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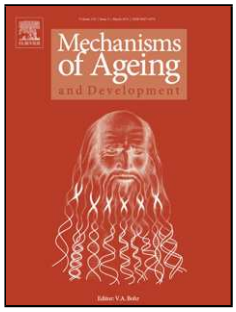

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Alzheimer's disease pathogenesis: Is there a role for folate?

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Highlights

- There is evidence that folate contributes to Alzheimer's disease aetiology and pathology via several mechanisms such as: secretase activity, tau phosphorylation, calcium homeostasis, oxidative stress and maintenance of neurotransmitters
- Components of one-carbon metabolism appear to have differential influences in pathology and could be used as therapeutic agents pending further investigation
- Biomarkers from molecular studies could be integrated into epidemiological studies

Abstract

Epigenetic modifications, including changes in DNA methylation, have been implicated in a wide range of diseases including neurological diseases such as Alzheimer's. The role of dietary folate in providing methyl groups required for maintenance and modulation of DNA methylation makes it a nutrient of interest in Alzheimer's. Late onset Alzheimer's disease is the most common form of dementia and at present its aetiology is largely undetermined. From epidemiological studies, the interactions between folate, B-vitamins and homocysteine as well as the long latency period has led to difficulties in interpretation of the data, thus current evidence exploring the role of dietary folate in Alzheimer's is contradictory and unresolved. Therefore, examining the effects at a molecular level and exploring potential epigenetic mechanisms could increase our understanding of the disease and aetiology. The aim

of this review is to examine the evidence for a role of folate which could contribute to Alzheimer's disease neuropathology and will focus on the effects of folate on DNA methylation which link to disease pathology, initiation and progression.

Key words

Alzheimer's disease, DNA methylation, epigenetics, folate, s-adenosylmethionine

Introduction

Alzheimer's disease (AD) is the most common form of dementia worldwide, bearing over two thirds of diagnoses (Alzheimer's, 2016). Overall it represents a high disease burden, more so in high-income countries (Mathers et al., 2008). In 2013 there were approximately 835,000 people living with dementia in the UK alone, with this figure projected to rise to over 1 million by 2025 due to an ageing population (Alzheimer's Society, 2014). The disease is categorised by amyloid plaques and neurofibrillary tangles that accumulate in the brain with an accompanying cognitive decline which worsens over time. The cause for sporadic AD is currently unknown, however many hypotheses exist which attempt to explain the disease aetiology. The central hypothesis is the Amyloid cascade hypothesis, which proposes that deposition of the β-amyloid (Aβ) is the central initiating event and driving force, with Aβ accumulation causing the resulting downstream processes seen in AD. However, drugs targeting β amyloid production/aggregation have had limited success alluding to alternative hypotheses (Herrup, 2015). Currently, there are four drugs in use for the treatment of AD. Three of these are acetylcholinesterase inhibitors, which inhibit acetyl choline breakdown after nerve stimulation and are prescribed for mild symptoms. Whilst Memantine, a NMDA antagonist which blocks glutamate receptors, is prescribed for moderate-to-severe symptoms.

Other than the above agents, there has been little progress in the way of drug interventions with none able to halt disease progression, but merely stall it. Therefore there is a need for new, alternative hypotheses capable of disentangling the many affected molecular pathways, which may reflect changes occurring at the epigenetic level.

There is a growing interest in the role of epigenetic changes in driving the development of Alzheimer's. The role of DNA methylation has been well documented in carcinogenesis (Das and Singal, 2004, Ehrlich, 2002) but interest continues to grow regarding its role neurodegenerative diseases (Mattson, 2003, Hwang et al., 2017). It has long been known that age is associated with a global loss of DNA methylation (Drinkwater et al., 1989, Bjornsson et al., 2008) however more recent developments in ageing research have begun using DNA methylation as a predictor of tissue age, known as the epigenetic clock (Marioni et al., 2016). AD varies in its onset, progression, severity and has a non-Mendelian aetiology, allowing the possibility of epigenetic mechanisms. Predisposition to AD via epigenetic alterations has been observed, including epigenetic drift (Wang et al., 2008), and discordant DNA methylation patterns have been detected independent of age-related changes (Bakulski et al., 2012). Studies on monozygotic twins discordant for neurological diseases have also demonstrated contrasting DNA methylation patterns between the healthy and diseased twin (Mastroeni et al., 2009, Ketelaar et al., 2012).

Developing an understanding of molecular events, particularly at the level of DNA methylation could provide further insights into disease aetiology and pathology and the identification of diagnostic biomarkers.

Dietary folate and Alzheimer's disease

A diet rich in plant-based foods is considered to be protective against many human diseases including neurological diseases, with high vegetable intakes associated with a slower rate of cognitive decline. Despite the fact that dietary folate inadequacy has been a matter of public health interest for many decades because of its association with neural tube defects in pregnancy (MRC Vitamin Study Research Group, 1991), in the UK, a deficiency of the dietary methyl donor folate is highly prevalent in older adults (Clarke et al., 2004). In particular, the Mediterranean diet, predominantly based on plant foods and fish was found to be the diet most protective against AD in a meta-analysis and is correspondingly likely to be rich in B vitamins, such as folate, B12 and B6 (Sofi et al., 2008). Low levels or insufficient conversion of these vitamins leads to elevated homocysteine levels and an associated increased risk of cardiovascular disease (El-Khairy et al., 2001), cognitive impairment and AD (Ravaglia et al., 2005). Indeed, adherence to

the Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diet, a combination of a Mediterranean diet and hypertension treatment diet, is protective against cognitive decline (Morris et al., 2014).

Folate and DNA methylation

Folate is found in its natural form in food or as folic acid, the stable compound in supplements and fortified products. Folate functions in one-carbon metabolism where it acts as a methyl donor and also plays a role in DNA synthesis. Methyl donors provide methyl groups (CH_3) for DNA methylation: an epigenetic mechanism that has the capacity to modulate gene expression and can be influenced by environmental factors such as diet and lifestyle (Jaenisch and Bird, 2003). It is a cellular regulatory mechanism that primarily involves the covalent addition of a methyl group (CH_3) on cytosine residues adjacent to guanine (CpG sites), catalysed by DNA methyltransferases (Suzuki and Bird, 2008). Clusters of CpG sites in gene promoter regions are referred to as CpG islands and generally are associated with low/no transcription or gene silencing (Suzuki and Bird, 2008). Recent research has also found non-CpG site methylation (CpA, CpT, and CpC) throughout the genome, but in particular levels are found to be high in neurons (Lister et al., 2013). This could suggest an important role in neurological disorders (Guo et al., 2014), and indeed non-CpG methylation has recently been identified in AD-related genes (Nicolia et al., 2017a, Nicolia et al., 2017b). Interest is growing in understanding the functional significance of non-CpG methylation, and once methodological measurement issues have been resolved with employment of non-biased methods it is likely that more studies will focus on non-CpG methylation (Patil et al., 2014 , Fuso et al., 2015).

The folate and methionine pathways are interlinked metabolic pathways. Dietary folate, through a sequence of reactions donates a methyl group to homocysteine (Hcy) forming Methionine and then SAM: a global methyl donor for numerous methylation reactions including DNA methylation and synthesis of phosphatidylcholine. When SAM is demethylated it forms s-adenosylhomocysteine (SAH) which is then converted back to Hcy thereby linking the cycle. The interactions of folate with other B-vitamins (B6, B12) and Hcy are complex and interrelated (Figure 1). The presence of these cofactors drive the formation of

SAM, therefore a deficiency in one nutrient can disrupt pathways at various points and ultimately reverse the cycle, leading to Hcy accumulation (Fuso et al., 2005). Methylenetetrahydrofolate reductase (MTHFR) is the rate limiting enzyme in the conversion of one form of folate to 5-methyltetrahydrofolate (5-MeTHF), which then drives the reaction of Hcy to methionine, thereby decreasing levels of Hcy in the system and increasing SAM availability which participates in methylation reactions.

Folate deficiency has also been found to lead to uracil misincorporation, DNA breaks, apoptosis and cell cycle arrest (Courtemanche et al., 2004, Blount et al., 1997) whilst hyperhomocysteinemia (HHCy) and B-vitamin deficiency have been linked to loss of brain volume and cognitive decline (Tangney et al., 2011, Vogiatzoglou et al., 2008). Studies of the hippocampus, the brain region involved in learning and memory which is particularly vulnerable to ageing, have found that Hcy increases susceptibility to excitotoxic and oxidative damage (Kruman et al., 2000) and that folate deficiency is detrimental (Partearroyo et al., 2013).

Folate deficiency can also lead to an increase or decrease in gene expression with associated alterations in DNA methylation and twinned changes in protein synthesis, which in turn will have downstream effects on metabolic pathways, signal transduction and cellular processes (Novakovic et al., 2005, Wolff et al., 1998). If these changes occur in genes implicated in Alzheimer's, this could play a role in disease aetiology and pathology.

Current understanding of the molecular pathways involved in Alzheimer's

There are multiple interrelated systems which contribute to the asymptomatic period of AD including alterations in protein processing, signalling, inflammation, lipid transport, apoptosis, oxidative damage and stress responses, tau pathology, neurodegeneration and energy metabolism affecting brain homeostasis (Hampel et al., 2011). These manifest as the early clinical symptoms of memory deficits, leading to cognitive decline and eventual dementia.

The amyloid plaques and neurofibrillary tangles characteristic of AD lead to neuronal cell loss and damage to the vascular system, causing reduced blood flow to the brain and consequential cognitive

impairment. Amyloid-β protein precursor (AβPP), found in high concentrations in neuron synapses is broken down by secretases to form smaller peptides: soluble amyloid precursor protein and amyloid beta peptide (AβP) (Zhang et al., 2012). AβP dissociates from the nerve cell and upon interaction with the parent protein forms oligomers which diffuse into synaptic clefts. A build-up of AβP is neurotoxic (sensitising neurons to excitotoxic damage) causing Neurofibrillary tangle (NFT) formation and neuronal cell death. NFTs are comprised of hyperphosphorlyated tau, a microtubule associated protein, phosphorylated by Glycogen synthase kinase-3 (GSK3) (Giese, 2009) and regulated by the calcium-dependent protease Calpain (Saito et al., 1993). AβP also disrupts intracellular calcium regulation leading to an increase in cytosolic calcium concentrations; a proposed cause of NFTs (Supnet and Bezprozvanny, 2010). The main pathways thought to be involved include amyloid aggregation, tau phosphorylation, calcium homeostasis and oxidative stress; each of these hallmarks are discussed further.

Expression of β and γ secretases can be regulated by methyl donor availability

γ, β and α secretase cleave AβPP at different positions along the protein length. Presenilins (PS1), encoded by the gene *PSEN1,* form part of the important catalytic subunit of the protease complex γ-secretase, with mutations in this gene the most common cause of familial AD (Kelleher and Shen, 2017). AβP represents a 40-42 amino acid fragment, following cleavage by β-secretase (BACE1) at the N terminus of AβPP and γ-secretase at the C terminus yielding the Aβ protein (Xia, 2003). Cleavage by an alternative protease: α-secretase, is considered to be part of the non-amyloidogenic pathway.

Due to their essential roles in amyloid processing Presenilin 1 and β-site AβPP cleaving enzyme (BACE1) are rational targets. Genome-wide association studies have found variable results, for example in AD post-mortem brains negative associations were identified between *BACE1* promoter methylation and Aβ load (Do Carmo et al., 2016a). Whilst another study found no change in *BACE1* but *PSEN1* hypomethylation in AD brain samples, along with great interindividual epigenetic variability of the genes involved in methylation homeostasis (*MTHFR* and *DNMT1),* supporting a role for epigenetic effects in disease development (Wang et al., 2008).

Other approaches altering the methylation potential in neuroblastoma cells through administration of the methyl donor SAM have been shown to down regulate *PSEN1* expression partially silencing the gene, preventing PS1 protein synthesis and hence reduced Aβ levels (6 fold), whilst Presenilin 2 protein synthesis remained unchanged (Scarpa et al., 2003). In both *in vitro* and *in vivo* models B vitamin deficiency was associated with *PSEN1* over-expression via promoter hypomethylation, which consequently led to increased cleavage and production of AβP (Fuso et al., 2011b). This suggests that methyl donor nutrient availability could affect *PSEN1* gene expression.

The results of previous work were validated with an experiment demonstrating that restriction of folate and B12 in cell culture caused a decrease in SAM, which led to specific increases in Presenilin-1 and β-secretase (α-secretases were unaffected) (Fuso et al., 2005), strengthening the links between nutritional deficits, the methylation cycle and AD (Figure 1). A useful feature of this work, in contrast to many other studies was the partial vitamin deprivation rather than total, as it is more representative of nutritional insufficiency. Similarly, a differential effect of folate and B-vitamin deprivation was found with *PS1* and *BACE1* up-regulation with increased Aβ, in neuroblastoma but not glioblastoma cells (Fuso et al., 2007).

Following on from *in vitro* work, similar observations were obtained *in vivo* with expression levels of *PS1* and *BACE1* shown to be increased (in both TgCRND8 and wild-type mice) by B-vitamin deficiency (leading to HHCy) in comparison to the control diet (Fuso et al., 2008). B vitamin deficiency accelerated plaque formation with around a 50% increase in number and an increase in size, however this change was not mirrored with equivalent cognitive impairment or neuronal apoptosis. Fuso and colleagues (2011), also demonstrated using neuroblastoma cells that the methylation pattern for the *PSEN1* 5' flanking region is site-specific and can be altered in response to external stimuli (i.e. folate deprivation and SAM supplementation), with demethylation leading to overexpression which can be counteracted by SAM administration (Fuso et al., 2011b).

The promoter region of *BACE1* contains distinguishable transcription start sites and transcription factor binding sites (Lahiri et al., 2006). It has multiple CpG sites across its length, including one in the SP1 transcription factor binding site of the promoter region (Christensen et al., 2009). An unmethylated SP1

site (due to lack of methyl donor availability) provides a site for transcription factor binding and hence *BACE1* expression, increasing levels of Aβ (Lahiri et al., 2006).

Folic acid has been shown to influence Aβ production through increasing α-secretase activity and decreasing PS1 and BACE1 protein expression (Tian et al., 2016). Folic acid exerted an inhibitory effect on Aβ accumulation in transgenic mice, with supplemented folic acid associated with increased methylation potential and DNMT activity and altered DNA methylation in *AβPP* and *PSEN1* promoters (Li et al., 2015).

SAM production could be impaired if methionine adenosyltransferase II (MAT2A), which catalyses the production of SAM from methionine and ATP is down regulated. The functional connection between AβPP and SAM levels were explored via a knockdown model of *AβPP* family members, which resulted in the down-regulation of MAT2A (Schrötter et al., 2012), with SAM concentrations found to be inversely proportional to MAT2A. *BACE1* and *PSEN1* expression were also examined with *BACE1* being upregulated and *PSEN1* downregulated in the knockdown cells, suggesting a self-regulatory mechanism as both enzymes participate in AβPP cleavage.

Further to this, the importance of SAM was highlighted in an experiment examining the effects of SAM supplementation on B-vitamin (folate) deficient transgenic vs. wild type mice and found that SAM was protective, reducing Aβ production and tau phosphorylation, improving spatial memory and inhibiting the B-vitamin induced *PSEN1* and *BACE1* expression (Fuso et al., 2012). An interesting finding of this experiment was that SAM reduced Aβ production and plaque-spread even on the control (complete) diet. It should be noted however, that direct measurement of NFTs was not possible in this mouse strain therefore Protein phosphatase 2A (PP2A) activity was used as a proxy.

Recent work using transgenic mouse models have discovered that early build-up of Aβ can lead to small changes *BACE1* promoter demethylation, however if SAM was administered this build up was prevented and cognitive ability was restored (Do Carmo et al., 2016b). Subsequent genome-wide association studies to assess whether similar results could be identified in human post-mortem brains also found an association between *BACE1* methylation and Aβ load in AD patients. Overall these results support a role for *BACE1* methylation and methyl donor availability in AD.

Tau phosphorylation

Tau is a cellular microtubule assembly protein found abnormally phosphorylated as the major component of the NFTs characteristic of AD (Grundke-Iqbal et al., 1986). Rat primary neuron cultures treated with methotrexate (a folate antagonist) increased levels of P-tau, AβPP and β-secretase and decreased levels of PP2A, the tau phosphatase (Yoon et al., 2007). Protein methylation of the C-terminal leucine residue (Leu309) in the catalytic subunit of PP2A (PP2Ac) can regulate its catalytic activity (Bryant et al., 1999). Obeid and colleagues studied the effects of HHcy on P-tau, NFTs and PP2A activity and found that despite increases in P-tau, activity levels of PP2A were unchanged, implying that the effect is not as a result of altered PP2A activity or protein level (Obeid et al., 2011).

MTHFR is the rate-limiting enzyme for the conversion of folate to active 5MeTHF, hence knockout mice have elevated Hcy and altered methylation potential (see Figure 1). In young and aged *Mthfr* knockout mouse models, it was demonstrated that mild MTHFR deficiency decreases methylation of PP2A in a region-specific manner (Sontag et al., 2014). The effects were exacerbated by folate deficiency which significantly decreased methylated PP2A levels associated with increased P-tau. The common *Mthfr* (677C>T) polymorphism reduces enzyme activity (Ueland et al., 2001) and as it is characteristic of a mild folate deficiency could be considered a proxy for nutritional insufficiency, suggesting that mild deficiency of folate could increase phosphorylated tau in both young and old populations.

Conversely, inducing high plasma Hcy levels (vs. mild as with (Sontag et al., 2014)), caused tau hyperphosphorylation at multiple sites, inhibition of PP2A activity and alterations in the PP2Ac subunit, with methylesterase activation leading to demethylation of PP2Ac (Zhang et al., 2008). Upon administration of folate and B12 the HHcy was reversed preventing tau hyperphosphorylation, inactivation of PP2A and modified activity of PP2Ac but no change was exhibited in GSK3. These findings highlight the complex and intricate links between folate and Hcy metabolism.

Another way in which methylation of the PP2A enzyme can be decreased in neuroblastoma cells is via incubation with SAH (Sontag et al., 2007). Demethylation of PP2A alters holoenzyme assembly which

subsequently does not affect PP2A activity but its substrate specificity through altering subunit composition. This is associated with a shift of AβPP processing towards the amyloidogenic pathways triggering P-tau accumulation and increased secretion of AβPP fragments (Tolstykh et al., 2000, Sontag and Sontag, 2014). These studies provide links between SAH (dietary folate deficiency) to decreases in methylation and subsequent regulation of AβPP processing.

In 2008, Chan et al., demonstrated that folate deprivation and elevated Hcy led to activation of calcium-dependent-kinase pathways and inactivation of phosphatases, with both leading to increased P-tau. This demonstrates that both pathways could contribute to increased tau phosphorylation following folate deprivation, with SAM attenuating the Hcy increases even in the presence of NMDA channel activation and PP2A inhibition (Chan et al., 2008b).

Folate deficiency alters calcium dynamics and stimulates apoptosis

In AD there is dysregulation of intracellular calcium signalling with changes not just confined to neurons, implying systemic alterations (reviewed (LaFerla, 2002)). Each major AD gene has been shown to alter intracellular calcium signalling, including those encoding presenilins. Additionally, the APOE e4 genotype that is strongly associated with AD risk, demonstrates higher free intraneuronal calcium levels in a dose-dependent manner in comparison to milder genotypes (APOE4>APOE3>APOE2); an effect aggravated by Aβ (Ohm et al., 2001).

A study using peripheral blood lymphocytes from AD patients and folate deficient (FD) cultured neuroblastoma cells found that folate deficiency led to promoter hypomethylation of *DR4* (death receptor 4), increasing its expression (1.5 fold in AD patients) and transducing a cell death signal (Wang et al., 2014). An increase in DNMT1 and 3a mRNA, along with an inhibition of cell growth was also observed in the FD media, demonstrating a direct effect of folate on potential neurodegeneration.

The disturbance of calcium homeostasis was shown in a mouse model of AD, whereby glutathione (GSH) depletion and increased Hcy levels (i.e. when folate levels are low) activated calcium entry into the hippocampal neurones via TRPM2, TRPV1, VGCC and NMDA channels, inducing cellular toxicity and

apoptosis (Övey and Naziroğlu, 2015). This offers a mechanism of how HHcy could induce damage via increased intracellular calcium concentration in neurons and links with oxidative stress pathways.

Folate can protect against oxidative stress

Folate is linked to oxidative stress in AD through pathways relating to Hcy. Oxidative stress, caused by elevated Hcy in neurons (Tjiattas et al., 2004, Cankurtaran et al., 2013), has been seen to be increased in late-onset AD (McCaddon et al., 2003, Zafrilla et al., 2006, Resende et al., 2008). Another effect of increased Hcy levels is increased cerebrovascular permeability and neurodegeneration with elevated levels of oxidative stress demonstrated by increases in lipid peroxidation and nitrite levels and low GSH (Kalani et al., 2014). In general antioxidants are protective against oxidative stress, however specific antioxidants (such as GSH (Ho et al., 2003), vitamin E and also folate (Aoyama et al., 2008, Dhitavat et al., 2005)) have also been shown to be protective against toxic compounds like Aβ. Effects of high Hcy have been shown to be reversed by folic acid supplementation, thereby providing a potential intervention to improve redox homeostasis of the brain (Kalani et al., 2014).

Culturing neuroblastoma cells in a FD medium caused increases in cytosolic calcium, ROS, P-tau and apoptosis (Ho et al., 2003). Hcy was proposed to play a role, as the Hcy inhibitor 3-deazaadenosine (DZA) prevented the ROS increase and upon addition of an NMDA agonist prevented Hcy induced Calcium influx. In terms of oxidative stress, the FD cultures had less reduced glutathione, which acts as an oxidative buffer, whilst high concentrations of folate prevented ROS generation.

Aβ oligomers can also modulate gene expression via altering redox and methylation potential and prevent cysteine uptake, in turn impeding GSH production (cysteine is the rate limiting precursor), causing dysfunction of the transsulphuration pathway (Hodgson et al., 2013). SAM deficiency inhibits glutathione transferase (GST), a GSH dependent enzyme, whilst SAM supplementation restored activity, prevented oxidative damage and cognitive impairment (Tchantchou et al., 2006). Similarly, a protective effect of SAM was seen in AD mice on a B-vitamin deficient diet, by restoring GST activity, indicating it is an important defence against oxidative damage (Fuso et al., 2005).

On the basis that dietary deficiencies of vitamin E are additive to the effects of folate deprivation induced neurotoxicity, it was hypothesised that treatments with agents of the methionine cycle would be neuroprotective to these effects (Tchantchou et al., 2004). Treatment with the S-adenosyl homocysteine (SAH) hydrolase inhibitor 3-deazaadenosine (DZA) decreased ROS to levels lower than the control, with maximal effects observed with a combined treatment encompassing DZA and SAM). Folate deficiency potentiated the effects of ApoE deficiency, a factor independently associated with AD and oxidative stress, with mice on the deficient diet scoring considerably worse on cognitive performance tests and had increased oxidative damage. However, the effects may have partially been due to the presence of the pro-oxidant iron, and deficiency in the antioxidant vitamin E. They highlight that although DZA inhibits Hcy formation from SAH, it was the combined treatment that demonstrated greater effects, indicating the importance of the overall methylation capacity in determining Hcy levels.

Additionally, there is evidence that 5MeTHF can itself be depleted as a result of oxidative stress (Farkas et al., 2013), which consequently will increase plasma Hcy and reduce SAM (and therefore methylation potential). This indicates a more complicated bi-directional relationship and the potential importance of adequate SAM.

Methyl donors are required for maintenance of neurotransmitters

SAM is required for the synthesis of phosphatidyl choline, the precursor to choline which is subsequently converted to Acetylcholine (Blusztajn et al., 1987). Changes in the cholinergic system in the peripheral nervous system have been exhibited in rats fed a folate-deficient diet (Crivello et al., 2010). A decrease in folate led to a decrease in choline (also involved in the methylation cycle, see Figure 1) and hence acetylcholine in a region-specific manner in the brain of adult rats fed a FD diet for 9 months. Both the young and the old groups of rats demonstrated increased cholinergic metabolic sensitivity to FD in the peripheral nervous system compared to the brain, however the younger group were more able to adapt.

Investigation into the role of folate in methyl-donor induced cognitive impairment in rats found that folate deficiency impaired spatial memory and decreased levels of methylated phospholipid

phosphatidycholine, but effects could be reversed in FD rats with methionine (Troen et al., 2008). In contrast to Li and colleagues (Li et al., 2015), there were no changes in the methylation potential between cognitively impaired and control rats, however the SAM:SAH ratio was significantly lower in the low folate/high methionine rats. Disruptions in 1-carbon metabolism depleted brain phosphatidylcholine and impaired specific aspects of cognition, however this was restored by L-methionine, suggesting that the correct balance of methyl donors is essential.

Folate can modulate DNA methylase activity

Aβ oligomers could themselves be responsible for alterations in gene expression, as they have been found to lower DNMT activity in hippocampal neuronal and HT-22 cells (Liu et al., 2016). In these cells folic acid dose-dependently promoted DNMT activity, demonstrating a potential mechanism by which it could prevent neuronal toxicity. Similarly, when AD transgenic mice were fed differing amounts of folic acid, again it dose-dependently stimulated DNMT activity and decreased *PSEN1* expression and AβPP production and the harmful effects of the Aβ oligomers were attenuated by folate (Li et al., 2015).

In neuroblastoma cells and transgenic mice, DNA demethylase activity was increased by B-vitamin (folate, B6, B12) deficiency and inhibited by SAM administration as measured via methyl incorporation (Fuso et al., 2011a). Sequence specific analysis identified preferential demethylation of CpG moieties in the *PSEN1* promoter, induced by B-vitamin deficiency compared to the control. The authors hypothesised a sequence of events leading to Aβ processing, with the B-vitamin deficiency leading to an increase in both HCY and SAH, inhibiting methyltransferase activity whilst unhampering demethylase activity, with this imbalance favouring *PSEN1* promoter demethylation, overexpression of *PSEN1* and up-regulation of gamma-secretase. This research demonstrated that activity of methylation enzymes can be regulated by B-vitamin availability, which also leads to changes in the PSEN1 promoter region, linking the implications to AD. It would be interesting to see if this then led to alterations characteristic to AD, Aβ accumulation or altered disease progression, to establish whether folate intake may be protective.

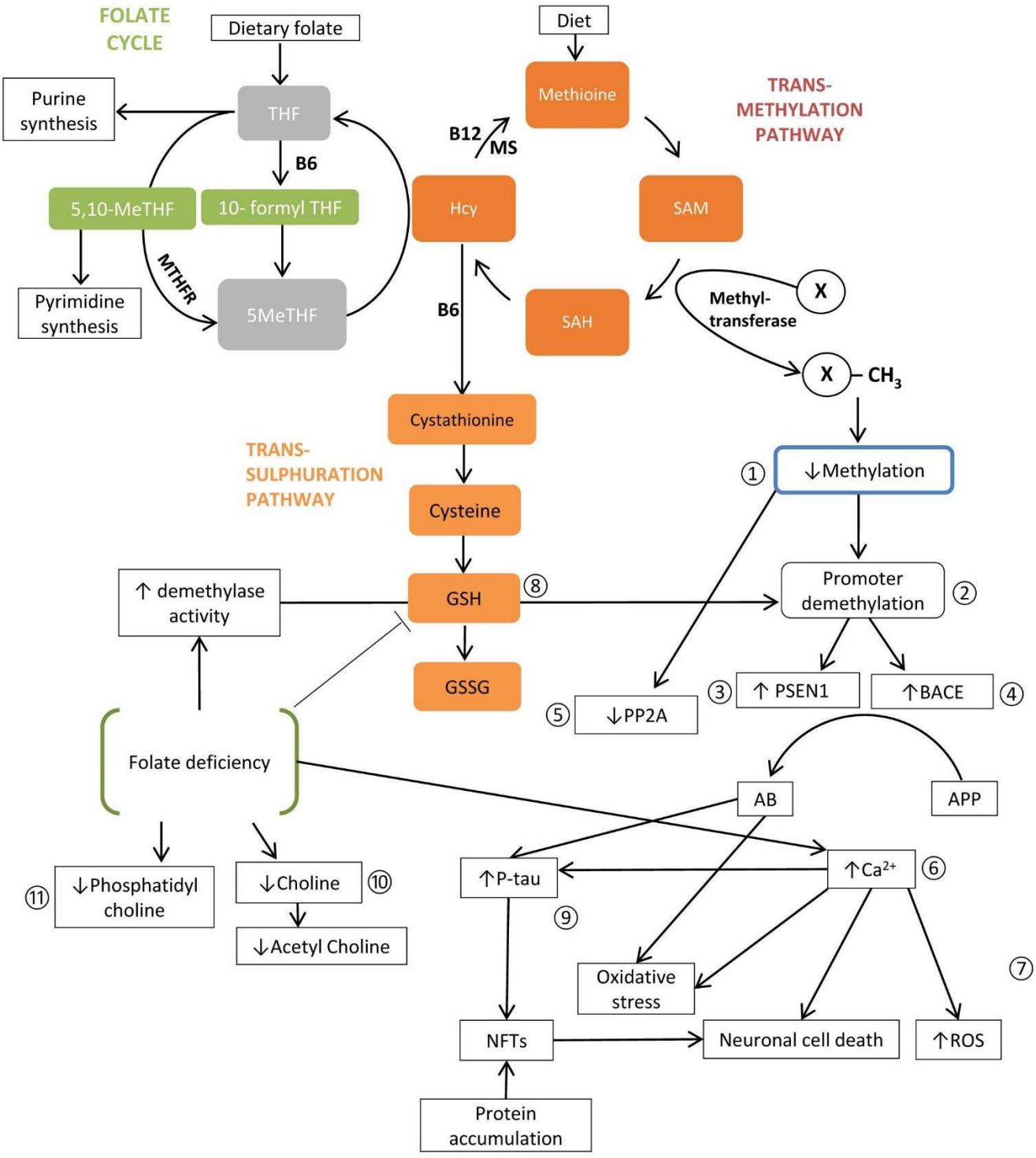

Figure 1 Diagrammatic summary of the findings of this review, linking the methylation cycle to events in Alzheimer's disease, with the numerous potential actions of folate on different pathways. The diagram shows how seemingly separate events occur but together they could accumulate damage that is summative to AD pathology. A decrease in dietary folate leads to a decrease in availability of methyl donors in the methylation cycle, which subsequently affects levels of SAM, and can be exacerbated by lack of B vitamins, which function as cofactors for the reactions in the SAM cycle. In a folate deficient state the equilibrium of the cycle favours Hcy accumulation and lack of methyl donor availability, causing a decrease in methylation. Promoter demethylation in the secretase genes (PSEN1 and BACE1) leads to Aβ accumulation, increased levels of P-tau and increased Ca^{2+}, with the downstream effects of increased ROS, NFTs, cell death and oxidative stress. Folate deficiency has separate but related effects of altering choline levels and hence availability of neurotransmitters. Table 1 links the figure references to those cited in the review. Cofactors/enzymes in bold Abbreviations- THF Tetrahdrafolate; 5,10-MeTHF 5,10-methylene tetrahydrafolate; 5 MeTHF 5-Methyl tetrahydrafolate; Hcy homocysteine; SAM S-adenosyl methionine; S-adenosyl homocysteine; MS methionine synthase; GSH glutathione; GSSG Glutathione disulphide; PP2A Protein phosphatase 2; PSEN1 Presenilin-1; BACE1 Beta-secretase 1; APP amyloid precursor protein; AB β-amyloid; P-tau phosphorylated tau; NFTs neurofibrillary tangles; ROS reactive oxygen species.

Pathways	References cited	Figure reference
Decreased methylation	(Chiang et al., 1996, Wang et al., 2008)	①
Promoter demethylation	(Scarpa et al., 2003, Lahiri et al., 2006, Scarpa et al., 2006)	②
Amyloid processing Presenilin expression/activity	(Scarpa et al., 2003, Schrötter et al., 2012)	③
β secretase expression/activity	(Scarpa et al., 2003, Fuso et al., 2005, Fuso et al., 2007, Schrötter et al., 2012)	④
Tau phosphorylation	(Fuso et al., 2012, Chan et al., 2008b)	
PP2A	(Sontag et al., 2014, Yoon et al., 2007, Obeid et al., 2011, Chan et al., 2008b)	⑤
Calcium homeostasis	(Övey and Naziroğlu, 2015, Ho et al., 2003)	⑥
Oxidative stress	(Tjiattas et al., 2004, Cankurtaran et al., 2013, Oikonomidi et al., 2016)	⑨
Reactive oxygen species	(Tchantchou et al., 2004, Ho et al., 2003)	⑦
GSH	(Övey and Naziroğlu, 2015, Aoyama et al., 2008, Dhitavat et al., 2005, Kalani et al., 2014, Hodgson et al., 2013)	⑧
Choline	(Crivello et al., 2010)	⑩
Phosphatidyl choline	(Troen et al., 2008)	⑪

Table 1 Summary of the pathways involved in AD and linked to one-carbon metabolism as reviewed here, with reference to Figure 1.

Evidence from epidemiological studies

Compared to healthy, age-matched controls, AD patients have been found to have high levels of Hcy and low blood levels of folate (Ravaglia et al., 2005, Clarke et al., 1998). A recent meta-analysis found that deficiencies in folate and B12 increase with age and correlate to AD risk (Shen and Ji, 2015). Additionally another systematic review and meta-analysis found significantly lower nutrient levels of folate and B12 (amongst others) in AD patients, unrelated to malnourishment and preceding the protein and energy malnutrition that accompany the disease, indicating that AD patients have impaired systemic availability of several nutrients (da Silva et al., 2014). However in studies assessing intake after disease onset, it is difficult to discern the direction of the association as the disease itself could lead to alterations in feeding behaviour and dietary intake.

Arguing for HHcy as a causative factor, results from a cross sectional study on subjects with very mild cognitive impairment suggested that folate deficiency precedes dementia onset, as those in the highest tertile for HHcy performed worse on the mini-mental state examination (Quadri et al., 2004). However as the disease develops over a prolonged time period, without subsequent brain examination it is unknown if these subjects are in the early stages of dementia. A cross-sectional study of 60 AD patients found a higher frequency of high Hcy plasma levels across AD patients and significantly lower levels of SAM in CSF, and hence a lower SAM: SAH ratio, which was found to be associated more so with APOE e4 allele carriers (Linnebank et al., 2009). Whilst another small study in adults with normal cognition found one-carbon metabolites (high Hcy, high SAH and low 5-MeTHF levels in CSF) to be associated with CSF levels of soluble AβPP and Aβ (Oikonomidi et al., 2016). No associations were seen for plasma folate, although levels were correlated with soluble α- and β-amyloid precursor protein. On the other hand, a large prospective cohort study with a 3.9 year follow-up found no association between dietary intakes of folate, B12 and B6 and AD (Morris et al., 2006). However, there is the possibility that the use of only a self-administered food frequency questionnaire to measure vitamin intake in a population with potential memory impairment may not be able to accurately capture intake. It is accepted that cross sectional

studies are not accurately able to address cause and effect due to the fact that they take measurements at the same time point.

In contrast, longitudinal studies are better equipped for studying the effects of dietary intake on disease development due to measures at multiple time points. Using data from the Cache study, dietary intakes of B-vitamins from foods and supplements were found to be unrelated to dementia/AD incidence (Nelson et al., 2009). However the authors acknowledged that the population was mostly Caucasian, religious and followed a healthy lifestyle (limited alcohol and tobacco use), which could affect outcomes. In the Baltimore Longitudinal study of Aging where participants were followed up for 14 years, a protective effect of a higher folate intake (levels above the recommended daily allowance) was found for AD. Although, again the demographic consisted of mostly Caucasian and well-educated volunteers not allowing the results to be generalised and Hcy levels were not measured (Corrada et al., 2005).

Additionally, folate deficiency would also be less likely in a US population due to the 1998 fortification program. Folate fortification has demonstrated protection from cognitive impairment (Ramos et al., 2005) but only in those with adequate B12, with detrimental effects observed in subjects with low B12 status (Morris et al., 2005). Increasing levels of folate in subjects with low B12, which is a cofactor for methionine synthase, 'traps' folate thereby preventing downstream methylation reactions.

Due to the role of B12 as a cofactor in one carbon metabolism, supplementation with folate has the capacity to mask B12 deficiency; therefore studies often administer supplements of both vitamins. This can lead to difficulty in differentiating the separate effects and drawing solid conclusions on the specific actions of the two, and despite each contributing to the same metabolic pathway, they differentially relate to individual cognitive consequences (Morris, 2012). A 2008 Cochrane review (Malouf and Grimley Evans, 2008) successfully separated the variables and found that there was a lack of consistency on the effects of folic acid supplementation on cognitive function with or without B12 on both healthy and cognitively impaired older adults. However there was evidence from one study of improvements in patient's response to cholinesterase inhibitors when supplemented with folic acid.

There have been other efforts in clinical studies using combinations of compounds shown to be effective in molecular studies (Chan et al., 2008a, Chan et al., 2009). A vitamin/nutriceutical formulation containing folate, vitamin B12 and S-adenosyl methionine has been piloted in small numbers of AD patients. Caregivers noted improvement in cognitive performance, behavioural and psychological domains for both early stage sufferers as well as a delay in decline for late stage sufferers (Remington et al., 2009). Larger phase II trials confirmed improvement or maintenance of cognitive performance and behaviour and was more effective for earlier stages of AD emphasising the importance of early intervention (Remington et al., 2015).

A similar approach has been proposed with Cerefolin NAC, a nutritional supplement composed of 5-MeTHF; N-acetylcysteine, precursor of the antioxidant glutathione; and methylcobalamin, the cofactor required for conversion of Hcy to methionine by methionine synthase (Figure 1) (McCaddon and Hudson, 2010). Preliminary studies suggest mild improvements in patients with HHcy, however properly conducted randomized, placebo-controlled trials are required (Hara et al., 2016). These nutriceutical formulations could be promising future treatments pending further trials.

Concluding comments

The associations noted for B vitamins and Hcy and cognitive decline suggest they are of some aetiological importance in AD. However, overall the epidemiological studies provide contradictory and inconsistent results regarding the effects of plasma Hcy, folate and B-vitamin levels and dietary intake and the links with cognitive decline. It could be due to the many potential confounding factors, the follow-up time period, population differences, dietary assessment methods or reporting issues (memory and recall). Additionally, dietary intakes may not always mirror serum levels. Study design can limit success in the ability to separate the variables and interactions, and intervention trials would be better suited determining causality. Additionally, demographics need to be representative in order to determine if folate intake is related to disease incidence or progression.

Perhaps there is scope for valuable predictive disease models (Lahiri et al., 2007) to provide biomarkers using early life exposures that might predetermine AD risk (Borenstein et al., 2006). This idea is proposed by the 'Latent Early-life Associated Regulation' (LEARn) model, which suggests a two-hit hypothesis in AD aetiology; developmental and environmental (Lahiri et al., 2007). The model proposes that nutritional remediation would significantly reduce AD risk in later life, for example via dietary supplementation (of folate) during the developmental period as a means to prevent adult neurodegeneration. It is an attractive theory supported by the developmental origins of health and disease hypothesis (Barker, 2007), with current interest on early life exposures which predispose to individuals to disease through epigenetic mechanisms. Longitudinal study designs integrated with epigenetic biomarkers and exposure data prior to disease onset could provide more insight, whilst another possible approach to assessing causality in molecular epidemiological studies could be Mendelian randomization (Smith and Ebrahim, 2003, Relton and Davey Smith, 2012).

Molecular studies have demonstrated more consistent findings when examining individual aspects of the disease as opposed to population-based studies, and could therefore be utilised to inform and be integrated into epidemiological studies.

In terms of the role low folate status plays is the resultant increase in Hcy, which could have undesirable effects in AD via different mechanisms. High Hcy levels can stimulate NDMA receptors leading to calcium influx and oxidative damage and downstream apoptosis providing evidence of neurotoxicity (Shea et al., 2002). Increased levels of Hcy also promotes DNA damage, which sensitises neurones to Aβ toxicity (Kruman et al., 2002).

Evidence also suggests that low folate can induce SAM reductions which can inhibit DNA methyltransferases, decreasing promoter methylation and thereby altering protein expression of genes related to AD pathogenesis. The protective effect of SAM has been presented in regards to oxidative stress (Tchantchou et al., 2006, Tchantchou et al., 2004), DNA methylase activity (Fuso et al., 2011a), decreasing Aβ (Fuso et al., 2011b, Fuso et al., 2012, Scarpa et al., 2003), and tau phosphorylation (Chan et al., 2008b) which could be due to lowering Hcy levels and/or an increase in methyl donor availability.

At this stage the evidence does not support a direct, sole responsibility for folate, but that it is necessary to have a fine balance of compounds in the methylation cycle. From the evidence presented it is likely that folate and the B-vitamin cofactors affect Hcy levels and consequently SAM:SAH ratio, thereby affecting methylation potential. The success of SAM in inhibiting AD progression stresses the importance of methyl donor availability for correct cellular functioning. Its ability to reverse the changes exhibited in AD as demonstrated in many studies makes it a potential therapeutic treatment to be explored further. The accumulation of these findings support a functional, deleterious capacity of folate deficiency in the cascade of epigenetic alterations and disturbances in cellular activity that lead to eventual neurodegeneration and AD. These effects appear to be additive or exacerbated by genotypic variation (APOE e4 allele or *MTHFR* polymorphism) and nutrient intakes.

Inducing a mild vitamin deprivation (Fuso et al., 2005, Sontag et al., 2014) is a useful tool as it represents model similar to that of nutritional insufficiency, producing more archetypal results. Additionally nutrients represent a modifiable risk factor in disease development; increasing understanding could lead to a simple preventative measure.

Both epidemiological and molecular studies have failed to find conclusive evidence that one component leads to AD, most probably because the balance/equilibrium of compounds is important and experimental design needs to be approached more holistically. Further investigations need to be made as to whether folate is associated with onset or progression of the disease, which is difficult to distinguish as the symptoms that accompany the disease lead to altered whole body metabolism, altered eating patterns and patterns of activity. Integration of the epidemiological evidence using at-risk populations and identifying potential epigenetic biomarkers from the appropriate disease model, when combined could aid in future diagnoses, prognosis or screening before disease onset.

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