, D.J., 1997. Modulation of phosphorylation of neuronal cytoskeletal proteins by neuronal depolarization. Cell. Mol. Neurobiol. 17, 129–140.
Mawal-Dewan, M., Henley, J., Van, d.V., Trojanowski, J.Q., Lee, V.M., 1994. The phosphorylation state of tau in the developing rat brain is regulated by phosphoprotein phosphatases. J. Biol. Chem. 269, 30981–30987.
Maynes, J.T., Perreault, K.R., Cherney, M.M., Luu, H.A., James, M.N., Holmes, C.F., 2004. Crystal structure and mutagenesis of a protein phosphatase-1:calcineurin hybrid elucidate the role of the beta12-beta13 loop in inhibitor binding. J. Biol. Chem. 279, 43198–43206.
Meberg, P.J., Ono, S., Minamide, L.S., Takahashi, M., Bamburg, J.R., 1998. Actin depolymerizing factor and cofilin phosphorylation dynamics: response to signals that regulate neurite extension. Cell Motil. Cytoskeleton 39, 172–190.
Meng, Y., Zhang, Y., Tregoubov, V., Janus, C., Cruz, L., Jackson, M., Lu, W.Y., MacDonald, J.F., Wang, J.Y., Falls, D.L., Jia, Z., 2002. Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron 35, 121–133.
Merrick, S.E., Trojanowski, J.Q., Lee, V.M., 1997. Selective destruction of stable microtubules and axons by inhibitors of protein serine/threonine phosphatases in cultured human neurons. J. Neurosci. 17, 5726–5737.
Miyamoto, Y., Torii, T., Yamamori, N., Ogata, T., Tanoue, A., Yamauchi, J., 2013. Akt and PP2A reciprocally regulate the guanine nucleotide exchange factor Dock6 to control axon growth of sensory neurons. Sci. Signal. 6, ra15.
Munoz, D.G., Greene, C., Perl, D.P., Selkoe, D.J., 1988. Accumulation of phosphorylated neurofilaments in anterior horn motoneurons of amyotrophic lateral sclerosis patients. J. Neuropathol. Exp. Neurol. 47, 9–18.
Nixon, R.A., Lewis, S.E., 1986. Differential turnover of phosphate groups on neurofilament subunits in mammalian neurons in vivo. J. Biol. Chem. 261, 16298–16301.
Nunbhakdi-Craig, V., Schuechner, S., Sontag, J.M., Montgomery, L., Pallas, D.C., Juno, C., Mudrak, I., Ogris, E., Sontag, E., 2007. Expression of protein phosphatase 2A mutants and silencing of the regulatory B alpha subunit induce a selective loss of acetylated and detyrosinated microtubules. J. Neurochem. 101, 959–971.
Oleinik, N.V., Krupenko, N.I., Krupenko, S.A., 2010. ALDH1L1 inhibits cell motility via dephosphorylation of cofilin by PP1 and PP2A. Oncogene 29, 6233–6244.
Oliver, C.J., Terry-Lorenzo, R.T., Elliott, E., Bloomer, W.A., Li, S., Brautigan, D.L., Colbran, R.J., Shenolikar, S., 2002. Targeting protein phosphatase 1 (PP1) to the actin cytoskeleton: the neurabin I/PP1 complex regulates cell morphology. Mol. Cell. Biol. 22, 4690–4701.
Ortega-Gutierrez, S., Leung, D., Ficarro, S., Peters, E.C., Cravatt, B.F., 2008. Targeted disruption of the PME-1 gene causes loss of demethylated PP2A and perinatal lethality in mice. PLoS One 3, e2486.
Ouimet, C.C., da Cruz e Silva, E.F., Greengard, P., 1995. The alpha and gamma 1 isoforms of protein phosphatase 1 are highly and specifically concentrated in dendritic spines. Proc. Natl. Acad. Sci. U. S. A. 92, 3396–3400.
Ozer, R.S., Halpain, S., 2000. Phosphorylation-dependent localization of microtubule-associated protein MAP2c to the actin cytoskeleton. Mol. Biol. Cell 11, 3573–3587.
Panda, D., Samuel, J.C., Massie, M., Feinstein, S.C., Wilson, L., 2003. Differential regulation of microtubule dynamics by three- and four-repeat tau: implications for the onset of neurodegenerative disease. Proc. Natl. Acad. Sci. U. S. A. 100, 9548–9553.
Pant, H.C., Veeranna, 1995. Neurofilament phosphorylation. Biochem. Cell Biol. 73, 575–592.
Papon, M.A., El Khoury, N.B., Marcouiller, F., Julien, C., Morin, F., Bretteville, A., Petry, F.R., Gaudreau, S., Amrani, A., Mathews, P.M., Hebert, S.S., Planel, E., 2013. Deregulation of protein phosphatase 2A and hyperphosphorylation of tau protein following onset of diabetes in NOD mice. Diabetes 62, 609–617.
Piperno, G., LeDizet, M., Chang, X.J., 1987. Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. J. Cell Biol. 104, 289–302.
Planel, E., Yasutake, K., Fujita, S.C., Ishiguro, K., 2001. Inhibition of protein phosphatase 2A overrides tau protein kinase I/glycogen synthase kinase 3 beta and cyclin-dependent kinase 5 inhibition and results in tau hyperphosphorylation in the hippocampus of starved mouse. J. Biol. Chem. 276, 34298–34306.
Price, N.E., Wadzinski, B., Mumby, M.C., 1999. An anchoring factor targets protein phosphatase 2A to brain microtubules. Brain Res. Mol. Brain Res. 73, 68–77.
Qian, W., Yin, X., Hu, W., Shi, J., Gu, J., Grundke-Iqbal, I., Iqbal, K., Gong, C.X., Liu, F., 2011. Activation of protein phosphatase 2B and hyperphosphorylation of Tau in Alzheimer's disease. J. Alzheimers Dis. 23, 617–627.
Rahman, A., Grundke-Iqbal, I., Iqbal, K., 2005. Phosphothreonine-212 of Alzheimer abnormally hyperphosphorylated tau is a preferred substrate of protein phosphatase-1. Neurochem. Res. 30, 277–287.
Reber, B.F., Bouron, A., 1995. Calyculin-A-induced fast neurite retraction in nerve growth factor-differentiated rat pheochromocytoma (PC12) cells. Neurosci. Lett. 183, 198–201.
Riederer, B.M., Moya, F., Calvert, R., 1993. Phosphorylated MAP1b, alias MAP5 and MAP1x, is involved in axonal growth and neuronal mitosis. Neuroreport 4, 771–774.
Roger, B., Al Bassam, J., Dehmelt, L., Milligan, R.A., Halpain, S., 2004. MAP2c, but not tau, binds and bundles F-actin via its microtubule binding domain. Curr. Biol. 14, 363–371.
Rudrabhatla, P., Albers, W., Pant, H.C., 2009. Peptidyl-prolyl isomerase 1 regulates protein phosphatase 2A-mediated topographic phosphorylation of neurofilament proteins. J. Neurosci. 29, 14869–14880.
Ruediger, R., Van Wart Hood, J.E., Mumby, M., Walter, G., 1991. Constant expression and activity of protein phosphatase 2A in synchronized cells. Mol. Cell. Biol. 11, 4282–4285.
Saito, T., Shima, H., Osawa, Y., Nagao, M., Hemmings, B.A., Kishimoto, T., Hisanaga, S., 1995. Neurofilament-associated protein phosphatase 2A: its possible role in preserving neurofilaments in filamentous states. Biochemistry 34, 7376–7384.
Sakaguchi, T., Okada, M., Kitamura, T., Kawasaki, K., 1993. Reduced diameter and conduction velocity of myelinated fibers in the sciatic nerve of a neurofilament-deficient mutant quail. Neurosci. Lett. 153, 65–68.
Sanchez, C., Tompa, P., Szucs, K., Friedrich, P., Avila, J., 1996. Phosphorylation and dephosphorylation in the proline-rich C-terminal domain of microtubule-associated protein 2. Eur. J. Biochem. 241, 765–771.
Sanchez, C., Diaz-Nido, J., Avila, J., 2000. Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. Prog. Neurobiol. 61, 133–168.
Schaar, B.T., Kinoshita, K., McConnell, S.K., 2004. Doublecortin microtubule affinity is regulated by a balance of kinase and phosphatase activity at the leading edge of migrating neurons. Neuron 41, 203–213.
Schneider, A., Biernat, J., von Bergen, M., Mandelkow, E., Mandelkow, E.M., 1999. Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments. Biochemistry 38, 3549–3558.
Schweiger, S., Schneider, R., 2003. The MID1/PP2A complex: a key to the pathogenesis of Opitz BBB/G syndrome. BioEssays 25, 356–366.
Sengupta, A., Kabat, J., Novak, M., Wu, Q.L., Grundke-Iqbal, I., Iqbal, K., 1998. Phosphorylation of tau at both Thr 231 and Ser 262 is required for maximal inhibition of its binding to microtubules. Arch. Biochem. Biophys. 357, 299–309.
Sents, W., Ivanova, E., Lambrecht, C., Haesen, D., Janssens, V., 2012. The biogenesis of active protein phosphatase 2A holoenzymes: a tightly regulated process creating phosphatase specificity. FEBS J. 280, 644–661.
Shao, J., Diamond, M.I., 2012. Protein phosphatase 1 dephosphorylates profilin-1 at ser-137. PLoS One 7, e32802.
Sharma, V.M., Litersky, J.M., Bhaskar, K., Lee, G., 2007. Tau impacts on growth-factor-stimulated actin remodeling. J. Cell Sci. 120, 748–757.
Shelden, E., Wadsworth, P., 1996. Stimulation of microtubule dynamic turnover in living cells treated with okadaic acid. Cell Motil. Cytoskeleton 35, 24–34.
Shi, Y., 2009. Serine/threonine phosphatases: mechanism through structure. Cell 139, 468–484.
Shmueli, A., Gdalyahu, A., Sapoznik, S., Sapir, T., Tsukada, M., Reiner, O., 2006. Site-specific dephosphorylation of doublecortin (DCX) by protein phosphatase 1 (PP1). Mol. Cell. Neurosci. 32, 15–26.
Short, K.M., Hopwood, B., Yi, Z., Cox, T.C., 2002. MID1 and MID2 homo- and heterodimerise to tether the rapamycin-sensitive PP2A regulatory subunit, alpha 4, to microtubules: implications for the clinical variability of X-linked Opitz GBBB syndrome and other developmental disorders. BMC Cell Biol. 3, 1.
Silverman-Gavrila, L.B., Praver, M., Mykles, D.L., Charlton, M.P., 2013. Calcium, calpain, and calcineurin in low-frequency depression of transmitter release. J. Neurosci. Off. J. Soc. Neurosci. 33, 1975–1990.
Sontag, E., 2001. Protein phosphatase 2A: the Trojan Horse of cellular signaling. Cell. Signal. 13, 7–16.
Sontag, J.M., Sontag, E., 2014. Protein phosphatase 2A dysfunction in Alzheimer's disease. Front. Mol. Neurosci. 7, 16.
Sontag, E., Nunbhakdi-Craig, V., Bloom, G.S., Mumby, M.C., 1995. A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J. Cell Biol. 128, 1131–1144.
Sontag, E., Nunbhakdi-Craig, V., Lee, G., Bloom, G.S., Mumby, M.C., 1996. Regulation of the phosphorylation state and microtubule-binding activity of Tau by protein phosphatase 2A. Neuron 17, 1201–1207.
Sontag, E., Nunbhakdi-Craig, V., Lee, G., Brandt, R., Kamibayashi, C., Kuret, J., White 3rd, C.L., Mumby, M.C., Bloom, G.S., 1999. Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. J. Biol. Chem. 274, 25490–25498.

Sontag, E., Luangpirom, A., Hladik, C., Mudrak, I., Ogris, E., Speciale, S., White, C.L., 2004. Altered expression levels of the protein phosphatase 2A AB alpha C enzyme are associated with Alzheimer disease pathology. J. Neuropathol. Exp. Neurol. 63, 287–301.

Sontag, E., Nunbhakdi-Craig, V., Sontag, J.M., Diaz-Arrastia, R., Ogris, E., Dayal, S., Lentz, S.R., Arning, E., Bottiglieri, T., 2007. Protein phosphatase 2A methyltransferase links homocysteine metabolism with tau and amyloid precursor protein regulation. J. Neurosci. 27, 2751–2759.

Sontag, J.-M., Nunbhakdi-Craig, V., Montgomery, L., Arning, E., Bottiglieri, T., Sontag, E., 2008. Folate deficiency induces in vitro and mouse brain region-specific downregulation of leucine carboxyl methyltransferase-1 and Protein phosphatase 2A B alpha subunit expression that correlate with enhanced tau phosphorylation. J. Neurosci. 28, 11477–11487.

Sontag, J.M., Nunbhakdi-Craig, V., Mitterhuber, M., Ogris, E., Sontag, E., 2010. Regulation of protein phosphatase 2A methylation by LCMT1 and PME-1 plays a critical role in differentiation of neuroblastoma cells. J. Neurochem. 115, 1455–1465.

Sontag, J.-M., Nunbhakdi-Craig, V., White III, C.L., Halpain, S., Sontag, E., 2012. The protein phosphatase PP2A/B alpha binds to the microtubule-associated proteins Tau and MAP2 at a motif also recognized by the kinase Fyn implications for Tauopathies. J. Biol. Chem. 287, 14984–14993.

Sontag, J.-M., Wasek, B., Taleski, G., Smith, J., Arning, E., Sontag, E., Bottiglieri, T., 2014. Altered protein phosphatase 2A methylation and Tau phosphorylation in the young and aged brain of methylenetetrahydrofolate reductase (MTHFR) deficient mice. Front. Aging Neurosci. 6.

Spence, E.F., Soderling, S.H., 2015. Actin out: regulation of the synaptic cytoskeleton. J. Biol. Chem. 290, 28613–28622.

Spillantini, M.G., Goedert, M., 2013. Tau pathology and neurodegeneration. Lancet Neurol. 12, 609–622.

Strack, S., Westphal, R.S., Colbran, R.J., Ebner, F.F., Wadzinski, B.E., 1997. Protein serine/threonine phosphatase 1 and 2A associate with and dephosphorylate neurofilaments. Brain Res. Mol. Brain Res. 49, 15–28.

Strack, S., Kini, S., Ebner, F.F., Wadzinski, B.E., Colbran, R.J., 1999. Differential cellular and subcellular localization of protein phosphatase 1 isoforms in brain. J. Comp. Neurol. 413, 373–384.

Swingle, M., Ni, L., Honkanen, R.E., 2007. Small-molecule inhibitors of ser/thr protein phosphatases: specificity, use and common forms of abuse. Methods Mol. Biol. 365, 23–38.

Tanabe, A., Shiraishi, M., Negishi, M., Saito, N., Tanabe, M., Sasaki, Y., 2012. MARCKS dephosphorylation is involved in bradykinin-induced neurite outgrowth in neuroblastoma SH-SY5Y cells. J. Cell. Physiol. 227, 618–629.

Tanaka, T., Takeda, M., Niigawa, H., Hariguchi, S., Nishimura, T., 1993. Phosphorylated neurofilament accumulation in neuronal perikarya by cyclosporin A injection in rat brain. Methods Find. Exp. Clin. Pharmacol. 15, 77–87.

Terrak, M., Kerff, F., Langsetmo, K., Tao, T., Dominguez, R., 2004. Structural basis of protein phosphatase 1 regulation. Nature 429, 780–784.

Tu, S., Okamoto, S., Lipton, S.A., Xu, H., 2014. Oligomeric Abeta-induced synaptic dysfunction in Alzheimer's disease. Mol. Neurodegener. 9, 48.

Ulloa, L., Diaz-Nido, J., Avila, J., 1993a. Depletion of casein kinase II by antisense oligonucleotide prevents neuritogenesis in neuroblastoma cells. EMBO J. 12, 1633–1640.

Ulloa, L., Dombradi, V., Diaz-Nido, J., Szucs, K., Gergely, P., Friedrich, P., Avila, J., 1993b. Dephosphorylation of distinct sites on microtubule-associated protein MAP1B by protein phosphatases 1, 2A and 2B. FEBS Lett. 330, 85–89.

Veeranna, D.S., Yang, J.H., Lee, K.Y., Vinod, P., Stavrides, N.D., Amin, H.C. Pant, Nixon, R.A., 2011. Declining phosphatases underlie aging-related hyperphosphorylation of neurofilaments. Neurobiol. Aging 32, 2016–2029.

Virshup, D.M., Shenolikar, S., 2009. From promiscuity to precision: protein phosphatases get a makeover. Mol. Cell 33, 537–545.

Wagner, O.I., Rammensee, S., Korde, N., Wen, Q., Leterrier, J.F., Janmey, P.A., 2007. Softness, strength and self-repair in intermediate filament networks. Exp. Cell Res. 313, 2228–2235.

Wang, Y., Mandelkow, E., 2016. Tau in physiology and pathology. Nat. Rev. Neurosci. 17, 5–21.

Wang, J., Tung, Y.C., Wang, Y., Li, X.T., Iqbal, K., Grundke-Iqbal, I., 2001. Hyperphosphorylation and accumulation of neurofilament proteins in Alzheimer disease brain and in okadaic acid-treated SY5Y cells. FEBS Lett. 507, 81–87.

Wang, Y., Yang, R., Gu, J., Yin, X., Jin, N., Xie, S., Wang, Y., Chang, H., Qian, W., Shi, J., Iqbal, K., Gong, C.X., Cheng, C., Liu, F., 2015. Cross talk between PI3K-AKT-GSK-3beta and PP2A pathways determines tau hyperphosphorylation. Neurobiol. Aging 36, 188–200.

Watkins, G.R., Wang, N., Mazalouskas, M.D., Gomez, R.J., Guthrie, C.R., Kraemer, B.C., Schweiger, S., Spiller, B.W., Wadzinski, B.E., 2012. Monoubiquitination promotes calpain cleavage of the protein phosphatase 2A (PP2A) regulatory subunit alpha4, altering PP2A stability and microtubule-associated protein phosphorylation. J. Biol. Chem. 287, 24207–24215.

Wei, Q., Holzer, M., Brueckner, M.K., Liu, Y., Arendt, T., 2002. Dephosphorylation of tau protein by calcineurin triturated into neural living cells. Cell. Mol. Neurobiol. 22, 13–24.

Williamson, T.L., Bruijn, L.I., Zhu, Q., Anderson, K.L., Anderson, S.D., Julien, J.P., Cleveland, D.W., 1998. Absence of neurofilaments reduces the selective vulnerability of motor neurons and slows disease caused by a familial amyotrophic lateral sclerosis-linked superoxide dismutase 1 mutant. Proc. Natl. Acad. Sci. U. S. A. 95, 9631–9636.

Xu, Y., Xing, Y., Chen, Y., Chao, Y., Lin, Z., Fan, E., Yu, J.W., Strack, S., Jeffrey, P.D., Shi, Y., 2006. Structure of the protein phosphatase 2A holoenzyme. Cell 127, 1239–1251.

Xu, Y., Chen, Y., Zhang, P., Jeffrey, P.D., Shi, Y., 2008. Structure of a protein phosphatase 2A holoenzyme: insights into B55-mediated Tau dephosphorylation. Mol. Cell 31, 873–885.

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., Mizuno, K., 1998. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature 393, 809–812.

Yoon, S.Y., Choi, H.I., Choi, J.E., Sul, C.A., Choi, J.M., Kim, D.H., 2007. Methotrexate decreases PP2A methylation and increases tau phosphorylation in neuron. Biochem. Biophys. Res. Commun. 363, 811–816.

Yoon, S.Y., Choi, J.E., Choi, J.M., Kim, D.H., 2008. Dynein cleavage and microtubule accumulation in okadaic acid-treated neurons. Neurosci. Lett. 437, 111–115.

Yu, D.Y., Tong, L., Song, G.J., Lin, W.L., Zhang, L.Q., Bai, W., Gong, H., Yin, Y.X., Wei, Q., 2008. Tau binds both subunits of calcineurin, and binding is impaired by calmodulin. Biochim. Biophys. Acta 1783, 2255–2261.

Yu, U.Y., Yoo, B.C., Ahn, J.H., 2014. Regulatory B subunits of protein phosphatase 2A are involved in site-specific regulation of Tau protein phosphorylation. J. Physiol. Pharmacol. 18, 155–161.

Yuan, A., Rao, M.V., Sasaki, T., Chen, Y., Kumar, A., Veeranna, R.K., Liem, J., Eyer, A.C., Peterson, J.P. Julien, Nixon, R.A., 2006. Alpha-internexin is structurally and functionally associated with the neurofilament triplet proteins in the mature CNS. J. Neurosci. 26, 10006–10019.

Zamora-Leon, S.P., Lee, G., Davies, P., Shafit-Zagardo, B., 2001. Binding of Fyn to MAP-2c through an SH3 binding domain. Regulation of the interaction by ERK2. J. Biol. Chem. 276, 39950–39958.

Zhang, C.E., Tian, Q., Wei, W., Peng, J.H., Liu, G.P., Zhou, X.W., Wang, Q., Wang, D.W., Wang, J.Z., 2008. Homocysteine induces tau phosphorylation by inactivating protein phosphatase 2A in rat hippocampus. Neurobiol. Aging 29, 1654–1665.

Zhou, X.W., Gustafsson, J.A., Tanila, H., Bjorkdahl, C., Liu, R., Winblad, B., Pei, J.J., 2008. Tau hyperphosphorylation correlates with reduced methylation of protein phosphatase 2A. Neurobiol. Dis. 31, 386–394.