The deregulation of Fyn kinase in Alzheimer's Disease

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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 material previously published or written by another person, except where due reference has been made. 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 provisions 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.

By signing below, I confirm that Goce Taleski contributed to the following publications entitled:

1. *Altered protein phosphatase 2A methylation and Tau phosphorylation in the young and aged brain of methylenetetrahydrofolate reductase (MTHFR) deficient mice*
2. *Methylenetetrahydrofolate reductase deficiency deregulates regional brain amyloid-β protein precursor expression and phosphorylation levels*
3. *The protein serine/threonine phosphatases PP2A, PP1 and calcineurin: A triple threat in the regulation of the neuronal cytoskeleton*
4. *Protein Phosphatase 2A and Tau: an orchestrated 'Pas de Deux'*
5. *Disturbances in PP2A methylation and one-carbon metabolism critically affect Fyn distribution and function* (submitted)

Outlined below are the items that the candidate has contributed towards:

- *Conduction of experiments (1, 2 and 5)*
- *Analysis of results (2 and 5)*
- *Review of literature (3 and 4)*
- *Prepared and organised the figures (2, 3 and 4)*

A/Prof Estelle Sontag

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LIST OF PUBLICATIONS

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2. Hoffman A, **Taleski G**, Qian H, Wasek B, Arning E, Bottiglieri T, Sontag JM, Sontag E. *Methylenetetrahydrofolate reductase deficiency deregulates regional brain amyloid-β protein precursor expression and phosphorylation levels.* J ALZHEIMERS DIS, 64(1) (2018)

3. Hoffman A, **Taleski G**, Sontag, E, *The protein serine/threonine phosphatases PP2A, PP1 and calcineurin: A triple threat in the regulation of the neuronal cytoskeleton.* MOL CELL NEUROSCI, 84 (2017)

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See Appendix for Publications 1-5.

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Sontag JM, Wasek B, **Taleski G**, Smith J, Arning E, Sontag E and Bottiglieri T. *Methylation of protein phosphatase 2A and phosphorylation of Tau protein in brain tissue from methylenetetrahydrofolate reductase deficient mice.* FASEB Conference on Folic Acid, Vitamin B12 and One-Carbon Metabolism, Steamboat Springs, Colorado, August 2014.

ORAL PRESENTATIONS AT CONFERENCES

Taleski G, Hoffman A, Qian H, Wasek B, Arning E, Bottiglieri T, Sontag JM, Sontag E. (Newcastle, Australia, 3rd August 2019) "A novel metabolic intervention for Tauopathies" **Centre for Brain and Mental Health Research, Hunter Medical Research Institute, 2019**.

Taleski G, van Hummel A, Ittner L, Sontag JM, Sontag E. (Newcastle, Australia, 2nd August 2018) "MTHFR deficiency and low dietary folate interact to promote the accumulation of phosphorylated amyloid protein precursor and tau" **Centre for Brain and Mental Health Research, Hunter Medical Research Institute, 2018**.

LIST OF AWARDS

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
Aβ	Amyloid beta
CNS	Central nervous system
CP13	Antibody against Tau phosphorylated at Ser202
FTLD-Tau	Frontotemporal Lobar Degeneration-Tau
Fyn^{CA}	Constitutively active Fyn
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
HET	*Mthfr* heterozygous knockout genotype
Hcy	Homocysteine
HTL	Homocysteine thiolactone
MCI	Mild cognitive impairment
Mthfr	Gene encoding methylenetetrahydrofolate reductase
MTHFR	Methylenetetrahydrofolate reductase
NFT	Neurofibrillary tangle
NULL	*Mthfr* homozygous knockout genotype
P-Tau	Hyperphosphorylated Tau
PHF-1	Antibody against Tau phosphorylated at Ser396 and Ser404
PP2A	Protein phosphatase 2A
PP2A/Bα	Protein phosphatase 2A holoenzyme containing Bα subunit
SAM	*S*-adenosylmethionine
SAH	*S*-adenosylhomocysteine
SFK	Src family kinase
TAU58/2	Transgenic mice overexpressing human Tau-P301S
THcy	Total homocysteine (homocysteine and derivatives)
WT	Wild-type

ABSTRACT

Sporadic Alzheimer's Disease (AD) is the prevailing form of dementia worldwide. Neuropathologically, this debilitating disorder is characterised by deposition of senile amyloid beta (Aβ) plaques and Tau-containing neurofibrillary tangles (NFTs). Aβ peptides are produced from the proteolytic processing of amyloid precursor protein (APP). There is strong experimental evidence that pathological accumulation of Aβ oligomers triggers a degenerative cascade mediated by Fyn kinase and abnormally phosphorylated Tau (p-Tau) proteins; this ultimately promotes synaptic and memory deficits in AD. The deregulation of Tau also prompts its self-aggregation into paired helical filaments that can aggregate further into NFTs. Significant clinical failures of Aβ-centric therapeutics for AD have recently shifted the industry towards development of Tau-targeting approaches that are currently under clinical investigation. However, the sole targeting of either Aβ or p-Tau may fail to address the underlying disease mechanisms, established risk factors, and complexity of this disorder. In an attempt to disentangle some of these issues, we considered the contribution of prevalent modifiable risk factors associated with AD, more specifically, low plasma folate levels and elevated plasma total homocysteine levels. Folate and homocysteine play a key role in one-carbon metabolism, a series of metabolic pathways that control cellular methylation potential. The integrity of one-carbon metabolism is critical for formation of methylation-dependent Protein Phosphatase 2A (PP2A) enzymes, which are major signalling mediators that become downregulated in AD-affected brain regions. Methylated PP2A holoenzymes are the predominant phosphatases that dephosphorylate p-Tau at many Ser/Thr epitopes. They also dephosphorylate APP phosphorylated at the Thr668 residue, which is linked to enhanced APP amyloidogenic processing. Here, we first demonstrated that both APP phosphorylation at Thr668 and Tau phosphorylation at AD-like epitopes are enhanced in various brain regions from mouse models with disturbances to one-carbon metabolism. These changes were associated with a concomitant reduction in methylated PP2A enzyme levels and increased activity of the Tau/APP kinase, GSK-3β. These findings support the involvement of altered one-carbon metabolism in AD pathogenic processes. Since Fyn kinase regulates both APP and Tau *via* Tyrosine (Tyr) phosphorylation, and becomes aberrantly activated in AD, we also deemed it important to address the impact of one-carbon metabolism and PP2A methylation on Fyn regulation. Our studies show that one-carbon metabolism and/or methylated PP2A enzymes regulate the distinct subcellular distribution of Fyn in neuronal cellular and *ex vivo* models. Fyn compartmentalisation was closely associated with its function in neurite outgrowth. Furthermore, we found that Fyn interacted with methylated PP2A enzymes, which may play a role in its targeting. In mice with altered one-carbon metabolism, the net activity and total protein expression levels of Fyn were dramatically altered in a brain region-specific manner; notably, Fyn was hyperactive in the cortex of these mice. Moreover, under conditions of increased Fyn signalling, we identified site-specific Tyr phosphorylation of PP2A catalytic

subunit (PP2Ac), which could have downstream ramifications for both APP and Tau regulation. Tyr phosphorylation of PP2Ac influenced physiological Fyn targeting and Fyn-dependent neurite outgrowth in N2a neuroblastoma cells. Together, our findings support the existence of a reciprocal functional interaction between Fyn and PP2A. Lastly, we demonstrated that a methyl group donor-based intervention ameliorated behavioural impairments, improved spatial memory, and reduced brain p-Tau accumulation in an AD-like mouse model. The mechanisms of action of this intervention likely included Fyn downregulation and the upregulation of methylated PP2A enzymes. Thus, the collective experimental work presented in this thesis unveils novel regulatory mechanisms of significance to AD pathogenesis. Besides opening up new avenues for AD research, our findings should also guide the development of rational therapeutics for delaying AD onset and/or disease progression.

Chapter 1 Introduction

1.1 Alzheimer's Disease (AD)

Alzheimer's disease (AD) is the prevalent form of dementia worldwide where it accounts for about 60-80% of total dementia diagnoses. Significantly, in Australia, dementia is currently the second leading cause of death in males and the first leading cause of death in females. About 1,800 new cases are diagnosed every week in Australia. If medical needs remain unmet, this figure is expected to rise to 7,400 by the year 2050 (Brown, Hansnata, & La, 2017), thus, creating significant socioeconomic challenges. Despite the introduction of many hypothesis-aligned and lesion-targeted therapeutics, there are currently no cure or effective disease-modifying treatments for AD (Golde, 2016). The significant failures and setbacks of numerous clinical investigations have made it paramount to enhance our understanding and insights into the precise and early molecular mechanisms underpinning this complex disorder that could lead to preventative or disease-modifying strategies.

1.2 AD symptomatic onset and clinical progression

AD is a progressive neurodegenerative disorder characterised by significant global cognitive decline and brain atrophy, and sulcal widening. The predominant form of AD occurring in ~95% of cases is referred to as sporadic or late-onset AD and has a symptomatic onset typically at 65 years of age or later. Nevertheless, subtle cognitive decline can be measured 20 years before dementia onset. Therefore, it is not surprising that the underlying neuropathological changes are known to occur three decades before symptoms manifest (Morris, 2005). Indeed, this long preclinical phase provides a critical opportunity for intervention with disease-modifying therapies. Eventually, individuals progress to mild cognitive impairment (MCI), a stage of subtle cognitive changes, which precedes AD dementia (Collie & Maruff, 2000). Typical early symptoms of AD include, frequent memory difficulties and confusion, visuospatial skill deficits, apathy, and personality and behavioural changes (mild AD). The latter symptoms prominently reflect global cognitive decline and include, challenges in problem solving and planning, agitation, and impairments to communication, sensory processing, reasoning and conscious thought (moderate AD). At this stage, memory difficulties and confusion have significantly worsened, and affected individuals may have hallucinations, delusions, nightmares and paranoia. Eventually, affected individuals lose the ability to communicate and function independently and require a caregiver (severe

AD). In most cases, mortality is due to secondary causes, such as pneumonia, cardiovascular complications and dysphagia-induced malnutrition and dehydration.

1.3 Modifiable and genetic risk factors of AD

Currently, advanced age is the strongest risk factor for AD development. Many modifiable and independent risk factors have been identified, such as B vitamin deficiencies, hyperhomocysteinemia, head trauma, diabetes, hypertension, obesity, dyslipidemia and metabolic syndrome.

The presence of the ε4 variant of the Apolipoprotein E (*ApoE*) gene (*ApoE4*) is currently the strongest genetic risk factor for sporadic AD, being expressed in over half of AD patients (Safieh, Korczyn, & Michaelson, 2019). ApoE is the most prevalent brain lipoprotein and plays a key role in lipid transport and cholesterol metabolism (Safieh et al., 2019). On the contrary, the ε2 allele of *ApoE* reduces AD risk (Corder et al., 1994). However, *ApoE4* is not a deterministic or disease triggering gene such as those invariably found in rare familial early-onset forms of AD, wherein symptoms usually develop in an individual's early 40s to mid-50s. Those include autosomal dominant mutations in the amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*) or presenilin-2 (*PSEN2*) genes, all of which invariably promote amyloidogenesis. Thus, in addition to harbouring *ApoE4*, additional risk genes/factors must be at play to contribute to the development of sporadic AD in *ApoE4* carriers.

Recently, the common functional C677T polymorphism of the Methylenetetrahydrofolate reductase gene (*Mthfr*), which causes hyperhomocysteinemia, was established as an AD risk factor (Rai, 2017; Stoccoro et al., 2017). Interestingly, this polymorphism has been shown to increase AD risk in *ApoE4* carriers (Peng et al., 2015).

In this thesis, we focused on identifying the underpinning molecular mechanisms of B vitamin deficiencies, hyperhomocysteinemia, and *Mthfr* functional polymorphisms in the context of AD.

1.4 Neuropathological hallmarks of AD

Invariably and within distinct brain regions, AD is neuropathologically characterised by synapse loss, amyloid beta (Aβ)-containing neuritic plaques, and aggregates of excessively phosphorylated (or 'hyperphosphorylated') Tau (p-Tau) proteins, termed neurofibrillary tangles (NFTs) (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011) (**Figure 1**). Similar p-Tau lesions also characterise a collection of dementias in the absence of an overt Aβ pathology, referred to as Tauopathies, with the most common

being Frontotemporal Lobar Degeneration-Tau (FTLD-Tau). The extent and pattern of distribution of NFTs in AD better correlates with severity and duration of disease relative to neuritic plaques (Perl, 2010) and forms the basis of Braak staging, which characterises disease progression into six immunohistochemistry-defined stages (Braak & Braak, 1991). Three-dimensional structural MRI studies (Vemuri & Jack, 2010) have revealed a similar progression of NFT pathology as described by Braak & Braak (1991). The neuropathology of AD begins in the transentorhinal cortex (stage I) and gradually involves the entorhinal cortex and hippocampus (stage II), which together play a key role in memory formation and retrieval. This parallels with memory decline being the earliest and predominant symptom. From here, the degenerative atrophy spreads to the basal temporal lobe and paralimbic cortical areas (stages III and IV) in the MCI phase. Ultimately, the disease process propagates to multimodal association areas of the cerebral cortex (stages V and VI) responsible for processing information from multiple sense modalities; the degeneration of this area manifests as AD dementia (Vemuri & Jack, 2010).

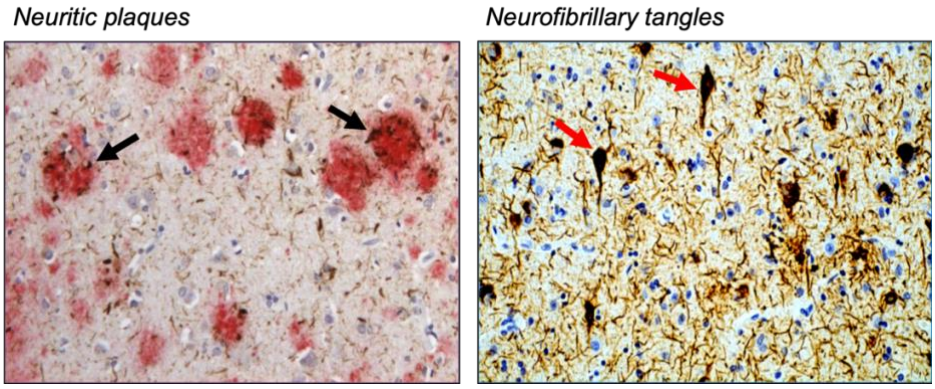

Figure 1. Major neuropathological lesions in AD-affected brain regions. (**Left**) Neuritic (or senile) plaques (red structures indicated by arrows; stained using "anti-APP, clone 22C11" antibodies directed to APP) are mainly found extracellularly and are composed of an abnormal aggregation of toxic prefibrillar oligomeric structures of Aβ that undergo fibrillation forming the central core, which is surrounded by abnormal neuronal processes or neurites (either dendrites or axons). These dystrophic neurites stain strongly with "anti-phospho-Tau-PHF-1" antibodies (brown staining), which label neurofibrillary tangles (NFTs) due to the presence of paired helical filaments (PHFs) of Tau. (**Right**) NFTs are abnormal fibrous inclusions of PHFs (brown structures indicated by arrows), which in turn are hyperphosphorylated Tau protein aggregates. These structures are found within the perikaryal cytoplasm surrounding the nucleus and extend to the apical dendrite of pyramidal neurons. NFTs significantly accumulate in the layer II neurons of the entorhinal cortex, the subicular and CA1 regions of the hippocampus, the amygdala and the deeper layers (III, IV, and superficial VI) of the neocortex (Perl, 2010). Both sections are counterstained with hematoxylin (blue) to label nuclei. Pictures provided by Drs. Estelle Sontag and Charles White III (UT Southwestern, Dallas, TX, USA).

Many experimental studies support the involvement of Aβ and p-Tau in initiating the underlying neurodegenerative cascade rather than simply being by-products of the disease process (Ittner & Gotz,

2011; Bloom, 2014). The advent of Aβ- and Tau-targeted tracers for use in AD brain imaging studies has delineated a temporal sequence for these pathologies. Notably, Aβ deposition is a prerequisite for Tau tangle pathology in cortical regions (Pontecorvo et al., 2019). This is supported by various experimental studies demonstrating Aβ oligomers as the *trigger* for p-Tau pathology, which in turn enhances Aβ toxicity at the postsynapse (the receiving end of neurons in synapses) *via* a positive feedback loop (Bloom, 2014). Accordingly, biomarker studies have unveiled that Aβ accumulates long before NFT pathology, and detectable changes in Aβ precede dementia by up to 30 years (Jansen et al., 2015). However, corresponding Aβ-positivity thresholds for positron emission tomography (PET) scans, which are often used in diagnoses, are only reached a decade before the onset of dementia. Interestingly, brain Aβ accumulation far below the threshold for PET-scan positivity is associated with subtle memory decline in an otherwise preclinical population (Landau, Horng, & Jagust, 2018).

Although Aβ accumulation can be found in elderly, non-AD individuals, it is associated with bilateral thinning of the prefrontal cortex and lower cognitive performance (Llado-Saz, Atienza, & Cantero, 2015). However, not all Aβ accumulation is inextricably linked to cognitive decline and p-Tau pathology. For instance, Aβ-containing plaques without the presence of abnormal neurites (only associated with neuritic plaques), termed diffuse plaques, are frequently encountered in the cerebral cortex of elderly individuals. They are not associated with lower cognition in various domains, or NFT pathology (Malek-Ahmadi, Perez, Chen, & Mufson, 2016). This is likely due to the molecular composition of Aβ aggregates - rather than their accumulation *per se* - since soluble Aβ oligomers only accumulate in AD and differ in properties such as aggregation and toxicity from the aggregates found in normal ageing (Piccini et al., 2005). These findings raise a question of paramount importance: *What drives the excessive formation of Aβ oligomers predisposing Tau pathology in AD?*

1.5 Amyloid Precursor Protein (APP) and its aberrant processing in AD

Aβ is a cleavage product of amyloid precursor protein (APP), a highly conserved cell transmembrane glycoprotein abundantly expressed in the healthy brain. The production of Aβ (**Figure 2**) is promoted by phosphorylation of APP at Thr668 in the cytoplasmic domain (Ando, Iijima, Elliott, Kirino, & Suzuki, 2001; Lee et al., 2003), which is elevated in AD brain (Lee et al., 2003). APP phosphorylation at Thr668 is mediated by various AD-implicated enzymes, including glycogen synthase kinase 3 beta (GSK-3β) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Kirouac, Rajic, Cribbs, & Padmanabhan, 2017). Physiologically, low levels of Aβ enhance memory and hippocampal acetylcholine secretion, and support hippocampal long-term potentiation (LTP), a postsynaptic mechanism involved in learning and memory processes (Morley et al., 2010).

Normally, the majority of APP cleavage is *via* the favourable non-amyloidogenic pathway initiated by α-secretase, which directly cleaves APP within the Aβ domain. This predominantly takes place at the plasma membrane (Minami, Hoe, & Rebeck, 2011), albeit some cleavage could also occur in the *trans*-Golgi network (Tan & Gleeson, 2019). Cleavage of APP by α-secretase yields the secreted sAPPα fragment, which has been shown to have neuroprotective and neurotrophic properties (Plummer, Van den Heuvel, Thornton, Corrigan, & Cappai, 2016). In contrast, amyloidogenic processing is dependent on endocytosis. It occurs within the endosomal/*trans*-Golgi network pathway in a sequential fashion by the major neuronal β-secretase, BACE1, and γ-secretase containing the PS1 (encoded by *PSEN1* gene) or PS2 (encoded by *PSEN2* gene) subunit. This pathway yields an Aβ peptide, with the 42 amino acid long Aβ peptide ($A\beta_{1-42}$) having a high propensity to self-aggregate into toxic oligomeric structures.

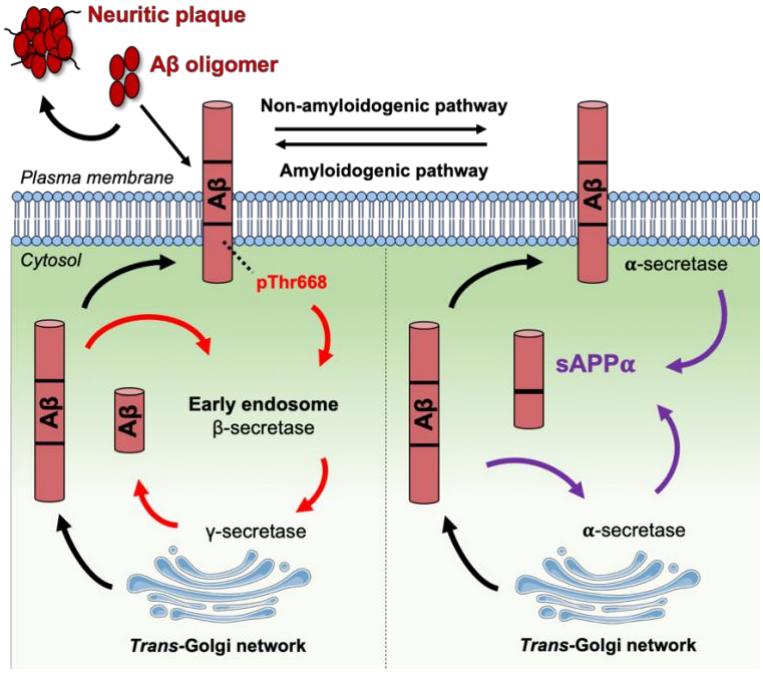

Figure 2. Functionally distinct APP proteolytic cleavage in the brain. Nascently-formed and plasma membrane-associated APP undergo proteolytic processing *via* one of two pathways. The amyloidogenic pathway (red arrows) is promoted by phosphorylation of APP on the cytoplasmic domain residue, Thr668, and occurs within the endosomal/*trans*-Golgi network pathway. In this pathway, APP is initially cleaved by the β-secretase, BACE1, which is found in lipid rafts and early endosomes, producing two fragments: sAPPβ and C99. Subsequently, the C99 fragment is cleaved in the trans-Golgi network by the γ-secretase enzymatic complex, which results in the release of the ectodomain, Aβ, ranging from 38 to 43 amino acids in length. $A\beta_{1-42}$ monomers self-aggregate into oligomeric structures (Aβo; dimers, trimers, and hexamers) and assemble readily *in vitro* in the order of seconds to minutes. High levels of toxic oligomeric structures are thought to aggregate further into fibrillary structures that are found in neuritic plaques. Alternatively, in the non-amyloidogenic pathway (purple arrows), APP is a substrate for α-secretase, predominantly found at the plasma membrane and *trans*-Golgi network, which cleaves APP within the

Aβ domain. This precludes Aβ production and instead generates the C83 fragment and a larger ectodomain, sAPPα, which is secreted. Figure adapted from Choy, Cheng, and Schekman (2012).

A small fraction of total APP localises to cholesterol and sphingolipids-enriched membrane microdomains, which are detergent-resistant membrane (DRM) fractions typically referred to as lipid rafts (Minami et al., 2011). Notably, lipid rafts represent a platform for compartmentalising protein-lipid and protein-protein interactions, the trafficking of proteins, and for cellular signalling events (Sontag, Nunbhakdi-Craig, & Sontag, 2013). Lipid rafts are thought to be a major site involved in the pathological processing of APP due to the presence of β- and γ- secretases (Parkin, Hussain, Karran, Turner, & Hooper, 1999; Riddell, Christie, Hussain, & Dingwall, 2001). Microscopy experiments have shown that the reallocation of APP and BACE1 to lipid rafts is followed by rapid endocytosis (Marquer et al., 2011). Building up on the latter, it was found that the majority of APP is cleaved in the endosomal pathway and *trans*-Golgi network (Choy et al., 2012). Interestingly, major components of lipid rafts such as cholesterol have been directly linked to Aβ deposition and AD through disturbances to lipid raft integrity (Hicks, Nalivaeva, & Turner, 2012). Together, these findings highlight the importance of understanding the mechanisms underlying the targeting of APP to the cell membrane, including its lateral shift in and out of lipid rafts, which can influence amyloidogenic processing.

Experimentally, Aβ oligomers have been found more toxic than their mature fibrillary forms found in neuritic plaques (Verma, Vats, & Taneja, 2015). Moreover, Aβ oligomers correlate better with disease progression; this is likely a result of their high solubility and tissue diffusivity compared to longer fibrils (Verma et al., 2015). At least in part, this differential toxicity could be due to the ability of Aβ oligomers to stimulate ERK signalling and GSK-3β activation, leading to phosphorylation of APP at Thr668, aberrant Tau phosphorylation and aberrant cell cycle activation in neurons (Kirouac et al., 2017). Besides mediating proteolysis by BACE1, APP phosphorylation at Thr668 increases its association with centrosomes; its aberrant phosphorylation may play a role in the impaired axonal transport and microtubule destabilisation present in AD (Kirouac et al., 2017).

Interestingly, amyloidogenic processing has been found to enhance Tau protein expression levels in human cerebral cortical neurons through its initial cleavage product, C99 (Moore et al., 2015). As such, inhibiting the sequential cleavage of C99 using a γ-secretase inhibitor results in the accumulation of C99, and consequently, increases Tau expression levels. In mice, high levels of Tau prompt pathological Tau phosphorylation and neurodegeneration (Adams et al., 2009). Hence, C99-induced Tau protein expression could explain the detrimental effects of γ-secretase inhibition on cognition in AD patients (Imbimbo & Giardina, 2011).

1.6 Tau and its excessive phosphorylation in AD

Tau is a major brain microtubule-associated protein (MAP) and has been a research focus in AD pathophysiology. In the adult brain, multiple isoforms of Tau exist (See **Publication 4**) and are generated through alternative splicing of the *MAPT* gene, yielding Tau species with either 0, 1, or 2 N-terminal projection domains (0N, 1N, or 2N), and either three (3R) or four (4R) carboxy-terminal repeat domains. All six isoforms are expressed in the adult brain with considerable regional variation (**Publication 4**). The shortest Tau isoform (0N3R-Tau) or fetal Tau is the only expressed isoform in the fetal human brain (**Publication 3**).

Tau is enriched in the axonal compartment where it plays a significant role in regulating the dynamic nature and stability of axonal microtubules, which in turn perform indispensable roles such as trafficking of cellular cargo (nutrients, mitochondria, synaptic vesicles…) (**Publications 3 and 4**). These cytoskeletal structures are also key determinants of neuronal morphogenesis and polarisation (Conde & Caceres, 2009). Importantly, Tau decorates microtubules in a non-uniform proximal to distal concentration gradient essential for enhanced organelle flux and maximal fast axonal transport (**Publication 4**). Under physiological conditions, Tau is also found in dendrites and is associated with and stabilises NR2B-containing N-Methyl-D-aspartate receptors (NMDAR), which play a key role in synaptic plasticity (**Publication 4**).

Like a majority of cellular proteins, Tau can be phosphorylated, which serves particular physiological functions (**Publication 4**). Its phosphorylation status is tightly controlled by a delicate balance of protein kinases and phosphatases (**Figure 3**).

Live imaging studies have shown that Tau is a rapidly moving molecule and interacts with microtubules in a very dynamic fashion described as a “kiss and hop” mechanism (Janning et al., 2014); this could explain why Tau does not interfere with axonal transport. The dynamic interaction of Tau with microtubules is due to a fine-tuned balance between Tau phosphorylation and dephosphorylation events at sites regulating microtubule binding. Of particular relevance to AD, the phosphatase battlefront is largely led by a distinct pool of Protein Phosphatase 2A (PP2A) enzymes (PP2A/Bα; **Figure 3**) that are responsible for the bulk of neuronal Tau dephosphorylation at Serine (Ser) and Threonine (Thr) sites (Sontag, Nunbhakdi-Craig, Lee, Bloom, & Mumby, 1996; Xu, Chen, Zhang, Jeffrey, & Shi, 2008) (**Publication 4**).

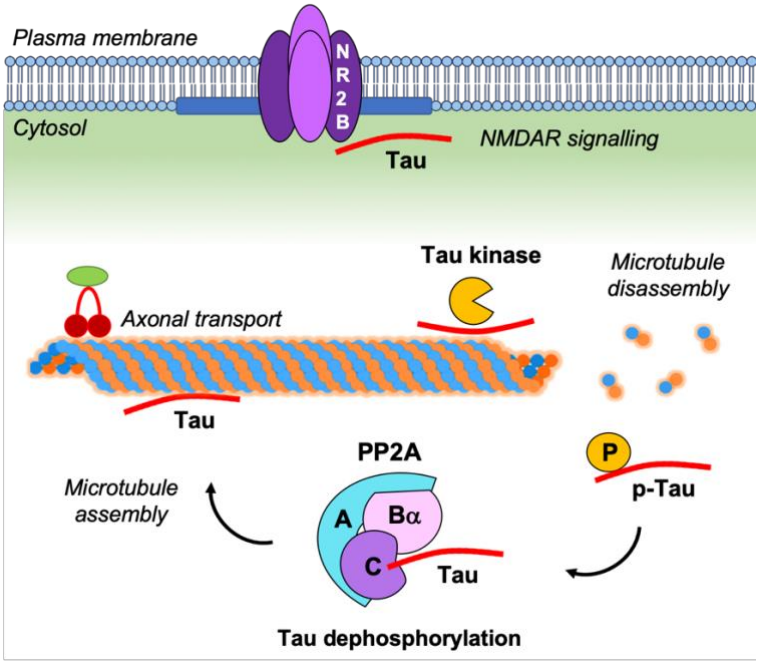

Figure 3. Regulation of Tau phosphorylation supports neuronal function and health. Spatial dephosphorylation events of Tau are influenced by and highly dependent on the regulated binding of the predominant Tau-specific phosphatase, Bα-containing protein phosphatase 2A (PP2A) holoenzyme, to neuronal microtubules. Tau binding to microtubules decreases their intrinsic dynamic instability and significantly influences bidirectional axonal transport of organelles and other critical cargo. PP2A/Bα enzymes are sequestered in an inactive state on microtubules and released upon microtubule depolymerisation. Once unbound, PP2A can directly associate with p-Tau in the cytosol to catalyse Tau dephosphorylation. Dendritic Tau phosphorylation status is likely controlled by a pool of PP2A found in postsynaptic density fractions and synaptic plasma membrane fractions. Figure adapted from **Publication 4**.

PP2A/Bα dephosphorylates Tau phosphorylated at various sites (**Figure 4**), which have been found hyperphosphorylated in AD in addition to many other Ser and Thr sites (Noble, Hanger, Miller, & Lovestone, 2013). Indeed, PP2A/Bα enzymes are deregulated in AD-affected brain regions (Sontag, Hladik, et al., 2004; Sontag, Luangpirom, et al., 2004). While PP2A/Bα is a predominant Tau phosphatase, it is worth noting that other PP2A isoforms and phosphatases also participate, directly or indirectly in the regulation of Tau phosphorylation state (**Publication 4**). Deregulation of Tau dephosphorylation events allows prominent Tau kinases implicated in AD such as GSK-3β, and ERK1/2, which are normally spatially regulated by PP2A, to override the equilibrium (**Publication 4**). This leads to hyperphosphorylation of Tau at specific sites, resulting in a net negative charge due to phosphate addition, reducing its electrostatic interaction with microtubules, which are anionic in nature (Jho, Zhulina, Kim, & Pincus, 2010). Invariably, perturbing these regulatory mechanisms promotes neuronal microtubule disassembly and consequently induces major defects in axonal cargo transport ultimately leading to synapse loss. In addition, p-Tau has the innate propensity to self-aggregate and sequester functional Tau

proteins in a prion-like fashion (Citron, 2010). In FTLD-Tau, various mutations in the *MAPT* gene have been identified (**Figure 4**), which predispose Tau to increased phosphorylation (**Publication 4**).

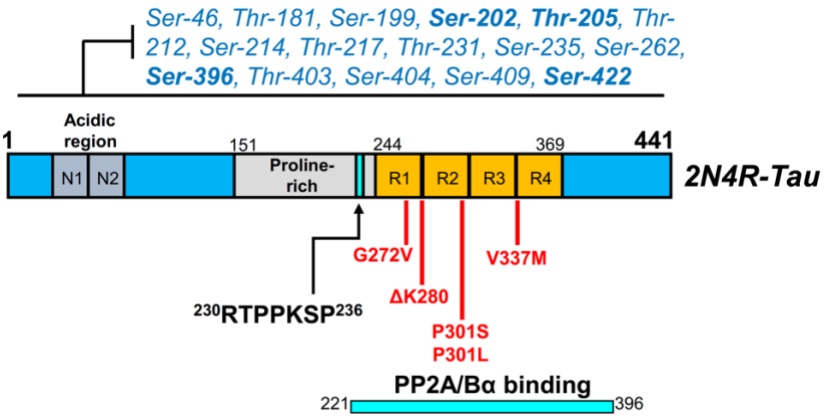

Figure 4. Mapping of Tau residues dephosphorylated by and interacting domains of PP2A/Bα. Depicted is the full-length isoform of Tau (2N4R-Tau). 2N4R-Tau consists of the following major domains: N-terminal projection domains (N1 and N2), and the microtubule-binding domain, comprised of a proline-rich region and repeat (R) domains, and a flanking region at the carboxy-terminal. PP2A/Bα interacts with a portion of Tau comprising the R domains and upstream proline-rich region, which contains the conserved RTPPKSP motif that is critical for PP2A binding. FTLD-Tau dementia-associated mutations in Tau (red text), such as P301S, are found within the PP2A-binding sequence. PP2A dephosphorylates Tau at various serine (Ser) and threonine (Thr) residues *in vitro* (all sites listed) and *in vivo* (sites in bold text). Figure from **Publication 4**.

It is generally accepted that hyperphosphorylation of Tau precedes and promotes the assembly of paired-helical filaments (PHFs) from oligomers, which eventually form the NFTs harboured by neurons in AD (Šimić et al., 2016) and by additional cell types in FTLD-Tau (Forrest et al., 2017). In line with this, GSK-3β-mediated phosphorylation prompts Tau filaments to mingle, forming tangle-like structures (Rankin, Sun, & Gamblin, 2007). In AD, Tau is phosphorylated on many sites, most of which are exclusively found in AD (Šimić et al., 2016). Vice versa, there are many other sites on which Tau is exclusively phosphorylated in the normal brain (Šimić et al., 2016). Many sites phosphorylated in AD can also be found in non-pathological human brain, although to a lesser extent. It is noteworthy that Tau can also become phosphorylated under physiological conditions. For instance, several ground-breaking studies from the Planel research group have shown that Tau phosphorylation is increased in hibernating animals, and during anaesthesia and hypothermic conditions (Reviewed in **Publication 4**). These findings illustrate Tau phosphorylation as a potential "defence mechanism" against insult. The toxic effects of aberrant Tau phosphorylation may be epitope-specific since phosphorylation at certain sites could be neuroprotective (Ittner et al., 2016). Thus, much remains to be learned about the role of epitope-specific Tau phosphorylation in Tau regulation. Interestingly, a recent study suggests that Tau hyperphosphorylation

in AD sequentially progresses in an N- to C-terminus direction and the apparent disappearance of earlier epitopes in later stages of disease could be due to conformational changes that hamper their detection (Regalado-Reyes et al., 2019).

In this thesis, we specifically looked at the Tau phospho-epitopes recognised by the PHF-1 and CP13 antibodies, which are widely used in AD research. Notably, the PHF-1 antibody recognises doubly phosphorylated Ser396 and Ser404, which is found in early pathological aggregates such as PHFs and early NFT-like structures (Mondragon-Rodriguez, Perry, Luna-Munoz, Acevedo-Aquino, & Williams, 2014). Indeed, this epitope is observed in the early stages of AD and is accumulated in severe stages. On the other hand, the CP13 antibody detects Tau phosphorylated on Ser202 and has high sensitivity for detecting pre-tangles and early intracellular NFTs compared to other Tau-directed antibodies (Espinoza, de Silva, Dickson, & Davies, 2008).

Like Aβ oligomers, recent studies suggest that p-Tau oligomers are the toxic entities leading to disease rather than their fibrillary forms such as NFTs. It has been proposed that these oligomers are released by neurons within exosomes or in a free state, allowing for propagation of p-Tau pathology to unaffected regions (Shafiei, Guerrero-Munoz, & Castillo-Carranza, 2017). For instance, Tau oligomers can be internalised through endocytosis, and hinder mitochondrial energy production and cause mitochondrial damage through direct interactions with mitochondrial proteins (Shafiei et al., 2017).

Under physiological conditions, Tau is predominantly confined to axons. It has been reported that hyperphosphorylation of Tau could be the underlying trigger for the abnormal compartmentalisation shift (or “missorting”) of Tau from axons to the somatodendritic compartment. Consequences of Tau missorting encompass the loss of dendritic spines, microtubule destabilization, mitochondrial dysfunction, axonal transport deficits, Tau aggregation, and cognitive impairment (Zempel & Mandelkow, 2014). Interestingly, site-specific pseudo-phosphorylation (substitution with glutamic acid to mimic phosphorylation) of Tau at classical AD phosphosites including PHF-1 is sufficient to increase targeting of Tau to dendritic spines (Xia, Li, & Gotz, 2015), wherein Tau is normally found albeit at much lower levels relative to the axonal compartment (Ittner et al., 2010). Being a site dephosphorylated by PP2A, the deregulation of PP2A in AD (Sontag, Hladik, et al., 2004; Sontag, Luangpirom, et al., 2004) could explain why Tau is found accumulated in dendritic compartments in AD (Ittner & Gotz, 2011).

Notably, PP2A also regulates the translocation of Tau pools to the plasma membrane (Sontag et al., 2013). The bulk of Tau present at the plasma membrane is found in a dephosphorylated state and in non-raft domains (Maas, Eidenmüller, & Brandt, 2000; Gloria Lee & Leugers, 2012). There, Tau may serve a signalling scaffold function (**Publication 4**).

While some small amounts of Tau are also present in lipid rafts under physiological conditions, it has been reported in neuronal cells that incubation with Aβ can promote the sequential phosphorylation of Tau at Tyrosine (Tyr) 18 and Ser396/404, and its association with lipid rafts (Kawarabayashi et al., 2004; Hernandez, Lee, Sjoberg, & Maccioni, 2009). In this context, there is strong evidence for a role of alterations in lipid rafts in AD (Mesa-Herrera, Taoro-González, Valdés-Baizabal, Diaz, & Marín, 2019). Thus, like APP, altered compartmentalisation of Tau in raft and non-raft membrane microdomains likely plays an important role in neurodegenerative cascades by affecting the formation of membrane-associated Tau scaffolds (Sontag et al., 2013).

In dendrites, Tau performs important functions that cannot be fulfilled by MAP2, another member of the MAP family, which is restricted to the dendritic shaft. This is due to the ability of Tau to enter the dendritic spine (Xia et al., 2015). There, Tau serves a critical role in synaptic plasticity by stabilizing NMDARs *via* promoting their complex formation with postsynaptic density protein 95 (PSD-95) (Ittner et al., 2010). PSD-95 is a major scaffolding protein involved in stabilising surface and synaptic expression of glutamate receptors (Won, Incontro, Nicoll, & Roche, 2016). This complex formation requires Fyn, another protein implicated in Tauopathies including AD, which is physiologically targeted to the postsynaptic density by Tau (Ittner et al., 2010).

1.7 Fyn, a major brain kinase implicated in AD

A key player linking Aβ and p-Tau pathologies is the Src-family Tyr kinase (SFK), Fyn. Fyn shares extensive sequence homology with the family's historical prototype, Src, but significantly varies within the N-terminus, which includes post-translational modifications (Okada, 2012). Fyn exists in two major splice variants, FynB (referred to as "Fyn" in this thesis) and FynT. FynB is the predominantly expressed isoform in the brain while FynT is mostly expressed in cells of hematopoietic lineage, especially T lymphocytes. Fyn activity is regulated by phosphorylation of particular Tyr residues (**Figure 5**).

At physiological levels in mature synapses, Fyn is required for LTP (Nygaard, van Dyck, & Strittmatter, 2014). Other fundamental roles include oligodendrocyte differentiation and myelination, synapse formation and regulation, spatial learning (Nygaard et al., 2014), and hippocampal development in mice (Grant et al., 1992).

In the AD brain, Fyn protein levels are significantly increased in the hippocampus and cerebellum (Zahratka et al., 2017). Fyn activity levels are also increased in neurons derived from AD patients (Lee et al., 2004).

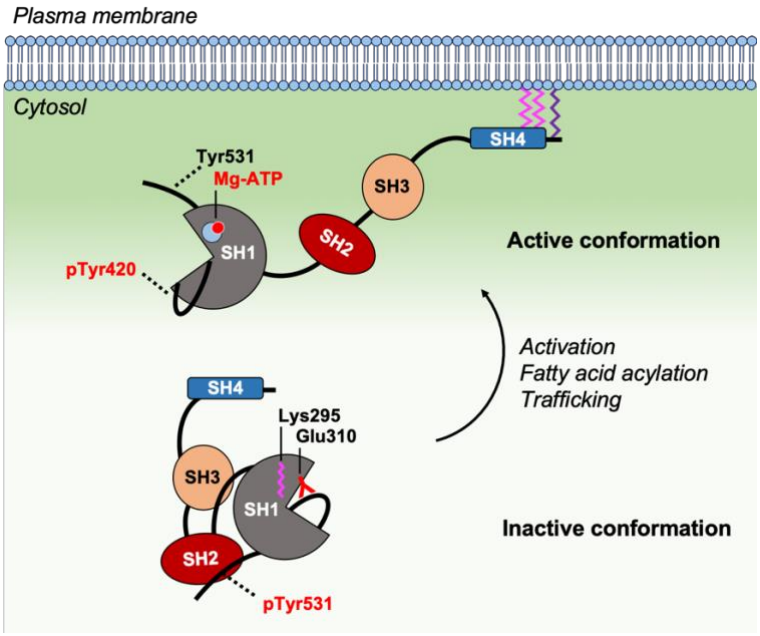

Figure 5. Regulation and targeting of Fyn to the plasma membrane. Fyn and other SFKs share a conserved domain architecture comprised of a myristoylated (on Glycine 2) unique N-terminal domain (SH4), followed by Src homology domains SH3, SH2, and SH1 (Tyr kinase domain), and a short C-terminal tail. The C-terminal tail harbours an autoinhibitory phosphorylation site, corresponding to Tyr531 on Fyn. Phosphorylation at this site promotes the required intramolecular interactions amongst the pTyr531 SH2, SH3, and Tyr kinase domains that constrain Fyn into an inactive conformation. Specifically, this conformation is stabilised through binding of pTyr531 to the SH2 domain and binding of the SH2-SH1 linker to the SH3 domain, which affects the configuration of the catalytic pocket. Dephosphorylation of this site induces a dramatic conformational change that allows hydrogen bonding between Glutamic acid (Glu) 310 and Lysine (Lys) 295 as required for Mg-ATP binding, and also exposes the regulatory site, Tyr420, with the activation loop. This allows the kinase to catalyse the intermolecular auto-phosphorylation of Tyr420, which locks the catalytic domain into an active conformation, allowing access of substrates to the active site. Fyn is also dual palmitoylated (magenta), and myristoylated (purple) within the N-terminal domain; these post-translational modifications promote its direct targeting to the plasma membrane. Figure adapted from Okada (2012).

However, it is unknown whether these changes are pathological or merely compensatory, for example, in response to synapse loss. Various experimental models have implicated Fyn in mediating the toxic effects of Aβ oligomers, leading to Tau hyperphosphorylation. Preclinical studies have shown that Fyn inhibition improves memory and synaptic function in an AD-like mouse model (Kaufman et al., 2015), consistent with the pathological role of Fyn under certain conditions (Ittner et al., 2010). In response, the clinical inhibition of Fyn using Saracatinib, an inhibitor of SFKs, was investigated for its efficacy in slowing cerebral metabolic decline (a measure of brain deterioration) in mild AD patients (van Dyck et al., 2019). However, this study found no difference between Saracatinib and placebo, thus showing no efficacy of this approach in mild AD. Moreover, subjects who received Saracatinib were prone to gastrointestinal disorders, which could worsen malnutrition in AD patients. Considering its multifunctional neuronal role

and involvement in myelination, long-term pharmacological targeting of Fyn - as would be needed for AD treatment - should also be viewed as a matter of concern.

1.7.1 Fyn compartmentalisation and functions

Uniquely, Fyn is present in an active state at the cell periphery in both basal conditions and growth factor-stimulated conditions (Sandilands, Brunton, & Frame, 2007). Fyn activation and membrane trafficking are dependent on RhoD-containing endosomes. Fyn translocation from the perinuclear region to the plasma membrane can be facilitated through interaction of nascent bundled actin filaments established between those spatial domains (Sandilands et al., 2007). Indeed, disruption of actin filaments using cytochalasin D inhibits the activation and plasma membrane targeting of Fyn. The kinase, therefore, stays in the perinuclear region in an inactive state (Sandilands et al., 2007).

A significant contributor to the direct plasma membrane translocation of Fyn upon synthesis and its association with RhoD endosomes, is palmitoylation of Cysteines (Cys) 3 and 6 of Fyn (Alland, Peseckis, Atherton, Berthiaume, & Resh, 1994). Palmitoylation is the covalent attachment of fatty acids to Cys residues in membrane-associated proteins. Prior to palmitoylation, Fyn is myristoylated on Glycine (Gly) 2 co-translationally on free ribosomes. Together, these modifications direct newly synthesised Fyn to the plasma membrane (Sato et al., 2009). Mutagenesis studies to abolish Fyn palmitate incorporation results in a dependence of Fyn on RhoB rather than RhoD endosomes for both its activation and membrane translocation (Sandilands et al., 2007) leading to its accumulation in the Golgi region (Sato et al., 2009). Moreover, there is a single report suggesting that Fyn targeting and function are regulated through methylation on specific Lysine (Lys) residues by an unidentified methyltransferase (Liang, Lu, Wilkes, Neubert, & Resh, 2004). However, to date, no other reports have confirmed these findings in any experimental model; thus, further investigation is warranted.

In mouse brain, the majority of Fyn localises to lipid rafts (Hoe et al., 2008; Minami et al., 2011). Fyn is also found strategically positioned in the postsynaptic density. There, it serves a critical physiological role in synaptic plasticity through NMDAR phosphorylation on Tyr1472 of NR2B subunit, which stabilises NMDARs by promoting their complex formation with PSD-95 (Ittner et al., 2010). Blocking phosphorylation at this site causes a rapid internalisation of NMDARs. Conversely, increased phosphorylation at this site is found following LTP induction and is associated with enhanced NMDAR surface expression (Prybylowski et al., 2005). Indeed, Fyn knockout mice have impaired LTP; normal LTP can be restored by reintroducing the Fyn transgene (Kojima et al., 1997). Moreover, mice expressing

the constitutively active mutant of Fyn have enhanced levels of Fyn in the dendritic spine, and consequently, increased NR2B phosphorylation and hyperactivity (Xia & Gotz, 2014).

1.7.2 The functional link between Fyn and APP

Fyn and APP co-localise and interact in primary hippocampal neurons (Minami et al., 2011). Multiple studies have demonstrated the involvement of Fyn in the regulation of APP. It has been reported in mouse brain and cultured cells that Fyn regulates the cell surface expression and translocation of APP in and out of lipid rafts *via* differential Tyr phosphorylation of APP and selected APP adaptor proteins. These Tyr phosphorylation events critically modulate the binding of APP to those adaptor proteins. In turn, changes in the compartmentalisation of APP affect its processing towards Aβ production or sAPPα secretion (Hoe et al., 2008; Minami et al., 2011; Minami, Clifford, Hoe, Matsuoka, & Rebeck, 2012).

Fyn specifically binds to the $_{682}$YENPTY$_{687}$ peptide domain of APP. This motif is known to be a conserved internalisation sequence, and mutations in this domain decrease endocytosis of APP and Aβ generation (Perez et al., 1999). Fyn directly phosphorylates APP at Tyr682, and pTyr682-APP species are concentrated in lipid rafts (Minami et al., 2011). Increased APP phosphorylation at Tyr682 and Fyn-APP binding have been found in AD brain and neural stem cells derived from AD patients, respectively (Poulsen et al., 2017). Moreover, phosphorylation of APP plays an important role in shaping the interactome of APP with important signalling molecules. Specifically, phosphorylation at Tyr682 creates a docking site for various proteins, including those with a SH2 domain such as SFKs, Shc and Grb2/7 proteins (involved in ERK signalling); of particular significance, these interactions are further enhanced when APP is phosphorylated at Thr668 (Tamayev, Zhou, & D'Adamio, 2009). Interestingly, these Tyr kinases and adaptors do not bind to unphosphorylated APP or when APP is only phosphorylated at Thr668. In this context, APP phosphorylation at Tyr682 acts as a signalling scaffold potentially forming a connection to various signalling pathways such as ERK signalling, which could have downstream consequences for Tau phosphorylation status.

Altogether, these earlier studies suggest that defective APP trafficking and enhanced APP compartmentalisation in lipid rafts, which boosts amyloidogenesis, could occur as a result of deregulated Fyn function and aberrant APP phosphorylation (Matrone, Iannuzzi, & Annunziato, 2019).

In a vicious cycle, Fyn-mediated deregulation of APP would promote the pathological accumulation of Aβ oligomers, which are known to stimulate a postsynaptic cascade leading to increased Fyn activity (Ittner et al., 2010) (**Figure 6**).

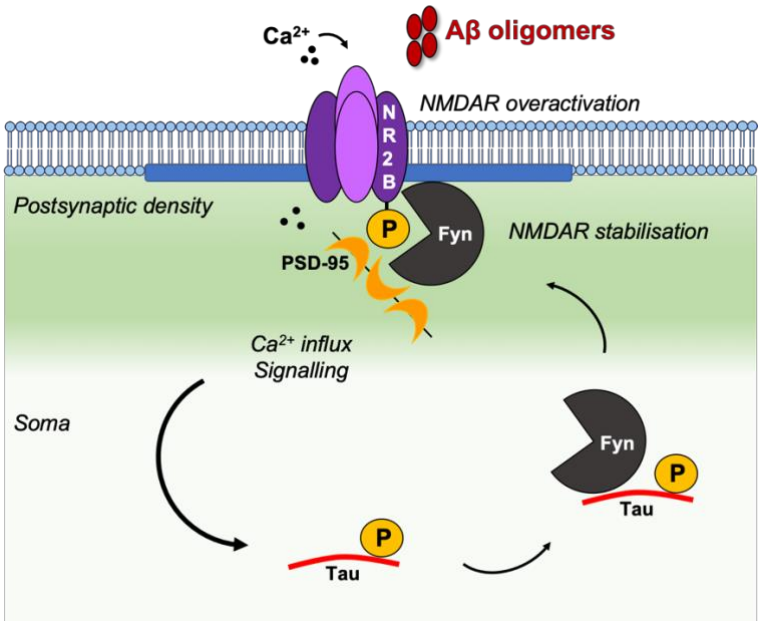

Figure 6. The Aβo-Fyn-Tau signalling cascade: a prominent disease model. Oligomeric Aβ (Aβo) binds with high-affinity to various surface-expressed proteins of the postsynapse. This interaction triggers an NMDAR-mediated signalling cascade that activates Fyn. Fyn in turn, phosphorylates dendritic Tau *via* direct and indirect mechanisms. Additionally, the NR2B subunit of the NMDAR is phosphorylated by Fyn on Tyr1472, which constitutes a signal for enhanced NMDAR activity by increasing its surface expression at synapses mediated through PSD-95 binding. Notably, NMDARs regulate intracellular levels of Ca^{2+}, a key contributor to excitotoxic signalling; a process whereby neurons are damaged and killed by the overactivation of their excitatory receptors. Downstream signalling increases non-dendritic Tau phosphorylation at specific sites (e.g. PHF-1), which enhances sorting of Tau to dendritic spines. Hence, this increases Tau-dependent trafficking of Fyn to the postsynaptic density and amplifies the effects of Aβo. Over-activation of postsynaptic NMDARs through this Aβo-Fyn-Tau feedback loop has emerged as a key mechanism leading to excitotoxicity and the synaptic dysfunction in AD that instigates memory and cognitive deficits. Figure adapted from Ittner et al. (2010).

Inevitably, upregulated Fyn activity leads to increased Tau phosphorylation through direct and indirect mechanisms, and Tau mislocalisation. Thus, in the context of overstimulation by Aβ oligomers, Fyn mediates a toxic feed-forward cycle (Nygaard, 2018). Fyn deregulation has thus the potential to perpetuate the continuous dysfunction of two central players in AD pathogenesis, Tau and APP.

1.7.3 Functional interactions between Fyn and Tau

Fyn directly phosphorylates Tau on the AD-exclusive site, Tyr18 (Lee et al., 2004; Šimić et al., 2016). Yet, Fyn also affects the Tyr phosphorylation of Tau *via* indirect mechanisms. Those involve the downstream activation of the novel Tau Tyr kinase, Pyk2, encoded by the *PTK2B* gene (Li & Gotz, 2018). A variant of this gene has been recently established as a risk allele for AD; it is associated with increased

Pyk2 mRNA levels, suggesting increased protein levels under disease conditions (Li & Gotz, 2018). Recently, Pyk2 has been reported to promote Aβ oligomer-induced suppression of synaptic plasticity; its deletion rescued synapse loss and learning/memory deficits in AD mouse models (Salazar et al., 2019). These studies suggest that aberrant Fyn activity in AD could have toxic consequences through downstream activation of Pyk2. The functional consequences of phosphorylated Tyr18 are unclear but could involve predisposing Tau to Ser/Thr phosphorylation (Hernandez et al., 2009). Transgenic mice expressing constitutively active Fyn display significant Tau phosphorylation at the AD-like AT8 epitope comprised of phosphorylated Ser202 and Thr205 (Xia & Gotz, 2014). Fyn knockdown has been reported to decrease p-Tau pathology in AD-like mouse models (Minami et al., 2012). Interestingly, a recent study showed that Tau phosphorylation at various Tyr sites, including Tyr18, abolishes its aggregation but inhibits its microtubule- and lipid-binding properties (Ait-Bouziad et al., 2020).

Besides inducing Tau phosphorylation, Fyn also mediates local protein translation of Tau within the somatodendritic compartment. Under conditions of oligomeric Aβ stimulation and Fyn overexpression, this function of Fyn prompts the somatodendritic accumulation of Tau, mimicking Tau pathology in AD (Li & Gotz, 2017). Glutamate and neural stimulation also induce the local translation of Tau within the somatodendritic compartment (Kobayashi, Tanaka, Soeda, & Takashima, 2019), probably through the functional association of Fyn with glutamate receptors.

In dendritic spines of hippocampal neurons, Tau regulates the organisation of Fyn into nanodomains/nanoclusters, likely lipid rafts (Padmanabhan, Martinez-Marmol, Xia, Gotz, & Meunier, 2019). Single-molecule tracking identified three distinct motion states of Fyn. Two of these are associated with nanodomains, including an immobile state wherein Fyn molecules are bound to their substrate, and a confined state wherein Fyn molecules are trapped in nanodomains. The other one is a free diffusive state where Fyn molecules move between synaptic and extrasynaptic compartments. Based on these observations, it is possible that nanodomains/lipid rafts, to some extent, act as reservoirs of the kinase, being essential in postsynaptic signalling. Interestingly, the FTLD-Tau-linked Tau P301L mutant immobilises Fyn in the dendritic spine (Padmanabhan et al., 2019). This confinement to an individual spine limits kinase signalling to neighbouring spines. It could promote aberrant Fyn signalling and interactions, which have been linked to neurotoxicity and neurodegeneration (Ittner et al., 2010).

A recent study demonstrated that Fyn-Tau interactions are primarily found in neurites of primary neurons and play a role in Aβ oligomer-induced toxicity (Rush et al., 2020). Earlier studies showed that Fyn-Tau interactions are increased following NMDAR-mediated Tau phosphorylation, which could explain why a small pool of phosphorylated Tau is found in dendrites under normal conditions (Mondragon-Rodriguez

et al., 2012). To prevent dendritic Tau hyperphosphorylation, the phosphorylation state of Tau is likely controlled by a pool of PP2A/Bα in the postsynapse (Chan & Sucher, 2001).

1.8 Methylated PP2A enzymes crucially regulate APP and Tau

PP2A is a major and omnipresent family of protein Ser and Thr phosphatases (Reviewed in detail in **Publications 3 and 4**). Collectively, PP2A enzymes represent up to 1% of total cellular proteins (Lin et al., 1998). They regulate an array of cellular processes, including signal transduction pathways, cell cycle regulation, apoptosis and development. The typical mammalian PP2A enzyme is a trimeric holoenzyme consisting of a scaffolding A subunit (Aα or Aβ), a catalytic C subunit (PP2Ac; Cα or Cβ), and one of 23 regulatory B subunits. Critically, the binding of certain B subunits to the AC core determines substrate specificity and subcellular localisation (Virshup & Shenolikar, 2009).

The majority of mature PP2A holoenzymes (over 90%) contain PP2Ac that is methylated on Leucine (Leu) 309 (Yu et al., 2001) by the *S*-adenosylmethionine (SAM)-dependent methyltransferase, leucine carboxyl methyltransferase-1 (LCMT1). Carboxymethylation is reversed by protein phosphatase methylesterase-1 (PME1). PME1 also associates with an inactive pool of PP2Ac subunits (Ogris, Du, et al., 1999; Longin et al., 2008), regardless of its esterase activity (Longin et al., 2008), probably to prevent any promiscuous activity of PP2Ac. Leu309 methylation is known to promote the assembly of substrate-specific trimeric PP2A holoenzymes, such as the formation of Tau-specific PP2A/Bα enzymes (Sontag & Sontag, 2014) (**Figures 3 and 7**). As mentioned previously, these enzymes account for the majority of brain Tau dephosphorylation events.

While Leu309 of PP2Ac is required for B subunit binding (Sontag & Sontag, 2014), methylation is not a prerequisite for the formation of all substrate-specific PP2A trimers; substrate-specific PP2A trimers comprising of a non-methylated PP2Ac exist. Besides methylation, PP2A can undergo other posttranslational modifications that have been reported to affect PP2A activity or stability (Janssens, Longin, & Goris, 2008).

Methylated PP2A enzymes also and significantly target APP for dephosphorylation at Thr668, a site dephosphorylated by PP2A in cultured cells (Sontag et al., 2007) and *in vivo* (Nicholls et al., 2016). Hence, downregulation of PP2A methylation results in increased phosphorylation of APP at Thr688, thereby affecting its processing and promoting amyloidogenesis (Sontag et al., 2007). In mice, overexpression of LCMT1 protects against Aβ-induced neurotoxicity, whereas PME1 overexpression sensitises mice to the detrimental effects of Aβ (Nicholls et al., 2016). Indeed, PP2A methylation, LCMT1, and PP2A/Bα

enzymes are downregulated in AD-affected brain regions (Sontag, Hladik, et al., 2004; Sontag, Luangpirom, et al., 2004), with likely important consequences for both APP and Tau.

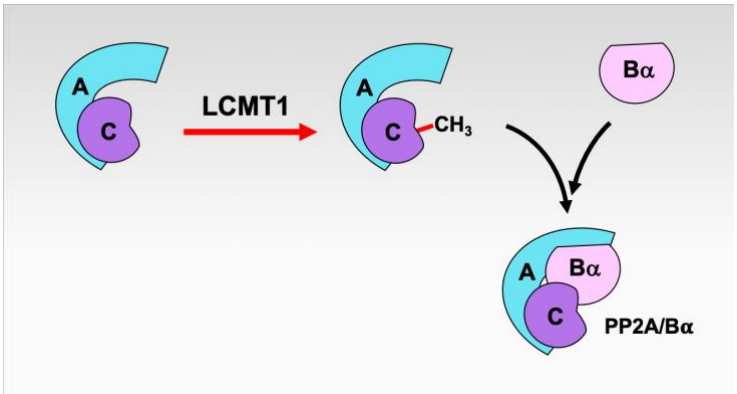

Figure 7. Methylation-dependent PP2A/Bα holoenzyme biogenesis. LCMT1-catalysed methylation (CH_3) of PP2A catalytic C subunit on Leu309 promotes the formation and stabilisation of PP2A trimeric holoenzymes containing the Bα regulatory subunit.

1.9 The delicate balance between Fyn-Tau and PP2A-Tau interactions, and possibly, Fyn-PP2A interactions

Specifically, Fyn binds to the SH3-binding, proline-rich RTPPKSP motif of Tau (depicted in **Figure 4**) and this inhibits the interaction of PP2A/Bα with Tau through competitive binding *in vitro* (Sontag, Nunbhakdi-Craig, White, Halpain, & Sontag, 2012) (**Publication 4**). Under physiological conditions, the balance between kinase and phosphatase activities maintains Tau in an overall low phosphorylation state (**Publication 4**). However, changes in Fyn-Tau interactions could contribute to Tau deregulation in Tauopathies. Indeed, increased Fyn-Tau (Bhaskar, Yen, & Lee, 2005) and decreased PP2A-Tau (Goedert et al., 2000) interactions have been observed with disease-associated Tau phosphorylation in the proline-rich region, as well as with FTLD-Tau-linked Tau mutations, which promote the accumulation of phosphorylated Tau at both Ser/Thr and Tyr residues. For instance, Tau phosphorylated at Thr231 decreases its affinity for PP2A, which could explain why this site is poorly dephosphorylated by PP2A/Bα (Sontag et al., 2012). Interestingly, Thr231 phosphorylation is increased in early stages of AD and is known to hamper PP2A/Bα-mediated dephosphorylation of other major phospho-epitopes increased in AD (Sontag et al., 2012). Moreover, Thr231 phosphorylation of Tau also reduces its affinity for Fyn-SH3 (Bhaskar et al., 2005). Based on these data, it could be speculated that reduced PP2A-Tau interactions and Fyn-catalysed Tyr18 phosphorylation of Tau are early events in AD.

Besides affecting PP2A-Tau protein-protein interactions, we hypothesised that Fyn can also promote Tau phosphorylation at Ser/Thr sites by directly inactivating PP2A through phosphorylation. This hypothesis is based on earlier data showing that the closely structurally related Src kinase can inactivate PP2A *via* Tyr phosphorylation of PP2Ac (Chen, Martin, & Brautigan, 1992; Chen, Parsons, & Brautigan, 1994). In this work, *in vitro* assays were used to show that Src phosphorylated full-length PP2Ac, but not truncated forms of PP2Ac lacking the Tyr307 residue. As a result, it was proposed that the Tyr phosphorylation of PP2A occurs solely on the Tyr307 site (Chen et al., 1992). Of particular relevance to AD, follow-up studies have proposed that inactivation of PP2Ac by Src-mediated Tyr307 phosphorylation leads to the accumulation of highly phosphorylated Ser/Thr Tau species (Arif, Kazim, Grundke-Iqbal, Garruto, & Iqbal, 2014; Xiong et al., 2013). Furthermore, PP2A was found to be highly phosphorylated at Tyr307 in AD brain tissue (Liu et al., 2008). However, it is worth mentioning that phosphorylation of endogenous PP2Ac at Tyr307 has never been confirmed *in vivo* using mass spectrometry (MS). In addition, several discovery-mode MS investigations have identified other PP2Ac phospho-Tyr residues in a variety of oncogenic/transformed cells and human cancers (www.phosphosite.org). Even more troubling, all the published studies examining PP2A phosphorylation at Tyr307 have primarily relied on the indirect utilisation of commercial polyclonal and monoclonal "anti-p-Tyr307" antibodies. In a collaborative effort, our group recently reported that all the "anti-p-Tyr307 antibodies" used collectively to date in these studies indeed lack any specificity for p-Tyr307; rather, recognition of PP2Ac by these antibodies is highly sensible to changes in PP2A methylation at Leu309 and/or phosphorylation at Thr304 (Frohner et al., 2020). Therefore, to clarify the regulation of PP2A, it is paramount to carefully re-examine and confirm the occurrence of PP2Ac phosphorylation at Tyr307. This is especially worthwhile in the context of aberrantly increased Fyn activity and phospho-Tau pathology in AD.

1.10 Folate and homocysteine metabolism and its clinical relevance in AD

Folate-dependent one-carbon reactions control various processes including amino acid metabolism, and the methylation of DNA bases, proteins (e.g. PP2A), phospholipids, neurotransmitters, and homocysteine (Hcy). Methylation of these molecules supports epigenetic maintenance, enzyme function, cell-membrane integrity and receptors, monoamine neurotransmitter biosynthesis, and the metabolism of homocysteine, respectively (Bottiglieri, 2013).

During normal conditions, Hcy is maintained at optimally low levels in serum due to the methylation cycle (or one-carbon metabolism) and transsulfuration pathway (**Figure 8**; Reviewed in Ducker and Rabinowitz (2017)). Hcy is a non-proteinogenic amino acid derived from the essential amino acid methionine. At high levels, Hcy is neurotoxic, as discussed below. Importantly, the remethylation of Hcy

to methionine in the brain is highly dependent on the availability of vitamin B12 (B12) and 5-methyltetrahydrofolate (5-MTHF), the blood brain permeable and biologically active form of dietary folate. 5-MTHF is exclusively produced in the liver by methylenetetrahydrofolate reductase (MTHFR; encoded by the *Mthfr* gene). In turn, methionine synthase catalyses methionine to *S*-adenosylmethionine (SAM), the universal methyl group donor in the brain. In a methylation reaction, SAM loses its methyl group, yielding S-adenosylhomocysteine (SAH), which is then hydrolysed to Hcy and adenosine. From here, Hcy can be remethylated to methionine or alternatively, it is metabolised by cystathionine β-synthase (CBS) to form cystathionine, which, through subsequent reactions, yields glutathione, the major antioxidant in the brain and a regulator of redox balance.

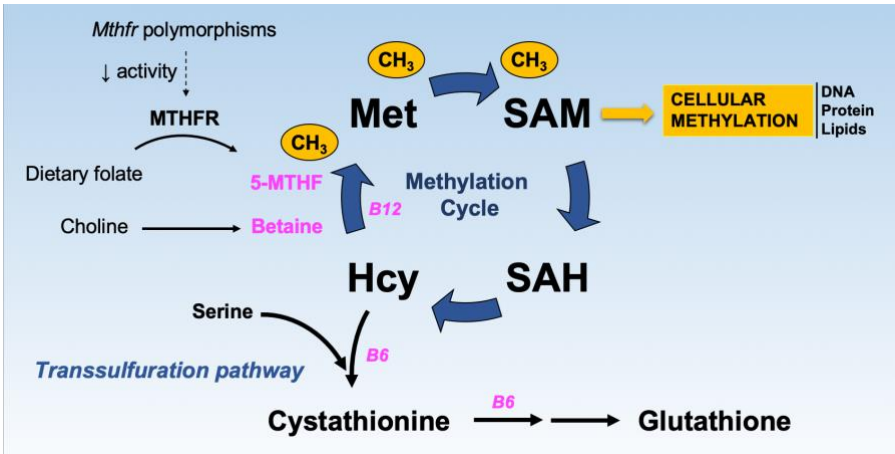

Figure 8. Overview of one-carbon metabolism. Methylenetetrahydrofolate reductase (MTHFR) produces 5-MTHF from an intermediate (5,10-MTHF) of dietary folate. 5-MTHF and vitamin B12 serve as the critical cofactors for the remethylation of homocysteine (Hcy) to methionine, which occurs both in the periphery and brain. Certain polymorphisms within the *Mthfr* gene encoding MTHFR decrease MTHFR activity, resulting in a lower production of 5-MTHF. The remethylation of Hcy is essential for the production of *S*-adenosylmethionine (SAM), which supports various cellular methylation reactions. Choline and betaine are also important sources of one-carbon units and promote the remethylation of Hcy to methionine, but this occurs exclusively in the periphery, not in the central nervous system (CNS). Alternatively, Hcy is irreversibly metabolised through the transsulfuration pathway, which occurs mainly in the liver and to some extent in the brain. It is condensed with serine by cystathionine β-synthase (CBS) to form cystathionine, in a vitamin B6-dependent reaction. Thereafter, cystathionine is converted to cysteine by cystathionine γ-lyase, for subsequent synthesis of glutathione. Figure adapted from Smith and Refsum (2016).

1.10.1 Functional significance of raised tHcy levels in cognitive impairment

Disturbances to one-carbon metabolism have been linked to many neurological and neuropsychiatric disorders (Bottiglieri, 2013). In AD, SAM levels are significantly decreased in the cerebrospinal fluid

(Linnebank et al., 2010). Other disturbances to one-carbon metabolism can be initiated by a variety of events such as: certain drugs, physiological problems, nutritional deficiencies in folate, vitamin B6 and/or B12, vitamin malabsorption, a high-methionine diet, and the presence of functional polymorphisms in genes like *Mthfr* and *Cbs* (Smith et al., 2018). Alterations in one-carbon metabolism are common in individuals over the age of 50. Interestingly, the National Health and Nutrition Examination Survey of the Centers for Disease Control and Prevention reported that 3.2% of adults over the age of 50 are clinically low in plasma B12 level, and up to 20% may have a borderline vitamin B12 deficiency. These various disturbances can independently lead to increased plasma levels of total Hcy (tHcy; Hcy and derivatives) or hyperhomocysteinemia, and are established risk factors for AD development (Smith & Refsum, 2016; Smith et al., 2018). When present at saturating levels, Hcy is reconverted back to SAH, a potent inhibitor of various methyltransferases, including LCMT1. Therefore, hyperhomocysteinemia restricts cellular (Bottiglieri, 2013) and PP2A (Sontag et al., 2007) methylation.

As mentioned previously, the common functional C677T polymorphism of *Mthfr*, resulting in an Alanine (Ala) to Valine (Val) substitution, was established as a risk factor by two independent meta-analyses (Rai, 2017; Stoccoro et al., 2017). Notably, there is a high prevalence of functional *Mthfr* polymorphisms in the general population of Australia with 60-70% of individuals harbouring a variant (C677T or A1298C). At least 40 mutations in *Mthfr* have been identified in individuals with homocystinuria and hyperhomocysteinemia. Symptoms of *Mthfr* polymorphisms include depression, anxiety, schizophrenia, and bipolar disorder. Most mutations cause amino-acid substitutions that decrease MTHFR enzyme activity. Specifically, CT and TT carriers of *Mthfr* C677T have about 60% and 30% of the wild-type (WT) enzyme's activity, respectively, due to thermolability (van der Put et al., 1998). The effects of a small decrease in MTHFR activity, as typically encountered in *Mthfr* C677T heterozygous carriers lead to mild elevation of tHcy levels that can usually be compensated by a folate-rich diet. However, functional *Mthfr* polymorphisms can ultimately lead up to the build-up of Hcy, especially in the context of low plasma folate status (for instance, due to dietary folate deficiencies or other factors). Hyperhomocysteinemia is prevalent in *Mthfr* C677T homozygous carriers, who require 5-MTHF supplementation to prevent severe adverse effects. Indeed, the 677C→T variant, the most frequent inborn error of folate metabolism, is recognised as the most common genetic cause of hyperhomocysteinemia.

As mentioned earlier, the *Mthfr* C677T polymorphism increases AD risk in *ApoE4* carriers (Peng et al., 2015), supporting again the idea of a strong functional interrelationship between one-carbon metabolism and AD pathogenic processes. This is also interesting since not all *ApoE4* carriers develop AD. Moreover, altered one-carbon metabolism could also be relevant to the ongoing conundrum that not all MCI patients develop AD. It is likely that multiple genetic and non-genetic risk factors for AD synergise with advanced ageing to affect disease onset and progression (Reitz & Mayeux, 2014). Indeed, the *Mthfr* C677T variant

has been linked to structural brain changes and brain atrophy in elderly populations with MCI (Rajagopalan et al., 2012).

Numerous robust studies, both experimental and clinical (including meta-analyses), have consistently associated hyperhomocysteinemia with neurodegenerative changes and/or cognitive decline (Smith & Refsum, 2016). This association is further emphasised in a recent consensus statement published by an international panel of experts (Smith et al., 2018). In AD patients, threshold effects of plasma tHcy levels were found such that patients with plasma tHcy levels >11.1 μmol/L had an increased rate of atrophy of the medial temporal lobe compared with those with plasma tHcy levels <11.1 μmol/L (Smith et al., 2018). Another prospective study found that every 5 μmol/L incremental increase in plasma tHcy levels is linearly associated with a 15% increase in relative risk of Alzheimer-type dementia (Zhou & Chen, 2019). Importantly, a causal role of Hcy in AD has also been convincingly established using a Mendelian randomisation method (Hu, Teng, Li, Hao, & Wang, 2016); this strategy avoids inherent limitations of epidemiological studies, such as confounding bias and reverse causation. Further excluding reverse causation, elevated plasma tHcy levels have been found in blood samples from elderly individuals taken eight years prior to the onset of dementia; they were similarly observed in midlife individuals, where they were associated with increased risk for AD development later in life (Smith et al., 2018).

Indeed, it has been established that Hcy at high levels exerts toxic effects and interferes with normal brain function by several mechanisms. Consequences of elevated Hcy levels include oxidative stress, exacerbation of Aβ and Tau pathology and cognitive deficits in AD-like mice, cerebral amyloid angiopathy, disruption of the endothelium in the cerebral vasculature, activation of Tau kinases, and toxic cell cycle activation in neurons (Smith and Refsum, 2016). In addition, Hcy is a known agonist for NMDARs and metabotropic glutamate receptors and can indirectly increase intracellular calcium levels, ultimately leading to excitotoxicity (Obeid and Herrmann, 2006). High plasma tHcy levels could also be significantly responsible for the deregulation of PP2A/Bα enzymes in AD (Sontag, Hladik, et al., 2004; Sontag et al., 2007). Supporting this hypothesis, our group has shown that elevated Hcy levels were associated with impaired LCMT1 function, resulting in decreased PP2Ac methylation and PP2A/Bα levels; Hcy also concomitantly enhanced both Tau phosphorylation at AD-like phospho-epitopes and APP Thr668-phosphorylation in various models (Sontag et al., 2007; Sontag et al., 2008).

1.10.2 The toxic erroneous conversion of homocysteine to homocysteine thiolactone

Potentially, the major toxic effects of elevated plasma tHcy levels arise from its metabolic conversion to the cyclic thioester form, Hcy thiolactone (HTL). This is catalysed by methionyl-tRNA synthetase

(MARS) in an error-editing reaction during protein synthesis whereby Hcy is selected in place of methionine (Jakubowski & Glowacki, 2011). HTL can produce cumulative effects through irreversible N-homocysteinylation (N-Hcy) exclusively of the primary amine group of Lys residues forming an isopeptide bond. This modification alters or impairs the structure and function of proteins. Hcy can also covalently bind proteins through its free thiol group forming a disulphide bond with another free thiol group of a Cys residue, a reversible modification termed S-homocysteinylation. This has a strong influence on the thiol-dependent redox status of proteins (Skovierova et al., 2016). The highly reactive HTL affects various major proteins in circulation, including LDL, HDL, and albumin, and inhibits Na^+K^+-ATPase in the rat hippocampus and cortex (Skovierova et al., 2016). Interestingly, increased gene copy number of MARS has been found in neural tube defect (NFD) and congenital heart defect (CHD) patients, and its inhibition lowered NFD and CHD onsets in hyperhomocysteinemia-induced animal models (Mei et al., 2020).

In the context of AD, HTL stabilises Aβ oligomers attenuating their aggregation into amyloid fibrils, and alters tubulin binding of Tau promoting Tau self-association and aggregation (Sharma, Kumar, Dar, & Singh, 2015). Interestingly, increased N-homocysteinylation of Tau has been reported in protein aggregates and tangles in brain tissue and CSF from AD patients (Bossenmeyer-Pourie et al., 2019). Specifically, N-homocysteinylation of Tau neutralises the positive charge of Lys residues critical for microtubule binding. This modification has the potential to impede Tau dephosphorylation by PP2A/Bα, but this remains to be determined. In dietary folate/vitamin B12-deficient rats with associated hyperhomocysteinemia, N-homocysteinylation accumulates in the hippocampal and cortex with age. Interestingly, this begins during foetal development and is not reversible with a normal diet after weaning (Bossenmeyer-Pourie et al., 2019). Other MAPs with dendritic roles such as the recruitment of NMDARs, have also been found N-homocysteinylated in AD brain tissue (Bossenmeyer-Pourie et al., 2019). These findings suggest that the cumulative and irreversible N-homocysteinylation of MAPs can affect synaptic plasticity and underlie cognitive decline, and could even enhance Aβ-mediated neurotoxicity.

Normally, HTL is hydrolysed by paraoxonase 1 (PON1). This enzyme is synthesized in the liver and kidney and released into circulation where it binds to HDL. Hydrolysis of HTL to Hcy occurs at the rate of 1mM of HTL to 0.7mM Hcy per 24 hours (Silla et al., 2019). However, this conversion is influenced by variations in *PON1* genotype/ PON1 activity that are known to modulate plasma HTL levels (Perla-Kajan, Borowczyk, Glowacki, Nygard, & Jakubowski, 2018).

1.11 The failure of numerous clinical interventions for AD - possibly due to the cumulative nature and toxic effects of raised homocysteine

The failure of various therapies in AD patients aimed at reducing Aβ levels, for instance, through γ-secretase inhibition or anti-Aβ monoclonal antibodies, could be attributed to various reasons (Mehta, Jackson, Paul, Shi, & Sabbagh, 2017). However, the strongest argument for these significant failures could simply be that the time of treatment was far too late to modify the already advanced disease process. Promising results *in vivo* have shifted the industry towards clinically investigating Tau-targeted immunotherapies (Congdon and Sigurdsson, 2018). With many trials still ongoing, recruiting, or waiting analysis, the tolerability and efficacy of Tau-targeting approaches in mild to moderate AD patients will become clearer over the next few years. While it is understood that Tau pathology correlates better than Aβ with disease severity in AD, the sole targeting of p-Tau or its aggregation fails to address the underlying disease mechanisms and complexity of the disorder.

Taking into consideration the cumulative neurotoxic effects of Hcy together with the established decades-long preclinical phase of AD, it seems logical that plasma tHcy levels should be monitored in individuals throughout their lifetime as a preventative strategy for the onset and development of AD. In support of this notion, the VITACOG Trial found that lowering plasma tHcy by ~30% through daily supplementation with folic acid, and vitamins B6 and B12 for 2 years, slowed the rate of whole brain atrophy by ~30%, including in MCI patients. These effects were associated with reduced cognitive decline. Furthermore, brain regions most severely affected in AD had a substantial reduced rate of atrophy by 88.5% (Smith et al., 2018). Higher doses of the same vitamin combination also benefited AD patients, but only in those with a higher Mini-Mental State Examination (MMSE) score at baseline. Consequently, it was concluded that B-vitamin treatment may be beneficial in patients with mild AD but not in those who have progressed to the moderate stage (Aisen et al., 2008).

1.12 Project background and aims

In order to delineate the pathogenesis of AD, which is still in its infancy, a thorough understanding of the regulation of major proteins affected in AD, and their interactions with established risk factors is essential. This will guide the development of rational disease-modifying therapeutics, and most importantly, preventative strategies for AD development.

One-carbon metabolism is critical for brain homeostasis and disturbances have been epidemiologically and experimentally linked to AD. A major aim of this thesis was to *further elucidate the link between altered one-carbon metabolism and AD*. Firstly, we investigated the effects of altered one-carbon metabolism on the regulation of PP2A, APP and Tau, all of which are significantly deregulated in AD. Secondly, we tested the hypothesis that altered one-carbon metabolism and/or PP2A methylation can participate in the deregulation of Fyn, a Tyr kinase central to AD neuropathological mechanisms. A better understanding of the upstream regulation of Fyn is important in the context of current drug development for AD, since Fyn regulates both APP processing and Tau phosphorylation. Thirdly, we investigated whether active Fyn can conversely regulate PP2A *via* phosphorylation on Tyr residues. This reciprocal regulation of PP2A by Fyn could have significant functional ramifications for PP2A-dependent APP and Tau regulation since Fyn becomes hyperactive in AD. Finally, we investigated the effectiveness of a methyl group donor-based intervention in alleviating p-Tau-related behavioural abnormalities and p-Tau accumulation in an AD-like mouse model.

The overall goal of the studies proposed in this thesis was to gain a better understanding of the regulation of Fyn, a major brain and AD kinase. Particular emphasis was given to uncover functional interactions between Fyn-dependent pathogenic cascades in AD and altered one-carbon metabolism, a significant risk factor for AD. Deciphering these relationships is a must to inform and accelerate the development of proper preventive and disease-modifying therapeutic strategies for AD.

Chapter 2 Materials and Methods

2.1 Chemicals and reagents

All chemicals/reagents used in this study were obtained from Sigma-Aldrich (Castle Hill, Australia) unless otherwise specified.

2.2 Antibodies

The antibodies used in this study (**Table 1**) were for biochemical analyses and/or confocal microscopy at dilutions recommended by the manufacturers.

Table 1. Antibodies.

Antibody	Immunogen	Host	Application	Source	Catalogue No.
Actin	Purified chicken gizzard actin.	Ms	WB	Sigma	MAB1501
APP	Peptide corresponding to sequence within human APP.	Rb	WB, IF	Abcam	ab32136
Phospho-APP (Thr668)	Peptide corresponding to residues surrounding Thr668 of human APP protein.	Rb	WB	CST	6986
Fyn	Peptide corresponding to N-terminus (amino acids 1-132) of human Fyn.	Ms	WB	BD TL	610163, 610164
Fyn	Peptide corresponding to N-terminus residues of human Fyn conjugated to KLH.	Rb	WB	Sigma	04-353
Phospho-SFK (Tyr416)	Phospho-peptide corresponding to residues surrounding Tyr419 of human Src protein.	Rb	WB, IF	CST	6943
HA epitope tag	Peptide CYPYDVPDYASL.	Ms	WB	Covance	MSS-101P
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle.	Ms	WB	Sigma	MAB374

GSK-3β	Recombinant protein specific to the carboxy-terminus of human GSK-3β protein.	Ms	WB	CST	9832
Phospho-GSK-3β (Ser9)	Phospho-peptide corresponding to residues surrounding Ser9 of human GSK-3β.	Rb	WB	CST	5558
p44/42 MAPK	Peptide corresponding to the sequence of p44/42 MAPK kinase.	Ms	WB	CST	9107
Phospho-p44/42 MAPK	Phospho-peptide corresponding to residues surrounding Thr202/Tyr204 of human p44 MAPK.	Rb	WB	CST	9101
	Recognises both phospho-epitopes of Thr202/Tyr204 and Thr185/Tyr187 of p44 MAPK and p42 MAPK, respectively.				
Methyl-Lysine	Methylated KLH.	Rb	WB	Enzo	ADI-KAP-TF121-2
PP2Ac	Peptide corresponding to amino acids 153-309 of human PP2Ac.	Ms	WB	BD TL	610556
PP2A Bα subunit (clone 2G9)	KLH conjugated synthetic peptide (CASGKRKKDEISVD) corresponding to amino acids 398-411 of human PP2A, B subunit.	MS	WB	Sigma	05-592
Demethyl-PP2Ac	Peptide corresponding to amino acids 295-309 of human PP2Ac.	Ms	WB	Upstate (FS)	05-421
Methyl-PP2Ac	Synthetic peptide corresponding to amino acids 302-309 of human PP2Ac conjugated to KLH.	Ms	WB	Sigma	04-1479
Phospho-PP2Ac ("Y2" site)	*Cannot disclose due to reasons of intellectual property.*	Alp	WB, IF	#	Non-commercial
Tau	Peptide corresponding to sequence found in all 6 isoforms of Tau.	Rb	WB	rPeptide	T-1308-1
Phospho-Tau (Ser202) – CP13	Epitope around phospho-Ser202.	Ms	WB	*	Non-commercial
Phospho-Tau (Ser396/Ser404) – PHF-1	Epitope around Ser396 and Ser404 phosphorylated sites.	Ms	WB	*	Non-commercial
Phospho-Tyr (100)	Phospho-Tyr-containing peptides.	Ms	WB	CST	9411
Phospho-Tyr (1000)	Phospho-Tyr-containing peptides.	Rb	WB	CST	8954

WB: Western blot; IF: Immunofluorescence; CST: Cell Signalling Technology, BD-TL: BD Transduction Laboratories, FS: Fisher Scientific, KLH: keyhole limpet haemocyanin. Alp: Alpaca, Ms: Mouse; Rb: Rabbit.

*Gift from Dr Peter Davies (Albert Einstein College of Medicine, New York. NY, USA).

#Gift from Dr Brian Wadzinski (Vanderbilt University School of Medicine, Nashville, TN, USA).

2.3 Plasmids

Plasmids used in this study were pcDNA5/TO Flag-Balpha, pAc CMV Fyn-GFP, pAc CMV Fyn Y531F-GFP, Rc/CMV Src Y529F, peCFP-APP, pBabe HA-LCMT1, pCMVTag3B Myc-LCMT1, pBabe Myc-PME1, pcDNA 3.1 HA-PP2Ac, pcDNA 3.1 HA-PP2Ac Leu309 deletion mutant (L309Δ), pcDNA5/TO HA_3-WT PP2Ac and three HA_3 Tyr→Phe substitution mutants of PP2Ac [pcDNA5/TO HA_3-PP2Ac Y1F, Y2F and F/F (Y1F/Y2F double mutant)]. The Tyr→Phe point mutants of PP2Ac were generated using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Australia). The plasmid expressing the constitutively active Fyn Y531F mutant (Fyn^{CA}) was generated using the Transformer Site-Directed mutagenesis kit (Clontech, USA). All plasmids sequences were verified.

2.4 *Mthfr* knockout mice

Mouse brain tissue from wild-type (WT) $Mthfr^{+/+}$, heterozygous (HET) $Mthfr^{+/-}$, and homozygous (NULL) $Mthfr^{-/-}$ female mice with a C57BL/6 background (Chen et al., 2001) were analysed here. Mice were bred, genotyped and housed in cages with a maximum of 4 mice per cage, maintained in a temperature-controlled animal facility on a 12 h light dark cycle, and were allowed access to food and water *ad libitum* (**Publications 1 and 2**). Regional brain homogenates were prepared from 5-week-old WT or *Mthfr* knockout mice (n = 6 mice/ group), and 16-month-old WT or HET mice (n = 8-10 mice/ group) fed a control diet containing 6.7 mg/kg folate (normal folate (NF) diet; Harlan Teklad, Madison WI) or a low folate diet (LF diet; Harlan Teklad, Madison, WI) containing 0.2 mg/kg folate for a period of 6 months prior to sacrifice by CO_2 asphyxiation. NF and LF diets contained succinylsulfathiazole (10 mg/kg) to inhibit gastrointestinal bacterial growth and prevent absorption of folate from this source. The diets did not induce any statistically significant changes in mouse body weight.

2.5 P301S-Tau transgenic (TAU58/2) mice

TAU58/2 mice express the human 0N4R Tau isoform with the P301S mutation under the control of the mouse Thy1.2 promoter. Transgenic mice were obtained, genotyped and housed using methods described previously (van Eersel et al., 2015). TAU58/2 mice have been extensively characterised and described previously (Przybyla et al., 2016; van Eersel et al., 2015). Here, 1-month-old female TAU58/2 mice were

fed a control amino acid-defined diet (CD) (Research Diets Inc.) or the same diet enriched with methyl group donors (MD) (Research Diets Inc.). Mouse body weights were measured every 2 months. At 5 months of age, mice were anesthetised and transcardially perfused with phosphate buffered saline (PBS, pH 7.4) to remove blood. Brains were removed and the hemispheres separated. One hemisphere was regionally dissected and then snap frozen for biochemical analysis. The other was immersion fixed in 4% paraformaldehyde (PFA) for immunohistochemical analyses.

All animal experiments with *Mthfr* knockout and TAU58/2 mice were approved by the respective University's Animal Care and Ethics Committee of our collaborators.

2.6 Homogenisation of mouse regional brain tissue

Briefly, total homogenates were prepared from the cortex, hippocampus, cerebellum, striatum or midbrain mouse brain regions at a ratio of 0.1g tissue/ ml of "Buffer 1" [25mM Tris pH 7.4, 150mM NaCl, 1mM sodium orthovanadate (Na_3VO_4), 1mM dithiothreitol (DTT), 0.5µM okadaic acid (OA), 5mM phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40 (NP-40), containing cOmplete Mini protease (Sigma, Australia) and PhosSTOP phosphatase inhibitor cocktail pills (Sigma, Australia)]. The samples were briefly sonicated on ice and centrifuged at 13,000 *g* to remove insoluble material, and the supernatants (total protein extracts) were flash frozen and stored at –80ºC.

2.7 Plasma and regional brain metabolite analyses

To confirm the intended effects of the genotypes (*Mthfr* knockouts) and the dietary interventions, plasma was collected before sacrifice and metabolite content was analysed by high pressure liquid chromatography (HPLC) and mass spectrometry. Regional brain cystathionine levels were also measured in all aged *Mthfr* knockout mice. These assays were performed by Dr. Teodoro Bottiglieri (Baylor Scott and White Research Institute, USA) in the case of the *Mthfr* mouse model studies and by a specialised vendor (Creative Proteomics, USA) in the case of the TAU58/2 mouse model analyses.

2.8 Behavioural testing in TAU58/2 mice

The following behavioural tests were conducted on all TAU58/2 mice at the age of 5-months, unless otherwise stated.

2.8.1 Morris Water Maze

Spatial memory testing was done as previously reported (van Hummel et al., 2016). Briefly, the apparatus consisted of a 1.2m diameter tank (50cm height) with a 40cm high Perspex platform (diameter 10cm), which was placed roughly 20cm from the edge of the wall. The tank was filled with water (19 - 22°C) to 0.5-1cm above the surface of the platform and a non-toxic, acrylic-based paint was added to obscure the platform. Four signposts with different shapes were placed equidistant around the pool as visual cues. Mice were acclimatized to the room for 1 hour prior to testing each day. Days 1 to 5 consisted of an acquisition phase, in which mice were placed in the quadrant opposite the platform at one of four starting positions along the outer edge and were given 60 seconds to locate the hidden platform. Mice that failed to find the hidden platform were guided to the escape platform and all mice remained on the platform for an additional 60 seconds before being removed from the maze. Mice underwent four trials per day. The starting position was randomised for all trials. On the final day, a flag was affixed on top of the platform, and visual cues were removed from outside of the pool, to exclude vision impairments and ascertain their ability to swim. Videos were recorded on a CCD camera and swim paths were analysed using the AnyMaze tracking software.

2.8.2 Challenge beam

To assess sensorimotor skills as previously done in TAU58/2 mice (van Eersel et al., 2015), mice were placed at one side of a horizontal dowel (18 cm) elevated over a basin of shallow water (to discourage voluntary drops and provide a softer landing) with their nesting house placed at the far end as an incentive to cross. The time to cross was measured with a maximum time of 120 seconds and the number of fore and hind limb slips were recorded. Mice underwent two training sessions before the test session, during which the best time was taken of two trials.

2.8.3 Pole test

To test grip strength and coordination as done previously (van Eersel et al., 2015), mice were placed at the apex of a vertical pole (47.5 cm length of dowel, diameter 0.8 cm) facing upward. The time taken to turn around, descend the pole, and reach the ground (with all four paws) was measured with a maximum time of 120 seconds. Mice underwent one to two training sessions before the test session, during which the best time was taken of two trials. Mice who were unable to descend the pole (slipped or fell) were given the maximum time.

2.9 Acute mouse brain slices

Brains were rapidly removed from 8 to 11-month-old female C57/BL6 mice that were sacrificed for another project approved by the Animal Care and Ethics Committee of the University of Newcastle. Mice were sacrificed *via* cervical dislocation. Brains were swiftly removed (Papouin & Haydon, 2018) and immersed in oxygenated and ice-cold sucrose substituted artificial cerebrospinal fluid (sACSF) containing: 250mM sucrose, 25mM $NaHCO_3$, 10mM glucose, 2.5mM KCl, 1mM NaH_2PO_4, 1mM $MgCl_2$ and 2.5mM $CaCl_2$ (Tadros, Farrell, Graham, Brichta, & Callister, 2015). The sACSF was constantly bubbled with 95% O_2 /5% CO_2 to maintain a pH of 7.3-7.4. While immersed, the olfactory bulbs and cerebellum were removed, and the remaining brain was positioned on a Styrofoam support block (caudal side down) and this was secured on the cutting stage with cyanoacrylate glue (Loctite 454, Loctite, Caringbah, NSW, Australia). The cutting stage was immediately transferred to the slicing chamber of the vibratome (Leica VT-1200S, Heidelberg, Germany). The slicing chamber was filled with an ice-cold slurry of oxygenated sACSF to submerge the brain and cutting blade. Coronal slices (400 μm thick) were obtained and transferred to an interface storage chamber containing oxygenated ACSF (118mM NaCl substituted for sucrose in sACSF) and allowed to recover for 1h at room temperature (~23°C) (Tadros et al., 2015). After recovery, slices were placed into room temperature oxygenated ACSF containing specific chemicals or vehicle for the determined time periods. Total protein extracts, detergent soluble and insoluble protein extracts were immediately prepared following treatment as described below. Extracts were stored at -80^{o}C for future analysis.

2.10 Cell culture and transfection

Neuro-2a (N2a) and SYF cells (deficient for Src, Yes, and Fyn) (American Type Culture Collection, USA) were maintained in DMEM (Thermo Fisher, Australia) containing 25 mM Hepes, pH 7.4, 10% fetal bovine serum (Bovogen Biologicals, Australia) and 10 μg/ml gentamicin (Thermo Fisher, Australia). N2a and SYF cells were transfected using Metafectene Pro (Biontex Laboratories, Germany), a liposomal transfection reagent, following manufacturer's instructions. In some instances, cells were co-transfected with two plasmids. The expression levels of transiently expressed recombinant proteins were determined by immunoblotting or immunofluorescence. Cells transiently transfected with pcDNA3.1, pcDNA5/TO or Rc/CMV empty vectors (EVs) were used as controls where indicated.

2.11 Cell treatment

All experiments were performed in cells grown to 80% confluence, in regular cell culture medium (DMEM) supplemented with the specified compound or vehicle (dimethylsulfoxide) for the specified time at 37°C in a humidified atmosphere with 5% CO_2.

2.12 Cell lysis and fractionation

2.12.1 Total cell extract preparation

Transfected and non-transfected cells were washed in ice-cold PBS, then harvested in Buffer 1. Homogenates were sonicated on ice and subsequently centrifuged for 5 min at 13,000 *g* at 4°C to remove insoluble material. The supernatant was collected and stored at -80°C for future analysis.

2.12.2 Detergent soluble and insoluble fractionation

Cells or acute brain slices were harvested in Buffer 1, then gently homogenised on ice using a microtube pestle. Homogenates were subsequently centrifuged for 90 min at 20,000 *g* at 4°C (Nunbhakdi-Craig et al., 2002). The supernatant (NP-40 detergent-soluble fraction) was collected and the remaining pellet (NP-40 detergent-insoluble fraction) was resuspended in the same buffer, then either sonicated or gently homogenised with a microtube pestle on ice, as required. Homogenates were centrifuged at 13,000 *g* at 4°C for 5 min. The supernatant was collected, and the pellet discarded. Both, detergent- soluble and insoluble fractions were stored at -80ºC for future analysis.

2.13 Immunoprecipitation

Cells transfected with plasmid cDNA encoding HA-tagged proteins or FLAG-tagged Bα (see "Plasmids") were rinsed with PBS and harvested in ice-cold Buffer 1 (without DTT) containing 0.5% sodium deoxycholate. Harvested cells were homogenized on ice using a microtube pestle. Insoluble material was cleared by centrifugation at 13,000 *g* for 5 min at 4ºC. Cleared lysates were added to PBS-prewashed 20µl of Rabbit-anti-HA antibody (Cell Signalling, Genesearch, Australia) or 25µl Mouse-anti-FLAG antibody (Sigma, Australia) magnetic bead slurry. Lysates were incubated at 4ºC overnight on a rotating platform.

Cell extracts were then removed, and beads were extensively washed in the same buffer used to harvest cells. Beads were resuspended in gel loading buffer for analysis by western blotting.

2.14 Western blotting

Briefly, aliquots of total cell homogenates (50µg), cell fraction homogenates (50µg), brain tissue homogenates (50µg) were run on NU-PAGE Bis-Tris 4%-12% gradient midi gels (Thermo Fisher, Australia). Proteins were then transferred overnight onto nitrocellulose membranes in a Tris-glycine-methanol transfer buffer. Western blotting was performed according to LI-COR protocol (LI-COR Biosciences, Millennium Science, VIC, Australia). Briefly, membranes were incubated for 1 hr in blocking buffer (Odyssey blocking buffer, LI-COR Biosciences, Millennium Science, VIC, Australia), followed by 1 hr incubation with a specific primary antibody diluted in the same blocking buffer containing 0.1% Tween20. Species-specific Infrared IRDye®-labelled secondary antibodies were then added for 1 hr. Results were visualized using the Odyssey™ Infrared imaging system (LI-COR Biosciences). Band intensity was determined using the Image Studio Lite version 5.0 Software (LI-COR Biosciences) to accurately quantify protein expression levels. Experiments requiring protein analysis by Western blot were performed at least 4 times.

2.15 Cell immunofluorescence

Cells were sparsely plated onto coverslips coated with Poly-L-Lysine, 24 hours after transfection. They were then rinsed with PBS prior to fixation with a 4% paraformaldehyde (PFA) solution for 20 min at room temperature. Fixed cells were immediately rinsed first with DMEM medium (to quench the PFA), then with PBS. Following this washing step, cells were permeabilised with a 0.1% Triton X-100 PBS solution for 5 min, then incubated with a 3% bovine serum albumin (BSA) PBS blocking solution for 1 hr. Cells were then exposed to the primary antibody diluted in a 1% BSA/PBS solution for 1 hr or overnight (as indicated). Primary antibodies were detected using Alexa Fluor (488 or 594) conjugated secondary antibodies incubated for 1 hr. Alexa Fluor 594 phalloidin was used to stain filamentous actin (F-actin). Images were acquired using a Nikon 80i Eclipse confocal microscope together with NIS-Elements image analysis software and exported to Image J.

2.16 Statistical analyses

Data are presented as Mean values ± SD or SEM as indicated. Differences in protein expression levels among two groups were analysed using an unpaired Student's *t*-test and verifying the normal distribution of data. For multiple group comparison, a one-way ANOVA with *post hoc* Tukey's or Dunnett's multiple comparison test was used. Differences with $p < 0.05$ were considered statistically significant. Data were analysed for normal distribution and statistical significance using GraphPad Prism 9.

Chapter 3. Further establishing the critical link between alterations in one-carbon metabolism and AD

3.1 Introduction

As previously mentioned, disturbances to one-carbon metabolism increase the risk for AD development. They can independently raise plasma tHcy levels that are associated with brain atrophy (Smith et al., 2018) and neuronal toxicity (Smith & Refsum, 2016). The frequently encountered *Mthfr* C677T functional polymorphism results in a critical amino-acid substitution resulting in a thermolabile and less functional variant of the encoded MTHFR protein. This enzyme is necessary for the production of 5-MTHF, the biologically active form of dietary folate. In the brain, 5-MTHF is critical for the remethylation of Hcy, which supports various methylation reactions through the production of SAM, the universal brain methyl group donor. Critical methylation reactions include PP2Ac methylation, which promotes the formation of methylated PP2A enzymes. These phosphatases contribute significantly to Tau phosphorylation status and APP phosphorylation at Thr668, which affects its processing (Sontag et al., 2007).

Here, *Mthfr* heterozygous (*Mthfr*$^{+/-}$ or HET) and homozygous (*Mthfr*$^{-/-}$ or NULL) knockout mice were used to model mild and severe MTHFR deficiencies associated with heterozygous and homozygous genotypes of the *Mthfr* C677T polymorphism, respectively. The specific activity of MTHFR in heterozygotes is 60-70% of that in WT mice (Chen et al., 2001), which is similar to that observed in human CT carriers of *Mthfr* C677T (van der Put et al., 1998). Our aim was to identify AD relevant pathological changes in these mice, henceforth to gain a better understanding of the involvement of the *Mthfr* C677T polymorphism and altered one-carbon metabolism in general in AD. Previous studies found that both *Mthfr* knockouts have significantly reduced SAM levels and/or increased SAH levels. This altered SAM/SAH ratio is indicative of altered cellular methylation potential, and was accompanied with global DNA hypomethylation (Chen et al., 2001). NULL mice are characterised by reduced survival (76.4% at 5 weeks of age), cerebellar abnormalities, motor and gait abnormalities or severe tremors (Chen et al., 2001). In addition, decreased whole brain and hippocampal volumes, reduced thickness of pyramidal cell layers, increased hippocampal apoptosis, altered choline metabolism, and memory impairments have also been described in these mice (Jadavji et al., 2012).

Specifically, we analysed 5-week-old (young) wild-type (WT), HET and NULL mice for changes in PP2A methylation, APP, Tau, and the major APP/Tau kinase GSK-3β, as seen in AD (**Figure 9**). In parallel, 16-month-old (aged) HET mice were fed either a normal diet containing normal folate levels (NF) or a diet with reduced folate content (or low folate, LF) for 6 months, then sacrificed. This study in older mice aimed to investigate whether changes in dietary folate alone or in combination with mild MTHFR enzyme deficiency and ageing can exacerbate changes in APP, Tau, PP2A and GSK-3β. Due to the significantly reduced lifespan of NULL mice (Chen et al., 2001), they could not be used in our dietary intervention study.

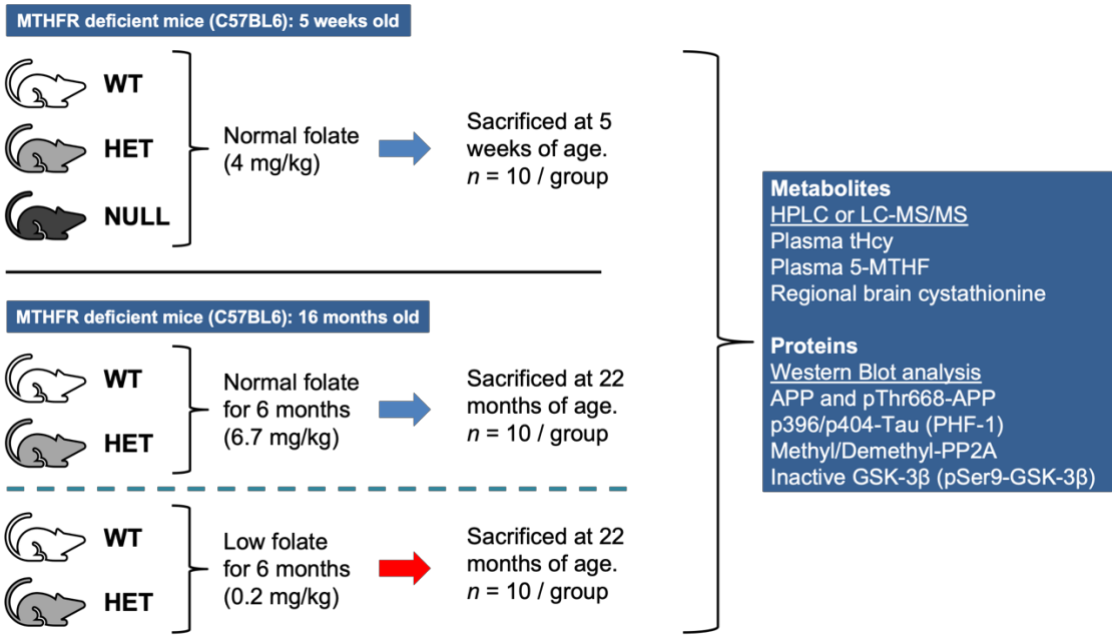

Figure 9. Overview of studies using mouse models of MTHFR and/or folate deficiencies. WT, HET and NULL female mice fed a NF diet were sacrificed at 5 weeks of age. Subgroups of 16-month-old WT and HET mice were fed a NF or LF diet for a period of 6 months before being sacrificed at 22 months of age. Brains were regionally dissected, and homogenates were prepared for Western blot analyses to look at Tau, APP and GSK-3β phospho-epitopes, as well as PP2Ac methylation levels. Blood was obtained to determine plasma tHcy levels by high pressure liquid chromatography (HPLC) with fluorescence detection. Plasma 5-MTHF levels and regional brain cystathionine levels were determined by stable-isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS).

3.2 MTHFR deficiencies alter Tau phosphorylation at the PHF-1 epitope and Thr668 phosphorylation and protein expression of APP in young *Mthfr* knockout mice.

Significant changes to plasma tHcy and/or 5-MTHF levels were observed in young *Mthfr* knockout mice and aged WT and HET mice subjected to a prolonged LF diet (**Table 2**).

Table 2. Plasma tHcy and 5-MTHF levels in young and aged WT, HET and NULL mice fed a normal folate (NF) or low folate (LF) diet.

	Female, 5-week-old			**Female, 22-month-old**			
Genotype	WT	HET	Null	WT	HET	WT	HET
Diet	NF	NF	NF	NF	NF	LF	LF
tHcy (μmol/L)	3.3±0.7	5.6±1.2*	32.8±8*	5.6±1.9	9.2±3.1*	35±12$^{\#}$	65±26$^{*\#}$
5-MTHF (nmol/L)	ND	ND	ND	64±20	47±12*	6.8±1.6$^{\#}$	5.8±2.1$^{\#}$

ND, not determined; $*$ $p < 0.05$, HET or NULL mice, relative to WT in same diet group; # $p < 0.05$, LF versus NF in the same genotype.

Specifically, the LC-MS/MS analyses showed that plasma tHcy levels in young HET and NULL mice were 1.6- and 10-fold higher than those in WT littermates, respectively (**Publications 1 and 2**).

Firstly, *via* Western blotting, we assessed Tau phosphorylation at the AD-like phospho-epitope PHF-1, which is observed in PHFs and early NFT-like structures; phosphorylation at this site is increased in early AD and correlates with disease progression (Mondragon-Rodriguez et al., 2014). After analysis, our studies showed that NULL *Mthfr* knockout mice have 30-50% higher Tau phosphorylation levels at the PHF-1 epitope in the hippocampus, cortex, and cerebellum relative to their WT littermates (**Figure 10A**). Conversely, Tau phosphorylation at this epitope was unaffected in all analysed brain regions from young HET mice.

We further investigated whether MTHFR deficiency in young mice could affect the regulation of APP. Thr668-APP phosphorylation can play an upstream role in promoting Tau phosphorylation at the PHF-1 epitope (Kirouac et al., 2017). Moreover, several studies have shown that Thr668-APP phosphorylation promotes $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ production (Ando et al., 2001; Lee et al., 2003). However, it is notoriously difficult to accurately measure Aβ levels in WT mouse models that do not overexpress APP. As expected, Western blot analyses showed that pThr668-APP levels were significantly increased in both young HET

and NULL mice relative to age-matched WT mice. This effect was seen in the cortex and hippocampus (**Figure 10B-C**), but not the cerebellum (See **Publication 2**).

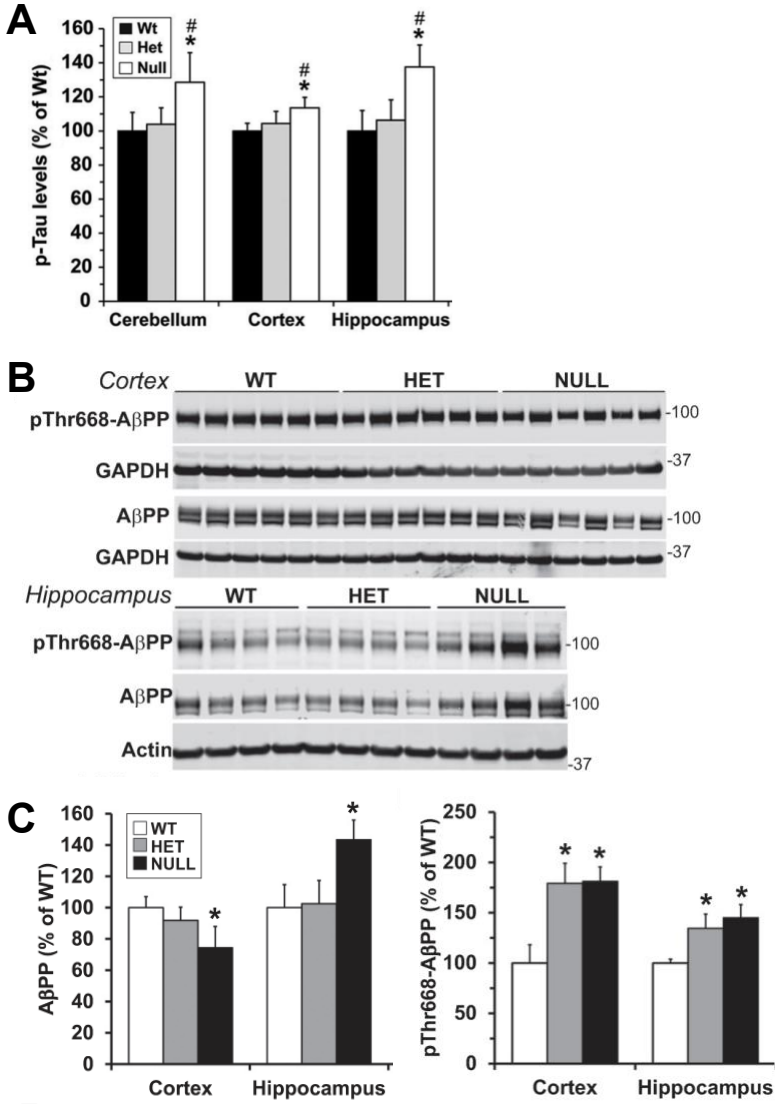

Figure 10. Brain region-specific effects of mild and severe MTHFR deficiencies on APP phosphorylation at Thr668 and protein expression levels, and Tau phosphorylation at the PHF-1 epitope in young *Mthfr* knockout mice. Regional brain homogenates were prepared from 5-week-old wild-type (WT), *Mthfr*$^{+/-}$ (HET), and *Mthfr*$^{-/-}$ (NULL) mice. (**A**) Relative levels of Tau phosphorylated at the PHF-1 epitope were quantified using densitometry after normalising for total Tau expression. (**B**) Representative Western blots prepared from total protein extracts probed for total or Thr668-phosphorylated APP ("AβPP") expression. (**C**) Relative APP and pThr668-APP expression levels normalised with actin or GAPDH and quantified using densitometry. Values represent Mean ± SD ($n = 6$ mice/ group); * $p < 0.05$, HET or NULL compared to WT; # $p < 0.05$, NULL compared to HET mice.

Since these changes in Tau and APP phosphorylation could be due to enhanced APP protein levels (Kirouac et al., 2017), we also quantified APP expression in regional brain homogenates (**Figure 10B-**

C). Western blot analyses revealed significant changes to APP expression, specifically, a ~25% decrease in the cortex and a ~45% increase in the hippocampus of young NULL mice relative to WT mice.

Significant changes to APP Thr668-phosphorylation and protein expression and Tau phosphorylation were also observed in the midbrain and striatum from young *Mthfr* knockouts (See **Publications 1 and 2**).

3.3 Changes in dietary folate intake and mild MTHFR deficiency in aged mice alter APP Thr668 phosphorylation and protein expression, as well as Tau phosphorylation at the PHF-1 epitope.

We next assessed the combined effects of dietary folate and MTHFR deficiencies on the basis that the clinical consequences associated with mild *Mthfr* polymorphisms can be aggravated by inadequate dietary folate intake (Li et al., 2017; Schwahn & Rozen, 2001). Indeed, low plasma folate levels are found in AD patients (Lopes da Silva et al., 2014). Parallel groups of aged WT and HET mice were fed a NF or LF diet for 6 months prior to sacrifice at 22 months of age. As shown in *Table 2*, aged NF-fed HET mice had plasma tHcy levels 1.6-fold higher than those in their WT counterparts. As expected, the prolonged LF diet alone significantly increased plasma tHcy levels by 6-fold in aged WT mice relative to NF-fed WT mice. Plasma 5-MTHF levels in aged NF-fed HET mice were reduced by 1.3-fold relative to WT counterparts. Interestingly, plasma 5-MTHF levels in aged WT and HET mice fed the LF diet were substantially reduced to similar extents. The observation that mild MTHFR deficiency failed to further reduce plasma 5-MTHF levels could be indicative that those were already maximally reduced in response to the prolonged LF diet in WT mice. Nevertheless, mild MTHFR deficiency in HET mice was able to aggravate the hyperhomocysteinemia induced by the LF diet. Furthermore, as expected, brain regional levels of cystathionine, produced by the transsulfuration pathway of Hcy (**Figure 8**), were significantly increased in aged mice in response to the LF diet and the *Mthfr*$^{+/-}$ genotype (**Figure 11**). Levels were further elevated in LF-fed HET mice.

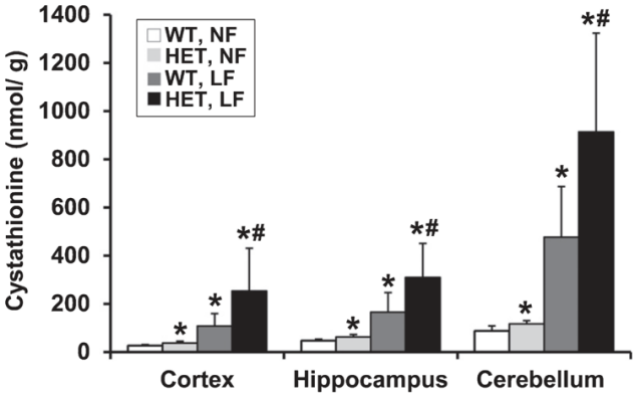

Figure 11. Regional brain cystathionine levels in aged WT and HET mice fed a normal (NF) or low folate (LF) diet. Cystathionine levels were determined in brain homogenates prepared from the cortex, hippocampus and cerebellum from 22-month-old mice WT and HET mice, which had been fed a NF or LF diet. Values represent Mean ± SD (n = 6-10 mice/ group); $* p < 0.05$, relative to WT mice fed on the NF diet; # $p < 0.05$, relative to HET mice fed the NF diet.

Under these experimental conditions, aged HET mice exhibited increased Tau phosphorylation at the PHF-1 epitope in various brain regions including the cortex and hippocampus (**Figure 12A**), which was not observed in their younger counterparts (**Figure 10A**). This effect was further exacerbated by the LF diet. The LF diet alone also greatly enhanced PHF-1-Tau levels in the cortex and hippocampus of WT mice to extents similar to LF-fed HET mice.

Western blot analyses of tissue homogenates of the cortex and hippocampus showed increased pThr668-APP levels in response to the LF diet for both WT and HET mice (**Figure 12B-C**). In contrast to the cortex, the hippocampus exhibited increased pThr668-APP levels in NF-fed HET mice.

Interestingly, dietary folate and mild MTHFR deficiencies increased APP expression levels in the hippocampus (**Figure 12B-C**). Conversely, APP expression levels were reduced in the cortex of WT and HET mice in response to the LF diet, which is similar to what was observed in the cortex of young HET mice (**Figure 10B-C**).

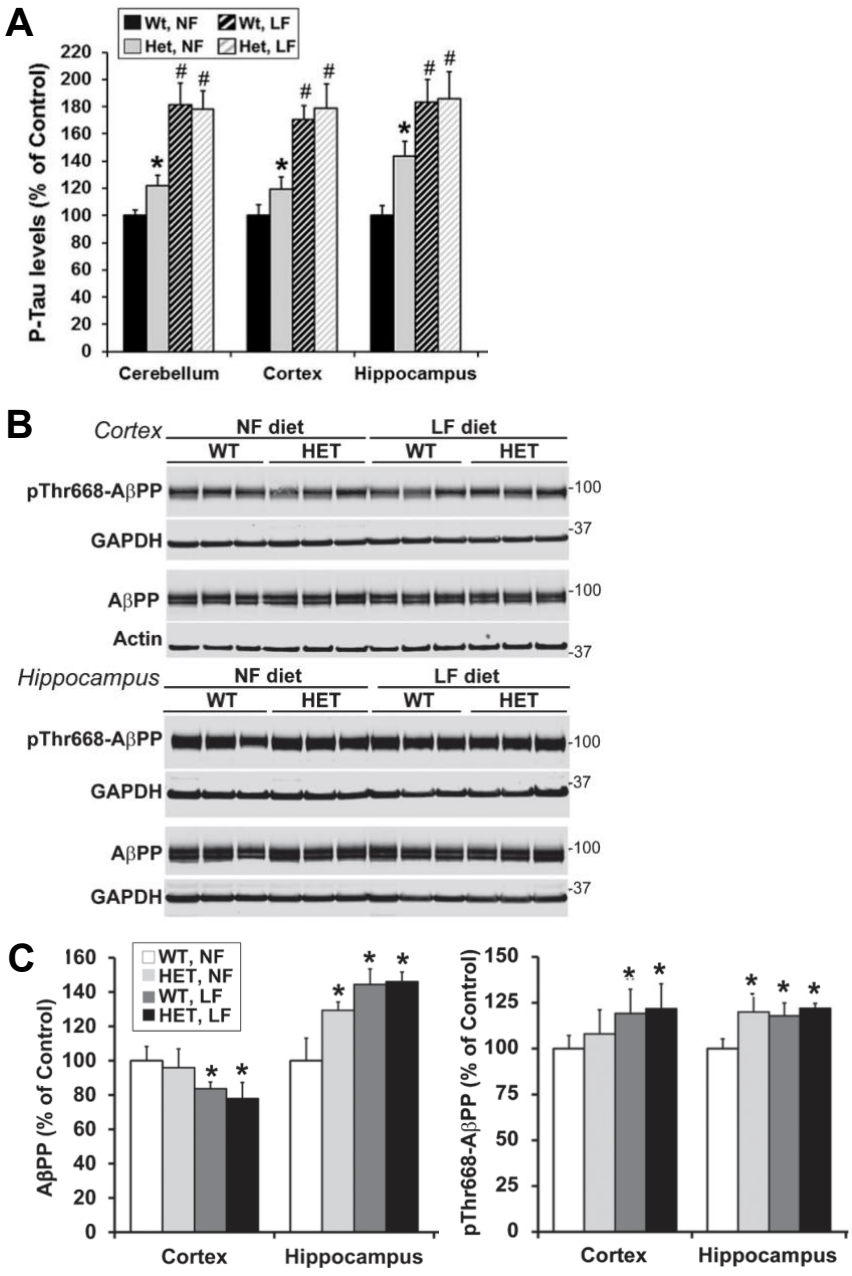

Figure 12. Dietary folate and mild MTHFR deficiencies alter total and Thr668-phosphorylated APP levels and Tau phosphorylation at the PHF-1 epitope in aged mice. Regional brain homogenates were prepared from 22-month-old WT and HET mice fed a normal folate (NF) or low folate (LF) diet for a period of 6 months prior to sacrifice. (**A**) Levels of PHF-1-Tau were quantified after normalising for total Tau expression. (**B**) Representative Western blots prepared from total cortical and hippocampal protein extracts probed for total and pThr668 APP ("AβPP") expression. Relative APP and pThr668-APP expression levels normalised with GAPDH and quantified using densitometry. (**C**) Relative APP and pThr668-APP expression levels normalised with GAPDH and quantified using densitometry. Values represent Mean ± SD for (n = 8 or 9 mice/ group); * $p < 0.05$, HET compared to WT; # $p < 0.05$, LF vs NF in the same genotype.

3.4 Enhanced APP and Tau phosphorylation in MTHFR and folate deficient mice are associated with enhanced PP2A demethylation and GSK-3β activation.

We next investigated what could be potential mechanisms mediating the observed changes to pThr668-APP and Tau PHF-1-phosphorylation levels in our mouse models. As discussed before, significant changes in PP2Ac methylation and PP2A/Bα levels are found in AD-affected brain regions (Sontag, Hladik, et al., 2004; Sontag, Luangpirom, et al., 2004), which could significantly contribute to enhanced APP and Tau phosphorylation in AD. Thus, we further analysed homogenates for PP2Ac methylation. Not surprisingly, we observed a significant increase in demethylated PP2Ac levels in the cortex and hippocampus of young HET and NULL mice (**Figure 13A-B**) and associated reduced levels of methylated PP2Ac (**Figure 13A**).

In addition, we analysed those tissue homogenates for GSK-3β activity, which is enhanced in AD brain. GSK-3β phosphorylates APP at Thr668 (Kirouac et al., 2017) and Tau at the PHF-1 epitope (Hernández, Lucas, Cuadros, & Avila, 2003). Significantly, inactivating-phosphorylation of GSK-3β at Ser9 was reduced in both young knockouts, relative to WT mice, indicative of increased GSK-3β activity (**Figure 13D-E**).

Similarly, both dietary intervention groups of aged HET mice had increased levels of demethylated PP2Ac in the cortex and hippocampus (**Figure 13C**). This was also observed in both aged WT and HET mice fed the LF diet.

In contrast to younger counterparts, pSer9-GSK-3β levels in aged HET mice were not significantly altered in the brain regions analysed (**Figure 13F-G**). However, the prolonged LF diet dramatically reduced Ser9-GSK-3β phosphorylation levels in both the cortex and hippocampus of aged WT and HET mice fed the LF diet relative to aged NF-fed WT mice.

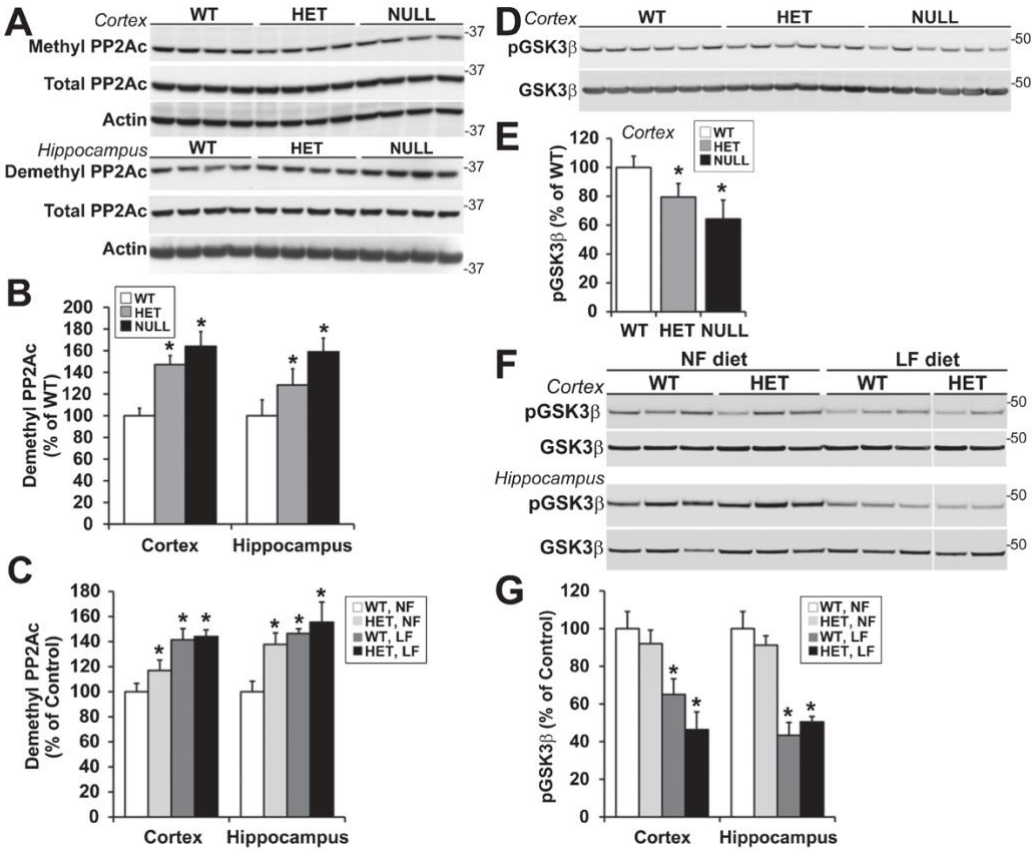

Figure 13. MTHFR and folate deficiencies decrease PP2Ac methylation and increase GSK-3β activity. Western blots prepared from young and aged mice were analysed for changes in PP2Ac methylation/demethylation (**A-C**) and for GSK-3β inactivating Ser9 phosphorylation (**D-G**). Signals for methylated, demethylated and total PP2Ac were normalised for protein loading with actin. Phospho-Ser9-GSK-3β levels were normalised for total GSK-3β expression levels (**D and F**). Values represent Mean ± SD (n = 6 mice/ group); * $p < 0.05$, young HET and NULL mice relative to WT mice, and aged WT and HET mice fed the LF diet relative to NF-fed WT mice.

3.5 Discussion

While there is solid experimental evidence that Aβ oligomers can trigger a neurodegenerative cascade leading to excessive phosphorylation of Tau (Bloom, 2014; Ittner & Gotz, 2011), the underlying causes of the shift towards enhanced amyloidogenic processing of APP in late-onset AD are not well understood. The complex and non-Mendelian aetiology of sporadic AD suggests that an epigenetic component is at play. In support of this notion, a CpG methylation analysis of AD brain found relatively large epigenetic differences in AD susceptibility genes, which included APP processing genes (*PSEN1* and *ApoE*) and *Mthfr* (Wang, Oelze, & Schumacher, 2008). Since MTHFR plays a significant role in cellular methylation homeostasis (**Figure 8**), we wanted to investigate if changes in MTHFR activity or 5-MTHF availability

could trigger hallmark molecular events observed in AD, using well-characterised *Mthfr* knockout mouse models. Indeed, these mice recapitulate the biochemical and clinical consequences of the *Mthfr* C667T polymorphism in humans (Chen et al., 2001; Jadavji et al., 2012).

As mentioned previously, reduced MTHFR activity, due to the *Mthfr* C677T polymorphism, raises plasma tHcy levels in humans (Jacques et al., 1996). Such elevation in tHcy has been linked to brain atrophy (Rajagopalan et al., 2012) and elevated risk for AD (Smith et al., 2018). Cementing this link, we show for the first time that reduced MTHFR activity can alter APP protein expression and Thr668-phosphorylation levels, and Tau phosphorylation at the AD-associated PHF-1 epitope, in a brain region- and age-dependent manner.

Mild and severe MTHFR deficiencies affect APP and Tau phosphorylation in young mice

Specifically, in young mice, we found that mild and severe MTHFR deficiencies substantially enhanced Thr668-APP phosphorylation in AD-susceptible regions, such as the hippocampus and cortex, but not the cerebellum, a region less affected in AD. This could be clinically relevant since accumulation of pThr668-APP species is found in both the AD brain and APP and Tau transgenic mouse models of AD (Shin et al., 2007). Other than its role in promoting Aβ production (Lee et al., 2003), the precise physiological significance of Thr668-APP phosphorylation remains to be further clarified. Nevertheless, it is known to induce neurotoxicity (Chang et al., 2006), potentially through mediating signalling cascades leading to aberrant Tau phosphorylation and detrimental cell cycle activation (Kirouac et al., 2017). In support of this, substitution of Thr668 with Alanine (non-phosphorylatable) prevents the development of memory and synaptic plasticity deficits in a mouse model of familial dementia (Lombino, Biundo, Tamayev, Arancio, & D'Adamio, 2013).

As expected, increased Tau PHF-1-phosphorylation was seen in the cortex and hippocampus of young NULL mice, but not in young HET mice, possibly due to the tight regulation of Tau phosphorylation. Thus, additional changes may be required to offset Tau phosphorylation status. For instance, elevated plasma tHcy levels have been shown to induce oxidative stress and excitotoxicity, which in turn, have been associated with increased APP and Tau phosphorylation (Smith & Refsum, 2016). There is an established threshold for plasma tHcy levels (>11.1 μmol/L) in humans for increasing the rate of brain atrophy, and marked hyperhomocysteinemia typically induces more severe deficits in the CNS (Smith et al., 2018). Accordingly, a massive elevation of tHcy in NULL mice, relative to their WT counterparts, was associated with an increase in both Thr688-APP and PHF-1-Tau phosphorylation. However, compared to WT, the generally small increase of tHcy observed in young HET mice (**Table 2**), and the

short duration of this elevation may have been insufficient to induce profound changes in Tau phosphorylation. HET mice may also be more resilient to certain effects of mild MTHFR deficiency due to compensatory effects of adequate folate in their NF diet, as observed in humans (Ashfield-Watt et al., 2002). Indeed, HET mice appear phenotypically normal relative to NULL mice (Chen et al., 2001). Yet, we cannot exclude the possibility that Tau was phosphorylated at earlier p-Tau epitopes in young HET mice. However, this could not be determined in this study due to insufficient amounts of brain tissue.

Dietary folate and mild MTHFR deficiencies alter APP and Tau phosphorylation in aged mice

Levels of APP phosphorylated at Thr668 in the cortex of aged HET mice was similar to those in aged WT mice, in contrast to what was observed in their younger counterparts. Conversely, increased pThr668-APP levels were observed in the hippocampus of aged NF-fed HET mice, as seen in younger counterparts. Although the observed increases in pThr668-APP levels were subtle and lower to what was observed in young *Mthfr* knockout mice, it is possible that basal levels of Thr668-APP phosphorylation in aged WT mice were already increased due to ageing alone. This is likely since plasma tHcy levels were higher in aged WT mice relative to their younger counterparts that were fed the same NF diet. In support of this hypothesis, cortical and hippocampal pThr668-APP levels were enhanced in aged and hyperhomocysteinaemic LF-fed WT mice, relative to aged NF-fed WT mice.

Tau phosphorylation at PHF-1 was increased in the cortex, hippocampus, and cerebellum of aged HET mice in contrast to their younger counterparts fed the same NF diet, which suggests an interaction between ageing and mild MTHFR deficiency. This could be due to the slightly higher plasma tHcy levels as previously discussed. It is also possible that young HET mice were predisposed to phosphorylation at earlier Tau epitopes, which primed Tau for subsequent phosphorylation at the PHF-1 epitope (Cho & Johnson, 2004). Of significance, PHF-1-Tau levels were enhanced in aged LF-fed WT mice. PHF-1-Tau levels were not further enhanced in LF-fed HET mice, relative to LF-fed WT mice, suggesting that Tau phosphorylation at this AD-like phospho-epitope was already maximally increased in response to the prolonged LF diet alone.

The similarities in pThr668-APP levels or PHF-1-Tau levels between aged WT and HET mice fed the LF diet could be due to the similarly reduced plasma 5-MTHF levels in these mice. The fact that the LF diet failed to further reduce plasma 5-MTHF levels in HET mice indicates that 5-MTHF levels were already maximally reduced in response to the prolonged LF diet.

Dietary folate and MTHFR deficiencies alter PP2Ac methylation and GSK-3β activity in young and aged mice

In young and aged *Mthfr* knockout mice, we have also found reduced PP2Ac methylation in various brain regions, including the cortex and hippocampus. Indeed, this coincided with a downregulation of Tau-specific PP2A/Bα and LCMT1 enzymes (See **Publication 1**). Similarly, these effects were observed in various brain regions from aged HET mice fed a NF diet, and were aggravated by the LF diet. Furthermore, these effects were seen in aged LF-fed WT mice. Interestingly, relative to aged LF-fed WT mice, HET mice fed the same diet had more pronounced LCMT1 downregulation (hippocampus, cortex and cerebellum), PP2A/Bα downregulation (hippocampus and cerebellum) and reduced cerebellar PP2Ac methylation (**Publication 1**) while plasma 5-MTHF levels remained similar. This could possibly be related to the higher plasma tHcy levels in HET mice induced by the LF diet, which was statistically significant. Thus, it is likely that impaired PP2Ac methylation due to LCMT1 downregulation is a significant contributor to the increased APP and Tau phosphorylation levels in *Mthfr* knockout mice and in response to the LF diet. Similarly, previous studies from our group found that Tau becomes phosphorylated at various sites including PHF-1 and AD-exclusive sites, in N2a cells under conditions of altered PP2Ac methylation (Sontag et al., 2007). In line with our findings, a recent study found significant retinal PP2Ac demethylation in a 3 to 4-month-old rat model with increased plasma tHcy levels. This correlated with an increase in retinal Tau phosphorylation at various Ser sites (Guo et al., 2019).

Enhanced PP2A demethylation also promotes the phosphorylation of APP at Thr668 in N2a cells (Sontag et al., 2007) and *in vivo* (Nicholls et al., 2016). Similarly, inhibition of PP2A activity in response to OA, a potent inhibitor of PP2A that also induces PP2A demethylation (Favre, Turowski, & Hemmings, 1997), enhances pThr668-APP levels in N2a cells (Sontag et al., 2007) and in degenerating neurons, where it accumulates in axonal swellings (Ahn et al., 2016). However, it is worth noting that while one-carbon metabolism specifically affects methylation-sensitive PP2A holoenzymes, OA not only binds to and inhibits PP2Ac, but also many PP2A-like phosphatases; thus, incubation with OA often leads to widespread and ultimately neurotoxic effects. Moreover, inhibiting PP2A can indirectly affect the activity of many cellular protein kinases. Thus, PP2A-dependent regulation of APP phosphorylation and processing likely results from a combination of direct and indirect effects (Sontag et al., 2007).

In our studies, we also found an upregulation of GSK-3β activity in response to MTHFR and dietary folate deficiencies. Studies suggest that GSK-3β activity plays a role in both early- and late-stage AD (Kirouac et al., 2017). GSK-3β phosphorylates Tau at the PHF-1 epitope as well as the Thr231 site, which decreases Tau-microtubules interactions (Cho & Johnson, 2003). Specifically, we found that GSK-3β activity was increased in the cortex of young HET and NULL mice, likely facilitating the enhanced

phosphorylation of both APP and Tau. The ability of GSK-3β to phosphorylate both APP and Tau has been described previously (Aplin, Gibb, Jacobsen, Gallo, & Anderton, 1996). This was also observed in response to dietary folate deficiency in the cortex and hippocampus of our aged mice regardless of genotype. In agreement with our findings, recent studies have shown that incubation of hippocampal slices with folate induces deactivating-phosphorylation (Ser9) of GSK-3β, which is accompanied with neuroprotective effects in the context of glutamate-induced excitotoxicity (Budni et al., 2018). Other neuroprotective mechanisms of folate and 5-MTHF include improving nitric oxide bioavailability and directly scavenging superoxide radicals (Budni et al., 2018; Stanhewicz & Kenney, 2017). Thus, folate deficiency induces oxidative stress and excitotoxicity through upregulation of GSK-3β activity, which in turn, increases APP phosphorylation at Thr668 (Kirouac et al., 2017). It has been proposed that, in response to Thr668-APP phosphorylation, Aβ oligomers are generated and stimulate a signalling cascade leading to more Thr668-APP phosphorylation through GSK-3β upregulation. In turn, this augments Tau phosphorylation, thus, creating a relentless and vicious cycle (Okada, 2012). It is worthy of speculation that this neuropathological cycle is initiated and maintained by the downregulation of PP2Ac methylation and PP2A/Bα enzymes, as found in AD-susceptible brain regions (Sontag, Hladik, et al., 2004).

Dietary folate and MTHFR deficiencies alter APP expression in young and aged mice

Despite its association with AD pathology, APP has many functions in neuronal physiology, such as synaptic plasticity, dendritic and spine morphogenesis, neuroprotection, and learning and memory (Del Turco et al., 2016). Indeed, APP knockout mice develop impairments in behaviour, LTP, dendritic branching and synaptic density (Guenette, Strecker, & Kins, 2017). Here, we observed that genetic-induced MTHFR deficiency and diet-induced folate deficiency affected total APP expression, however, in a complex and brain-region specific manner. Due to the shortage of brain tissue, we were unable to determine whether these effects were epigenetically driven since MTHFR supports DNA methylation reactions. Alternatively, these effects could involve changes to the transcriptional regulation of APP by stress factors, inflammatory cytokines and Rho family GTPases (Chen, Zhang, Xu, & Bu, 2013). For instance, young NULL mice and all aged mice fed the LF diet had reduced cortical APP expression levels. This could possibly contribute to the neurodevelopmental delays and cognitive deficits in humans affected by severe MTHFR deficiency (Smith & Refsum, 2016). In contrast, hippocampal APP expression levels were upregulated in young NULL mice, aged NF-fed HET mice, and all aged mice fed the LF diet. Interestingly, overexpression of APP in mice has been associated with early memory impairment and loss of critical dendritic and postsynaptic proteins, even though Aβ levels remain unaffected (Simon et al., 2009). This could, in part, be due to the kinase- and cell cycle- activating pathways mediated by APP

(Kirouac et al., 2017). The latter study also found increased PHF-1-Tau levels in the hippocampus of APP-overexpressing mice, which is consistent with our results. Interestingly, the accumulation of APP has been reported to occur at sites of axonal injury in the brain and is a useful marker for the detection of injured axons, such as in head trauma (Sherriff, Bridges, Gentleman, Sivaloganathan, & Wilson, 1994). This is in agreement with the significant hippocampal apoptosis exhibited by NULL mice (Jadavji et al., 2012). Increased APP expression is also found in the cerebrovascular and brain tissue of nitric oxide-deficient mice (Katusic & Austin, 2014). Since, folate deficiency is known to induce nitric-oxide impairment (Stanhewicz & Kenney, 2017; Budni et al., 2018), this could be an underlying mechanism for the increased APP expression we observed in MTHFR- and dietary folate-deficient mice.

In accordance with our findings, a remarkable increase in hippocampal APP expression has been found previously in a rat model of slightly elevated plasma tHcy levels. Interestingly, this was observed in the absence of changes to Tau phosphorylation (Guo et al., 2019), which reflects the temporal pattern of Aβ and p-Tau pathology development in AD patients (Pontecorvo et al., 2019).

Elevated brain regional cystathionine levels

Significantly, brain regional levels of cystathionine were increased in aged mice in response to the LF diet and these were more pronounced with the *Mthfr*$^{+/-}$ genotype. This reflects the particular importance of folate-driven Hcy remethylation in the brain, wherein the transsulfuration pathway is significantly less active. This is due to the relatively low brain expression of the cystathionine-breakdown and cysteine biosynthetic enzyme, CSE, which is predominately expressed in the liver (Kabil, Vitvitsky, Xie, & Banerjee, 2011). The consequences of elevated brain cystathionine or depleted brain cysteine levels *per se* are unknown; however, CSE deficiency has been linked to the neurodegeneration in Huntington's disease (Paul et al., 2014).

Our studies show for the first time that diet- and/or genetic-induced folate (or 5-MTHF) deficiency leads to complex changes in APP expression, and significant changes to APP and Tau phosphorylation at AD-implicated sites. These changes could be attributed to the perturbance of critical APP and Tau regulating enzymes such as PP2A and GSK-3β. Together, these alterations in folate metabolism could have detrimental effects through various mechanisms, ultimately resulting in neurodegeneration (**Figure 14**). Our findings strongly support the existence of significant links between altered folate and Hcy metabolism and AD. Furthermore, they provide a compelling argument for the clinical monitoring of plasma 5-MTHF and tHcy levels in the aged population, as well as the implementation of Hcy-lowering therapies as preventative measures for AD development.

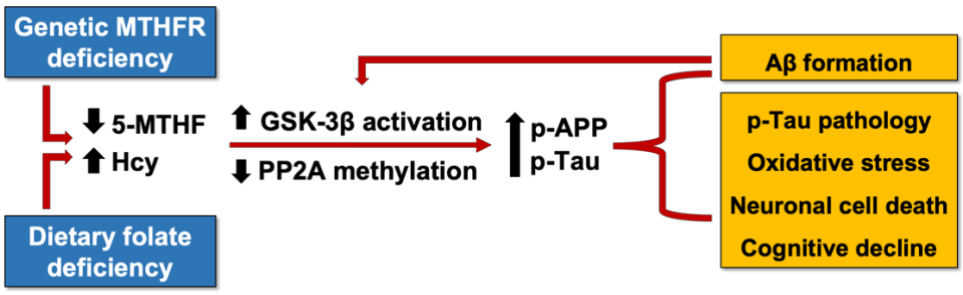

Figure 14. Proposed mechanisms linking dietary folate deficiency and the *Mthfr* C667T polymorphism to AD. Adequate production of 5-MTHF, and thus, normal Hcy metabolism, is dictated by dietary folate intake and MTHFR activity. MTHFR activity is hindered by functional polymorphisms, such as C667T. Hence, reduced dietary folate intake and less active MTHFR variants impede the remethylation of Hcy to methionine, leading to enhanced tHcy levels. Elevated Hcy levels and low levels of 5-MTHF can lead to oxidative stress-induced GSK-3β activation, and also reduce the methylation of PP2A. Consequently, these perturbances promote uncontrolled phosphorylation of APP at the Thr668 and Tau phosphorylation at various disease-associated epitopes. These events can trigger Aβ formation, p-Tau pathology, oxidative stress and neuronal cell death. This process gradually occurs over years, and ultimately manifests as cognitive decline.

Chapter 4. Disturbances to one-carbon metabolism and PP2A methylation critically affect Fyn distribution and function

4.1 Introduction

As mentioned in details in *Chapter 1*, Fyn is strategically positioned at the postsynaptic plasma membrane where it facilitates various physiological processes, including LTP, NMDAR integrity, glutamate receptor-mediated signalling and myelination. The bulk of Fyn is found in lipid rafts (Kramer, Klein, Koch, Boytinck, & Trotter, 1999; Shima, Nada, & Okada, 2003; Minami et al., 2011; Hanafusa & Hayashi, 2019). Lipid raft-associated Fyn stimulates neurite outgrowth (Beggs, Soriano, & Maness, 1994; Brackenbury et al., 2008) and regulates APP surface expression and processing (Hoe et al., 2008; Minami et al., 2011). Lipid raft-associated Fyn activity is modulated by various events such as neural differentiation (Hanafusa & Hayashi, 2019), myelination (Kramer et al., 1999), and cell spreading (Shima et al., 2003). Indeed, various studies have demonstrated that Fyn is enriched in detergent-insoluble extracts obtained after lysing tissue or cells with non-ionic detergents such as Triton X-100 (Bertagnolli, Hudson, & Stetsenko, 1999; Maekawa, Toyama, Yasuda, Yagi, & Yuasa, 2002) or NP-40 (Shima et al., 2001); those typically contain lipid raft membrane microdomains in addition to cytoskeletal proteins.

Mass spectrometric analyses have suggested that Fyn is trimethylated on Lys-7/9, and mutational analyses of these sites abolished the targeting of Fyn to the plasma membrane as well as its function in cell adhesion and spreading in fibroblasts (Liang et al., 2004). However, to date, Fyn methylation has never been confirmed in any other studies, and the dedicated Fyn methyltransferase has not been identified. Regardless, these observations suggest that altering one-carbon metabolism has the potential to directly affect Fyn methylation state, and thereby affect its subcellular targeting.

There are examples of post-translational methylation being involved in the targeting of major signalling enzymes to the plasma membrane, including Ras (Chiu et al., 2004) and PP2A (Sontag et al., 2013). Of particular interest to this thesis, carboxyl methylation of PP2Ac targets PP2A holoenzymes to lipid rafts. Moreover, it influences the translocation of Tau to the plasma membrane (primarily non-raft membrane microdomains) by promoting Tau dephosphorylation (Sontag et al., 2013). Based on these findings, it is

plausible that Tau-dependent trafficking of Fyn to postsynaptic densities (Ittner et al., 2010) is influenced by methylated PP2A enzymes. Significantly, methylated PP2A enzymes regulate the actin cytoskeleton (**Publication 3**), which itself, is essential for plasma membrane targeting of Fyn (Sandilands et al., 2007).

Collectively, these findings led us to investigate whether modulating one-carbon metabolism and/or PP2A methylation influence the steady-state distribution of Fyn, and thus, its function. Furthermore, we explored the possibility that Fyn and methylated PP2A enzymes interact based on earlier reports showing that PP2A binds to Src, a SFK structurally closely related to Fyn (Ogris, Mudrak, Mak, Gibson, & Pallas, 1999; Yokoyama & Miller, 2001). To that end, we used neuroblastoma N2a cells and acute mouse brain slices, which have been widely used in AD research. Furthermore, we analysed regional brain homogenates from *Mthfr* knockout mice (*Chapter 3*), to determine the effect of long-term one-carbon metabolic disturbances on Fyn regulation.

4.2 One-carbon metabolism regulates Fyn cellular distribution and Fyn-dependent neurite outgrowth in N2a cells

We first performed experiments to establish whether one-carbon metabolism regulates Fyn targeting under acute conditions that result in enhanced/decreased methylated PP2A enzymes levels in N2a cells (Sontag et al., 2007). Interestingly, we found that treatment with the universal methyl group donor SAM led to a pronounced increase in Fyn levels in detergent-insoluble extracts, wherein Fyn is known to primarily fractionate (**Figure 15A and C**). Alternatively, N2a cells were treated with either Hcy, SAH, or the SAH hydrolase inhibitor 3-deazaadenosine (3-DZA) that inhibits the conversion of SAH to Hcy. Cells were also incubated in folate-deficient (FD) medium to induce short-term folate deficiency. These treatments hinder one-carbon metabolism through unique mechanisms of action. However, they all promote an elevation of intracellular Hcy levels, which alters the cellular SAM/SAH ratio that controls cellular methylation potential (Fowler, 2005). In contrast to SAM, we found that these treatments induced a substantial decrease in detergent-insoluble Fyn levels (**Figure 15A-C**). Total cell lysates and detergent-insoluble fractions were also probed in parallel with commercial anti-p-Tyr416-SFK antibodies that recognise activating Tyr phosphorylation of SFKs. Of note, the numbering of this Tyr residue varies according to the nature of the SFK and species; the activating site in mouse and human Fyn corresponds to Tyr420. All treatments prompted parallel changes in anti-p-Tyr416-SFK immunoreactivity, indicating that active Fyn was redistributed (**Figure 15A-C**). After normalization, there were no statistically significant changes in net Fyn activity or total Fyn protein expression levels between treated and untreated N2a cells.

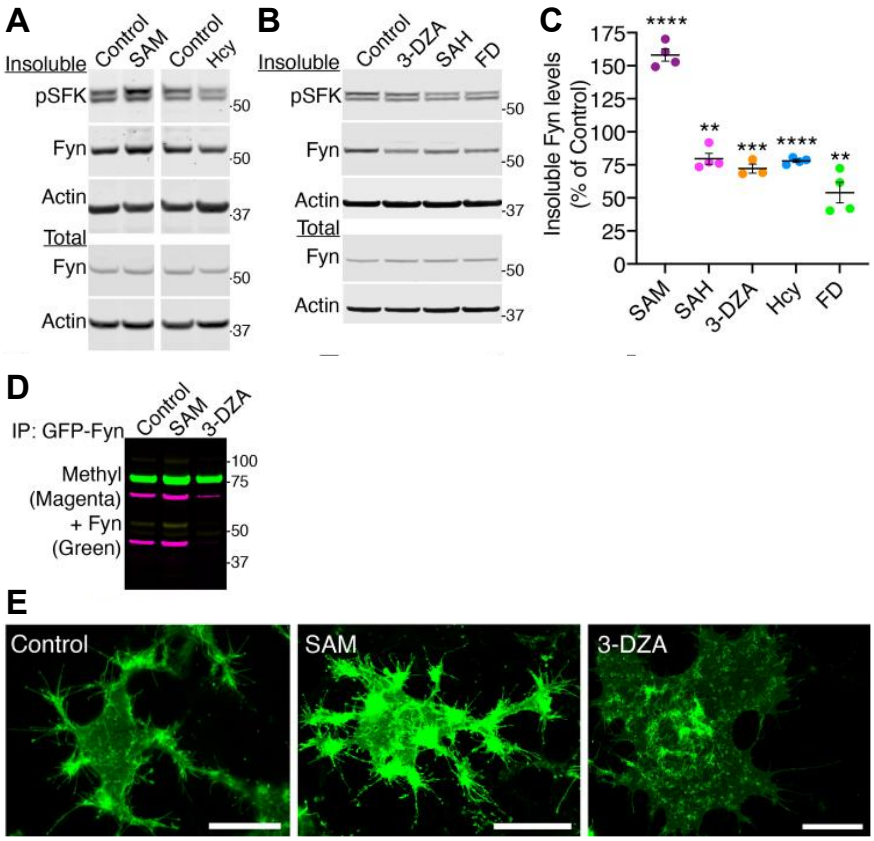

Figure 15. Fyn distribution and Fyn-dependent process outgrowth are dependent on one-carbon metabolism in N2a cells. (**A**) Immunoblot analysis of Fyn and its activating-phosphorylation (pSFK; bottom band is pTyr420-Fyn, recognised by anti-p-Tyr416-SFK antibodies) in total lysates and detergent-insoluble fractions from N2a cells that were incubated for ~16 h with either 100 µM SAM, 100 µM Hcy or vehicle (Control). (**B**) Representative immunoblots of Fyn and pSFK in total lysates and detergent-insoluble fractions from N2a cells that were incubated for ~16 h with either 50 µM 3-Deazaadenosine (3-DZA), 100 µM SAH or vehicle (Control). A subset of cells was incubated for 4 h in folate-deficient medium (FD). (**C**) Detergent-insoluble Fyn levels were quantified in these cells and are expressed as percent of levels in control cells. Data shown are Mean ± SEM (n = 3-4 independent experiments); **p < 0.01; ***p < 0.001; ****p < 0.0001. (**D**) GFP-Fyn immunoprecipitates were prepared from transfected N2a cells and analysed by Western blot using anti-methyl-lysine (Magenta) followed by reprobing with anti-Fyn (Green) antibodies. (**E**) Confocal images of GFP-Fyn in transfected N2a cells that were incubated for ~18 h in low-serum medium in the presence of 100 µM SAM, 50 µM 3-DA or vehicle (Control) prior to fixation. All figure panels are representative of at least 3 independent experiments.

To address the previous reporting that Fyn is methylated on Lys residues (Liang et al., 2004), we also analysed GFP-Fyn immunoprecipitates prepared from transfected N2a cells that had been treated either with SAM or 3-DA to alter cellular methylation potential, or treated with vehicle (control cells). Immunoprecipitates were analysed by Western blotting with validated anti-methyl-lysine antibodies. While immunoreactivity to other proteins was present in GFP-Fyn immunoprecipitates, we were unable to detect any immunoreactivity of GFP-Fyn itself despite potent manipulations to cellular methylation (**Figure 15D**).

We next assessed whether altered one-carbon metabolism affects Fyn-dependent neurite outgrowth, considering it affects the specific compartmentalisation of the kinase. Cells were incubated in a low-serum medium to induce differentiation (Sontag, Nunbhakdi-Craig, Mitterhuber, Ogris, & Sontag, 2010). Interestingly, incubation with SAM greatly enhanced the number of filopodia and processes that develop in GFP-Fyn-expressing N2a cells (**Figure 15E**). Furthermore, SAM-treated cells exhibited regions of high local concentration of GFP-Fyn molecules, which are likely zones of interactions with actin-filaments or actin-rich structures according to previous studies (Chen, Bailey, & Fernandez-Valle, 2000; Calaminus, Thomas, McCarty, Machesky, & Watson, 2008). In striking contrast, GFP-Fyn-dependent process outgrowth was abolished in cells treated with 3-DZA, wherein GFP-Fyn staining appeared more central, diffuse, disorganised and was absent from the cell periphery (**Figure 15E**).

Collectively, our results clearly indicate that one-carbon metabolism is an important regulator of Fyn compartmentalisation and Fyn-dependent neurite outgrowth in N2a cells.

4.3 Methylated PP2A enzymes co-immunoprecipitate with Fyn

Since Fyn was not directly methylated under our experimental conditions, we wanted to see if methylated PP2A enzymes could be the link between one-carbon metabolism and Fyn regulation. We first assessed whether methylated PP2A enzymes and Fyn co-immunoprecipitate. To that end, we analysed HA-tagged PP2Ac (WT PP2Ac) and HA-tagged L309Δ (L309Δ; methylation-incompetent PP2Ac mutant) immunoprecipitates from transfected N2a cells for immunoreactivity to Fyn (**Figure 16A-B**). Our results showed that endogenous Fyn was present in WT PP2Ac immunoprecipitates, but not in L309Δ immunoprecipitates (**Figure 16A**). Notably, the L309Δ mutant is unable to associate with regulatory B-type, including Bα, subunits in N2a cells (Nunbhakdi-Craig et al., 2007). Hence, our results indicate that Fyn preferentially associates with methylation-sensitive B-containing PP2A holoenzymes. To confirm that Fyn can more specifically form a complex with Bα-containing PP2A holoenzymes, we analysed FLAG-tagged Bα immunoprecipitates from N2a cells co-transfected with GFP-Fyn or EV, for immunoreactivity to Fyn. We opted for this approach since there are no suitable antibodies that efficiently pull down PP2A/Bα holoenzymes, and because of the close proximity of endogenous Bα, Fyn and immunoglobulins on blots. Significantly, GFP-Fyn was present and concentrated in FLAG-tagged Bα immunoprecipitates (**Figure 16B**). These immunoprecipitates also contained proportional amounts of PP2Ac to FLAG-tagged Bα, which further confirmed that Fyn and PP2A/Bα holoenzymes co-immunoprecipitated.

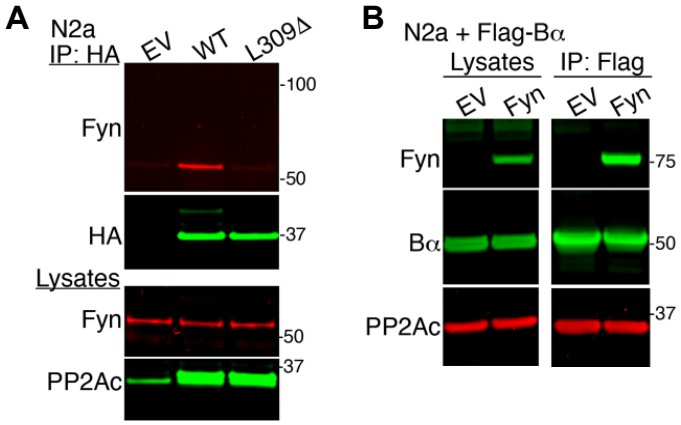

Figure 16. Methylated PP2A enzymes and Fyn co-immunoprecipitate. (**A**) Total cell lysates and HA-immunoprecipitates prepared from N2a cells co-transfected with plasmids encoding for HA-tagged PP2Ac, HA-tagged L309Δ or an empty vector (EV). HA-immunoprecipitates were analysed by Western blot for potential PP2A/Fyn interactions with the indicated antibodies. (**B**) Total cell lysates and FLAG-immunoprecipitates prepared from N2a cells co-transfected with plasmids encoding for FLAG-tagged Bα and GFP-Fyn^{Wt} or an EV. FLAG-immunoprecipitates were analysed by Western blot for potential PP2A/Bα-Fyn interactions with the indicated antibodies. As indicated by the double bands in total cell lysates, anti-B subunit antibodies recognised both endogenous (bottom band) and FLAG-tagged Bα (top band). Panels were assembled from the same blot. (**A**-**B**) Representative experiments from at least three separate immunoprecipitation assays.

4.4 PP2A methylation regulates Fyn distribution and Fyn-dependent neurite outgrowth in N2a cells

Based on the findings that methylated PP2A enzymes are targeted to lipid rafts (Sontag et al., 2013) and interact with Fyn (**Figure 16**), we next wanted to confirm whether manipulating PP2A methylation, through various means, impacts the steady-state distribution of endogenous Fyn in N2a cells. To that end, NP-40 detergent-insoluble fractions were prepared from N2a cells transfected with either EV (controls), WT PP2Ac, L309Δ, LCMT1 or PME1. Previously, it has been shown that WT PP2Ac-expressing or LCMT1-expressing N2a cells exhibit a ~30% increase in levels of total and membrane-associated methylated PP2A enzymes and PP2A/Bα in N2a cells (Sontag et al., 2013). Conversely, L309Δ-expressing or PME1-expressing N2a cells are characterised by a stark reduction in overall methylated PP2Ac and Bα levels (Sontag et al., 2007). Western blot analyses showed that basal detergent-insoluble Fyn levels were increased by ~30% in WT PP2Ac-expressing N2a cells compared to controls (**Figure 17**). Similarly, overexpression of LCMT1 enhanced basal detergent-insoluble Fyn levels (**Figure 17**).

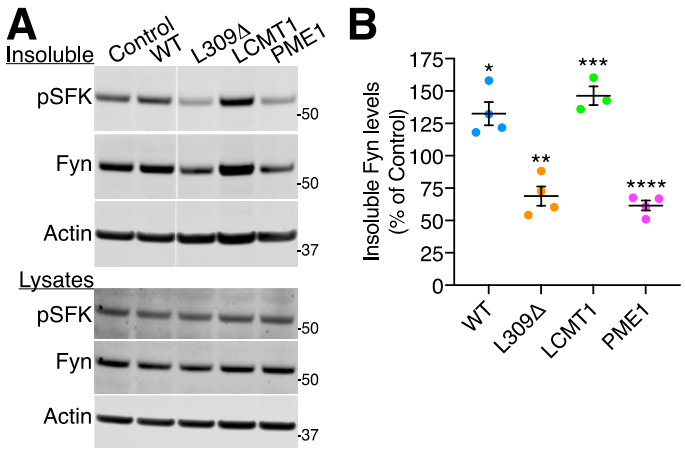

Figure 17. **Changes in PP2A methylation influence the membrane distribution of Fyn in N2a cells.** (**A**) Representative immunoblots of Fyn, pSFK and actin in total lysates and NP-40 detergent-insoluble fractions prepared from N2a cells stably expressing either WT PP2Ac, the L309Δ PP2Ac mutant (methylation incompetent), LCMT1, PME1, or empty vector (Control). Panels in detergent-insoluble fractions originated from the same blot. (**B**) Quantification of Fyn levels in NP-40 detergent-insoluble fractions from these cells. Data (Mean ± SEM from n = 3-4 independent experiments) are expressed as percent of Fyn levels in control cells; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

In contrast, in L309Δ-expressing N2a cells, detergent-insoluble Fyn levels were significantly reduced (**Figure 17**). Similarly, detergent-insoluble Fyn levels were decreased in PME1-expressing N2a cells (**Figure 17**). Statistically significant differences in total Fyn protein expression or activating-phosphorylation between conditions were not observed. Parallel immunoblotting of detergent-insoluble extracts showed that pSFK levels changed proportionally to Fyn levels, indicating that the majority of redistributed Fyn protein was active.

Collectively, our results indicate that intact PP2A methylation is required for the specific compartmentalisation of active Fyn to detergent-insoluble fractions and is a link between one-carbon metabolism and Fyn compartmentalisation.

We next checked whether changes in PP2A methylation driven Fyn compartmentalisation affects the function of Fyn in neurite outgrowth. To that end, N2a cells were co-transfected with GFP-Fyn and either WT PP2Ac, L309Δ, LCMT1, PME1 or EV (controls). A subset of GFP-Fyn-expressing control cells were treated with the PME1 inhibitor, AMZ-30, which enhances PP2A methylation (Bachovchin et al., 2010). Interestingly, WT PP2Ac expression enhanced the outgrowth of elongated and highly branched processes compared to cells expressing GFP-Fyn alone (**Figure 18**), reminiscent to SAM-treated cells (**Figure 15E**). Similarly, AMZ-30 induced highly elongated processes and secondary branching, however, more prominently. Likewise, LCMT1-expressing N2a cells exhibited Fyn-enriched, highly elongated axonal-like processes; however, they were not as abundant per cell. Significantly, the ability of overexpressed

GFP-Fyn to induce neurite outgrowth was hampered in L309Δ-expressing or PME1-expressing N2a cells. In both transfected cells, GFP-Fyn was associated with short filamentous spikes and/or restricted to cytoplasmic and perinuclear vesicular patches rather than the cell periphery.

These results clearly show that PP2Ac methylation functions upstream of Fyn compartmentalisation and participates in Fyn-dependent neuritogenesis.

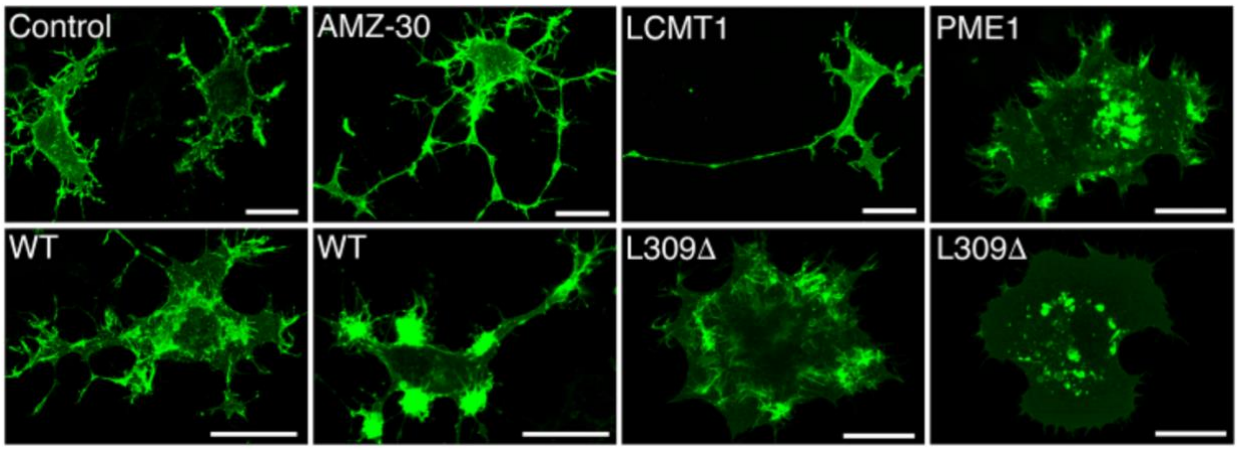

Figure 18. **PP2A methylation state affects Fyn-dependent process initiation and outgrowth.** Representative confocal images of the distribution of GFP-Fyn in Control, WT-, L309Δ-, LCMT1- or PME1-expressing N2a cells co-transfected with GFP-Fyn. Cells were incubated for ~18 h in low-serum medium to initiate differentiation prior to fixation. A subset of control cells was incubated in differentiation medium containing 1 μM AMZ-30, a PME1 inhibitor. Two distinct patterns of GFP-Fyn distribution were observed in WT- and L309Δ-expressing cells, irrespective of transfected Fyn levels. Images shown are representative of three separate experiments. Scale bars, 20 μm.

4.5 PP2A methylation concomitantly regulates F-actin organisation and Fyn compartmentalisation in N2a cells

It is well established that the bulk of Fyn is localised along actin-rich structures at the plasma membrane corresponding to either focal adhesions, filopodia or ruffles, depending on cell stimuli. Small pools of the kinase can sometimes also be detected in perinuclear endosomes, which are involved in Fyn trafficking (Sandilands et al., 2007). Several studies have demonstrated that Fyn interacts with actin-rich structures in both neuronal (Chen et al., 2000; Shima et al., 2001; Porro et al., 2010) and nonneuronal (Bertagnolli et al., 1999; Maekawa et al., 2002; Calaminus et al., 2008) cells or tissue. Significantly, a functional and intact actin cytoskeleton is mandatory for the trafficking of Fyn to the periphery (Sandilands et al., 2007). In the context of PP2Ac methylation, our group has previously reported that LCMT1 overexpression induces a reorganisation of F-actin (filamentous actin) during N2a cell differentiation (Sontag et al.,

2010). The distribution pattern of Fyn in differentiated N2a cells (**Figure 18**) suggests that altering PP2A methylation could influence Fyn distribution at least in part by affecting actin cytoskeletal dynamics. To test this hypothesis, we thus comparatively analysed the organisation of F-actin and GFP-Fyn under conditions of enhanced/reduced PP2A methylation (as described in **Figure 17**). To that end, N2a cells cultured in normal medium (to prevent differentiation) were stained with fluorophore-conjugated phalloidin to visualise F-actin organisation. In agreement with previous studies (Sandilands et al., 2007), the majority of GFP-Fyn was localised at peripheral focal contact sites and along actin-rich filopodia in N2a control cells (**Figure 19A**). A similar distribution of GFP-Fyn was found in WT PP2Ac- and LCMT1-coexpressing N2a cells, which also displayed prominent filopodia, rich in both GFP-Fyn and F-actin, compared to controls. In comparison, F-actin organisation and GFP-Fyn distribution were significantly disorganised in both L309Δ- and PME1-coexpressing N2a cells. The central and diffuse distribution of GFP-Fyn in these cells is similar to that in cells treated with 3-DZA-treated (**Figure 15E**) and in cells with a disrupted actin cytoskeleton (Sandilands et al., 2007). F-actin and GFP-Fyn still co-localised to a significant degree in these cells; however, this was mainly in cytoplasmic patches rather than filopodia.

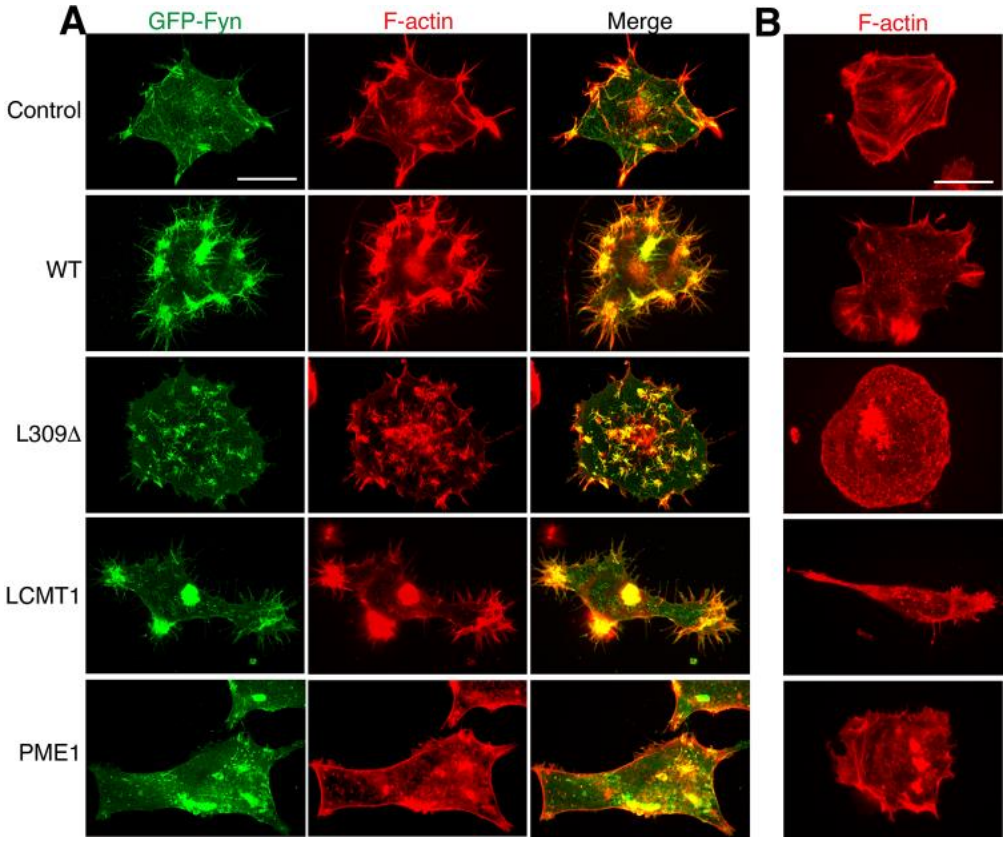

Figure 19. **Altering PP2A methylation induces defects in Fyn localisation in N2a cells.** (**A**) Representative confocal images of the distribution of Fyn and F-actin in control and WT PP2Ac, L309Δ, LCMT1- or PME1-expressing N2a cells co-transfected with GFP-Fyn. (**B**) F-actin distribution in the same cell lines in the absence of GFP-Fyn. Images in (**A**-**B**) are representative of three separate experiments. Scale bars, 20 μm.

Confocal analyses of N2a cells expressing "PP2A regulatory" constructs in the absence of GFP-Fyn (**Figure 19B**), clearly demonstrated that changes in PP2A methylation are sufficient to profoundly affect F-actin organisation. Hence, these results suggest that an intact PP2A methylation is critical for Fyn distribution and Fyn-induced neuritic morphology.

4.6 Elevated levels of homocysteine or homocysteine thiolactone induce a redistribution of Fyn and PP2A in acute mouse cortical slices

To complement our studies in N2a cells, we looked at the effects of acute disturbances to one-carbon metabolism on Fyn distribution in acute mouse brain slices, which maintain many aspects of *in vivo* biology. To that end, NP-40 detergent-insoluble extracts were prepared from acute mouse cortical slices incubated with oxygenated artificial cerebrospinal fluid (ACSF) supplemented with Hcy or its derivative, Hcy thiolactone (HTL). Compared to vehicle-treated cortical slices, treatment with Hcy or HTL induced a ~30% decrease in detergent-insoluble Fyn levels (**Figure 20A-C**).

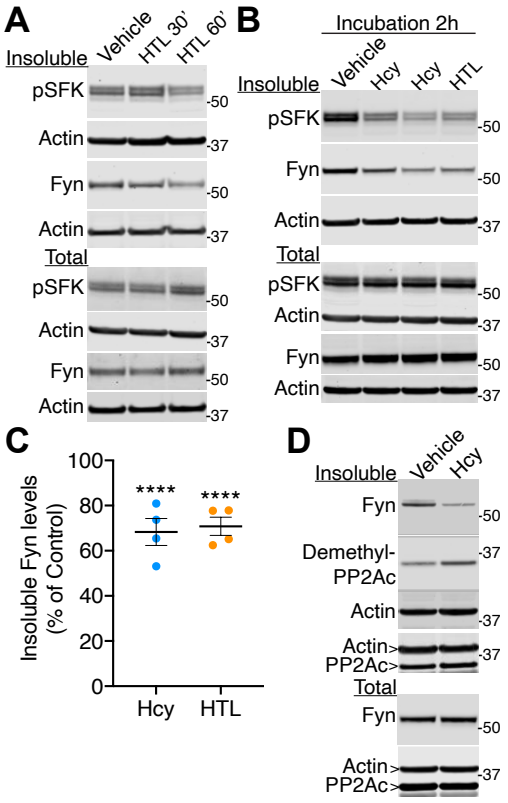

Figure 20. **Elevated levels of homocysteine affect the presence of methylated PP2A and Fyn enzymes in detergent-insoluble fractions prepared from acute mouse brain slices.** (**A**) Representative immunoblots of Fyn and pSFK (SFK activating-phosphorylation) in total extracts (Total) and detergent-insoluble (Insoluble) fractions prepared from acute mouse cortical slices incubated for 30 or 60 minutes

with 200 μM HTL. (**B**) Representative immunoblots of pSFK and Fyn in acute brain slices incubated for 2 h with either 200 μM Hcy or 200 μM HTL. Slices shown were from the same animal. (**C**) Quantification of detergent-insoluble Fyn levels in Hcy- or HTL-treated slices, expressed as percent of vehicle-treated, control slices. Data shown are Mean ± SEM from 4 separate mouse brain tissue; ****p <0.0001, relative to vehicle-treated slices. (**D**) Comparative immunoblot analysis of demethylated PP2Ac, total PP2Ac and Fyn in total extracts and detergent-insoluble fractions prepared from acute mouse cortical slices incubated for 2 h with Hcy. Quantitative analyses of the immunoblots showed a ~58 ± 9% increase (n = 3 separate experiments; p > 0.0001) in demethylated PP2A in detergent-insoluble fractions from Hcy-treated, relative to vehicle-treated slices.

In these detergent-insoluble extracts, the pSFK signal was proportionally reduced, indicating that active Fyn was lost, as seen in N2a cells (**Figures 15 and 17**). There were no statistically significant changes in net Fyn activity in total or detergent-insoluble extracts or total Fyn protein expression levels between treatments. Concomitantly, demethylated PP2Ac levels were increased by ~60% in detergent-insoluble fractions from Hcy-treated slices, relative to vehicle-treated slices (**Figure 20D**). These results are consistent with our findings in N2a cells, and support the hypothesis that elevated Hcy levels can induce PP2A demethylation and concomitant redistribution of active Fyn in the brain.

4.7 Fyn expression and/or activity is altered in brain regions from MTHFR knockout mice

So far, our findings in N2a cells and/or mouse brain slices demonstrated significant effects of *acute* alterations in one-carbon metabolism and PP2A methylation on Fyn distribution and function in neurite outgrowth. However, these studies did not address whether Fyn becomes deregulated following more *chronic* disturbances in one-carbon metabolism. To start addressing this question, we re-analysed regional brain homogenates from our 5-week-old *Mthfr* deficient mice (*Chapter 3*) for potential changes in Fyn expression and phosphorylation. Due to the shortage of hippocampal brain tissue, we could only analyse total extracts prepared from the cortex and cerebellum from young WT, HET ($Mthfr^{+/-}$) and NULL ($Mthfr^{-/-}$) mice. Interestingly, Western blot analyses showed that Fyn protein expression levels and/or net Fyn activity were significantly altered (**Figure 21**). More specifically, Fyn expression levels were significantly downregulated in the cerebellum of HET mice (~35%) and to a greater extent in NULL mice (~70%). Cerebellar Fyn activity in HET mice did not change relative to WT mice. Due to the substantial loss of Fyn (and associated faint signals on Western blots), we were unable to accurately quantify cerebellar Fyn activity in NULL mice. In contrast, analyses of cortical homogenates showed that Fyn protein expression levels were significantly increased in NULL but not HET mice, relative to their WT

counterparts. Moreover, net Fyn activity was enhanced in cortical homogenates from HET and NULL mice by ~30% and ~50%, respectively.

These results indicate that long-term disturbances in one-carbon metabolism can have significant effects on Fyn protein expression levels and activity, which were not readily observed in our models of acute alterations in one-carbon metabolism.

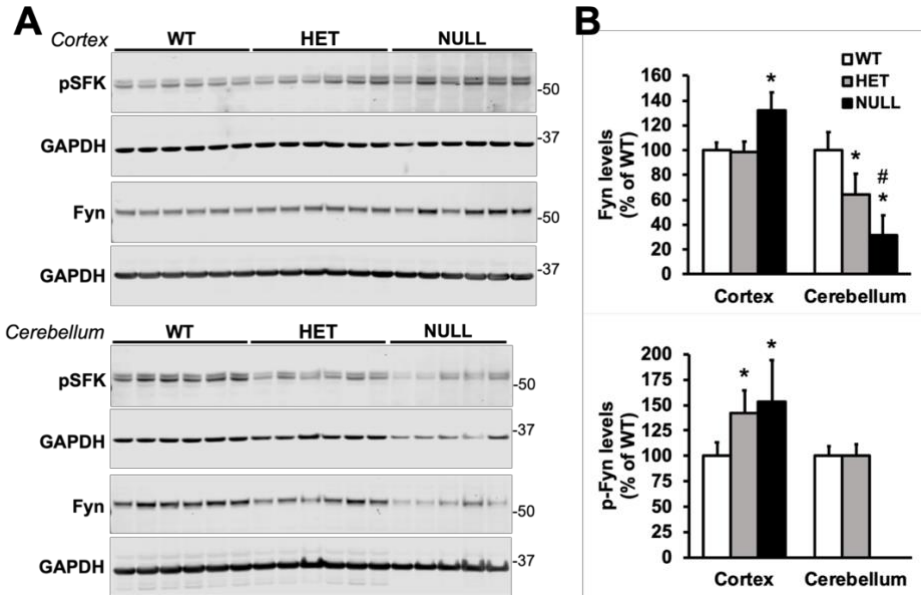

Figure 21. Brain region-specific effects of mild and severe MTHFR deficiencies on Fyn expression and/or activity in young *Mthfr* knockout mice. Cortical and cerebellar homogenates were prepared from 5-week-old WT, HET (*Mthfr*$^{+/-}$), and NULL (*Mthfr*$^{-/-}$) mice. (**A**) Western blots prepared from duplicate aliquots of total protein extracts probed for total Fyn or pSFK. (**B**) Total Fyn and p-Fyn (apportioned to Fyn) expression levels were quantified using densitometry after normalising with GAPDH. Values represent Mean ± SD for $n = 6$ mice/ group; * $p < 0.05$, HET or NULL compared to WT; # $p < 0.05$, NULL compared to HET mice.

4.8 Discussion

Together, studies have shown that the membrane targeting of active Fyn is directed by N-terminal fatty acylation; Fyn trafficking is highly dependent on specific endosomes and the integrity of actin (Sandilands et al., 2007). In neuronal cells, it is also influenced by Tau phosphorylation state (Ittner et al., 2010). Here, we unveil one-carbon metabolism and PP2A methylation as other critical mechanisms involved in regulating Fyn targeting. Our findings (**Publication 5**) support the hypothesis that the influence of one-carbon metabolism on Fyn distribution can be attributed, at least in part, to its effects on PP2A methylation. In contrast to the initial study suggesting that Fyn is methylated on Lys sites, which directs

its plasma membrane targeting in COS-1 cells (Liang et al., 2004), we were unable to detect Lys-methylated Fyn in N2a cells under conditions of diminished cellular methylation potential. Hence, we speculate that the reported failure of Lys mutants of Fyn to associate with the plasma membrane (Liang et al., 2004) could be due to the substitution of these potentially critical Lys sites - which are within the structurally important acylation domain - rather than their methylation *per se*. However, we cannot completely exclude the possibility that Fyn is methylated in a cell-type specific manner or under other experimental conditions.

Interestingly, we found that enhanced PP2A methylation accentuated Fyn-dependent neurite outgrowth and branching, which was otherwise diminished or abolished under conditions of decreased PP2A methylation. These results support earlier reports showing that Fyn compartmentalisation in rafts is critical for its function in neurite outgrowth (Beggs et al., 1994; Brackenbury et al., 2008). Interestingly, the effect of decreased PP2A methylation on Fyn-dependent neurite outgrowth is reminiscent to the loss of Fyn, which impairs neurite formation and the branching number of migrating cortical neurons (Huang et al., 2017). It is likely that the mis-targeting of Fyn can spatially restrict its interaction with key substrates or ability to induce signalling cascades involved in neurite outgrowth. For instance, Fyn is known to activate the plasma membrane-targeted signalling protein Ras, which mediates ERK activation, being essential for neurite outgrowth in N2a cells (Wang et al., 2011). Interestingly, Ras targeting to the plasma membrane is also dependent on its carboxyl methylation (Chiu et al., 2004), thus, Ras may be 'trapped' in the cytoplasm under conditions of altered one-carbon metabolism, aggravating the functional consequences of redistributed Fyn.

Interestingly, we found that Fyn distribution closely mirrored the state of the actin cytoskeleton, which was considerably disorganised under conditions of increased PP2A demethylation. Notably, F-actin plays a key role in neurite initiation, elongation and branching (Luo, 2002), and dendritic protein trafficking and morphology (Konietzny, Bar, & Mikhaylova, 2017). While it has been established that PP2A activity critically regulates the actin cytoskeleton (Reviewed in **Publication 3**), the underlying mechanisms and contribution of specific PP2A isoforms to these effects remain for the most part unknown. Fyn is also known to regulate cytoskeletal dynamics particularly in migrating neurons (Huang et al., 2017). Earlier studies have shown that Fyn is localised along actin-rich peripheral structures associated with adhesion and migration (Calaminus et al., 2008; Sandilands et al., 2007) and interacts with the actin cytoskeleton (Bertagnolli et al., 1999; Maekawa et al., 2002). This association could occur through binding to β-adducin, which co-immunoprecipitates with Fyn from detergent-insoluble extracts prepared from mouse brain (Shima et al., 2001). β-adducin is a highly expressed cytoskeletal junction protein that is regulated by Fyn (Gotoh et al., 2006) and binds/regulates actin-filaments. Furthermore, this protein is a constituent of synaptic structures, such as dendritic spines and growth cones, and is required for synaptic plasticity

(Porro et al., 2010). Critically, actin filaments are essential for the trafficking of Fyn to the plasma membrane; their depolymerisation with cytochalasin D leads to the accumulation of the kinase in the perinuclear region (Sandilands et al., 2007). Interestingly, Fyn distribution in cytochalasin D-treated nonneuronal cells is similar to those observed in N2a cells under our conditions of disturbed one-carbon metabolism and increased PP2A demethylation.

An intact actin cytoskeleton is also critical for the establishment of lipid rafts (Plowman, Muncke, Parton, & Hancock, 2005), where Fyn resides. Cholesterol-dependent protein nanoclustering in lipid rafts fail to form upon disruption of the actin cytoskeleton (Gómez-Llobregat, Buceta, & Reigada, 2013). Lipid raft fractions are enriched with cytoskeletal proteins, suggesting a structural interplay between lipid rafts and the actin cytoskeleton. Hence, it is plausible that lipid raft formation is affected under conditions of altered one-carbon metabolism or decreased PP2A methylation, which we found critically affects F-actin organisation. Notably, actin filaments are disrupted by high levels of Hcy (Pan, Yu, Huang, Zheng, & Xu, 2017); such disruption could have significant biological and clinical implications, in part due to the disruption of signalling platforms offered by lipid rafts.

In contrast to our models of acutely disturbed one-carbon metabolism, Fyn activity and protein expression levels were significantly affected in 5-week-old *Mthfr* knockout mice, which may reflect the long-term consequences of altered cellular methylation potential. Due to the shortage of brain tissue, we were unable to assess levels of detergent-insoluble Fyn, which are likely reduced considering that PP2A demethylation is increased in these mice. Significantly, we observed increased Fyn protein expression levels and activity in the cortex of NULL mice, while only cortical Fyn activity was enhanced in HET mice. This concomitant increase in protein expression and activity could provide an explanation for the increased PHF-1-Tau levels seen in NULL mice only (*Chapter 3*, **Figure 11**). Notably, increased Fyn signalling induces Tau phosphorylation on Tyr18 (Lee et al., 2004; Xia & Gotz, 2014), which could predispose Tau to phosphorylation at the PHF-1 epitope (Hernandez et al., 2009), and affects APP interaction with key adaptor proteins, and thus, its processing (Hoe et al., 2008; Minami et al., 2011). Conversely, we found a substantial loss in cerebellar Fyn protein levels in young HET mice, which was further enhanced in NULL mice. Such severe perturbations in Fyn levels likely contribute to the cerebellar, motor and gait abnormalities in NULL mice (Chen et al., 2001).

Further studies will be required to investigate how exactly chronic and long-term alterations in one-carbon metabolism affect Fyn protein expression levels. One possibility is regulation at the level of gene transcription. For instance, the compounding effects seen in the cerebellum of NULL mice could indicate epigenetic changes, since MTHFR participates in methylation metabolism. One could also speculate that changes in Fyn expression and activity are compensatory responses to the mis-trafficking of the kinase.

For instance, increases to Fyn expression and activity could be intended to recover Fyn signalling that is lost from lipid rafts. It is also possible that cells would compensate for continuously increased Fyn signalling in the wrong compartment by promoting its removal through targeted degradation *via* the ubiquitin system (Rao et al., 2002). Reducing Fyn protein expression would thus prevent aberrant signalling and interaction with non-typical substrates.

What could be the ramifications of mis-localised Fyn in the brain? This would likely entail altered synaptic plasticity, NMADR integrity and glutamate signalling; increased Fyn-Tau interactions promoting Tau hyperphosphorylation and mislocalisation; disrupted APP trafficking and enhanced amyloidogenic processing; and abnormal neuronal migration, oligodendrocyte maturation and differentiation. Notably, Fyn induces cell cycle arrest in neuroblastoma cells (Berwanger et al., 2002). Therefore, it would be worthwhile to see whether Fyn becomes mis-localised in AD neurons, since aberrant APP-induced proliferative signalling is a prominent mechanism leading to AD neurodegeneration (Kirouac et al., 2017).

We also showed for the first time that PP2A/Bα and Fyn enzymes co-immunoprecipitate. Interactions between these enzymes could play a role in the targeting of Fyn to lipid rafts, wherein PP2A/Bα is also found (Sontag et al., 2012). On the other hand, PP2A/Bα−Fyn interactions may only occur in lipid rafts. However, these protein-protein interactions are likely to be indirect since purified forms of these proteins fail to bind *in vitro* (Sontag et al., 2012). In this context, it is possible that PP2A methylation-driven Fyn targeting involves Fyn-Tau interactions since methylated PP2A enzymes also regulate Tau targeting to the plasma membrane (Sontag et al., 2013). Hence, PP2A methylation could be involved in Tau-dependent Fyn targeting (Ittner et al., 2010). Significantly, the PXXP motif in the proline-rich region of Tau is critical for Fyn binding through its SH3 domain and directs postsynaptic density targeting of Fyn (Ittner et al., 2010).

In summary, the findings presented in this chapter provide further evidence for the involvement of folate- and homocysteine-metabolic disturbances in AD. Collectively, our results show that neuronal Fyn is critically regulated by one-carbon metabolism and PP2A methylation state.

Chapter 5. Identification of a Fyn-PP2A signalling module

5.1 Introduction

While our experimental work in Chapter 4 uncovered a critical link between altered PP2Ac methylation and Fyn deregulation, in this chapter, we tested the hypothesis that PP2A could also function downstream as a substrate of Fyn. The possible cross-regulation of these major signalling enzymes is of significance to AD, since both can regulate two central players in AD, Tau and APP. Moreover, PP2A and Fyn are positioned at the crossroads of Ser/Thr and Tyr signalling pathways.

As mentioned in *Chapter 1*, it was initially reported that PP2Ac is phosphorylated on Tyr by receptor and non-receptor protein Tyr kinases. This was shown in murine fibroblasts or T cells transformed with oncogenic and catalytically active Src (Src^{CA}) or Lck (another SFK), and *in vitro* using purified oncogenic Src and epidermal growth factor and insulin receptors. In the initial study, investigators also showed that oncogenic Src catalyses thiophosphorylation of PP2Ac at an unidentified site(s), which leads to reduced PP2A activity. Further analyses suggested that the Tyr residue targeted by oncogenic Src was Tyr307. Indeed, *in vitro* assays indicated that Src can phosphorylate full-length PP2Ac but not trypsin-truncated forms ($PP2Ac_{1-302}$ and $PP2Ac_{1-293}$), which were missing the Tyr307 residue, being the only Tyr residue within the cleaved C-terminus (Chen et al., 1992). Based on these observations, it was concluded that Tyr307 is the sole PP2Ac residue phosphorylated by Src. It is important to note that these experiments were done using purified PP2Ac but not PP2A holoenzymes, the typical enzymes found intracellularly. Moreover, the truncated forms of PP2Ac were also missing Leu309, which is important for regulatory B subunit binding (Sontag & Sontag, 2014); however, the authors did not demonstrate whether their truncated forms were capable of B subunit binding. Furthermore, it is unknown whether the B subunit is critical for Src binding, and thus, Src-mediated phosphorylation of other PP2Ac Tyr sites.

Outside of this initial suggestion that Tyr307 is a Src-mediated phosphorylation site, no other work has been done to confirm Tyr307 phosphorylation by MS, until very recently (Frohner et al., 2020). However, it is worth mentioning that, in these studies, phosphorylation of endogenous PP2Ac at Tyr307 could not be detected by discovery MS. Indeed, pTyr307 was detected at very low abundance, and only when using targeted MS studies of cells overexpressing PP2Ac and active Src. As such, the stoichiometry and

functional significance of PP2Ac phosphorylation at Tyr307 remains to be further validated (Frohner et al., 2020).

Despite the absence of confirmatory MS studies, it was universally accepted in the past two decades that phosphorylation of PP2Ac occurs solely at Tyr307 and triggers its inactivation. Consequently, a series of "anti-pTyr307-PP2Ac" antibodies were developed and extensively used to evaluate PP2A activity in normal cell homeostasis and in disease (Frohner et al., 2020). In a collaborative effort, we demonstrated that those antibodies completely lacked specificity for pTyr307 but were rather sensitive to L309 methylation or Thr304 phosphorylation, or even cross-reacted with unrelated phospho-Tyr residues (Frohner et al., 2020). Moreover, discovery MS studies have identified many potential phosphorylation sites on PP2Ac in a variety of cell models (see for instance "Phosphosite"). Altogether, these observations prompted our group to completely re-examine the regulation of PP2A by phosphorylation.

More specifically, we first tested here the hypothesis that Fyn can phosphorylate PP2Ac on Tyr residue(s) in light of its structural similarity with Src, and the fact that Fyn and methylated PP2A enzymes co-immunoprecipitate (*Chapter 4*; **Figure 16**). Since Fyn is aberrantly activated in AD, enhanced phosphorylation of PP2A could have functional ramifications for PP2A-dependent regulation of APP and Tau. In this chapter, we also investigated in N2a cells whether PP2A Tyr phosphorylation affects Fyn distribution and Fyn-dependent neurite outgrowth (as seen with PP2A methylation in *Chapter 4*).

5.2 Fyn phosphorylates PP2Ac on tyrosine residues in N2a cells

Firstly, we performed experiments to see if Fyn phosphorylates PP2Ac on Tyr. To demonstrate this, we immunoprecipitated recombinant HA-tagged PP2Ac from N2a cells overexpressing both recombinant PP2Ac and constitutively active GFP-tagged Fyn-Y531F (Fyn^{CA}). Immunoprecipitates from total cell extracts were analysed by Western blot using well-characterised and validated commercial anti-phospho-Tyr monoclonal antibodies. Since Src-mediated PP2Ac Tyr phosphorylation has been reported before in fibroblasts (Chen et al., 1992; Frohner et al., 2020), immunoprecipitates from Src^{CA}-expressing N2a cells were also loaded on the gel in parallel to serve as positive controls. We also treated subsets of cells with pervanadate, an effective inhibitor of protein Tyr phosphatases; this inhibitor markedly increases overall cellular protein Tyr phosphorylation, including SFK activity (Secrist, Burns, Karnitz, Koretzky, & Abraham, 1993). It also induces PP2A Tyr phosphorylation in fibroblasts (Frohner et al., 2020). Western blot analyses of HA-immunoprecipitates revealed that Fyn^{CA} or Src^{CA} overexpression induced Tyr phosphorylation of a protein that precisely co-migrates with HA_3-tagged PP2Ac (**Figure 22**).

Furthermore, treatment with pervanadate further augmented Tyr phosphorylation of PP2Ac in Fyn^{CA}- and Src^{CA}-expressing cells.

These findings demonstrated that PP2Ac is a target for Fyn-mediated Tyr phosphorylation.

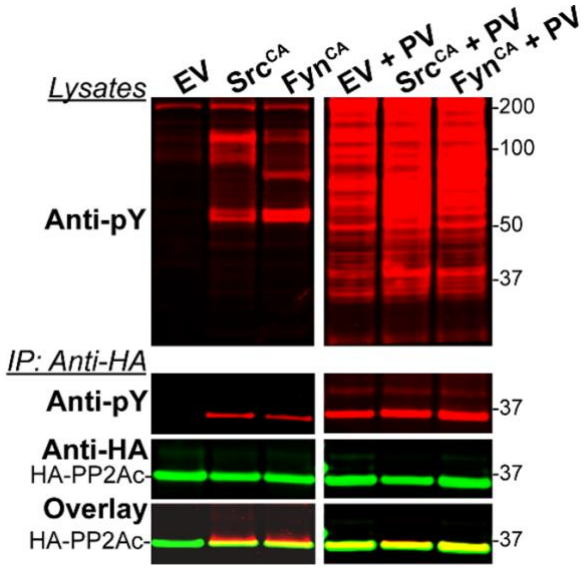

Figure 22. HA_3-tagged PP2Ac undergoes tyrosine phosphorylation in N2a cells expressing either Src^{CA} or Fyn^{CA}, and treated with pervanadate. Total cell lysates and HA-immunoprecipitates were prepared from N2a cells expressing HA_3-tagged PP2Ac, that were co-transfected with either constitutively active GFP-Fyn (Fyn^{CA}), Src (Src^{CA}), or an empty vector (EV). Prior to harvesting, a subset of these cells was treated with pervanadate (+ PV) for 15 min. Anti-phospho-tyrosine (pY) antibodies were used to verify the increase in Tyr phosphorylation of endogenous proteins in response to expression of SFKs. Note the increase in PP2Ac Tyr phosphorylation following the expression of either GFP-Fyn^{CA} or Src^{CA}, or treatment with pervanadate (Overlay). Similar results were observed in four separate experiments.

5.3 Identification of PP2Ac phospho-acceptor sites targeted by Fyn

In a collaborative effort with the research group of Dr. Brian Wadzinski (Vanderbilt University, USA), we next combined MS and biochemical approaches to identify potential PP2Ac phosphosites that are targeted by Fyn (Manuscript in preparation). For proprietary reasons, we are calling those amino acids Y1 and Y2 since those results are not yet published. To confirm the existence of these sites, plasmids encoding HA_3-tagged WT and Tyr→Phe mutants of PP2Ac (single site mutants; Y1F and Y2F, or their combination; F/F) were generated and transiently co-transfected with Fyn^{CA} into N2a cells. Consistent with our hypothesis, Western blot analyses of HA-immunoprecipitates from total cell lysates revealed a significant reduction in Tyr phosphorylation for each single site PP2Ac mutant, which was almost absent

for the PP2Ac F/F double mutant when compared to HA_3-tagged PP2Ac (**Figure 23**). These results clearly indicated that Y1 and Y2 are major phospho-acceptor sites of PP2Ac targeted by Fyn.

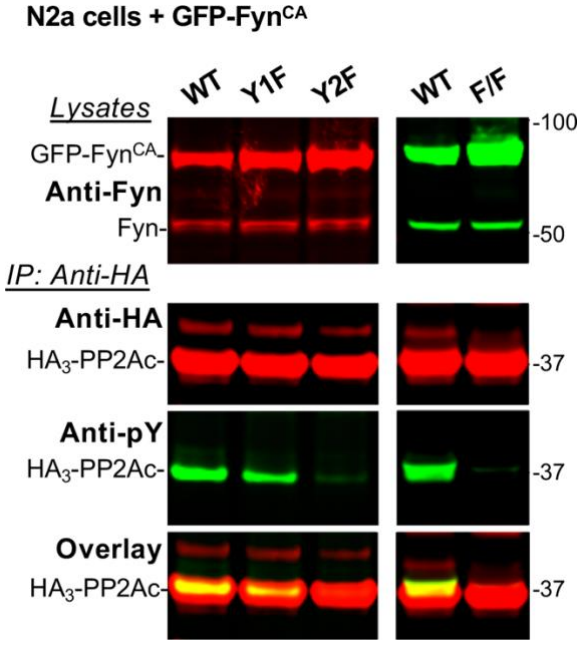

Figure 23. Identification of HA_3-tagged PP2Ac phospho-mutants that can block phosphorylation of PP2Ac by Fyn^{CA}. Total cell lysates and HA-immunoprecipitates were prepared from GFP-tagged Fyn^{CA}-expressing N2a cells that were transfected with HA_3-tagged PP2Ac wild-type (WT) and one of three Tyr→Phe mutants: Y1F, Y2F, and the double mutant F/F. Representative Western blots with the indicated antibodies are shown. The expression of GFP-Fyn^{CA} was validated using an anti-Fyn antibody. Note the decreased or near absence of PP2Ac Tyr phosphorylation following the expression of the phospho-Tyr mutants (Overlay). Similar results were observed in four separate experiments.

5.4 PP2Ac F/F double mutant impedes Fyn^{CA}-mediated Tau phosphorylation at AD-like sites and ERK activation

Since it was reported that PP2Ac Tyr phosphorylation reduces its catalytic activity (Chen et al., 1992), we wanted to see if PP2Ac Tyr-phosphorylation by Fyn was changing/inhibiting its capacity to dephosphorylate Tau. Specifically, we looked at Tau phosphorylation changes at the PHF-1 and CP13 phospho-epitopes, which can be dephosphorylated by PP2A *in vitro* and *in vivo*, respectively (*Chapter 1;* **Figure 4**). It is well known that under basal conditions, Tau is mostly in a dephosphorylated state. Accordingly, there were very low, if not background levels of immunoreactivity for PHF-1 or CP13 in EV-transfected control N2a cells. Tau was also similarly dephosphorylated at these epitopes in control N2a cells expressing HA_3-tagged WT PP2Ac (**Figure 24A**).

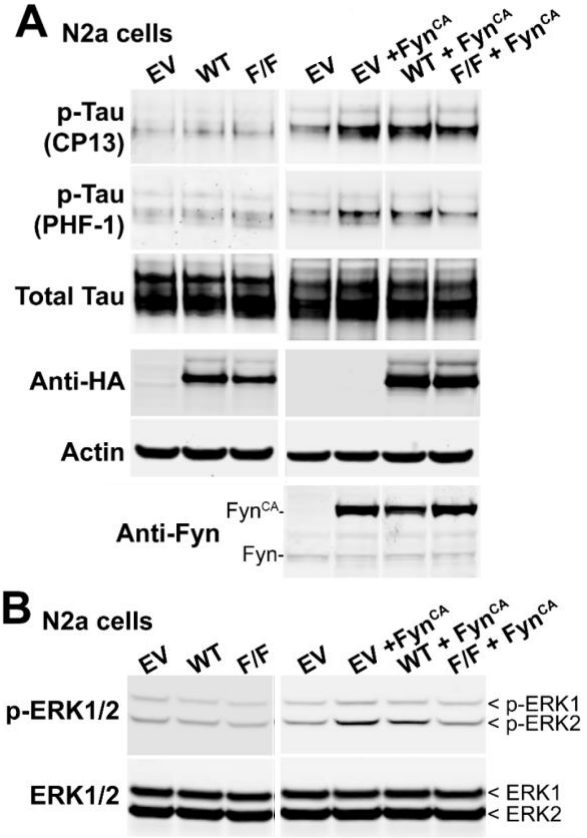

Figure 24. The PP2Ac F/F mutant inhibits Fyn^{CA}-mediated Tau phosphorylation and ERK activation. Total cell homogenates were prepared from control or Fyn^{CA}- expressing N2a cells that were transfected with either an empty vector (EV), PP2Ac wild-type (WT) or the PP2Ac phospho-mutant F/F. (**A**) Tau-purified fractions prepared from total cell lysates were analysed by Western blot for Tau phosphorylation at the CP13 and PHF-1 phospho-epitopes. (**B**) Aliquots of the same total cell lysates were analysed in parallel by Western blot for active ERK (p-ERK1/2) using validated antibodies against the total phosphorylated forms of ERK1 (or p42-MAPK) and ERK2 (or p44-MAPK), and total ERK1/2. Representative Western blots are shown. Similar results were observed in four separate experiments.

It is important to note that cellular PP2Ac expression levels are very tightly autoregulated; consequently, ectopic HA-tagged PP2Ac cannot be significantly "overexpressed" over basal endogenous levels as normally found with other transfected proteins. Instead, a portion of endogenous pools of PP2Ac is replaced by the recombinant species (Sontag et al., 2007). A relatively small increase in total cellular PP2Ac, together with the absence of notable phosphorylation of Tau at the CP13 and PHF-1 AD-like epitopes under basal conditions, likely explain why it is not possible to detect further dephosphorylation of Tau in HA_3-tagged PP2Ac-expressing cells relative to EV-transfected N2a cells.

Tau was dephosphorylated to a similar extent in N2a cells expressing PP2Ac F/F (**Figure 24A**), indicating that the mutant is catalytically active toward phosphorylated Tau. Expression of Fyn^{CA} induced a ~1.5- to 2-fold increase in endogenous Tau phosphorylation at both the CP13 and PHF-1 epitopes. Interestingly, while a similar enhancement of Tau phosphorylation was observed in cells co-expressing Fyn^{CA} and HA_3-

tagged PP2Ac, co-expression of Fyn^{CA} and PP2Ac F/F inhibited this stimulatory effect of Fyn^{CA} on Tau phosphorylation at both epitopes (**Figure 24A**).

We next analysed the activity of ERKs, since both PP2A (Sontag et al., 1993) and Fyn (Lovatt et al., 2006) exert a regulatory control on ERK signalling, respectively. It is widely known that ERK1 and ERK2 enzymes are activated by stimulus-dependent phosphorylation at Thr202/Tyr204 and Thr185/Tyr187, respectively. In agreement with the ability of PP2A to dephosphorylate ERKs (Sontag et al., 1993), our analyses showed that ERK1/2 activity remained unchanged in N2a cells expressing either HA_3-tagged PP2Ac or PP2A F/F relative to unstimulated cells transfected with EV (**Figure 24B**). As observed with p-Tau, these data suggested that both PP2Ac species were catalytically active toward ERK1/2. As expected from earlier studies (Li & Gotz, 2017), ERK1/2 phosphorylation increased upon Fyn^{CA} expression. Similar results were obtained when cells were transfected with Fyn^{CA} in combination with HA_3-tagged PP2Ac. However, ERK1/2 activity decreased by ~2-fold in Fyn^{CA}-expressing N2a cells co-transfected with PP2A F/F, relative to cells co-transfected with EV or HA_3-tagged PP2Ac.

Collectively, these findings suggest that PP2A-mediated Tau dephosphorylation is hampered by Fyn activity, leading to increased Tau phosphorylation and ERK1/2 activating-phosphorylation. In contrast, the double F/F PP2Ac phospho-mutant is unaffected by Fyn^{CA}-induced inhibition and still able to trigger Tau dephosphorylation and ERK inactivating-dephosphorylation.

5.5 Confirmation of Fyn-mediated PP2Ac phosphorylation at "Y2" site, using a validated antibody

While Fyn phosphorylates PP2Ac at both Y1 and Y2 residues, mutant analyses indicated that Y2 may be a more sensitive/preferred site targeted by Fyn (**Figure 23**). To further confirm that this amino acid is indeed a Fyn phosphosite, we tested a novel affinity-purified antibody directed against Y2-phosphorylated PP2Ac, that was developed by Dr. Wadzinski. The specificity of this antibody was validated by Dr. Wadzinski using a combination of dot blot and peptide adsorption assays. We repeated some of the experimental conditions described in **Figure 23** and looked at specificity of recognition of the previously observed phosphosites with the anti-phospho-Y2-PP2Ac antibody. Briefly, HA-immunoprecipitates were prepared from N2a cells co-expressing HA-tagged PP2Ac and either Fyn^{CA} or EV, and analysed by Western blot. Results showed that, indeed, Fyn^{CA} mediated phosphorylation of HA_3-tagged PP2Ac at the Y2 site (**Figure 25**).

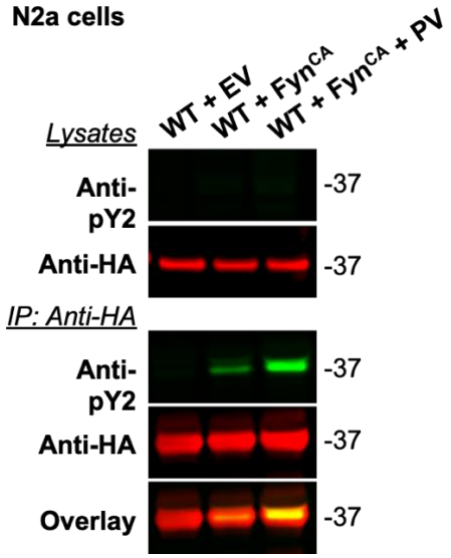

Figure 25. Fyn^{CA} and pervanadate mediate PP2Ac phosphorylation at the Y2 site. Total cell lysates and HA-immunoprecipitates were prepared from N2a cells that were co-transfected with HA_3-tagged PP2Ac wild-type (WT) and either GFP-Fyn^{CA} or an empty vector (EV). A subset of cells expressing WT and Fyn^{CA} were treated with pervanadate (+ PV). Representative Western blots with the indicated antibodies are shown. Note the presence of PP2Ac Y2-phosphorylation following the expression of GFP-Fyn^{CA} and increased signal following treatment with pervanadate (Overlay). Representative results from at least three separate experiments are shown.

As observed with anti-phospho-Tyr antibodies, PP2Ac Y2-phosphorylation was significantly increased by additional treatment of cells with pervanadate (**Figure 25**). Moreover, since Tyr phosphorylation of PP2Ac is not readily detected in total cell lysates, this suggests that only a small pool of PP2Ac undergoes this modification, which becomes enriched upon HA-immunoprecipitation.

5.6 Fyn^{CA} and Y2-phosphorylated PP2Ac co-localise

Next, we used the anti-phospho-Y2-PP2Ac antibody to visualise the distribution of Y2-phosphorylated PP2Ac. We wanted to get confirmation that only small, spatially restricted pools of PP2A are phosphorylated in the steady-state, as suggested by our inability to visualise Tyr phosphorylated PP2Ac in total cell extracts (**Figure 25**). Based on our findings that Fyn and methylated PP2A enzymes co-immunoprecipitate (*Chapter 4*; **Figure 16**), we also expected some co-localisation between the enzyme and its substrate; however, it was also possible that phosphorylation may detach Y2-phosphorylated PP2Ac from the Fyn protein complex. To study the distribution of pY2-PP2Ac, we used SYF cells, which are mouse embryo fibroblasts (MEFs) deficient in the SFKs, Src, Yes and Fyn. Cells transfected with EV (Controls) or GFP- Fyn^{CA} were comparatively analysed by confocal microscopy for immunoreactivity with the anti-phospho-Y2-PP2Ac antibody. Using this cell model allowed us to have a "clean slate" to

specifically study Fyn-dependent PP2Ac phosphorylation without having to worry about potential interference from endogenous Src-mediated PP2Ac phosphorylation. Invariably, relative to controls, SYF cells expressing GFP-Fyn^{CA} exhibited an overall migratory phenotype consistent with the known roles of Fyn in differentiation and migration. We found that expressed GFP-Fyn^{CA} significantly co-localised with Y2-phosphorylated endogenous PP2Ac in specific restricted areas of SYF cells (**Figure 26**).

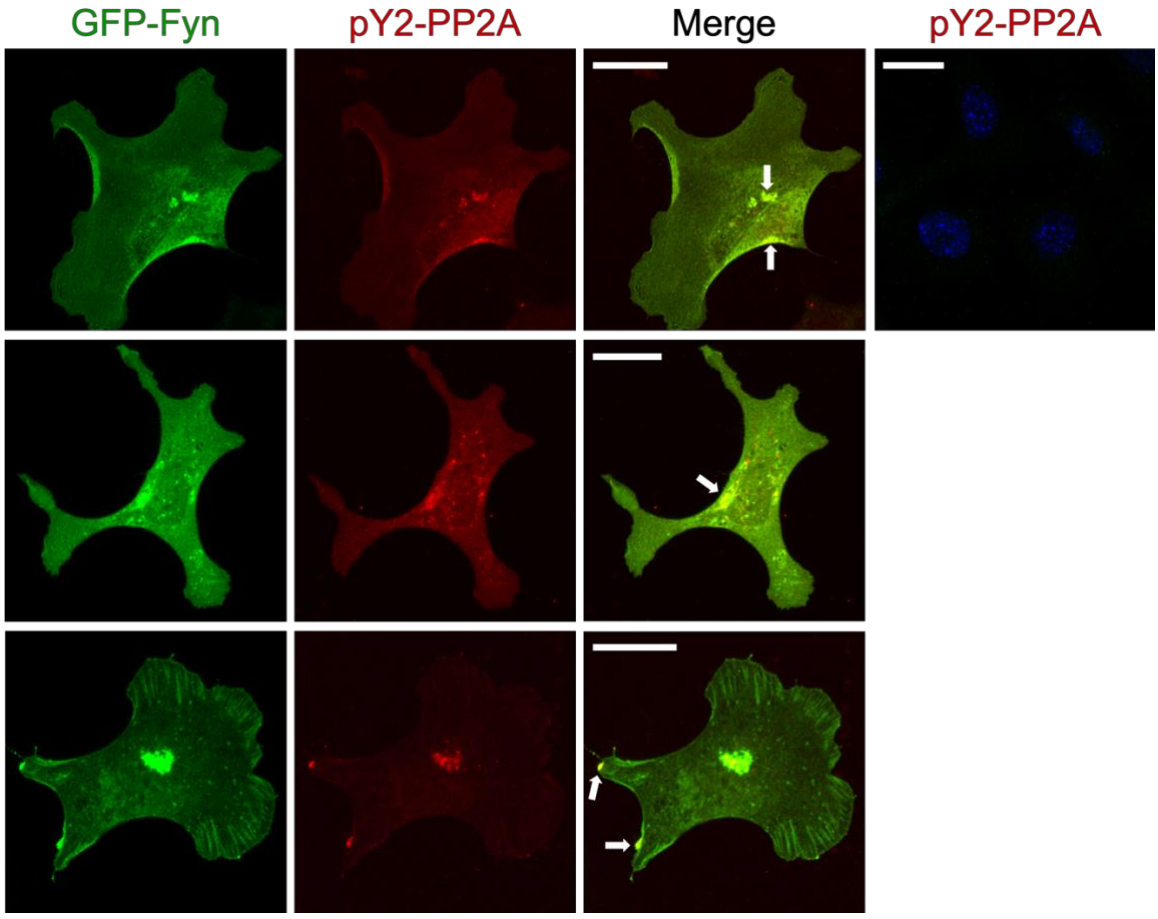

Figure 26. Y2 phosphorylated-PP2Ac co-localises with Fyn^{CA} in SYF cells. SYF cells were transfected with GFP-Fyn^{CA} or EV (controls; top right panel). Cells were fixed, permeabilised and incubated sequentially with the anti-phospho-Y2-PP2Ac antibody (pY2-PP2A), goat anti-alpaca and donkey anti-goat AlexaFluorPlus (594) antibodies. Arrows show examples of co-localisation between endogenous Y2-PP2Ac and GFP-Fyn. Fluorescence in either the red or green channel was not observed in EV-transfected SYF cells (top right panel) stained with the nuclear stain, DAPI. Representative images from three separate experiments are shown. Scale bars = 20 µm.

This co-localisation was predominantly along the peripheral membrane, and in cytoplasmic endosome-like structures (which are known to contain Fyn). In agreement with our biochemical findings in N2a cells (**Figure 25**), there was no phospho-Y2-PP2Ac signal in control SYF cells. Moreover, our confocal analyses in SYF cells support the observations in N2a cells (**Figure 25**) that only small pools of endogenous PP2A are phosphorylated at the Y2 site at steady-state.

5.7 Fyn distribution and function in neurite outgrowth is severely hindered by PP2Ac F/F

Both Fyn (Beggs et al., 1994; Brackenbury et al., 2008) and ERK activity within cells (Wang et al., 2011) are essential for neurite outgrowth in N2a cells. Since Fyn-mediated ERK1/2 activating-phosphorylation is significantly impaired by PP2Ac F/F expression (**Figure 24**), we next examined the effects of this mutant on Fyn-dependent neuritogenesis. N2a cells were allowed to differentiate using the same conditions as described previously (*Chapter 4*; **Figures 15 and 18**). As expected, N2a cells co-expressing GFP-Fyn^{CA} and HA$_3$-tagged PP2Ac exhibited prominently extended neurites compared to N2a cells expressing GFP-Fyn^{CA} alone (**Figure 27**).

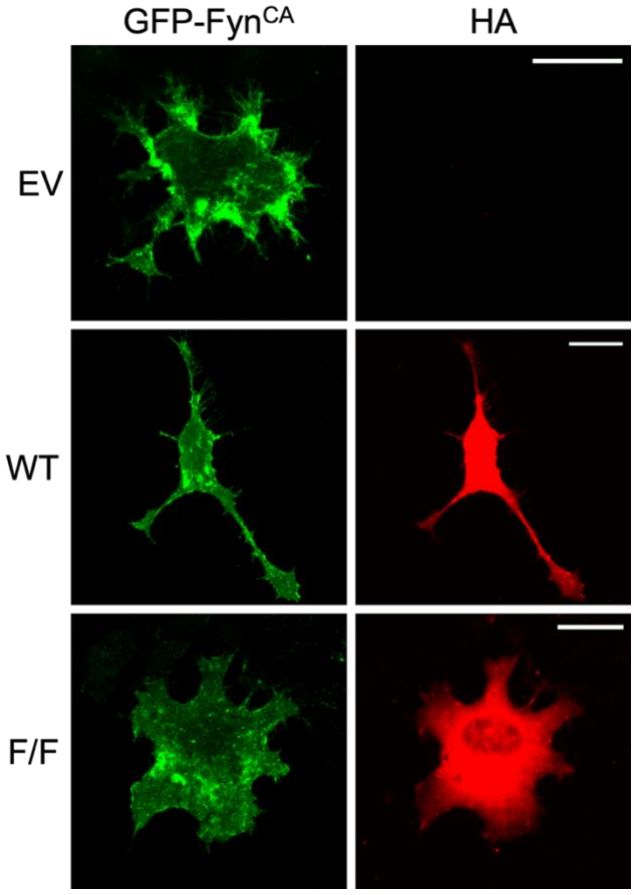

Figure 27. PP2Ac F/F affects Fyn compartmentalisation and function in neurite outgrowth. N2a cells were co-transfected with GFP-Fyn^{CA} and either empty vector (EV; control), HA-tagged PP2Ac wild-type (WT) or HA-tagged F/F. Representative images are shown. Scale bars = 20 µm.

In contrast, N2a cells co-expressing PP2Ac F/F and GFP-Fyn^{CA} spread but failed to extend neuritic-like processes; this inhibitory effect was strikingly similar to what we observed when PP2Ac methylation was downregulated (*Chapter 4;* **Figure 18**). Moreover, in F/F expressing cells, GFP-Fyn^{CA} distribution was

more central, diffuse and in cytoplasmic patches, reminiscent to the pattern observed in L309Δ- and PME1-expressing cells (*Chapter 4;* **Figure 18**). Inhibition of neuritogenesis in response to PP2Ac F/F expression could potentially result from such deficits in Fyn^{CA} localisation and/or downstream effects on ERK activity.

5.8 Discussion

Fyn and PP2A play pivotal roles in signal transduction, and therefore, contribute significantly to neuronal homeostasis. Hence, understanding their regulation should prompt the development of therapies for use in AD, wherein these vital enzymes are dramatically deregulated. For the first time, our findings demonstrate a reciprocal functional regulation between PP2A and Fyn, which could have significant implications for APP processing and Tau phosphorylation in AD. Through various means, we have identified Fyn as a mediator of PP2Ac phosphorylation on certain Tyr residues.

While MS studies identified endogenous PP2Ac phosphorylated at Y1 and Y2 sites, we were only able to detect by Western blotting Tyr phosphorylation of PP2A in immunoprecipitates, but not in total lysates from Fyn^{CA}-overexpressing cells. There are several possible explanations for this observation: 1) Phosphorylation may only occur in a spatiotemporally restricted manner in specific small areas of the cells, as shown by our confocal analyses. 2) The phosphorylation of PP2Ac is extremely labile, due to autodephosphorylation (Chen et al., 1992); by overexpressing the active kinase or treating with pervanadate, we may tilt the balance towards phosphorylation; and 3) Only very specific sub-pools of PP2A and/or less abundant PP2A isoforms may undergo phosphorylation, so that these phospho-species get diluted in total cell lysates.

The overexpression of Fyn^{CA} mimicked pathological conditions such as AD and cancer, where there is aberrant activity or expression of active Fyn. Yet, it remains to be determined whether Fyn-mediated Tyr phosphorylation of PP2Ac also occurs under physiological conditions known to modulate Fyn activity, such as neural differentiation (Hanafusa & Hayashi, 2019), myelination (Kramer et al., 1999), cell spreading (Shima et al., 2003) and stimulation of glutamate receptors (Um et al., 2013).

While expression of the PP2Ac F/F mutant inhibited Fyn-mediated ERK1/2 activation and Tau phosphorylation, future studies will be required to establish whether Tyr phosphorylation directly affects PP2A catalytic activity, substrate binding, or the assembly of substrate-specific PP2A enzymes. Indeed, we were unable to quantitatively measure the activity of Tyr-phosphorylated PP2A in immunoprecipitates prepared from our cells since PP2A is rapidly autodephosphorylated in the absence of its potent inhibitor,

OA (Chen et al., 1992). Additional studies will need to be performed to understand how exactly each of our identified Y1 and Y2 phosphosites regulate PP2A function.

It is particularly noteworthy that the PP2Ac F/F mutant inhibited Fyn-dependent Tau phosphorylation at both PHF-1 and CP13 epitopes. Hence, Fyn-induced PP2Ac phosphorylation could be a mechanism of Fyn-mediated Tau phosphorylation at Ser/Thr sites (Lee et al., 2004; Xia & Gotz, 2014). Besides affecting PP2A-dependent Tau regulation, Fyn-mediated PP2Ac phosphorylation has also the potential to influence PP2A-dependent signalling. It would also be worthwhile to investigate whether the PP2Ac F/F mutant alleviates mouse Tau pathology in the context of Fyn hyperactivity.

In *Chapter 4*, we observed significant co-immunoprecipitation of GFP-Fyn^{WT} and FLAG-Bα-containing PP2A complexes (**Figure 16**). We concluded that this is likely an indirect interaction based on previous experiments, which failed to detect a direct association between purified Fyn and bovine brain PP2A/Bα (Sontag et al., 2012). However, it cannot be ruled out that Fyn directly interacts with Tyr-phosphorylated PP2Ac through its phospho-Tyr binding SH2 domain. In fact, our confocal microscopic analyses revealed significant co-localisation between Fyn^{CA} and Y2-phosphorylated PP2Ac in SYF cells.

Interestingly, overexpression of the PP2Ac F/F mutant significantly impaired Fyn-dependent neurite initiation in N2a cells. In part, this hindrance could be attributed to the negative effect of PP2Ac F/F on ERK activation. Notably, ERK activity mediates neurite outgrowth in N2a cells (Wang et al., 2011). PP2A F/F-mediated alterations in the distribution of Fyn could hamper its interaction with substrates, such as the ERK signalling transducer Ras (Abe & Berk, 1999), with downstream consequences for ERK-dependent neurite initiation and outgrowth. It is also possible that the PP2Ac F/F mutant impacts the actin cytoskeleton, which is normally regulated by PP2A (Reviewed in **Publication 3**). Alterations to the actin cytoskeleton could certainly alter Fyn compartmentalisation as found previously (*Chapter 4*). Interestingly, the PP2Ac F/F-expressing N2a cells have a comparable phenotype and distribution of Fyn as N2a cells which have impaired PP2Ac methylation (*Chapter 4*). Lastly the F/F mutant could also induce detrimental effects on the microtubule cytoskeleton, which is required for proper neurite outgrowth. Indeed, impaired PP2A methylation affects microtubule stability (**Publication 3**).

In conclusion, our findings clearly demonstrate that Fyn and PP2A regulate one another through mechanisms that require further exploration. We show for the first time that PP2A is regulated by Fyn through phosphorylation of certain Tyr residues. Tyr phosphorylation is known to typically affect formation of signalling scaffolds *via* the complex regulation of protein-protein interactions. Active Fyn enhances Tau phosphorylation at Tyr; in addition, our data suggest that Fyn-mediated phosphorylation of PP2A can also promote Tau phosphorylation at AD-like Ser/Thr phospho-epitopes. Significantly, this

mechanism could occur in AD where Fyn is hyperactive, as well as under conditions of altered one-carbon metabolism, which affects Fyn activity (*Chapter 4*). Furthermore, our data suggest that Tyr-phosphorylated PP2A plays an important regulatory role in Fyn distribution and Fyn-dependent neuritogenesis and ERK activation.

Chapter 6. Can we revert AD-like pathology with a diet enriched in methyl group donors?

The following project is a collaboration with the research group of Professor Lars Ittner at Macquarie University (Sydney, Australia). The dietary intervention was designed by A/Prof. Estelle Sontag and Dr. Jean-Marie Sontag at the University of Newcastle. For reasons of intellectual property, the compounds of interest within the test diet (including metabolite analyses) will not be disclosed. I participated in the animal behavioural tests conducted by Dr. van Hummel and analysed all regional brain homogenates.

6.1 Introduction

In this chapter, we investigated whether a diet high in particular methyl group donors aimed at boosting one-carbon metabolism in the brain, can improve behavioural abnormalities and reduce Tau phosphorylation in TAU58/2 mice, a model of severe Tauopathy (van Eersel et al., 2015; Przybyla et al., 2016). TAU58/2 mice overexpress the human 0N4R Tau isoform with the P301S mutation under the control of the mouse Thy1.2 promoter. This mutation predisposes Tau to increased phosphorylation, and in humans, causes familial FTLD-Tau. Tau-P301S carriers clinically present with dementia, motor abnormalities, and inappropriate behaviour (van Eersel et al., 2015). TAU58/2 mice have been well characterised and rapidly develop p-Tau pathology and behavioural impairments (van Eersel et al., 2015; Przybyla et al., 2016). In addition, these mice recapitulate various aspects of human AD pathology, including frequent neurofilament-positive axonal swellings, progressive NFT formation (present at 3 months of age), and activated microglia in close proximity to Tau-P301S-expressing neurons (van Eersel et al., 2015).

Various studies, in both animals and humans, have demonstrated therapeutic effects of methyl group donors in the brain. For instance, treatment with SAM has been shown to protect against nerve injury-induced cognitive impairment in mice (Gregoire et al., 2017). Moreover, maternal supplementation with choline in a mouse model of Down Syndrome, an AD risk factor, significantly improved spatial cognition and adult hippocampal neurogenesis, and protected basal forebrain cholinergic neurons, which are typically lost early in AD (Strupp et al., 2016). It has been demonstrated that methyl group donors can influence the expression of genes associated with synaptic plasticity (Strupp et al., 2016), memory and cognitive function, and histone modification (Strupp et al., 2016; Bekdash, 2019). In normal elderly

individuals, folic acid supplementation was found to slow the rate of decline in information processing, and improve memory and global cognitive functions (Smith et al., 2018). Moreover, folic acid, in combination with vitamins B6 and B12 (co-factors in one-carbon metabolism; **Figure 8**), has been reported to slow whole brain atrophy and improve cognition in MCI patients. Interestingly, beneficial therapeutic effects of folic acid supplementation on cognition have also been seen in a cohort of probable AD from mild to moderate severity (Chen et al., 2016). Yet, further investigation into the therapeutic potential of methyl group donors in treating or preventing AD is warranted, including the delineation of molecular mechanisms, to cement the involvement of altered one-carbon metabolism in the disease process and/or progression.

Based on our data emphasising the role of one-carbon metabolism in Fyn regulation, we hypothesised that an early methyl group donor-enriched dietary intervention could help prevent the accumulation of hyperphosphorylated Tau species, and as a result, normalise behavioural and motor deficits in AD-like mice. In this study, we used female TAU58/2 mice (**Figure 28**). Mice were fed a control amino acid-defined diet (CD) or the same diet enriched with methyl group donors (MD).

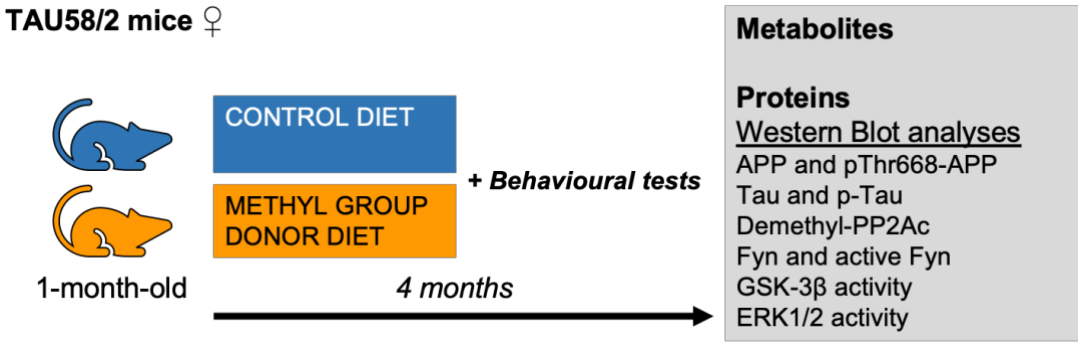

Figure 28. Methyl group donor supplement intervention in TAU58/2 mice. At 1 month of age, female TAU58/2 mice (*n* = 12 mice/ group) were fed a control diet or a methyl group donor-enriched diet for 4 months. At 5 months of age, all mice underwent the following behavioural tests: Morris water maze (spatial memory performance), Pole test (grip strength and coordination), and Challenge beam (sensorimotor skills). Brains were then regionally dissected, and homogenates were prepared for protein analyses *via* Western blot (*n* = 5-6 mice/ group). Blood plasma was collected for metabolite analyses (Creative Proteomics, USA).

6.2 Our methyl group donor-enriched diet reverses motor deficits and improves spatial memory in TAU58/2 mice.

TAU58/2 mice progressively develop motor deficits, which first appear at 2 months of age (van Eersel et al., 2015). In addition, they display neuropsychiatric symptoms found in AD, such as hyperactivity and

disinhibition-like behaviour (Przybyla et al., 2016). Recently, learning and memory impairments were also found in TAU58/2 mice (Przybyla el al., 2020). Several neurodegenerative diseases are characterised by both cognitive and motor deficits and are accompanied by the accumulation of p-Tau. In AD, motor symptoms are commonly observed and progress rapidly (Scarmeas et al., 2004). Therefore, we comparatively assessed behavioural and motor skills in 5-month-old TAU58/2 mice fed either the CD or MD.

Aliquots of plasma from CD- and MD-fed TAU58/2 mice were first analysed by specialised MS for a panel of one-carbon metabolites (Creative Proteomics, USA), which confirmed that the custom MD induced the intended effects. Importantly, prior to behavioural testing, we also verified that there were no differences in body weight among the CD and MD mouse groups (**Figure 29A**). This showed that there were no overall detrimental effects of the diet on mouse growth, as well as ensured that body size would not interfere with performance during behavioural testing.

Next, we assessed behavioural and motor skills in 5-month-old TAU58/2 mice fed the CD or MD. Firstly, we assessed sensorimotor skills, using the challenge beam test. In a previous study, longer crossing times were found in 6-month-old TAU58/2 mice relative to WT mice (van Eersel et al., 2015). Significantly, we observed shorter crossing times in MD-fed TAU58/2 mice; they were reduced by ~3-fold relative to CD-fed TAU58/2 mice (**Figure 29B**). We also noticed that MD-fed TAU58/2 mice were more likely to stay upright on the beam, whereas CD-fed TAU58/2 mice tended to go underneath during their crossing. Collectively, these data indicate improved motor coordination in the MD-treated group.

We further evaluated motor performance using the pole test, which is a more complex task that assesses grip strength and spatial coordination skills. Confirming what we observed with the beam test, MD-fed TAU58/2 mice also took significantly less time to manoeuvre into a downward position and descend the vertical pole (by ~3.7-fold for both parameters) relative to CD-fed TAU58/2 mice (**Figure 29C**). Typically, we observed that CD-fed TAU58/2 mice failed to grasp the pole and slid to the bottom as soon as they were released, which has been reported previously in these mice (van Eersel et al., 2015).

Finally, we determined whether spatial learning and memory formation was improved in MD-fed TAU58/2 mice. To that end, the standard Morris Water Maze (MWM) paradigm was used. Both groups had a comparable progressive reduction in the latency to escape onto the submerged and obscured platform in the presence of proximal visual cues over five consecutive days, suggesting similar memory formation ability (**Figure 29D**). However, on the fifth day, MD-fed TAU58/2 mice found the escape platform relatively quicker, indicating enhanced spatial memory ability. On the final day ("Flag"), both groups of mice found the escape platform rapidly when equipped with a flag and in the absence of

proximal visual cues as used during the acquisition phase, suggesting normal vision, swimming abilities and cue-associative learning.

Collectively, these results establish our MD intervention as a potential treatment to alleviate p-Tau-associated behavioural and motor impairments.

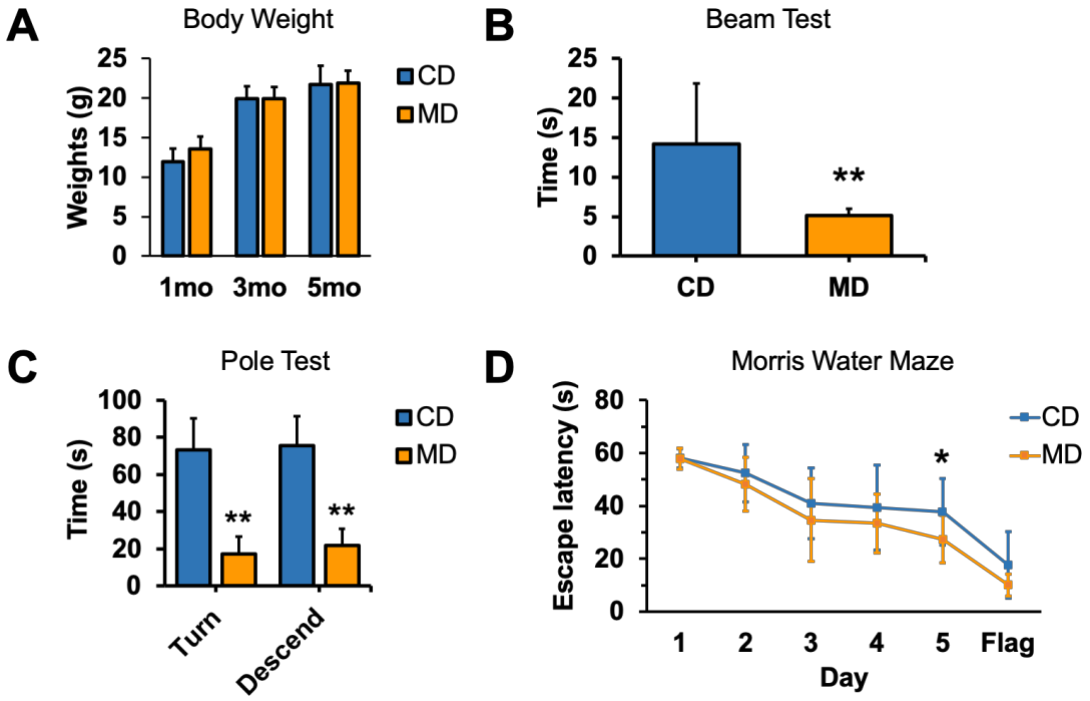

Figure 29. Our methyl group donor-enriched diet reverses motor deficits and improves spatial learning in TAU58/2 mice. One-month-old female TAU58/2 mice were randomised to receive either a control amino acid defined diet (CD) or the same diet enriched in methyl group donors (MD) for 4 months before being sacrificed. (**A**) Body weights of mice from 1- to 5-months (mo) of age. (**B**) Quantification of time required to cross a suspended beam in challenge beam test. (**C**) Quantification of time required to manoeuvre into a downward position once placed at the apex of a vertical pole facing upwards ("Turn") and descend the vertical pole ("Descend") in pole test. (**D**) Quantification of time required to find the submerged platform on the fifth day of the acquisition phase and on the following day ("Flag") where the submerged platform was flagged, and proximal visual cues were removed in Morris Water Maze test. Values represent Mean ± SD for n = 11-12 mice per group; MD *vs* CD: *$p < 0.05$; **$p < 0.01$.

6.3 Our methyl group donor-enriched diet reduces PP2A demethylation in TAU58/2 mice.

We next investigated what could be potential mechanisms mediating the observed improvements to behaviour in MD-fed TAU58/2 mice. While PP2A methylation is impaired in models of altered one-carbon metabolism, boosting the SAM/SAH ratio *via* manipulation of one-carbon metabolism enhances

LCMT1-mediated PP2A methylation in N2a cells (Sontag et al., 2007) (See also *Chapter 3*). Thus, we first assessed whether PP2Ac methylation was increased in MD-fed, relative to CD-fed TAU58/2 mice. Indeed, demethylated PP2Ac levels were significantly and similarly reduced (by ~30%) in homogenates prepared from the cortex, hippocampus or cerebellum of MD-fed mice relative to CD-fed TAU58/2 mice (**Figure 30**). These findings confirmed that the MD enhanced basal brain methylation potential in the brain.

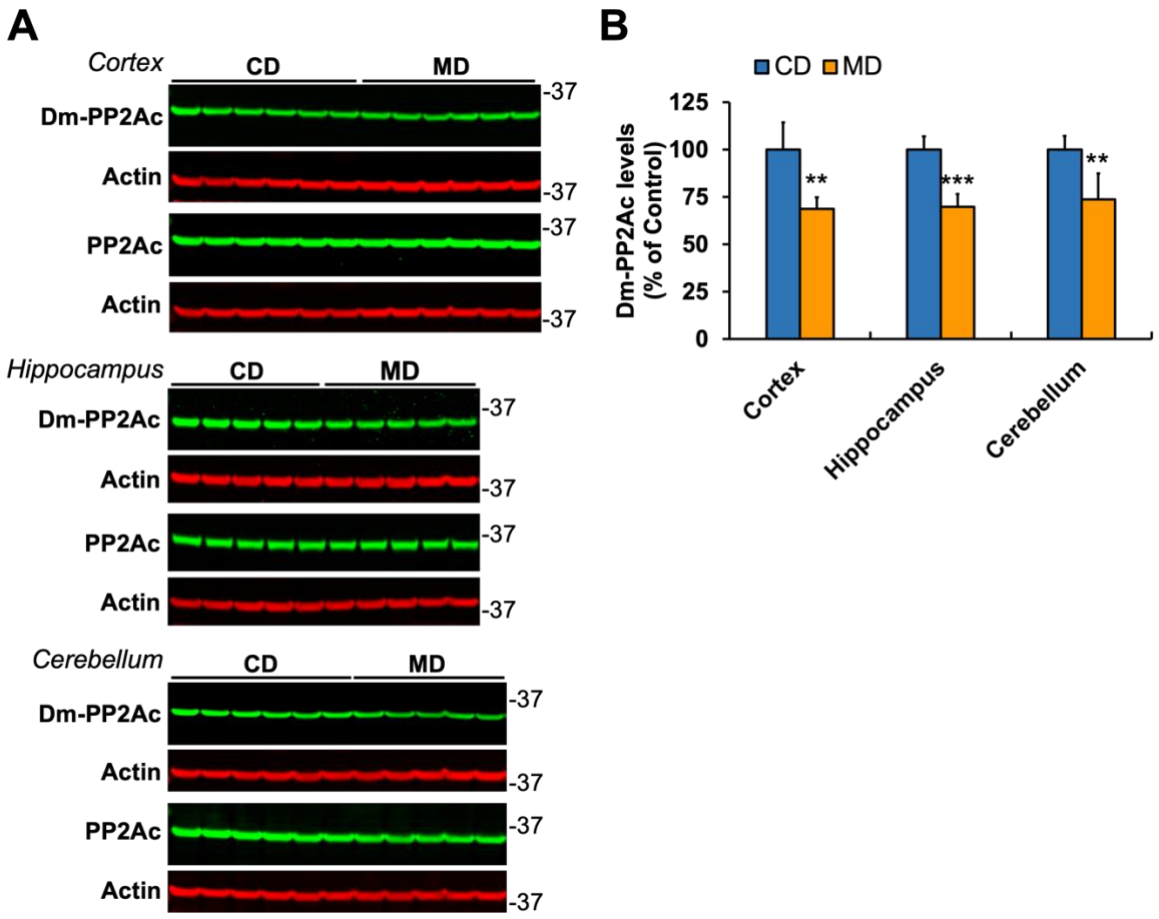

Figure 30. Our methyl group donor-enriched diet reduces demethylated PP2Ac levels in various brain regions in TAU58/2 mice. (**A**) Total cell extracts were prepared from the cortex, hippocampus and cerebellum of TAU58/2 mice and analysed for PP2Ac methylation *via* Western blot. Duplicate blots were prepared and probed for demethylated PP2Ac (dm-PP2Ac) or total PP2Ac. (**B**) Demethylated PP2Ac levels were quantified after normalisation with actin. Values represent Mean ± SD for n = 5-6 mice/ group; MD *vs* CD: $^{**}p < 0.01$ or $^{***}p < 0.001$.

6.4 Our methyl group donor-enriched diet reduces p-Tau levels in TAU58/2 mice.

In the same brain regions, we next investigated Tau phosphorylation at AD-like sites to check whether it is reduced in response to the MD intervention. A reduction in phospho-Tau levels would be in line with the observed decrease in PP2A demethylation. Enhanced methylation of PP2Ac is associated with the stabilisation and elevation of Tau-specific PP2A/Bα enzymes (Sontag et al., 2007). In turn, reduced levels of pathologically phosphorylated Tau could explain the behavioural improvements observed in MD-fed TAU58/2 mice. To verify this hypothesis, regional brain homogenates were processed to enrich for Tau as previously described (**Publication 1**). Specifically, we assessed Tau phosphorylation at the CP13 and PHF-1 epitopes, which are dephosphorylated by PP2A/Bα (Sontag et al., 2007) and are found in early pathological aggregates of Tau, such as PHFs and early NFT-like structures (Espinoza et al., 2008; Mondragon-Rodriguez et al., 2014). Cortical and hippocampal NFTs progressively increase over time in TAU58/2 mice (van Eersel et al., 2015). Indeed, we observed a significant reduction in levels of Tau phosphorylated at either CP13 or PHF-1 epitopes when comparing Tau enriched-extracts prepared from the cortex of MD-fed TAU58/2 mice relative to their CD-fed counterparts (**Figure 31**).

A subtle reduction (~12%) in CP13-Tau levels was also observed in the hippocampus of MD-fed TAU58/2 mice; however, this downward trend ended up being not statistically significant. Confirming our biochemical approach, immunohistochemistry (IHC) analyses of cortical sections (performed by the research group of Professor Lars Ittner) revealed that phospho-Tau levels were significantly reduced in response to the MD, namely at Ser214 and Ser422, which are other sites regulated by methylated PP2A enzymes (Sontag et al., 2007). Due to the lack of tissue and suitable antibodies, we were unable to assess Tau phosphorylation status at other sites, especially in the hippocampus.

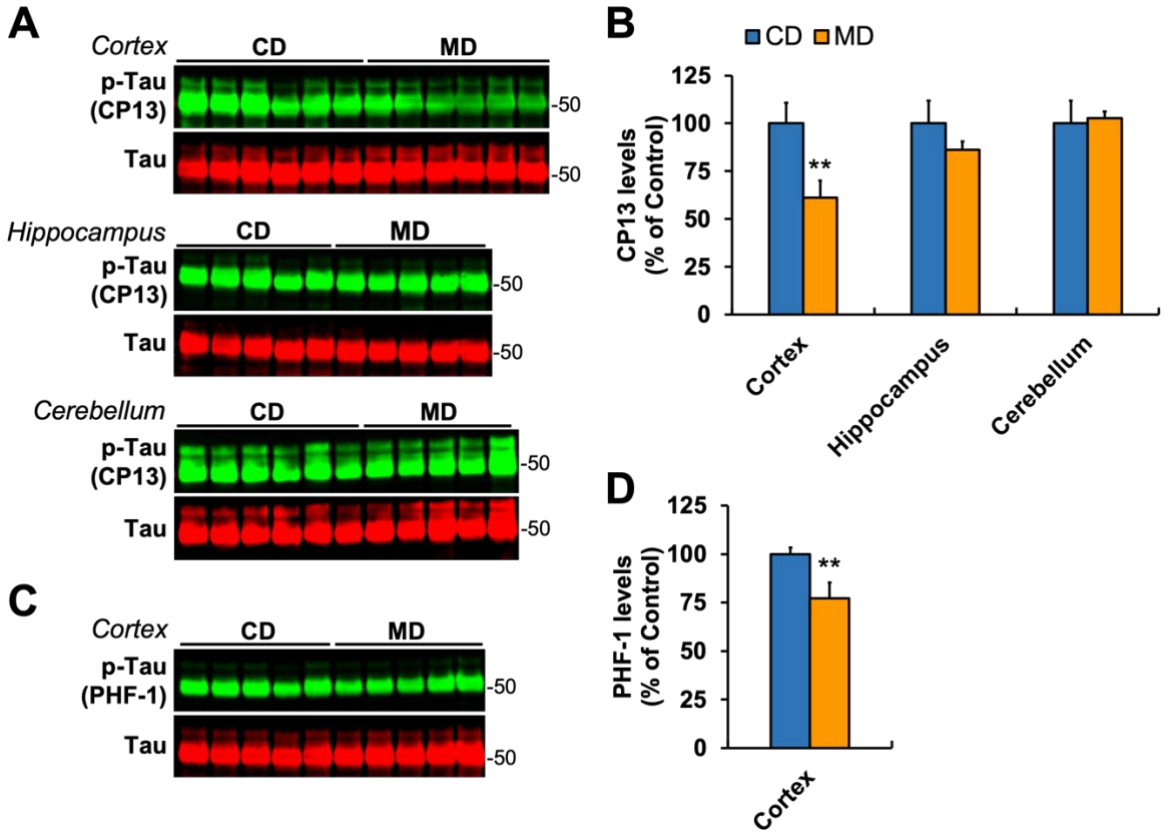

Figure 31. Our methyl group donor-enriched diet reduces brain p-Tau levels in TAU58/2 mice. Brain homogenates were prepared from the cortex, hippocampus and cerebellum of 5-month-old CD- or MD-fed TAU58/2 mice. (**A**) Representative Western blots prepared from Tau-enriched samples probed for total Tau and CP13-Tau. (**B**) Quantification of CP13 levels after normalising for total Tau expression. (**C**) Representative Western blots for total Tau and PHF-1-Tau. (**D**) Quantification of PHF-1 levels after normalising for total Tau expression. Values represent Mean ± SD for n = 5-6 mice/ group; MD *vs* CD: **$p < 0.01$.

6.5 Our methyl group donor-enriched diet has no effect on APP total protein expression or phosphorylation at Thr668.

Earlier, we showed that one-carbon metabolism affects APP protein expression and Thr668-phosphorylation levels in *Mthfr* knockout mice and WT mice fed a LF diet (*Chapter 3*). Based on these findings, and data that demonstrate that one-carbon metabolism and PP2A methylation are critical regulators of APP (Sontag et al., 2007), we also checked for regional changes in steady-state APP protein expression and/or Thr668 phosphorylation levels (**Figure 32**). Looking again at the cortex, hippocampus and cerebellum of CD- and MD-fed TAU58/2 mice, we could not see any statistically significant changes in either APP protein expression or Thr668-phosphorylation levels.

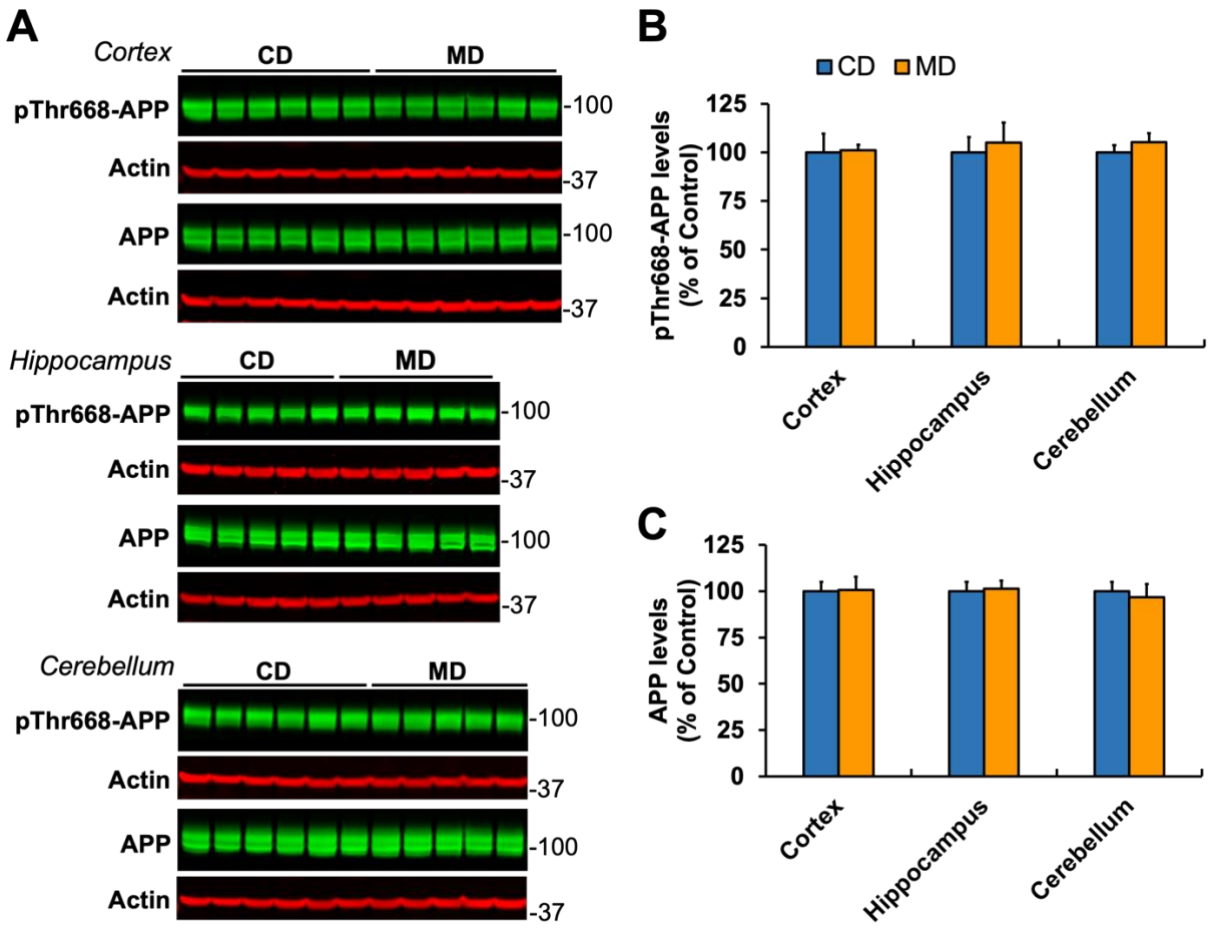

Figure 32. APP total protein expression and Thr668-phosphorylation are not affected in TAU58/2 mice fed our methyl group donor-enriched diet. Regional brain homogenates from TAU58/2 mice fed were reanalysed by Western blot for the relative expression of total APP and APP phosphorylated at Thr668 (pThr668-APP). (**A)** Representative immunoblots. (**B**) Relative pThr668-APP expression levels were quantified after densitometric analysis and normalisation for total APP expression levels. (**C**) Relative total APP expression levels were quantified after normalisation with actin. Values represent Mean ± SD for n = 5-6 mice/ group; MD *vs* CD: *p < 0.05.

6.6 Our methyl group donor-enriched diet reduces Fyn protein expression levels in the cortex and hippocampus of TAU58/2 mice.

We next assessed Fyn protein expression levels and activity in regional brain homogenates. Notably, FTLD-Tau-like mutations and disease-related Tau phosphorylation increase Fyn-Tau interactions (Bhaskar et al., 2005) while decreasing PP2A-Tau interactions (Goedert et al., 2000) (**Publication 4**). Moreover, we have shown that one-carbon metabolism and PP2A methylation critically influence Fyn regulation in N2a cells (*Chapter 4*). Hence, we hypothesised that Fyn protein expression levels and/or activity are decreased in the cortex of MD-fed TAU58/2 mice wherein reduced PP2A demethylation and

p-Tau levels are observed (**Figures 30 and 31**). Indeed, we observed a significant reduction in cortical Fyn protein expression levels in MD-fed TAU58/2 mice relative to CD-fed counterparts (**Figure 33A-B**). Similarly, we observed a decrease in Fyn protein expression levels in the hippocampus of MD-fed TAU58/2 mice. In the cerebellum, total Fyn expression levels were unaffected in response to the MD, suggesting a brain-region specific effect of the diet. Parallel immunoblotting with anti-pSFK antibodies, recognising Fyn activating-phosphorylation at Tyr420, showed that pSFK levels changed proportionally to Fyn protein levels. In line with this, there was no statistically significant difference in Fyn Tyr420-phosphorylation between groups (**Figure 33A and C**). Thus, the MD led to an overall loss of activated Fyn proteins in cortical and hippocampal regions, without affecting net Fyn activity. Due to the shortage of brain tissue, we were unable to prepare NP40 detergent-insoluble extracts to assess the distribution of Fyn.

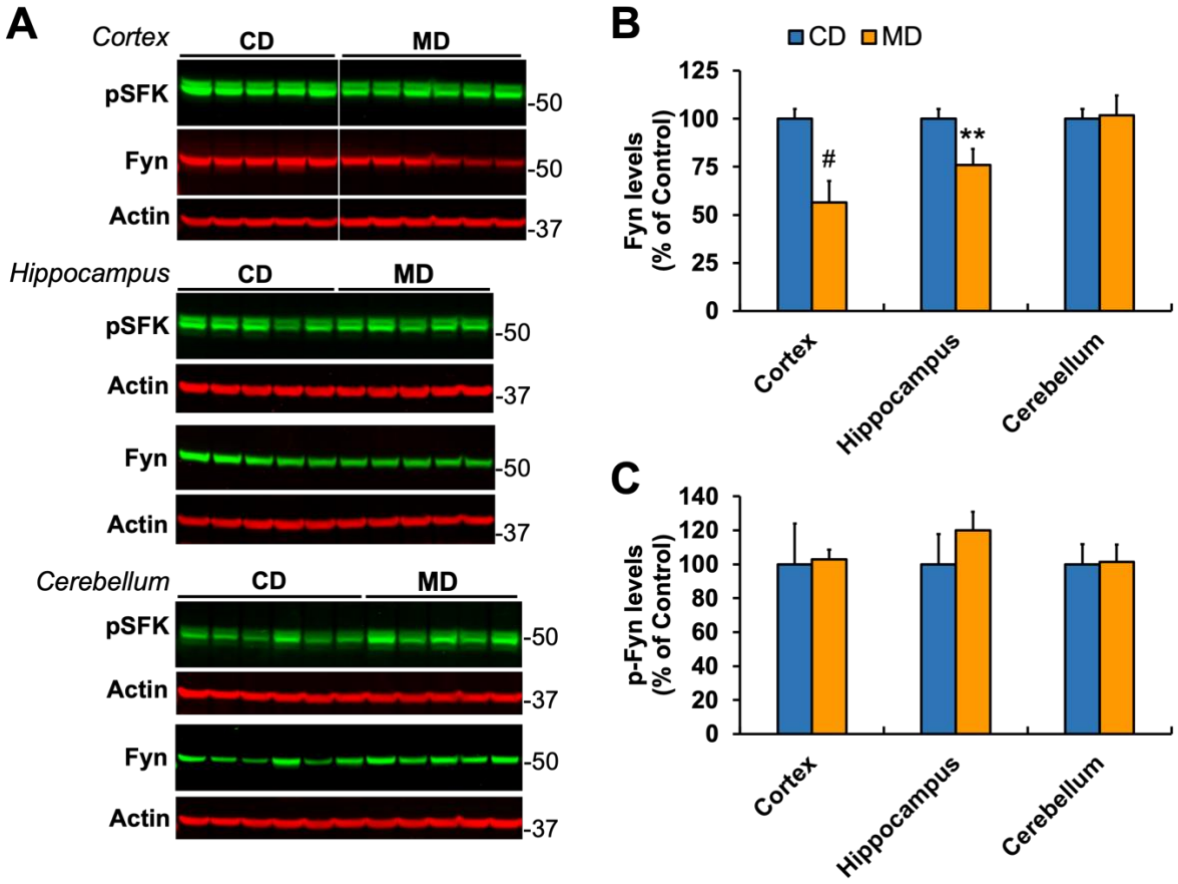

Figure 33. Our methyl group donor-enriched diet reduces cortical and hippocampal Fyn protein expression in TAU58/2 mice. (**A**) Homogenates were prepared from the cortex, hippocampus or cerebellum of TAU58/2 mice and analysed for Fyn total protein expression and activating-phosphorylation at Tyr420 (recognised by the anti-pSFK antibody) *via* Western blot. For the cortex, panels were assembled from the same blot. (**B**) Fyn expression levels were quantified after normalisation with actin. (**C**) Fyn activating-phosphorylation levels were quantified by normalising the pSFK signal (apportioned to Fyn) with total Fyn expression levels. No statistically significant difference in Fyn activity

was found between groups. Values represent Mean ± SD for n = 5-6 mice/ group; MD *vs* CD: $^{**}p < 0.01$ or $\# p < 0.0001$.

6.7 Our methyl group donor-enriched diet has no effect on ERK1/2 or GSK-3β activity in TAU58/2 mice

In *Chapter 3,* we demonstrated that GSK-3β activity is enhanced in mouse models of genetic- or diet-induced alterations in one-carbon metabolism. As mentioned previously, GSK-3β is a major Tau protein Ser/Thr kinase; its over-activation in AD prompts Tau phosphorylation at pathological epitopes and formation of NFT-like structures (Rankin et al., 2007), which are exhibited by TAU58/2 mice (van Eersel et al., 2015). Thus, decreased GSK-3β activity could further explain the observed decrease in p-Tau levels in MD-fed TAU58/2 mice. Based on these observations, we next comparatively analysed our regional brain homogenates for changes in GSK-3β activity in response to the MD. In parallel, we also assessed the activity of ERK1/2, which is a downstream target of SFKs in rat brain slices (Jin, Mao, & Wang, 2019) and of PP2A (Sontag et al., 1993). This was warranted, since ERK1/2 also regulate Tau phosphorylation (Qi et al., 2016), and Fyn expression levels and PP2A demethylation were significantly reduced by the MD intervention. However, our Western blot analyses revealed no changes in either GSK-3β or ERK1/2 activity in all brain regions analysed from MD-fed, relative to CD-fed TAU58/2 mice (**Figure 34**). Changes to GSK-3β or ERK1/2 total expression levels in response to the MD were not observed either.

These results indicate that the decrease in p-Tau levels in response to the MD is not primarily mediated through the suppression of these major Tau kinases.

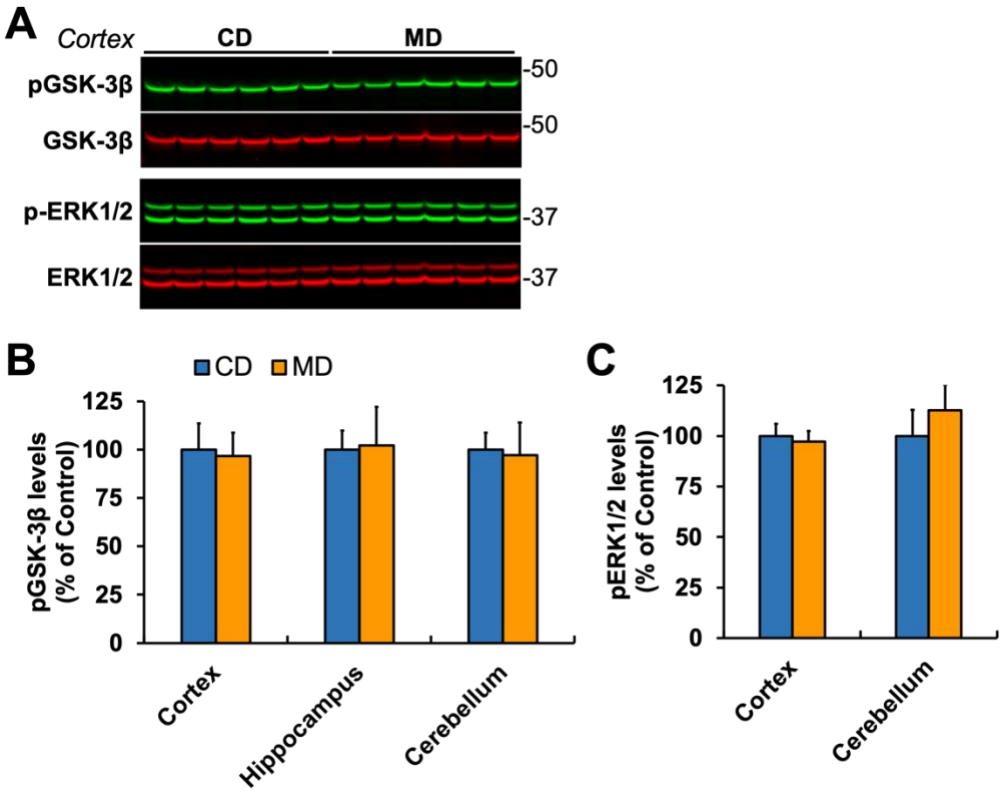

Figure 34. Our methyl group donor-enriched diet has no effect on brain GSK-3β or ERK1/2 activity in TAU58/2 mice. (**A**) Regional brain homogenates were analysed by Western blot for changes in deactivating phosphorylation (pSer9) of GSK-3β and activating-phosphorylation of ERK1/2. (**B**) Relative levels of Ser9-phosphorylated GSK-3β and (**C**) activating-phosphorylation of ERK1/2 were quantified after densitometric analyses. Values represent mean ± SD for 4-6 mice/ group. No statistically significant differences between groups were found (MD *vs* CD: $p > 0.05$).

6.8 Discussion

For the first time, we show that an early methyl group donor-based intervention is effective in alleviating or preventing behavioural impairments prompted by the hyperphosphorylation-prone Tau-P301S mutant in TAU58/2 mice. Five-month-old TAU58/2 mice fed our MD over a period of 4 months exhibited seemingly normal motor functions (spatial coordination and grip strength) as indicated by their strong performance in pole and beam testing relative to their CD-fed counterparts, which performed poorly in comparison. Interestingly, these results suggest that methyl group donors could be effective in alleviating motor dysfunction symptoms as found in AD and FTLD-Tau. Supplementation with particular methyl group donors has been shown to influence the expression of genes associated with synaptic plasticity (Strupp et al., 2016). In line with this, MD-fed TAU58/2 mice exhibited an improvement to spatial learning in MWM testing, which is a robust and reliable test for learning and memory since it is strongly correlated with hippocampal synaptic plasticity and NMDA receptor function (Qi et al., 2016). These

behavioural improvements in response to the MD intervention could, at least in part, be attributed to the observed reduction in cortical Tau phosphorylation at both CP13 and PHF-1 epitopes. However, before we can solely attribute the behavioural improvements to the decreases in cortical p-Tau levels, our MD intervention would need to be comparatively assessed in WT mice to exclude the possibility that this intervention enhances motor performance and spatial memory in general.

Unsurprisingly, the MD intervention showed significant reduction of demethylated PP2Ac levels in all brain regions analysed. It was somewhat expected because of the previously observed significant increase of demethylated PP2Ac levels in *Mthfr* knockout mice as well as mice fed folate deficient diets (**Publications 1 and 2**). Since steady-state methylation levels of PP2Ac are ~90% *in vivo* (Yu et al., 2001) and significantly high in human brain homogenates (Sontag, Hladik, et al., 2004), it is plausible that our MD intervention maximally increased methylated PP2A holoenzyme levels, and thus, Tau-specific PP2A/Bα levels. Therefore, the reduction in cortical p-Tau levels in MD-fed TAU58/2 mice could, in part, be attributed to enhanced methylated PP2A-mediated signalling and stabilisation of PP2A/Bα in the cortex. Despite demethylated PP2Ac levels being similarly reduced in the hippocampus and cerebellum of MD-fed TAU58/2 mice, Tau phosphorylation at the CP13 epitope was not significantly affected. A small decrease in CP13-Tau levels was observed in the hippocampus of MD-fed TAU58/2 mice, however, this was not statistically significant, probably due to the inherent limitations of quantitative Western blotting in measuring subtle changes. Due to the shortage of brain tissue, we were unable to assess Tau phosphorylation at other common disease-related phospho-epitopes in these brain regions, which should be addressed in future studies.

Here, we provide novel *in vivo* evidence that brain Fyn protein expression levels can be affected by manipulating one-carbon metabolism. To the best of our knowledge, this is the first demonstration of a non-pharmaceutical intervention that targets Fyn protein expression levels. Changes in Fyn expression levels likely occur through epigenetic mechanisms, but regulatory processes involved in the endocytosis and/or degradation of Fyn could also be involved (See *Chapter 4*). More specifically, we found reduced Fyn protein expression levels in the cortex and hippocampus of MD-fed TAU58/2 mice. This is in agreement with the observation that cortical Fyn protein expression levels are upregulated in young *Mthfr* knockout mice, which are deficient in major methyl group donors (*Chapter 4*; **Figure 21**). Conversely, in the cerebellum, Fyn protein expression levels were unaffected by the MD, as was the case with p-Tau levels (**Figure 31**), despite reduced PP2A demethylation (**Figure 30**). These findings suggest that regional differences in p-Tau levels in MD-fed mice are, to some extent, influenced by Fyn protein expression levels *per se*. Interestingly, it has been established that disease-associated Tau phosphorylation and FTLD-Tau-associated Tau mutations increase Fyn-Tau (Bhaskar et al., 2005) and decrease PP2A-Tau (Goedert et al., 2000) interactions. For instance, the P301S-Tau mutant overexpressed in TAU58/2 mice reduces

PP2A-Tau interactions by ~20-30% *in vitro* (Goedert et al., 2000). Moreover, the similar P301L-Tau mutant has a ~42-fold greater affinity for the SH3 domain of Fyn compared to WT Tau (Bhaskar et al., 2005). Thus, reducing Fyn protein expression levels may be a necessary approach to counteract Tau pathology in Tauopathies, in order to enhance PP2A-Tau interactions, and subsequently PP2A-dependent Tau dephosphorylation (**Publication 4**). Our findings further demonstrate the importance of one-carbon metabolism in Fyn regulation, which is a critical puzzle piece in delineating the pathogenesis of AD, wherein Fyn is found intensely upregulated in p-Tau-affected neurons (Shirazi & Wood, 1993).

Despite the significant changes in Fyn protein expression and PP2A demethylation, ERK1/2 and GSK-3β activities were unaffected in all brain regions examined from MD-fed mice, relative to CD-fed TAU58/2 mice. Hence, the observed decreases to cortical p-Tau levels may not have involved the inhibition or downregulation of these major Tau kinases. Likewise, there were no alterations in endogenous APP expression levels and phosphorylation at Thr668, which is not surprising since TAU58/2 mice do not normally display Aβ pathology. Notably, ERK1/2 and GSK-3β activities are significantly influenced by APP expression and Thr668-phosphorylation state (Kirouac et al., 2017). Accordingly, this was reflected in our *Mthfr* mice, which displayed both increased Thr668-phosphorylated APP and GSK-3β activity (*Chapter 3*). Consequently, our research group is currently assessing the MD intervention in transgenic APP mouse models of AD, to gain a better understanding of our intervention's potential to mitigate deregulation of APP phosphorylation and Aβ production.

Our MD intervention clearly had beneficial effects in TAU58/2 mice. Supplementation with various methyl group donors has been deemed safe in various clinical trials performed in the context of other human diseases. Hence, our experimental pilot work with selected methyl group donor compounds provides a solid foundation for the conduct of human trials where they could be used as a basic supplement as part of a multipronged, preventive or disease-modifying AD treatment. In this context, boosting one-carbon metabolism could synergise and increase efficacy of drugs currently being used for symptomatic relief in AD (NMDAR antagonists and cholinergic treatments); it may even be possible to reduce the dosage of these drugs, therefore preventing unwanted side-effects. Based on earlier B-vitamin trials (Smith et al., 2018), supplementation with methyl group donors alone could potentially counteract MCI and/or halt AD progression. These "metabolic restoring approaches" may also prove to be clinically superior to current symptomatic approaches, since they address important risk factors and can target key proteins affected in AD. Indeed, our results indicate that a methyl group donor-based intervention could be beneficial to all individuals affected by Tauopathies, regardless of their one-carbon metabolism status. Considering that female mice were only used herein, future studies will need to address whether similar effects of the MD intervention occur in male TAU58/2 mice. It would also be worthwhile exploring

whether our MD intervention has beneficial effects in TAU58/2 mice when given following the onset of AD-like pathology.

In summary, our findings indicate that an early methyl group donor-based intervention could be effective in protecting against the accumulation of p-Tau and its detrimental effects to behavioural and motor abilities. The mechanisms of action of the MD intervention in prompting behavioural improvements and/or cortical Tau dephosphorylation include enhancing PP2A methylation and downregulating Fyn expression, but additional mechanisms are likely at play.

Chapter 7 General Discussion

7.1 Thesis overview

A thorough understanding of the regulation of major proteins implicated in AD pathophysiology, and their interaction with established risk factors, is key in delineating the pathogenesis of this complex disorder. Undoubtedly, this would guide the development of a successful therapeutic intervention and "bench-to-bedside" translatability. Despite significant clinical investigative efforts over the past decade, current AD patients can only be prescribed treatments for symptomatic relief such as acetylcholinesterase inhibitors and Memantine, an extrasynaptic NMDAR antagonist, which offer very limited efficacy and only in some patients. Of particular concern, it has recently been reported that Memantine can significantly increase Tau phosphorylation at various AD-like epitopes in mice exposed to prolonged stress (Liu et al., 2019). Moreover, prior studies found that Memantine inhibits NMDAR-mediated truncation of GSK-3β in a dose-dependent manner in primary cortical neurons (Goni-Oliver, Avila, & Hernandez, 2009). Of course, such treatments do not target the underpinning disease mechanisms or address known risk factors.

This body of work unveils one-carbon metabolism and PP2A methylation as novel mechanisms of Fyn regulation. Predominant models of AD pathophysiology position Fyn centrally in the disease process. Uniquely, our findings shed light on upstream mechanisms of Fyn regulation; when deregulated, those could potentially contribute to set in motion the pathological and erroneous signalling events prompting AD pathophysiology.

In this study, we first identified various AD-like changes in AD vulnerable brain regions from mouse models of altered one-carbon metabolism. More specifically, we found increased APP phosphorylation at the Aβ-promoting Thr668 site, altered APP protein expression levels, and increased Tau phosphorylation at the AD-like PHF-1 epitope. These effects coincided with a deregulation of critical regulators, including reduced PP2A methylation and increased GSK-3β activity. Collectively, these changes strongly emphasise the crucial role of one-carbon metabolism in neuronal homeostasis, and further establish altered one-carbon metabolism as a significant contributor to AD development (*Chapter 3*).

Furthermore, we showed that one-carbon metabolism can regulate Fyn compartmentalisation to detergent-insoluble domains in N2a cells, which was verified in acute mouse brain slices. These effects were mediated in part by changes in PP2A methylation. Of course, the specific subcellular distribution of Fyn

is critical for its function. This was strongly apparent under conditions of altered one-carbon metabolism and PP2A demethylation, which resulted in the cytosolic 'trapping' of Fyn, hampering its ability to stimulate neurite outgrowth. Our data support an underlying mechanism involving disruption of F-actin organisation, which is significantly influenced by PP2A methylation in N2a cells. Interestingly, we identified Fyn-methylated PP2A and Fyn-PP2A/Bα interactions, which could play a role in Fyn targeting or lipid raft retention. The analyses of chronic mouse models of altered one-carbon metabolism revealed significant alterations to Fyn net activity and protein expression levels, which reflect the long-term consequences of aberrant Fyn deregulation. Furthermore, Fyn deregulation could be a link between altered one-carbon metabolism and increased APP and Tau phosphorylation in these mice (*Chapter 4*).

Looking at the Fyn-PP2A relationship in more depth, we identified a reciprocal functional interaction between Fyn and PP2A enzymes. Firstly, we found in N2a cells that PP2A is a target of Fyn-mediated phosphorylation on Y2, and to a lesser extent on Y1 sites, in N2a cells. Interestingly, the substitution of these sites with non-phosphorylatable residues hampered Fyn-mediated Tau phosphorylation in N2a cells, suggesting that Fyn regulates PP2A-mediated Tau dephosphorylation. Moreover, our data indicate that phosphorylated PP2A species only represent a small fraction of total cellular PP2A enzymes. Small pools of endogenous Y2-phosphorylated PP2A co-localised with expressed GFP-Fyn in SYF cells. In contrast, Tyr phosphorylation of PP2A was not present in control SYF cells that do not express Fyn, and was not detectable in N2a cells under basal conditions. Thus, it is likely that Tyr phosphorylation of PP2A is highly transient and labile, as previously proposed (Chen et al., 1992). It may preferentially occur under certain physiological conditions or pathological events wherein Fyn activity is enhanced. Of relevance to AD, Fyn activity becomes upregulated in particular brain regions of *Mthfr* knockout mice (*Chapter 4*) and in response to aberrant NMDAR activation (Um et al., 2013). Enhanced Fyn-mediated PP2A phosphorylation could contribute to the deregulation of APP and Tau, further worsening the effects of deregulation of PP2A methylation. Future studies should evaluate the potential cross-talk between methylation and phosphorylation in the modulation of PP2A enzymes. Lastly, we demonstrated that the Y1 and Y2 sites of PP2Ac are critical for physiological Fyn compartmentalisation and function in neurite outgrowth in N2a cells, at least in part through their influence on ERK signalling (*Chapter 5*).

Having established that one-carbon metabolism and PP2A methylation can regulate Fyn, we hypothesised that a methyl group donor-based intervention could reduce AD-like pathology and ameliorate its behavioural consequences. Indeed, we found that a diet enriched in methyl group donors significantly reduced cortical p-Tau levels at AD-like phospho-epitopes and improved motor deficits and spatial learning in mouse models of severe Tauopathy (TAU58/2). Tau phosphorylation levels at the CP13 site were subtly reduced in the hippocampus as well in response to the MD intervention. The mechanisms of action of the MD intervention likely include enhancing PP2A methylation and downregulating Fyn

expression. This chapter emphasises the critical involvement of methyl group donors in Tau phospho-regulation *in vivo*, which provide an explanation for their effects in normalising behaviour and motor dysfunction in TAU58/2 mice. Undoubtedly, these findings will open avenues for clinical exploration into the effectiveness of methyl group donors in AD (*Chapter 6*).

Together with the novel data presented herein linking one-carbon metabolism with the regulation of major proteins affected in AD, these findings provide a compelling mechanism depicted below (**Figure 35**).

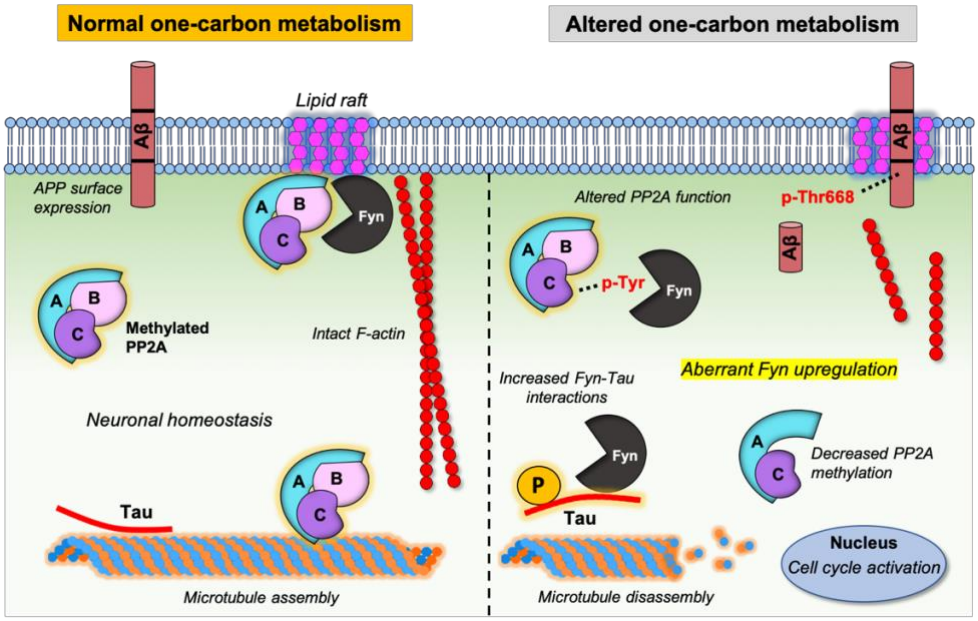

Figure 35. Proposed model for the role of intact one-carbon metabolism and PP2A methylation, and the functional ramifications of their deregulation for Fyn signalling. *Normal one-carbon metabolism:* Fyn is trafficked to the plasma membrane, an event dependent on an intact F-actin organisation. Methylation targets subpools of PP2A to lipid rafts, where Fyn is enriched. Cytoplasmic methylated PP2A enzymes bind to and maintain Tau in a dephosphorylated state, promoting its binding to microtubules. APP at the plasma membrane is predominantly present in non-raft microdomains, where it is primarily processed *via* the non-amyloidogenic pathway (See **Figure 2** in *Chapter 1* for details). *Altered one-carbon metabolism:* Altered one-carbon metabolism promotes PP2A demethylation and alters trafficking of Fyn to lipid rafts. Fyn protein expression levels and activity become upregulated, possibly through epigenetic or compensatory mechanisms intended to restore normal basal Fyn functions. Enhanced activation of Fyn and PP2A demethylation promote the recruitment of APP to lipid rafts, where amyloidogenic processing is favoured. Demethylation of PP2A leads to decreased PP2A/Bα-Tau interactions and increased disease-associated Ser/Thr phosphorylation of Tau, which falls off microtubules. These events further enhance Fyn-Tau interactions, leading to the accumulation of Tau phosphorylated at both Tyr and Ser/Thr epitopes. Fyn upregulation induces PP2Ac Tyr-phosphorylation, affecting PP2A signalling and further hampering PP2A-mediated Tau dephosphorylation. The deregulation of Fyn and PP2A ultimately lead to the aberrant phosphorylation, mis-trafficking, mislocalisation and deregulation of both Tau and APP. This vicious cycle ultimately aggravates Aβ production and elicits aberrant proliferative signalling and neurodegeneration.

7.2 Significance

Altogether our findings show that one-carbon metabolism can exert important regulatory effects on major players in AD pathogenic cascades, i.e. Tau, APP and Fyn, at least in part *via* its influence on PP2A methylation. Overall, our studies provide compelling evidence that normalising or enhancing one-carbon metabolism is a potential avenue for mitigating AD onset and progression. As previously mentioned, disturbances to one-carbon metabolism are frequently observed in the elderly and AD patients. They are also found in individuals with FTLD-Tau (Engelborghs et al., 2004; Lovati et al., 2007), which is another leading cause of dementia. As found in AD-affected brain regions (Sontag, Hladik, et al., 2004), LCMT1 expression and methylated PP2Ac levels are also reduced in affected brain regions in FTLD-Tau (Park et al., 2018). Importantly, our findings can also be extended to medical conditions outside the realm of Tauopathies. For instance, low plasma folate levels and elevated plasma tHcy levels are also found in patients with Parkinson's disease (PD) (Rodriguez-Oroz et al., 2009). They are closely associated with white matter hyperintensities (WMHs; brain tissue damage lesions) and the postural instability gait disorder phenotype in PD (Shen et al., 2019). Hence, it would be worthwhile investigating the effectiveness of our methyl group donor-based intervention in PD patients, especially since we found that it can improve/prevent motor deficits in TAU58/2 mice (*Chapter 6*). Likewise, elevated plasma tHcy levels are associated with WMHs in a psychiatric cohort (Scott et al., 2004).

Our findings may also be applicable to the field of cancer. Enhanced expression and/or activity of Fyn is associated with many cancers, including neuroblastoma, glioblastoma, melanoma, and breast and prostate carcinomas (Elias & Ditzel, 2015). Fyn is involved in both cancer progression and resistance to cancer drug treatment (Elias & Ditzel, 2015). Notably, changes in Fyn localisation can serve as a prognostic marker in some of these cancers (Elias & Ditzel, 2015). Of particular significance for our studies, hyperhomocysteinemia has been shown to be closely associated with cancer (Hasan et al., 2019). Regardless of Fyn involvement, there is increasing evidence that global hypomethylation leads to genome instability, a hallmark of cancer, making methyl group donor supplementation a potential preventative strategy (Mahmoud & Ali, 2019). However, it should be noted that boosting one-carbon metabolism may not always be an adequate strategy, since folate has been reported to promote cancer progression (Oliai Araghi et al., 2019; Pelucchi et al., 2005).

The inhibitor of folate metabolism, methotrexate, is currently used as treatment for certain cancers. Not surprisingly, this drug has been found to induce dementia symptoms (Dautzenberg, Jessurum, Dautzenberg, & Keijsers, 2015) and CNS toxicity (Wernick & Smith, 1989) in humans. It also induces cognitive and hippocampal proliferation deficits in mice (Elens, Dekeyster, Moons, & D'Hooge, 2019).

Methotrexate is also widely used to treat autoimmune diseases, and causes hyperhomocysteinemia at the prescribed dose, which is lower than what is used in chemotherapy (van Ede et al., 2002). Recently, a retrospective study using primary medical records reported that rheumatoid arthritis patients taking methotrexate had their dementia risk reduced by half (Judge et al., 2017), which is questionable. However, amongst various limitations, this study did not consider whether methotrexate users were taking folic or folinic acid supplements in combination with the drug, which is a common and recommended practice to reduce side-effects.

Despite the overwhelming evidence implicating altered one-carbon metabolism in dementia (Smith et al., 2018), the World Health Organization (WHO) has officially strongly recommended against the use of B vitamin supplementation in reducing the risk of cognitive decline and dementia (See, *Risk reduction of cognitive decline and dementia*, 2019 Guidelines by WHO). Surprisingly, this followed the release of an International Consensus Statement conducted by a panel of experts, which emphasised the causal role for raised plasma tHcy levels and B-vitamin deficiencies in dementia (Smith et al., 2018). Moreover, WHO's guidelines disregard the prevalence of B-vitamin deficiencies and functional *Mthfr* polymorphisms and their links to dementia. Unfortunately, those conclusions were based on the inefficacy of B-vitamin supplementation studies performed in non-demented individuals or individuals with cardiovascular disease. Many past negative trials also failed to account for background genetic polymorphisms and absorption issues that could severely compromise a proper interpretation of the outcomes. Furthermore, a recent paper has provided a molecular explanation for the failure of B-vitamin trials, especially in the context of AD (Bossenmeyer-Pourie et al., 2019). Hence, these guidelines should be re-evaluated, and novel, better designed trials should be conducted.

Considering the decades-long preclinical phase of AD and the cumulative effects of Hcy through its metabolite HTL, which irreversibly modifies Tau in AD (Bossenmeyer-Pourie et al., 2019), it seems logical to monitor and maintain plasma tHcy levels in individuals throughout their lifetime as a preventative strategy for AD development. Moreover, this would be beneficial for overall health, and even reduce the risk for other hyperhomocysteinemia-linked diseases. As demonstrated herein, long-term effects of altered one-carbon metabolism could also impact Fyn-driven processes, affecting homeostasis in various cell types in the brain. Plasma tHcy levels are currently not routinely measured despite being a reliable indicator of an individual's one-carbon metabolism status. Currently, the "normal" clinical levels of plasma tHcy ranges from 4 to 15 µmol/L following a 12 hour fast or after an oral methionine load (100 mg/kg body weight). However, this may need to be reconsidered since plasma tHcy >11.1 µmol/L is associated with increased rate of atrophy of the medial temporal lobe in humans (Smith et al., 2018). Interestingly, it has been found that methylation of the *Mthfr* gene promoter is significantly higher in the brain of sporadic AD patients relative to normal controls (Wang et al., 2008). DNA methylation typically

acts to suppress gene transcription. Moreover, the MTHFR enzyme itself is functionally regulated through C-terminal binding of SAM; it is a target of GSK-3β-mediated phosphorylation at multiple Ser sites of the N-terminus, which allosterically inhibits the enzyme (Wan et al., 2018). Indeed, these constraints to 5-MTHF production could contribute to elevated tHcy levels in AD patients, in addition to *Mthfr* polymorphisms.

Our work has also uncovered complex inter-relationships between Fyn and PP2A (methylation and phosphorylation). There is an increasing interest in developing Fyn inhibitors and "PP2A activators" for AD and cancer. However, based on their multifaceted cellular functions and our data, these compounds are likely to induce off-target effects, especially in the context of disturbed one-carbon metabolism. We believe that some of these approaches fail to address the complexity of the causal and regulatory mechanism(s) underlying these complex diseases (For details, see **Publication 4** and *Chapter 4*: **Discussion**).

7.3 Future directions

Our overall goal is to further our understanding of AD pathogenesis by studying major key players in AD, like Fyn and PP2A, and their interactions under both homeostatic and disease conditions. Additional studies should be carried out in the future to cement our hypothesis that altered one-carbon metabolism and PP2A methylation-driven Fyn regulation is an early event preceding and aggravating AD pathophysiology. For instance, experiments should investigate in primary neurons the functional ramifications of the redistribution of Fyn protein on axonal Tau, to test our hypothesis that it increases Tau phosphorylation at Ser/Thr sites through outcompeting PP2A for Tau binding (Sontag et al., 2012; **Publication 4**). It would be also interesting to determine the fate of the redistributed Fyn protein (i.e. is it inactivated or degraded?) and the response of neurons to a loss of lipid raft-associated Fyn. Assessing Fyn stability and transcriptional regulation should help to clarify the mechanisms underlying the changes in Fyn protein expression levels observed in our *Mthfr* knockout mice. We hypothesise that those are a matter of epigenetic influence considering one-carbon metabolism crucially supports DNA methylation. Additionally, it would be informative to see whether Fyn compartmentalisation is also affected in *Mthfr* knockout mice.

A potential limitation to our hypothesis that one-carbon metabolism and PP2A methylation regulate Fyn targeting, particularly in the brain, is that elevated Hcy levels could be over-stimulating glutamate receptors that are functionally linked to Fyn. This could trigger a compensatory internalisation of Fyn. This may be addressed in additional acute brain slice experiments, in which glutamate receptor antagonists

will be concomitantly used with Hcy or HTL. Moreover, brain slices can be treated with SAM to assess whether it can boost Fyn targeting to detergent-insoluble compartments as seen in N2a cells. In addition, to increase the physiological significance of our findings, it may be worthwhile to comparatively analyse the role of one-carbon metabolism in AD mouse models that have been crossed or not with Fyn knockouts. Conversely, the functional links between PP2A and Fyn could be further studied in LCMT1 and PME1 mouse models of PP2A demethylation (Nicholls et al., 2016).

We also intend to further explore the relevance of Fyn-PP2A regulatory interactions, for instance in the context of their trafficking to lipid rafts. It will also be important to confirm and assess the functional significance of Fyn-mediated PP2A Tyr phosphorylation *in vivo*, since all our studies so far were carried out in cultured cells. Indeed, our group, in collaboration with Dr. Delerue (Macquarie University) tried to generate a PP2Ac F/F mutant mouse model using both CRISPR-Cas9 or TALEN genome editing techniques. Unfortunately, despite multiple attempts, those approaches led to embryonic lethality. This is likely due to the essential biological role of PP2Ac. Nevertheless, we plan to analyse cortical and hippocampal samples of *Mthfr* knockout and AD mouse models, where Fyn activity is elevated. It is also worthwhile investigating whether the PP2Ac F/F mutant alleviates mouse Tau pathology in the context of Fyn hyperactivity. Furthermore, additional experiments are needed to understand the regulatory effects of Tyr phosphorylation on PP2A holoenzyme catalytic activity, targeting and function. The observation that PP2Ac F/F-expressing N2a cells have a phenotype and Fyn distribution reminiscent to those of N2a cells with impaired PP2Ac methylation (*Chapter 4*) suggest overlapping mechanisms of action. Thus, it may be interesting to investigate whether Tyr phosphorylation of PP2Ac impedes Leu309 methylation or B subunit binding.

Lastly, we plan to further assess our MD intervention in WT mice to see whether this intervention improves behavioural test performances in general, in order to confirm that the observed behavioural improvements in TAU58/2 mice were solely related to diminished p-Tau pathology. Additionally, it would also be interesting to determine the effects of the diet on Fyn-Tau *vs* PP2A-Tau interactions, and phosphorylation of Tau at other epitopes in our TAU58/2 models. As mentioned previously, our research group is currently assessing the MD intervention in transgenic mice with APP pathology, to determine its potential in counteracting excessive Aβ production. In light of the encouraging effects of our safe MD intervention in TAU58/2 mice, our group is currently planning a clinical trial in MCI and/or mild AD patients, to investigate the disease-modifying therapeutic potential of our intervention.

7.4 Concluding remarks

Overall, this thesis work provides novel insights and underscores the role of one-carbon metabolism and PP2A methylation in the regulation of Fyn compartmentalisation, protein expression and/or activity. These novel regulatory pathways could be fundamental in dissecting the underpinning molecular mechanisms instigating pathological changes to APP and Tau in AD, wherein one-carbon metabolism is commonly altered. We also unveiled that increased Fyn signalling occurred in mouse models of altered one-carbon metabolism and mediated phosphorylation of PP2A on Tyr in N2a cells, which could have significant ramifications not only for APP and Tau regulation, but also cellular signalling in general. Studies presented in this thesis broaden the current understanding of how risks factors and deregulation of important signalling molecules (Fyn, PP2A) could interact in AD pathogenic pathways, which despite considerable effort, remain poorly understood. By further supporting the link between altered one-carbon metabolism and AD development, our findings open up new avenues for research and design of preventive or therapeutic strategies for AD and other Tauopathies. Indeed, we believe that our MD intervention could serve as a supportive therapeutic strategy for delaying AD onset and/or disease progression. Significantly, this intervention downregulated Fyn protein expression and enhanced methylated PP2A enzymes, therefore counteracting their toxic deregulation in AD.

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Appendix

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AGING NEUROSCIENCE

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Altered protein phosphatase 2A methylation and Tau phosphorylation in the young and aged brain of methylenetetrahydrofolate reductase (MTHFR) deficient mice

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Common functional polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene, a key enzyme in folate and homocysteine metabolism, influence risk for a variety of complex disorders, including developmental, vascular, and neurological diseases. MTHFR deficiency is associated with elevation of homocysteine levels and alterations in the methylation cycle. Here, using young and aged *Mthfr* knockout mouse models, we show that mild MTHFR deficiency can lead to brain-region specific impairment of the methylation of Ser/Thr protein phosphatase 2A (PP2A). Relative to wild-type controls, decreased expression levels of PP2A and leucine carboxyl methyltransferase (LCMT1) were primarily observed in the hippocampus and cerebellum, and to a lesser extent in the cortex of young null $Mthfr^{-/-}$ and aged heterozygous $Mthfr^{+/-}$ mice. A marked down regulation of LCMT1 correlated with the loss of PP2A/Bα holoenzymes. Dietary folate deficiency significantly decreased LCMT1, methylated PP2A and PP2A/Bα levels in all brain regions examined from aged $Mthfr^{+/+}$ mice, and further exacerbated the regional effects of MTHFR deficiency in aged $Mthfr^{+/-}$ mice. In turn, the down regulation of PP2A/Bα was associated with enhanced phosphorylation of Tau, a neuropathological hallmark of Alzheimer's disease (AD). Our findings identify hypomethylation of PP2A enzymes, which are major CNS phosphatases, as a novel mechanism by which MTHFR deficiency and *Mthfr* gene-diet interactions could lead to disruption of neuronal homeostasis, and increase the risk for a variety of neuropsychiatric disorders, including age-related diseases like sporadic AD.

Keywords: Alzheimer's disease, folate, LCMT1, MTHFR, methylation, PP2A, Tau phosphorylation

INTRODUCTION

5,10-methylenetetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme for converting folate to its active form, methylfolate (5-MTHF). 5-MTHF is a necessary co-factor for remethylation of homocysteine to methionine, which is essential for production of S-adenosylmethionine (SAM), the universal methyl donor (**Figure 1**). Severe MTHFR deficiency is the most common inborn error of folate metabolism, and leads to decreased red cell folate levels, elevated plasma total homocysteine (tHcy) levels, homocysteinuria, hypomethioninemia, and impaired cellular methylation potential (Goyette et al., 1996). It is associated with variable clinical outcomes, ranging from early neonatal demise to later-onset damage to the nervous and vascular systems, resulting in varying degrees of developmental delay, neurological impairment, motor dysfunction, gait abnormalities, seizures, and thrombotic events. Notably, there is a relatively high prevalence of functional genetic *Mthfr* polymorphisms in the general population (Botto and Yang, 2000). Among them, the common human *Mthfr* 677C→T gene polymorphism is associated with mild MTHFR deficiency, and is the most frequent cause of hyperhomocysteinemia (Leclerc and Rozen, 2007). *In vitro*, the C to T change at nucleotide position 677 leads to production of thermolabile MTHFR enzymes with ∼35% and ∼65% decreased MTHFR activity in heterozygous 677CT and homozygous 677TT individuals, respectively (Rozen, 1997). Of particular relevance to the CNS, the *Mthfr* 677CT polymorphism has been clinically identified as a risk factor for development neural tube defects (Botto and Yang, 2000), vascular disease and stroke (McNulty et al., 2012), and a variety of neuropsychiatric diseases, including Down syndrome (Hobbs et al., 2000), epilepsy (Wu et al., 2014), migraine (Liu et al., 2014), depression, schizophrenia and bipolar disorder (Gilbody et al., 2007). Homozygosity for *Mthfr* 677TT is also associated in selected populations with age-related neurodegenerative diseases such as Alzheimer's disease (AD; Kageyama et al., 2008; Wang et al., 2008; Hua et al.,

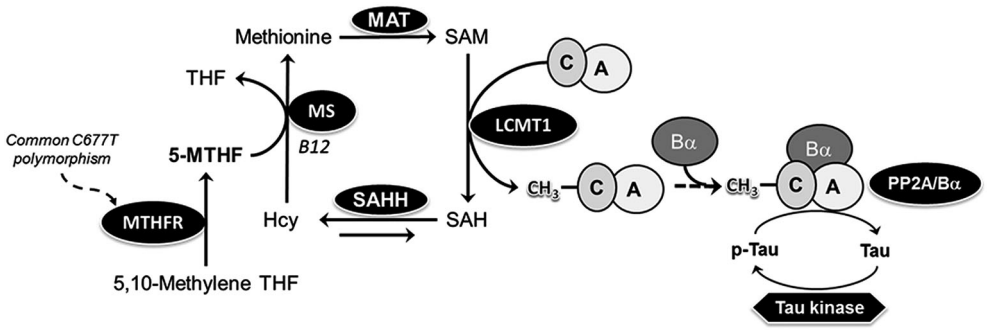

FIGURE 1 | Model of the link between MTHFR, folate, PP2A methylation and Tau phosphorylation. 5-MTHF, produced via the action of MTHFR, is essential for remethylation of homocysteine (Hcy) to methionine, the precursor of S-adenosylmethionine (SAM), the universal methyl donor. Methylation of PP2A catalytic subunit on Leu-309 by the dedicated SAM-dependent LCMT1 methyltransferase promotes the biogenesis of PP2A/Bα heterotrimers, the primary Tau Ser/Thr phosphatases in the brain. Common C677→T polymorphisms in the *mthfr* gene induce mild MTHFR deficiency, leading to reduced 5-MTHF levels. Folate deficiency promotes accumulation of Hcy, which may be converted back to SAH, a potent inhibitor of LCMT1 activity. *MAT, methionine adenosine transferase; MS, methionine synthase; MTHF, methyltetrahydrofolate; SAHH, SAH hydrolase; THF, tetrahydrofolate.*

2011; Coppede et al., 2012) and Parkinson's disease (Wu et al., 2013).

Despite numerous clinical studies, the effects of MTHFR deficiency at the molecular level are not well understood. By affecting folate and homocysteine metabolism, MTHFR polymorphisms have the potential to disrupt the transfer of one-carbon units, thereby influencing many methylation-sensitive targets (**Figure 1**). We hypothesized that one of them might be protein phosphatase 2A (PP2A), a large family of essential Ser/Thr protein phosphatases (Sontag and Sontag, 2014). Significantly, PP2A is regulated by Leucine carboxyl methyltransferase 1 (LCMT1)-dependent methylation of its catalytic C subunit (PP2Ac), which influences PP2A holoenzyme biogenesis and substrate specificity (De Baere et al., 1999; Leulliot et al., 2004; Stanevich et al., 2011; Tsai et al., 2011). We have reported *in vivo* that low folate status (Sontag et al., 2008) and hyperhomocysteinemia (Sontag et al., 2007), lead to down regulation of expression levels of LCMT1, PP2A methylation, and PP2A holoenzymes containing the regulatory Bα (or PPP2R2A) subunit (PP2A/Bα). This correlates with increased phosphorylation of specific PP2A/Bα substrates, such as Tau proteins (Sontag and Sontag, 2014). The accumulation of phosphorylated Tau (p-Tau) species, a neuropathological hallmark of AD and other tauopathies, mediates neurotoxicity in AD mouse models (Wang et al., 2013). Here, using young and aged *Mthfr* knockout mouse models, we investigated whether MTHFR deficiency and *Mthfr*-low folate gene-diet interactions can potentially affect the methylation state of PP2A, and its substrate, Tau.

MATERIALS AND METHODS

MATERIALS

Unless indicated, all chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO and Castle Hill, Australia).

Mthfr MICE AND FOLATE DEFICIENCY

All experiments with mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Baylor Research Institute. Wild-type (Wt) $Mthfr^{+/+}$, heterozygous (het) $Mthfr^{+/-}$ and homozygous (null) $Mthfr^{-/-}$ knockout mice in the C57Bl/6 background (Chen et al., 2001) were obtained from Dr. Rima Rozen (The Research Institute of the McGill University Health Center, Montreal, Quebec, Canada). Mice were bred, genotyped and housed in cages with a maximum of 4 mice per cage, maintained in a temperature-controlled animal facility on a 12-h light dark cycle, and were allowed access to food and water *ad libitum*. At ∼5 weeks of age, a subset of mice (n = 10 female per genotype) fed a normal chow diet was sacrificed by CO_2 asphyxiation. At ∼16 months of age, subsets of wt mice were placed for 6 months on an amino acid defined diet (n = 10 female per group) with either a normal folate (6.7 mg/kg, NF) or low folate (0.2 mg/kg, LF) content. These custom diets (Harland Teklad) also contained succinylsulfathiazole (10 g/kg) to inhibit gastrointestinal bacterial growth and prevent absorption of folate from this source (Sontag et al., 2008). The diets did not induce any statistically significant changes in mouse weight in any of the group studied (mean weight in grams ± SD: Wt mice on NF diet, 37 ± 9 at baseline, 40 ± 7 at 22 months; het mice on NF diet, 43 ± 7 at baseline, 41 ± 6 at 22 months; wt mice on LF diet, 35 ± 7 g at baseline, 34 ± 3 g at 22 months; het mice on LF diet, 46 ± 14 g at baseline, 40 ± 10 g at 22 months). At ∼22 months of age, mice were sacrificed by CO_2 asphyxiation. Blood was obtained by cardiac puncture and brain tissue rapidly removed for regional dissection (Sontag et al., 2008). Tissues were stored at −80°C until time of analysis.

PLASMA METABOLITE ANALYSIS

Plasma tHcy was determined by high pressure liquid chromatography with fluorescence detection (Ubbink et al., 1991). Plasma

5-MTHF was measured by liquid chromatography mass spectrometry as previously described (Nelson et al., 2004).

DETERMINATION OF PROTEIN EXPRESSION AND PP2A METHYLATION LEVELS

Total brain homogenates (0.1 g tissue/ml of buffer) were prepared from each brain region exactly as described previously (Sontag et al., 2007, 2008). Aliquots were analyzed immediately for PP2A methylation or kept frozen at −80°C for future analyses. Equivalent aliquots (5 μl) of brain homogenates were resolved on 4–12% Bis-Tris gels using the NU-PAGE system (Thermo Fisher Scientific) followed by quantitative Western blotting. Precision Plus Protein™ Standards (BIO-RAD) were used as molecular weight standards. Antibodies against LCMT1 (clone 4A4, Merck Millipore #05-592), Bα (clone 2G9, Merck Millipore #05-849), total Tau (rabbit anti-Tau T-1308-1, rPeptide) and Tau phosphorylated at the PHF-1 epitope (Greenberg et al., 1992) were used to assess protein expression levels exactly as described previously (Sontag et al., 2007, 2008; Bottiglieri et al., 2012). Methylation of PP2A was determined using two methods. First, western blots of brain tissue samples were probed with monoclonal anti-methyl specific PP2A antibodies (clone 2A10, Merck Millipore #04-1479), followed by re-probing with methylation-insensitive antibodies (clone 46, BD Biosciences #610556) (Sontag et al., 2007). In the second method, equivalent aliquots of brain tissue homogenates were incubated for 30 min at 37°C in the absence or presence of 0.2 N sodium hydroxide (NaOH). Alkaline treatment results in complete demethylation of PP2Ac at Leu-309. NaOH-treated and untreated samples were then analyzed by NU-PAGE electrophoresis followed by Western blotting with monoclonal anti-demethylated PP2Ac antibodies (clone 1D6, Merck Millipore #05-421) to detect demethylated (NaOH-untreated samples) and total PP2Ac (NaOH-treated samples) (Bottiglieri et al., 2012). All the blots were re-blotted with monoclonal anti-actin antibodies (clone 4, MAB1501 Merck Millipore) to allow normalization for protein loading. Western blotting was performed using Infrared IRDye®-labeled secondary antibodies and the Odyssey™ Infrared imaging system (LI-COR Biosciences). Band intensity was determined using the associated Image Studio Lite version 3.1 Software (LI-COR Biosciences) to accurately quantify protein levels. Some samples were also analyzed in duplicate by Western blotting using chemiluminescence detection system (Pierce), followed by densitometry (Sontag et al., 2007).

STATISTICS

Data were analyzed using one-way ANOVA with *post hoc* Tukey's multiple comparison test. Differences with p-values < 0.05 were considered statistically significant.

RESULTS

MTHFR DEFICIENCY IN YOUNG MICE IMPAIRS BRAIN LCMT1 PROTEIN EXPRESSION AND PP2A METHYLATION IN A REGION-SPECIFIC MANNER

To investigate whether MTHFR deficiency can influence the methylation of PP2A and its substrate Tau, we first analyzed young murine models of mild ($Mthfr^{+/-}$) and severe ($Mthfr^{-/-}$) MTHFR deficiency. These well-characterized knockout mice have been shown to reproduce the biochemical and clinical consequences of *Mthfr* 677C→T polymorphisms in human (Chen et al., 2001; Ghandour et al., 2004). Relative to their 5-week-old wt littermates, there was an average ∼1.6-fold increase in plasma tHcy levels in het $mthfr^{+/-}$ mice, while null $Mthfr^{-/-}$ mice exhibited a ∼10-fold increase in plasma tHcy levels (**Figure 2**), in agreement with earlier studies (Chen et al., 2001). It is well established that impairment of MTHFR activity can lead to reduced 5-MTHF levels and elevated tHcy levels that compromise SAM-dependent methylation reactions (Chen et al., 2001). Significantly, the activity of LCMT1, the sole PP2A methyltransferase (MacKay et al., 2013), is dependent on SAM supply (Lee et al., 1996; Leulliot et al., 2004; Sontag et al., 2007; Tsai et al., 2011). Accordingly, we found that, relative to wt mice, methylation of PP2Ac was reduced in all the brain regions examined from het or null *mthfr* knockouts (**Figure 3A**). Of note, the extent of decrease in methylated PP2Ac levels was variable among regions, being highest in the hippocampus, and lowest in the striatum. We have previously reported that, besides affecting SAM-dependent LCMT1 methyltransferase activity, *in vivo* alterations in plasma folate and/or tHcy levels can also result in down regulation of brain LCMT1 at the protein level, through a yet unresolved mechanism (Sontag et al., 2007, 2008; Bottiglieri et al., 2012). Interestingly, a marked decrease in LCMT1 expression levels was observed in the hippocampus and cerebellum, and to a lesser extent in the cortex, but not in the midbrain and striatum from young het $Mthfr^{+/-}$ mice, relative to wt mice (**Figures 3B,C**). Compared to their wt littermates, lower LCMT1 protein amounts were observed in all brain regions of null $Mthfr^{-/-}$ mice, with the exception of the striatum. Notably, the down-regulation of LCMT1 expression was most prevalent in the hippocampus, wherein the loss of methylated PP2Ac was maximal (**Figure 3A**),

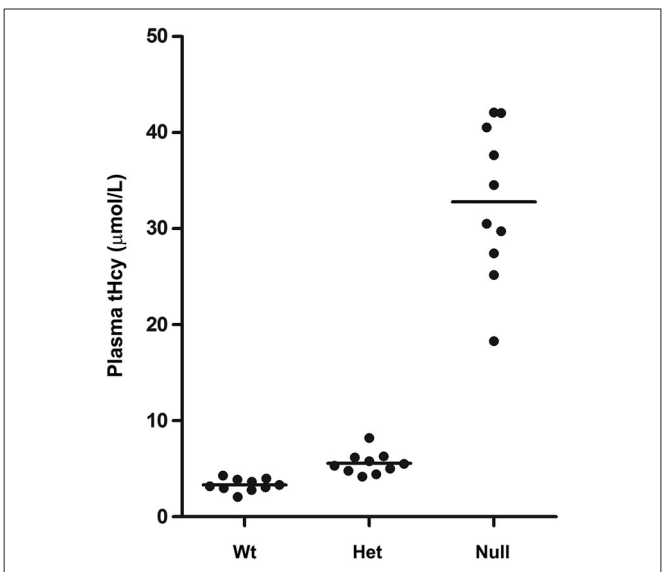

FIGURE 2 | Plasma tHcy levels in *Mthfr* knockout mice. Plasma tHcy levels were measured in 5-week-old wt $mthfr^{+/+}$, het $mthfr^{+/-}$ and null $mthfr^{-/-}$ mice (n = 10 mice/ group).

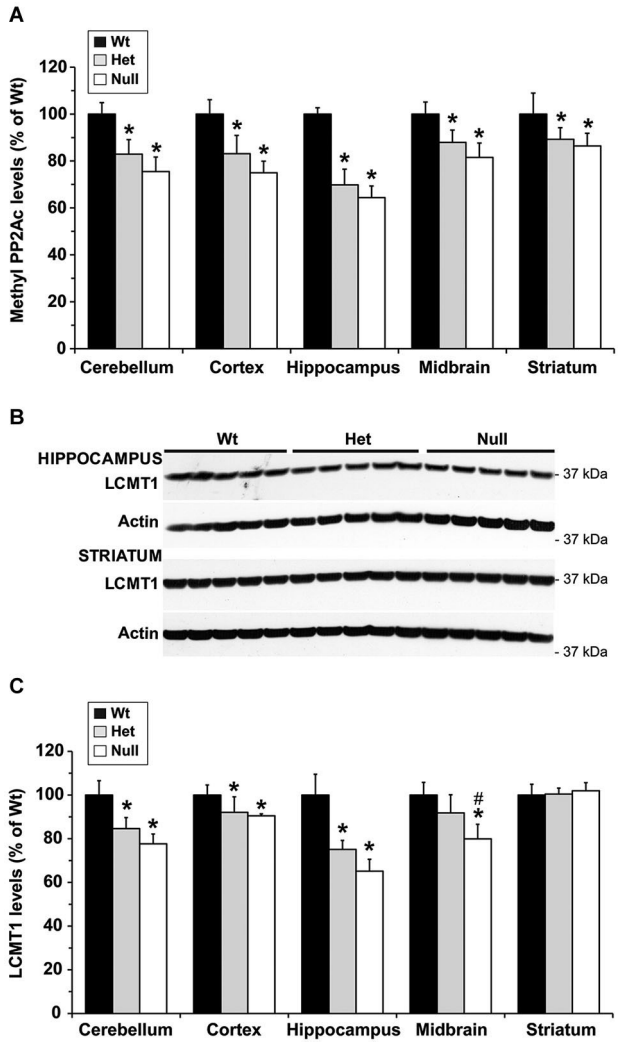

FIGURE 3 | Alterations in regional brain levels of methylated PP2Ac and LCMT1 in young mice models of mild and severe MTHFR deficiency. Regional brain homogenates from 5-week-old wt *mthfr*$^{+/+}$ (black bars), het *mthfr*$^{+/-}$ (gray bars), and null *mthfr*$^{-/-}$ (white bars) mice were analyzed by quantitative Western blotting for relative protein expression levels of methylated PP2Ac **(A)** and LCMT1 **(B,C)**. Values represent mean ± SD for 6 mice per group; * $p < 0.05$, het or null compared to wt mice; # $p < 0.05$, null vs. het mice. Representative LCMT1 blots of two brain regions are shown in **(B)**.

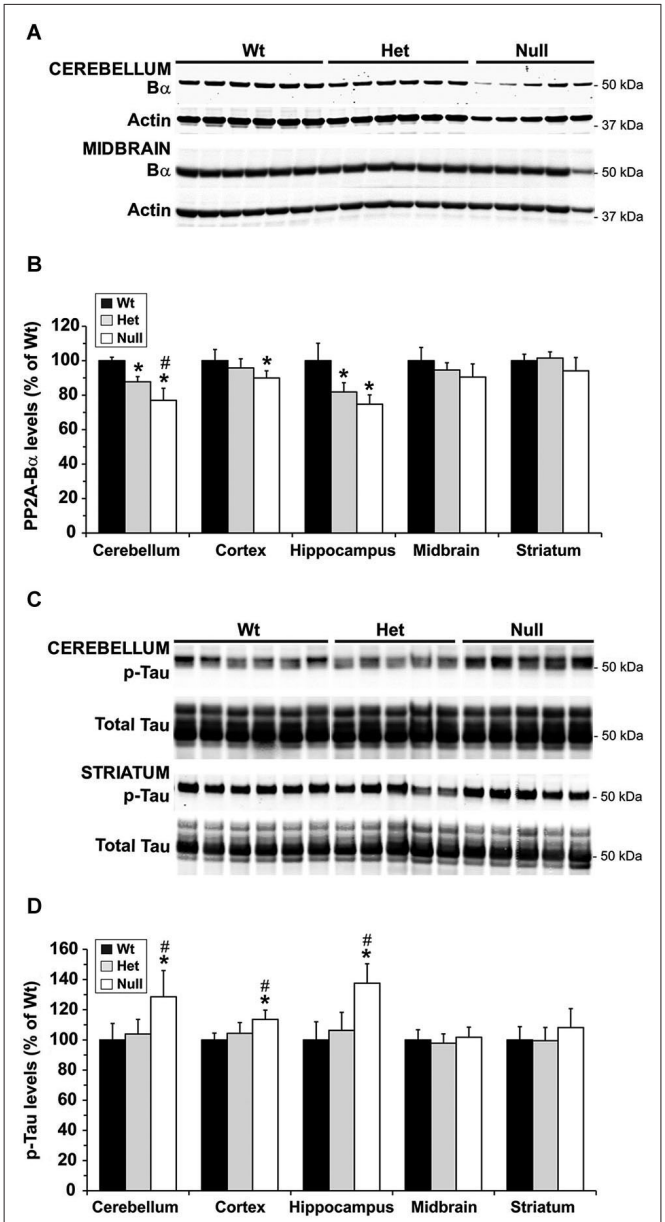

FIGURE 4 | Regional alterations in PP2A/Bα and p-Tau levels in young mice models of mild and severe MTHFR deficiency. Regional brain homogenates from the same mice described in **Figure 3** were comparatively analyzed by quantitative Western blotting for expression levels of PP2A/Bα **(A,B)** and Tau phosphorylated at the PHF-1 epitope **(C,D)**. Bar shading and symbols as in **Figure 3**. Representative blots of two regions are shown in **(A)** and **(C)**.

in line with the observation that knock down of LCMT1 results in a significant reduction in PP2Ac methylation (Lee and Pallas, 2007; Sontag et al., 2008; MacKay et al., 2013). Together, these results suggest that MTHFR deficiency affect LCMT1 and PP2A methylation in a brain region-specific manner.

SEVERE MTHFR DEFICIENCY IN YOUNG MICE INDUCES A DOWN REGULATION OF PP2A/Bα AND CONCOMITANT ENHANCEMENT OF p-Tau LEVELS IN THE HIPPOCAMPUS AND CEREBELLUM, AND TO A LESSER EXTENT, IN THE CORTEX

We and others have shown that, by reducing the formation of PP2A/Bα heterotrimers, down regulation of LCMT1 is accompanied with a preferential loss of endogenous PP2A/Bα isoforms (Lee and Pallas, 2007; Sontag et al., 2008). Likewise, the region-specific decrease in LCMT1 expression levels (**Figure 3C**) correlated with a similar reduction in PP2A/Bα expression levels (**Figures 4A,B**) in het and null *Mthfr* mice. As observed with LCMT1, the down regulation of PP2A/Bα expression levels was more prevalent in the hippocampal and cerebellar regions. Since the PP2A/Bα heterotrimer is a major Tau phosphatase

(Sontag et al., 1996; Xu et al., 2008), we next investigated whether the reduction in PP2A/Bα amounts correlated with enhanced phosphorylation of endogenous Tau at the AD-like p-Ser396/Ser404 PHF-1 epitope. Levels of p-Tau (PHF-1) in each brain region were similar in wt and het MTHFR mice (**Figures 4C,D**). However, relative to control wt mice, a ∼30–50% enhancement of Tau phosphorylation was observed in the hippocampus and cerebellum of null mice, the only regions wherein PP2A/Bα levels were markedly decreased (**Figure 4B**). A minor increase in p-Tau levels, which correlated with a smaller loss of PP2A/Bα (**Figure 4B**) was also observed in cortical homogenates of null mice. In contrast, relative to controls, there was no change in p-Tau or PP2A/Bα amounts measured in the midbrain and striatum. Together, these data point to an inverse relationship between PP2A/Bα and p-Tau levels. They also suggest that a certain threshold of LCMT1 and PP2A/Bα down regulation, which selectively occurs in the hippocampal, cerebellar and cortical regions of young null mice, is required to achieve a significant increase in Tau phosphorylation.

AGING WORSENS THE EFFECTS OF MILD MTHFR DEFICIENCY ON p-Tau IN SUSCEPTIBLE REGIONS

Notably, *Mthfr* polymorphisms have been identified as a risk factor for AD, an age-related disorder (Wang et al., 2005, 2008; Kageyama et al., 2008; Hua et al., 2011; Coppede et al., 2012; Mansouri et al., 2013). Thus, we next addressed the hypothesis that normal aging could aggravate the detrimental effects of MTHFR deficiency on LCMT1, PP2A methylation and p-Tau. To that end, we comparatively analyzed regional brain homogenates from 22-month-old wt and het *Mthfr* mice fed an amino acid defined diet with normal folate content (normal folate diet, NF). Null $Mthfr^{-/-}$ mice could not be used in these studies, since mice with severe MTHFR deficiency die prematurely as a result of atherosclerosis and other complications (Chen et al., 2001; Lawrance et al., 2011). Similar to the effects of MTHFR deficiency in young $Mthfr^{+/-}$ mice (**Figure 2**), plasma tHcy levels were increased by an average of ∼1.6-fold in old $Mthfr^{+/-}$ mice, relative to wt controls (**Figure 5A**). Besides promoting hyperhomocysteinemia, MTHFR deficiency leads to altered folate distribution and reduction in plasma and brain 5-MTHF levels (Chen et al., 2001; Ghandour et al., 2004). Accordingly, plasma 5-MTHF levels were significantly decreased in old het vs. wt animals (**Figure 5B**). These metabolic changes were associated with a marked decrease of PP2Ac methylation in all the brain regions examined from het mice (**Figure 6A**), reinforcing the hypothesis that alterations in plasma tHcy and 5-MTHF levels can lead to inhibition of SAM-dependent, LCMT1-mediated PP2A methylation in the brain. Relative to controls, there was also a small but statistically significant down regulation of LCMT1 protein expression levels in all brain regions examined from old het $MTHFR^{+/-}$ mice (**Figures 6B,C**); again, the loss of LCMT1 and methylated PP2Ac was more pronounced in the hippocampus. As observed in young $Mthfr^{+/-}$ het mice, reduced PP2A/Bα levels (**Figures 7A,B**) correlated with enhanced p-Tau phosphorylation at the PHF-1 epitope (**Figures 7C,D**) in the hippocampus, cerebellum and cortex from old het $Mthfr^{+/-}$ mice.

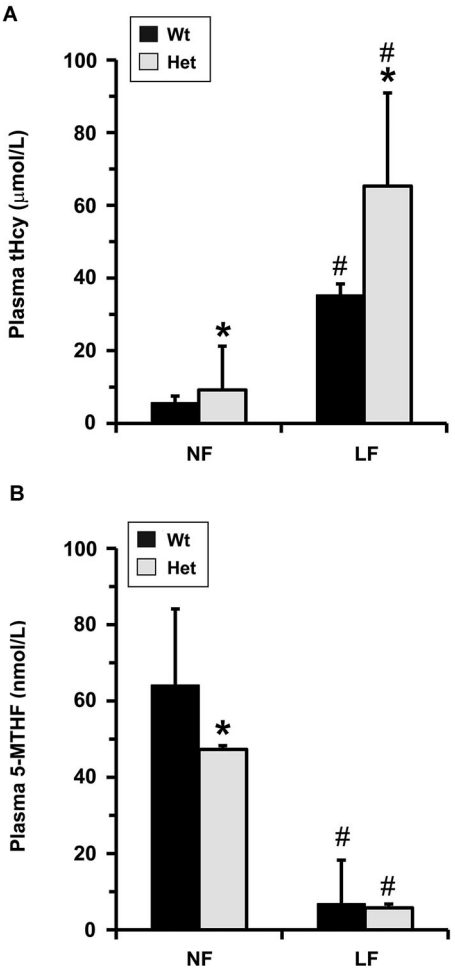

FIGURE 5 | Plasma metabolite levels in old mice models of mild MTHFR deficiency. Plasma tHcy **(A)** and 5-MTHF **(B)** levels were determined in 22-month-old wt $mthfr^{+/+}$ and het $mthfr^{+/-}$ mice that had been fed for 6 months an amino acid defined diet with either a normal folate (NF) or a low folate (LF) content. Values represent mean ± SD for 10 mice per group; * $p < 0.05$, het vs. wt in the same diet group; # $p < 0.05$, LF vs. NF in the same genotype.

Overall, our data indicate that the MTHFR genotype very similarly influenced LCMT1, methylated PP2Ac and PP2A/Bα protein expression levels in regional brain homogenates from young and old het $MTHFR^{+/-}$ mice, relative to their age-matched wt littermates. However, aging appeared to slightly worsen the effects of mild MTHFR deficiency on p-Tau, which selectively accumulated in the hippocampus, cerebellum and cortex of old, but not young het $Mthfr^{+/-}$ mice.

MILD MTHFR DEFICIENCY WORSENS THE EFFECTS OF FOLATE DEFICIENCY ON LCMT1 AND PP2A IN SUSCEPTIBLE BRAIN REGIONS

Interestingly, the relatively mild functional clinical consequences of *Mthfr* 677C→T polymorphisms are aggravated by deficiencies in dietary folate intake (Botto and Yang, 2000; Rozen, 2000; Schwahn and Rozen, 2001; Leclerc and Rozen, 2007). By

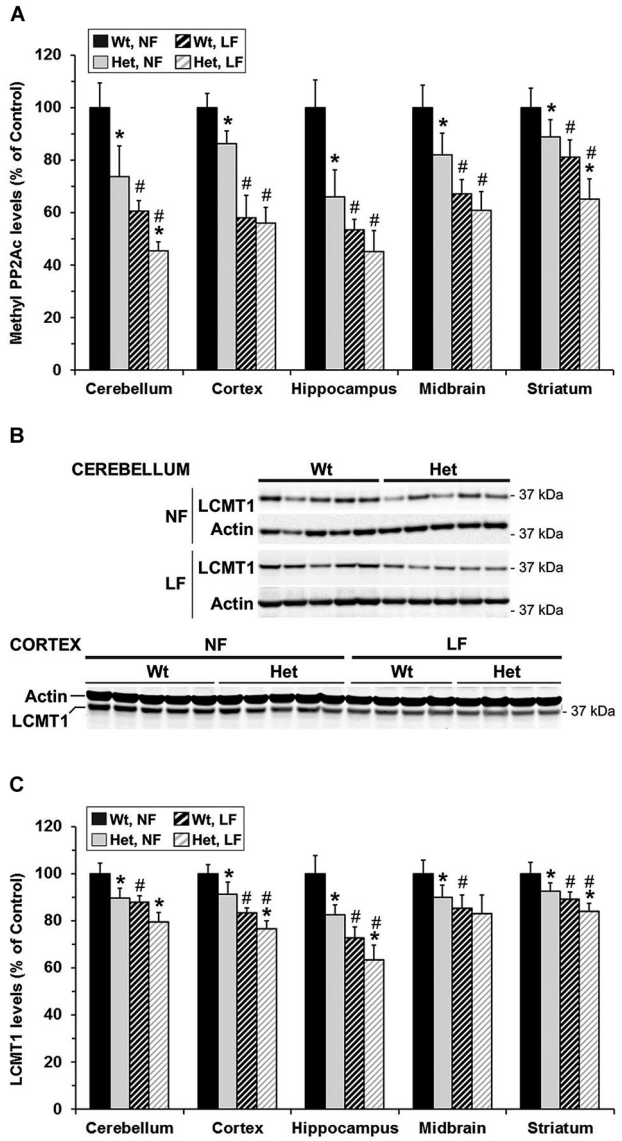

FIGURE 6 | Brain region-specific effects of folate and mild MTHFR deficiencies on PP2A methylation and LCMT1 expression levels. 22-month-old wt *mthfr*$^{+/+}$ and het *mthfr*$^{+/-}$ mice were fed for 6 months either a NF diet (Black bars, wt mice; gray bars, het mice) or a LF diet (hatched black bars, wt mice; hatched light gray bars, het mice). Relative methylated PP2Ac **(A)** and LCMT1 **(B,C)** protein expression levels were determined after quantitative Western blotting. Values represent mean ± SD for 9 mice per group; * $p < 0.05$, het vs. wt in the same diet group; # $p < 0.05$, LF vs. NF in the same genotype. Representative LCMT1 blots for two regions are shown in **(B)**.

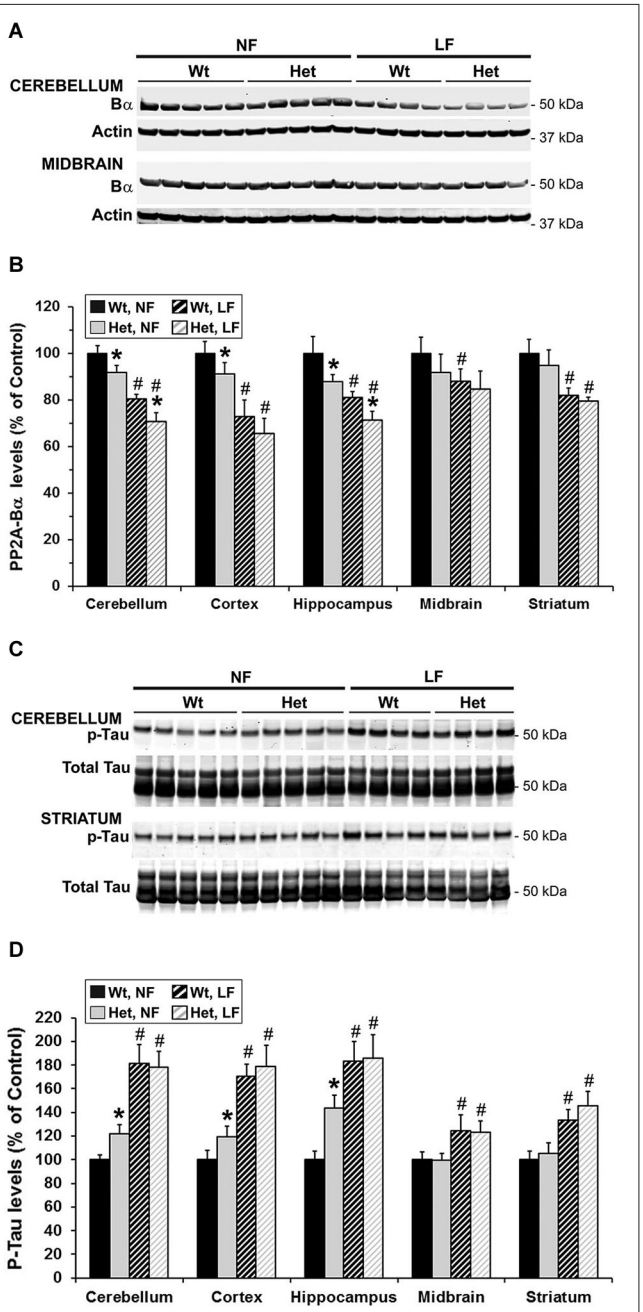

FIGURE 7 | Brain region-specific effects of folate and mild MTHFR deficiencies on PP2A/Bα and p-Tau expression levels. Regional brain homogenates from the same mice described in **Figure 6** were comparatively analyzed by quantitative Western blotting for relative protein expression levels of PP2A/Bα **(A,B)** and Tau phosphorylated at the PHF-1 epitope **(C–D)**. Bar shading and symbols as in **Figure 6**. Representative blots for two regions are shown in **(A)** and **(C)**.

functionally affecting the activity of methionine synthase, a key enzyme of the folate and methylation cycle (**Figure 1**), folate deficiency restricts Hcy metabolism, resulting in elevation of plasma tHcy levels (Fowler, 2005). Of particular significance, low folate status and elevated tHcy levels are independent risks factors for AD (Zhuo et al., 2011; Morris, 2012), and patients with AD have lower plasma folate levels (Lopes da Silva et al., 2014). Based on these critical observations, we examined whether dietary folate deficiency can affect the levels of LCMT1, PP2A and p-Tau levels in old mice, either by itself or in synergy with mild MTHFR deficiency. Low folate status was induced by feeding ∼16-month-old mice an amino acid defined diet with reduced folate content

(low folate diet, LF) for 6 months. As predicted, plasma tHcy levels were significantly increased (**Figure 5A**), and 5-MTHF levels were dramatically reduced (**Figure 5B**) in 22-month-old wt mice fed the LF diet, compared to age-matched control animals receiving the NF diet. Mild MTHFR deficiency in het *Mthfr*$^{+/-}$ mice aggravated the hyperhomocysteinenia induced by the LF diet (**Figure 5A**, het vs. wt mice). However, it failed to further affect plasma 5-MTHF levels (**Figure 5B**, het vs. wt mice), probably because those were already maximally decreased in response to the prolonged dietary folate deficiency. Comparative Western blot analyses of tissue homogenates from wt mice fed a NF or LF diets showed that folate deficiency alone led to a significant down regulation of methylated PP2Ac (**Figure 6A**), LCMT1 (**Figures 6B,C**) and PP2A/Bα (**Figures 7A,B**), and concomitant accumulation of p-Tau (**Figures 7C,D**) in all brain regions examined. Again, the LF diet-induced enhancement of Tau phosphorylation was especially prominent in the hippocampus, cerebellum and cortex. Comparative analysis of het mice showed that mild MTHFR deficiency further exacerbated the effects of folate deficiency on PP2Ac methylation (**Figure 6A**), LCMT1 (**Figure 6C**) and PP2A/Bα expression levels (**Figure 7B**) in a brain region-specific manner. Despite inducing a greater reduction in the amounts of PP2A/Bα levels in susceptible regions (hippocampus, cerebellum), there was no apparent effect of the *Mthfr*$^{+/-}$ genotype on p-Tau levels in mice fed the LF diet (**Figures 7C,D**). This suggests that endogenous Tau phosphorylation had already peaked to its maximum level in response to the dietary folate deficiency alone.

DISCUSSION

SEVERE MTHFR DEFICIENCY AFFECTS LCMT1-DEPENDENT PP2A METHYLATION, PP2A/Bα AND p-Tau EXPRESSION LEVELS IN THE HIPPOCAMPUS, CEREBELLUM AND CORTEX

It is well established that disturbances in folate metabolism exert a detrimental effect on the brain by affecting the supply of methyl groups that are critical for normal homeostasis; indeed, individuals with severe MTHFR deficiency have a variety of neurological problems and decreased mental abilities (Watkins and Rosenblatt, 2012). Null *Mthfr*$^{-/-}$ mice have been found to have abnormalities in the size and/or structure of the cerebellum, cortex and hippocampus, and to exhibit memory impairment and other behavioral anomalies reminiscent of those encountered in patients with severe MTHFR deficiency (Chen et al., 2001, 2005; Jadavji et al., 2012). Here, using *Mthfr*$^{-/-}$ knockout mice, we show for the first time that severe MTHFR deficiency can impair LCMT1-dependent PP2A methylation. PP2A is a family of abundant brain Ser/Thr phosphatases that collectively participate in nearly all aspects of neuronal homeostasis. Of particular interest, biogenesis of major PP2A/Bα isoforms that are primary brain enzymes that dephosphorylate Tau, is critically influenced by LCMT1-mediated PP2Ac methylation (Lee and Pallas, 2007; Sontag et al., 2008). Decreased PP2A methylation and PP2A/Bα expression levels correlate with enhanced tau phosphorylation in several mouse models of altered one-carbon metabolism Reviewed in Sontag and Sontag (2014) and in diabetic mice (Papon et al., 2013). Notably, altered phosphorylation of Tau is a central pathological event believed to initiate Tau aggregation and dysfunction, ultimately resulting in neurodegeneration and cognitive decline in AD and other tauopathies. There is also substantial evidence that the accumulation of p-Tau is a central mediator of amyloid-β toxicity and synaptic deficits in AD (Liao et al., 2014). Significantly, we demonstrate *in vivo* that severe MTHFR deficiency is associated with down regulation of LCMT1 and PP2A/Bα, and concomitant enhancement of Tau phosphorylated at the AD-like PHF-1 epitope in the hippocampus and cerebellum, and to a lesser extent in the cortex. Alterations in p-Tau in the hippocampus, a region critically involved in spatial learning and memory, could contribute to some of the cognitive deficits previously reported in *Mthfr*$^{-/-}$ mice (Chen et al., 2001, 2005; Jadavji et al., 2012). Because of the prominent position of PP2A in neuronal signaling, deregulation of LCMT1-dependent PP2A methylation also provides a novel insight into the mechanisms by which MTHFR deficiency can negatively impact neuronal function in specific susceptible regions (hippocampus, cerebellum, cortex) identified in earlier *Mthfr*$^{-/-}$ mouse studies (Chen et al., 2001, 2005; Jadavji et al., 2012). Interestingly, the hippocampus and cortex are especially vulnerable to AD-related pathological p-Tau changes, while the cerebellum is traditionally spared in this neurodegenerative disorder. Accordingly, using a large series of neuropathologically confirmed autopsy cases of AD, we have shown that down regulation of LCMT1, PP2Ac methylation (Sontag et al., 2004a) and PP2A/Bα (Sontag et al., 2004b) occurs in AD-affected cortical and hippocampal regions, where it correlates with the accumulation of p-Tau. In contrast, we have not observed a concomitant loss of these enzymes in the AD cerebellum, a region wherein neuronal p-Tau lesions do not accumulate. Yet, down regulation of PP2A methylation in wt mice increases cerebellar p-Tau levels (Bottiglieri et al., 2012) and excitoxicity influences PP2A-dependent tau phosphorylation in cerebellar granule cells (Kuszczyk et al., 2009), further demonstrating the tau regulatory function of PP2A in the cerebellum. While MTHFR deficiency-induced cerebellar p-Tau changes may not directly relate to the neurodegenerative process in AD, it is worth mentioning that low concentrations of 5-MTHF in the cerebrospinal fluid have been linked to cerebellar atrophy and neurological disorders in children (Grapp et al., 2012).

We noticed that severe MTHFR deficiency affected PP2Ac methylation in all brain regions, albeit with distinct intensity. Yet, this did not automatically translate into the loss of PP2A/Bα, which better correlated with the down regulation of LCMT1. These data suggest the existence of additional brain-region specific regulation of these enzymes- for instance transcription, synthesis, degradation- and/or compensatory mechanisms that will need to be investigated in future studies. Alternatively, a certain threshold loss of LCMT1 activity/expression and PP2Ac methylation may need to be reached to observe an effect on PP2A/Bα expression levels. Inherent limitations of quantitative Western blotting may preclude the measurement of subtle changes in the expression of these PP2A isoforms. Moreover, while both down regulation of SAM-dependent LCMT1 activity and LCMT1 protein expression levels can inhibit PP2Ac methylation (Sontag et al., 2008), alterations in the protein amounts of PME-1, the dedicated PP2A methylesterase, could also contribute to the observed accumulation of demethylated PP2Ac. For instance,

previous studies have shown that incubation of primary neurons with folate antagonists can induce an up-regulation of PME-1 that correlates with an increase of endogenous demethylated PP2A (Yoon et al., 2007). We have also observed changes in PME-1 levels in wt mice fed for 2 months on a folate deficient diet (Sontag et al., 2008). Unfortunately, due to shortage of tissue material, we were unable in the present study to measure expression levels of PME-1 in corresponding brain regions of our *mthfr* knockouts.

MILD MTHFR DEFICIENCY AFFECTS LCMT1, PP2A METHYLATION, PP2A/Bα AND p-Tau (PHF-1) IN A BRAIN-REGION SPECIFIC MANNER

There is substantial evidence that common single human *Mthfr* polymorphisms that have a frequency of up to 50% in certain populations (Zappacosta et al., 2014), modify the risk for numerous diseases (Nazki et al., 2014). Of particular interest here, *Mthfr* polymorphisms that result in mild MTHFR deficiency have been identified as a risk factor for AD in selected populations (Wang et al., 2005, 2008; Kageyama et al., 2008; Hua et al., 2011; Coppede et al., 2012; Mansouri et al., 2013). Moreover, epigenetic mechanisms involving *Mthfr* and altered methylation homeostasis may predispose to development of late-onset AD (Wang et al., 2008). Interestingly, we found that mild MTHFR deficiency was able to induce a variable down regulation of PP2Ac methylation in all brain regions examined, and the intensity of these effects was comparable in young or old $Mthfr^{+/-}$ mice, relative to age-matched wt littermates. A similar small loss in LCMT1 protein levels was observed in the hippocampal, cerebellar and cortical regions of young and old *Mthfr* knockouts. In 22-month-old $Mthfr^{+/-}$ mice, reduced expression of PP2A/Bα again correlated with increased levels of p-Tau, in agreement with the role of this isoform as a primary Tau phosphatase. In contrast, we were unable to detect an increase in Tau phosphorylation at the PHF-1 epitope in young $Mthfr^{+/-}$ animals, relative to controls, despite the small decrease in PP2A/Bα levels observed in the hippocampus and cerebellum. It is possible that Tau protein Ser/Thr kinases could compensate for the small regional decrease of PP2A/Bα and maintain a steady state of Tau phosphorylation in the brain of 5-week-old mice. Those compensatory mechanisms might become compromised with aging, or the small loss of PP2A/Bα could have a compounding effect with time, resulting in the increased tau phosphorylation observed in older $Mthfr^{+/-}$ mice. Another alternative is that Tau could become phosphorylated at other epitopes not studied here, due to the lack of sufficient amounts of regional brain tissue. The PHF-1 is considered a late phospho-Tau epitope in AD, and PP2A methylation regulates many other "earlier" Tau phosphorylation sites (Sontag et al., 2007) that could be potentially sensitive to the effects of mild MTHFR deficiency in young animals. However, we were unable to detect increased phosphorylation of Tau at pSer422 in cortical homogenates from young het *Mthfr* mice (data not shown).

INTERACTIONS BETWEEN THE *Mthfr* GENOTYPE AND DIETARY FOLATE DEFICIENCY DIFFERENTIALLY AFFECT LCMT1, PP2A AND p-Tau IN DISTINCT BRAIN REGIONS

We have previously reported that LCMT1, PP2Ac methylation and PP2A/Bα become down regulated, and Tau phosphorylation is increased in the cerebellum and cortex, and to a lesser extent in the midbrain and striatum of 4-week-old wt mice fed for 2 months a LF diet (Sontag et al., 2008). Here, we found similar results in 22-month-old wt mice that had been fed for 6 months a LF diet (**Figures 6**, **7**), except that the prolonged diet induced more intense effects. We also show here that the hippocampus is especially sensitive to the effects of dietary folate deficiency, which resulted in nearly 30% loss of LCMT1, 50% loss of methylated PP2Ac, and concomitant doubling of p-Tau (PHF-1) levels in wt mice fed the LF diet, relative to control animals fed the NF diet. Whether down regulation of LCMT1 results either from prolonged inhibition of its methyltransferase activity -due to hyperhomocysteinemia and decreased cellular methylation potential- or other regulatory mechanisms remains to be defined. Of note, folate deficiency aggravated the negative effects of mild MTHFR deficiency on LCMT1 and PP2A methylation in a region-specific manner. Dietary folate deficiency also synergized with the $Mthfr^{+/-}$ genotype to further exacerbate the loss of PP2A/Bα in the hippocampus and cerebellum. However, this did not lead to a further enhancement of p-Tau levels in these regions, probably because p-Tau was already maximally increased in response to the LF diet alone. Altogether, these findings suggest brain-region specific responses to the effects of both dietary folate and MTHFR deficiencies. Further studies will be required to determine whether those are related to either differential expression patterns of all proteins involved, or compensatory mechanisms. Nevertheless, our findings clearly establish deregulated LCMT1 and PP2A as novel brain intermediates of the detrimental effects of MTHFR deficiency. Our results also unveil for the first time a link between MTHFR deficiency and deregulation of Tau phosphorylation, which could explain -at least in part- why epidemiological studies have identified *Mthfr* polymorphisms as risk factors for AD. Our experimental data also reinforce the notion that this risk may be modulated by folate intake. Significantly, the age-related decline of brain 5-MTHF levels (Bottiglieri et al., 2000) is associated with increased levels of p-Tau and cognitive decline (Herrmann and Obeid, 2007). Besides promoting tau phosphorylation, down regulation of LCMT1 and PP2A methylation can also affect amyloid protein precursor phosphorylation and processing (Sontag et al., 2007). Whether MTHFR polymorphisms also influence AD risk by altering amyloidogenesis remains to be investigated.

Due to the critical and ubiquitous cellular functions of PP2A enzymes, MTHFR-mediated PP2A dysfunction is likely to have functional consequences reaching far beyond the scope of Tau deregulation and AD. For instance, *Mthfr* polymorphisms influence the risk for many other neurological, vascular and developmental disorders that affect the CNS, as well as the pharmacodynamics of antifolates and effectiveness and toxicity of many therapeutic drugs (Schwahn and Rozen, 2001).

AUTHOR CONTRIBUTIONS

Jean-Marie Sontag, Estelle Sontag and Teodoro Bottiglieri participated in the design and conceptualization of the study, analysis and interpretation of data, and editing of the manuscript for intellectual content.

Estelle Sontag wrote the manuscript; Jean-Marie Sontag, Goce Taleski and Josephine Smith participated in the acquisition and interpretation of Western blot data; Brandi Wasek did all the experimental mouse work; Erland Arning and Brandi Wasek performed metabolite analyses.

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Methylenetetrahydrofolate Reductase Deficiency Deregulates Regional Brain Amyloid-β Protein Precursor Expression and Phosphorylation Levels

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Abstract. Deregulation of the amyloid-β protein precursor (AβPP) plays a critical role in the neurodegenerative cascade of Alzheimer's disease (AD). Significantly, common functional polymorphisms in the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene are a risk factor for the development of late-onset AD. Reduced MTHFR activity is associated with alterations in folate and homocysteine metabolism. Here, we first show that in young *MTHFR* knockout mice, mild and severe MTHFR deficiency markedly increase cortical and hippocampal AβPP phosphorylation at the regulatory Thr668 site. However, the hippocampus is especially vulnerable to the effects of aging and mild MTHFR deficiency. Notably, the effects of severe MTHFR deficiency in young mice are recapitulated by prolonged dietary folate deficiency in old mice, which leads to regional brain accumulation of cystathionine due to impaired methylation of homocysteine. The incremental AβPP phosphorylation at Thr668 mediated by severe genetic-or diet-induced impairment of the folate cycle correlates with enhanced accumulation of demethylated protein phosphatase 2A (PP2A), and activation of glycogen synthase kinase-3β (GSK-3β). Lastly, we show that severe disturbances in folate metabolism can also affect AβPP expression levels in a brain region specific manner. Together our findings identify a novel link between genetic MTHFR deficiency, activation of GSK-3β, demethylation of PP2A, and enhanced phosphorylation of AβPP at Thr668, which is known to critically influence neuronal AβPP function and pathological amyloidogenic processing. Deregulation of AβPP provides a novel mechanism by which common human *MTHFR* polymorphisms may interact with dietary folate deficiency to alter neuronal homeostasis and increase the risk for sporadic AD.

Keywords: Alzheimer's disease, amyloid-β protein precursor, cystathionine, folate, glycogen synthase kinase 3β, methylation, MTHFR, phosphorylation, protein phosphatase 2A

INTRODUCTION

The metabolically active form of folate called 5-methyltetrahydrofolate (5-MTHF) is a critical cofactor in one-carbon metabolic networks that integrate

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nutrient input from the environment to regulate many cellular processes essential for neuronal homeostasis. Folate-dependent single-carbon reactions support amino acid metabolism, protein methylation, epigenetic maintenance, redox balance, and a variety of critical biosynthetic pathways involved in the generation of DNA, RNA, membrane lipids, and neurotransmitters [1]. Not surprisingly, deregulation of folate metabolism is associated with an array of neurological disorders. Of particular interest, several epidemiological studies have linked alterations in one-carbon metabolism with an increased risk for cognitive decline, brain atrophy and Alzheimer's disease (AD) [2]. Disturbances in folate metabolism exert damaging effects in the AD brain, in part by inducing a deficit in the supply of methyl groups necessary for protein methylation and epigenetic modifications [3, 4]. Altered folate metabolism is also typically associated with elevation of plasma total homocysteine (tHcy) levels, which exerts direct neurotoxic effects via several mechanisms, including oxidative damage and excitotoxicity [2]. Low folate status and hyperhomocysteinemia have been established as independent risk factors for AD [5, 6]. Recent meta-analyses further support a causal link between low folate status, elevated tHcy levels and AD [7, 8].

The most common genetic cause of hyperhomocysteinemia in human is due to polymorphisms in the *MTHFR* gene encoding 5,10-methylenetetrahydrofolate reductase (MTHFR) [9]. MTHFR is an important enzyme that catalyzes the conversion of the 5,10-methylenetetrahydrofolate metabolite derived from dietary folate into 5-MTHF, which functions as a methyl-group donor involved in the conversion of homocysteine to methionine. In turn, methionine is an important precursor of S-adenosylmethionine (SAM), the methyl donating compound for enzymatic transmethylation reactions [1] (Fig. 1). Thus, normal MTHFR enzyme activity is crucial to maintain the pool of circulating 5-MTHF and methionine, and to prevent the accumulation of tHcy. Indeed, the *MTHFR* 677C⟶T allele is associated with a ∼40% reduced MTHFR enzymatic activity, decreased concentrations of circulating folate and mildly increased plasma tHcy levels, especially when folate levels are low. The more severe TT genotype, which decreases MTHFR activity by ∼70%, leads to reduced methionine synthase activity, hyperhomocysteinemia [10, 11] and elevated serum cystathionine levels [12, 13] (Fig. 1).

Besides its role in the cellular methylation cycle, 5-MTHF displays high antioxidant activity via its

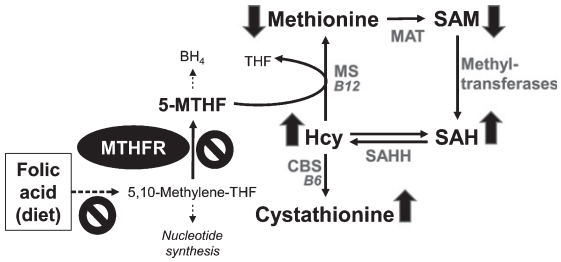

Fig. 1. Schematic diagram showing how severe alterations in folate metabolism can lead to deregulation of key metabolites of the methylation cycle in the brain. MTHFR catalyzes the conversion of the 5,10-methylenetetrahydrofolate metabolite derived from dietary folic acid into 5-MTHF. Genetic *MTHFR* and dietary folate deficiencies can lead to decreased levels of 5-MTHF, thereby decreasing the remethylation of Hcy to methionine. Severe remethylation deficits have been linked to decreased production of methionine and SAM, the universal methyl donor. They promote accumulation of Hcy, which can either be converted back to SAH, a potent inhibitor of methyltransferases, or condense with serine to form cystathionine. 5-MTHF deficiency also decreases the bioavailability of BH_4, an important cofactor for enzymes involved in nitric oxide production and synthesis of monoaminergic neurotransmitters. BH_4, tetrahydrobiopterin; CBS, cystathionine-β-synthase; Hcy, homocysteine; MAT, methionine adenosine transferase; MS, methionine synthase; MTHF, methyltetrahydrofolate; SAH, S-adenosylhomocysteine; SAHH, SAH hydrolase; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

ability to directly scavenge free radicals. It also serves indirect antioxidant function in cells, for instance by preventing the oxidation and enhancing the bioavailability of tetrahydrobiopterin (BH_4), an essential cofactor for nitric oxide synthase (NOS) activity; BH_4 enhances NOS coupling and nitric oxide (NO) production, thereby reducing intracellular superoxide generation due to uncoupled NOS [14]. Indeed, altered 5-MTHF status has been associated with oxidative stress and NO-mediated dysfunction of the endothelium in the cerebral vasculature [2, 14]. Interestingly, BH_4 is also a vital co-factor for synthesis of monoamine neurotransmitters such as serotonin, so that severe 5-MTHF deficiency plays a role in the pathophysiology of depression [1, 15].

Significantly, the common human *MTHFR* C677T polymorphism is linked with increased risk for late-onset AD [16–18]. Yet, the molecular mechanisms by which altered MTHFR activity may contribute to AD pathogenesis are not fully elucidated. The abnormal accumulation of oligomers of amyloid-β (Aβ) peptides derived from β- and γ-secretase cleavage of the amyloid-β protein precursor (AβPP) is believed to trigger the neurodegenerative cascade in AD [19]. Interestingly, hyperhomocysteinemia induced either by cystathionine-β-synthase *(Cbs)* [20] or dietary B-vitamin [21, 22] deficiencies increase amyloidoge-

nesis in AβPP mouse models of AD. Many studies in a wide range of models have shown that Aβ production and neurodegeneration are promoted by AβPP phosphorylation at the Threonine 668 (Thr668) site [23–30]. AβPP phosphorylation at Thr668 is elevated in the brain from hyperhomocysteinemic *Cbs* mouse models [31] and homocysteine-injected rats [32], supporting an important link between deregulation of homocysteine metabolism and AβPP phosphorylation at Thr668.

Based on these observations, we examined here whether genetic-induced mild or severe MTHFR deficiency is sufficient to influence AβPP phosphorylation at Thr668 in specific brain regions from well-characterized young and old *MTHFR* knockout mice. We also assessed whether the effects of mild MTHFR deficiency could be modulated by dietary folate deficiency in old mouse cohorts.

MATERIALS AND METHODS

Experimental mouse models

The brain tissue homogenates analyzed here were obtained from a previous study performed in wild-type (WT) $MTHFR^{+/+}$, heterozygous (HET) $MTHFR^{+/-}$, and homozygous (NULL) $MTHFR^{-/-}$ female mice [33]. Briefly, regional brain homogenates were obtained from 5-week old WT, HET and NULL mice that had been fed with a control diet containing 6.7 mg/kg folate (NF diet; Harlan Teklad, Madison WI). In addition, 16-month-old WT and HET mice were fed for 6 months with either the NF diet or a low folate diet (LF diet; Harlan Teklad, Madison, WI) containing only 0.2 mg/kg folate, prior to sacrifice. For this experiment in old mice, NF and LF diets also contained succinylsulfathiazole (10 mg/kg) to inhibit gastrointestinal bacterial growth and prevent absorption of folate from this source. Plasma tHcy was determined by high pressure liquid chromatography with fluorescence detection [33] and plasma 5-MTHF was determined by stable-isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described [34]. Regional brain cystathionine levels were measured by LC-MS/MS as previously described [35].

Total homogenates were prepared from the cortex, hippocampus, cerebellum, or midbrain mouse brain regions at a ratio of 1 g tissue/10 ml buffer [Tris 25 mM pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 0.5 μM okadaic acid, 5 mM PMSF, 1% NP-40, containing a cocktail of protease and phosphatase inhibitors]. The samples were briefly sonicated and centrifuged at 13,000 *g* to remove insoluble material, and the supernatant flash frozen and stored at –80°C. Unless indicated, all chemicals used in this study were obtained from Sigma-Aldrich.

Western blot analyses of regional brain homogenates

Protein concentration was determined using the Bradford protein assay kit™ (BIO-RAD). Duplicate aliquots (~50 μg proteins/lane) were separated on NU-PAGE Bis-Tris 4%–12% gradient midi gels (ThermoFisher Scientific). Proteins were transferred overnight onto nitrocellulose membranes, and analyzed by western blot following standard protocols (LI-COR Biosciences, Millennium Science, VIC, Australia). Blots were first probed with rabbit anti-phospho-AβPP (Thr668) antibodies (Cell Signaling Technology #6986 or ThermoFisher Scientific #44-336 G). In some experiments, blots were directly re-probed with mouse anti-AβPP 22C11 antibodies to assess total AβPP expression levels (ThermoFisher Scientific #14-9749-80). In most experiments, duplicate blots were analyzed with rabbit anti-AβPP antibodies (ThermoFisher Scientific # 36–6900 or Cell Signaling Technology #2452). Protein phosphatase 2A (PP2A) methylation status was determined as previously reported [31] using mouse antibodies directed against demethylated (clone1D6, Merck Millipore), methylated (clone 2A10, Merck Millipore) and total (clone 46, BD Biosciences) PP2A catalytic subunit (PP2Ac). Inhibitory phosphorylation of glycogen synthase kinase-3β (GSK-3β) at Ser9 was assessed using rabbit anti phospho-GSK-3β (Ser9) (clone D85E12) and re-blotting with mouse anti-GSK-3β antibodies (clone 3D10; Cell Signaling Technology). Re-probing with antibodies directed either against α-actin (clone 4, MAB1501, Merck Millipore) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; clone 6C5, MAB374, Merck Millipore) was used to normalize for protein loading. Precision Plus Protein™ Standards (BIO-RAD) were used as molecular weight standards. Western blotting was performed using Infrared IRDye®-labelled secondary antibodies and 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. Total AβPP protein levels were determined

after normalization for GAPDH or actin. The levels of phosphorylated AβPP were determined after normalization for protein loading and total AβPP levels.

Statistical analyses

Data are presented as mean values ± SD. Differences in AβPP phosphorylation and/or expression levels were analyzed using one-way ANOVA with Dunnett's test. Differences with p-values < 0.05 were considered statistically significant.

RESULTS

MTHFR deficiency is associated with brain region specific elevation of AβPP Thr668 phosphorylation in young transgenic MTHFR knockout mice

To test the hypothesis that genetic alterations in MTHFR activity can affect the regulation of AβPP, we first analyzed tissue homogenates from different brain regions obtained from young transgenic *MTHFR* knockout mice fed a normal folate-containing diet (NF diet). Mouse models with one (heterozygous $MTHFR^{+/-}$, HET) or no (homozygous $MTHFR^{-/-}$, NULL) *MTHFR* allele 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 [36–39]. It has been demonstrated that levels of 5-MTHF are decreased in both the plasma and brain of MTHFR deficient mice, which results in elevation of tHcy and concomitant alterations in metabolites related to the methylation cycle [36, 37] (Fig. 1). According to previously published data [36], the 5-week-old HET mice used in this study had significant but mildly elevated tHcy levels, a ∼1.6-fold increase over basal levels measured in their wild-type ($MTHFR^{+/+}$, WT) littermates (Table 1). Compared to age-matched WT mice, NULL mice were hyperhomocysteinemic, showing a ∼10-fold increase in tHcy levels (Table 1).

Western blot analysis of regional brain homogenates revealed that, relative to WT animals, Thr668-phosphorylated AβPP (pThr668-AβPP) levels were significantly increased in the cortex, hippocampus and midbrain, but not in the cerebellum, of both HET and NULL mice (Fig. 2).

These results suggest that, in brain regions susceptible to AD-related neurodegenerative processes, mild MTHFR deficiency in young mice is sufficient to induce the deregulation of signaling enzymes that control neuronal AβPP phosphorylation state at Thr668.

Mild MTHFR deficiency is associated with enhanced pThr668-AβPP levels in the hippocampus of old mice

Since the *MTHFR* C677T polymorphism is a risk factor for AD [17, 18], and sporadic AD is primarily an age-related disorder, we also analyzed AβPP phosphorylation state in 22-month-old WT and HET *MTHFR* mice that had been fed a NF diet. Of note, aged NULL mice could not be studied here due to known premature mortality [38, 39]. As a consequence of decreased MTHFR activity, plasma total folate levels were expectedly reduced ($p < 0.05$) in 22-month-old HET mice, compared to age-matched WT mice fed the same NF diet (Table 1). Mild MTHFR deficiency in old HET mice also correlated with a small elevation of plasma tHcy ($p < 0.01$) levels, relative to WT controls. Interestingly, we noticed 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 cohorts (Table 1), suggesting an effect of aging on this metabolite.

As observed in young animals (Fig. 2), the $MTHFR^{+/-}$ genotype had no effect on pThr668-AβPP levels in the cerebellum of aged mice (Fig. 3). Comparable amounts of pThr668-AβPP accumulated in hippocampal homogenates from old (Fig. 3) and young (Fig. 2) HET mice, relative to WT con-

Table 1
Regional brain metabolites in young and old *MTHFR* mice (n = 8–10/group) fed a normal folate (NF) or low folate (LF) diet

	MTHFR mice, female, 5-week-old			MTHFR mice, female, 22-month-old			
Genotype	WT	HET	NULL	WT	HET	WT	HET
Diet	NF	NF	NF	NF	NF	LF	LF
Plasma tHcy (μmol/L)	3.3 ± 0.7	5.6 ± 1.2*	32.8 ± 8*	5.6 ± 1.9	9.2 ± 3.1*	35 ± 12#	65 ± 26*,#
Plasma 5-MTHF (nmol/L)	ND	ND	ND	64 ± 20	47 ± 12*	6.8 ± 1.6#	5.8 ± 2.1#

ND, not determined; *$p < 0.05$, HET or NULL mice, relative to WT in same diet group; #$p < 0.05$, LF versus NF in the same genotype.

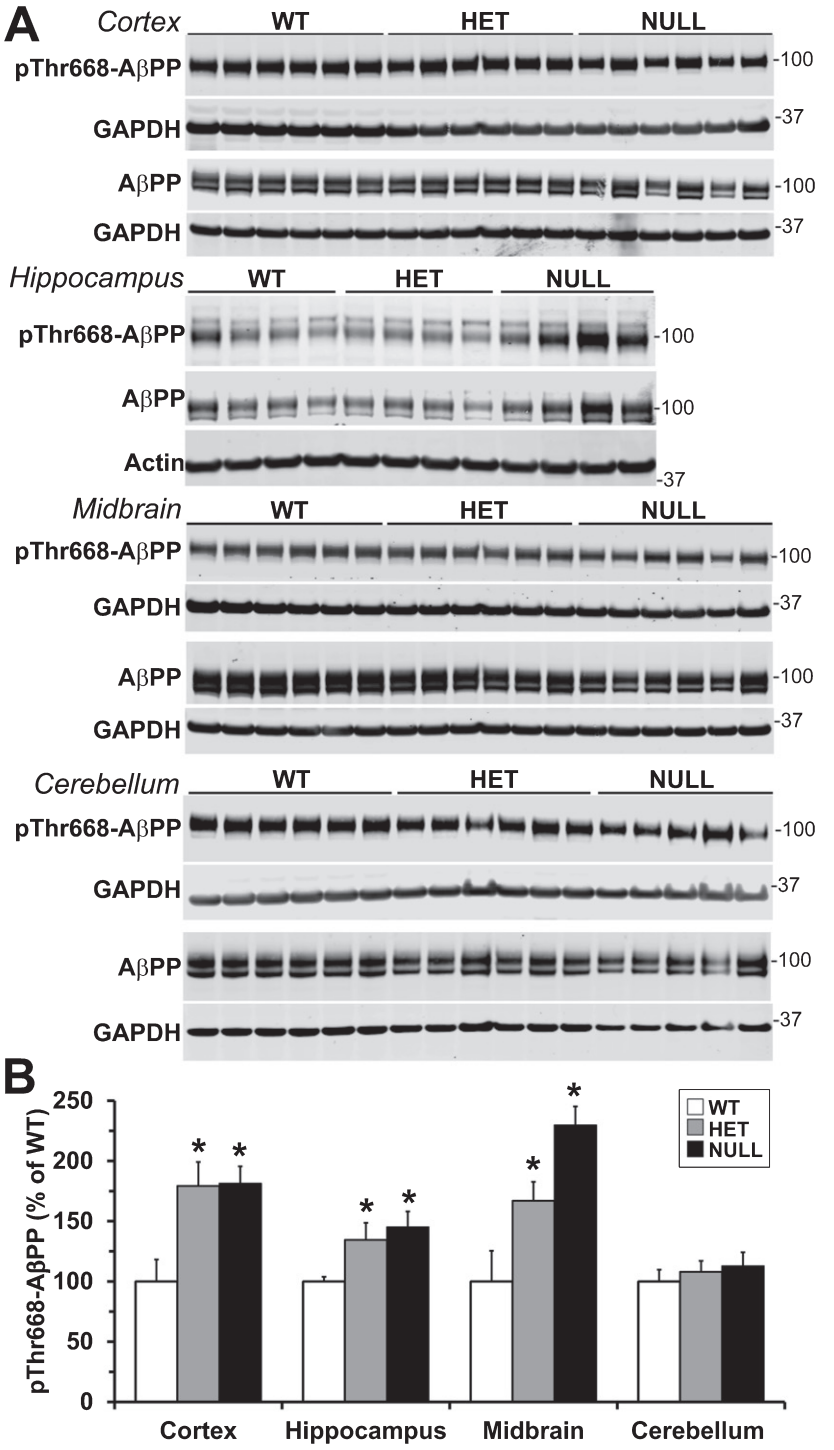

Fig. 2. Brain region-specific effects of mild and severe MTHFR deficiencies on AβPP phosphorylation at Thr668 in young *MTHFR* knockout mice. Regional brain homogenates were prepared from 5-week-old WT $MTHFR^{+/+}$ (white bars), HET $MTHFR^{+/-}$ (gray bars), and NULL $MTHFR^{-/-}$ (black bars) mice fed a normal folate-containing diet. Duplicate aliquots of total extracts were analyzed by western blot for the relative expression of total AβPP and AβPP phosphorylated at Thr668 (pThr668-AβPP). GAPDH or actin were used to normalise each blot for protein loading. A) Representative immunoblots. B) Relative pThr668-AβPP expression levels were quantified after densitometric analysis of the blots and normalization for total AβPP expression levels. Values represent mean ± SD for $n = 6$ mice/group; $^{*}p < 0.05$, HET or NULL compared to WT mice.

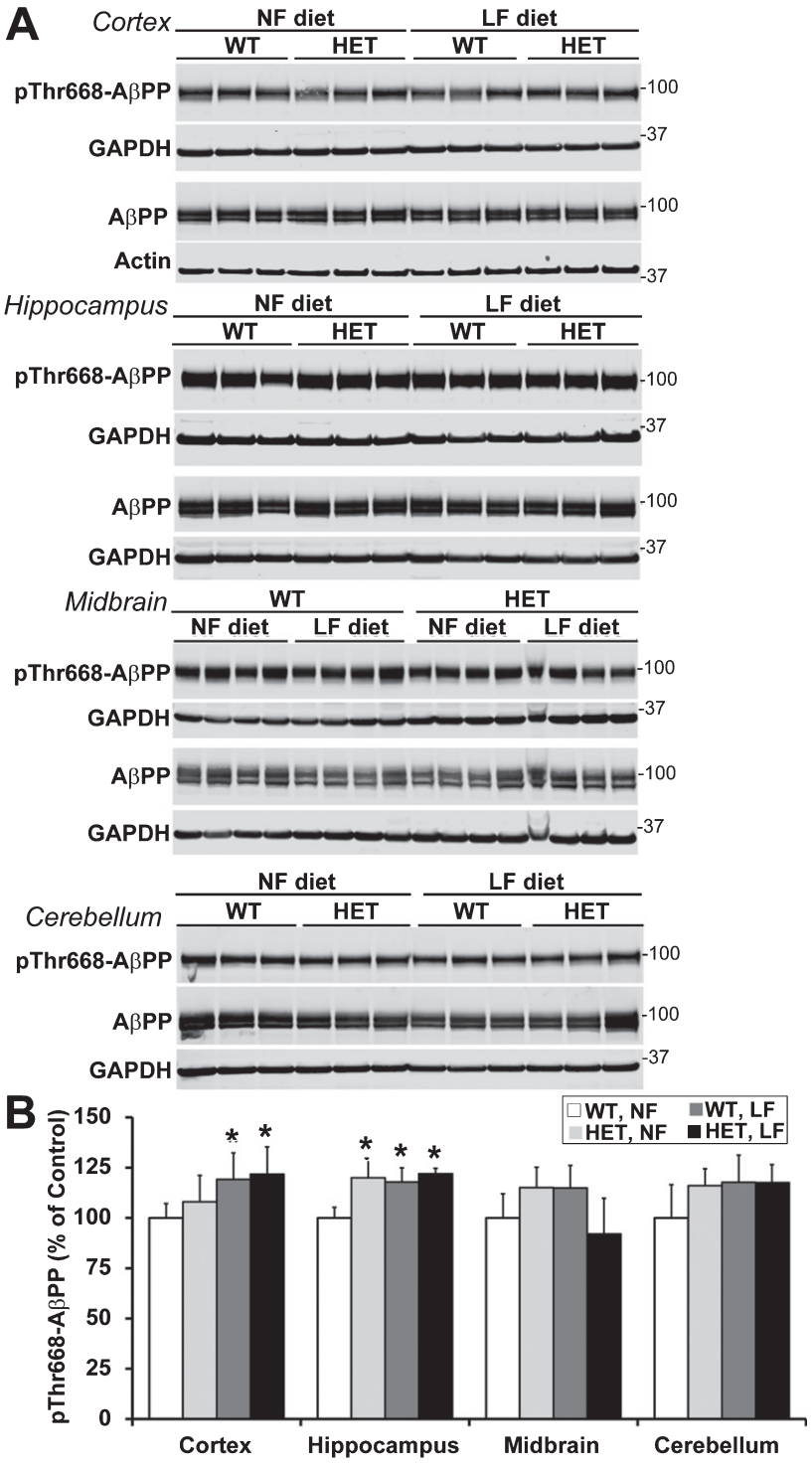

Fig. 3. Brain region-specific effects of mild MTHFR and dietary folate deficiencies on Thr668-phosphorylated AβPP levels in old mice. Regional brain homogenates were prepared from 22-month-old WT *MTHFR*$^{+/+}$ and HET *MTHFR*$^{+/-}$ mice that had been fed for 6 months either a normal folate (NF) diet (WT mice, white bars; HET mice, light gray bars) or a low folate (LF) diet (WT mice, darker gray bars; HET mice, black bars). Aliquots of regional brain homogenates were analyzed by western blot for total or Thr668-phosphorylated AβPP. A) Representative immunoblots. B) Relative pThr668-AβPP expression levels were quantified after densitometric analysis of the blots. Values shown are mean ± SD ($n = 8$ mice/group; $^{*}p < 0.05$, relative to WT mice fed on the NF diet).

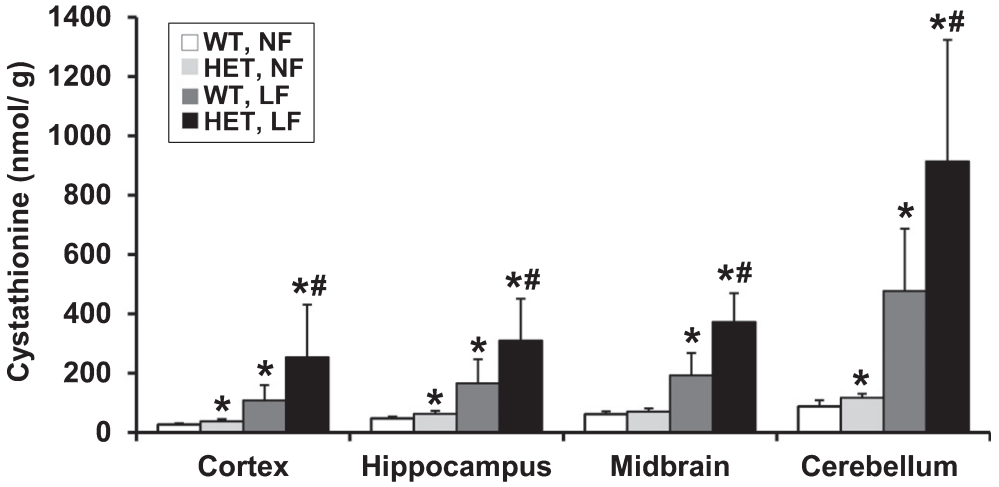

Fig. 4. Mild MTHFR and dietary folate deficiencies synergize to increase regional brain cystathionine levels in old mice. Cystathionine levels were determined in brain homogenates from the same old WT and HET mice described in Fig. 3, which had been fed either NF or LF diets (Bar shading as in Fig. 3). Values shown are mean ± SD from $n = 6$–10 mice/group; $^{*}p < 0.05$, relative to WT mice fed on the NF diet; $^{\#}p < 0.05$, relative to HET mice fed the NF diet.

trols. In contrast to what was observed in young HET mice (Fig. 2), there was no net increase in pThr668-AβPP in the cortex and midbrain of aged HET mice.

These findings suggest that, in regards to AβPP phosphorylation, the hippocampus is more sensitive than other brain regions to the combined effects of aging and mild MTHFR deficiency.

Dietary folate deficiency in old mice increases cortical and hippocampal AβPP phosphorylation

Since it is known that the functional consequences of *MTHFR* C677T polymorphisms can be aggravated by inadequate dietary folate intake [9], we next assessed how dietary folate deficiency can influence the effect of the *MTHFR* genotype on AβPP regulation. To that end, parallel cohorts of aged WT and HET mice were fed for 6 months NF or LF diets. Unsurprisingly, feeding either WT or HET mice the LF diet resulted in greatly decreased plasma folate levels (Table 1). As expected (Fig. 1), the loss of folate correlated with a dramatic increase in tHcy levels, relative to mice fed the NF diet. Dietary folate deficiency in WT mice was also associated with a significant elevation of cystathionine levels (∼3 to 5-fold over mean basal levels measured in WT mice fed the NF diet) in all brain regions examined (Fig. 4). Moreover, mild MTHFR deficiency synergized with dietary folate deficiency to further enhance regional brain cystathionine levels (∼6 to 10-fold increase in mean levels in HET mice fed the LF diet, relative to WT mice fed the NF diet). These experimental findings are in agreement with earlier clinical studies showing that severe disturbances in folate metabolism -due either to dietary folate deficiency [12] or severe inborn *MTHFR* deficiency [13]- are associated with hallmark elevation of cystathionine levels due to impaired methylation of Hcy (Fig. 1).

Dietary folate deficiency induced a small and similar increase in AβPP phosphorylation in the cortex of both WT and HET mice, relative to cohorts fed the control NF diet (Fig. 3). Likewise, pThr668-AβPP 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. 2) and old (Fig. 3) HET mice fed a NF diet. However, there was no significant change in the amounts of normalized pThr668-AβPP levels in the midbrain or cerebellum of WT or HET mice fed the LF diet, relative to mice fed the NF diet (Fig. 3).

Together, these data indicate that dietary folate or genetic MTHFR deficiencies can significantly affect the phosphorylation of AβPP at Thr668, but these effects are brain region specific and age-dependent.

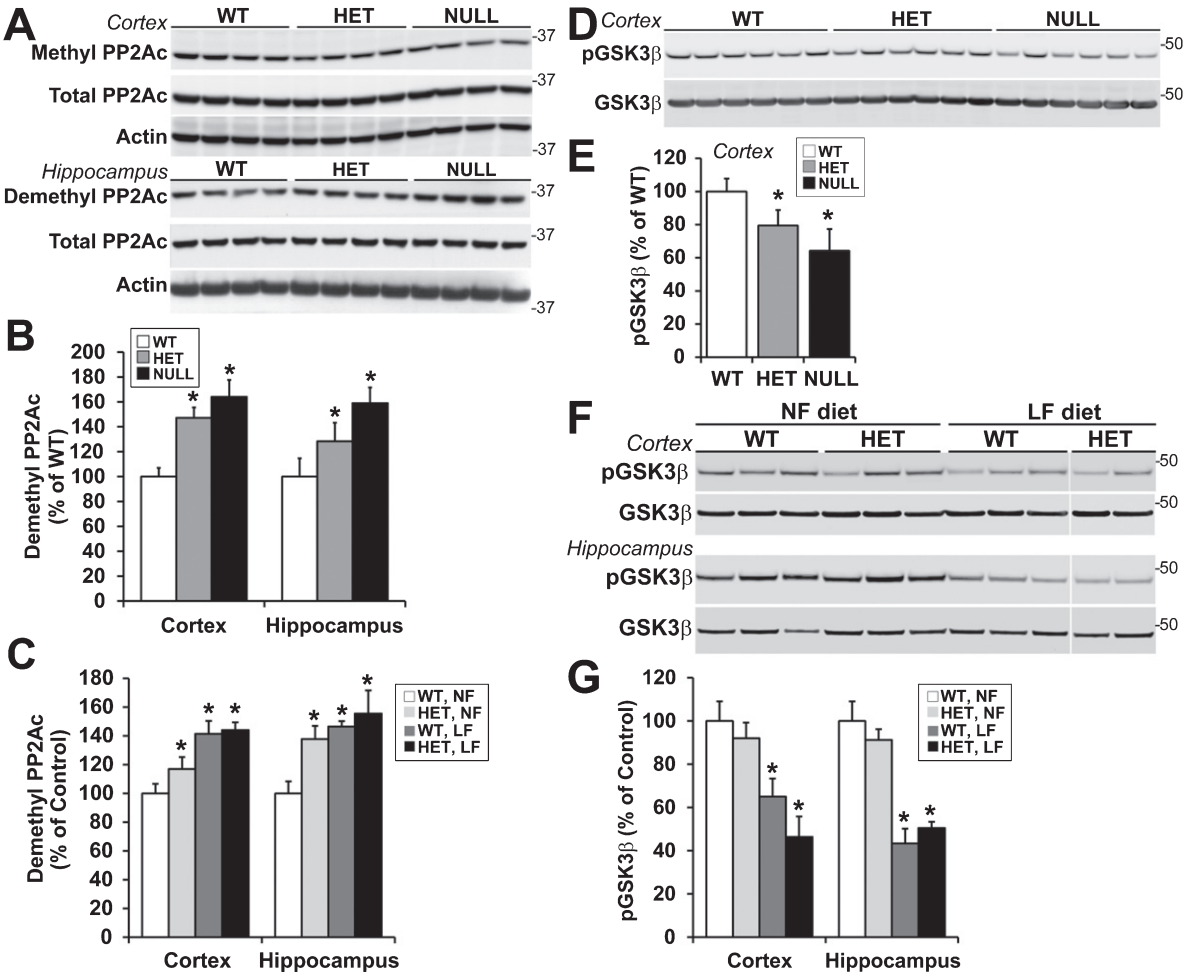

Fig. 5. MTHFR and dietary folate deficiencies promote the accumulation of demethylated PP2A and dephosphorylation of GSK-3β in the mouse cortex and hippocampus. The same regional brain homogenates described in Figs. 2 and 3 were re-analyzed by western blot for PP2A methylation state and GSK-3β phosphorylation at Ser-9. Values shown are mean ± SD; $^{*}p < 0.05$, relative to WT mice fed on the NF diet. A) Representative immunoblots of methylated, demethylated and total PP2A catalytic subunit (PP2Ac) in young WT, HET and NULL mice fed a NF diet. B) Relative levels of demethylated PP2Ac in these young mice. C) Relative levels of demethylated PP2Ac in old WT and HET mice fed either a NF or LF diet. D) Representative immunoblots of phosphorylated (Ser9) and total GSK-3β in young WT, HET and NULL mice fed a NF diet. E) Relative levels of phosphorylated GSK-3β in young mice. F) Representative immunoblots of phosphorylated (Ser9) and total GSK-3β in old WT and HET mice fed either a NF or LF diet. G) Relative levels of phosphorylated GSK-3β in old mice.

Enhanced AβPP phosphorylation at Thr668 in MTHFR and folate deficient mice is associated with enhanced PP2A demethylation and GSK-3β activation

We next investigated what could be potential mechanisms underlying the increased phosphorylation of AβPP at Thr668 that we observed in our mouse models. In this context, we and others have previously reported that dietary folate deficiency and hyperhomocysteinemia can independently promote the demethylation of PP2A, a major brain Ser/Thr phosphatase [31, 40–42]. Significantly, altered methylation of PP2A catalytic subunit Leu309 residue is known to affect PP2A substrate specificity [4]. It is sufficient to promote the phosphorylation of AβPP at Thr-668, a site dephosphorylated by PP2A, in both cultured cells and *in vivo* [43, 44]. Based on these findings, we tested the hypothesis that enhanced PP2A demethylation could underlie the increase in cortical and/or hippocampal pThr668-AβPP that we measured in MTHFR mice. Indeed, re-analysis of the same cortical and/or hippocampal homogenates used in Figs. 2 and 3 to assess pThr668-AβPP showed that

MTHFR and dietary folate deficiencies were indeed associated with the accumulation of demethylated PP2A in both young (Fig. 5A, B) and old animals (Fig. 5C).

Besides affecting PP2A methylation, impaired folate and homocysteine metabolism could also enhance the activity of protein kinases known to phosphorylate AβPP. We focused our attention on GSK-3β, because aberrant activation of this Ser/Thr kinase plays a central role in AD-related neurodegenerative processes [45], and has been linked to hyperphosphorylation of AβPP at Thr668 [30]. Western blot analysis of cortical homogenates from young mice revealed that phosphorylation of GSK-3β at the Ser9 site, which negatively regulates its kinase activity, was significantly decreased in response to MTHFR deficiency (Fig. 5D, E). Likewise, dietary folate deficiency was associated with a marked downregulation of the inhibitory phosphorylation of GSK-3β in the cortex and hippocampus of old mice (Fig. 5F, G).

These results suggest that enhanced demethylation of PP2A and GSK-3β activation likely contribute to the increased phosphorylation of AβPP that occurs in response to severe alterations in folate metabolism in our mice.

MTHFR and dietary folate deficiencies are also associated with brain region specific changes in AβPP expression

Lastly, since elevated levels of pThr668 phosphorylation could also be linked to enhanced AβPP protein levels, we quantified AβPP expression in the regional brain homogenates from our young (Fig. 6A) and old (Fig. 6B) mouse models. We found that the *MTHFR* genotype or LF diets had no effect on total AβPP expression levels in the cerebellum of either young or old animals. In contrast, deregulation of total AβPP expression was observed in other brain regions; the extent of these AβPP changes followed a trend that generally correlated with incremental impairment of folate metabolism. Indeed, mild MTHFR deficiency in HET mice had no or less effects on AβPP than severe MTHFR deficiency in MTHFR$^{-/-}$ knockouts; moreover, the effects of severe MTHFR deficiency on AβPP expression were closely recapitulated by long term feeding of mice with LF diets. Surprisingly, such profound disturbances in folate metabolism were associated with decreased AβPP levels in the cortex (∼25%) and midbrain (∼35%), but significantly enhanced (∼45%) AβPP amounts in the hippocampus, relative to WT mice fed a NF diet (Fig. 6). We also noted that there was no major effect of aging on the relative effects of the MTHFR$^{+/-}$ genotype, except in the hippocampus wherein aging and mild MTHFR deficiency interacted to increase AβPP expression; this was reflected by a ∼30% increase in AβPP in old HET mice, relative to age-matched WT littermates and young HET cohorts. By comparison, mild MTHFR deficiency did not affect cerebellar or cortical AβPP expression, and similarly decreased AβPP in the midbrain of young and old animals.

Thus, diet- or genetic-induced alterations in folate metabolism have the potential to induce a brain region specific deregulation of AβPP expression.

DISCUSSION

While it is well recognized that abnormally enhanced production of Aβ triggers the neurodegenerative process in AD, much remains to be learned on the fine regulation of AβPP. Significantly, there is increasing evidence that alterations in folate and homocysteine metabolism are important risk factors for sporadic AD [2, 5–8]. As such, there is growing interest in elucidating underlying mechanisms. Here, we show for the first time that genetic-induced MTHFR deficiency can alter AβPP expression and/or phosphorylation at Thr668 in a brain region- and age-dependent manner. In these studies, we used well-characterized *MTHFR* knockout mice known to recapitulate the effects of MTHFR deficiency, which is associated with the common human C677T polymorphism, a risk factor for sporadic AD [16–18].

First, we found in young mice that MTHFR deficiency was sufficient to markedly enhance AβPP phosphorylation at Thr668 in AD susceptible brain regions such as the hippocampus and cortex, but not in the cerebellum, a region typically less involved in AD. These results are significant since neuron-specific phosphorylation at Thr668 [46] is thought to be important for the regulation of AβPP protein-protein interactions, trafficking and function, although its exact physiological significance remains to be clarified [47]. AβPP phosphorylation at Thr668 is also highly relevant to AD-related pathological processes, as highlighted by numerous studies demonstrating the role of this posttranslational modification in facilitating the amyloidogenic processing of AβPP and neurodegeneration [23–30]. Mutation of Thr668 to Alanine in AβPP prevents the devel-

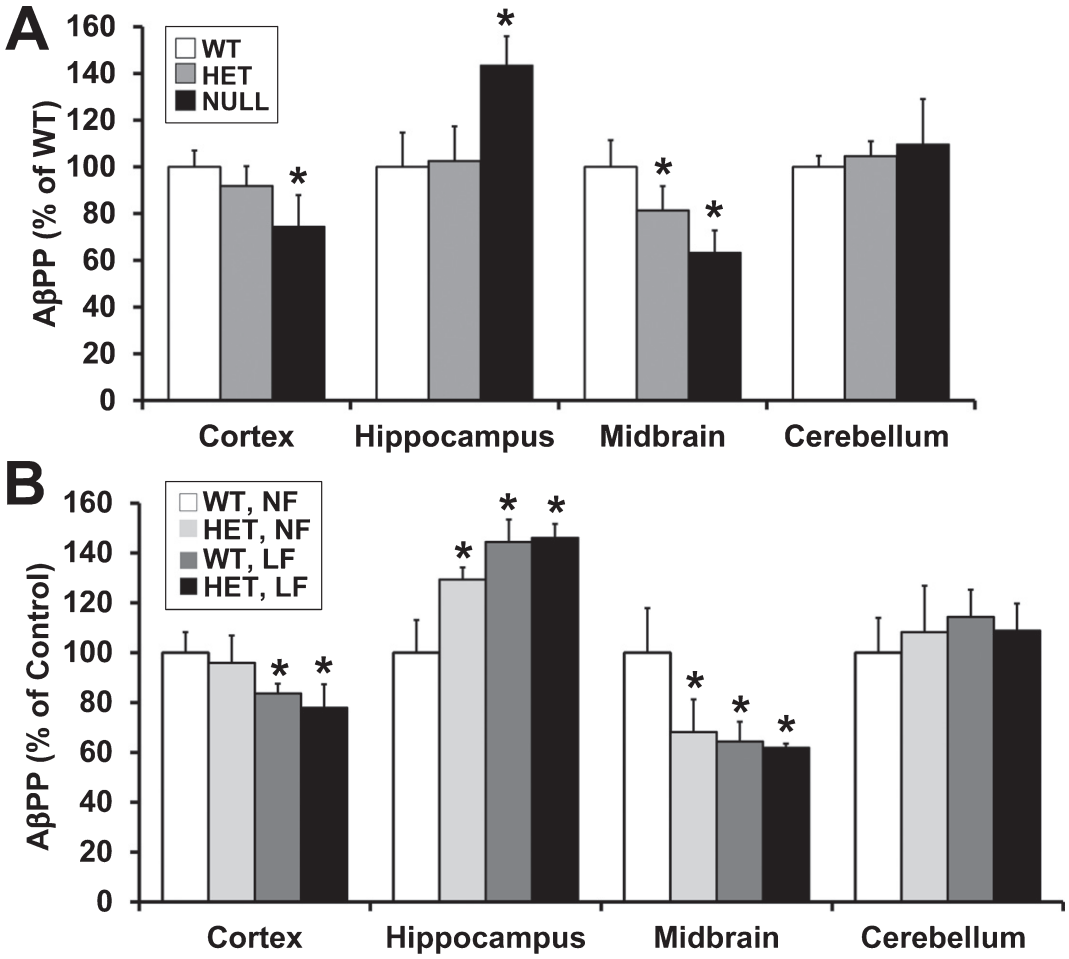

Fig. 6. MTHFR and dietary folate deficiencies also induce brain-region specific alterations in AβPP expression. Regional brain homogenates from young (Fig. 2) and old (Fig. 3) *MTHFR* mice were analyzed by western blot for total AβPP expression levels, which were determined after densitometric analysis of the blots and normalization for protein loading. Representative blots are shown in Figs. 2A and 3A. A) Relative total AβPP expression levels in regional brain homogenates from young WT, HET and NULL mice fed a NF diet (mean ± SD; $n = 6$ mice/group; $^{*}p < 0.05$, HET or NULL compared to WT). B) Relative total AβPP expression levels in 22-month old WT and HET mice fed either a NF or LD diet (mean ± SD; $n = 8$ mice/group; $^{*}p < 0.05$, relative to WT mice fed on the NF diet).

opment of memory and synaptic plasticity deficits in mice [29]. Moreover, AβPP phosphorylation is upregulated in AD, and postmortem investigations have shown that pThr668-AβPP species are associated with Aβ lesions in human AD brain tissue and mouse models of AD [24, 25, 30, 48]. While AβPP phosphorylation at Thr668 promotes amyloidogenesis, the accumulation of Aβ oligomers can in turn further promote phosphorylation of AβPP at Thr668, thereby amplifying the neurodegenerative cycle [30]. In this context, it is interesting that earlier *in vivo* studies have not only connected hyperhomocysteinemia with AβPP phosphorylation at Thr668 [31, 32], but also facilitation and exacerbation of amyloidogenesis [20–22, 32]. Thus, by enhancing pThr668-AβPP, genetic MTHFR deficiency could promote Aβ production. Unfortunately, this assumption could not be validated here since it is well known that WT mice do not innately develop age-associated Aβ pathology. In this regard, future studies examining the effect of MTHFR deficiency in more appropriate amyloidogenic mouse models of AD will be informative. It is also worth mentioning that the studies presented herein were performed only in female *MTHFR* mice. Interestingly, it has previously been reported that hyperhomocysteinemia increases Aβ production, but only in female mouse models of AD [20]. Thus, future studies will need to address whether similar deregulation of AβPP phosphorylation occurs in male *MTHFR* mice.

In contrast to what was observed in young animals, there was no major effect of genetic-induced mild MTHFR deficiency on the phosphorylation of AβPP in the cortex and midbrain of older HET mice, relative to their age-matched WT counterparts. We hypothesize that these discrepancies could result from age-related enhancement of basal levels of pThr668-AβPP in 22-month-, relative to 5-week-old WT mice; however, we were unable to test this hypothesis due to exhaustion of our brain tissue homogenates. It is also possible that decreased regional amounts of pThr668-AβPP reflect age-dependent alterations in regulatory phosphorylation signaling cascades. Furthermore, there may be time-dependent compensatory mechanisms aimed at counteracting potential toxic effects of excessive AβPP phosphorylation or altered AβPP expression. At the same time, we found that as observed in young animals, pThr668-AβPP levels were increased in the hippocampus of 22-month-old HET mice, relative to WT controls. These findings are in agreement with previous reports highlighting the particular sensitivity of the hippocampus -a region especially vulnerable to early AD damage- to MTHFR deficiency [33, 39]. In contrast to the hippocampus, we did not observe any changes in relative AβPP phosphorylation in the cerebellum of either young or old MTHFR deficient mice, despite the fact that structural cerebellar abnormalities have been reported in MTHFR knockouts [36]. These observations further emphasize that the regulation of AβPP is brain region specific.

We found that dietary folate deficiency in old mice mimicked the effects of severe MTHFR deficiency in young mice, and augmented AβPP phosphorylation at Thr668 in the hippocampus and cortex of WT mice (Fig. 3). However, these effects were not boosted in HET mice with mild MTHFR deficiency; this likely reflects the effect of prolonged feeding of mice with the LF diet, which induced a maximal and similar decrease in plasma folate levels in both genotypes (Table 1).

What could be the underlying mechanisms linking genetic *MTHFR* and dietary folate deficiencies and pThr668-AβPP? First, severe disturbances in folate metabolism can lead to compromised cellular methylation reactions [1] (Fig. 1). Indeed, it has been reported that while total folate levels are unchanged, 5-MTHF levels are decreased, and non-methylfolate species are increased in the brain of MTHFR deficient mice; this altered distribution of folate derivatives affects the flux of one-carbon units between methylation reactions and nucleotide synthesis [37] (Fig. 1). Consequently, MTHFR deficiency induces defects in cellular methylation potential in mice [36]. Accordingly, we found that PP2A methylation was impaired in the cortex and hippocampus of MTHFR deficient mice (Fig. 5A-C). It was also impaired in mice fed for 6 months a LF diet (Fig. 5C), in agreement with our earlier report showing that short-term dietary folate deficiency promotes an alteration in the cortical SAM/SAH ratio, and concomitant accumulation of demethylated PP2A [41]. Since PP2A is a major neuronal enzyme that dephosphorylates AβPP at the Thr668 site, and PP2A demethylation is sufficient to promote the accumulation of p-Thr668 AβPP in cells and *in vivo* [31, 44], it is likely that impairment of PP2A methylation is a major contributor to the increased phosphorylation of AβPP observed in our studies.

Besides affecting cellular methylation potential, it is well established that MTHFR deficiency is associated with elevation of plasma tHcy in human [10, 11] and mice [36, 37]. It is noteworthy that PP2A demethylation and AβPP phosphorylation at Thr668 are increased in the brain of homocysteine-injected rats [32] and hyperhomocysteinemic *Cbs* deficient mouse models [31]. Elevated tHcy levels, as observed in our MTHFR deficient mouse models (Table 1), have been linked with oxidative stress and glutamate-mediated excitotoxicity [2]. Likewise, reduced 5-MTHF levels can directly or indirectly promote oxidative stress [14], while folic acid protects against glutamate-induced excitoxicity [49]. Such mechanisms have the potential to influence neuronal AβPP phosphorylation state in a PP2A-independent manner. For instance, oxidative stress and excitoxicity can enhance GSK-3β activity, which in turn promotes AβPP phosphorylation at Thr668 and ultimately induces neurodegeneration [30, 45]. Thus, it is likely that similar mechanisms are related to the upregulation of GSK-3β and pThr668-AβPP in our hyperhomocysteinemic MTHFR deficient mice (Fig. 5E). Our results showing an upregulation of GSK-3β activity in response to folate deficiency are in complete agreement with recent results showing that folic acid induces antidepressant and neuroprotective effects, at least in part by causing the phosphorylation (Ser9) and inhibition of GSK-3β [15, 49]. Interestingly, GSK-3β activation may further decrease PP2A methylation, while PP2A methylation does not affect GSK-3β activity [50].

Besides playing a pathological role in AD, AβPP exerts an important function in neuronal and synaptic

plasticity and maintenance, but its regulation remains somewhat poorly understood [47]. Strikingly, we observed that genetic- or diet-induced folate deficiency affected total AβPP expression in our mouse models, but in a complex, brain region specific manner. For instance, severe MTHFR and dietary folate deficiencies were associated with upregulation of hippocampal and downregulation of cortical AβPP levels. Future comprehensive studies will need to be performed to determine whether these changes are linked to epigenetic mechanisms or changes in the activity of numerous regulatory factors that control AβPP gene expression in response to a variety of inputs, including growth factors, stress and inflammation [51]. Many AβPP regulatory signaling pathways may become deregulated following impairment of PP2A methylation [4] and elevation of tHcy [2] that both occur in response to alterations in folate metabolism in these mice. Interestingly, the accumulation of AβPP has been reported to occur at sites of axonal injury in the brain. In fact, upregulation of AβPP is considered a marker of central nervous system axonal injury [52]. Likewise, in *MTHFR* knockouts, we found increased AβPP levels (Figs. 2 and 4) in the hippocampus, which shows many biochemical and structural changes, including increased apoptosis, in response to MTHFR deficiency [33, 39]. Moreover, decreased 5-MTHF and elevated Hcy levels can also independently promote oxidative stress and inhibit endothelial NOS via distinct mechanisms, resulting in impaired synthesis and vasodilatory action of NO, and altered cerebral blood flow [14, 53]. Notably, these deficits have been associated with enhanced hippocampal AβPP expression levels and Aβ production *in vivo*, and cognitive decline [54]. Thus, by reducing 5-MTHF and elevating tHcy levels, MTHFR and dietary folate deficiencies could synergize to impair endothelial NOS activity, and ultimately enhance AβPP expression in the hippocampus.

On the other hand, we found that severe MTHFR deficiency induced a downregulation of AβPP in the cortex and midbrain of young mice. Downregulation of AβPP expression can occur acutely after a head trauma [55], illustrating that complex and yet unresolved causal and compensatory mechanisms are involved in modulating AβPP transcriptional regulation in a time-dependent manner. Since AβPP is required for neuronal homeostasis and synapse formation [47], a loss of cortical AβPP could contribute to neurodevelopmental delays and cognitive deficits in human with severe MTHFR deficiency [1, 2, 9].

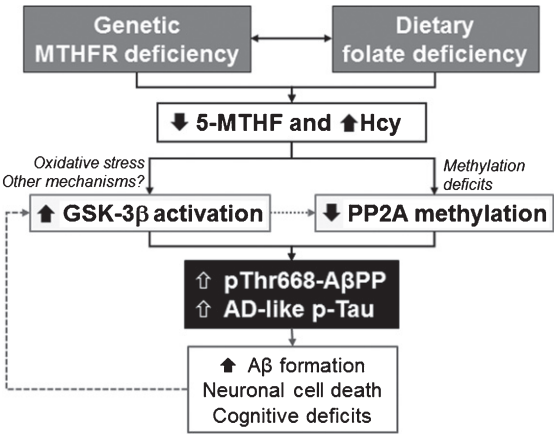

Fig. 7. Proposed mechanisms linking MTHFR deficiency with altered phosphorylation of AβPP and tau in AD. 5-MTHF deficiency can be mediated by genetic MTHFR deficiency and/or dietary-induced alterations in folate metabolism, leading to accumulation of Hcy and a decrease in the SAM/SAH ratio that controls cellular methylation reactions. Elevated Hcy and SAH levels can both induce demethylation of PP2A, which results in enhanced phosphorylation of AβPP at Thr668. Reduced 5-MTHF and elevated Hcy levels can also promote oxidative stress via distinct and independent mechanisms, resulting in activation of protein kinases, such as GSK-3β. Activated GSK-3β can phosphorylate AβPP on Thr668, and further induce PP2A demethylation. Both GSK-3β activation and PP2A demethylation stimulate tau phosphorylation at AD-like phosphoepitopes. In AD, AβPP Thr668 phosphorylation is associated with enhanced Aβ production. In turn, Aβ can promote oxidative stress and GSK-3β activation, thereby inducing a vicious cycle that ultimately results in neurodegeneration. See text for details.

In conclusion, we show here for the first time that complex regional alterations in AβPP expression and/or phosphorylation occur in young and aged cohorts of mice with genetic- or diet-induced folate deficiency. These results strongly support a link between severe alterations in folate metabolism and deregulation of AβPP, a major protein involved in neuronal homeostasis. We have previously reported that MTHFR and/or dietary folate deficiencies also increase the cortical and hippocampal accumulation of pathologically phosphorylated tau proteins [33, 41], which play a critical role in mediating Aβ neurotoxicity in AD [19]. Furthermore, we show that severe genetic- or diet-induced disturbances in folate metabolism also promote the activating dephosphorylation (Ser9) of GSK-3β in these brain regions. Significantly, over-activation of GSK-3β in AD is not only linked to Aβ production, but also to tau hyperphosphorylation, cognitive decline and neurodegeneration [45]. Together with the novel data presented herein linking MTHFR deficiency with enhanced accumulation of Thr668-phosphorylated

AβPP in the hippocampus, these findings provide a compelling mechanism (Fig. 7) for explaining how the human *MTHFR* C677T polymorphism is a risk factor for AD and cognitive decline.

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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

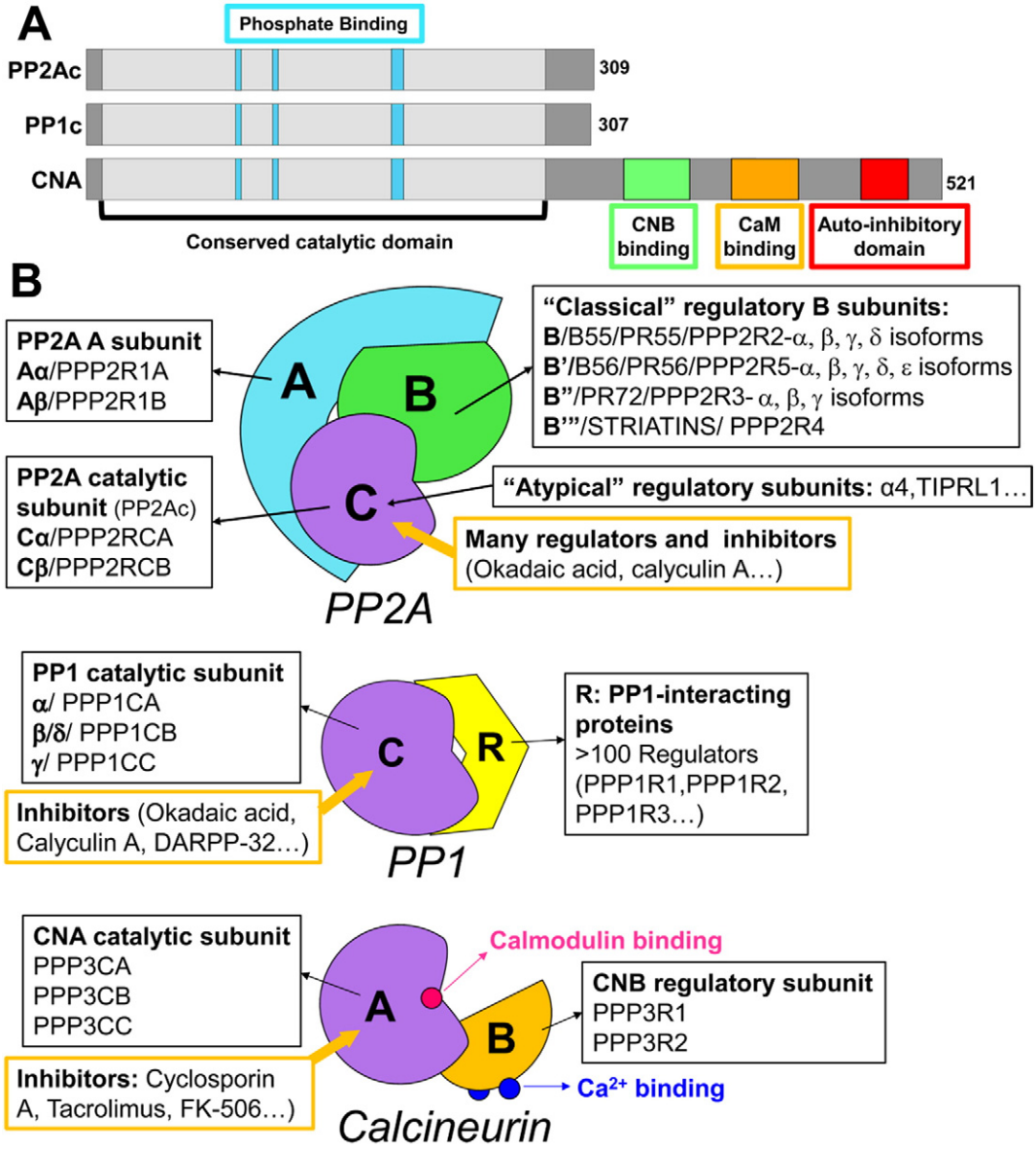

Fig. 1. Schematic structure of PP2A, PP1 and calcineurin enzymes. **A**. These phosphatases all share a ~ 30 kDa conserved catalytic domain. The calcineurin A subunit contains additional B subunit- and calmodulin (CaM)-binding domains, and an auto-inhibitory domain. **B**. Subunit interactions play a 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

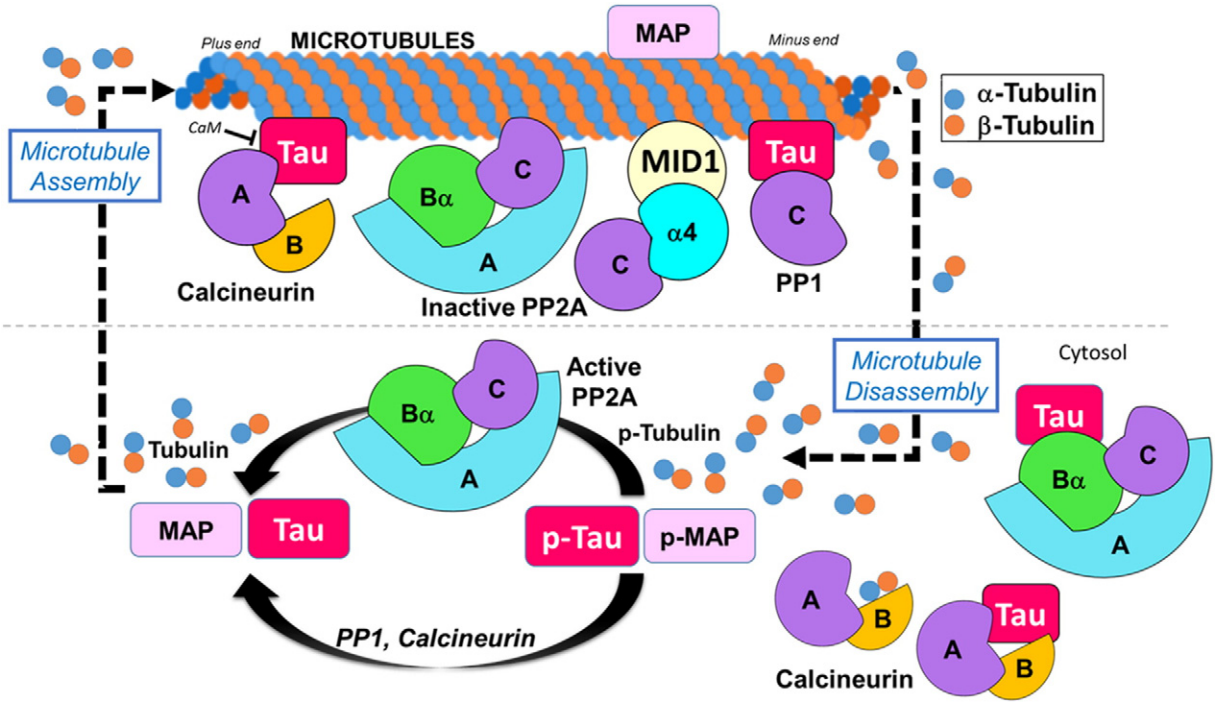

Fig. 2. The regulated binding of PP2A, PP1 and calcineurin to neuronal microtubules influences the spatial dephosphorylation of tubulin and MAPs. PP2A/Bα and α4/PP2Ac enzymes are sequestered in an inactive state on MTs. MT depolymerisation releases PP2A (and MAPs), resulting in enhanced dephosphorylation 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

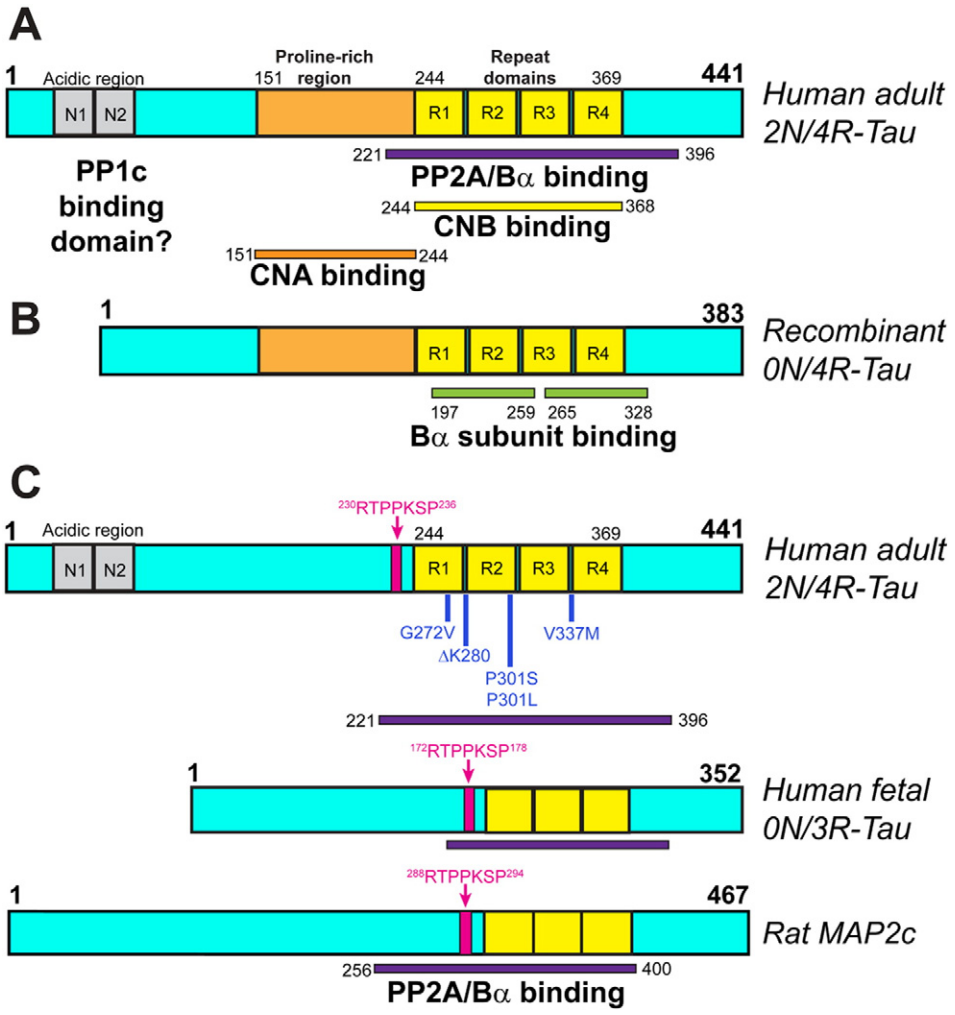

Fig. 3. PP2A, PP1 and calcineurin interact with tau. **A**. The PP2A/Bα trimeric enzyme interacts with a domain encompassing the MT-binding repeats and upstream proline-rich region of the longest adult tau 2N/4R–Tau isoform containing 2 N-terminal inserts (2N) and four MT-binding repeats (4R). Calcineurin 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 P*X*X*P* 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 P*X*X*P* 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

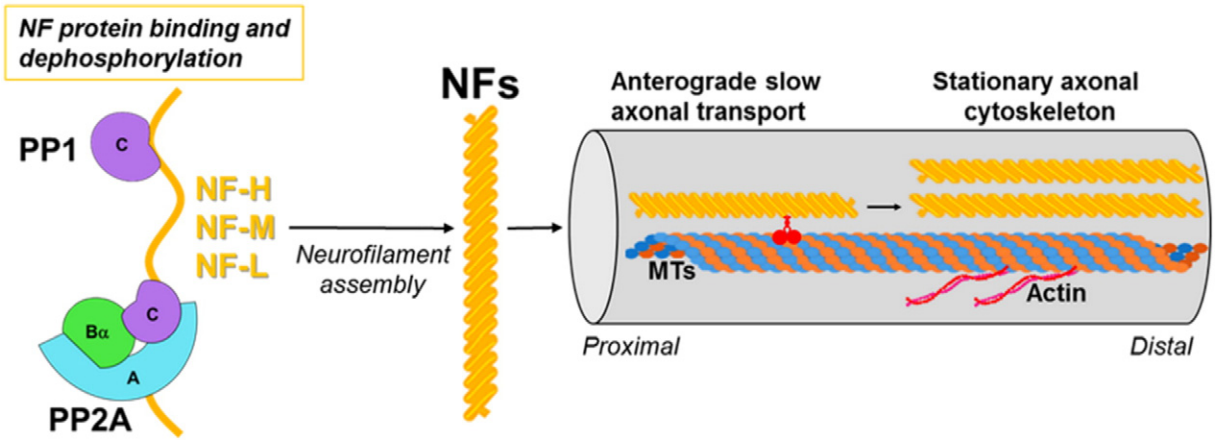

Fig. 4. PP2A- and PP1-dependent regulation of neurofilament architecture. Neurofilaments (NFs) are intermediate filaments that abundantly occupy axons and constitute the fundamental axonal cytoskeletal framework. Both PP2A and PP1 can interact with and dephosphorylate NF proteins, and play an essential 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),

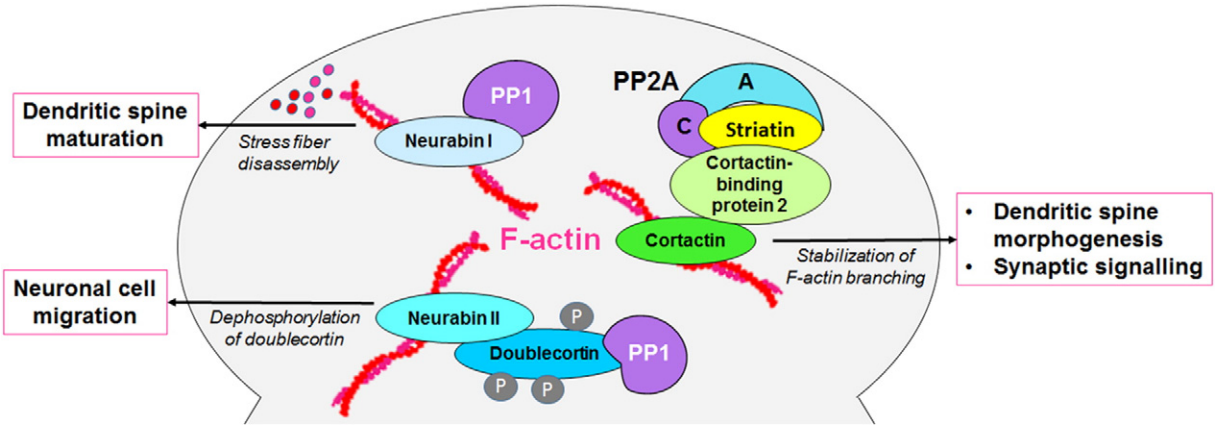

Fig. 5. Targeting of PP2A and PP1 to the actin cytoskeleton. Selected PP2A and PP1 enzymes have been found to be recruited to the F-actin network by specific actin-binding proteins in dendritic spines. This leads to actin rearrangements that modulate synaptic function and spine morphogenesis. Neuronal 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.

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REVIEW ARTICLE

Protein phosphatase 2A and tau: an orchestrated 'Pas de Deux'

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The neuronal microtubule-associated protein tau serves a critical role in regulating axonal microtubule dynamics to support neuronal and synaptic functions. Furthermore, it contributes to glutamatergic regulation and synaptic plasticity. Emerging evidence also suggests that tau serves as a signaling scaffold. Tau function and subcellular localization are tightly regulated, in part, by the orchestrated interplay between phosphorylation and dephosphorylation events. Significantly, protein phosphatase type 2A (PP2A), encompassing the regulatory PPP2R2A (or Bα) subunit, is a major brain heterotrimeric enzyme and the primary tau Ser/Thr phosphatase *in vivo*. Herein, we closely examine how the intimate and compartmentalized interactions between PP2A and tau regulate tau phosphorylation and function, and play an essential role in neuronal homeostasis. We also review evidence supporting a strong link between deregulation of tau–PP2A functional interactions and the molecular underpinnings of various neurodegenerative diseases collectively called tauopathies. Lastly, we discuss the opportunities and associated challenges in more specifically targeting PP2A–tau interactions for drug development for tauopathies.

Keywords: dephosphorylation; PP2A; protein interactions; tau; tauopathies

Tau proteins are major brain microtubule-associated proteins (Reviewed in Ref. [1]). There are multiple tau isoforms (See Fig. 1) generated *via* alternative splicing of the encoding *MAPT* gene. All six isoforms are expressed in the adult brain with considerable regional variation, but tau mRNA and protein levels are two-fold enriched in the neocortex relative to white matter and cerebellum [2]. It is well established that tau functions to maintain microtubule stability, which contributes to its role in microtubule-dependent transport of cellular cargo. In addition to supporting synapse function through this role, tau also contributes to postsynaptic signaling scaffolds and synaptic plasticity. In particular, hippocampal long-term depression (LTD) is dependent on the presence of tau [3]. Tau proteins have attracted significant attention due to their central pathological role in a variety of neurodegenerative diseases designed as 'tauopathies', wherein characteristic aggregates of 'hyperphosphorylated' tau proteins deposit in affected brain regions (Reviewed in Ref. [1]). Hence, historically, tau phosphorylation has been principally studied in the general neuropathological context of its excessive or aberrant phosphorylation, which was considered as a prelude to its toxic aggregation in the dementia brain. In contrast, comparatively little progress has been made in understanding the physiological relevance of site-specific tau phosphorylation and dephosphorylation events. How such processes precisely affect the normal function of tau in neuronal homeostasis—beyond its canonical

Abbreviations
AD, Alzheimer disease; FTDP-17, frontotemporal dementia with parkinsonism-17; GSK3 β, glycogen synthase kinase 3β; LCMT1, leucine carboxyl methyltransferase 1; LTD, long-term depression; NMDARs, N-Methyl-D-aspartate receptors; PP2A, protein phosphatase 2A; PSD-95, postsynaptic density protein 95; PSDs, postsynaptic densities; Ser, Serine; Thr, Threonine.

role in regulating microtubule dynamics—remain, for the most part, to be clarified.

The phosphorylation status of tau is tightly controlled by a delicate balance between protein kinases' and phosphatases' activities. Among the many brain protein kinases that regulate tau (Reviewed in Ref. [4]), by far, glycogen synthase kinase 3β (GSK 3β) is the major Ser/Thr tau kinase. It contributes to the pathology of several neurodegenerative diseases [5]. Importantly, GSK3β phosphorylates the Ser-396 residue of tau, which is critical for LTD [3,6]. Of particular relevance, the phosphatase battlefront is largely led by a distinct pool of protein phosphatase 2A (PP2A) enzymes that are responsible for the bulk of neuronal tau dephosphorylation [7,8]. PP2A is a highly conserved, essential, and omnipresent family of protein Serine (Ser) and Threonine (Thr) phosphatases that play a central role in signal transduction pathways and cell homeostasis [9].

Here, we more specifically review how PP2A enzymes directly or indirectly regulate tau phosphorylation state, thereby impacting tau function under various physiological and pathological conditions. In contrast to the reductionist view that PP2A only regulates tau *via* its role as a potent Ser/Thr tau phosphatase, recent studies emphasize that PP2A also serves an important function in controlling tau subcellular targeting and scaffolding function. Indeed, we discuss how direct protein–protein interactions between PP2A and tau can also modulate tau function, and how pathological disruption of such interactions could contribute to tauopathies. Based on the critical importance of PP2A in tau regulation, and its known dysfunction in neurodegenerative diseases

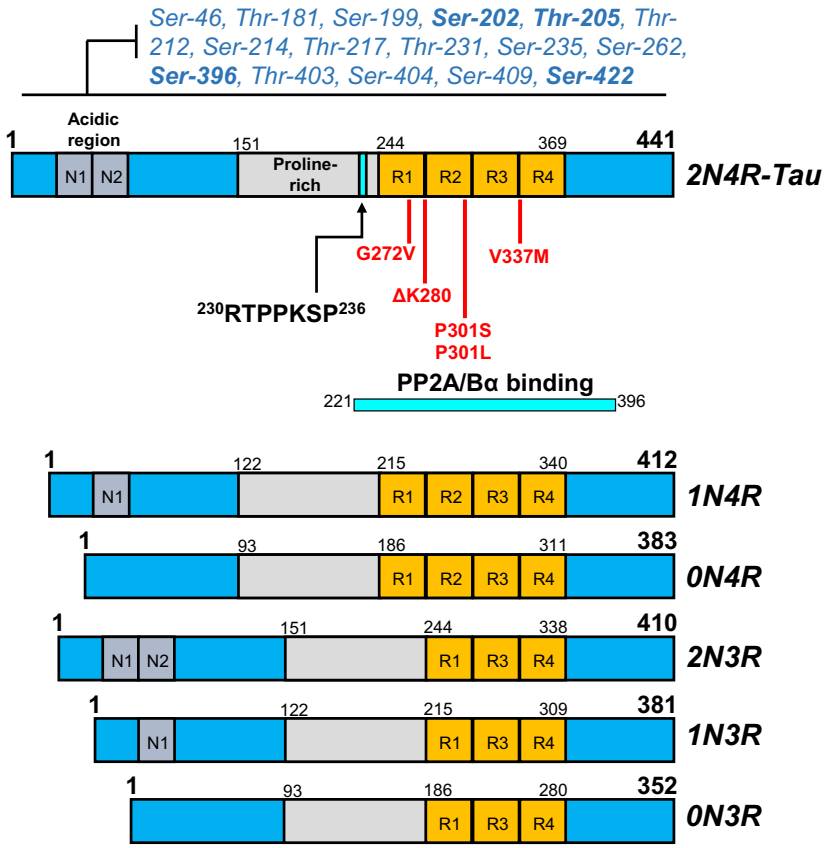

Fig. 1. Mapping of the interaction of PP2A/Bα with human brain tau isoforms. Major domains in the tau protein include the N-terminal projection domain, which projects away from microtubules, and the microtubule-binding domain, comprised of a proline-rich region, repeat (R) domains and a flanking region at the carboxy-terminal. The six human brain tau isoforms shown are generated through alternative splicing, yielding tau species with either 0, 1, or 2 amino-terminal inserts (0N, 1N, or 2N), and either three (3R) or four (4R) carboxy-terminal repeat domains. The PP2A/Bα enzyme interacts with all tau isoforms *via* a domain encompassing the MT-binding repeats and upstream proline-rich region, which harbors the conserved RTPPKSP motif that is critical for PP2A binding. As shown (blue text), PP2A dephosphorylates tau at various serine (Ser) and threonine (Thr) sites *in vitro* (all sites listed) and *in vivo* (sites in bold text). Frontotemporal dementia-associated tau mutations (red), harbored in the PP2A-binding sequence inhibit the interaction of PP2A with tau.

(Reviewed in Ref. [10]), we will also debate whether targeting PP2A could be a viable option to develop therapeutic strategies for tauopathies.

PP2A: A major regulator of tau phosphorylation

PP2A: The primary brain tau Ser/Thr phosphatase

For long, PP2A was only considered as a housekeeping enzyme. However, as expected for a key modulator of many diverse cellular processes, PP2A activity is tightly and intimately regulated by multiple mechanisms, including post-translational modifications and association of PP2A catalytic subunit with an extensive array of regulators [11–13]. The typical mammalian holoenzyme exists in a heterotrimeric complex between a scaffolding 'A' (PPP2R1A or PPP2R1B) subunit and a catalytic 'C' (PPP2CA or PPP2CB) subunit, that is associated with one of a plethora of regulatory 'B' subunits, of which 23 isoforms have been identified [9]. The binding of selective 'B' subunits and other regulators contributes to PP2A substrate specificity and subcellular localization, aimed at preventing unwanted promiscuous activity (Reviewed in Ref. [14]). Of key interest, the prevalent neuronal PP2A holoenzyme encompassing the Bα (or PPP2R2A or PR55) regulatory subunit (PP2A/Bα) is the isoform with the strongest affinity for tau and most potent tau phosphatase activity [7,15,16].

Direct site-specific dephosphorylation of tau by PP2A

'Protein phosphatase 2A' accounts for ~71% of total tau phosphatase activity in the human brain [8], and modulates tau phosphorylation at multiple Ser/Thr phosphosites *in vitro* and *in vivo* (Fig. 1). *In vitro* assays with purified PP2A enzymes show direct dephosphorylation of tau at preferential phosphosites [8,17,18]. Yet, as further discussed in this review, site-specific dephosphorylation of tau is governed by a convoluted regulatory network that involves specific PP2A enzymes and regulators, protein kinases, cytoskeletal proteins, and compartmentalized protein–protein interactions.

PP2A/Bα: The primary PP2A isoform that dephosphorylates tau

Among other PP2A holoenzymes, the PP2A/Bα isoform is by far the most efficient tau phosphatase *in vitro* [7,15,16]. PP2A/Bα binds to the microtubule-binding domain of tau [16] (Fig. 1), and this interaction promotes tau dephosphorylation at multiple sites [7]. As a result of these interactions, PP2A can only bind to tubulin-unbound tau (Reviewed in Ref. [9]). PP2A/Bα has also greater affinity for adult 4 repeat (4R-) containing tau isoforms than fetal tau [16], suggesting differential dephosphorylation of human tau isoforms by this enzyme.

As supported by protein crystallization studies, the propeller design of Bα makes extensive interactions with the ridge of PP2A Aα subunit, while having few interactions with the C subunit [15]. Interestingly, targeting PP2A using the viral SV40 small tumor antigen, which displaces regulatory B subunits from the A subunit, results in increased tau phosphorylation [7]. Further emphasizing the importance of PP2A/Bα in tau dephosphorylation, selective downregulation of Bα or expression of a PP2A catalytic subunit mutant with reduced affinity for Bα substantially increase tau phosphorylation in cells and *in vivo* [19,20]. Conversely, Bα overexpression protects cells against folate deficiency-induced tau phosphorylation [19].

PP2A isoforms indirectly control tau phosphorylation *via* their action on tau kinases

Besides PP2A/Bα, other PP2A isoforms have been found to regulate phospho-tau homeostasis *via* indirect mechanisms, by modulating upstream tau protein kinases [18,21]. For instance, PP2A/Bα holoenzymes can regulate the activities of many Ser/Thr tau kinases, such as ERK [22]. In addition, B56δ subunit-containing PP2A isoforms influence tau phosphorylation through dephosphorylation and subsequent activation of GSK3β [23]; knockout of B56δ results in progressive tau phosphorylation at pathological sites implicated in tauopathies [21]. In support of the critical role of PP2A in the regulation of tau phosphorylation state, PP2A inhibition can also overcome the inhibition of key tau kinases (cdk5 and GSK3β), and promote tau hyperphosphorylation in the hippocampus of starved mouse [24].

PP2A–tau interactions play a key role in tau regulation

Mapping of the tau interaction domain in PP2A

Without the Bα subunit, the PP2A AC core enzyme only weakly binds to tau and partially dephosphorylates tau [7], further cementing the role of Bα in promoting PP2A substrate selectivity. Studies using tau

mutants have shown that the microtubule binding domain and the upstream proline-rich region in tau are important for PP2A binding [16]. Further structural studies have described a highly acidic groove harbored in the top face of the seven-bladed β-propeller of Bα; this allows the Bα subunit to bind to two nonoverlapping peptide fragments (residues 197–259 and 265–328 of the 0N4R tau isoform shown in Fig. 1) enriched in positively charged amino acids, which fall within the MT-binding repeats of tau [15]. The presence of more than one PP2A/Bα-binding site in tau strategically maximizes the efficiency of PP2A-dependent tau dephosphorylation, by enhancing the presentation of the multiple and dispersed Ser/Thr phosphorylated residues in tau to PP2A catalytic subunit [15].

Disruption of PP2A–tau interactions by PP2A deregulation

Of particular relevance to PP2A-dependent tau dephosphorylation, several studies indicate that the assembly and stabilization of PP2A/Bα holoenzymes are promoted by methylation of PP2A catalytic C subunit at Leu309 by leucine carboxyl methyltransferase 1 (LCMT1). Decreased PP2A methylation levels can lead to a loss of PP2A/Bα and concomitant changes in endogenous PP2A subunit composition that affect PP2A substrate specificity and targeting (Reviewed in Ref. [10]). Accordingly, downregulation of PP2A methylation is associated with the disruption of PP2A–tau protein–protein interactions, thereby precluding PP2A-mediated tau dephosphorylation [25,26]. Significantly, decreased LCMT1 and methylated PP2A levels [27] correlate with reduced PP2A/Bα amounts and the severity of phospho-tau pathology in Alzheimer disease (AD)-affected brain regions [28]. Thus, by affecting intracellular pools of PP2A/Bα, changes in PP2A methylation, for instance in response to disturbances in one-carbon metabolism, have the potential to offset the delicate equilibrium between tau kinases' and phosphatases' activities. Such alterations may render tau more susceptible to phosphorylation events and even increase its interaction with certain kinases (See below).

Protein phosphatase 2A is also targeted by numerous endogenous regulators such as SET (or I_2PP2A), a potent inhibitor that binds to PP2A C subunit resulting in inhibition of its tau phosphatase activity [29]. SET is primarily nuclear, but can translocate to the cytoplasm under certain conditions. Cytoplasmic retention of SET is associated with reduced PP2A activity and methylation, and enhanced tau phosphorylation at Ser202 [30]. The cytoplasmic translocation of SET has been observed in susceptible neurons of AD [31] and Down syndrome [32] patients, and could represent one of the multiple mechanisms for the abnormally elevated levels of phosphorylated tau accompanying these disorders.

Regulation of PP2A–tau interactions by tau phosphorylation

Although deregulation of PP2A/Bα can affect its ability to interact with tau and consequently tau (de)phosphorylation state, phosphorylation of tau at specific sites has also the potential to affect its ability to associate with the phosphatase. Indeed, AD-like pseudophosphorylation of several tau sites in the N-terminal proline-rich region of tau inhibits the association of tau with PP2A [33]. More specifically, phosphorylation of Thr231 in the proline-rich ^{230}RTPPKSP^{236} motif of tau significantly decreases binding of tau to PP2A *in vitro* [34], which provides a plausible explanation for the poor dephosphorylation of pThr231-Tau by PP2A/Bα [35]. Of clinical significance, phosphorylation of tau at Thr231 is found in early AD and prevents further dephosphorylation of pathological pS202/pThr205 tau sites by PP2A/Bα [35].

Disruption of PP2A–tau interactions by tau mutations

Many mutations of the *MAPT* gene have been described in familial non-AD tauopathies. They cause dementia by various mechanisms, with changes in alternative splicing, phosphorylation state, interaction with tubulin and self-assembly into filaments being the most significant contributing factors to the underlying tau pathology [36]. Notably, frontotemporal dementia with parkinsonism-17 (FTDP-17)-associated missense mutations G272V, ΔK280, P301L, P301S, S305N, V337M, G389R, and R406W are harbored within the PP2A/Bα interacting domain of tau and inhibit the binding of tau to PP2A/Bα by ~20–70% [37] (Fig. 1). As discussed earlier, the reduced binding of mutated tau to PP2A could favor the accumulation of phosphorylated tau, by interfering with tau dephosphorylation processes.

Influence of other proteins on PP2A–tau interactions

Like tau, pools of PP2A/Bα are also anchored to the microtubule wall, but both PP2A and tau bind at distinct sites on polymerized tubulin [16]. Interestingly,

the association of PP2A with microtubules could act as a regulatory mechanism to sequester the phosphatase and circumvent the untimely dephosphorylation of microtubule-associated proteins, considering that this cytoskeletal anchoring induces an inhibitory effect on the tau phosphatase activity of PP2A [16]. PP2A/Bα is also influenced by microtubule dynamics since tubulin assembly decreases PP2A/Bα activity *in vitro* [16]. Together, these findings infer that tau and PP2A only interact when they both dissociate from the microtubule cytoskeleton (Reviewed in Ref. [9]).

Other cytoskeletal components such as neurofilaments may also indirectly interfere with PP2A–tau interactions. It is well established that the neurofilament architecture that supports axonal caliber and function is regulated by PP2A activity (Reviewed in Ref. [9]). Since Bα-containing PP2A complexes associate quite strongly with neurofilaments [38], it is possible that this cytoskeletal interaction also serves to isolate PP2A/Bα pools from tau, so that neurofilament dynamics could also indirectly influence PP2A's tau phosphatase activity.

Of importance to tauopathies, certain protein kinases could also affect PP2A/Bα activity toward phosphorylated tau. For instance, Fyn, a Src family nonreceptor tyrosine kinase, binds *in vitro* to the ^{230}RTPPKSP^{236} motif of tau, thereby inhibiting the interaction of PP2A with tau through competitive binding [34]. It is noteworthy that increased Fyn–tau and decreased PP2A–tau interactions have been observed with disease-associated tau phosphorylation in the proline-rich region, as well as with frontotemporal dementia-associated tau mutations (Reviewed in Ref. [9]). Future studies will likely uncover additional modulators of PP2A–tau interactions.

PP2A: A chief conductor of tau subcellular distribution

Phosphorylation of tau orchestrates its subcellular targeting

Physiologically, tau is enriched in axons of mature neurons and serves various crucial functions not confined to the axoplasm. A key finding that has dramatically enhanced our understanding of tau is that pseudophosphorylation of selective Ser/Thr residues leads to its detachment from microtubules and subsequent redistribution to somatodendritic compartments, in particular, dendritic spines [39]. When deregulated, this mechanism could result in synaptic impairment [40]. Furthermore, deletion of the microtubule-binding repeat domain or P301L mutations in tau, both of which affect PP2A binding, target tau to dendritic spines [39].

Besides its primary residence in axons, tau is also present in the nucleus, predominantly in a hypophosphorylated state [41]. It is likely that nuclear PP2A/Bα pools present in neurons [38] could contribute to maintain nuclear tau in this dephosphorylated state, which underlies its DNA-protective functions [42,43] (Fig. 2).

Tau proteins are also targeted to the plasma membrane (Fig. 2) mostly in a dephosphorylated state [44]. Notably, downregulation of PP2A methylation leads to a loss of membrane-associated tau, and concomitant cytoplasmic accumulation of phosphorylated tau [45]. These findings suggest that tau phosphorylation state, at least in part, regulates its association with plasma membrane microdomains enriched with PP2A/Bα [45] and other key signaling molecules. Tau likely carries out a major scaffolding function in the assembly of these signaling complexes.

Physiological significance of PP2A-dependent tau regulation

Microtubules decorated with tau have altered dynamics

The most recognized function of tau is its ability to regulate microtubule dynamics (Reviewed in Ref. [9]). Tau binding to microtubules is regulated by site-specific phosphorylation, and is therefore overly dependent on PP2A/Bα (Fig. 2). Indeed, specific PP2A inhibition disrupts microtubule binding and stability, and is invariably associated with increased tau phosphorylation [7].

An important role of tau in axonal transport and synaptic plasticity

The distribution of tau along the axonal length is critical for the translocation of motor proteins, and thus, the flux of organelles and other cellular cargo. In healthy axons, tau distribution is nonuniform owing to a proximal to distal (with respect to the axon) increase in the concentration gradient [46]. Importantly, this enhances organelle flux and maximizes fast axonal transport toward the synapses [47,48]. The motor proteins dynein and kinesin frequently encounter tau patches along their path, which hinder their motility, leading to the reversal of direction or detachment, respectively [49]. Kinesin efficiently binds tau in the cell body wherein tau concentration is minimal, initiating anterograde transport; ultimately, the motor protein is released at the distal end of the axon

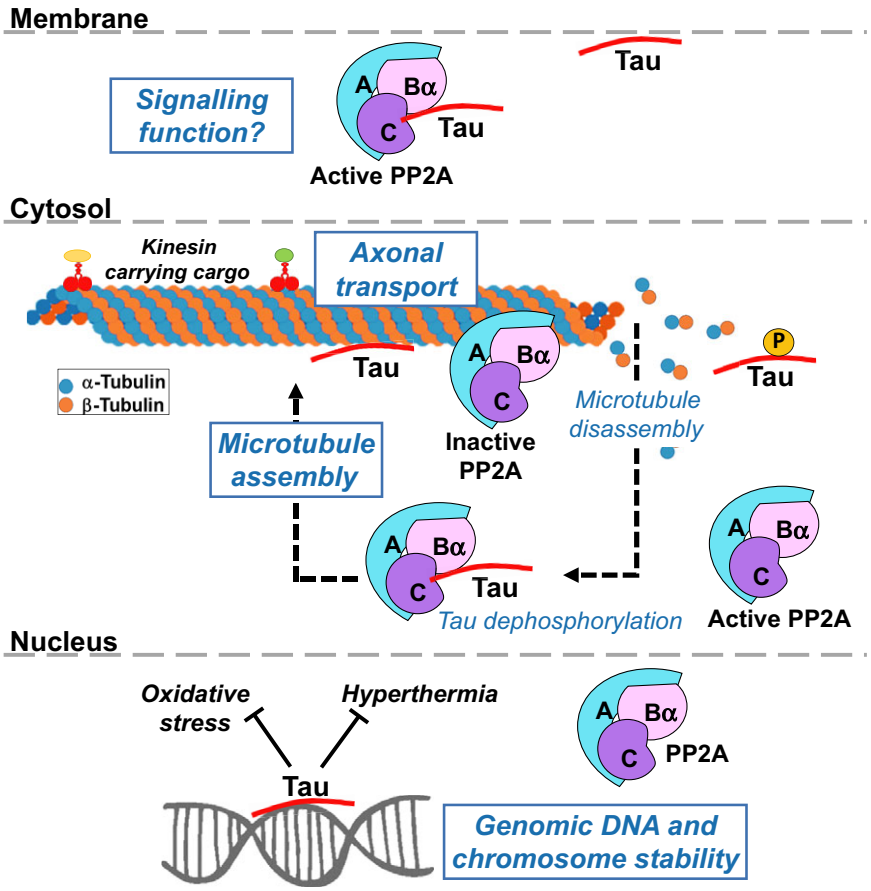

Fig. 2. Compartmentalization and PP2A-dependent regulation of tau function. In the axon where tau is enriched, spatial dephosphorylation of tau is influenced by the regulated binding of PP2A/Bα to neuronal microtubules. Tau binding to microtubules decreases their intrinsic dynamic instability and significantly influences bidirectional axonal transport. PP2A/Bα enzymes are sequestered in an inactive state on microtubules and released upon microtubule depolymerization. The direct association of PP2A/Bα with tau in the cytosol induces tau dephosphorylation. Tau may also exert other compartmentalized functions. For instance, nuclear pools of hypophosphorylated tau protect DNA from damage induced by thermal and oxidative stress. The proline-rich and R2 motifs anchor tau to DNA; this interaction is facilitated by tau dephosphorylation, which could be promoted by nuclear PP2A/Bα enzymes. Furthermore, PP2A methylation is required for the targeting of PP2A/Bα holoenzymes and tau to the plasma membrane [45], wherein tau could serve as a scaffold for signaling molecules. The tau–membrane interaction is inhibited by site-specific phosphorylation and involves tau's N-terminal projection domain [44].

characterized by higher tau concentrations [49]. Relative to kinesin, dynein can bind microtubules distally because of its lower sensitivity to tau, and initiates dynein-driven retrograde transport. It is likely that this phenomenon governed by the distinct occupancy of tau along axons can be fine-tuned by modulating phosphorylation and dephosphorylation within tau's microtubule-binding domain.

A signaling function of tau

Beyond its traditional role confined to axons, there is an increasing interest in noncanonical functions of tau such as facilitating glutamate signaling (Fig. 3). It appears that under normal physiological conditions, tau strategically traffics Fyn to postsynaptic densities (PSDs). There, tau serves a critical role in synaptic plasticity by stabilizing N-Methyl-D-aspartate receptors (NMDARs) by promoting their complex formation with the scaffolding protein, postsynaptic density protein 95 (PSD-95) [50]. From a neuropathological context, this role of tau boosts excitotoxicity and increases amyloid-β-mediated neurotoxicity during AD pathogenesis [51]. Upon NMDAR activation, tau dissociates from GluN2B subunit-containing NMDAR-PSD-95 scaffolds leading to increased tau phosphorylation at various Ser/Thr sites, while weakening its interaction with PSD-95 [52]. Tau leaves the NMDAR-PSD-95 complex with increased affinity for Fyn [52]. In this context, tau phosphorylation could

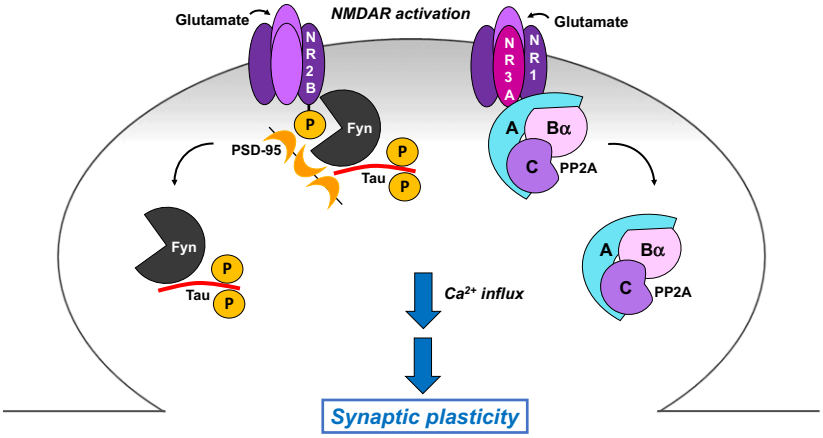

Fig. 3. An important role for tau in synaptic plasticity. Hetero-multimeric NMDA receptors (NMDARs) play a key role in synaptic plasticity, and their overactivation has been linked to excitotoxicity in Alzheimer disease. A small pool of endogenously phosphorylated tau is present in dendrites and synapses. In this model, tau interacts with a multifaceted complex including NR2-containing NMDAR, Fyn and PSD-95; this interaction is governed by the phosphorylation state of tau and tau-Fyn binding. NMDAR activation increases tau phosphorylation at Ser/Thr sites resulting in dissociation of tau and Fyn from the postsynaptic scaffold; this facilitates long-term depression (LTD). Interestingly, PP2A/Bα functionally interacts with the NR3 subunit-containing NMDAR complexes; it dephosphorylates Ser-897 on the NR1 subunit, which is known to regulate NMDAR activity. Upon NMDAR activation, PP2A dissociates in an inactive state. PP2A has been shown to be required for LTD. PP2A also competes with Fyn for binding to tau, and could therefore participate in modulating this glutamatergic cascade, by regulating Fyn–tau interactions and/or inducing tau dephosphorylation at Ser/Thr sites. Deregulation of normal tau–Fyn–PP2A interactions could contribute to the pathological accumulation of phosphorylated tau proteins, which has been shown in many studies to enhance NMDAR-dependent Ca^{2+} influx and promote glutamate excitotoxicity.

prevent excitotoxicity through destabilizing these multifaceted excitatory complexes by abrogating their interaction with Fyn. Interestingly, overexpressing a phosphomimetic tau species significantly enhances NMDAR currents and facilitates hippocampal LTD [52]. In support of this finding, a recent study in hippocampal slices has demonstrated that tau phosphorylation at Ser396 is required for LTD [6]. Hence, the phosphorylation of tau in response to NMDAR activation could explain why a small pool of phosphorylated tau is found in dendrites [52].

To prevent excessive, neurotoxic phosphorylation, the phosphorylated state of dendritic tau is likely controlled by a pool of PP2A, which is also found in PSDs and synaptic plasma membrane fractions. There, PP2A functionally associates with NR3 subunit-containing NMDAR complexes [53]. PP2A dissociates in a catalytically inactive state from these particular complexes following NMDAR activation [54] (Fig. 3). Thus, delineating the regulation of tau and PP2A in postsynaptic signaling events is essential for understanding the mechanisms leading to the pathological accumulation of phosphorylated tau species, which in turn enhances NMDAR-dependent Ca^{2+} influx and promotes glutamate excitotoxicity [51].

Tau has also been reported to negatively regulate ERK signaling, an important cascade for neuronal survival; depletion of tau induced ERK activation in mouse cortical neurons following extrasynaptic NMDAR stimulation [55]. Tau overexpression also primes Src for activation and influences Src-mediated actin remodeling in growth factor-stimulated cells [56].

A role for tau in genomic DNA protection and repair

Interestingly, tau also functions in the nucleus to protect DNA against oxidative stress and denaturation by hyperthermia [42,43] (Fig. 2). Foci of phosphorylated histone H2AX, a double-strand DNA break marker, have been found in tau-knockout mice during hyperthermic conditions [57]. Both genomic DNA and RNA integrity are altered under hyperthermic conditions in tau depleted hippocampal neurons [57]. It has been observed that nuclear tau is predominantly hypophosphorylated [41], which may promote its binding to the DNA backbone [42,58–60]. Like tau, PP2A functions in the nucleus to mediate DNA repair, and suppression of specific B subunits, including Bα, sensitizes cells to DNA damage-inducing agent [61–64]. PP2A/B56ε enzymes function in the repair process of double-strand breaks by dephosphorylating phosphorylated histone H2AX [61]. It is thus tempting to speculate that alterations in tau phosphorylation state could

impair its nucleic acid safeguarding function and potentiate DNA and RNA oxidative damage in AD brain, and this could be mediated in part by the loss of PP2A/Bα enzymes and PP2A dysfunction in AD [28].

Ribosomal function of tau

Tau was recently shown to associate with ribosomes [65]. This association is enhanced in AD brain extracts, as evidenced by the detection of tau phosphorylated at Ser396/Ser404 in ribosomal fractions; increased ribosome–tau interactions decrease RNA translation and impair PSD-95 synthesis [65]. These findings suggest that ribosomal tau functions to regulate protein translation, thereby influencing synaptic function, which relies on constant protein synthesis.

Emerging functions of non-neuronal tau

Tauopathies are also characterized by aggregates of tau in glial cells [66]. In oligodendrocytes, tau's interaction with Fyn promotes process outgrowth and could be important for initiating myelination [67]. Tau is also present in platelets from peripheral blood in cognitively normal subjects, and exists in high molecular weight or oligomeric forms in AD platelets [68]. Surprisingly, high molecular weight phosphorylated tau species have also been found in low quantities in non-neuronal cells, including several cancer cell lines [69]. Non-neuronal cytoplasmic and nuclear tau share functional features with brain tau such as the capacity to promote tubulin polymerization, and microtubule binding and stabilization [70]. However, they may also exert additional specific functions that remain to be uncovered.

Significance of PP2A-dependent tau phosphorylation under altered physiological conditions

Many changes to physiologically relevant metabolic processes deregulate PP2A, resulting in increased tau phosphorylation in various animal models. For instance, several studies have linked alterations in glucose metabolism, such as those found in diabetes and hypothermia, to increased tau phosphorylation [71–74]. Hypothermia is also associated with anesthesia, and increased tau phosphorylation is observed in mice sedated with anesthetic compounds (Reviewed in Ref. [75]). Notably, low temperatures exponentially decrease the activity of brain-extracted PP2A, while the activities of tau kinases are linearly reduced [72]. On the other hand, increased tau phosphorylation is also apparent in mice sedated under normothermic conditions [76,77], probably as a result of deregulation of tau kinases [77,78]. Such mechanisms could be relevant to public health since anesthesia is widely used and can be associated with postoperative cognitive dysfunction; repeated exposure may even increase AD risk [77].

Brain tau phosphorylation is also increased in a reversible way in hibernating animals [79]. Increased phosphorylated tau levels coincide with downregulation of brain PP2A methylation and activity in hibernating Arctic ground squirrels [80]. In hibernating golden hamsters, soluble hyperphosphorylated tau is associated with spine regression of hippocampal apical dendrites, but does not lead to memory impairment [81]. In this context, the physiological purpose of increased tau phosphorylation, especially at the AD-like PHF-1 epitope, remains unclear. It could represent a neuroprotective mechanism to circumvent NMDA-mediated hyperexcitation in response to gradual cooling of the brain during hibernation [79].

Other metabolic alterations such as hyperhomocysteinemia and low folate/vitamin B_{12} status disturb the brain methylation potential and are independent risk factors for sporadic AD and cognitive decline in human populations. Notably, they lead to the downregulation of PP2A methylation and increased tau phosphorylation *in vivo* (Reviewed in Ref. [10]).

The accumulation of hyperphosphorylated tau proteins has also been described following traumatic brain injury, and is a substantial risk factor for neurodegeneration [82].

Lastly, there is increasing evidence for a contribution of stress pathways in enhancement of tau phosphorylation. It is well established that mental stress has a priming role in the development of various psychopathologies (Reviewed in Ref. [83]), and may even increase the risk for tauopathies. In support of this, the major stress hormones, glucocorticoids, can induce GSK3-mediated tau phosphorylation at Ser-396 in the hippocampus, leading to the impairment of synaptic function; this involves activation of synapse weakening pathways that facilitate LTD [84]. To add to this mechanism, reduced levels of synaptic scaffolding proteins and dendritic spine loss are triggered by glucocorticoids in hippocampal neurons [85]; those are especially vulnerable to stress due to high expression of glucocorticoid receptors [83]. As mentioned earlier, the pSer-396 tau phosphosite is dephosphorylated by PP2A *in vivo*, and PP2A also regulates GSK3 activity. Thus, an imbalance between GSK3 and PP2A activities could result in enhanced susceptibility of neurons to stress, and in turn, facilitate phospho-tau-mediated development of neurodegenerative processes.

Significance of altered tau phosphorylation in tauopathies

Disease-associated tau mutations

Frontotemporal dementia with parkinsonism-17-associated mutations of tau not only impede its binding to PP2A but also to microtubules, reducing its ability to promote MT assembly [86]. It is noteworthy that FTDP-17 patients have increased 4R-tau expression. Disease-related tau phosphorylation and missense mutations increase the interaction of 4-repeat tau (4R-tau) and Fyn [87], while decreasing its binding to PP2A [34]. Thus, deregulation of PP2A/tau/Fyn interactions likely contributes to promote the accumulation of phosphorylated tau in those tauopathies.

The toxic role of tau 'hyperphosphorylation'

Although the toxicity of abnormally phosphorylated tau has been debated for years, it is generally accepted that enhanced tau phosphorylation, especially at selected sites, precedes and promotes the assembly of tau into toxic aggregates in tauopathies (Reviewed in Ref. [88]). 'Hyperphosphorylated' tau species accumulate in somatodendritic compartments and instigate neurodegenerative cascades *via* several mechanisms. For instance, the redistribution of axonal tau to the soma, which alters its functionally strategic axonal distribution, could impair axonal transport by inhibiting tau-sensitive kinesin-1 binding to microtubules [49]. Another mechanism could involve excessive tau-dependent sorting of Fyn to the dendrites augmenting excitotoxic signaling induced by amyloid-β in AD [51]. Hyperphosphorylated tau also sequesters normal tau, leading to its toxic coaggregation and decreased supply of 'physiological' tau [88], ultimately impairing normal synaptic function.

The phosphorylation state of tau also modulates its proteolytic cleavage [89]. Several studies support a role for caspase-mediated tau cleavage as an early event in AD pathology [89–92]. Caspase-2-mediated cleavage of tau at Asp314 produces the Δtau314 product, which impairs cognitive and synaptic function [90]. Interestingly, specific PP2A subpopulations can indirectly suppress caspase-2 activity [93], and inhibition of PP2A by okadaic acid alters tau cleavage during neuronal apoptosis [94]. Moreover, GSK3β, which can be activated by PP2A-mediated Ser9 dephosphorylation [18], is found upstream of caspase-2 activation [95]. Thus, altered PP2A–tau interactions in AD could affect tau cleavage, leading to accumulation of dysfunctional phosphorylated tau in degenerating AD neurons.

A protective role for tau phosphorylation?

A pivotal discovery supports a novel neuroprotective role of site-specific tau phosphorylation, while challenging the historical perception that enhanced tau phosphorylation is systematically associated with neurotoxicity. In this study, tau phosphorylation at Thr205 by p38γ kinase, inhibited amyloid-β-induced neuronal death and excitotoxicity [96] by disrupting postsynaptic PSD-95/tau/Fyn complexes [50]. Another study using phosphomimetic tau mutants support a role for phosphorylation at Thr205 in compromising tau's interaction with PSD-95 [52]. Notably, PP2A/Bα can dephosphorylate this site *in vitro* [18], and this dephosphorylation might allow to revert tau to its basal low phosphorylated state, so that it can respond to future 'stresses'. The neuroprotective role of pThr205 tau is proposed to occur at the onset of AD pathogenesis, and progressively become overwhelmed by repeated stresses. PP2A has the potential to significantly influence this physiological mechanism, since PP2A directly associates with, dephosphorylates, and inhibits p38 activity *in vitro* [97] and *in vivo* [98]. PP2A dysfunction in AD (Reviewed in Ref. [10]) could contribute to derail these tau-dependent neuroprotective mechanisms.

So, should we target PP2A to treat tauopathies?

PP2A-centric therapies: promises

Together, the leading tau regulatory role and dysfunction of PP2A in AD provide at first sight a compelling rationale for developing PP2A-centric therapies for tauopathies. Targeting the major tau phosphatase is an attractive strategy to normalize or restore the phosphorylation state of pathological tau species. Ideally, this would require early intervention since site-specific phosphorylation of tau can inhibit PP2A phosphatase activity toward other key sites implicated in AD [35]. Many compounds have already been found to enhance basal PP2A activity by several—most often indirect—mechanisms, thereby facilitating tau dephosphorylation (Table 1). However, as outlined below, there are several obstacles for the clinical use of such compounds.

Current PP2A-targeting compounds

Patients with mild and moderate AD have been recruited to evaluate the clinical response to and efficacy of sodium selenate, an inorganic compound that enhances PP2A activity *in vitro,* reduces phospho-tau

Table 1. Compounds that have been shown to mitigate aberrant tau phosphorylation in a PP2A-dependent manner. Note that most of these compounds, which reduce tau phosphorylation levels, act indirectly on PP2A. In many cases, the specificity of these compounds for PP2A has not been fully validated, and/or underlying PP2A regulatory mechanisms are unknown. ↑: increased; ↓, decreased; ND, not determined or unknown.

Compounds	Target	Effect on PP2A	Experimental model and additional effects
Sodium selenate	PP2A C?	↑ Activity	AD mouse models [99,100]—Reverses memory deficits
Metformin	PP2A C	↓ Degradation ↑ Activity	Cortical neurons and tau transgenic mice [110]
COG112	SET [101]	↑ Activity [101]	AD mouse models [102]—Protects against impaired neurogenesis
Memantine	SET	↑ Activity	SET-overexpressing PC12 cells [31]
Lovastatin	ND	↑ Activity	Rat primary neurons [119]
Clioquinol	ND	↑ Activity	Tau transgenic mice [120]
SEW2871	ND	↑ Methylation	Rat hippocampal slices [109]
Folic acid + B_{12}	PP2A C	↑ Activity	Aged hyperhomocysteinemic rats [103]—↓ memory deficits
Folic acid	LCMT-1	↑ Methylation	Streptozotocin-treated (diabetic) mice [104]
S-adenosylmethionine	LCMT-1	↑ Methylation	N2a cells [25]
Forskolin	ND	↑ Activity and expression	At high concentrations in rats [121]—also improves memory deficits at high concentration
EHT	PME1	Inhibits demethylation	Truncated SET-expressing rats—Prevents memory and cognitive impairment [105]
Resveratrol	ND	↑ Activity	Formaldehyde-treated N2a cells [122]
Ginsenoside Rd	ND	↑ Activity	Okadaic acid-treated rats [123]— Protects against cytoskeletal disruption in cortical neurons
Ginkgo biloba extract	ND	↑ Activity	Hyperhomocysteinemic rats [124]—Protects against spatial memory deficits
Xanthoceraside	ND	↑ Expression	Streptozotocin-treated rats [125]—Protects against impaired learning and memory

pathology, improves spatial learning and memory, and mitigates motor deficits in tauopathy transgenic mouse models [99,100]. However, its exact mechanism of action and specificity toward PP2A enzymes has not been formally demonstrated.

There are also efforts to target SET, the redistribution of which is a major mechanism for abnormal cytoplasmic tau phosphorylation in AD [31]. Memantine, an uncompetitive NMDAR antagonist used to symptomatically treat AD, inhibits SET-mediated PP2A inhibition and reverses SET-induced abnormal phosphorylation of tau in PC12 cells [31]. The apolipoprotein E mimetic, COG112, also increases PP2A activity by inhibiting its interaction with SET [101] and reduces the phosphorylation and somatodendritic accumulation of hyperphosphorylated tau in the hippocampus and cerebral cortex of AD mouse models [102].

The loss of PP2A/Bα holoenzymes in AD-affected brain regions [28] parallels the downregulation of PP2A methylation and LCMT1, which is normally abundantly expressed in neurons throughout the cortex [27]. Significantly, alterations in homocysteine and folate metabolism and associated deficits in brain methylation metabolites have been described in AD; they induce a downregulation of LCMT1 and enhanced tau phosphorylation *in vivo* (Reviewed in Ref. [10]). Thus, restoring neuronal methylation potential using dietary methylation donors and/or directly targeting the enzymes that control PP2A methylation (Table 1) could facilitate the assembly of PP2A/Bα holoenzymes and increase PP2A-mediated tau dephosphorylation. Indeed, folate and vitamin B_{12} supplementation in aged rats prevents hyperhomocysteinemia-mediated PP2A inhibition, tau hyperphosphorylation, and memory deficits [103]. Folic acid supplementation reduces tau phosphorylation by affecting PP2A methylation in diabetic mice [104]. The coffee-derived compound EHT, which decreased the rate of PP2A demethylation by the methylesterase, PME-1, mitigates tau hyperphosphorylation and cognitive impairment in SET-expressing rats [105]. However, it remains to be established whether this compound could overcome the loss or metabolic deregulation of LCMT1 in AD.

Various compounds of interest for cancer treatment have also been designated as 'PP2A activators', such as forskolin, a cAMP pathway activator, and FTY720, a sphingosine-1-phosphate receptor agonist [106]. Yet, it is worth mentioning that the underlying activating mechanism of such compounds fails to align with the true definition of an *enzyme activator*, since they indirectly target PP2A. For instance, forskolin indirectly enhances PP2A activity *via* activation of cAMP

signaling [107, 108]. Like COG112 [97], FTY720 disrupts PP2A–SET interactions, resulting in 'increased cellular PP2A activity' [106]. Of note, SEW2871, a more selective sphingosine-1-phosphate receptor agonist than FTY720, can enhance PP2A methylation and reduce tau phosphorylation [109].

Issues with specificity and 'off-target' effects of some of these PP2A regulatory compounds

Interestingly, metformin, a drug used to treat diabetes, can decrease tau phosphorylation in a PP2A-dependent manner (Table 1), by disrupting the interaction of PP2A C subunit with specific regulatory subunits, which normally promotes its degradation [110]. Yet, follow-up studies have found that, despite inducing tau dephosphorylation, metformin promotes tau aggregation and exacerbates abnormal behavior in tauopathy mouse models [111].

While forskolin 'activates' PP2A [107,108], it affects cAMP signaling that orchestrates a number of cellular processes, thus increasing the risk of unwanted side effects. Moreover, it enhances phosphorylation of tau at particular sites in primary hippocampal neurons [108]. Forskolin induces PKA-dependent phosphorylation of tau *in vivo*, which makes tau a more favorable substrate for the major tau kinase GSK-3β [112]. Pharmaceutically targeting SET to 'disinhibit' PP2A catalytic subunit could also pose significant side effects. For instance, FTY720 was found to potentially increase the risk for malignancies in recipients (For instance, see [113]), and apolipoprotein E-mimetic peptides downregulate p38 activity [114].

Protein phosphatase 2A being a family of tailored heterotrimers displaying exquisite substrate specificity, the clinical implication of simplistically randomly 'reactivating' cellular pools of PP2A enzymes should be viewed as a matter of concern rather than a therapeutic opportunity. In considering the comprehensive cellular role of the PP2A family of enzymes, and their broad range of substrates, indiscriminately activating 'PP2A' with potent compounds could be toxic. Furthermore, physiological modulators of PP2A ensure a sophisticated regulation to prevent untimely, and thus, detrimental phosphatase activation prior to holoenzyme biogenesis [115]. For instance, PME-1, targeted by EHT [100], is known to stabilize a nuclear pool of inactive PP2A enzymes [116]. Mechanistically, it is also not clear how 'PP2A activating compounds' could compensate for the loss of the major tau phosphatase, PP2A/Bα, in AD-affected regions, simply by enhancing the catalytic activity of remaining neuronal PP2A isoforms.

However, development of drugs targeting more selectively PP2A regulatory subunits, or disrupting specific PP2A protein signaling complexes could be promising, especially when the target signaling pathways are deregulated in AD. For instance, orally bioavailable small activators of PP2A termed SMAPs, drive activation-inducing conformational changes in PP2A by binding to the Aα subunit, and inhibit ERK signaling [117]. Similarly, the natural anticancer product withaferin A, which also targets Aα, leads to activation of PP2A and inhibition of AKT signaling [118].

Still, a major challenge will be the toxicity of long-term use of PP2A/tau-targeting compounds, a must for AD patients due to the extended course of this disorder. As mentioned earlier, phosphorylated tau, like PP2A, is also present in other cell types, which could also be a problem for systemic PP2A/tau-directed strategies.

Conclusions and Perspectives

Like many other cytoskeletal and cellular proteins, tau harbors a multitude of validated and predicted phosphorylation sites. Regulated phosphorylation of tau can significantly modulate its distribution, interaction with cytoskeletal components, and cellular functions. While tau proteins are phosphorylated to a certain degree under physiological conditions, abnormally increased phosphorylation has been extensively used as a direct measure or readout of tau toxicity and disease progression in numerous tauopathy studies. Consequently, decreasing overall tau phosphorylation, for instance by inhibiting major tau kinases, has been proposed as a valid approach to counteract tau toxicity in these disorders. Taking into consideration that PP2A enzymes dephosphorylate a majority of tau phosphosites *in vivo*, control the activity of tau kinases, and are significantly downregulated in AD-affected brain regions, there is increasing interest in developing PP2A-activating compounds to counteract tau pathology. There is a strong rationale for this approach since the deregulation and loss of tau-dedicated PP2A/Bα holoenzymes can directly contribute to deregulation of tau in various experimental models, and likely plays a role in the pathogenic processes underlying tauopathies.

Yet, we argue that the approach of simply activating PP2A-dependent tau dephosphorylation is likely flawed. While a blanket activation of PP2A can induce tau dephosphorylation, PP2A enzymes exert widespread functions. Restoring normal tau function will more specifically require to re-establish normal PP2A/Bα-tau and kinase/tau protein–protein interactions. Those interactions, which critically regulate

tau distribution and support tau function as a signaling scaffold, become altered in tauopathies. Therapeutic strategies also need to take into account that correcting common age- and genetic-related metabolic deficits that deregulate PP2A (Reviewed in Ref. [10]) is essential for restoring neuronal homeostasis. Furthermore, there is increasing evidence that site-specific phosphorylation differentially modulates tau function and toxicity. Indeed, tau phosphorylation at particular sites is important for axonal transport, signaling and neuroprotection. Thus, a better understanding of the role of phosphorylation/dephosphorylation processes in the regulation and cellular functions of tau is paramount on the challenging and tangled path to development of effective drugs for tauopathies.

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JBC RESEARCH ARTICLE

Disturbances in PP2A methylation and one-carbon metabolism compromise Fyn distribution, neuritogenesis, and APP regulation

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The nonreceptor protein tyrosine kinase Fyn and protein Ser/Thr phosphatase 2A (PP2A) are major multifunctional signaling molecules. Deregulation of Fyn and altered PP2A methylation are implicated in cancer and Alzheimer's disease (AD). Here, we tested the hypothesis that the methylation state of PP2A catalytic subunit, which influences PP2A subunit composition and substrate specificity, can affect Fyn regulation and function. Using Neuro-2a (N2a) neuroblastoma cell models, we first show that methylated PP2A holoenzymes containing the Bα subunit coimmunoprecipitate and copurify with Fyn in membrane rafts. PP2A methylation status regulates Fyn distribution and Fyn-dependent neuritogenesis, likely in part by affecting actin dynamics. A methylation-incompetent PP2A mutant fails to interact with Fyn. It perturbs the normal partitioning of Fyn and amyloid precursor protein (APP) in membrane microdomains, which governs Fyn function and APP processing. This correlates with enhanced amyloidogenic cleavage of APP, a hallmark of AD pathogenesis. Conversely, enhanced PP2A methylation promotes the nonamyloidogenic cleavage of APP in a Fyn-dependent manner. Disturbances in one-carbon metabolic pathways that control cellular methylation are associated with AD and cancer. Notably, they induce a parallel loss of membrane-associated methylated PP2A and Fyn enzymes in N2a cells and acute mouse brain slices. One-carbon metabolism also modulates Fyn-dependent process outgrowth in N2a cells. Thus, our findings identify a novel methylation-dependent PP2A/Fyn signaling module. They highlight the underestimated importance of cross talks between essential metabolic pathways and signaling scaffolds that are involved in normal cell homeostasis and currently being targeted for cancer and AD treatment.

Fyn is a member of the Src family kinases (SFKs) of nonreceptor protein tyrosine kinases that modulate a plethora of key cellular functions, including growth, survival, adhesion, migration, and differentiation (1). Expectedly, deregulation of these important signaling enzymes is associated with numerous pathological conditions, including cancer (2). Of particular interest, deregulation of Fyn also participates in Alzheimer's disease (AD) pathogenesis. In AD, the abnormal accumulation of amyloid-β (Aβ) peptides derived from enhanced β- and γ-secretase cleavage of amyloid precursor protein (APP) is believed to be a key event in initiating hallmark neurodegenerative cascades (3). Pathological levels of oligomeric Aβ species are linked to aberrant overstimulation of postsynaptic Fyn-dependent signaling pathways, ultimately leading to impaired synaptic and cognitive functions, and neurotoxicity in several animal models of AD (4). Overactivation of Fyn also induces an abnormal elevation of APP phosphorylated at Tyr^{682}, causing its mistrafficking and missorting in neurons, with important consequences for Aβ production (5). Inhibition of Fyn activity can counteract memory and synaptic deficits in AD mice (6), further demonstrating the essential link between Fyn deregulation and AD pathogenic pathways.

SFKs transduce signals from a variety of receptors *via* their ability to form complexes with numerous cytoskeletal and signaling proteins at the plasma membrane (1, 2). The spatial localization and signaling activity of SFKs is tightly controlled by endocytic trafficking (7, 8). In neuronal cells, the myristoylated and palmitoylated Fyn kinase is preferentially enriched and activated in sphingolipid- and cholesterol-enriched plasma membrane microdomains traditionally referred to as lipid or membrane rafts (9–11). These specialized microdomains serve a key role in cell signaling and function by compartmentalizing and regulating interactions of key membrane proteins (12). For instance, activation of raft-associated Fyn stimulates neurite outgrowth (13–15) and regulates the targeting of APP to lipid rafts (11). Notably, membrane microdomain switching is a key determinant of APP processing and function (16–19). Under normal physiological conditions, a majority of APP undergoes proteolytic processing by α-secretase, which precludes Aβ formation and generates neurotrophic-secreted soluble amyloid precursor protein α (sAPPα) fragments. There is strong support that the α-secretase cleavage of APP preferentially occurs in nonraft membrane microdomains, while its amyloidogenic processing primarily takes place in lipid rafts (17–19).

Another major signaling molecule deregulated in cancer (20) and AD (21) is protein Ser/Thr phosphatase 2A (PP2A).

This article contains supporting information.

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The "PP2A family" encompasses multimeric enzymes with the typical mammalian holoenzyme being composed of a catalytic "C" subunit (PP2Ac) associated with a scaffolding "A" subunit and a variable regulatory "B" subunit. PP2A biogenesis, stability, and substrate specificity can be modulated by leucine carboxyl methyltransferase 1 (LCMT1)-dependent methylation of PP2Ac on the Leu309 residue; conversely, PP2Ac is demethylated by the methylesterase, PME1 (22). We have previously reported that reduced PP2A methylation is associated with a loss of PP2A/Bα holoenzymes that contain the regulatory Bα (or PPP2R2A) subunit and altered dephosphorylation of PP2A/Bα substrates, including APP phosphorylated at the Thr^{668} site, in Neuro-2a (N2a) cells and *in vivo* (23, 24). Phosphorylation of APP at Thr^{668} and Tyr^{682} regulates APP interactions (25), subcellular localization, processing, and function, so that abnormally enhanced phosphorylation of APP in AD likely contributes to APP dysfunction (3, 5). PP2A methylation becomes downregulated in AD and after alterations in one-carbon metabolism in cells and *in vivo* (21). Disturbances in one-carbon metabolism that promote toxic elevation of plasma homocysteine (Hcy) and its oxidized derivatives and inhibition of cellular methylation are strongly associated with AD (26, 27) and cancer (28).

In this study, using neuroblastoma N2a cell models, we show that intact PP2A methylation is essential for the formation of PP2A/Bα-Fyn protein complexes and their codistribution in membrane rafts. Altered PP2A methylation promotes a redistribution of Fyn and inhibits Fyn-dependent neuritogenesis. It affects the compartmentalization of Fyn and APP in membrane microdomains, which regulates APP processing. Manipulations of one-carbon metabolism that modulates PP2A methylation state also affect Fyn distribution. Our findings identify a novel mechanism of regulation of Fyn at the crossroads of metabolism and signaling.

Results

PP2A coimmunoprecipitates with Fyn in a methylation-dependent manner

Based on the reported *in vitro* binding of PP2Ac to Src (29), an SFK structurally closely related to Fyn, we first assessed the existence of PP2A–Fyn protein complexes using a series of coimmunoprecipitation assays. Western blot analyses showed that endogenous PP2Ac was present in Fyn immunoprecipitates prepared from mouse cortical homogenates (Fig. 1*A*). However, endogenous Fyn was not detected in corresponding PP2Ac immunoprecipitates. Although these data suggest that PP2A is a major Fyn interacting protein and Fyn is a minor interacting partner of PP2Ac, it is worth noting that anti-PP2Ac subunit antibodies are ineffective at quantitatively pulling down native heterotrimeric PP2A holoenzymes, the predominant species in mammalian cells (30). To circumvent this problem, anti-hemagglutinin (anti-HA)–conjugated beads were used to effectively immunoprecipitate PP2A holoenzymes from well-characterized N2a cell lines stably expressing hemagglutinin (HA)-tagged WT PP2Ac or the L309Δ PP2Ac mutant, as described previously (23). In contrast to the WT, the L309Δ mutant is methylation incompetent and unable to

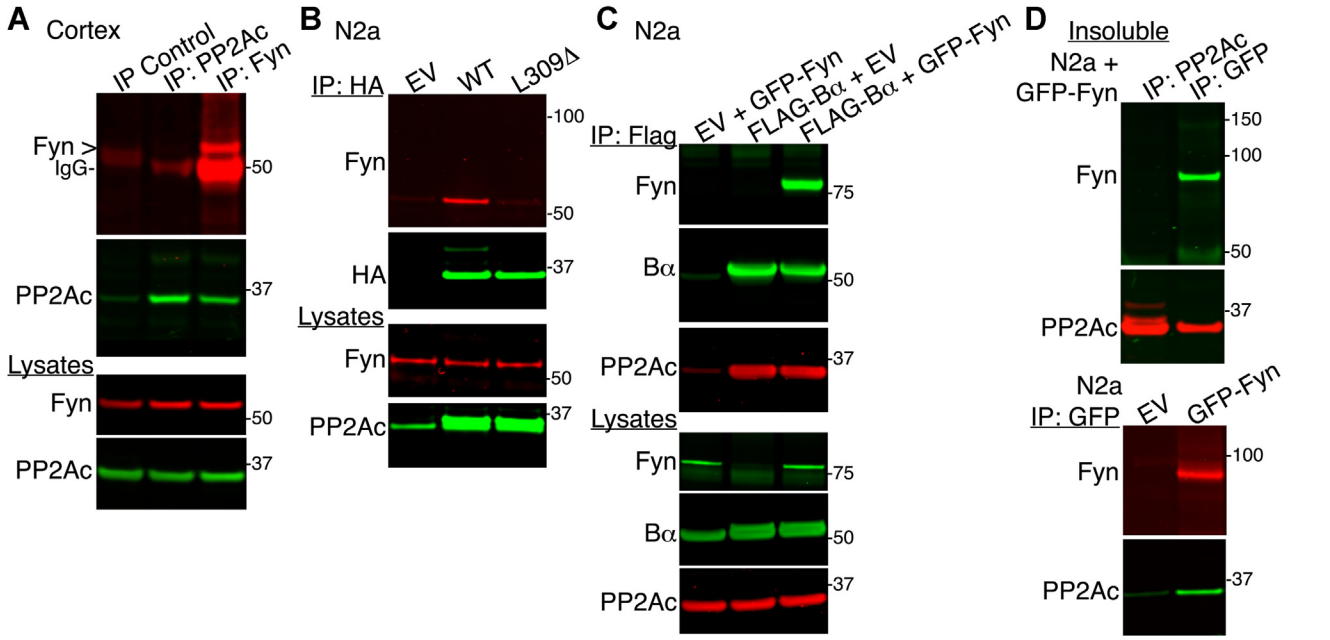

Figure 1. Methylated PP2A/Bα enzymes coimmunoprecipitate with Fyn. *A*, coimmunoprecipitation of endogenous PP2Ac and Fyn from total mouse cortical lysates. *B*, HA immunoprecipitates and total lysates from N2a cells stably expressing HA-tagged PP2Ac (WT), the methylation-incompetent L309Δ PP2Ac mutant (L309Δ), or empty vector (EV) were immunoblotted for Fyn, HA, or PP2Ac. *C*, flag immunoprecipitates and total lysates from N2a cells transfected with the indicated plasmids were immunoblotted for Fyn, PP2Ac, and PP2A-Bα. *D*, the whole NP-40 detergent-insoluble fraction prepared from GFP–Fyn–expressing N2a cells was divided into two equal parts. GFP or PP2Ac immunoprecipitates were prepared from each aliquot and analyzed for the presence of Fyn and PP2Ac (*top* panel). No Fyn or PP2Ac was found in control GFP immunoprecipitates carried out in EV-transfected, compared with, GFP–Fyn–expressing N2a cells (*bottom* panel). Representative blots from 3 separate experiments are shown in panels *A*–*D*. HA, hemagglutinin; IgG, immunoglobulin; PP2A, PP2A, phosphatase 2A; PP2Ac, catalytic "C" subunit of PP2A; N2a, Neuro-2a.

associate with regulatory B-type subunits in N2a (31) and other cell lines (22). Cells transfected with an empty vector (EV) were used as controls. Western blot analyses revealed that endogenous Fyn was present in HA–PP2Ac immunoprecipitates prepared from WT but not L309Δ-expressing or control N2a cell lysates (Fig. 1*B*), suggesting that Fyn preferentially associates with B-containing methylated PP2A holoenzymes. To confirm the existence of PP2A/Bα–Fyn protein complexes, immunoprecipitates were prepared from N2a cells coexpressing Flag–Bα and GFP–Fyn. We chose this approach based on the lack of suitable antibodies to pull down PP2A/Bα holoenzymes and the close proximity of endogenous Bα, Fyn, and immunoglobulins on gels. GFP–Fyn was clearly concentrated with the Bα subunit in Flag–Bα immunoprecipitates prepared from these cells but was absent in parallel control immunoprecipitations (Fig. 1*C*). The concomitant presence of PP2Ac in these immunoprecipitates further confirmed that PP2A/Bα holoenzymes were successfully pulled down under our experimental conditions.

In agreement with the preferential targeting of Fyn to membrane rafts, Fyn is enriched in detergent-insoluble fractions obtained after lysing tissue or cells with the nonionic detergent, NP-40 (32). Typically, NP-40 detergent-insoluble fractions contain highly insoluble lipid rafts and cytoskeletal components, whereas the detergent-soluble fraction largely consists of cytosolic proteins and extracted proteins "less tightly" attached to membranes. Endogenous PP2Ac coimmunoprecipitated with Fyn in NP-40 detergent-insoluble fractions prepared from GFP–Fyn–transfected but not EV-transfected N2a cells (Fig. 1*D*), supporting the existence of membrane-associated PP2A–Fyn protein complexes. Together, these findings indicate that methylation-dependent PP2A/Bα holoenzymes are important for the formation of PP2A–Fyn protein complexes.

PP2A methylation state affects the levels of membrane-associated Fyn in N2a cells

In light of the importance of PP2A methylation and subunit composition for its membrane targeting (33) and interaction with Fyn (Fig. 1*B*) in N2a cells, we next assessed whether modulating PP2A methylation state has any influence on the steady-state distribution of Fyn. To that end, total lysates and Fyn-enriched NP40-detergent insoluble fractions were first prepared from our N2a cell models and comparatively analyzed by Western blotting for relative changes in endogenous Fyn levels. Compared with controls, detergent-insoluble Fyn levels were overall increased in WT-expressing N2a cells (Fig. 2, *A* and *B*), which display a proportional ∼30% increase in total cellular and membrane-associated PP2Ac amounts (33). In contrast to the WT, there was a decrease in insoluble Fyn levels in L309Δ-expressing N2a cells. In these cells, there is a ∼35 to 45% reduction of methylated PP2Ac and Bα subunit levels and concomitant accumulation of demethylated PP2Ac (23); these methylation deficits induce a pronounced loss of PP2A/Bα enzymes targeted to the plasma membrane (33). Further demonstration of the regulatory role of PP2A methylation in Fyn distribution was obtained by deregulating PP2A methyltransferase and methylesterase enzymes. Overexpression of LCMT1, which promotes a ∼30% increase in the steady-state levels of total and membrane-associated methylated PP2Ac and PP2A/Bα in N2a cells (23, 33), markedly enhanced the amounts of Fyn in detergent-insoluble fractions (Fig. 2, *A* and *B*). Conversely, those were reduced after overexpression of PME-1, which promotes the accumulation of demethylated PP2Ac, downregulation of PP2A/Bα, and concomitant decrease in membrane levels of methylated PP2A enzymes (23, 33). Notably, this reduction in detergent-insoluble Fyn levels was recapitulated by inducing partial, siRNA-mediated LCMT1 downregulation in N2a cells (Fig. 2, *C* and *D*). This submaximal reduction in LCMT1 levels is associated with a pronounced loss of methylated PP2Ac and PP2A/Bα (34), and membrane-associated LCMT1 and PP2Ac in N2a cells (33). Thus, our findings suggest that PP2A methylation state concomitantly affects the membrane targeting of PP2A/Bα and Fyn.

Parallel immunoblotting of total cell lysates confirmed that the differential enrichment of Fyn in NP-40 detergent-insoluble fractions in our cell models was not related to changes in total Fyn protein expression levels (Fig. 2*A*, Fig. S1*A*). To assess whether PP2A-mediated alterations in detergent-insoluble Fyn levels are associated with modulation of Fyn activity state, cell fractions were also probed with validated antibodies recognizing SFKs phosphorylated (pSFK) at the conserved regulatory Tyr^{416} (numbering depending on species), a readout of SFK activity (1). Basal levels of active pSFK were observed in control N2a cells cultured in a "normal" serum-containing medium (Fig. 2*A*). Relative to controls, there was a marked increase in the pSFK signal in insoluble fractions that closely mirrored the increase in insoluble Fyn protein expression levels in WT- and LCMT1-expressing cells. Conversely, a similar reduction in the levels of pSFK and Fyn was observed in insoluble fractions from L309Δ- and PME1-expressing N2a cells. Yet, after normalizing the pSFK signal (apportioned to Fyn) for Fyn protein expression levels, we found no changes in the net phosphorylation of Fyn in any of the cell lines examined, relative to controls (Fig. S1*B*). Thus, under our experimental conditions, PP2A methylation influenced steady-state levels of active Fyn in detergent-insoluble fractions *via* mechanisms that do not implicate overall changes in Fyn activity or protein turnover.

Because neuronal Fyn is primarily concentrated in lipid rafts that resist extraction by nonionic detergents (9–11), perturbing PP2A methylation could more specifically alter the targeting of Fyn to these membrane microdomains. In this context, we have previously shown that pools of LCMT1 and methylated PP2Ac and PP2A/Bα holoenzymes are concentrated in lipid rafts, whereas demethylated PP2Ac is preferentially distributed in nonraft membrane microdomains purified from N2a cells (33). These observations suggest that Fyn and methylated PP2A/Bα enzymes are present in the same lipid raft compartment in N2a cells. To confirm this hypothesis, we reanalyzed fully characterized lipid raft and nonraft fractions obtained after membrane fractionation of N2a cells in an earlier study (33). Indeed, Fyn copurified with endogenous

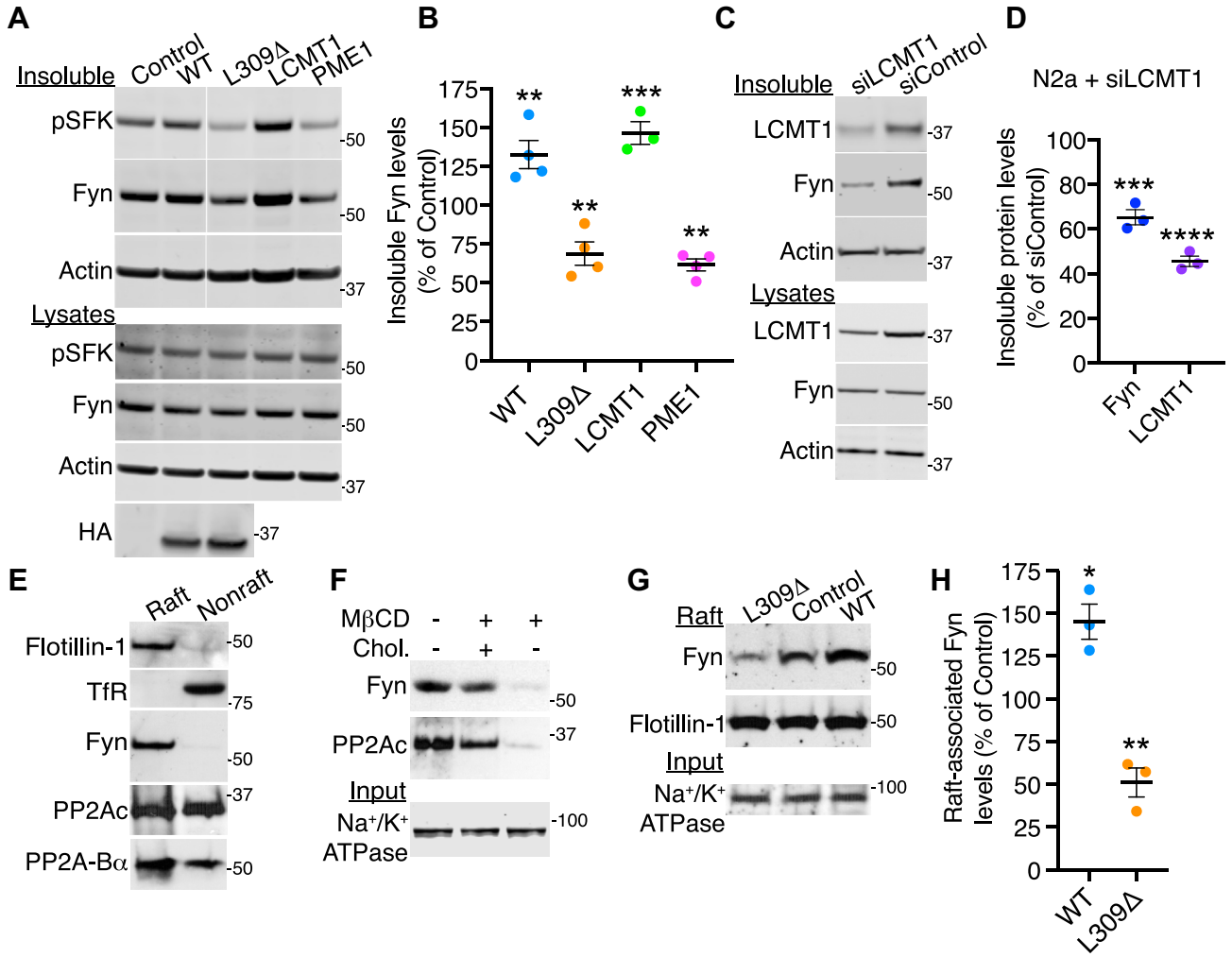

Figure 2. Changes in PP2A methylation influence the discrete membrane distribution of Fyn in N2a cells. *A*, representative immunoblots of pY416-SFK (pSFK), Fyn, and actin in total lysates and NP-40 detergent–insoluble fractions prepared from N2a cells stably expressing WT PP2Ac, the L309Δ PP2Ac mutant, LCMT1, PME1, or empty vector (control). Panels in detergent-insoluble fractions originated from the same blot. *B*, quantification of Fyn levels in NP-40 detergent–insoluble fractions from these cells. Data (mean ± SEM from $n = 3$–4 independent experiments) were appraised using one-way ANOVA ($F(4, 14) = 33.44$; $p < 0.0001$) with Dunnett's post hoc test. $^{**}p < 0.01$, $^{***}p < 0.001$, *versus* control. *C*, total lysates and NP-40 detergent–insoluble fractions purified from N2a cells transfected with a validated siRNA targeted to LCMT1 (siLCMT1) or a mismatch siRNA control (siControl) were analyzed by Western blotting for the presence of Fyn and LCMT1. *D*, Fyn and LCMT1 protein levels were decreased in detergent-insoluble fractions from siLCMT1 relative to siControl-transfected N2a cells. Data (mean ± SEM; $n = 3$ separate experiments) were analyzed using a student *t*-test. $^{***}p < 0.001$, $^{****}p < 0.0001$. *E*, representative immunoblots of PP2ABα and PP2Ac subunits, Fyn, flotillin-1, and transferrin receptor (TfR) in raft and nonraft membrane fractions purified from N2a cells. Similar results were obtained in three separate purifications. *F*, representative distribution of PP2Ac and Fyn in aliquots (15 μg) of raft fractions purified from N2a cells that were incubated for 15 min in a serum-deficient medium in the absence (–) or presence (+) of the cholesterol depletion agent MβCD or cholesterol (Chol). Total membrane fractions (input) from these cells were probed with an antibody against the membrane marker, sodium potassium adenosine triphosphatase (Na^+/K^+ ATPase). *G*, representative Western blot analysis of total membrane fractions (input) and rafts purified from EV-, WT-, or L309Δ-transfected N2a cells. *H*, relative levels of raft-associated Fyn were quantified in EV-, WT-, and L309Δ-expressing N2a cells. Data (mean ± SEM from $n = 3$ separate purifications) were analyzed using a student *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$, *versus* control. EV, empty vector; LCMT1, leucine carboxyl methyltransferase 1; N2a, Neuro-2a; MβCD, methyl-β-cyclodextrin; PP2A, PP2A, protein phosphatase 2A; PP2Ac, catalytic "C" subunit of PP2A.

PP2Ac and Bα subunits in these flotillin-1–positive membrane rafts isolated from N2a cells (Fig. 2*E*). As expected, membrane cholesterol depletion by methyl-β-cyclodextrin (MβCD) induced the loss of both PP2A (33) and Fyn from these fractions (Fig. 2*F*). This effect was reversed by subsequent cholesterol replenishment using a preformed cholesterol–MβCD complex, demonstrating the cholesterol-dependent microdomain association of PP2A and Fyn. We next assessed how disrupting the integrity of PP2A methylation affects the levels of Fyn in N2a cell membrane rafts. We have previously reported that raft-bound PP2Ac levels are increased by ∼30% in WT-expressing N2a cells, compared with controls (33). We observed a similar pattern for Fyn. Expression of the WT enhanced the relative levels of raft-associated Fyn (Fig. 2, *G* and *H*), in agreement with the increase in Fyn amounts found in detergent-insoluble cell fractions (Fig. 2, *A* and *B*). In contrast, there was a marked reduction in raft-associated Fyn levels after expression of the methylation-incompetent L309Δ mutant. Unlike its WT counterpart, the L309Δ mutant is excluded from rafts, and raft-associated pools of PP2A become downregulated in L309Δ-expressing N2a cells (33). The decrease in raft-associated Fyn was also reminiscent of the loss

of Fyn in detergent-insoluble fractions from L309Δ-expressing cells (Fig. 2, *A* and *B*). These findings indicate that altering PP2A methylation can negatively influence the targeting of Fyn to membrane rafts in N2a cells. The parallel loss of raft-associated PP2A (33) and Fyn (Fig. 2*G*) in L309Δ-expressing cells likely point to close spatial and functional interrelationships between the phosphatase and the kinase.

Changes in PP2A methylation induce profound concomitant changes in the distribution of Fyn and F-actin in N2a cells

To complement our biochemical approach, we compared by confocal microscopy the distribution of expressed GFP–Fyn in our N2a cell models. Cells were stained in parallel with phalloidin to reveal the organization of the F-actin cytoskeleton and cell shape. Earlier studies have established that Fyn is primarily localized at the cell plasma membrane, along actin-rich peripheral structures corresponding to focal adhesions, filopodia, or ruffles, depending on cell stimuli. Being subjected to important endocytic trafficking, small pools of the kinase can also be detected in perinuclear endosomes (7). Upon plating, neuronal cells also typically develop actin filopodial structures that grow into immature neurites during early stages of the differentiation process (35). In agreement with these studies, we observed that the bulk of GFP–Fyn was distributed along short actin-rich filopodia in control N2a cells (Fig. 3*A*). A similar colocalization of GFP–Fyn and F-actin was found in WT- and LCMT1-expressing N2a cells (Fig. 3, *A* and *B*). However, relative to controls, these cells displayed longer filopodia (Fig. 3*C*) where GFP–Fyn and F-actin coclustered. In addition, actin aggregates, which are associated with neurite initiation (35), were often present in these cells. In contrast, the distribution patterns of GFP–Fyn and F-actin were greatly disturbed in L309Δ- and PME1-expressing N2a cells. More central, diffuse, and disorganized GFP–Fyn staining alongside very short actin filamentous spikes were observed in N2a cells stably expressing the L309Δ mutant (Fig. 3, *A*–*C*). In PME1-expressing N2a cells, GFP–Fyn and F-actin colocalized in diffuse cytoplasmic patches rather than filopodia. GFP–Fyn was also absent from the cell periphery showing labeling of a cortical actin ring.

Thus, our findings uncover a concurrent reorganization of GFP–Fyn and the F-actin cytoskeleton after alterations in PP2A methylation. Because intact F-actin is critically required for proper peripheral membrane targeting of SFKs (36), we hypothesized that upsetting PP2A methylation homeostasis

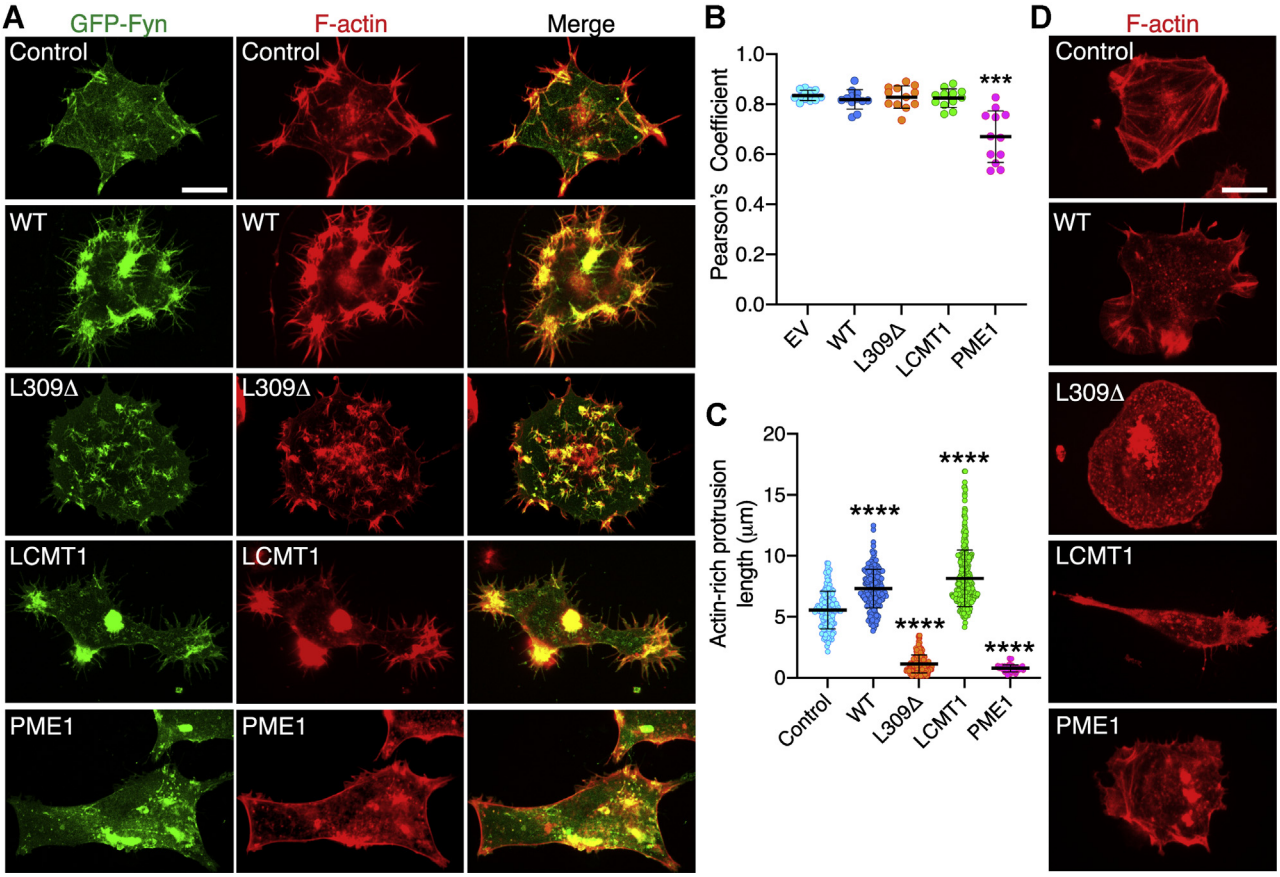

Figure 3. Altering PP2A methylation induces defects in Fyn localization and F-actin organization in N2a cells. *A*, representative confocal images of the distribution of GFP–Fyn and F-actin in EV (control), WT-, L309Δ-, LCMT1-, or PME1-expressing N2a cells cotransfected with GFP–Fyn. *B*, Pearson's correlation coefficients (mean ± SD, $n = 12$ cells/transfection from three separate experiments) showing colocalization of Fyn and F-actin in these cells. Data were analyzed using one-way ANOVA ($F(4, 55) = 18.62$, $p < 0.0001$) with post hoc Dunnett's test; ***$p < 0.001$, *versus* EV. *C*, cells were also analyzed for the length of actin-positive protrusions. Data (mean ± SD) were appraised using one-way ANOVA ($F(4, 928) = 785$; $p < 0.0001$) with post hoc Dunnett's test. ****$p < 0.0001$, *versus* control. *D*, F-actin distribution in the indicated N2a cell lines in the absence of GFP–Fyn. Images in panels *A* and *D* are representative of three separate experiments. Scale bars, 5 μm. EV, empty vector; N2a, Neuro-2a; LCMT1, leucine carboxyl methyltransferase 1; PP2A, protein phosphatase 2A.

can influence the subcellular distribution of Fyn, at least in part by inducing a remodeling of the actin cytoskeleton. In support of this hypothesis, alterations in PP2A methylation state in our stable cell models were also associated with a profound rearrangement of the F-actin cytoskeleton and cell shape changes, in the absence of expressed GFP–Fyn (Fig. 3*D*).

The interplay between PP2A methylation and Fyn regulates process outgrowth in N2a cells

Actin dynamics and reorganization are critical for neuritogenesis (35). Furthermore, one important role of Fyn clustering and activation in membrane rafts is to stimulate neurite outgrowth (13–15). In light of the effects of compromised PP2A methylation on the distribution of Fyn (Fig. 2) and F-actin (Fig. 3), we further investigated how changes in PP2A methylation affect Fyn-dependent process outgrowth. To that end, our N2a cell models were transfected with either GFP–Fyn or EV and incubated for ∼18 h in a low-serum medium to induce differentiation (37). Cells were comparatively analyzed for process outgrowth by assessing the distribution of transfected GFP–Fyn (Fig. 4*A*) or labeling neurites with the specific marker, βIII-tubulin (Fig. 4*B*). As expected, expression of GFP–Fyn in control N2a cells correlated with the outgrowth of highly branched Fyn-positive processes (Fig 4*A*), relative to EV-transfected N2a cells, which grew shorter neurites under the same differentiation conditions (Fig. 4, *B* and *C*). The stimulatory effects of Fyn on neuritogenesis were further accentuated in WT-transfected N2a cells. Cells coexpressing GFP–Fyn and LCMT1 displayed highly elongated processes. Likewise, incubation of GFP–Fyn–expressing N2a cells with AMZ-30, a specific PME1 inhibitor that blocks PP2A demethylation and enhances cellular levels of methylated PP2A (38), induced the formation of well-developed arrays of extended neurites. In contrast, expression of GFP–Fyn failed to induce process outgrowth in L309Δ- or PME1-expressing N2a cells (Fig. 4, *A* and *C*), which retained their characteristic rounded morphological appearance (see Fig. 3*A*). In these cell populations, GFP–Fyn was either distributed on short filamentous spikes as observed in undifferentiated cells (Fig. 3*A*) or retained in cytoplasmic and perinuclear vesicular patches. L309Δ- or PME1-transfected N2a cells also failed to differentiate in the absence of GFP–Fyn (37). Although we have previously reported that expression of WT and LCMT1 in N2a cells stimulates process outgrowth (37), expression of GFP–Fyn clearly promoted the growth of longer neurites in these cells, and in AMZ-30–treated N2a cells (Fig. 4, *B* and *C*). Conversely, neuritogenesis was completely abolished when cells were incubated in differentiation medium containing the Fyn inhibitor, PP2. These results indicate that intact PP2A methylation is essential for Fyn-dependent neuritogenesis while Fyn is required for PP2A methylation-dependent differentiation in N2a cells.

PP2A methylation state affects the partitioning of APP in membrane microdomains and Fyn-dependent APP processing

Besides regulating process outgrowth, Fyn critically regulates APP trafficking (5) and compartmentalization in membrane microdomains (11, 39). We thus hypothesized that, by affecting Fyn levels and distribution in membrane microdomains (Fig. 2), manipulating PP2A methylation could also alter the membrane distribution of APP in our N2a cell models. We first observed that, relative to controls, expression of WT and LCMT1 increased, whereas expression of L309Δ and PME1 decreased the levels of endogenous APP in plasma membrane fractions purified from N2a cells (Fig. 5, *A* and *B*). Quantification of Western blot analyses of total homogenates from these stable N2a cell lines confirmed that altering PP2A methylation state did not induce statistically significant changes in endogenous APP expression levels (Fig. 5*B*), as reported previously (23). These findings prompted us to further assess whether altering PP2A methylation promotes APP membrane microdomain switching in our models. To that end, we analyzed endogenous APP expression levels in aliquots of the same purified raft and nonraft preparations probed earlier for the presence of Fyn and PP2A (Fig. 2*E*). In contrast to Fyn (Fig. 2*E*), the bulk of endogenous APP partitioned in nonraft membrane fractions prepared from control N2a cells (Fig. 5, *C* and *D*), in agreement with earlier studies performed in neuronal tissue and cells, including N2a cells (16–18). Relative to controls, WT expression increased the levels of APP copurifying with nonraft membrane fractions. In contrast, the methylation-incompetent L309Δ mutant enhanced the relative proportion of APP segregating in rafts *versus* nonrafts. Thus, disrupting the integrity of PP2A methylation in N2a cells leads to increased partitioning of APP in membrane rafts.

Changes in the compartmentalization of APP in membrane microdomains critically affect APP processing (16–19). Indeed, L309Δ-mediated redistribution of APP in rafts (Fig. 5, *C* and *D*) correlates with enhanced amyloidogenic processing of APP in L309Δ-expressing N2a cells (23). Conversely, we have previously shown that enhanced PP2A methylation in N2a cells stimulates the nonamyloidogenic processing of APP (23). Because the α-secretase cleavage of APP is also regulated by Fyn (39), we further examined whether Fyn inhibition can affect PP2A-mediated sAPPα secretion. Expression of either WT or LCMT1 in N2a cells boosted the release of sAPPα species (Fig. 5, *E* and *F*), in agreement with our earlier studies (23). Notably, these stimulatory effects were inhibited when cells were incubated in the presence of the PP2 inhibitor, but not PP3 (drug control). These findings suggest that PP2A methylation–dependent APP cleavage is dependent on Fyn.

Disturbances in one-carbon metabolism that downregulate PP2A methylation alter the distribution of Fyn and Fyn-dependent process outgrowth in N2a cells

All cellular methylation reactions depend on the availability of the universal methyl donor, SAM, whose levels are tightly regulated by one-carbon metabolism (40). We have previously reported that alterations in one-carbon metabolism inhibit LCMT1-dependent PP2Ac methylation in N2a cells and *in vivo*, thereby affecting PP2A subunit composition and substrate specificity (23, 34, 41). To further explore the PP2A methylation/Fyn connection, we investigated the effects of manipulating one-carbon metabolism on Fyn regulation. N2a cells were

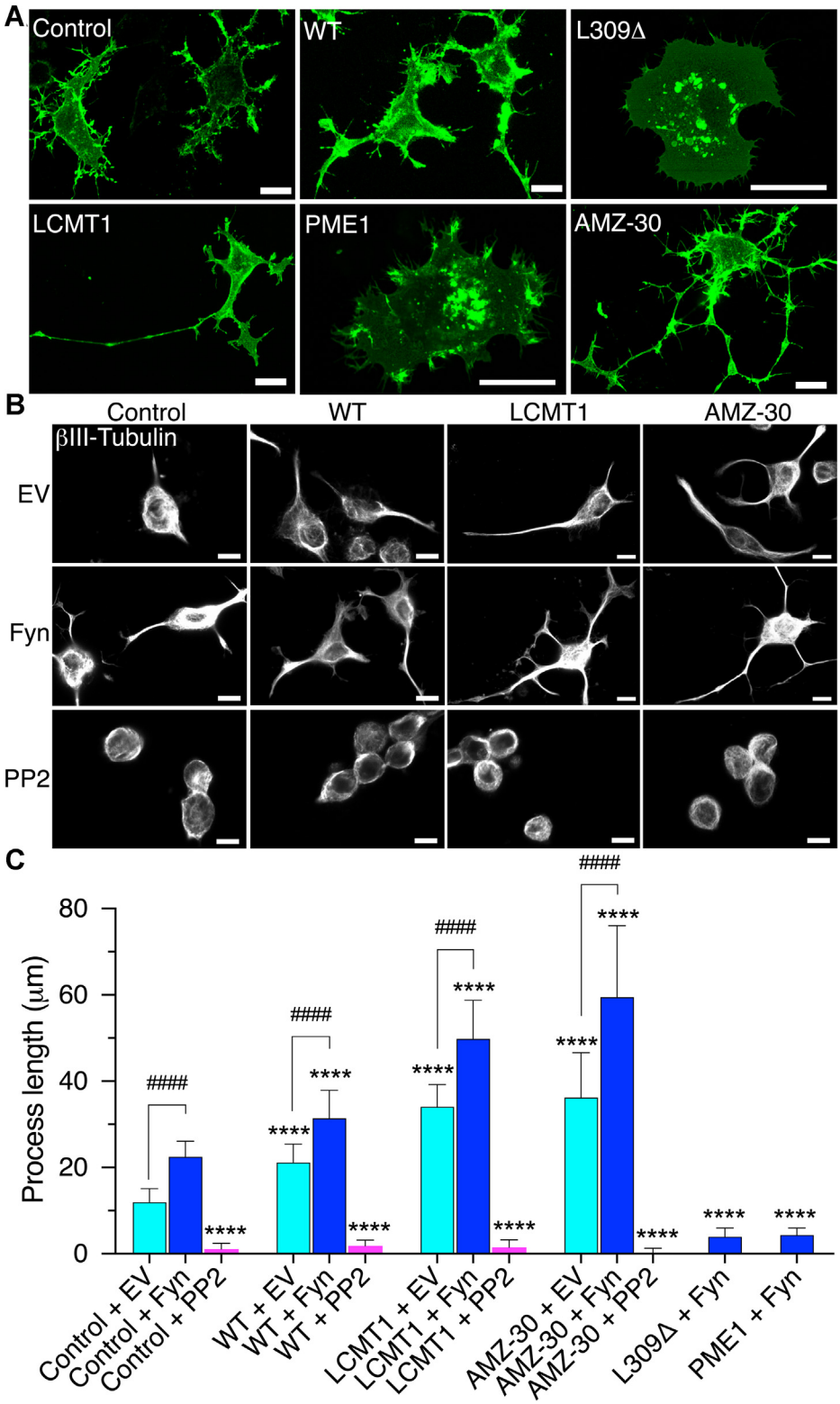

Figure 4. PP2A methylation state affects Fyn-dependent process outgrowth. *A*, distribution of GFP–Fyn in control or WT-, L309Δ-, LCMT1-, or PME1-expressing N2a cells cotransfected with GFP–Fyn. Cells were incubated for ∼18 h in a low-serum medium to initiate differentiation before fixation. A subset of control cells was incubated in differentiation medium containing 1-μM AMZ-30. *B*, βIII-tubulin staining was used to assess neurite outgrowth in control or WT- or LCMT1-expressing N2a cells that were transfected with either EV or GFP–Fyn and incubated for ∼18 h in a low-serum medium in the absence or presence of PP2. Subsets of control cells were also incubated in the differentiation medium containing 1-μM AMZ-30. Confocal images shown in panels *A–B* are representative of three separate experiments. Scale bars, 10 μm. *C*, cells were comparatively analyzed for neuritic process length. Data are mean ± SD from $n = 3$ separate experiments and were appraised using two-way ANOVA (effect of PP2A methylation: $F = 333.5$, $p < 0.0001$; effect of Fyn–PP2: $F = 2506$, $p < 0.0001$; interaction: $F = 113.8$, $p < 0.0001$) with Tukey's post hoc multiple comparisons test. $^{****}p < 0.0001$, *versus* control; $^{\#\#\#\#}p < 0.0001$. N2a, Neuro-2a; PP2A, protein phosphatase 2A.

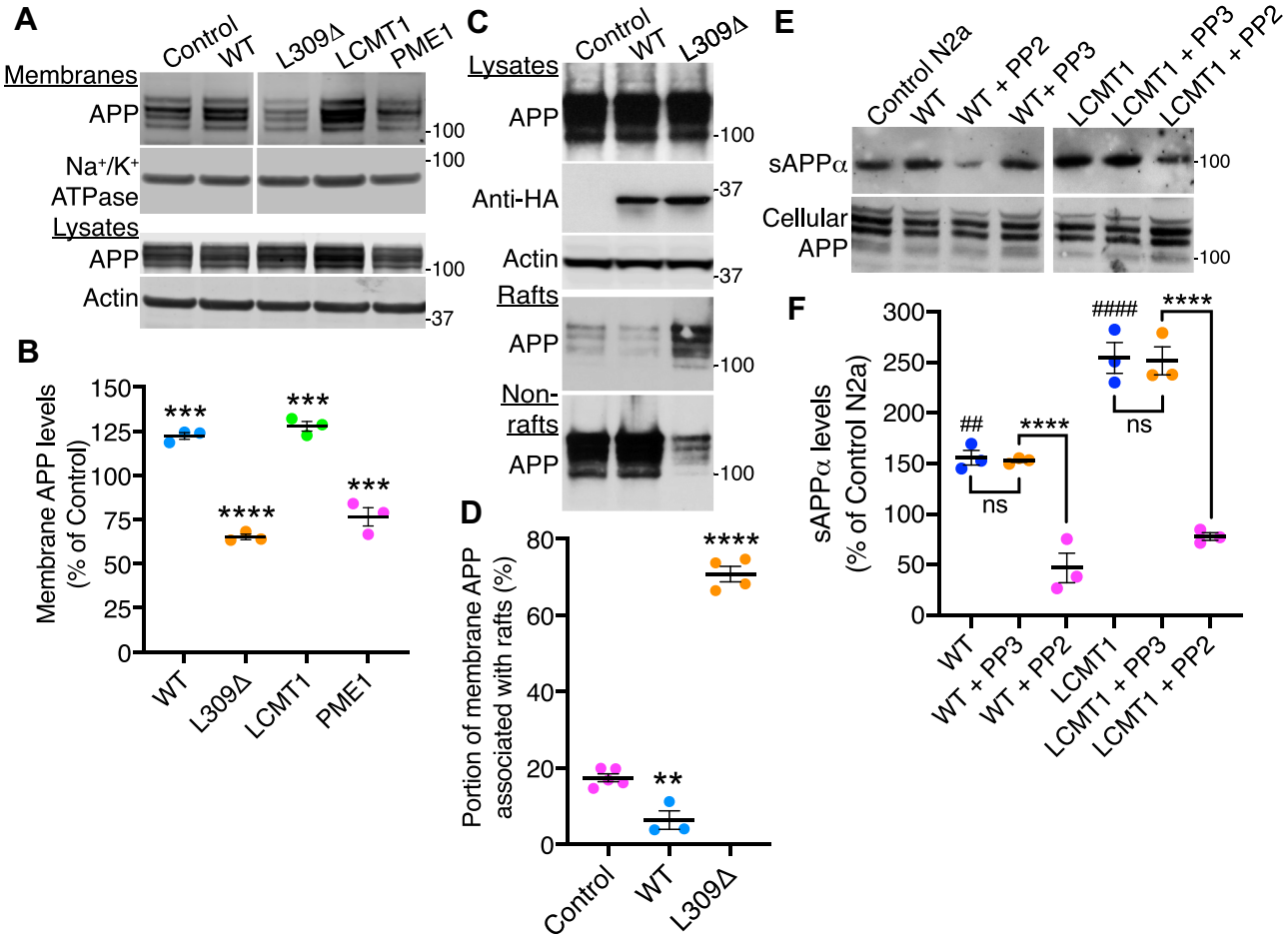

Figure 5. PP2A methylation state affects APP membrane distribution and Fyn-dependent APP processing in N2a cells. *A*, representative immunoblots of endogenous APP distribution in purified membrane fractions from control or WT-, L309Δ-, LCMT1-, or PME1-expressing N2a cells. Quantitative analyses of the immunoblots from $n = 4$ separate experiments confirmed that there were no statistically significant changes in APP expression levels ($p > 0.05$) in total lysates from these cells. *B*, relative levels of membrane-associated APP in these cells. Data are mean ± SEM from $n = 3$ separate purifications and were analyzed using one-way ANOVA ($F(4, 10) = 99.2$, $p < 0.0001$) with post hoc Dunnett's test. $^{***}p < 0.001$, $^{****}p < 0.0001$, *versus* control. *C*, representative immunoblots of APP distribution in aliquots of the same purified N2a cell raft and nonraft fractions analyzed in Figure 2*E*. Levels of expressed HA-tagged proteins and APP in corresponding total cell lysates are shown for reference. *D*, relative levels of raft-associated APP were quantified in EV-, WT-, and L309Δ-expressing N2a cells and are expressed as the percent of total membrane-associated APP. Data (mean ± SEM from $n = 3$–5 separate purifications) were appraised using one-way ANOVA ($F(2, 9) = 382.5$, $p < 0.0001$) with post hoc Dunnett's test. $^{**}p < 0.01$, $^{****}p < 0.0001$, *versus* control. *E*, levels of secreted sAPPα species and corresponding cellular APP levels were comparatively analyzed by Western blot in WT- and LCMT1-expressing N2a cells, relative to control N2a cells, after incubation for 4 h in a conditioned media in the absence or presence of 5-μM PP2 or PP3. *F*, the release of sAPPα was quantified in these cells after normalization for total cellular APP levels. Data shown are the mean ± SEM from 3 separate assays and were analyzed using two-way ANOVA (cell line: $F = 73.57$, $p < 0.0001$; treatment: $F = 112.7$, $p < 0.0001$; interaction: $F = 6.46$, $p = 0.01$) using Tukey's post hoc multiple comparisons test. $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$, *versus* control N2a; $^{****}p < 0.0001$; ns, not significant. APP, amyloid precursor protein; HA, hemagglutinin; LCMT1, leucine carboxyl methyltransferase 1; N2a, Neuro-2a; PP2Ac, catalytic "C" subunit of PP2A.

first incubated in an SAM-enriched medium, which boosts LCMT1-mediated PP2Ac methylation (23) and increases amounts of methylated PP2A enzymes targeted to the plasma membrane (33). Incubation with SAM enhanced the basal levels of endogenous Fyn in detergent-insoluble cell fractions, relative to vehicle-treated controls (Fig. 6, *A* and *C*). Next, N2a cells were subjected to various treatments known to deregulate one-carbon metabolism and interfere with the normal methylation cycle. Cells were incubated with Hcy, SAH, or a SAH hydrolase inhibitor (3-deazaadenosine [3-DZA]); subsets of cells were also switched to a folate-deficient (FD) medium to induce short-term folate deficiency (Fig. 6, *A* and *B*). Albeit their mechanisms of action are distinct, these treatments culminate to elevate intracellular Hcy and alter the cellular SAM/SAH ratio that controls cellular methylation potential (40). In turn, decreased SAM availability and/or increased cellular levels of SAH, a potent inhibitor of methyltransferases, induce the inhibition of LCMT1-dependent PP2A methylation and loss of membrane-associated methylated PP2A enzymes in N2a cells (23, 33, 34). Likewise, these treatments led to a pronounced reduction in detergent-insoluble Fyn levels (Fig. 6, *A*–*C*). As observed when deregulating PP2A methylation (Fig. 2*A*), manipulations of one-carbon metabolism induced parallel changes in pSFK immunoreactivity and Fyn expression levels in detergent-insoluble cell fractions (Fig. 6, *A*–*C*). After normalization, there were no statistically significant changes in either net Fyn activity or total Fyn expression levels in untreated or treated N2a cells.

Notably, incubation of N2a cells in the FD medium caused a time-dependent parallel loss of LCMT1, methylated PP2A, and Fyn enzymes from detergent-insoluble fractions (Fig. 6*D*).

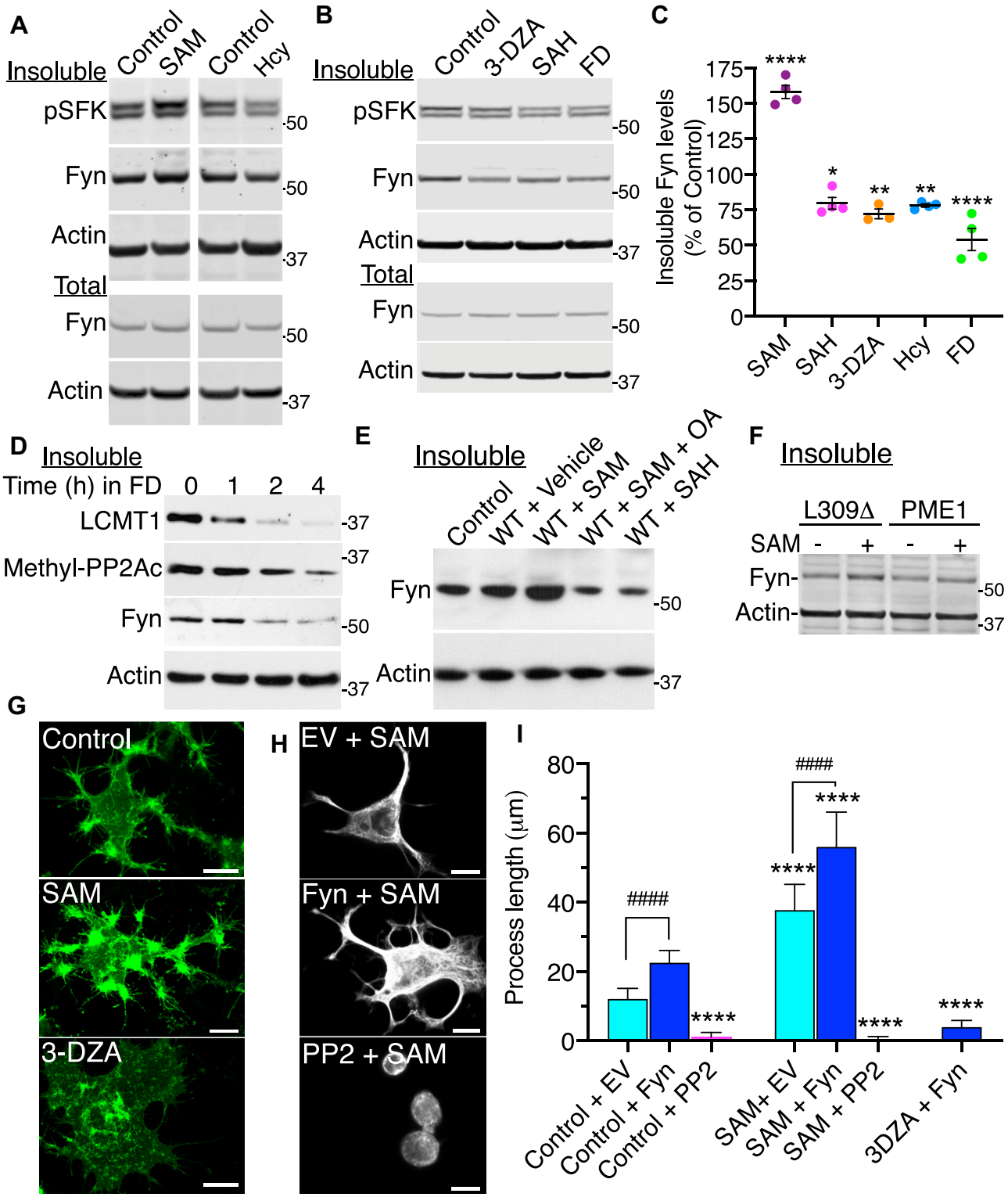

Figure 6. Fyn distribution and Fyn-dependent process outgrowth are dependent on one-carbon metabolism in N2a cells. *A*, immunoblot analysis of Fyn and pY416-SFK (pSFK) in total lysates (total) and detergent-insoluble (insoluble) fractions from N2a cells that were incubated for ∼16 h with 100-μM SAM, 100-μM Hcy or vehicle (control). *B*, representative immunoblots of Fyn and pY416–SFK (pSFK) in total lysates and detergent-insoluble fractions from N2a cells that were incubated for ∼16 h with 50-μM 3-deazaadenosine (3-DZA), 100-μM SAH, or vehicle (control). A subset of cells was incubated for 4 h in a folate-deficient (FD) medium. Quantitative analyses of the immunoblots from $n = 3$ to 4 separate experiments revealed that incubation with either SAM, Hcy, SAH, 3-DZA, or FD did not induce any statistically significant changes ($p > 0.05$) in total Fyn protein expression levels, or total or detergent-insoluble Fyn phosphorylation levels, relative to vehicle-treated N2a cells. *C*, detergent-insoluble Fyn levels were quantified in these cells. Data shown are mean ± SEM from $n = 3$ to 4 independent experiments and were analyzed using one-way ANOVA ($F(5, 17) = 69.37$, $p < 0.0001$) with post hoc Dunnett's test. *$p <$ 0.05, **$p < 0.01$, ****$p < 0.0001$, *versus* control. *D*, time-dependent changes in detergent-insoluble levels of Fyn, methylated PP2Ac, and LCMT1 enzymes in N2a cells switched for the indicated time from normal folate-containing medium to FD medium. *E*, immunoblot analysis of detergent-insoluble Fyn levels in control or WT-expressing N2a cells incubated for 16 h with 100-μM SAM, 100-μM SAH, or a combination of 100-μM SAM and 5-nM okadaic acid (OA).

Moreover, treatment of N2a cells with SAM further synergized with WT expression to enhance insoluble Fyn levels, whereas SAH blocked the ability of WT to increase insoluble Fyn levels (Fig. 6*E*). SAM-mediated increase in insoluble Fyn levels was also abolished when cells were coincubated with the PP2A inhibitor, okadaic acid (OA). However, incubation with SAM was unable to rescue the loss of insoluble Fyn levels induced by L309Δ or PME1 expression in N2a cells (Fig. 6*F*). Together, these findings indicate that the status of one-carbon metabolism can concurrently influence the membrane distribution of PP2A and Fyn. They support the hypothesis that metabolic-induced deregulation of PP2A methylation is a major contributor to Fyn deregulation. In this context, it has been reported in non-neuronal COS-1 cells that Fyn is trimethylated at $Lys^{7/9}$ (42). This observation raised the possibility that altering one-carbon metabolism could also impact Fyn regulation in a PP2A-independent manner, by directly affecting its methylation state. To address this hypothesis, GFP–Fyn immunoprecipitates were prepared from transfected N2a cells treated with SAM (to enhance methylation), 3-DZA (to inhibit methylation), or vehicle and then analyzed by Western blot with validated anti-methyl-lysine antibodies (Fig. S2). Although methyl-sensitive proteins were clearly present in the GFP–Fyn immunoprecipitates, we were unable to detect any immunoreactivity of GFP–Fyn with these antibodies under our experimental conditions.

We next investigated whether Fyn-dependent process outgrowth was susceptible to changes in the SAM/SAH ratio. Relative to vehicle-treated controls, incubation with SAM enhanced GFP–Fyn–mediated differentiation of N2a cells (Fig. 6, *G–I*). This stimulating effect and the pattern of GFP–Fyn distribution in SAM-treated cells were highly reminiscent of those induced by AMZ-30, WT, and LCMT1 (Figs. 3 and 4). Notably, SAM-mediated N2a cell differentiation was abolished by PP2. Fyn-dependent process outgrowth was also inhibited by 3-DZA. The distribution of GFP–Fyn in 3-DZA–treated cells was strikingly similar to that observed in L309Δ-expressing cells (Figs. 3*A* and 4*A*). Thus, the integrity of one-carbon metabolism is essential for Fyn-dependent process formation in N2a cells.

Elevated Hcy levels influence the amounts of methylated PP2A and Fyn enzymes in detergent-insoluble fractions prepared from acute mouse brain slices

To validate the studies performed in N2a cells, we examined the effects of disturbing Hcy metabolism on the detergent insolubility of Fyn in acute mouse brain slices. Fyn-enriched detergent-insoluble fractions were prepared from cortical slices that had been incubated for up to 2 h in the presence of Hcy or its metabolite, Hcy thiolactone (HTL) (Fig. 7, *A–C*). This short treatment was preferentially chosen to prevent putative protein loss due to ultimate Hcy neurotoxicity. Relative to vehicle-treated slices, incubation with either Hcy or HTL induced a time dependent loss of detergent-insoluble Fyn levels, with a mean ∼30% loss found at the 2-h time point. As observed in N2a cells (Figs. 2 and 6), the loss of Fyn was accompanied by a similar and parallel decrease in the signal for pY416–Fyn in detergent-insoluble fractions. Indeed, after normalization, there were no significant changes in net insoluble Fyn activity in Hcy- or HTL-treated compared with vehicle-treated slices. The loss of Fyn also coincided with a large increase in demethylated PP2A in detergent-insoluble fractions from Hcy-treated slices, relative to vehicle-treated slices (Fig. 7*D*). These results indicate that the abnormal elevation of Hcy can induce a concomitant demethylation of PP2A and redistribution of active Fyn enzymes.

Discussion

Methylation is a key regulatory post-translational mechanism that controls biogenesis of PP2A/Bα holoenzymes and PP2A subunit composition, thereby influencing PP2A targeting, substrate specificity, and interactions with numerous proteins and regulators (22). Here, using N2a cells, we first show that manipulating PP2A methylation affects the distribution and function of Fyn, a major signaling enzyme deregulated in AD and cancer. The subcellular distribution of Fyn is regulated by trafficking to and internalization from the plasma membrane; owing to N-terminal lipid modifications, Fyn is preferentially targeted to membrane rafts, regardless of its activity (7, 8, 43–45). We found that enhanced cellular PP2A methylation was associated with increased levels of Fyn in detergent-insoluble N2a cell fractions and membrane rafts and enhanced clustering of Fyn along peripheral actin-rich filopodia. Conversely, the accumulation of demethylated PP2A promoted a steady-state loss of membrane- and raft-associated Fyn and its mislocalization. These effects were not associated with changes in net Fyn activity or expression levels; rather, they closely correlated with a reorganization of the F-actin cytoskeleton. Although general PP2A activity has been implicated in the complex regulation of actin dynamics, underlying mechanisms and contribution of specific PP2A isoforms remain poorly characterized (46). Nevertheless, an intact actin cytoskeleton is critically required for proper peripheral membrane targeting of Fyn (36). Our findings suggest that altering PP2A methylation in N2a cells interferes with the normal distribution of Fyn, likely in part by deregulating F-actin dynamics. This hypothesis is further supported by the strong functional link between cytoskeletal reorganization and

F, immunoblot analysis of detergent-insoluble Fyn and actin levels in L309Δ- and PME1-expressing N2a cells incubated for ∼16 h with 100-μM SAM or vehicle. For panels *D–F*, similar results were observed in three separate experiments. *G*, representative confocal images of GFP–Fyn in transfected N2a cells that were incubated for ∼18 h in a low-serum medium in the presence of 100-μM SAM, 50-μM 3-DZA, or vehicle (control) before fixation. Scale bars, 10 μm. *H*, N2a cells transfected with either EV or GFP–Fyn were incubated for ∼18 h in the differentiation medium in the absence or presence of 5-μM PP2 and labeled with anti-βIII-tubulin antibodies. Scale bars, 10 μm. *I*, quantification of the neurite length in these cells. Data shown are mean ± SD from cells from 3 separate experiments and were analyzed with two-way ANOVA (effect of SAM: $F = 1152$, $p < 0.0001$; effect of Fyn–PP2: $F = 1600$, $p < 0.0001$; interaction: $F = 342.1$, $p < 0.0001$) with post hoc Tukey's test. ****$p < 0.0001$, *versus* control + EV or control + Fyn; $^{\#\#\#\#}p < 0.0001$. EV, empty vector; FD, folate-deficient; Hcy, homocysteine; LCMT1, leucine carboxyl methyltransferase 1; N2a, Neuro-2a; PP2Ac, catalytic "C" subunit of PP2A.

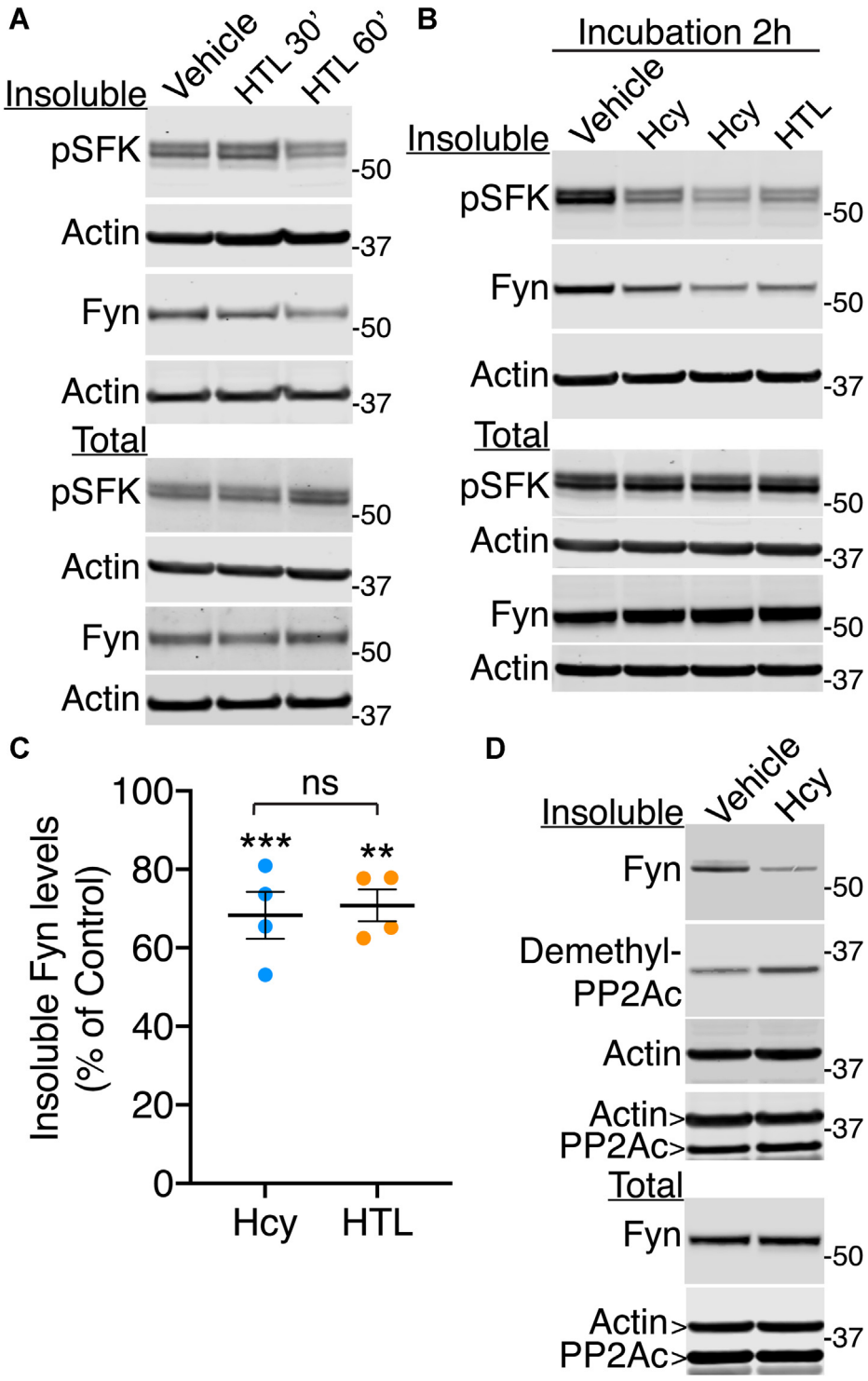

Figure 7. Elevated levels of Hcy or its thiolactone derivative induce concomitant PP2A demethylation and alterations in Fyn distribution in acute mouse brain slices. *A*, representative immunoblots of Fyn and pSFK in total extracts (total) and detergent-insoluble (insoluble) fractions prepared from acute mouse cortical slices incubated for 30 or 60 min with 200-μM Hcy-thiolactone (HTL). *B*, representative immunoblots of pSFK and Fyn in acute brain slices incubated for 2 h with either 200-μM Hcy or HTL. Two separate slices treated with Hcy are shown. Quantitative analyses of the immunoblots from n = 4 separate experiments revealed that total Fyn protein expression levels, or total or detergent-insoluble Fyn phosphorylation levels were not statistically significant different ($p > 0.05$) in Hcy or HTL-treated, relative to, vehicle-treated slices. *C*, quantification of detergent-insoluble Fyn levels in Hcy- or HTL-treated slices, expressed as percent of vehicle-treated, control slices. Data shown are mean ± SEM from 4 separate mouse brain tissue and were analyzed using one-way ANOVA (F (2, 9) = 17.88, p = 0.0007) with post hoc Dunnett's test; $^{**}p < 0.01$, $^{***}p < 0.001$, relative to vehicle-treated slices. *D*, comparative immunoblot analysis of demethylated PP2Ac, Fyn, and actin in total extracts and detergent-insoluble fractions prepared from acute mouse cortical slices incubated for 2 h with 200-μM Hcy. Duplicate blots were probed for total PP2Ac levels and actin. Quantitative analyses of the immunoblots showed a ∼58 ± 9% increase (n = 3 separate experiments; $p < 0.0001$; student t-test) in demethylated PP2A levels in detergent-insoluble fractions from Hcy-, relative to, vehicle-treated slices. Hcy, homocysteine; HTL, Hcy thiolactone; PP2Ac, catalytic "C" subunit of PP2A; pSFK, phosphorylated SFK.

clustering of proteins in membrane rafts, which serve as signaling platforms regulating adhesion, differentiation, and polarity (47). These membrane microdomains are also involved in protein sorting, endocytosis, and recycling from/to the plasma membrane (48). Moreover, deregulation of PP2A promotes the endocytosis of E-cadherin by inducing F-actin disassembly (49). The pattern of Fyn distribution in L309Δ- and PME1-expressing N2a cells suggests that enhanced PP2A demethylation could similarly promote Fyn internalization *via* actin-dependent mechanisms. Yet, defects in Fyn trafficking could also occur by other mechanisms, based on the role of PP2A in dephosphorylating adaptor proteins regulating clathrin-mediated endocytosis (50).

We also observed that increasing PP2A methylation stimulated Fyn-dependent process outgrowth, whereas altering PP2A methylation abolished it. Because activation of raft-associated Fyn (13–15) and actin remodeling (35) are intimately linked with neuritogenesis, it is likely that PP2A influences Fyn-dependent N2a cell differentiation by affecting F-actin dynamics and Fyn targeting to rafts. For instance, the reorganization of cortical actin into aggregates and filopodia, which is required for neurite initiation (35), was prevalent in WT- and LCMT1- but absent in PME1-expressing N2a cells. However, additional direct and indirect mechanisms, such as PP2A-induced changes in microtubule stability (46) and Fyn-mediated F-actin dynamics (1), could also be involved.

Our data also indicate that deregulation of PP2A in N2a cells influences the clustering of APP in membrane microdomains, which governs APP processing (17–19). Accordingly, we found that enhanced association of APP with rafts (Fig. 5*D*) coincided with enhanced β-secretase cleavage of APP (23) in L309Δ-expressing N2a cells. Conversely, expression of the WT in N2a cells increased the relative levels of endogenous APP in nonraft plasma membrane microdomains and sAPPα secretion (Fig. 5). In agreement with decreased sAPPα release in Fyn KO mice (39), inhibiting Fyn abolished both WT- and LCMT1-mediated sAPPα secretion in N2a cells. These data further cement the existence of a close methylated PP2A/Fyn functional inter-relationship in regulating APP.

Phosphorylation-dependent protein–protein interactions also shape APP localization and processing (3, 25). Fyn-mediated Tyr phosphorylation regulates the association of APP with adaptor proteins and promotes the sorting of APP to lipid rafts (11). Aberrant Fyn activation in AD has been linked to enhanced Fyn–APP interactions, deficits in APP sorting and trafficking, and amyloidogenesis (5, 51). We found that PP2A/Bα holoenzymes copurified in membrane rafts and coimmunoprecipitated with detergent-insoluble Fyn. This indicates the existence of membrane-bound Fyn–PP2A complexes; however, these protein–protein interactions may be indirect because purified Fyn and PP2A/Bα holoenzymes do not associate *in vitro* (52). Relative to control N2a cells, expression of the WT enhanced Fyn levels in rafts while increasing the ratio of APP partitioning in nonraft membrane microdomains, suggesting that WT promotes the segregation of Fyn from its substrate, APP. In contrast to WT, the methylation-incompetent and B binding–incompetent L309Δ mutant failed to associate with Fyn and promoted the sorting of APP into rafts. Whereas the expression of L309Δ decreased total Fyn levels in rafts, enhanced targeting of APP to rafts may increase the potential for functional interactions of APP with the active kinase still present in these microdomains. Enhanced PP2A demethylation in N2a cells (23) also promotes phosphorylation of APP at Thr^{668}, which impacts the APP interactome (25) and APP distribution (3). In neurons, $pThr^{668}$–APP species are concentrated in endosomes, favoring APP β-secretase cleavage (3). Based on these findings, it is tempting to speculate that PP2A methylation regulates the formation of localized protein scaffolds that play a crucial role in directing the trafficking and processing of APP toward the competing amyloidogenic or nonamyloidogenic pathways. In this context, it is noteworthy that the L309Δ mutant fails to associate not only with Fyn (Fig. 1*B*) but also with tau proteins (23). Enhanced formation of Fyn–tau scaffolds plays a key role in mediating Aβ-induced synaptic dysfunction and excitotoxicity in AD (4). Because PP2A/Bα and Fyn compete for tau binding, disruption of normal PP2A–Fyn and PP2A–tau protein–protein interactions as a result of PP2A demethylation would enhance the potential for neurotoxic Fyn–tau interactions (53). Thus, interfering with homeostatic PP2A methylation has the potential, *via* several intricate mechanisms, to deregulate the function of key players in AD pathogenesis.

Using N2a cells and mouse brain slices, we also established a link between one-carbon metabolism and the regulation of Fyn distribution. Metabolic disturbances that lead to elevated Hcy levels and altered cellular methylation potential induced a concomitant loss of methylated PP2A and Fyn enzymes from detergent-insoluble fractions; conversely, boosting cellular methylation led to their coenrichment. In an earlier mass spectrometry study, Fyn was reported to be trimethylated on Lys residues within the SH4 domain; a regulatory link between methylation and Fyn targeting and function was further proposed based on the use of Lys mutants in COS-1 cells (42). These observations could provide a plausible mechanism by which altering one-carbon metabolism can directly affect Fyn methylation state and thereby affect its distribution. However, to the best of our knowledge, Fyn methylation has never been confirmed in any follow-up studies, and the identity of the Fyn methyltransferase remains unknown to date. Proper validation of protein Lys methylation is challenging, requiring several approaches to avoid pitfalls (54). Assigning effects of Lys mutants to changes in Fyn methylation (53) may be confounded by the fact that Fyn also undergoes acylation in the same N-terminal domain, which controls Fyn association with membrane rafts (44). Under our experimental conditions, we were unable to detect Fyn methylated on Lys residues, arguing against a prevalent direct role of Lys methylation in regulating Fyn distribution. Yet, we do not exclude the possibility that Fyn undergoes methylation on other yet unidentified amino acids, which could render Fyn directly susceptible to alterations in one-carbon metabolism. Nevertheless, our findings in N2a cells (Fig. 6, *E* and *F*) and brain slices (Fig. 7) strongly support the hypothesis that altered PP2A methylation

actively contributes to deregulation of Fyn in response to disturbances in the methylation cycle. Yet, PP2A- and methylation-independent mechanisms, such as oxidative stress (26), could also participate in Fyn dysregulation in response to altered folate and Hcy metabolism.

The crosstalk between Hcy metabolism and the major signaling molecules, PP2A and Fyn, is of particular importance for the AD and cancer fields. Hyperhomocysteinemia is an established risk factor for AD (27) and is experimentally associated with the development of hallmark pathological features of AD, including tau and APP phosphorylation, and amyloidogenesis (26). Disturbed Hcy metabolism is strongly associated with cancer (28). Hyperhomocysteinemia promotes PP2A demethylation *in vivo* (23, 55). Alterations in PP2A methylation are found in patients with AD (21) and cancer (56). In our neuroblastoma model, they promote a loss of Fyn from membrane rafts. Because the confinement of Fyn in membrane rafts limits its ability to promote cell transformation (44, 57), it is possible that deregulation of Fyn contributes to the role of PP2A in cancer.

Collectively, our findings demonstrate that the integrity of one-carbon metabolism and PP2A methylation are essential for proper regulation of Fyn and APP. Our study identifies an important link between metabolic pathways and multifunctional signaling molecules currently being targeted for AD and cancer therapies.

Experimental procedures

Materials and reagents

Unless indicated, all chemicals and drugs were from Sigma-Aldrich/Merck Millipore. Primary antibodies used in this study included the following: Rabbit anti-HA clone C29F4, anti-pSrc Tyr416 clones 100F9 and D49G4, anti-Na^+/K^+ ATPase #3010, and anti-GFP clone D5.1 (Cell Signaling Technology); rabbit Anti-APP clone Y188 (Abcam); mouse anti-HA clone 16B12 (Covance); rabbit anti-actin #AAN01 (Cytoskeleton Inc); anti-transferrin receptor clone H68.4 (Thermo Fisher Scientific); rabbit anti-methylated Lysine (Enzo Life Sciences); mouse anti-Fyn clone 25 #610163, anti-flotillin-1 clone 18, and anti-$PP2Ac_\alpha$ clone 46 (BD Transduction Laboratories); rabbit anti-Fyn clone EPR5500, mouse anti-Bα clone 2G9, anti-actin clone C4, anti-LCMT1 clone 4A4, anti-APP clone 22C11 (MAB348), and anti-demethyl PP2Ac clone 1D6 (Merck Millipore).

Cell culture and transfection

Mouse N2a neuroblastoma cells were obtained from the American Type Culture Collection. N2a cells stably expressing myc-tagged PME-1, HA-tagged LCMT1, HA-tagged WT PP2Ac, or the HA-tagged methylation site L309Δ C subunit mutant have been extensively characterized in previous studies (23, 31, 33, 34). Control and stable cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) containing 2.5-mM Hepes, pH 7.4, 10% fetal bovine serum (FBS, Bovogen Biologicals), and 10-μg/ml gentamycin (Thermo Fisher Scientific). In some experiments, control and stable cells lines were transiently transfected with the indicated plasmids using METAFECTENE PRO reagent, following the manufacturer's instructions (Biontex laboratories, Germany). Plasmids used in this study included the following: pAcCMV Fyn–GFP plasmid encoding GFP-tagged human Fyn (OriGene); peCFP-APP plasmid encoding human WT APP695 (gift from Dr Ottavio Arancio, Columbia University, New York, NY); and Bα/pcDNA5/TO plasmid encoding Flag-tagged PP2A Bα (PPP2R2A) subunit (58) (gift from Dr Brian Wadzinski, Vanderbilt University, Nashville, TN). All plasmids were verified by sequencing. Cells mock-transfected with EVs were used as "controls." Partial knockdown of endogenous LCMT1 in N2a cells was performed using transient transfection with small interfering RNA (siLCMT1) shown to specifically target mouse LCMT1; cells transfected with mismatch siRNA (siRNA control) were used as controls. Experimental conditions were optimized to prevent cell death ultimately caused by the complete or prolonged loss of LCMT1 (33, 34).

Cell treatment and differentiation

Unless otherwise indicated, all experiments and incubation with compounds were performed in ∼80% confluent cells cultured in a regular cell culture medium. To assess the role of one-carbon metabolism, cells were incubated for ∼16 h with 100-μM SAM, 100-μM SAM + 5-nM 100 OA, 100-μM SAH, 50-μM 3-DZA, 1 μM AMZ-30, or vehicle (33). Folate deficiency was induced by switching N2a cells cultured in normal folate-containing medium to folate-free RPMI-1640 medium supplemented with 2% dialyzed FBS (Thermo Fisher Scientific) (34). To assess the role of cholesterol, subsets of cells were incubated at 37 °C for 15 min in a serum-free medium with 1% MβCD premixed or not with 100 μg/ml cholesterol, before harvesting for membrane raft purification (33). To assess the role of Fyn, cells were incubated for the indicated time in the presence of 5-μM PP2 or PP3 (control drug for the SFK inhibitor, PP2). sAPPα secretion was analyzed 4 h after incubation of N2a cells in the conditioned media, exactly as described previously (23). To study neurite outgrowth, cells were plated in a regular medium 24 h after transfection onto poly-L-lysine–coated glass coverslips. Five hours after plating, cells were switched to DMEM containing 0.5% FBS in the absence or presence of the indicated drugs and incubated for ∼18 h before fixation.

Confocal microscopy

To visualize F-actin, cells were fixed for 20 min with 4% paraformaldehyde, permeabilized for 5 min with PBS containing 0.1% Triton X-100, washed, and incubated for 1 h in PBS containing 3% bovine serum albumin. Cells were labeled with Alexa $Fluor^{594}$ conjugated phalloidin to detect F-actin (Thermo Fisher Scientific). GFP–Fyn was directly visualized in fixed cells. To label neurites, N2a cell lines were fixed for 5 min at −20 °C with absolute methanol before staining with rabbit anti-βIII-tubulin antibodies (Abcam #18207) followed by Alexa $Fluor^{594}$–conjugated secondary antibody (Thermo Fisher Scientific #A27034) (37). After washing in PBS, all

samples were mounted with Fluoromount (ProSciTech) and examined on a Nikon Eclipse 80i confocal microscope using a 60x objective. Captured images (z-stacks) were exported to NIH ImageJ/Fiji for analyses of either the protrusion length or protein colocalization. Images were transferred to Adobe Photoshop/Illustrator 2020 (Adobe Systems Incorporated) for figure preparation.

Cell lysis and subcellular fractionation

After washing with PBS, total N2a cell homogenates (100-mm dishes) were prepared in 400-μl buffer 1 [10-mM Tris, pH 7.4, 150-mM NaCl, 1-mM dithiothreitol, 0.5-μM OA, 5-mM PMSF, 1% NP-40, Sigma Protease Inhibitor Cocktail, and Sigma Phosphatase Inhibitor Cocktail] using a mortar and pestle. In some experiments, total cell lysates were further centrifuged for 90 min at 20,000*g* to generate NP-40 detergent–soluble (supernatant) and NP-40 detergent–insoluble (pellet) fractions. The detergent-insoluble cell pellet was resuspended in 200 μl of buffer 2 (buffer 1 + 0.5% sodium deoxycholate) and carefully homogenized for 80 s using a mortar and pestle. For immunoprecipitation assays, homogenized total and insoluble fractions were cleared by centrifugation at 13,000*g* for 3 min at 4 °C. For Western blot analyses, total homogenates and detergent-insoluble fractions were further sonicated before clarification. The protein concentration was determined in diluted aliquots of homogenates using the Bradford protein assay kit (Bio-Rad). Previously purified NP-40–insoluble fractions from N2a cells transiently transfected with validated siLCMT1 or siRNA control (33) were also reanalyzed here by immunoblotting. Purification of the plasma membrane from N2a cells was carried out by ultracentrifugation (33). Validated detergent-free procedures based on fractionation of the plasma membrane by centrifugation on an OptiPrep gradient were used to purify raft and nonraft membrane microdomains from N2a cells (33). Aliquots of the same N2a cell membrane microdomain preparations characterized in a previous study (33) were reanalyzed here by Western blot for the presence of Fyn and APP.

Immunoprecipitation

Immunoprecipitates were prepared from total homogenates or detergent-insoluble fractions from N2a cells or mouse cortical tissue (∼500 μg proteins/assay). Immunoprecipitation of transfected proteins was performed by incubating samples overnight at 4 °C with either anti-Flag–coupled (clone M2, Sigma #M8823), anti-GFP–coupled (clone RQ2, MBL International #D153–9), or anti-HA–coupled (clone C29F4; Cell Signaling Technology #11846) magnetic beads. When immunoprecipitating endogenous proteins, homogenates were precleared for 1 h at 4 °C before overnight incubation with the indicated antibodies. Samples were then incubated for 1 h at 4 °C with PureProteome Protein A/G mix magnetic beads (Merck Millipore). Magnetic beads were washed 5 times in buffer 2 before being resuspended in a gel loading buffer. Input fractions (∼50 μg proteins) and corresponding immunoprecipitates were analyzed by Western blotting.

Mouse brain tissue analyses

Brains were rapidly removed from 8- to 11-month-old female C57/BL6 mice that were sacrificed for another project approved by the Animal Care and Ethics Committee of the University of Newcastle. For the preparation of acute slices, brains were immediately immersed in ice-cold, oxygenated, sucrose-substituted artificial cerebrospinal fluid (250-mM sucrose, 25-mM $NaHCO_3$, 10-mM glucose, 2.5-mM KCl, 1-mM NaH_2PO_4, 1-mM $MgCl_2$, and 2.5-mM $CaCl_2$) (59). Cortical coronal slices (∼400 μm thick) were obtained using a vibratome (Leica VT-1200S, Heidelberg, Germany) and transferred to an interface storage chamber containing oxygenated artificial cerebrospinal fluid (118-mM NaCl substituted for sucrose in sucrose-substituted artificial cerebrospinal fluid). Slices were allowed to recover for 1 h at 22 to 24 °C before incubation for the indicated time into RT oxygenated artificial cerebrospinal fluid containing the indicated compounds or vehicle. Slices were then harvested for further Western blot analyses. Total homogenates and NP-40 detergent-insoluble fractions were prepared from either acute slices or fresh mouse cortical tissue as described above for N2a cells.

Gel electrophoresis and Western blotting

Protein samples (∼50-μg proteins/lane) were resolved on NuPAGE 4 to 12% Bis-Tris gels (Thermo Fisher Scientific). Prestained Protein Standards (Bio-Rad) were used as molecular weight markers. Membrane raft fractions were analyzed by immunoblotting using chemiluminescence as described previously (33). Other Western blot analyses were performed using the indicated primary antibodies, followed by Infrared IRDye-labeled secondary antibodies, and visualized using the Odyssey Infrared imaging system (LI-COR Biosciences). In most cases, blots were cut between molecular weight markers to allow simultaneous immunostaining and reprobing of the top and bottom parts with distinct antibody species. Band intensity was determined using the associated Image Studio Lite, version 5.0, Software (LI-COR Biosciences) to accurately quantify protein expression levels. Anti-actin antibodies were used to normalize for protein loading. Anti-$pTyr^{416}$ SFK antibodies were used to assess the phosphorylation state of Fyn, which was determined after normalizing the pSFK signal corresponding to Fyn (determined as being the fastest migrating band) for total Fyn expression levels and protein loading. PP2A demethylation was assessed as described previously (33).

Statistics

Data were analyzed for normal distribution and statistical significance using GraphPad Prism 9.

Data availability

All the data supporting our conclusions are presented in this article. All materials are available upon request.

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Author contributions—E. S. and J.-M. S. conceived and managed the project, and designed experiments. E. S. wrote the manuscript and prepared figures. E. S. and J.-M. S. revised the manuscript. G. T., D. S., H. S., and J.-M. S. performed experiments. All authors carried out data analyses and approved the final article.

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Abbreviations—The abbreviations used are: 3-DZA, 3-deazaadenosine; Aβ, amyloid-β; AD, Alzheimer's disease; anti-HA, anti-hemagglutinin; APP, amyloid precursor protein; EV, empty vector; FBS, fetal bovine serum; FD, folate-deficient; HA, hemagglutinin; Hcy, homocysteine; HTL, Hcy thiolactone; LCMT1, leucine carboxyl methyltransferase 1; MβCD, methyl-β-cyclodextrin; N2a, Neuro-2a; OA, okadaic acid; PP2A, protein phosphatase 2A; PP2Ac, catalytic "C" subunit of PP2A; pSFK, phosphorylated SFK; SFKs, Src family kinases.

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