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Effect of Experimental Hyperhomocysteinemia on Brain Methylation and Neurodegenerative Markers in Rats

DISSERTATION

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I Abbreviations

ABαC	Dominant brain isoform of protein phosphatase 2A
AD	Alzheimer's disease
Adas-cog	Alzheimer's Disease Assessment Scale-cognitive subscale
ACT	Alpha-1-antichymotrypsin
APP	Amyloid precursor protein
Aβ	Amyloid beta
BACE	β-site of APP cleaving enzyme
BHMT	Betaine homocysteine methyltransferase
CAT	Computed axial tomography
CBS	Cystathionine beta synthase
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Coefficient of variation
Cys	Cystathionine
DLB	Dementia with Lewy bodies
DTT	Dithiotrilae
FTD	Frontotemporal dementia
HCY	Homocysteine
HHCY	Hyperhomocysteinemia
HRP	Horseradish peroxidase
LCMT-1	Leucine carboxyl methyltransferase-1
LC-Tandem-MS	Liquid chromatography tandem massspectrometry
L-DOPA	L-3,4-dihydroxyphenylalanine
MCI	Mild cognitive dysfunction
Met	Methionine
MMA	Methylmalonic acid
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
MS	Methionine synthase
MTHF	Methylenetetrahydrofolate
MTHR	Methylenetetrahydrofolate reductase

m/z	Mass-to-charge ratio
NFT	Neurofibrillary tangles
n.s.	Not significant
PDD	Parkinson's disease with dementia
PHF	Paired helical filaments
PME-1	Protein phosphatase methylesterase-1
PNF-H	Phosphorylated neurofilament H
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
PPMT	Protein phosphatase 2A methyltransferase
PS1	Presenilin 1
PS2	Presenilin 2
P-tau	Phosphorylated tau
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SD	Standard deviation
SPE	Solid phase extraction
TMB	Tetramethylbenzidine
tHcy	Total homocysteine
THF	Tetrahydrofolate
TNF-α	Tumor necrosis factor alpha
VaD	Vascular dementia
WML	White matter lesions

II Summary

Many studies show a significant relationship between elevated plasma total homocysteine concentration (tHcy) and age associated neurodegenerative diseases like dementia, but it is not yet possible to link these two causally because of study limitations and pending treatment studies. Plasma tHcy is a nutritional metabolic marker. Mild to moderate hyperhomocysteinemia (HHCY; tHcy 12-30 µmol/l) is an indicator for deficiencies in B-vitamins (folate, B_{12}, B_6). HHCY is associated with elevated plasma concentrations of S-adenosyl homocysteine (SAH). SAH is the demethylation product of S-adenosyl methionine (SAM) which functions as a methyl group donor. The remethylation of homocysteine (HCY) to methionine is an important source for the generation of SAM. A disturbed cellular methylation potential is one important pathomechanism in HHCY and plays an important role in the development of neurodegenerative disease.

The goal of the study was to determine in vivo if HHCY leading to a disturbed methylation status is linked to neurodegenerative alterations. In this study we examined the effect of experimentally induced HHCY on accumulation of phosphorylated tau (P-tau) protein, phosphorylated Neurofilament H (pNF-H), tumor necrosis factor alpha (TNF-α), and protein phosphatase 2A (PP2A) activity adjusted for total PP2A protein level in rat cerebral tissue.

We induced HHCY with a homocystine (1.7%) and a methionine (2.4%) enriched diet in Wistar rats. After euthanizing the animals, the brain tissue was extracted and the accumulation of P-tau protein was measured by immunofluorescence. Metabolites were measured by liquid chromatography tandem mass spectrometry (LC-Tandem-MS). Concentrations of pNF-H, TNF-α, and PP2A activity and level were measured in cerebral cortex extracts. Results were normalized for total protein.

The in vivo study showed that plasma tHcy in Wistar rats increased by more than 10-fold after five months of the methionine and homocystine diets when compared to baseline concentrations. Furthermore the dietary intervention led to a reduced methylation status reflected by elevation of SAH concentration, as well as a significant decrease in SAM/SAH ratio in two different brain regions (frontal cortex and brain stem). Plasma concentrations of folate and vitamin B_{12} were significantly correlated to brain SAM/SAH ratio. We noted a considerably increased accumulation of P-tau protein in the cerebral frontal cortex of rats nourished with both of the HHCY inducing diets. P-tau accumulation in the frontal cortex was positively related to plasma tHcy and negatively related to folate concentration. Folate status rather than activity of PP2A or level of PP2A in brain tissues was related to phosphorylated

protein forms like P-tau and pNF-H. TNF-α concentration in frontal cortex did not differ according to diet.

Our study showed that methionine and homocystine treated diets fed to Wistar rats are strongly effective in inducing a pronounced experimental HHCY. Requirements and turnover of methyl groups were different according to brain region and may reflect different regional biochemical pathways. Plasma concentrations of folate and vitamin B_{12} were significantly correlated to brain SAM/SAH ratio proving that better B-vitamin status is directly related to better brain methylation status. The accumulation of P-tau protein in the rat brain was related to HHCY and enhanced folate consumption or requirement. Plasma folate was lowered by supplementation of methionine or homocystine, thus causing increased P-tau and pNF-H. The effect of the diet on P-tau and pNF-H seemed not to be explained by lower activity of the catalytic unit of PP2A or protein level of the enzyme. Low folate, hyperhomocysteinemia, hypomethylation in the brain caused hyperphosphorylation of functional proteins involved in neurodegeneration. TNF-α in cerebral cortex does not explain HCY neurotoxicity in our study.

These results must be verified in further studies examining the exact neurodegenerative mechanisms. In addition, it must be determined what effect mild to moderate HHCY, over a considerably longer period of time, has on P-tau, pNF-H, and PP2A activity. Because our study cannot be directly extrapolated to humans, it would be interesting to explore the effect of treatment with B-vitamins, leading to an improved methylation status, on concentration of P-tau protein, pNF-H, and PP2A activity and to test if this treatment has a direct impact on the cessation or delay of demential development.

Zusammenfassung

In vielen Studien zeigt sich eine signifikante Beziehung zwischen einem erhöhten Plasmaspiegel an Gesamthomocystein (tHcy) und altersassoziierten neurodegenerativen Erkrankungen, insbesondere Demenz. Jedoch kann man bisher noch nicht von einer kausalen Beziehung sprechen wegen Studienlimitationen und ausstehenden Therapiestudien. Plasma tHcy ist ein metabolischer Marker, der sich ernährungsabhängig verhält. Milde bis moderate Hyperhomocysteinämie (HHCY; tHcy 12-30 µmol/l) ist ein Indikator für B-Vitaminmangel (Folat, B_{12}, B_6). HHCY steht im Zusammenhang mit einer erhöhten Plasmakonzentration an S-Adenosylhomocystein (SAH), wobei SAH das Demethylierungsprodukt von S-Adenosylmethionin (SAM) darstellt, welches als Methylgruppenspender fungiert. Eine entscheidende Quelle der SAM-Bildung ist die Remethylierung Homocysteins (HCY) zu Methionin. Ein gestörtes zelluläres Methylierungspotential ist ein grundlegender Pathomechanismus bei HHCY und spielt für die Entwicklung neurodegenerativer Erkrankungen eine wichtige Rolle.

Das Untersuchungsziel bestand in der Aufklärung, ob in vivo HHCY, die zu einem gestörten Methylierungsstatus führt, in Verbindung mit neurodegenerativen Veränderungen steht. In dieser Studie untersuchten wir die Wirkung experimentell induzierter HHCY auf die Anreicherung von Tau-Protein (P-Tau), phosphoryliertem Neurofilament H (PNF-H), Tumornekrosefaktor (TNF-α) und Protein-Phosphatase 2A (PP2A) Aktivität und Level im Rattengehirn.

Durch eine mit Homocystin (1,7%) und eine mit Methionin (2,4%) angereicherten Diät konnnten wir bei Wistar-Ratten eine HHCY erzeugen. Nach Euthanasieren der Tiere wurde das Gehirngewebe entnommen und die Akkumulation von Tau-Protein mittels Immunfluoreszenz untersucht. Die untersuchten Metabolite wurden mittels Flüssigkeitschromatografie-Tandem-Massenspektrometrie (LC-Tandem-MS) bestimmt. Konzentrationen von PNF-H, TNF-α und PP2A Aktivität und Level wurden im Extrakt des Rattengehirns bestimmt. Ergebnisse wurden normalisiert für Gesamtprotein.

Unsere in vivo Studie zeigt, dass Plasma tHcy bei Wistar Ratten sowohl bei einer mit Methionin wie auch mit Homocystin angereicherten Diät im Zeitraum von fünf Monaten um mehr als das 10-fache ansteigt. Darüber hinaus führte diese diätetische Intervention zu einem Anstieg der SAH-Konzentration im Gehirn, sowie zu einer signifikanten Erniedrigung des SAM/SAH Quotienten in zwei verschiedenen Gehirnregionen (Frontaler Kortex und

Hirnstamm). Folsäure und Vitamin B_{12} Plasmakonzentrationen korrelierten signifikant mit dem SAM/SAH Quotienten. Wir beobachteten bei den Ratten unter Methionin und Homocystin angereicherten Diäten eine deutliche Verstärkung der phosphorylierten Tau-Proteinfärbung im frontalen Kortex. Die Anreicherung von P-Tau im frontalen Kortex stand in positiver Beziehung zum Plasmaspiegel von tHcy und in negativer Beziehung zur Folsäurekonzentration im Plasma. Der Folsäure Status der Ratten im Plasma stand eher in Beziehung zu phosphorylierten Proteinformen wie P-tau und PNF-H als die PP2A-Aktivität oder –Level. Konzentrationen von TNF-α im frontalen Kortex unterschieden sich nicht in Bezug auf die diätetische Intervention.

Unsere Studie belegte, dass Methionin und Homocystin angereicherte Diäten in Wistar Ratten eine ausgeprägte experimentelle HHCY hervorrufen. Der Bedarf und Umsatz von Methylgruppen unterschieden sich in Anhängigkeit von der Gehirnregion und könnte regionale Unterschiede in biochemischen Stoffwechselwegen wiederspiegeln. Folsäure und Vitamin B_{12} Plasmakonzentrationen korrelierten signifikant mit dem SAM/SAH Quotienten und beweist hiermit, dass ein verbesserter B-Vitamin Status direkt mit einem verbesserten zerebralen Methylierungsstatus zusammenhängt. Die Anreicherung von P-Tau-Protein im Rattengehirn stand im kausalen Zusammenhang mit HHCY und erhöhtem Folsäurebedarf oder –umsatz. Folsäure Plasmaspiegel wurden erniedrigt durch den Zusatz von Methionin und Homocystin und korrelierte mit vermehrtem P-Tau und PNF-H. Die Auswirkung der Diät schien nicht erklärbar durch eine verringerte Aktivität oder Level der katalytischen Dimer-Untereinheit des PP2A Enzyms. Niedriger Folsäurestatus, Hyperhomocysteinämie und Hypomethylierung im Gehirn scheint Hyperphosphorylierung von funktionellen Proteinen zu verursachen, die bei Neurodegeneration involviert sind. TNF-α im frontalen Kortex erklärt die neurotoxische Wirkung HCYs nicht.

Diese Ergebnisse müssen in weiterführenden Untersuchungen, die exakten neurodegenerativen Mechanismen betreffend, bestätigt werden. Außerdem sollte man Auswirkungen auf P-Tau, PNF-H und PP2A Aktivität untersuchen, wenn eine milde bis moderate HHCY über deutlich längeren Zeitraum aufrechterhalten wird. Da unsere Studie nicht direkt auf den Menschen übertragen werden kann, wäre es interessant in klinischen Studien die Wirkung einer Behandlung mit B-Vitaminen, insbesondere Folsäure und somit eines verbesserten Methylierungsstatus auf die Konzentration von phosphoryliertem Tau-Protein, PNF-H und die PP2A Aktivität zu testen und ob diese Behandlung eventuell einen direkten Einfluss auf das Aufhalten oder Verzögern einer dementiellen Entwicklung hat.

III Introduction

Approximately 4.5 million new cases of dementia are predicted worldwide for each year. The world's population is aging; the prevalence of dementia will increase considerably in the next 40 years when the world will be dealing with more than 110 million patients with dementia (38). The risk of developing dementia, a form of neurodegeneration, is strongly age-related. However, dementia explicitly is not an obligatory part of aging but is a true disease. There are different forms of dementia each having different pathomechanisms. In Alzheimer's disease (AD) tau becomes hyperphosphorylated (P-tau) and aggregates into paired helical filaments (PHF) which accumulate in neuronal cell bodies as neurofibrillary tangles (NFT), these correlating with cognitive deficits, neurodegenerative disorder, and dementia. Vascular Dementia (VaD) is understood as a heterogeneous syndrome in which the underlying cause is a form of cerebrovascular disease such as multi-infarct dementia. Dementia is caused by several genetic and non-genetic risk factors. Many cross-sectional studies and prospective studies (46;112;132;146;165;181) on more than 10,000 subjects have shown significant associations between cognitive deficit or dementia and homocysteine (HCY) and/or B-vitamins. Different mechanisms have been proposed to account for these associations, but a definite causal pathway has yet to be shown. Hyperhomocysteinemia (HHCY) has been linked in many studies to diseases of the central nervous system (CNS). It is an important goal to find prognostic markers allowing early detection of disease or populations at risk for dementia. Raised plasma total homocysteine (tHcy) is a strong prognostic marker of future cognitive decline and dementia, and is common in world populations. Low-normal concentrations of the B-vitamins, the main determinant of HCY concentrations, are also common and occur in particularly susceptible phases in life, such as in infancy and at a more advanced age. An improved pathobiochemical understanding of effects of HHCY on hypomethylation and hyperphosphorylation will aid in developing strategies for early prevention. A decrease in the risk for the development of neurodegenerative diseases by lowering the concentration of tHcy by sufficient alimentary vitamin supply or supplementation in the case of vitamin deficiencies was shown in some studies (13;30;195) but not in others (103) and needs to be evaluated further. Research at this time calls for large-scale multi-centered randomized trials of HCY-lowering vitamins to see if a proportion of dementia in the world can be prevented.

1 Dementia, an Age-Associated Neurodegenerative Disease

Neurodegeneration is a term used to describe the process of pathological and destructive changes occurring in the brain. Neurodegenerative diseases include a wide group of disorders of various etiologies and clinical features and are mostly present in the second half of life and probably take decades to develop. They all have in common structural and functional protein modifications, resulting in deterioration of certain nerve cells or neurons. The clinical correlate of age-related neurodegeneration is cognitive decline and can lead to dementia. Mild cognitive impairment (MCI) represents the intermediary stage between normal aging and dementia. MCI is an age-dependent disorder characterized by impairment of memory and/or another cognitive domain. This reduction of previous level of function is by definition not severe enough to interfere with daily function, independence, social, or professional abilities. However, compared to people without memory decline, patients with MCI, older than 65 years, have a risk of 10-15%/year to develop dementia in comparison to the healthy population with a risk of 2%/year (28;155). Understanding this is essential in order to identify risk factors and markers for the development or progression of MCI and/or dementia. Identifying extrinsic factors that accentuate the effects of aging and eliminating these, would then lead to successful aging, in which extrinsic factors play a neutral or positive role. HHCY is an example for a prognostic marker; an inverse relationship has been shown for cognitive function. Studies have shown HHCY to accelerate the progression of mild cognitive impairment to dementia as in this recent study where tHcy measured at baseline predicted the rate of cognitive decline in patients with AD (137).

1.1 Epidemiology of Dementia

A very high proportion of elderly people develop dementia and the probability increases with age. Worldwide prevalence of dementia is strongly related to age: 0.5% in those between 60 and 65 years, 1.5% between 65 and 69 years, 3% between 70 and 74 years, 6% between 75 and 79 years, 12% between 80 and 84 years, and 25% in those aged 85 to 89 years. The prevalence reaches almost 50% in those over 95 years (42). Figure 1 demonstrates how prevalence of dementia according to age in Germany increases approximately by two-fold every 5 years after the age of 65 years. Although the development of dementia is strongly age-related, dementia is not an inevitable part of aging. It is a true disease caused by exposure to genetic as well as non-genetic risk factors.

The world prevalence of dementia for the year 2000 was estimated at 25.5 million by Wimo et al. (204). The authors estimated that in the year 2000 numbers for dementia were 11.9 million in Asia, 7.4 million in Europe, 3.1 million in North America, 1.7 million in Latin America, 1.25 million in Africa. However, the number for North America may have even been estimated rather low since for the U.S. alone there was an estimated 4.5 million Americans with AD (65). AD is the most common form of chronic dementia but accounts only for 60 to 80 percent of dementias.

Due to the predicted demographics showing a steady percentile increase of the elderly generation, the prediction of future worldwide prevalence for dementia suggests that there may be as many as 114 million patients with dementia by the year 2050. The Delphi consensus study (38) largely is in agreement with these predictions Wimo et al. (204) made and estimated that each year there will be 4.5 million new cases of dementia worldwide. Another concerning feature of the worldwide prevalence estimates is that, in 2000–2001, 52% to 60% of those with dementia lived in less-developed regions (38), by 2050 there will be as many as 74% (84 million) living in the less-developed regions (204). These alarming predictions for the next 40 years underline the importance of research on this subject matter to find new possibilities for prevention. Preventing dementia will become a realistic possibility when non-genetic risk factors can be identified and targeted.

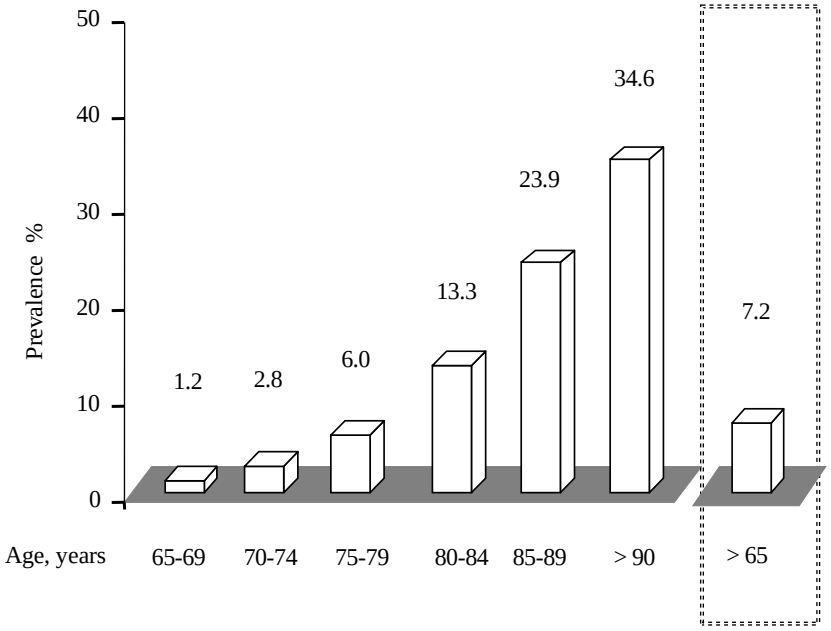


Figure 1
Prevalence of dementia according to age in Germany (year 2004); Source: German Alzheimer Society (Deutsche Alzheimer Gesellschaft)

1.2 Different Forms of Dementia

Dementia is the most common form of neurodegeneration and is a disorder characterized by impairment or loss of memory and at least one other cognitive domain (aphasia, apraxia, agnosia, or executive function). These must show a reduction of the previous level of function and be severe enough to interfere with daily function and independence (2). Progression of disease leads to significant personality changes, loss of both motor control and coordination, and an increasing inability to take care of oneself causing the patient's loss of independence.

The major dementia syndromes include: AD, VaD, dementia with Lewy bodies (DLB), Parkinson's disease with dementia (PDD), frontotemporal dementia (FTD) like Morbus Pick, and reversible dementias like Morbus Wilson. The most common forms of dementia are AD and VaD and will be discussed briefly.

AD, the leading cause of dementia, is an incurable disease and is the classic example for cortical dementia. In contrast to subcortical dementias where the main problem is loss of functional motor skills, the main symptom in AD is the dementia itself. Upon autopsy a loss of cortex neurons is found especially in the temporo-basal (hippocampus) and temporoparietal region. The microscopic examination reveals necrosis of cells and deposition of senile plaques and Alzheimer fibrilles. An Amyloid angiopathy is frequently associated. AD can only be diagnosed by the presence of Amyloid plaques, NFT, synaptic and neuronal loss, and atrophy of the brain in certain cerebral regions by autopsy. Cerebral computed axial tomography (CAT) scan can show brain atrophy, but present studies show no clear correlation with clinical findings. A likely diagnosis can be made by the evaluation of cognitive tests like the Mini-Mental State Examination (MMSE) (40), in the living patient and by excluding other diseases such as stroke or hypothyroidism.

VaD is the second most common form of dementia and is mainly caused by subcortical lacunary infarcts on the basis of cerebral microangiopathy (subcortical atherosclerotic encephalopathy) or less commonly caused by several cortical-subcortical infarcts on the base of macroangiopathy. These two forms are often combined. The localization and volume of the lesions are significant for the extent and progression of the demential syndrome. The entity of VaD is no longer understood as one distinct disorder but rather as a heterogeneous syndrome in which the underlying cause is some form of cerebrovascular disease like sequelae of recurrent strokes "multi-infarct dementia" and its ultimate manifestation is dementia. The three most important pathophysiological mechanisms are: large artery infarctions, usually cortical, sometimes also or exclusively subcortical in location; small artery infarctions or

lacunes, exclusively subcortical, in the distribution of small penetrating arteries, affecting the basal ganglia, caudate, thalamus, and internal capsule as well as the cerebellum and brain stem; and finally chronic subcortical ischemia occurring in the distribution of small arteries in the periventricular white matter. Neuroimaging should be performed in patients with suspected VaD, either cerebral CAT scan or the more accurate magnetic resonance imaging (MRI) scan. MRI will show cortical and subcortical infarctions as well as the presence of subcortical ischemic changes or leukoaraiosis. Diagnosis by radiographic finding is limited due to these white matter lesions (WML) being non-specific: it is impossible to differentiate between post-stroke patients with or without dementia.

1.3 Risk Factors for Dementia

The development of dementia is multifactorial, influenced both by genetic and environmental risk factors. Genetic factors seem to be involved in some cases as in mutations of the amyloid precursor protein (APP) and play a role mostly when referring to early-onset disease. Another strong genetic risk factor for AD is the presence of the epsilon 4 (e4) allele of the apolipoprotein E (apoE) gene (6). The vast majority of AD cases is late-onset and is probably influenced mainly by environmental factors. Besides age, around 20 non-genetic risk factors have been suggested for dementia. Unfortunately very few of them have been confirmed by randomized intervention trials. Elevated plasma tHcy and low-normal concentrations of B-vitamins (folate, vitamin B_{12}, and vitamin B_6) have been postulated as risk factors for two most common causes of dementia Alzheimer's disease (AD) and vascular dementia (VaD). Some of the environmental risk factors that are determined by lifestyle such as smoking increase not only the risk for dementia but increase plasma tHcy causing HHCY in turn a risk factor in itself for dementia. Intensified risk factor evaluation is important because other risk factors may be magnified in subjects with elevated plasma tHcy. The interpretation is that, even if raised total homocysteine turns out itself not causally related to dementia, it is a strong prognostic marker and subjects with HHCY should be intensely treated for conventional risk factors like hypertension.

Table 1 shows the most important environmental, lifestyle factors and other diseases that modulate the risk for dementia.

Table 1. Environmental, lifestyle factors, and other diseases can increase the risk for dementia.

Risk factor	Key references cited are prospective studies
Low blood pressure	Qiu, 2005 (142)
Hypertension	Bunce, 2005 (14)
Diabetes	Biessels, 2006 (8)
Cholesterol	Shobab, 2005 (166)
Obesity	Whitmer, 2005 (203)
Low intake of antioxidants	Engelhart 2005 (35)
Lack of fish in diet	Morris, 2003 (122); Huang, 2005 (78)
Low physical activity (midlife)	Yoshitake, 1995 (208); Fratiglioni, 2004 (43); Rovio, 2005 (154); Larson, 2006 (101)
Myocardial Infarction	Luchsinger, 2005 (108)
Atherosclerosis	Honig, 2005 (77)
Lack of alcohol consumption	Mukamal, 2003 (128), Luchsinger, 2004 (109)
Smoking	Piyathilake, 2003 (141)
Low testosterone in men	Henderson, 2004 (66); Hogervorst, 2004 (76)
Hormone replacement therapy in women over 65	Craig, 2005 (25)
Lack of NSAID use	Szekely, 2004 (180)
Increased blood markers of inflammation	Engelhart, 2004 (34)
Dietary copper intake	Morris, 2006 (121)
Lack of flavanoid-rich substances (tea, wine, chocolate)	Nurk, 2009 (131)
Lack of social or mental activities	Karp, 2004 (90)
Low education status	Karp, 2004 (90)
Depression	Green, 2003 (56)
HHCY and B-vitamin deficiency	See table 2 and 3

1.4 Clinical Studies Validate Homocysteine's Position as a Prognostic Marker for Cognitive Decline and Dementia

HCY is a sulfur-containing and non-protein forming amino acid and occurs naturally in all humans. HCY acts as a cytotoxin and can be recycled into methionine or converted into cysteine with the aid of B-vitamins. Plasma tHcy is a nutritional metabolic marker. Mild to moderate HHCY (tHcy 12-30 µmol/l) is a marker for deficiencies in B-vitamins (folate, B_{12}, B_6).

Neurodegenerative diseases include a wide group of disorders of various etiologies and clinical features. They all have in common structural and functional protein modifications, resulting in deterioration of certain nerve cells or neurons. Dementia, AD, and stroke are all examples of neurodegenerative diseases associated with HHCY.

A causal link between HHCY and CNS disorders was first described in patients with cystathionine beta synthase (CBS) deficiency. These patients suffer from mental retardation, cerebral atrophy, seizures, and cognitive dysfunction in addition to severely elevated plasma concentrations of tHcy (>70µmol/l) (57;126). In a systematic review of nine case-control studies higher concentrations of tHcy and lower concentrations of folate and vitamin B_{12} in AD patients (193) were verified. VaD is caused mainly by micro- and macrovascular lesions reflected by the presence of silent brain infarction and extensive white matter hyperintensity. The Northern Manhattan Study reported a concentration of HHCY >15µmol/l to be a strong independent risk factor for VaD and cerebral ischemia. (205). The Rotterdam Scan Study, in which 1077 healthy elderlies were examined, showed WML to be significantly associated with age and hypertension as well as with elevated tHcy (194). Another study showed HHCY in patients with VaD increased the danger for subcortical vascular encephalopathy even more than established risk factors like smoking, hyperlipidemia, and high blood pressure (206).

The inverse relation between HHCY as well as in some cases folate deficiency and cognitive impairment and/or dementia have been shown in the following prospective studies and listed in Table 2 and 3. One of the first prospective studies was on patients with a clinical diagnosis of AD in a study by Clarke (23) where it was found that the baseline level of total homocysteine was related to the rate of disease progression. Several community-based prospective studies have since been completed in initially dementia-free subjects. When discussing epidemiological studies, it is important to consider which factors should be included as covariates. All studies should include gender, age and, in relation to the risk of dementia, additional risk factors such as the genetics (like presence of apoE4), years of

education, race, and possibly smoking, depression, and socioeconomic status should be considered. While some of these, notably age, gender, and smoking are also related to total homocysteine concentrations (149), there will be a risk of over-adjusting if very strong determinants of total homocysteine, such as intake or blood concentrations of folate and vitamin B_{12}, are included. Still, in the following prospective studies and listed in Table 2 one or more B-vitamins were included in the analysis, and the prospective association between tHcy and cognitive deficit remained significant and were able to validate this relationship.

In a study with 32 healthy elderly individuals, age >65 years, by McCaddon et al. (112) concentrations of tHcy were able to predict cognitive decline showing a maximal effect on spatial copying skills tested by MMSE and Alzheimer's Disease Assessment Scale-cognitive subscale (Adas-Cog). Using Data from the Framingham study including 1092 dementia free subjects with a mean age of 76 years Seshadri et al. (165) demonstrated that after an 8 year follow-up an elevation of 5 µmol/l in plasma tHcy concentration increased the risk for AD by 40 percent (Figure 2).

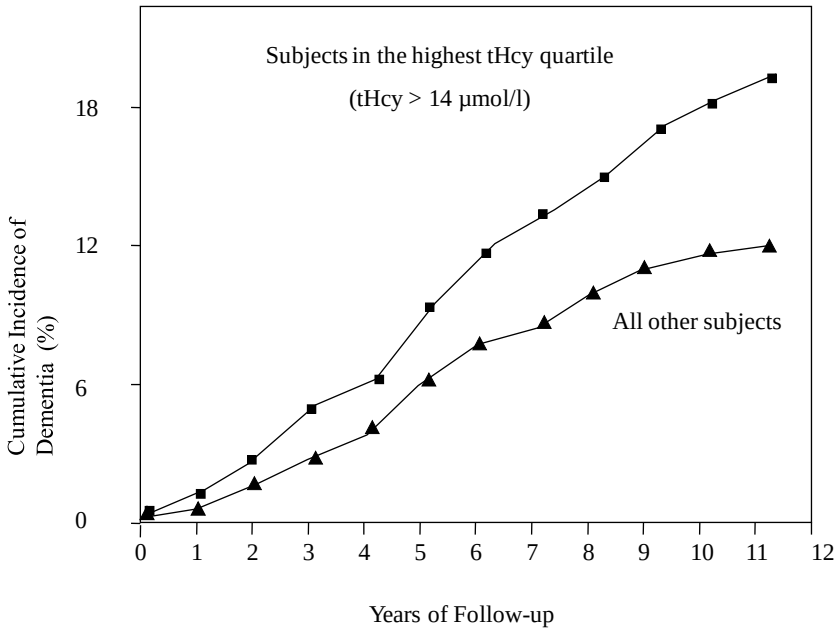


Figure 2
Data from the Framingham Study on the increase in the incidence of dementia in elderly subjects with tHcy > 14 µmol/l. The Framingham study is a follow-up study for 12 years that included 1092 subjects aged (68-97 years) without dementia at baseline.

Again, an inverse correlation between tHcy and attention and memory was demonstrated by Teunissen in 144 subjects over a follow-up of 6 years after adjusting for several factors, including folate (181). Even over a shorter follow-up of only 2.3 years Garcia et al. confirmed that tHcy was inversely correlated with executive function in 180 subjects. Increase in total homocysteine over time was associated with decline in test scores for executive function and learning. Analysis was adjusted for multiple factors, including folate and vitamin B_{12} (46). In a further study by Nurk et al., a higher risk for memory deficits after 6 years was demonstrated in 2189 subjects, aged 65-67, when baseline concentration of tHcy was in the highest quintile and folate and vitamin B_{12} in the lowest quintile. Subjects with memory deficits at baseline showed higher tHcy and lower folate status (132). Another study with 819 subjects by Ravaglia et al. (146) calculated a hazard ratio for AD of 1.54 for each SD in tHcy showing a strongly dose-related effect. The most recent study by Zylberstein et al. (212) examined mid-life HHCY and evaluated 1368 women 35 yrs later and is until now the only study with a follow-up longer than 8 yrs. This study showed that the highest tHcy tertile was related to a hazard ratio of 1.7 (95% CI 1.1-2.6) for developing any dementia, 2.1 (95% CI 1.2-3.7, n=100) for AD and 2.4 (95% CI 1.3-4.7, n=68) for AD without cerebrovascular disease and confirmed midlife HHCY as a risk factor.

Table 2. Prospective studies linking tHcy to cognitive decline or dementia

Subjects	Age	Follow up	Tests	Results	Study
32 healthy subjects (22 female)	> 65 yrs	5 yrs	MMSE, ADAS-Cog	tHcy predicted cognitive decline in healthy elderly, maximal effect on spatial copying skills	McCaddon, 2001 (112)
1092 non-demented subjects (667 female)	Mean 76 yrs	8 yrs	Cases with dementia and Alzheimer disease were identified after 8 years	Relative risk 1.9 (1.3 to 2.8) for dementia and 1.9 (1.2 to 3.0) for AD. Increase in tHcy of 5 µmol/l increased the multivariable-adjusted risk of AD by 40 percent	Seshadri, 2002 (165)
144	30-80yrs	6 yrs	Tests of cognitive speed, attention, verbal memory	Inverse correlation between tHcy and attention and memory. Adjusted for several factors, including folate	Teunissen, 2003 (181)

180	>65 yrs	2.3 yrs	MMSE, verbal learning, dementia rating, executive function	tHcy inversely correlated with executive function. Increase in tHcy over time associated with decline in test scores for executive function and learning. Multiple adjustments, including folate and vitamin B_{12}	Garcia, 2004 (46)
816	74 yrs	3.8 yrs	Incident dementia, including Alzheimer's disease	Hazard ratio for AD of 1.54 for each SD in tHcy. Strongly dose-related. Multiple adjustments, including cardiovascular disease, folate and vitamin B_{12}	Ravaglia, 2005 (146)
2189 subjects	65-67 yrs	6 yrs	Memory performance (Kendrick Object Learning Test)	Mean tHcy was higher and folate was lower in subjects with memory deficit at baseline. Baseline tHcy in the highest quintile, folate in the lowest quintile and vitamin B_{12} in the lowest quintile predicted a higher risk for memory deficit after 6 yrs.	Nurk, 2005 (132)
1368 women	38-60 yrs	35 years	MMSE, ADAS-Cog, and Clinical Dementia Rating	The highest tHcy tertile was related to a hazard ratio of 1.7 (95% CI 1.1-2.6) for developing any dementia, 2.1 (95% CI 1.2-3.7, n=100) for AD and 2.4 (95% CI 1.3-4.7, n=68) for AD without cerebrovascular disease. HHCY in midlife is an independent risk factor for the development of late-life Alzheimer dementia in women	Zylberstein, 2009 (212)

The link between B-vitamin deficiency and cognitive dysfunction and/or dementia is more controversial. The following recent prospective studies on B-vitamins and subsequent impairment or dementia have shown striking findings and are listed in Table 3.

Wang et al. (199) found that in subjects with a high baseline MMSE (≥ 26) and serum folate concentrations <12 nmol/L or serum vitamin B_{12} concentrations < 250 pmol/L, the relative risk of incident AD was increased versus those with normal concentrations of both vitamins. A study by Kado et al. demonstrated that after 7 years 370 elderly subjects aged 70-79 with folate in the bottom quartile (<3.15 ng/ml) had a 1.6-fold higher risk of being in the worst quartile for cognitive performance (86). The Veterans Affairs Normative Vitamin B Aging Study (185) found that the cognitive test that best related to B vitamin status and total homocysteine involved constructional praxis (a spatial copying task). The changes in scores

on this test over 3 years were strongly related to folate status even when tHcy and the other plasma B-vitamin concentrations were included in the regression. Corrada et al. (24) found that a higher intake than the recommended daily allowance of folate was associated with a significantly reduced risk of AD in 579 subjects when followed over 9.3 years, but no effect of vitamin B12 on risk reduction. In the prospective Conselice study with 816 subjects in an elderly community in Italy by Ravaglia et al. in 2005 (146) a strong dose-related inverse relationship was found between incident dementia and serum folate. Low serum folate (<11.8 nmol/l) at baseline was associated with 2-fold risk of AD after adjustment for tHcy. No association was found with vitamin B_{12} concentrations. Nurk et al. tested episodic memory scores in 2173 subjects over a follow-up of 6 years (132) and discovered that baseline serum folate was dose-related to test score; a decrease in folate over 6 yr associated with lower test score. No prospective association with vitamin B_{12} was found. Another study in 2006 by Ravaglia et al. (145) including 165 subjects with MCI a hazard ratio for conversion to AD was 3.11 for those with low folate (<10.4 nmol/l) was found.

When trying to summarize the results on B-vitamins we encounter inconsistent findings. On the one hand, several vitamin B_{12} prospective studies were able to show an inverse relationship with cognitive decline or the incidence of dementia like the study by Wang et al. (199), on the other hand there are a number of studies finding no association with vitamin B_{12} when adjusting for other B-vitamins and tHcy (24;86;185) We must keep in mind though that this may be a result of different populations studied, different tests, assays, and endpoints. A further factor may be that concentrations of serum vitamin B_{12} don't automatically reveal the amount of vitamin B_{12} available for cellular function which is carried by transport protein transcobolamin-II. Better functional markers might be tHcy, although not specific for vitamin B_{12} status, methylmalonic acid and/or holotranscobolamin-II (67;105;157).

We can conclude that in several very different population studies, low-normal blood folate concentrations were associated with a risk of developing dementia (24;86;132;145;146;185;199) However some studies that need to be considered were not able to confirm these results, on the contrary one reported that higher blood concentrations of folate were associated with a greater rate of global cognitive decline, even though in the same cohort a strong positive cross-sectional relationship was found between folate and cognition (120). These conflicting findings need to be examined further and the possibly harmful effect of high folate supplementation (183) must be taken into consideration especially in the context of low vitamin B_{12} status (123).

Table 3. Prospective studies linking B-vitamins (low folate status) and subsequent cognitive impairment or dementia

Subjects	Age	Follow up	Tests	Results	Study
370	>75 yrs	3 yrs	Incident dementia and AD	Low folate (< 10 nM) alone, or low folate and low vitamin B_{12} (< 150 pmol/l) were associated with incident AD, adjusted for age, gender, education. Only significant in subjects with baseline MMSE > 26	Wang, 2001 (199)
370	74 yrs	7 yrs	Multiple cognitive domains: scores summed	Baseline folate and vitamin B_6 inversely related to cognitive decline when adjusted for age and gender, but only folate significant when other covariates including tHcy were added	Kado, 2005 (86)
321 men		3 yrs	MMSE, working memory, verbal memory, verbal fluency, constructional praxis	Baseline plasma concentrations and dietary intake of folate, vitamin B_6, and vitamin B_{12} related to praxis; baseline dietary folate related to verbal fluency. Change in praxis scores over 3 yrs was dose-related to folate and vitamin B_6 concentrations and intake. Multiple adjustments, including education. Folate remained associated when all 3 vitamins and tHcy were included.	Tucker, 2005 (185)
579	70 yrs	9,3 yrs	Incidence of possible or probable AD	Higher intake (> RDA) of folate (RR 0.4) or vitamin B_6 (RR 0.4) associated with reduced risk of AD. No effect of vitamin B_{12}. Multiple adjustments. When folate and vitamin B_6 included together, only folate remained significant	Corrada, 2005 (24)
816	74 yrs	3.8 yrs	Incident dementia, including Alzheimer's disease	Low serum folate (<11.8 nmol/l) at baseline associated with 2-fold risk of AD after adjustment for tHcy and many covariates, including cardiovascular disease, ApoE4. Dose-related inverse relationship of folate with cumulative incidence. No association with vitamin B_{12} concentrations	Ravaglia, 2005 (146)
2,173	65–67 yrs	6 yrs	Episodic Memory scores	Baseline serum folate dose-related to test score; increase (decrease) in folate over 6 yr associated with higher (lower) test score. No prospective association with vitamin B_{12}. Multiple adjustments.	Nurk, 2005 (132)
165 with MCI		2,8 yrs	Conversion to AD	Hazard ratio for conversion to AD was 3.11 for those with low folate (<10.4 nmol/l). Multiple adjustments.	Ravaglia, 2006 (145)

The following examples of retrospective studies further verify the link between HHCY and cognitive decline and are listed below in Table 4 for further review.

Elevated plasma tHcy (>13 µmol/l) in 200 healthy postmenopausal women, aged 56-67, was shown to be linked to poor performance of combined verbal and working memory independent of age and hormone therapy (21). It has also been demonstrated in 209 subjects with a mean age of 76 that both tHcy and methylmalonic acid (MMA) were independently and inversely correlated with movement and cognitive performance (102). In stroke free subjects MMSE scores were negatively correlated to tHcy in subjects >65 years, and a concentration of tHcy >15 µmol/l was associated with 3.3 less points on MMSE test (205). Concentration of tHcy correlated negatively to cognitive performance in 812 dementia and stroke free subjects after adjusting for possible confounding factors (32). In the Framingham Offspring Study with 2096 dementia and stroke free subjects aged >40 years, concentrations of tHcy were inversely related to 9 of 12 cognitive measures tested and was significant in subject 60 years or older (33). These results were confirmed by other studies on elderly people (117;147;163). Serum concentrations of markers of vitamin B_{12} deficiency were also related to certain cognitive functions (72;114). Also in healthy individuals, B-vitamin plasma concentrations seemed to be inversely related to deficits in neuro-cognitive tests (54).

Table 4. Examples for retrospective studies linking tHcy to cognitive decline or dementia

Subjects	Age	tHcy, vitamins	Tests	Results	Study
200 healthy women	56-67 yrs	Mean tHcy= 10 µmol/l	California Verbal Learning Test-II, Ten unrelated words, WAIS Letter-Number Sequencing	tHcy > 13 µmol/l was associated with poor performance of combined verbal and working memory independent of age, and hormone therapy	Clark (21)
209 subjects	76 yrs	Means tHcy= 17.2 µmol/l MMA= 220 nmol/l Folate= 16.0 nmol/l B_{12}= 325 pmol/l	Postural-Locomotor-Manual test A battery of cognitive tests	tHcy and MMA correlated independently with movement and cognitive performance	Lewerin (102)
2871 subjects free of stroke	40 yrs	Means tHcy= 10.2 µmol/l MMA= 214 nmol/l 17% had MMA > 271 nmol/l	MMSE scores	MMSE scores were negatively related to tHcy in subjects > 65 yrs. 3.3 lower MMSE points in subjects with tHcy > 15 µM	Wright (205)
812 subjects free of dementia and stroke		Means tHcy= 10.0 µmol/l Folate= 38.5 nmol/l B_{12}= 389 pmol/l	Visual spatial organization, working memory, scanning tracking, abstract reasoning	tHcy was negatively related, and vitamin B_6 was positively related to cognitive performance even after adjusting for possible confounding factors	Elias (32)
2096 subjects free of dementia and stroke	> 40 yrs	Mean tHcy= 10.32 µmol/l in 705 subjects aged 60-82 years	Multiple measures of cognitive function	tHcy was inversely related to 9 of 12 cognitive measures tested. This was significant in subjects who were 60 years or older	Elias (33)
1789 elderly,	≥ 60 yrs	Median tHcy= 9.8 µmol/l	The modified MMSE test, and 6 other cognitive tests	A modest, significant correlation was observed between tHcy and several measures of cognitive function	Miller (117)
650 healthy, free of dementia	≥ 65 yrs	Mean tHcy was higher in subjects with lower MMSE scores (11.9 vs. 14.5 µmol/l for scores > 28 vs. < 26	MMSE test	tHcy is an in depended and graded risk factor for cognitive decline with age	Ravaglia (147)

When interpreting the studies described above, no matter how statistically accurate they have been conducted, they are observational and not able to differentiate causality from reverse causality. Elevated concentration of tHcy can be lowered by supplementing B-vitamins. To show causality between B-vitamins, or tHcy and dementia it is necessary to carry out randomized clinical trials with doses of B vitamins that lower tHcy in the elderly. Several

studies have tested the hypothesis that lowering tHcy might improve cognitive function and/or decrease the rate of cerebral atrophy. Some studies were able to confirm this (13;30;132;195) others were not (103). One study on elderly adults with vascular disease supplemented with 2.5 mg folic acid and 0.5 mg vitamin B_{12} for the duration of a year showed no improvement of cognitive function even though tHcy was significantly decreased (178). In a further study including elderly patients with vitamin B_{12} deficiency who were treated with folic acid and vitamin B_{12} during a time frame of 24 weeks failed to show positive results, as well (36).
Taken together, the associations between HHCY and cognitive dysfunction have been validated. HHCY position as a prognostic marker for cognitive decline and dementia has been demonstrated. Elevated concentrations of tHcy can be lowered by supplementing B-vitamins. Yet, their effect on cognitive function in vitamin intervention studies remains inconclusive. Because dementia is developed over several decades, vitamin treatment for a short duration might not be sufficient and will have to be initiated early in the disease process and last for several years in order to show an effect on cognitive decline in order to prove causality.

1.5 Pathobiochemical Mechanisms in Dementia

Upon autopsy and radiographic examinations the major dementia syndromes each manifest differently. A multifactorial pathogenesis seems likely when considering the number of risk factors associated with the development of dementia. Deposition of Amyloid beta (Aβ) in the brain and phosphorylation of tau leading to accumulation of NFT in neuronal cell bodies are associated with AD. Influencing functional proteins that enhance these processes seem to be an important link between risk factors and neurodegeneration.

Amyloid beta hypothesis

Aβ is a peptide of 39–43 amino acids that appears to be the main constituent of amyloid plaques in the brains of AD patients. As part of the normal aging process Aβ is deposited extensively in the brain. One of the characteristics in patients with AD is the widespread deposition of Aβ within senile plaques and in cerebral and meningeal blood vessels (52;111). The endoplasmatic reticulum protein (Herp) has recently received attention because of its capacity to enhance the γ-secretase activity and thereby the Aβ 1-40 accumulation in the brain (156). There is a relation to cognitive decline and low concentrations of Aβ in cerebrospinal fluid (CSF) samples from AD patients and VaD (41).

Tau hypothesis

Tau is an important protein in the human brain implicated in memory decline and dementia. It is an intracellular microtubule-associated protein, stabilizing and promoting the polymerization of microtubules (202). Microtubules play an important role in the protection of cellular architecture of neurons. Normally tau is found in the axons of neurons (9). In AD tau becomes hyperphosphorylated and aggregates into PHF which accumulate in neuronal cell bodies as NFT. These correlate with cognitive deficits, neurodegenerative disorder and dementia. NFT (1;96) cause neuron degeneration and neuron loss in the hippocampus, substantia innominata, locus coeruleus, and in the temporal, parietal, and frontal cortex. Making tau detached and accessible for proteolysis is probably key in neuroprotection. The enzyme protein phosphatase 2A (PP2A) can dephosphorylate P-tau in PHF (201). Activation of kinases with the capacities of phosphorylating tau (GSK3beta, phosphatidylinositol 3-kinase (PI3K), MAP kinase) may be stimulated by increased Aβ (37). Increased P-tau may be related to lower phosphatase activity or to an increased kinase activity. Some studies were able to show a decreased expression or activity of PP2A in cerebral tissue of patients with AD (196) (169). In fibroblasts of patients with AD, the expression of PP2A protein was reduced; simultaneously mRNA for PP2A was increased. This suggests the defect or failure lies not in the pretranslational pathway but possibly in posttranslational modifications or in the protein stability itself (210). A therapeutic potential for AD may involve influencing these phosphatases or kinases.

Mixed dementia (AD with cerebrovascular disease) refers to the concurrence of AD and VaD and is increasingly recognized as a condition requiring independent consideration. It is often difficult to distinguish AD from VaD, let alone to determine which is etiologically most important when both are present. The high prevalence of both AD and VaD is such that their concurrence is common. Whether these vascular lesions are coincidental or causal in the pathogenic processes of AD remains to be defined. In autopsy series, vascular pathology was found in 34 to 50 percent of those with pathologic AD. About one third of the patients diagnosed with VaD had AD pathology at autopsy (88). In addition to their frequent coincidence, there is increasing evidence that AD and VaD may share etiologic or pathogenic features and/or influence each other's course.

2 Homocysteine

2.1 Overview

HCY is a cytotoxin which is why this product should be eliminated from the cell or converted to less harmful substances. A relationship between HHCY and pathologic alteration in the human organism was first described in 1962. Carson and Neil reported elevated HCY concentrations in the urine of mentally retarded children in Belfast (17). This disease was named homocystinuria and was discovered by two other groups simultaneously in Philadelphia and Milwaukee (49;173). Pathological examination showed distinct vascular lesions, thrombosis and fatty degeneration of the liver (51). Biochemical analysis showed that a deficiency of vitamin B_6-dependant enzyme CBS forms the basis of homocystinuria (125). Today over 100 different mutations have been identified in the gene encoding this enzyme (97). Homocystinuria is a rare genetic disease associated with a severe HHCY (plasma tHcy 100-250 µmol/l). Mild to moderate HHCY (plasma tHcy 12-30 µmol/l) is common in elderly people (69;84;93;176). The cause is often a folate, vitamin B_{12}, and/or vitamin B_6 deficiency (22;45;55;69;93;176), as well as a decline of renal function with increased age (70;130;190). Moderate HHCY in the adult is a risk factor for atherosclerotic (68;176;198), thrombotic (92), pregnancy complications (115), and neurodegenerative diseases (47;143).

Concentrations of tHcy between 10-12 µmol/l are considered normal. HHCY is either mild when tHcy is between 16-30 µmol/l, moderate when tHcy is between 31-100 µmol/l, or severe when tHcy > 100 µmol/l (81). Chemical structures of homocysteine, methionine, and cystathionine are shown in Figure 3.

Homocysteine	$HS\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$
Methionine	$CH_3\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$
Cystathionine	$HOOC\text{-}CH(NH_2)CH\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$

Figure 3
Chemical structures of homocysteine, methionine, and cystathionine

2.2 Biochemical Aspects of Homocysteine

HCY is a sulfur-containing, non-protein forming amino acid and a product in the metabolism of the essential amino acid methionine (118). Methionine enters the body by alimentary animal proteins. HCY is a small molecule (molecular weight: 135 Dalton) and not well soluble in H_2O. The sulfur containing amino acid methionine enters the human organism exclusively via alimentary proteins. It functions as a universal methyl group donor in the human body when activated to S-adenosyl methionine (SAM) by the methionine adenosyltransferase (Figure 4). These methyl groups are transferred to a wide spectrum of acceptors, such as melatonin, DNA, RNA, protein, epinephrine, myelin, creatine, and phospholipids. When one of these acceptors is methylated, S-adenosyl homocysteine (SAH) is formed out of SAM by S-adenosylmethionine-dependent methyltransferase. SAH in turn can then be hydrolyzed to form HCY in a reversible reaction mediated by SAH-hydrolase.

Because HCY is toxic, a low cellular HCY concentration is necessary. The cell has three possibilities to achieve this: elimination from the cell, remethylation to methionine, or the irreversible degradation via the transsulfuration pathway (Figure 4). HCY can be remethylated to form methionine by accepting the methyl group of 5-methyltetrahydrofolate (5-MTHF) by the vitamin B_{12}-dependant enzyme methionine synthase (MS). Alternatively this can be achieved by receiving the methyl group from betaine by the betaine homocysteine methyltransferase (BHMT). However this reaction is primarily limited to the liver and consequently plays a secondary role as determinant of tHcy concentrations.

If there is an excess of methionine, like after a protein rich meal, HCY flows preferably via the transsulfuration pathway. HCY and serine are condensed to form cystathionine (Cys) by CBS and then to cysteine by γ-cystathionase (CS) and then to glutathione, both enzymes being irreversible and requiring vitamin B_6 as a cofactor. Folate, vitamin B_{12}, and vitamin B_6 are essential in maintaining a constantly low tHcy concentration. Because these B-vitamins are important cofactors for HCY catabolism, an elevated concentration of tHcy can indicate B-vitamin deficiency (39;167).

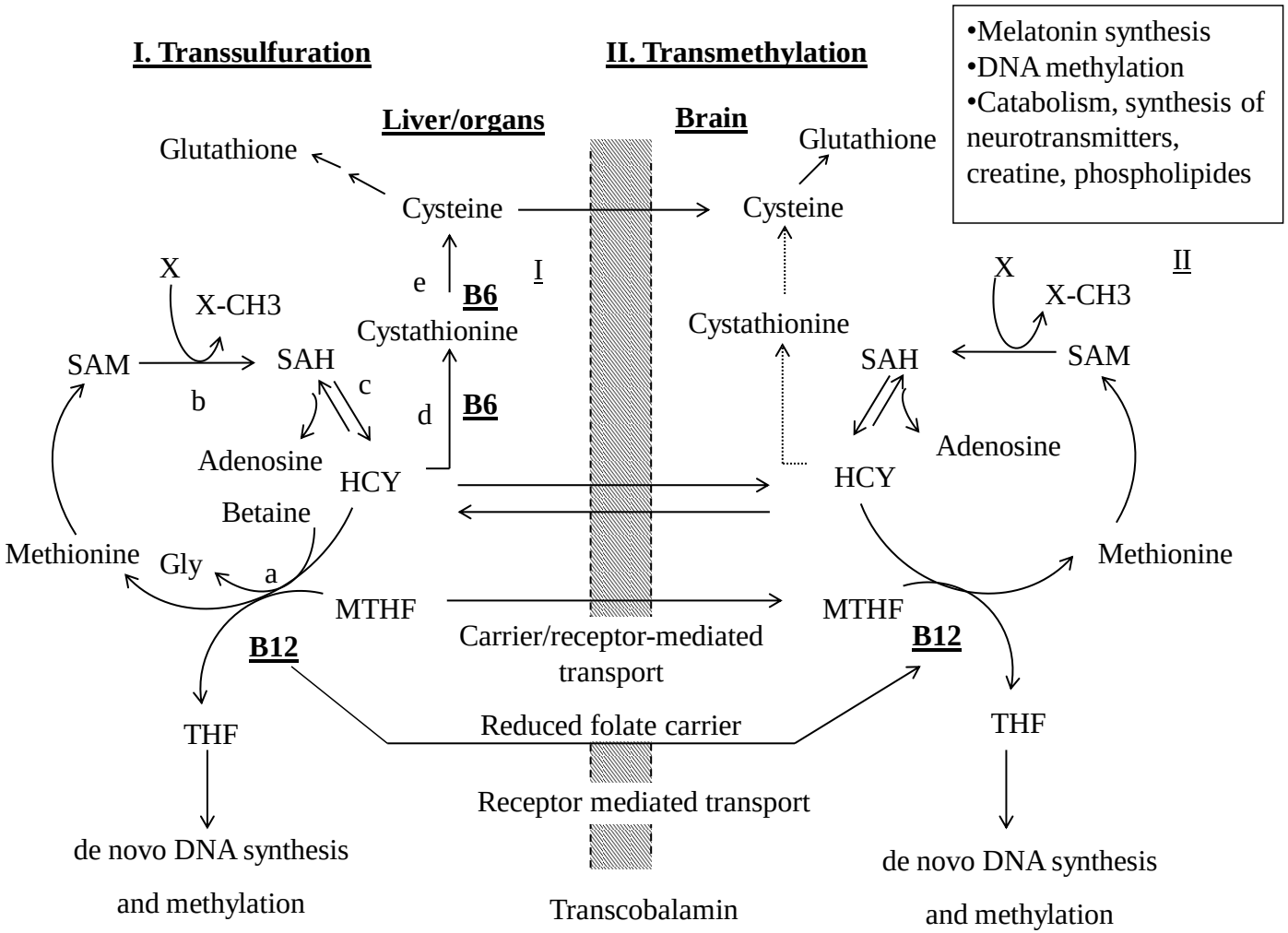


Figure 4
Homocysteine metabolism, pathways, and enzymes: a: methionine synthase (MS) and betaine homocysteine methyltransferase (BHMT), b: SAM-dependent methyltransferase, c: SAH-hydrolase, d: cystathionine beta synthase (CBS), e: γ-cystathionase

Besides being important cofactors for enzymes in HCY metabolism, these vitamins have further independent characteristics (7;20;152;197). Folate deficiency not only induces HHCY, but is also responsible for hypomethylation, DNA damage, and disturbed cellular proliferation (94;175). Vitamin B_{12} is a cofactor for MS and is hereby linked with folate utilization. Therefore an isolated vitamin B_{12} deficiency, in spite of a sufficient folate supply, can reduce HCY-remethylation and hereby cause hypomethylation. Thereby HCY is increased, and folate becomes functionally deficient, despite its normal concentration in plasma. Deficiencies in folate, vitamin B_{12}, and vitamin B_6 limit enzyme activity, thereby blocking degradation of HCY and consecutively cause an intracellular increase of HCY concentration (29;83).

HCY can be actively transported out of the cell and into the plasma where less than 2% can be found in its free and reduced form. Only 0.29% of HCY are found in the form of HCY-Thiolacton which is significant because of its high toxicity. HCY is mainly bound to proteins in two different variations. The first binds via SH-group and the second via amino group (148). The N-protein bound HCY is associated with functional damage of proteins and cannot be detected by current assays (82). The main component though is made up of disulfide complexes where HCY is bound by SH-groups of plasma proteins, like albumin. Mixed disulfides are formed also with cysteine, as well as symmetric disulfides with HCY producing homocystine (187). Free reduced HCY is not very stable and not soluble at neutral pH levels (60). Therefore the concentration of free HCY is extremely low (only 1-2% of concentration of tHcy in the physiological range of < 12µmol/l) (150). When HCY is reduced, it can be dissolved out of its disulfide form. This is usually used in many in vitro assays designed for quantification of tHcy concentrations in plasma. Table 5 shows the distribution of main forms of HCY in vivo in percent.

Table 5. Different components of plasma HCY

Reduced form:	
Free Homocysteine (HCY)	1-2%
Oxidized form:	
Homo-Disulfide; Homocystine	5-10%
Mixed Disulfides:	
Protein-bound Homocysteine	80-90%
Cysteine-Homocysteine	5-10%

Approximately 70% of HCY is metabolized in the epithelia of the kidneys. Only small amounts of HCY are generally detected in the urine of healthy individuals. When kidney function is reduced, lower levels of remethylation and transsulfuration are found, thereby leading to accumulation of HCY and consecutive HHCY (190-192). This may also explain why especially older patients with reduction of renal function often have HHCY.

2.3 Homocysteine Metabolism in the Central Nervous System

The HCY metabolism described above gives a general overview but probably does not occur in full except in a few tissues like the liver or kidney (39). In the brain the metabolism is limited (16). BHMT has not been detected in brain tissue. In the liver about 50% of cysteine for glutationine synthesis is produced by the transsulfuration pathway. However this is not the case in the brain. CBS expression and activity is observed in the brain, but limited brain cystathionase activity is detected in only a few studies with wide regional span leading to the conclusion that expression and function of cystathionase in the brain seems questionable.

The metabolism of HCY is an important source of SAM (162). This methyl donor is important for the synthesis and catabolism of neurotransmitters, phospholipids (phosphatidylcholine being the methylated product of phosphatidylethanolamine), DNA methylation, and activation of several essential enzymes in the brain. The role of SAM in the brain corresponds with SAM reducing apoptosis by 50% caused by HCY in cortical neuronal cells (75). SAM supplementation after ischemia was able to improve the blood brain barrier and neuronal survival (144).

A specific cellular receptor seems to be responsible for the transport of HCY in brain cells (58). Transfer between different brain regions and between brain and blood has not yet been well studied. The integrity of the blood brain barrier seems to play an important role in the distribution of HCY. Severe HHCY in patients with CBS deficiency is associated with a 10-fold increase in CSF tHcy (179). Even though HCY remethylation doesn't occur with the aid of betaine in the brain, lowering the concentration of plasma tHcy with betaine causes a significant decrease of CSF tHcy (179) suggesting HCY can be transported across the blood brain barrier in both directions. Several neurological diseases are associated with increased brain and CSF concentrations of tHcy or increased concentrations of its precursor SAH (80;151). In general, plasma tHcy concentrations are 20- to 100-fold higher than CSF concentrations and plasma tHcy is positively correlated to brain or CSF concentrations (135). Therefore an increased plasma tHcy concentration may reflect an increased brain or CSF tHcy

concentration. HHCY leads to increased SAH, a potent competitive inhibitor of many SAM-dependent methyltransferases in the human brain. Hypomethylation in the brain is related to numerous functional and biochemical defects. The importance of methylation for the CNS has been demonstrated. Accordingly, supplementing SAM after ischemia improves the blood brain barrier and neuronal survival and protects against disturbances in brain phospholipids (87;184). Other limiting factors for HCY metabolism in the brain are that folate must be reduced before entering the blood-brain barrier, and the majority of brain cysteine and vitamin B_{12} must be imported from the blood.

3 Factors leading to Hyperhomocysteinemia

3.1 Genetics in Hyperhomocysteinemia

It is important to differentiate between the autosomal recessive form of severe HHCY and the mild HHCY which is induced by heterozygote form, and minor defects of the CBS, 5,10-methylenetetrahydrofolate reductase (MTHFR), or MS. The severe form of HHCY develops because of an autosomal recessive genetic deficiency in CBS or MTHFR. The incidence for homozygosity in CBS deficiency is documented as 1:200.000-1:344.000. Heterozygote's plasma tHcy is increased by 2- to 4-fold in comparison with healthy individuals. While a deficiency of CBS can be treated by high dose vitamin B_6 in combination with folic acid, so far no therapeutic options exist for MTHFR deficiency, explaining why its prognosis is far worse (127). The MTHFR can also be affected by different point mutations, in which a partial activity of the enzyme is preserved. The most important factor for the development of mild HHCY is the thermolabile variant of MTHFR. It results from the point mutation (C677T) in the encoding region for the binding place of the MTHF in the MTHFR molecule. The amino acid alanine is replaced by valine. The consequence of this mutation is the reduced specific activity and catalytic function at 37°C of the TT-enzyme in comparison with the CC-genotype. Prevalence for homozygosity of this MTHFR gene mutation is between 5-18%, depending on population and race (12;119). A mild form of HHCY is only acquired when folate is deficient or low. When folate has an increased but still normal or elevated concentration, HHCY does not develop, because the high folate concentrations stabilize the TT enzyme (63).

3.2 Hyperhomocysteinemia and Vitamin Deficiency

Deficiencies in vitamins functioning as co-enzymes in HCY metabolism like folate, vitamin B_6, or vitamin B_{12} can induce HHCY. A substitution can normalize their plasma concentrations (11;89;174).

3.3 Diseases Associated with Hyperhomocysteinemia

Disease like renal failure, hypothyroidism, pernicious anemia and several malignant tumors are associated with elevated tHcy concentrations (62;104;153).

3.4 Influence of Medication and Lifestyle on Total Plasma Homocysteine

There are a large number of substances influencing tHcy. Some are direct antagonists of cofactors and enzyme activity; others have indirect effects i.e. by inducing malabsorption in the gastrointestinal tract or enzyme induction. Table 6 and Table 7 show the influence on plasma tHcy caused by different medications and lifestyle factors.

Table 6. Influence of medication on total plasma homocysteine

Medication	↑↓	Mechanism	Reference
Theophylline	↑	Vitamin B_6 antagonist	(186)
Nitrousoxide	↑	Inactivation of MS	(26)
Methotrexate	↑	Competitive inhibition of folate	(61)
Fibrate	↑	Peroxisome proliferator-activated receptor (PPAR)	(85)
Niacin	↑	Vitamin B_6 antagonist	(48)
L-DOPA	↑	Substrate for SAM-dependant methylation	(211)
Antiepileptics	↑	Decrease of folate and vitamin B_6	(4)
N-acetylcysteine	↓	Cleavage of disulfide bridges	(161)
Proton pump inhibitors	↑	Increase of stomach pH, causes vitamin B_{12} deficiency	(159)
Estrogen, progesterone	↓	Hormone function	(116)
Tamoxifen	↓	Partial agonist of estrogen receptor	(18)
Metformin	↑	Inhibition of intrinsic factor secretion	(15)

Table 7. Influence of lifestyle factors on plama homocysteine

Lifestyle	↑↓	Mechanism	Reference
Smoking	↑	Interference with cobalamin, pyridoxine, and folate; production of radicals	(15)
Alcoholism	↑	General malnutrition, block of methylation reaction	(160;182)
Coffee consumption	↑	Vitamin B_6 antagonism	(59)
Vegetarian Diet	↑	Vitamin B_{12} deficiency	(71)

4 Pathomechanisms linking Homocysteine to Neurodegeneration

There are several mechanisms by which HHCY can damage the brain. Elevated concentrations of plasma tHcy therefore either have a direct neurotoxic effect or cause damage by disturbing the methylation potential (SAM/SAH) of the brain.

HHCY can cause lowered SAM and elevated SAH, the potent competitive inhibitor for methyltransferases. Increased concentration of tHcy is associated with increased production of SAH via the reversible reaction mediated by SAH-hydrolase. The importance of the transmethylation pathway in the brain can be recognized from studying patients with SAH-hydrolase deficiency. This rare disorder was associated with a diffuse white matter atrophy, restricted myelination of some brain regions, abnormal spectroscopy of cerebral white matter, and developmental delay. The neurological symptoms and brain abnormalities in the described patients were probably attributed to the severe SAH elevation (>100-fold). In contrast, plasma tHcy was only slightly elevated (14.5-15.9 µmol/l) (5). Methyl groups are required for synthesis and catabolism of neurotransmitters as well as maintaining DNA methylation and hence are important in maintaining neurological health. A lowered ratio in SAM/SAH causes DNA damage and apoptosis demonstrating one important mechanism of neurotoxicity (98).

Experimental HHCY leads to increased concentrations of tHcy and SAH in rat brain (50). Neurological damage has been reported in CBS enzyme deficient mice (CBS -/+ or CBS -/-), where tHcy increased by approximately 2- to 50-fold in comparison to wild type mice, depending on the genotype and the type of diet. These animals show alterations in neuronal plasticity, suffered from severe retardation, and died early. Animals exposed to HCY accumulate this compound in the brain, suffer from restricted growth, neural or cognitive dysfunction, and impaired brain energy metabolism.

4.1 Homocysteine and Hypomethylation

Hypomethylation is one important mechanism explaining the toxic effect of elevated tHcy. Metabolic markers of methylation in blood like SAM and SAH reflect tissue methylation. (94;175). HCY increases neuronal death and DNA damage (98). PS1 gene expression is one important example that has been tested in relation to DNA methylation (44;158;164). Presenilins are a family of transmembrane proteins that function as a part of the γ-secretase protease complex. PS1 mediates the formation of Aβ from APP. When DNA is

hypomethylated, APP processing and hence Aβ production is accelerated by an up-regulation of PS1 gene. Interestingly, Aβ production can be reduced by silencing of the PS1 gene by exogenous SAM. These results suggest a silencing of the PS1 gene may be a target therapy for AD patients (158;164) and that the treatment of folate and vitamin B_{12} deficiency might protect against Aβ accumulation in dementia by supplying methyl groups.

Factors that lower phosphorylation or enhance dephosphorylation of certain proteins may offer an approach to prevent some forms of neurodegeneration like in AD. Under physiological conditions, tau phosphorylation is regulated by a dynamic equilibrium between the kinases and phosphatases acting on the molecule. An important biological reaction where hypomethylation may increase damage to the brain in AD is the formation and clearance of P-tau (Figure 5). The dephosphorylation of P-tau is accomplished by the enzyme protein phosphatase 2A (PP2A). PP2A is regulated by the enzyme protein phosphatase 2A methyltransferase (PPMT) which is SAM-dependent. PP2A is composed of three subunits A, B and C. The regulatory subunit B is involved in substrate recognition. Methylation of the subunit C at Leu-309 by means of a specific SAM-dependent methyltransferase (PPMT) facilitated assembly of the AC-dimer subunit and forming the PP2A holoenzyme. This means reduced methylation capacity can increase P-tau by decreasing dephosphorylation. Dephosphorylation is important because it enables the detachment of tau, making proteolysis accessible. In the brain tissue of AD patients expression or activity of PP2A was reduced compared to controls (169;196). Furthermore fibroblasts of AD patients showed a low expression of PP2A (210).

Clinical studies have not yet tested the effect of methylation on the pathophysiology of tau or Aβ.

Another mechanism which may accelerate P-tau accumulation is the increase of Aβ by the activation of the kinases that phosphorylate tau (GSK3beta, phosphatidylinositol 3-kinase (PI3K), MAP kinase) (37).

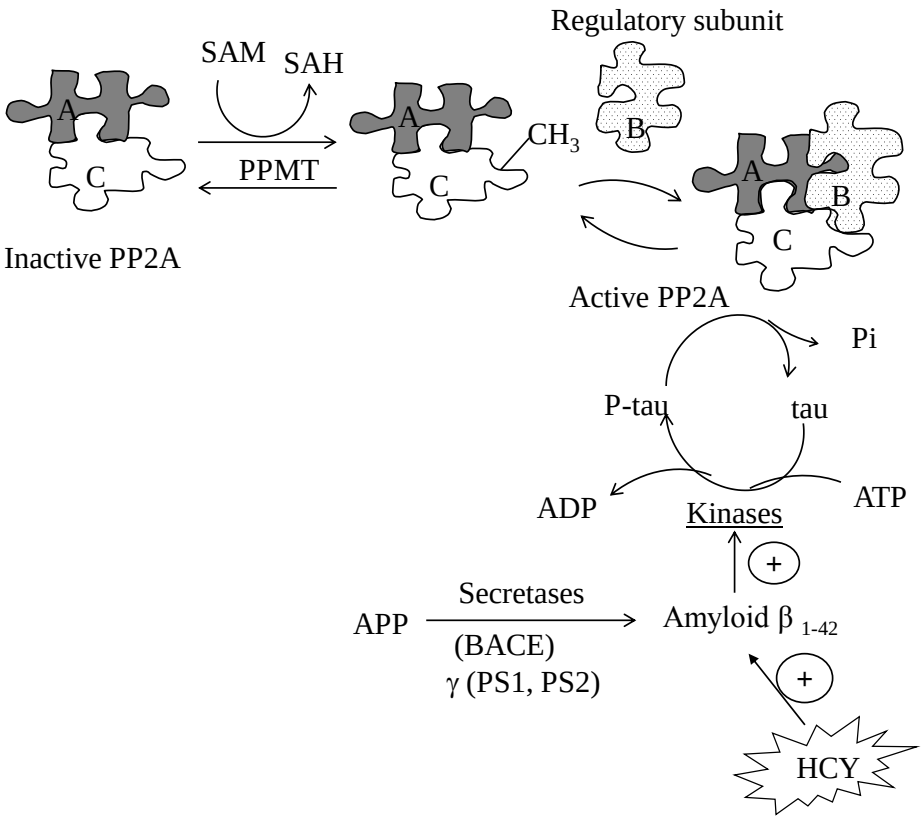


Figure 5
SAM, SAH, and HCY influence the dephosphorylation of P-tau and Aβ metabolism.

There seems to be a positive association between plasma concentrations of tHcy and that of Aβ 1-40 (79). The endoplasmatic reticulum protein (Herp) is responsive to HCY and is capable of enhancing the γ-secretase activity and thereby the Aβ 1-40 accumulation in the brain (156). Along with this direct mechanism there is evidence that HCY can act neurotoxic by potentiating Aβ neurotoxicity (74;99). Neuronal cells are susceptible to not only intracellular but also extracellular accumulation of Aβ 42 induced by homocysteic acid (64). HCY also increased Aβ toxicity and caspase-3 activation in vascular smooth muscle cells dose-dependently. When taking into account the current data published on this subject matter, HCY seems to accelerate dementia by stimulating Aβ deposition in the brain.

4.2 Homocysteine and Glutamate Receptors

HCY is capable of acting on N-methyl-D-aspartate receptor subtype as a glutamate receptor agonist. Brain cells produce homocysteic acid from HCY oxidation which is then released when stimulated. Homocysteic acid can function as an excitatory neurotransmitter through the activation of the NMDA receptor. Hence the neurotoxicity can be blocked by NMDA receptor antagonists (106).

4.3 Homocysteine and Inflammation

There are few studies examining HHCY's effect on inflammation. Some studies were able to show that a negative association between higher HCY and cognitive function was strongest in persons with a high level of IL-6 (207), CRP, and Alpha-1-antichymotrypsin (ACT) (189).

5 Aim of Work and Study Hypothesis

Although there is clear evidence that there is an association between HHCY and neurodegenerative disease, the causal link between the two is still not well studied. Of the wide range of risk factors that have been postulated, HHCY is one of the few that could easily be influenced. HHCY is a marker for vitamin deficiency and is the main reason for hypomethylation. The aim of our work was to contribute further to risk factor evaluation by identifying neurodegenerative changes in the presence of HHCY and B-vitamin depletion and to further improve pathobiochemical understanding by clarifying the influence HHCY has on phosphorylation and methylation.

The causal effects of HCY metabolism on neurodegeneration in vivo remain elusive. Therefore we tested the hypothesis that increased tHcy is causally related to accumulation of P-tau protein in brain tissues via affecting PP2A activity or level. For this purpose, we studied Wistar rats fed methionine or homocystine rich diets, compared to those fed a control diet. In addition we proposed that changes in phosphorylation caused by HHCY can lower the activity of PP2A and thereby causing enhanced tau phosphorylation and decreased dephosphorylation of other functional proteins like PNF-H in rat brain. We also studied a marker for inflammation, tumor necrosis factor alpha (TNF-α), in brain tissue extracts in relation to the metabolic conditions caused by the diets. Hopefully, this study can promote impulses for future strategies for large-scale multi-centered randomized clinical trial studies with HCY-lowering vitamins like folate to establish if a proportion of dementia in the world can be prevented.

IV Materials and Methods

1 Study Design

We induced HHCY in Wistar rats by two different approaches and tested the effect on blood and brain methylation, neurodegenerative markers, and functional protein activity. Our study consisted of a feeding experiment with three different treatment groups. Nineteen animals received a homocystine enriched diet (1.7%), 18 received a methionine rich diet (2.4%), and 18 received a control diet. The rats' age at recruitment was 8 weeks and the duration of the study was 5 months. Body weight was determined before and at the end of the experiment. After the end of the 5 months, animals were euthanized. The brain was removed for further analysis.

2 Animal Model

Photo 1
A female Wistar rat, 7 months old at the end of the dietary intervention

We used 8 week old female Wistar rats purchased from Charles River (Sulzfeld, Germany) with similar starting body weight of approximately 200g. To ensure an acclimatization of the animals after their arrival, they were fed several days with a standard diet and tap water. Subsequently the animals were randomized into three treatment groups. For randomization 25 animals were put into a large cage. From the middle of the cage, a rat was removed and assigned to one of the groups. In the first cycle rats were sorted into groups 1-3; the second cycle was distributed in reverse order, continuing until the cage was empty. The remaining animals were randomized by the same procedure ensuring group equality.

All rats were marked with specific ear hole combinations composed of complete and semi-complete holes, and their tails were marked with red and black color combinations. In

addition, every cage was labeled with a specific symbol assigned to a certain animal diet group, and the food was colored according to the diet, allowing a definite identification and an accurate administration of the pellets. Animals were placed in an air-conditioned room where an automatic timer simulated a 12 hour to 12 hour day and night cycle. Humidity and temperature were regulated at a constant 52 ± 10 % and 22 ± 2 °C, respectively. Animals had free access to water and food. The amount ingested was recorded after periodic weighing of the leftover food. The study was approved by the Institutional Animal Care and Use Committee at the University of the Saarland.

3 Dietary Intervention

Basis of homocystine, methionine, and control diet was an herbal standard diet (Altromin C1100, Gesellschaft für Tierernährung GmbH, Deutschland) in pellet form. The purified control diet C1100 contained all essential food components in defined quantities. The content of B vitamins and energy were as follow:

Vitamin B_1: 18 mg/kg

Vitamin B_2: 12 mg/kg

Vitamin B_6: 9 mg/kg

Vitamin B_{12}: 0.024 mg/kg

Folic acid: 2.904 mg/kg

L-methionine: 3.772 g/kg (0.38%)

Energy content: 2490 055 kcal/kg

4 Narcotics

The rats were anesthetized with a ketamin-rompun (1:0.24) mixture and dosed with 0.2 ml per 100 mg bodyweight. Rompun (Bayer HealthCare®, Leverkusen, Germany) was available as 2% and Ketamin (Pharmacia®, Erlangen, Germany) as 0.1 % solution. The intraperitoneal injection was administered in supine position and oblique head inclination.

5 Collection of Blood and Tissue Samples

Capillary blood from the retrobulbar venous plexus of the eye was collected before beginning the diet. Venous blood from the right ventricle of the heart was gathered at the end of the dietary intervention under anesthesia.

We isolated, sectioned the brain, and prepared the tissue samples for different purposes: measurement of SAH and SAM concentrations, as well as their ratios were determined in brain tissues, measurement of concentrations of phosphorylated Neurofilament H (pNF-H), tumor necrosis factor alpha (TNF-α), PP2A activity, in addition to protein expression of P-tau protein.

First the rat brain and connecting proximal 2 mm brain stem were removed from the skull.

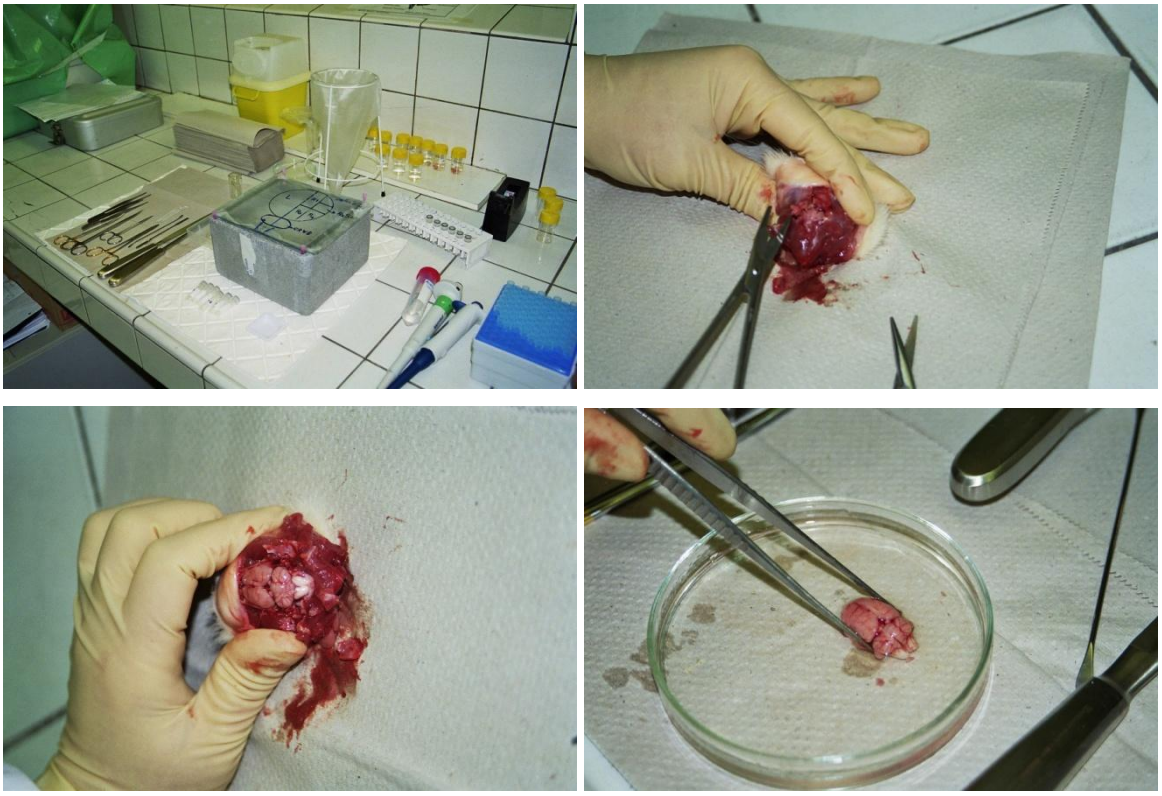
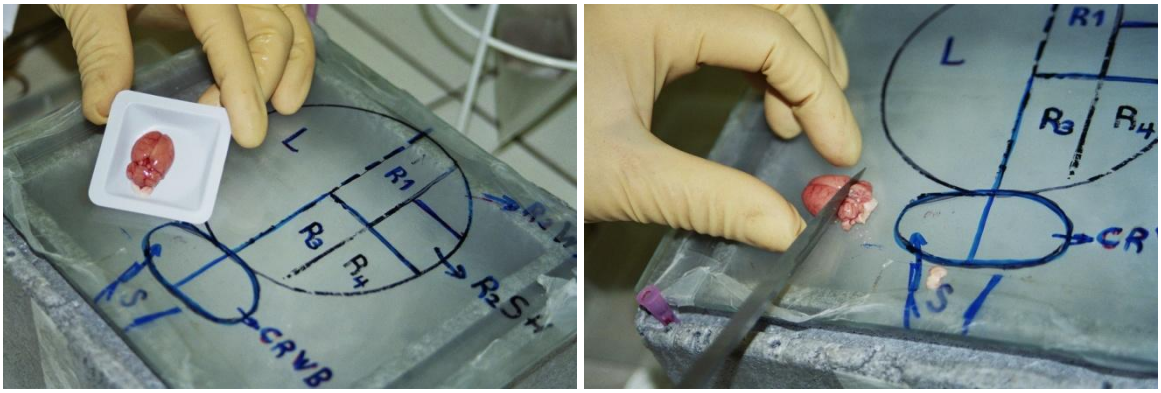


Photo 2-5
Tools and instruments used, dissection of rat brain, removal from skull, PBS rinsing

Next the brain was rinsed in cooled PBS solution and weighed. Then each brain was immediately dissected with clean tools on a glass plate cooled on ice. The brain stem (S) was separated from the cerebellum, and the later was sectioned in median-sagital plane as shown in Photos 6 and 7.



Photos 3 and 4
Sectioning of the brain

The left (L) and right hemisphere were separated. Sagital and frontal plane sectioning divided the right hemisphere into quarters (R1, R2, R3, and R4). The (R2) of the right hemisphere was then sectioned in a frontal plane (R2WB and R2SH).

Table 8 shows which methods were used and which parameter measured according to the different brain sections.

Table 8

Method, Parameter	Brain sections	Conservation	Storage
LC-Tandem-MS SAH/SAM measurement	R2SH, S	0.1g brain tissue/ml acetic acid 1M, homogenized, mixed, sonicated on ice 10s	Transfer to -80°C
ELISA pNF-H, TNF-α	R1	Snap frozen in screw eppendorfs on liquid nitrogen	Transfer to -80°C
ELISA PP2A activity, and total PP2A level	R3	Snap frozen in screw eppendorfs on liquid nitrogen	Transfer to -80°C
Immunohistochemistry P-tau	L	Immersed in 4% formaldehyde, parafinized	Room temperature

Abbreviations:
R2SH: frontal lateral cortex section, S: brain stem, R1, R3: frontal medial cortex sections, L: left hemisphere

6 SAH and SAM Measurement in Brain Tissue and Plasma

We used a modified stable-isotope dilution liquid chromatography tandem mass spectrometry method described by Kirsch et al (95).

Preparation of brain tissue for SAH and SAM measurement:

The frontal lateral R2SH and brain stem section S were used for SAH and SAM measurement. Tissue samples each weighing approximately 50 mg were immediately treated with cold 1 M acetic acid to a final concentration of 0.1 g tissue/ml (for 50 mg/500 µl acetic acid). Brain tissue was homogenized on ice in screw eppendorf containers. These homogenates were vortexed and sonicated on ice, five times 10 s each, and then frozen at –80 °C.

Acidified tissue samples were defrosted, mixed, and centrifuged at 879 g for 7 min (Sigma 3K 12 Centrifuge®, West-Germany). Two hundred µl of the supernatants were then used for SAM/SAH assay. The supernatants were first prepared for solid phase extraction (SPE) for SAM/SAH assay as follows:

Solid Phase extraction (SPE) was performed using a centrifuge 5810 R® (Eppendorf®, Hamburg, Germany) on a constant setting at 8 °C. SPE columns (Varian inc.®) containing phenylboronic acid, which specifically binds cis-diol groups at a pH of 7-8 were preconditioned by addition of 5 x 1 ml of mobile phase (4.24 M acetic acid, pH 2.636) and 5 x 1 ml of wash buffer (20 mmol/l ammonium acetate, pH 7.42). Columns were centrifuged after each addition at 500 g for 1 min. Before SPE separation the acidified 200 µl tissue extracts were neutralized with 20 µl of 1 M ammonia to a pH of 7.4-7.5 and were mixed with 50 µl of internal standards (3 µmol/l for ^{2}H3-SAM and 0.8 µmol/l of ^{13}C5-SAH) (labeled SAM: Dr. Ehrendorf GmbH®, Augsburg; labeled SAH: VU Medical Center®, Metabolic Laboratory, Amsterdam).

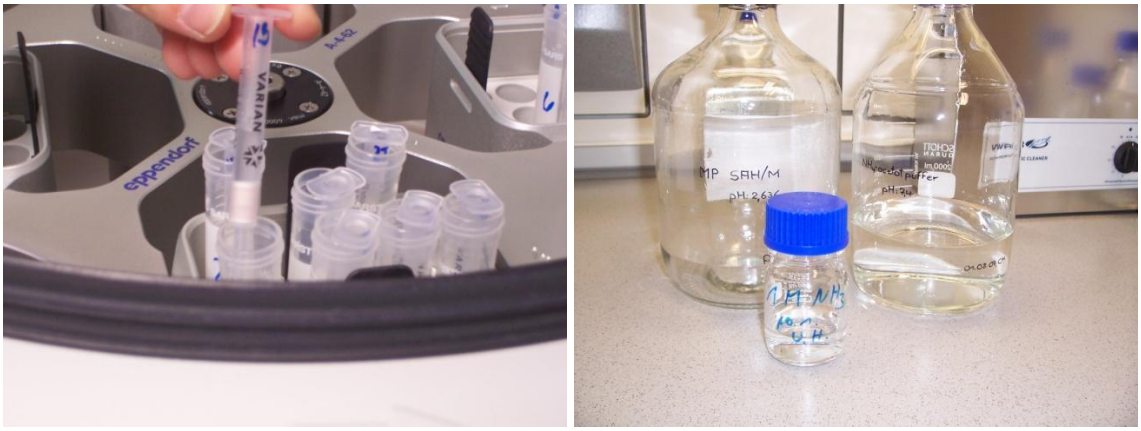


Photo 5-6
Materials, Instruments used for SAH/SAM sample preparation

Calibrators (SAM and SAH), high control, and low control were analyzed in each run. The stock solutions SAM 12.0 µmol/l and SAH 3.2 µmol/l were diluted with wash buffer (20 mmol/l ammonium acetate, pH 7.42) to concentrations of 0, 750, 900, 1500, 1800 nmol/l for SAM and 0, 100, 200, 300, and 400 nmol/l for SAH as shown in Table 9. A high control of 1500 nmol/l for SAM, 350 nmol/l for SAH and a low control at 800 nmol/l for SAM, and 150 nmol/l for SAH were run each time to determine the coefficient of variations (CVs). 400 µl of each calibrator was mixed with 50 µl of internal standards and 40 µl wash buffer, the latter added for volume adjustment. The samples were mixed with 20 µl NH_3 and 220 µl ammonium acetate for volume adjustment (Table 9).

Table 9. SAH and SAM assay in brain tissue supernatants

Calibrator SAM/SAH, in nmol/l and sample	Volume of calibrator or sample, in µl	Internal standard 3 µmol/l $^{2}H_3$-SAM and 0.8 µmol/l $^{13}C_5$-SAH, in µl	20 mmol/l ammonium acetate (pH 7.42), in µl	1 mmol/l ammonia NH3, in µl
1800/400	400	50	40	
1500/300	400	50	40	
750/100	400	50	40	
0	0	50	440	
HC 1500/350	400	50	40	
LC 800/150	400	50	40	
Sample	200	50	220	20

Abbreviations: HC: high control; LC: low control

The samples and calibrators were then applied to the SPE columns for binding of SAM, SAH, and their corresponding internal standards. Loaded columns were then centrifuged for 2 min at 250 g and then washed twice with 1 ml of the wash buffer, centrifuged for 1 min at 500 g to remove non-specifically bound compounds. SAH and SAM were eluted with 3 x 350 µl of mobile phase and centrifuged at 250 g for 2 min after each addition. After SPE the eluted samples containing SAH and SAM were either measured directly or stored for later assay with LCMS/MS at –80 °C.

Liquid Chromatography Tandem Mass Spectrometer measurement

Concentrations of SAH and SAM were measured on a Waters® 2795 Alliance HT HPLC System with a Micromass Quattro Micro API Tandem Mass Spectrometer. The mobile phase was aqueous acetic acid (pH 2,636) set to a flow rate of 0.3 ml/min. The samples (20 µl) were injected on SymmetryShield® RP_{18} Column (3.5 µm, 2.1x100 mm) with a corresponding Guard Column (3.5 µm, 2.1x10 mm) allowing the full injection of the sample into the electrospray injection chamber. Run time was 3 min per analysis. The retention times were 1.99 min for SAH, 1.96 min for $^{13}C_5$-SAH, 1.01 min for SAM, 0.98 min for $^{2}H_3$-SAM. SAM and SAH concentrations were measured by multiple reaction monitoring (MRM) LC-ESI-MS/MS in the positive-ion (ESI+) mode. The optimal conditions in ESI+ mode yielded following transitions: mass-to-charge ratio (m/z) 398.9 → 250.2 for SAM, m/z 402.9 → 250.2 for $^{2}H_3$-SAM, m/z 385.1 → 136.1 for SAH, and m/z 390.1 → 136.1 for $^{13}C_5$-SAH. Concentrations of SAH and SAM were tested in acidified EDTA plasma samples just like in tissue samples (described above) with slight modifications (Table 10).

Table 10. SAH and SAM assay in EDTA plasma sample, pool plasma: 103 nmol/l SAM, 15.6 nmol/l SAH

Calibrator SAM/SAH, in nmol/l and sample	Volume of calibrator or sample, in µl	Internal standard 3 µmol/l $^{2}H_3$-SAM and 0.8 µmol/l $^{13}C_5$-SAH, in µl	20 mmol/l ammonium acetate (pH 7.42), in µl	1 mmol/l ammonia NH_3, in µl
200/48	400	50	40	
100/24	400	50	40	
50/12	400	50	40	
25/6	400	50	40	
0	0	50	440	
Pool Plasma	400	50	40	20
EDTA plasma sample	400	50	40	20

Kirsch et al. (95) compared HPLC method with other studies. The determined concentrations of SAM and SAH obtained by Acquity UPLC BEH C18 column are comparable to the results

obtained by SymmetryShield RP18 with correlation coefficients (UPLC–MS/MS vs. HPLC–MS/MS method) of r = 0.96 (SAH) and r = 0.83 (SAM). Mean SAM plasma concentrations (85.5 nmol/L) measured by UPLC–MS/MS method are in between those reported by Gellekink et al. (94.5 nmol/L) and Struys et al. (74.7 nmol/L). SAM plasma concentrations reported by Stabler et al. (109 nmol/L (71–168 nmol/L, 95% CI)) were approximately 22% higher. SAH plasma concentrations (13.3 nmol/L) are comparable with that obtained by Gellekink et al. (12.3 nmol/L), 13% lower than that obtained by Stabler et al. (15 nmol/L (8–26 nmol/L, 95% confidence interval)) and approximately half of the concentration than that reported by Struys et al. (26.2 nmol/L) Precision was evaluated; inter-assay and intra-assay CVs <6 %.

7 ELISA PNF-H, TNF-α, PP2A Activity and Level in Rat Brain Tissue

Preparation of rat brain tissues for ELISA

Brain samples from the frontal medial cortex sections R1 and R3 were collected in screw Eppendorf containers and immediately snap frozen in liquid nitrogen for several minutes and then transferred to –80 °C and stored.

Later the R1 tissue samples were defrosted and homogenized in a buffer containing 4 M urea, 1 mmol/l EDTA, 1 mmol/l EGTA, and 0.2 mmol/l PMSE in 10 mmol/l Tris-HCL at a pH of 7.22. We used approximately 10 mg of wet weight of brain tissue per 1 ml buffer. After homogenization with insulin needles and sonication, the material was centrifuged in microfuge tubes for 5 minutes at top speed in an Eppendorf type centrifuge. We used 2 µl of homogenized CNS tissue for the ELISA.

We used the sandwich ELISA for pNF-H (Chemicon International®) kit, Enzyme Immunoassay kit for TNF-α (assay designs® Catalog number: 900-086A), sandwich ELISA assay for PP2A activity in cell lysates (DuoSet IC® ELISA) from R&D System®. Total PP2A concentrations also measured using (DuoSet® IC ELISA) from R&D System.

The pNF-H assay system depends on using a 96-well plate coated with chicken polyclonal antibodies generated against pNF-H to capture pNF-H from the sample. Captured pNF-H is detected using pNF-H specific rabbit polyclonal antibodies and a goat anti-rabbit alkaline phosphatase conjugate. After addition of the substrate solution, the amount of pNF-H is determined. The standard curve demonstrates a direct relationship between optical density and pNF-H concentration. The calibration standards for pNF-H were set at 0.12, 0.23, 0.47, 0.94, 1.86, 3.75, 7.5 ng/ml. Sensitivity: 0.0585 ng/ml. Range of Detection: 0.0293 ng/ml to 15

ng/ml. Total protein concentration in tissue extract was measured by using BCA method and pNF-H results were normalized for total protein.

The TNF-α assay depends on using wells coated with a monoclonal antibody that binds rat TNF-α in the samples. A solution of biotinylated polyclonal antibody is added and binds to rat TNF-α. A solution of horseradish peroxidase (HRP) conjugate is added which binds to the rat TNF-α polyclonal. Tetramethylbenzidine (TMB) substrate solution is added. The substrate generates a blue color when catalyzed by HRP. Stop solution is added, and the resulting color is red at 450 nm. The standard curve demonstrates a direct relationship between optical density and TNF-α concentration. The calibration standards for TNF-α were 25, 31.3, 50, 62.5, 100, 125, and 250 pg/ml. Specificity: There was no significant cross reactivity. Sensitivity was determined to be 12.0 pg/ml in Assay Buffer 28. Inter-assay and intra-assay precision both had CVs <12%.

The activity of PP2A was measured in brain frontal cortex samples using sandwich ELISA assay for rat PP2A activity in cell lysates (DuoSet IC ELISA) from R&D System®. For this assay, R3 brain sections were extracted by using 50 mmol/l HEPES, 0.1 mmol/l EGTA, 0.1 mmol/l EDTA, 120 mmol/l NaCl, 0.5% NP-40 (pH 7.5), 25 µg/ml Leupeptin, 25 µg/ml Pepstatin, 2 µg/ml Aprotinin, 1 mmol/l PMSF. An immobilized capture antibody specific for the catalytic subunit C of PP2A binds both active and inactive PP2A. After washing out unbound material a synthetic phosphopeptide substrate is added. The phosphate release in the brain extract was then proportional to the active PP2A. This substrate is then dephosphorylated by active PP2A enabling the generation of free phosphate and unphosphorylated peptide. The free phosphate then is detected by a sensitive dye-binding assay using malachite green and molybdic acid. By calculating the rate of phosphate release, the activity of PP2A is determined and normalized to total protein content in the extract. The Duoset IC activity is specific for PP2A and was demonstrated by Western blot analysis of protein immunoprecipitated with the anti-PP2A antibody supplied in the assay kit.

Total PP2A concentrations were also measured in brain homogenesates using (DuoSet® IC ELISA) from R&D System. The assay depends on using an immobilized capture antibody specific for the catalytic subunit of PP2A. After washing the unbound material, a biotinylated detection antibody specific for PP2A is used. The detection is completed using a standard Steptavidin-HRP system.

8 Biochemical Blood Analysis

We analyzed concentrations of SAH, SAM, tHcy, folate, vitamin B_{12}, and B_6 in the rats' plasma samples in the control and interventionary diets. The first sample was collected by extracting capillary blood from the retrobulbar venous plexus of the eye. The second sample was extracted by collecting venous blood from the right ventricle of the heart under anesthesia.

9 Plasma SAH, SAM measurement

Plasma SAH and SAM were selectively measured with our modified version of stable-isotope dilution liquid chromatography tandem mass spectrometry method described above in section 5.2.

10 Total Plasma Homocysteine Measurement

Concentrations of tHcy were measured by enzymatic Fluorescence-Polarization-Immunoassay (FPIA) on an AxSYM® analysis device (Abbott®, Wiesbaden, Deutschland). This is a method used for routine use and suitable for laboratories requiring HCY high-output testing capabilities. The analytical performance of the AxSYM® tHcy FPIA has been compared to the well established high-performance liquid chromatography (HPLC) and IMx® tHcy FPIA methods and the two methods have corresponding mean differences. The method has been validated and compared to HPLC assay (107) and showed a correlation coefficient of r=0.985. Inter-assay and intra-assay precision both had CVs <5% according to protocol of National Committee for Clinical Laboratory Standards.

11 Folate Measurement

Folate was measured by competitive Chemilluminescence immunoassay on a Centaur® analysis device (Bayer Diagnostics®, Fernwald, Germany). To release folate bound to endogenous proteins, the sample is pretreated with a deliberation reagent. The folate in the sample competes with acridinium-marked folate in the reagent for a limited number of folate-binding proteins covalently bound to paramagnetic particles. Finally these paramagnetic particles are washed and treated with a reagent leading to a Chemilluminescence reaction producing light. The intensity of this light reaction is inversely proportional to the folate concentration in the sample. The test used has been compared to the alternative method using chemilluminescence and showed a correlation coefficient of r=0.941. Traceability is

guaranteed by calibration to an internal standard which was produced by using highly purified N-5-methyltetrahydrofolate. Inter-assay and intra-assay precision both had CVs <8%.

12 Vitamin B_{12} Measurement

Vitamin B_{12} was measured by competitive Chemilluminescence immunoassay on a Centaur® analysis device (Bayer Diagnostics®, Fernwald, Germany). To release vitamin B_{12} bound to transcobalamins, the sample is pretreated with sodium hydroxide and dithiotrilae (DTT). The vitamin B_{12} in the sample competes with acridinium-marked vitamin B_{12} in the reagent for a limited amount of intrinsic factor covalently bound to paramagnetic particles. Finally these paramagnetic particles are washed. A starter reagent is added thereby activating the chemilluminescence reaction. The detected relative light units are inversely proportional to the concentration of vitamin $B_{12.}$ The test used has been compared to the alternative method using ADS:180 VB12-test and showed a correlation coefficient of r=0.99. Traceability is guaranteed by calibration to an internal standard which was produced using material of the United States Pharmacopeia (USP). Inter-assay and intra-assay precision both had CVs <5%.

13 Vitamin B_6 (Pyridoxal-5'-Phosphate) Measurement

Vitamin B_6 was measured in the plasma on the Gynkotek® HPLC with an appropriate kit (Immundiagnostik®). The compositions of the flow agent, derivatisation, and precipitation reagent were not disclosed by Immundiagnostik. The HPLC method is isocratic (constant composition of the mobile phase). A fluorescence detector was used to detect the signal. Intra-assay and inter-assay CVs were <4.1%.

14 Immunohistochemistry

We isolated and sectioned the brain, as described above in 5. The left hemisphere was fixed in 4% formalin. Then three parts of tissue (A, B, C) sectioned by transversal plane were embedded in paraffin. We chose the middle section B for our assay. To mount samples onto slides, silanization was necessary. In order to do this, slides were placed in acetone for 1 min, next in 2% silane in acetone (3-aminopropyl) triethoxysilane (Sigma® A-3648) for 1 min, dipped in acetone for 10 s and then dH_2O for 10 s. The slides were dried overnight at 37 °C.

We sectioned the middle section of the left hemisphere in transversal plane in slices of 5 µm thickness. The samples were floated atop a water bath to be rolled out and teased apart, always maintaining the same direction. Then the slices were transferred to a warm water bath

(50-55 °C) to smooth and stretch out the sample before floating the section onto the slide treated with silane. Slides then were dried in an oven at 60 °C overnight. For deparafinisation we first incubated the slides in two cuvettes filled with Xylene (Fluka® Cat NR 95692) five minutes each. Next the slides were moved into two cuvettes of 100 % Ethanol abs (Hedinger®) 3 min each. Slides were then incubated in 96 % Ethanol (2 min), 70 % Ethanol (2 min), 50 % Ethanol, dH_2O (at least 30 s) and in PBS (10 min at room temperature), making sure not to dry out slides between transfers.

The slides were incubated for 3 min in 1 mmol/l pH 6.0 citrate buffer in a plastic cuvette at 560 W in a microwave accompanied by a plastic cuvette of dH_2O to minimize evaporation. Incubation in microwave was repeated 7 times.

For immunohistochemical stain we blocked unspecific bonds with 5% BSA in PBS for 30 min. Then the slide was dried around the mounted sample, and a circle was carefully drawn with a paraffin marker. The samples were incubated with the first antibody (Mouse Anti Phospho Tau (ser396) 1:500 PBS (Invitrogen® Cat NR 355300) in a closed box lined with damp paper towels overnight at 4 °C. Next the slides were rinsed in PBS, and then washed 3 times for 5 minutes each in a cuvette of PBS. Then samples were incubated with second antibody Alexa flour 488 goat anti mouse IgG 1:500 PBS (Invitrogen® Cat NR A-11001) for 1 hour in the dark. The slides were washed again in PBS 3 times for 5 min. In the last step we trickled a drop of Mowiol on the slide, placed a cover glass on top, and let them dry overnight in the dark at room temperature. The fluorescence intensity in 4 independent photos from each section were calculated using AxioVision® program from Carl Zeiss®, and the mean of the intensities was considered for this section of a corresponding animal.

15 Reagents, Materials, and Instruments

Reagent	Company	Catalog Number
Herbal standard diet	Tierernährung GmbH, Deutschland	Altromin C1100
Ketamin (0.1% solution)	Pharmacia®, Erlangen, Germany	
Rompun (2% solution)	Bayer HealthCare®, Leverkusen, Germany	
Acetic Acid	Aldrich®	338826
Ammonium acetate	Biosolve®	01244156
Ammonia	Merck®	1-05432.2500
$^{13}C_5$-SAH	VU Medical Center®, Metabolic Laboratory, Amsterdam	
$^{2}H_3$-SAM	Dr. Ehrendorf GmbH, Augsburg	
Urea	Sigma®	U-6504
EDTA (ethylenedinitrilotetraacetic acid disodium salt dehydrate)	Merck®	1.08418
EGTA (ethylene glycol tetraacetic acid)	Sigma®	095K5447
PMSE (phenylmethylsulfonylfluoride)	Sigma®	P-7626
Tris (hydroxymethyl) aminomethane	Merck®	1.08382.1000
Liquid nitrogen	Air liquide®, Oberhausen	
Enzyme immunoassay kit for tumor necrosis factor alpha (TNF-α)	Assay designs®	900-086A
Phosphorylated neurofilament H (PNF-H) kit	Chemicon International®	NS170
DuoSet IC® ELISA kit (PP2A activity)	R&D System®	
DuoSet IC® ELISA kit (total PP2A concentration)	R&D System®	
Dulbecco's® Phosphate Buffered Saline (x1)	PAA Laboratories®	H21-002
Formalin	Fischar®	26053025
Acetone	Hedinger®	
Silane (3-Aminopropyltriethoxysilane)	Sigma®	A-3648
Xylene	Fluka®	Cat NR 95692
Ethanol abs	Hedinger®	
1 mmol/l ph 6.0 Citrate Buffer	Fluka®	82568
BSA	Sigma®	A 7906-100G
Paraffin marker	Dako®	S 200230-2
Mouse Anti Phospho Tau (ser396)	Invitrogen®	Cat NR 355300
Mouse APP (anti Alzheimer precursor protein A4)	Chemicon®	Bat NR MAB348
Polyclonal Rabbit Anti-Mouse Immunoglobulin/ HRP	DakoCytomation®	P0161
2nd antibody (Alexa flour® 488 goat anti mouse IgG)	Invitrogen®	Cat NR A-11001
Mowiol	Calbiochem®	9002-89-5

Instruments and Materials	Company	Manufactured in
Vortex VF 2	Janke und Kunkel GmbH	
Sonicator 12	Bransonic®	
3K 12 Centrifuge	Sigma®	
5810 R Centrifuge	Eppendorf®	Hamburg, Germany
Solid phase extraction columns containing phenylboronic acid	Varian inc®	
HT 2795 HPLC System with a Micromass Quattro Micro API Tandem Mass Spectrometer	Waters®	
Equilibrated (mobile phase) SymmetryShield® RP18 Column (3.5 µm, 2.1x100mm)	Waters®	
Guard Column (3.5 µm, 2.1 x 10mm)	Waters®	
Spectrometer Polarstar Optima®	BMG Labtech®	
Microwave	Panasonic®	
Microscope Axiovert® 40CFL	Zeiss®	
ELISA Optical Densitometry	Fluostar Optima®	
Immunofluorescence Densitometry	Axio Vision® 4.4	

16 Statistics

Data analyses were performed by using SPSS Version 18.0 (SPSS®, Chicago, USA). Results are shown as geometric mean (SD). Continuous variables were compared between the 3 dietary groups using ANOVA test followed by Tamhane-T test when ANOVA was significant. Simple correlations between different parameters were tested using Spearman test. All p values <0.05 were considered statistically significant.

V Results

1 Main Characteristics and Blood Markers Assayed in Plasma and Brain

Main characteristics and blood markers in study animals at start are shown in Table 11. All 3 group sizes were comparable. The control group consisted of 18 rats, homocystine group of 19, and the methionine group was made up of 18 rats. All animals had a comparable starting body weight with a mean of 202 g. Folate concentration before dietary intervention was 277.4 nmol/l (SD=61.1) for homocystine, 254.3 nmol/l (50.3) for methionine, and 311.7 nmol/l (48.3) for the control diet. Vitamin B_{12} concentration was 784.9 pmol/l (239.4) for homocystine, 1013.7 pmol/l (220.4) for methionine compared to 649.7 pmol/l (173.5) for the control diet. Before randomization, the methionine group had significantly lower folate and higher plasma concentrations of vitamin B_{12} compared to the control group. However, plasma concentrations of tHcy were only slightly lower than the control group (mean 7.0 versus 8.8 µmol/l; p=0.054) allowing this finding to be interpreted as coincidental. The concentrations of tHcy at start were not correlated to those of vitamin B_{12} or folate. Furthermore, concentrations of plasma folate and vitamin B_{12} in rats were negatively related before randomization (R=-0.49, p=0.001). These unexpected correlations between folate and vitamin B_{12} might be related to differences in the content of the mother's diet (before birth) or the rat's diet shortly after birth.

Table 11. Main characteristics and blood markers of study animals before dietary intervention

	Control (n=18)	Homocystine (n=19)	Methionine (n=19)	p
Rat weight, in g	202.9 (9.3)	202.0 (9.6)	202.2 (8.1)	0.949
Plasma tHcy, in µmol/l	8.8 (2.7)	7.2 (2.1)	7.0 (1.8)*	0.039
Plasma folate, in nmol/l	311.7 (48.3)	277.4 (61.1)	254.3 (50.3)**	0.032
Vitamin B_{12}, in pmol/l	649.7 (173.5)	784.9 (239.4)	1013.7 (220.4)***	<0.001

Data are geometric mean (standard deviation). *p=0.054 compared to control, **p=0.028 compared to control, and ***p<0.001 compared to control and homocystine groups. ANOVA followed by post-hoc Bonferroni test were applied.

Table 12 demonstrates the main characteristics and blood markers of study animals after dietary intervention according to diet. At the end of the 5 month dietary intervention, the rats from the homocystine group showed a significantly lower body weight mean (SD) 321.4 g (25.6), as did the animals from the methionine group 280.8 g (25.1) in comparison to the rats from the control diet 364.0 g (42.3). The weight of the brain after dietary intervention did not vary between the three groups 2.05 g (0.96) for the control group, 2.04 g (0.97) for the homocystine group, and 2.04 g (0.65) for the methionine group. Concentrations of tHcy were significantly increased after homocystine and methionine enriched diets over 5 months in comparison to the control diet. They caused a more than 10-fold increase in tHcy for homocystine diet and more than 15-fold increase for methionine diet.

Moreover, homocystine and methionine enriched diets both showed a significant increase ($p<0.05$) for SAH and SAM and a significant reduction ($p<0.05$) of SAM/SAH ratio concentrations in the frontal cortex and brain stem as well as the plasma compared to concentrations found in the control rats. Effects in cerebral tissue are also shown in Figure 7.

Surprisingly, though the dietary intervention was not vitamin deficient, the addition of homocystine and methionine for 5 months induced a higher folate turnover with lower folate plasma concentrations 192.6 nmol/l (34.9) for homocystine diet and 203.7 nmol/l (29.8) for methionine diet, compared to the control diet 222.5 nmol/l (41.8), as well as a significant reduction of vitamin B_{12} plasma concentration 492.6 pmol/l (64.8) for homocystine diet, 459.5 pmol/l (69.9) for the methionine diet in comparison to 591.5 pmol/l (98.6) for control diet.

Animals receiving the hyperhomocysteinemic diets showed a reduction in plasma vitamin B_{12} concentrations compared to the control animals. The median difference between pre and post treatment of vitamin B_{12} was 75 pmol/l in the control, 218 pmol/l in the homocystine, and 620 pmol/l in the methionine group. Plasma folate medians were lowered by 96 nmol/l in the control, 101 nmol/l in the homocystine, and 36 nmol/l in the methionine group when compared to baseline concentrations prior to dietary intervention.

Table 12. Main characteristics and blood markers of study animals after dietary intervention according to diet (1.7% homocystine diet and 2.4% methionine diet)

	Control diet	Homocystine diet	Methionine diet	$p_{C\text{-}Hcy}$	$p_{C\text{-}Met}$	$p_{Met\text{-}Hcy}$
Rat weight, in g	364.0 (42.3)	321.4 (25.6)	280.8 (25.1)	<0.05	<0.05	<0.05
Rat weight difference, in g	158.6 (40.1)	117.9 (24.0)	76.9 (20.9)	<0.05	<0.05	<0.05
Brain weight, in g	2.05 (0.96)	2.04 (0.97)	2.04 (0.65)	n.s.	n.s.	n.s.
Plasma tHcy, in µmol/l	7.0 (2.5)	74.4 (26.6)	112.9 (40.3)	<0.001	<0.001	0.088
Plasma folate, in nmol/l	222.5 (41.8)	192.6 (34.9)	203.7 (29.8)	<0.05	>0.05	n.s.
Plasma vitamin $B_{12,}$ in pmol/l	591.5 (98.6)	492.6 (64.8)	459.5 (69.9)	0.003	<0.001	0.382
Plasma vitamin $B_{6,}$ in nmol/l	1474.5 (542.7)	1399.4 (452.7)	1264.8 (509.3)	n.s.	n.s.	n.s.
Plasma SAH, in nmol/l	18.5 (6.9)	172 (85)	494 (199)	<0.05	<0.05	<0.05
Plasma SAM, in nmol/l	270 (53)	266 (44)	712 (153)	<0.05	<0.05	<0.05
Plasma SAM/SAH ratio	14.6 (2.9)	1.5 (0.8)	1.4 (0.5)	<0.05	<0.05	n.s.
Frontal cortex SAH, in nmol/g	2.20 (0.36)	6.32 (1.32)	3.89 (1.85)	<0.001	<0.001	0.001
Frontal cortex SAM, in nmol/g	23.42 (4.62)	24.80 (3.15)	27.59 (12.7)	0.64	0.27	0.57
Frontal cortex SAM/SAH ratio	10.65 (2.71)	3.92 (0.96)	7.09 (2.2)	<0.001	<0.001	<0.001
Brain stem SAH, in nmol/g	1.49 (0.56)	5.37 (1.97)	4.3 (3.05)	<0.001	<0.001	0.46
Brain stem SAM, in nmol/g	21.11 (10.3)	25.05 (3.94)	29.42 (8.78)	0.25	0.035	0.28
Brain stem SAM/SAH ratio	14.2 (2.39)	4.67 (1.47)	6.85 (3.14)	<0.001	<0.001	0.016

All data are geometric mean (standard deviation). P-values: $p_{C\text{-}Hcy}$ testing for significance between control diet and homocystine diet, $p_{C\text{-}Met}$ between control diet and methionine diet, $p_{Met\text{-}Hcy}$ between methionine and homocystine. P according to ANOVA and post-hoc Tamhane-T test. The abbreviation n.s. stands for not significant.

2 Effect of Diets on Total Plasma Homocysteine in Rats

Concentrations of tHcy were significantly increased after homocystine (n=19) and methionine (n=18) enriched diets over 5 months in comparison to the control diet (n=18). They caused more than a 10-fold increase in tHcy for homocystine and more than 15-fold increase for methionine diet. The medians were 86.2 µmol/l for the homocystine diet group, 129.8 µmol/l for the methionine group, and 6.8 µmol/l for the control group (Figure 6).

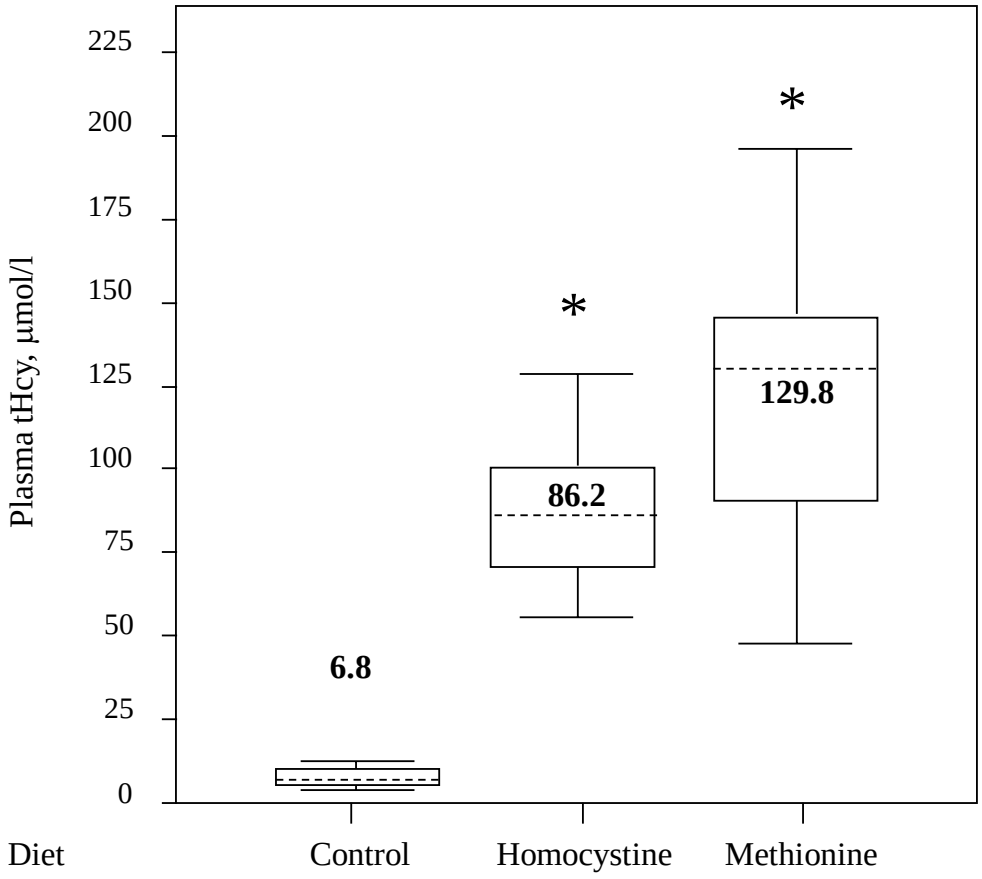


Figure 6
Median (75th-25th percentile) plasma tHcy in rats according to diet after 5 months of dietary intervention. *p<0.05 compared to controls (Mann-Whitney Test).

3 Effect of Diet on SAH, SAM, and SAM/SAH Ratio in Two Brain Regions

Figure 7 shows median (75^{th}-25^{th}) percentiles of SAM, SAH, and their ratio in brain frontal cortex and brain stem according to the type of diet. Homocystine (n=19) and methionine (n=18) enriched diets showed a significant increase in SAH ($p<0.05$) and a significant reduction ($p<0.05$) of SAM/SAH ratio concentrations in brain stem and cortex compared to concentrations found in the corresponding brain area of the control rats (n=18). Median concentrations of SAH were 2.2 nmol/g for control group, 6.6 nmol/g for homocystine group, and 4.1 nmol/g for methionine group in the cortex. Median concentrations of SAH were 1.5 nmol/g for control group, 5.4 nmol/g for homocystine group, and 4.3 nmol/g for methionine group in the brain stem. In the cortex median concentrations of SAM were 24.4 nmol/g for homocystine, 27.8 nmol/g for methionine, and 22.7 nmol/g for control. In the brain stem there was a significant increase ($p<0.05$) 25.5 nmol/g for homocystine diet, 33.7 nmol/g for methionine diet, and 21.2 nmol/g for control diet (Figure 7).

Median SAM/SAH ratio was 10.2 for control rats, 4.0 for homocystine group, and 7.7 for methionine group in the cortex. SAM/SAH ratio was 13.9 for control rats, 6.5 for homocystine group, and 4.7 for methionine diet in the brain stem.

In the control and the homocystine fed animals, samples of brain stem showed lower concentrations of SAH compared to brain cortex. However comparable concentrations of SAM between the cortex and brain stem caused higher SAM/SAH ratio in the brain stem compared to the brain cortex. In contrast, in the methionine fed animals there were no significant differences in SAH, SAM, or their ratio between the frontal cortex and brain stem.

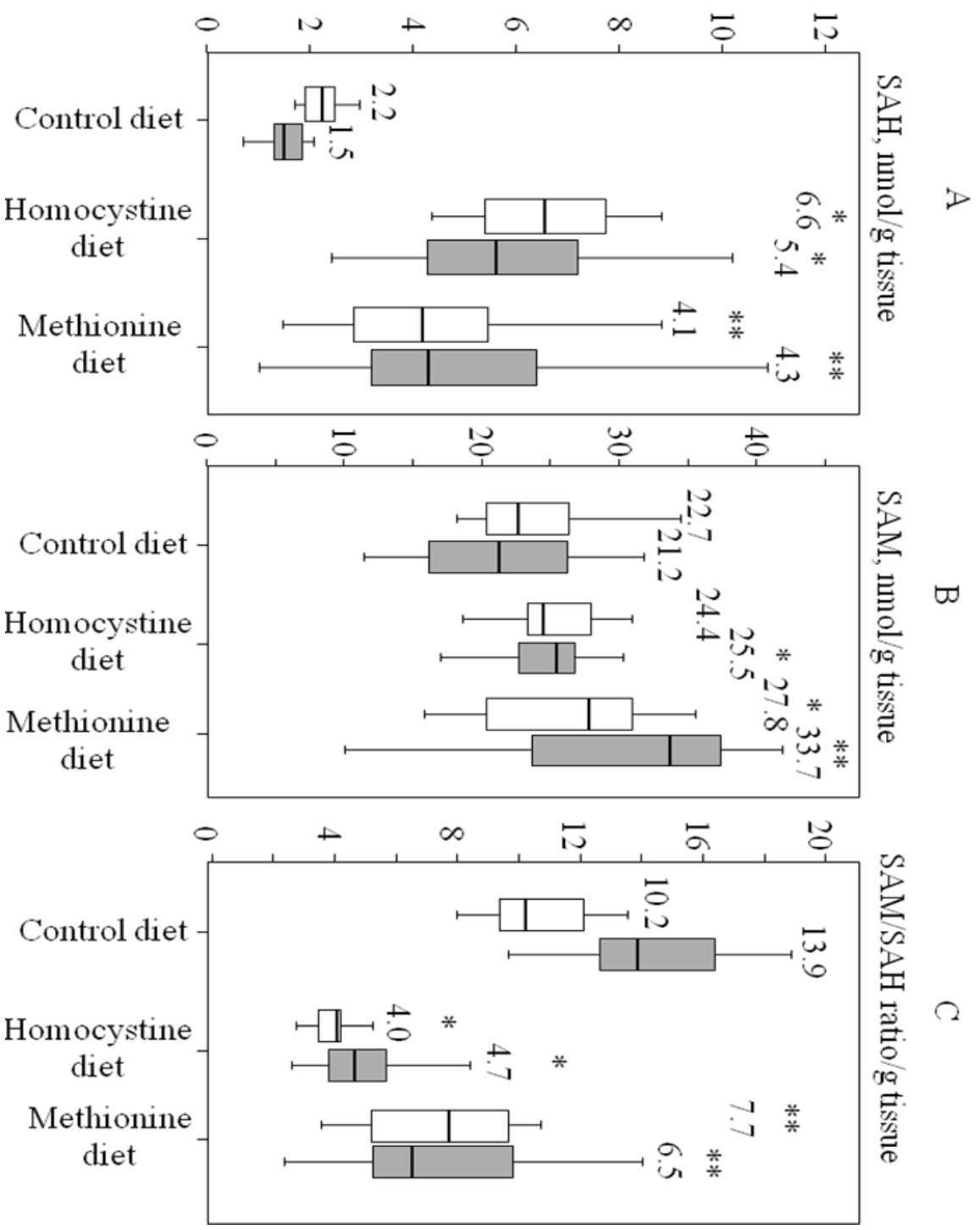


Figure 7
Concentrations of SAH (A), SAM (B), and their ratio (C) per gram of rat brain tissue according to the type of diet. All data are median (75^{th}-25^{th} percentile), (*) p<0.05 compared to control group, (**) p<0.05 compared to homocystine and to control group. ☐ frontal cortex, ▬ brain stem.

4 Plasma Folate and Vitamin B_{12} and Association with Methylation Index

As shown in Figure 8 plasma folate is positively correlated to methylation potential in the brain stem (Spearman correlation coefficient R=0.30, p=0.025) and the frontal cortex (R=0.36, p=0.007). In Figure 9 Plasma vitamin B_{12} is positively correlated to methylation potential reflected by SAM/SAH ratio in the brain stem (R=0.44, p=0.001) and the frontal cortex (R=0.37, p=0.006).

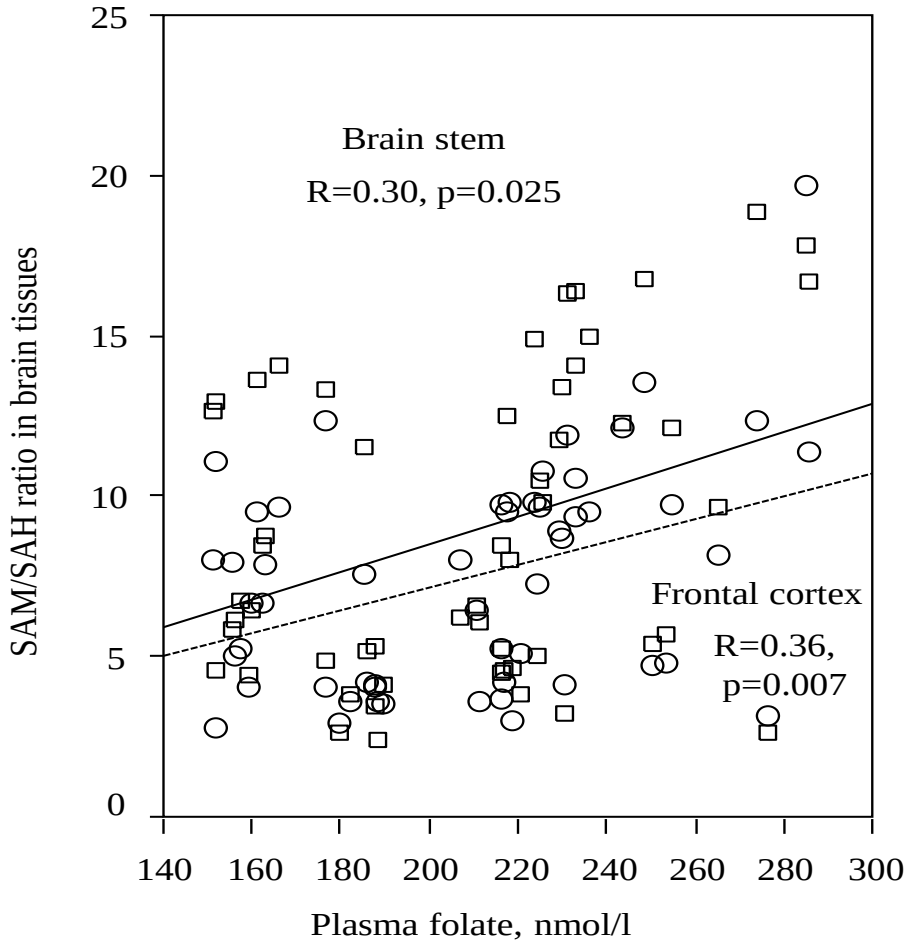


Figure 8
Correlation between plasma folate and SAM/SAH ratio after dietary intervention (Spearman test). Total number of rats analyzed=55; O (circles); dotted line: frontal cortex, □ (squares); continuous line: brain stem.

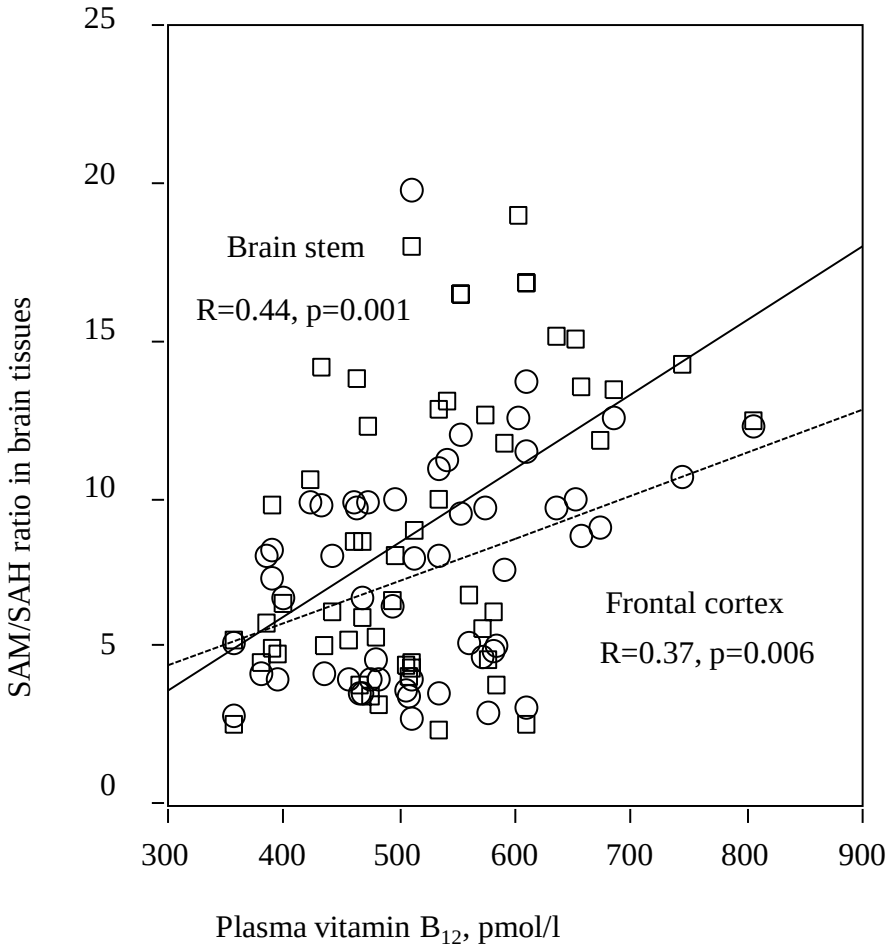


Figure 9
Correlation between plasma vitamin B12 and SAM/SAH ratio after dietary intervention; Spearman test. Total number of rats analyzed=55; O (circles); dotted line: frontal cortex, □ (squares); continuous line: brain stem.

5 Experimental Hyperhomocysteinemia and P-tau in Rat Brains

We examined P-tau accumulation in frontal cortex from a total of 21 rats (9 controls, 6 methionine, and 6 homocystine). The median optical density measured in pixel in the frontal cortex of the left hemisphere probed with antibody against P-tau phosphorylated at serine 396 and then with a secondary antibody labeled with Alexa fluor® 488 was significantly ($p<0.05$) increased in rats receiving methionine (n=6) and homocystine (n=6) enriched diets over 5 months in comparison to the control group (n=9) (Figure 10) (Photos 12-17). The methionine and homocystine-fed animals did not differ in P-tau(ser396) staining. We used Axio Vision® Rel 4.4 Carl Zeiss® computerized image analysis system.

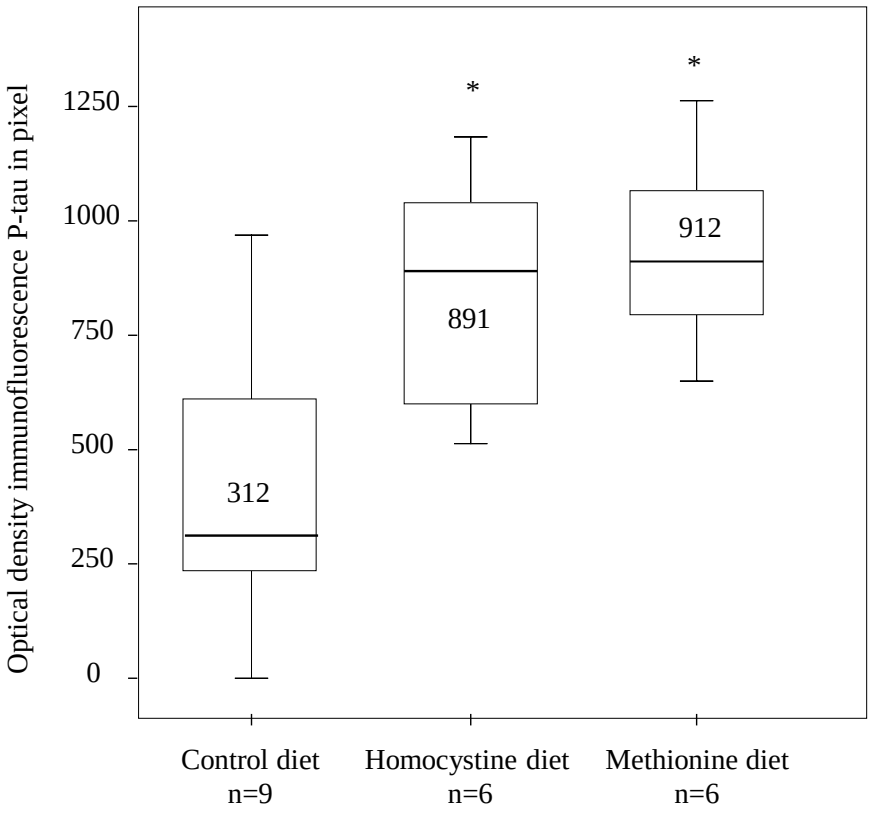
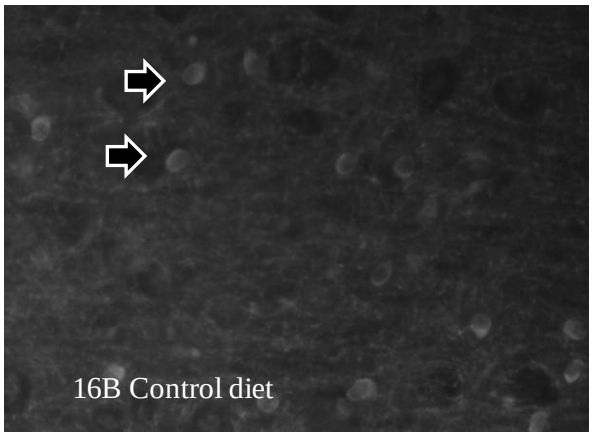
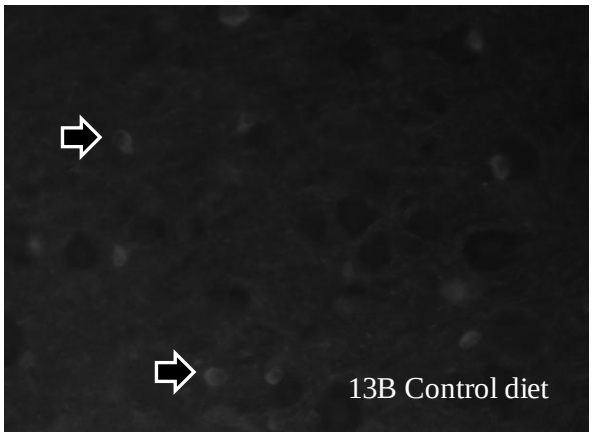
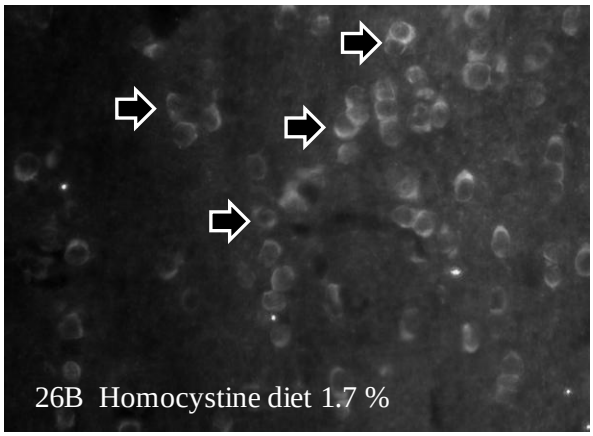
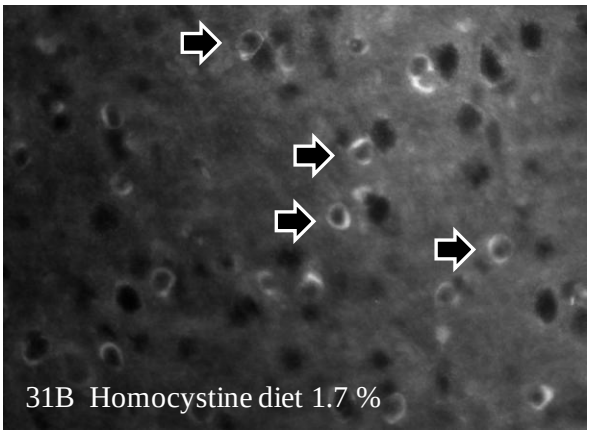


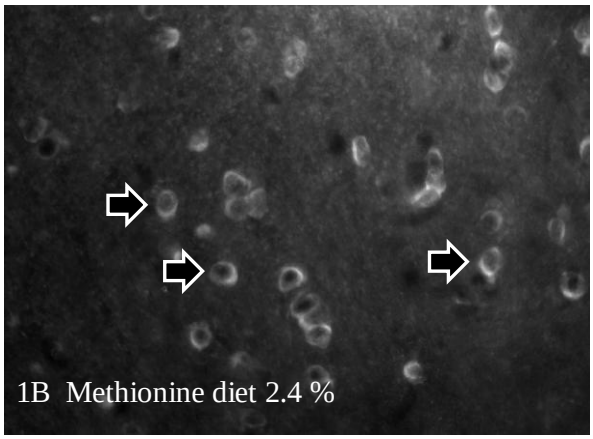
Figure 10
Optical density of P-tau immunofluorescence in brain tissue in pixel. Primary antibody was P-tau phosphorylated at serine 396. Measured with Axio Vision® Rel 4.4 Carl Zeiss®. * p<0.001 in comparison to control diet (ANOVA and Bonferroni tests).

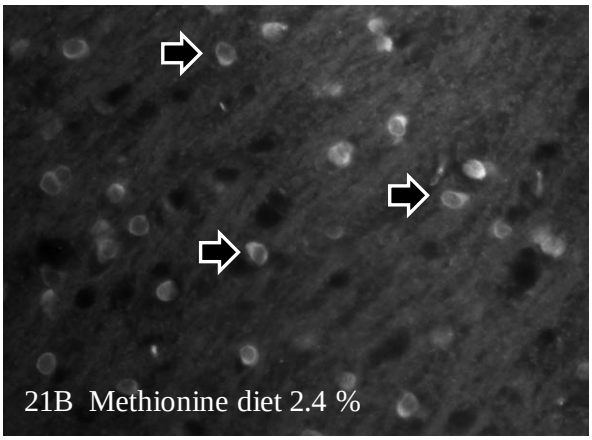












Photos 12-17
Representative sections in control diet group, homocystine, and methionine group in rat brain frontal cortex, left hemisphere. Fluorescence detection using P-tau at serine 396 and Alexa flour®, Axio Vision® Rel 4.4 Carl Zeiss® in all photos.

6 P-tau in Relation to Total Plasma Homocysteine and Plasma Folate

Figure 11 shows the correlations between concentrations of plasma folate and tHcy and optical density in brain sections probed with antibody against P-tau phosphorylated at serine 396 measured in pixel using Axio Vision® Rel 4.4 Carl Zeiss®. The staining of P-tau (quantitative optical density) correlated strongly and positively to plasma levels of tHcy (R= 0.68, p=0.001, n=21) and negatively to plasma folate (R=-0.567, p=0.007, n=21). The correlations remained significant after adjusting for baseline metabolic markers (P-tau with tHcy R=0.68; and with folate R=- 0.76; p<0.01). Accumulation of P-tau in rat brain did not correlate to plasma concentrations of vitamin B_6 or vitamin B_{12}.

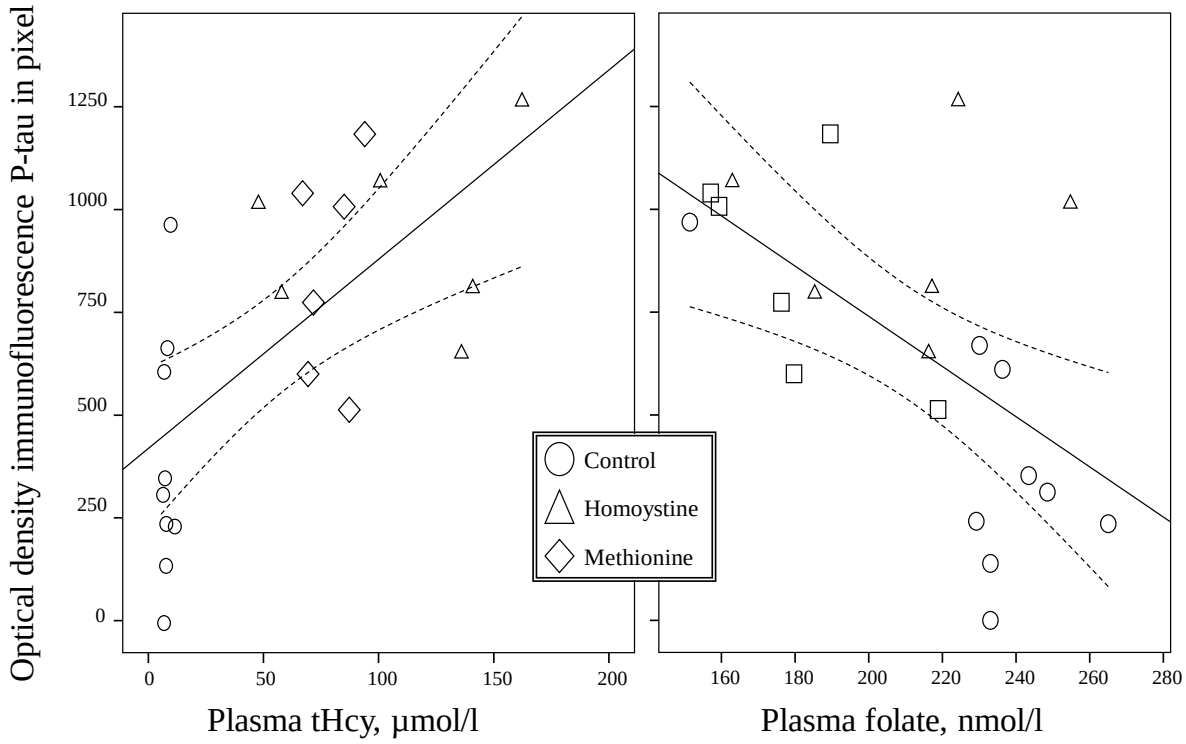


Figure 11
Correlation between optical density (pixel) obtained from immunofluorescence microscope images of brain sections probed with antibody against P-tau (y-Axis), and plasma tHcy (R=0.68, p=0.001, n=21) on the left and plasma folate (R=-0.567, p=0.007, n=21) on the right.

7 TNF- α, a Marker for Inflammatory Reaction

The hyperhomocysteinemic diets had no significant effect on concentrations of TNF-α extracted from the brain tissue (Figure 12). This indicates the diet did not induce an inflammatory reaction in brain tissues.

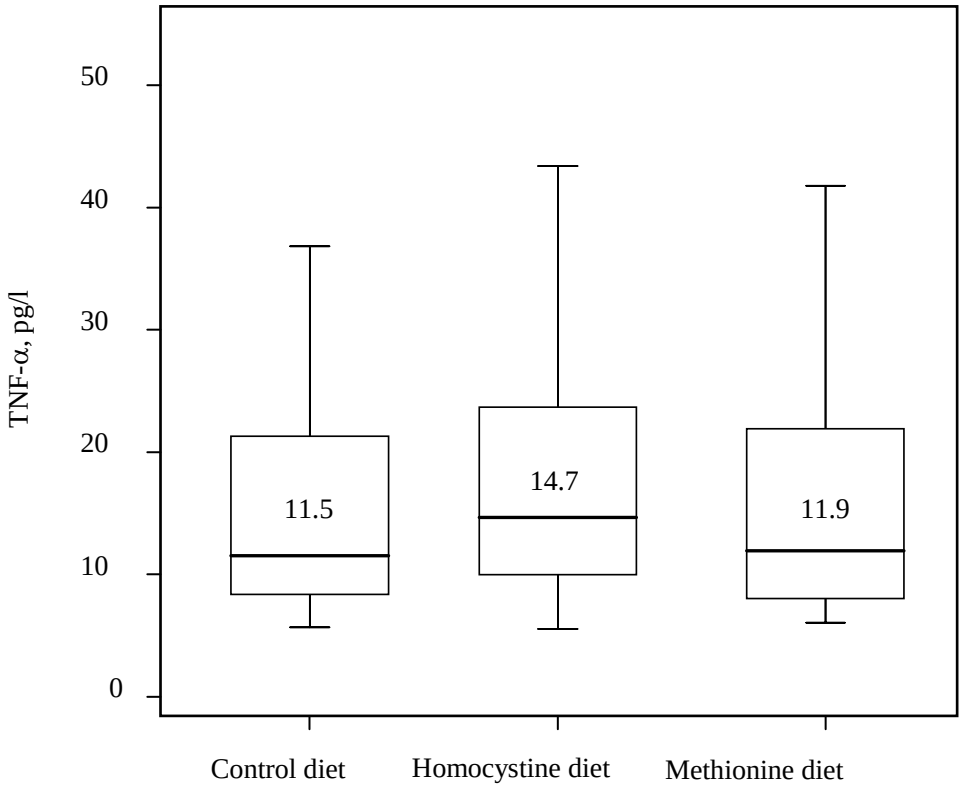


Figure 12
Medians (75th-25th) percentiles of TNF-α in rat brain frontal cortex according to the diet. No significant differences in concentrations were found between the rats according to the diet. The study included 55 rats. Concentrations were not adjusted for total protein in the extracted tissues and measured by Enzyme Immunoassay kit for TNF-α (assay designs®).

8 PP2A Activity and Level in Extracts of Frontal Cortex

We further studied the link between the metabolic conditions associated with HHCY and PP2A activity and/or level of PP2A protein in brain samples. Therefore, we tested the activity of the catalytic subunit of PP2A extracted from frontal cortex samples. Data on immunostaining for P-tau was available from 21 animals. Neither the activity of the active PP2A (the catalytic methylated subunit) when corrected for protein level nor the protein level itself of the catalytic subunit of PP2A differed according to diet (Table 13).

Table 13. PP2A activity, level, and pNF-H of study animals after dietary intervention according to diet (1.7% homocystine diet and 2.4% methionine diet)

	Control diet	Homocystine diet	Methionine diet	$p_{C\text{-}Hcy}$	$p_{C\text{-}Met}$	$p_{Met\text{-}Hcy}$
PP2A activity, nmol phosphate relase/ng PP2A protein/30 min*	13.6 (6.1)	12.1 (7.0)	12.7 (10.8)	n.s.	n.s.	n.s.
PP2A protein level, ng/mg protein*	1.24 (0.35)	1.05 (0.34)	1.07 (0.36)	n.s.	n.s.	n.s.
pNF-H, ng/mg protein*	0.53 (0.3)	0.73 (0.58)	3.47 (12.0)	n.s.	n.s.	n.s.

All data are geometric mean (standard deviation). P-values: $p_{C\text{-}Hcy}$ testing for significance between control diet and homocystine diet, $p_{C\text{-}Met}$ between control diet and methionine diet, $p_{Met\text{-}Hcy}$ between methionine and homocystine. P according to ANOVA and post-hoc Tamhane-T test. The abbreviation n.s. stands for not significant. * Marker measured in same extract of frontal cortex.

We did observe a moderate negative correlation between PP2A activity in frontal cortex (expressed as nmol/µg protein/30min) and staining of P-tau protein (p=-0.51, p=0.013). Yet no significant correlation was found between PP2A activity of the methylated catalytic subunit when corrected for total catalytic PP2A level and P-tau.

Plasma folate, but not the type of diet or the methylation markers (in tissues or in plasma) was related to PP2A. According to this as demonstrated in Figure 13, we observed a weak positive correlation between PP2A activity of the methylated catalytic subunit corrected for total catalytic PP2A level and total protein and plasma folate (R=0.282, p=0.045, n=51).

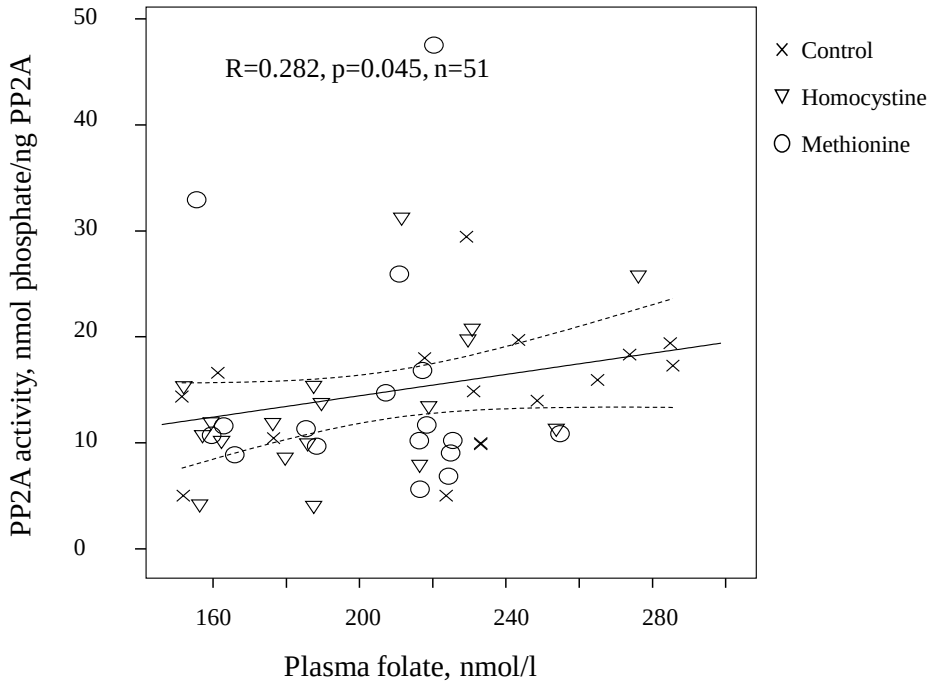


Figure 13
PP2A activity in relation to plasma folate (p=0.045) showing weak positive correlation (R=0.282) for n=51; corrected for total PP2A protein and total protein.

9 Phosphorylated Neurofilament H, a Marker of Axonal Degeneration

We found no significant effect of the diet on concentrations of the marker for axonal degeneration, pNF-H, in brain tissues (Figure 14). Adjusting for total protein in the same extract of frontal cortex did not change the results (Table 13).

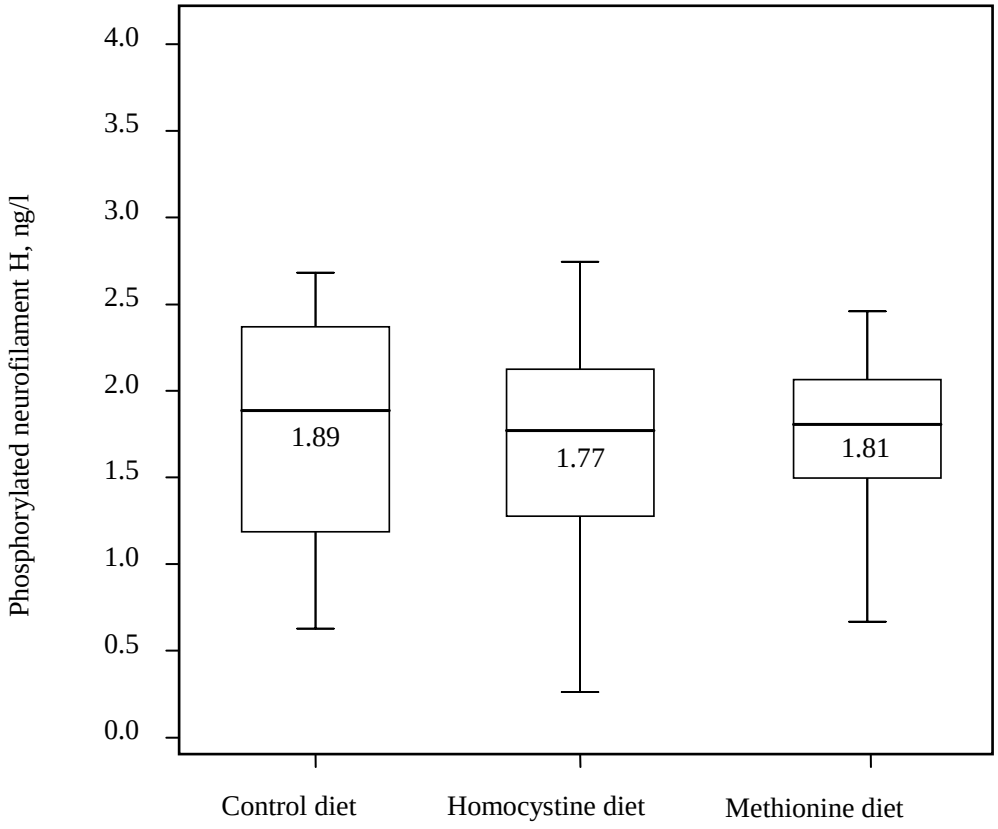


Figure 14
Medians (75-25th percentile) percentiles of pNF-H in rat brain frontal cortex according to diet. No significant differences in concentrations were found. The study included 55 rats. Concentrations of pNF-H adjusted for total protein in the extracted tissues and measured by ELISA (CHEMICON®).

10 PNF-H in Extracts of Frontal Cortex correlates with Folate

We proposed that lower PP2A activity might be related to increased pNF-H because of the decrease in its dephosphorylation. Figure 15 demonstrates the negative significant correlation between pNF-H from brain extracts and plasma folate for the whole group (R=-0.30, p= 0.031, n=52). When taking into consideration only the animals on the HHCY diets, pNF-H showed an even stronger negative correlation to plasma folate concentration (R=-0.46, p=0.005, n=36). PNF-H showed only a week negative correlation to the activity of the PP2A enzyme in brain tissues after correcting for PP2A protein level (R=-0.27, p=0.052, n=53), but a positive correlation with brain staining for P-tau (R=0.50, p=0.021, n=21).

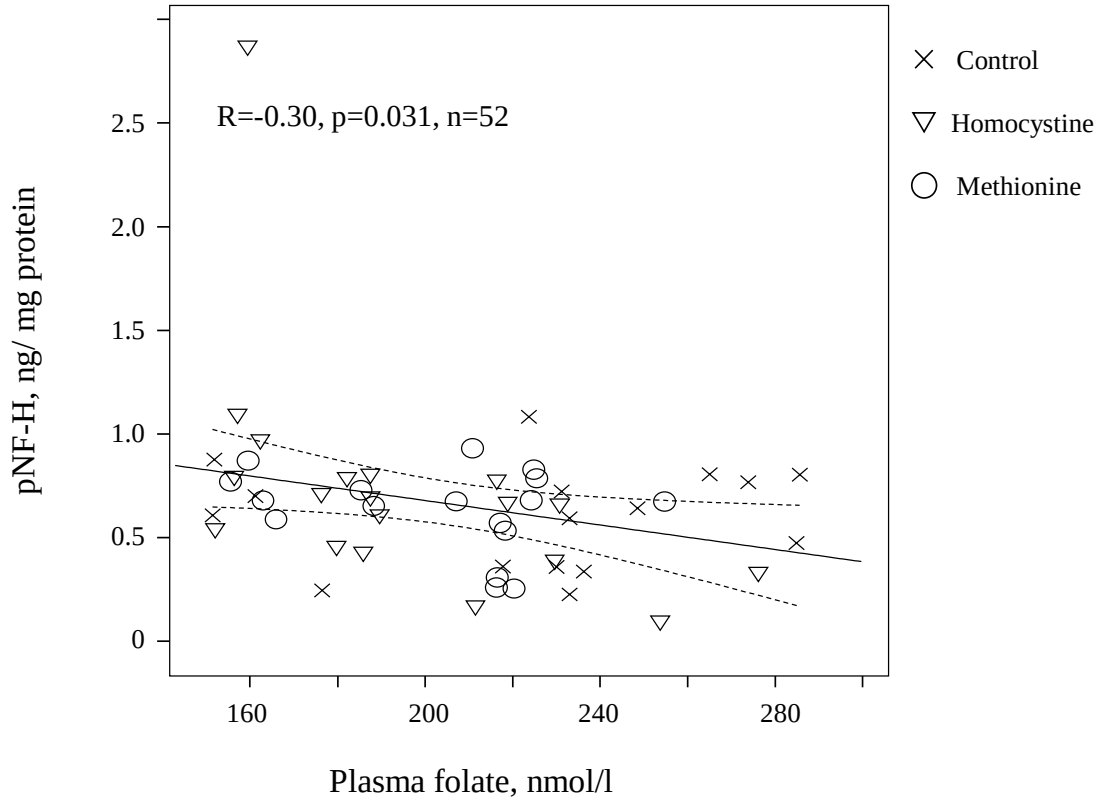


Figure 15
PNF-H in relation to plasma folate showing negative correlation (R=-0.30; p=0.031).

11 Ratio of P-Tau to PP2A activity in Relation to SAH, tHcy, and Folate

We calculated the ratio of P-tau staining to PP2A activity per ng PP2A protein in the 21 samples for which the immunohistochemistry results were available. Figure 16 shows strong correlations found between this ratio and plasma concentrations of SAH in brain cortex (R=0.53, p=0.019), tHcy (R=0.65, p=0.002), plasma folate (R=-0.63, p=0.003)

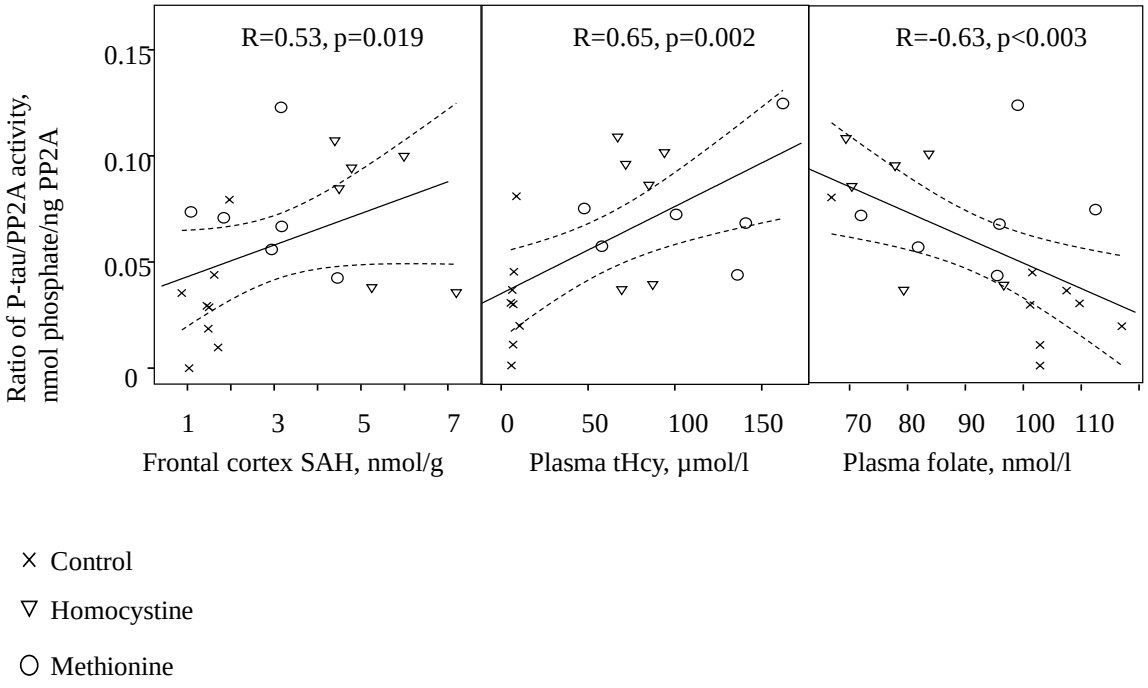


Figure 16
The correlation between ratio of P-tau staining/PP2A activity per ng PP2A protein and brain cortex SAH, plasma tHcy and plasma folate in 21 rats (9 controls, 6 methionine, 6 homocystine). R is correlation coefficient according to Spearman test.

VI Discussion

In the search for pathomechanisms that lead to neurodegeneration, in particular AD associated molecular abnormalities, much attention has been focused on micronutrients. Retrospective (21;32;33;102;205) as well as prospective clinical studies (46;112;132;146;165;181;212) have linked HHCY to neurodegenerative disease. However, treatment studies until now showed no consistent results, a causal relationship is therefore still outstanding. A possible link between impaired methylation and the etiology of AD has been proposed (169;188). The presumption is that HHCY is linked to neurodegenerative disease by mechanisms including hypomethylation. Vafai and Stock (169;188) were the first who suggested this link between impaired methylation and NFT formation. HHCY is known to cause lowered SAM, increased SAH concentrations, and therefore lowered SAM/SAH ratio. Several observational studies from cerebrospinal fluid and brain tissue of AD patients (91;124;134) support this view. An earlier report from 2007 found a negative association between concentrations or P-tau, folate and the SAH/SAM ratio in the CSF (134). The diet is one important source of methyl donors, therefore dietary recommendations for prevention of dementia could be an attractive approach.

In our study homocystine and methionine diets fed to Wistar rats lead to a plasma tHcy elevation by more than 10-fold. Blood and brain concentrations of SAH and SAM were increased and their ratio lowered in rats fed a HHCY diet. Despite not using a diet deficient in folate or B-vitamins, animals fed with HHCY diets seemed to have higher requirements for folate and vitamin B_{12}. Rats with higher plasma folate and plasma vitamin B_{12} concentrations showed a better methylation status. This was reflected by the positive correlation between plasma folate ($R=0.36$; $p=0.007$) (Figure 8) as well as plasma vitamin B_{12} ($R=0.37$; $p=0.006$) (Figure 9) and SAM/SAH ratio in two different brain regions. These results suggest that folate and/or vitamin B_{12} might increase availability of methyl groups in the brain.

Our results are in accordance with one study by Sontag et al. (172) utilizing a folate deficient diet and another study by McCampbell et al. (113) using a methionine rich diet to generate HHCY in a mouse model. The magnitude of increase in concentrations of plasma tHcy and cerebral tissue SAH which we achieved by HHCY diets hardly differed in comparison. The concentrations of SAH, SAM and SAM/SAH ratio in brain tissue in rats nourished with our

control diet were similar to those on the control diet in the folate deficient mouse model described by Sontag et al. (172)

Homocystine and methionine diets in Wistar rats lead to a plasma tHcy elevation by more than 10-fold and depletion of folate and vitamin B_{12}. Both approaches leading to HHCY induced an impaired methylation status and folate depletion reflected by low SAM/SAH ratio in different brain areas. These animals showed an increased staining for P-tau in the frontal cortex compared to the control diet, and this may well be related to a disturbed methylation index. Our study demonstrated a negative correlation between plasma folate concentration and P-tau staining ($R=- 0.76$; $p<0.01$) even after adjusting for baseline metabolic markers.

Vitamin deficient conditions can promote neurodegeneration (44;133;158;172) either via inducing hypomethylation, homocysteine, or a direct effect. For example in neuroblastoma cells, reduced methylation of PP2A (171) and PPMT activity (27) after SAH exposure or in a folate deficient medium (19) were reversible after adding SAM to the medium. PP2A can be demethylated by a methylesterase (PME-1) (136). PME-1 knock-out caused lower demethylation of PP2A but did not reduce cell death caused by folate deficiency (172). This suggests that folate deficiency has a unique mechanism inducing cell death. The negative relationship between folate on the one hand and P-tau or pNF-H on the other hand suggests that folate might protect against neurodegeneration by lowering protein phosphorylation. Our findings suggest that plasma tHcy and folate may be a predictor for the severity and/or progression of the accumulation of P-tau in the brain and higher blood folate might protect the brain from disorders in methyl group metabolism. This assumption is in agreement with the recent clinical study done by Oulhaj et al. in 2010 (138).

In our study, SAH increase was associated with a substantial increase in SAM and a reduction of SAM/SAH ratio. The level and activity of the methylated catalytic subunit of PP2A were not lowered after our dietary intervention, but were negatively related to folate. Methyltransferases are inhibited either by decreased SAM concentrations or by increase of the competitive inhibitor SAH. In neuroblastoma cells adding SAH lowered PP2A methylation, and thereby substrate specificity (171). Moreover, mice fed on a high methionine/low folate diet had higher brain SAH and lower expression of PPMT thus leading to decrease of PP2A (171). Animal studies have shown that decreased methylated PP2A subunit in the brain tissues was associated with tau hyperphosphorylation (172). Clinical studies have been able to support this idea by showing that SAM was reduced in brain tissue and CSF in patients with AD (10;91;124;171). The role of SAM in dephosphorylation of tau suggests a relation

between plasma tHcy or folate and P-tau in the brain. Furthermore, an increase in concentration of SAH in AD patients was associated with lower activities in catechol-O-methyltransferase and phenylethanolamine-N-methyltransferase (91). Other studies on AD brains have shown that the decrease in PP2A activity was related to a lower PP2A methylation and the expression of the regulatory subunit B of the enzyme (169). In addition, brains of patients with AD have shown lowered mRNA for PP2A (196), and elevated SAH suggesting a link between these conditions.

Animals fed the homocystine and methionine diets in our study showed increased staining for P-tau. Our results revealed a positive correlation between plasma tHcy and optical density of P-tau accumulation in frontal cortex of cerebral tissue in rats ($R=0.61$, $p=0.005$). Recent in vivo studies are in accordance with our results. The study by Zhang et al. in 2008 (209) showed that high plasma tHcy induced by vena caudalis injection for 2 weeks was able to induce tau hyperphosphorylation at multiple sites in rat brain hippocampus. Folate and vitamin B_{12} supplementation lowered tHcy and tau phosphorylation in this animal model (209). In our study HHCY did not lead to changes in PP2A-mC activity or protein level of the total catalytic C subunit as listed in Table 13. In 2004 Sontag et al. proposed that the PP2A methylation system could be the link relating elevated plasma tHcy to Alzheimer disease (169). The enzyme PP2A is responsible for dephosphorylation of P-tau. It is likely that SAM-dependant PP2A methyltransferase is inhibited by an increase in SAH hence impeding the degradation of P-tau protein. Zhang et al. (209) demonstrated how HCY induces tau phosphorylation by inhibiting the activity of PP2A and also activating methylesterase which stimulates demethylation of PP2A(C). In hippocampal slices of the HCY injected-rats and of the AD patients, the demethylated but not the methylated PP2A(C) was co-localized with the hyperphosphorylated tau. The study by Luo et al. in 2007 (110) investigated the effect of HCY on phosphorylation of tau by injecting HCY into the lateral cerebral ventricle of rats. They found that level of the hyperphosphorylated tau (ser396) was elevated prominently at 6, 9, and 12 h after the injection, and recovered to normal at 24 h. Simultaneously, the level of PP2A catalytic subunit was reduced markedly when compared with control. The recent study by Sontag et al. (172) examined how folate deprivation over 8 weeks effects hyperphosphorylation of tau protein, PP2A enzyme, and the methyltransferase necessary for its activation. The folate deprived diet lead to disturbed methylation potential in cerebral tissue of mice and the involvement of downregulation of the methyltransferase LCMT-1, and consequently the loss of ABαC (dominant brain isoform of protein phosphatase 2A) seems

related to tau phosphorylation and cell death. The authors were able to show that folate deficiency enhanced expression of non-methylated form of PP2A enzyme but was not able to change the methylation status of pre-existing PP2A suggesting that binding of B subunits to the methylated core enzyme stabilize it against demethylation by PME-1 (protein phosphatase methylesterase-1). However one in vitro study by Kuszczyk et al. is in contrast to the assumption that HHCY is a risk-factor for AD in which abnormal hyperphosphorylation of tau leads to neurofibrillary degeneration, and showed that acute exposure of primary rat cerebellar granule cell cultures to HCY at millimolar concentrations actually induced a glutamate receptor mediated decrease in tau phosphorylation (100).

Taking into consideration different models that all shared similar metabolic conditions with increased SAH and reduced SAM (19;171) we proposed that the effect on P-tau protein was not directly related to tHcy increase. Our results suggest that the accumulation of P-tau may be linked to elevation of SAH. Although SAH seems to act by binding the active sites of methyltransferases and thereby impeding SAM attachment and thereby inhibiting PP2A, this may be an oversimplification because many more factors seem to be involved.

Recently, McCampbell et al. (113) used a high methionine diet or a folate deficient diet over the course of 10 weeks to induce HHCY. APP mice fed a high methionine diet but not those fed a folate deficient diet showed increased brain total tau and p-tau. No changes in either total or methylated PP2A protein were observed. In accordance with the results by McCampbell at al. we propose that the effect of the diet used in our study on P-tau was not directly related to decreased dephosphorylation of P-tau by PP2A.

In general the accumulation of P-tau might be related to enhanced phosphorylation or decreased dephosphorylation (Figure 17). The interactive regulation of different kinase and phosphatase activities are thought to be a complex cellular process and alterations in PP2A activity are capable of affecting activity of protein kinases leading to changes in copious cellular functions (168). Increased P-tau under HHCY conditions without significant changes affecting PP2A suggests that P-tau accumulation might be related to enhanced formation mediated by augmentation of kinase pathways responsible for the phosphorylation in the brain. The effect of folate deficiency on P-tau-protein may be related to enhanced activity of different kinases involved in tau phosphorylation. The kinases' activity seems to be sensitive to and inducted by calcium influx mediated via HCY-dependant activation of NMDA receptors (19). Another in vitro study (100) treated neurons acutely with HCY at neurotoxic concentrations. To reduce this effect an uncompetitive NMDA receptor antagonist was used

and a rapid decrease of P-tau staining was reported. This study demonstrates the link between NMDA receptor, a glutamate receptor, and P-tau accumulation.

Hypomethylation seems to enhance abnormal tau phosphorylation by activation of glycogen synthase kinase 3 beta (GSK3beta) in addition to lowering PP2A (129). For example, GSK3beta gene expression and protein levels were increased under vitamin deficient conditions. This is in accordance with our finding on the positive correlation between P-tau and plasma folate and the reverse correlation between P-tau and plasma tHcy.

Ho et al. demonstrated that increased tHcy or decreased folate concentrations in cultured neuron can promote tau phosphorylation (73) possibly mediated through oxidative stress via increased reactive oxygen species. Another explanation for P-tau increase without change in PP2A is protein-N-homocysteinylation that has been found to cause amyloid like protofibrils (139). P-tau might be N-homocysteinylated with HHCY causing PP2A to be unable to bind this substrate. This protein homocysteinylation might be the reason for a modification in substrate affinity of the enzyme. Furthermore, tau protein may be hyperphosphorylated at multiple sites causing the dissociation of this protein and the inability to bind to PP2A (170). The failure of interacting with phosphatases is a main pathomechanism in taupathies (31).

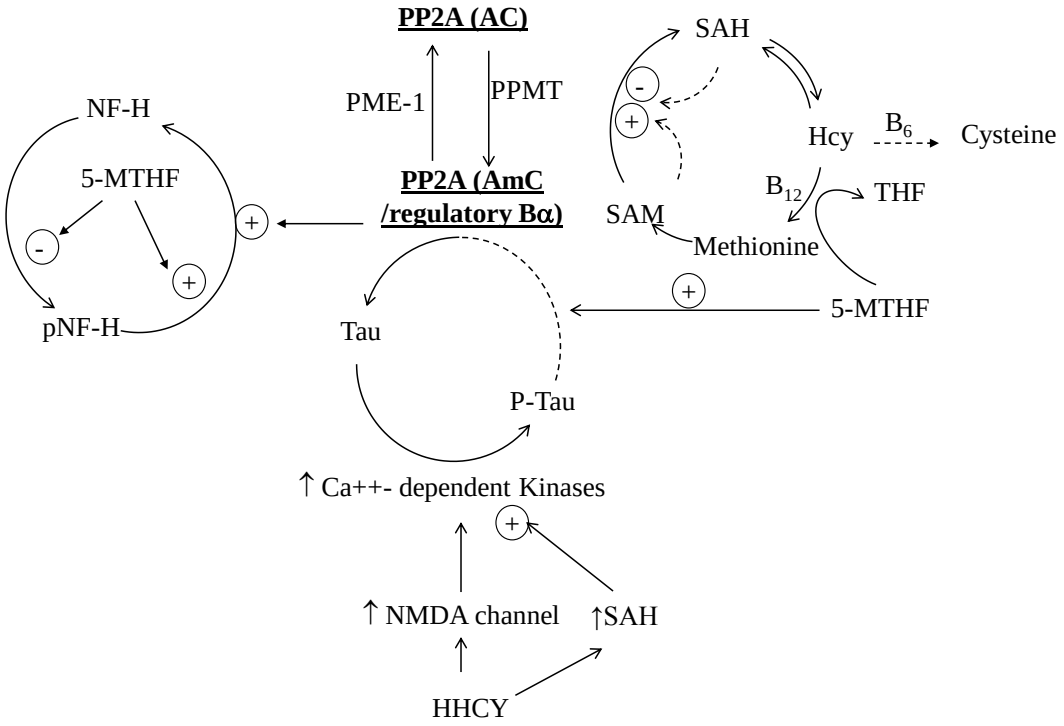


Figure 17
Pathomechanisms affecting the phosphorylation of P-tau and pNF-H: Ca-dependent kinases phosphorylate tau and are enhanced by NMDA receptors, HHCY and SAH, PP2A activity can be enhanced by SAM and inhibited by SAH; Folate has a positive effect on dephosphorylation of P-tau.

PNF-H is an important protein involved in neurodegeneration. The majority of studies in the past have concentrated on hyperphosphorylation of tau as the major cytoskeletal pathology in AD. PNF-H though is also a component of aggregates in the AD brain (177;200).

Our study documented a weak correlation between decreased PP2A activity and increased pNF-H concentrations. In one study, the inhibition of PP2A by ocaidic acid induced an increase in the pNF-H throughout the neuron showing that pNF-H is a substrate for PP2A (53). HHCY was not directly related to pNF-H. In our study however, plasma folate is lowered by supplementation of methionine or homocystine and this was related to increased pNF-H. Folate status seems to be a link to increased pNF-H in cerebral tissue, especially in rats subjected to HHCY diets. One study compared the pNF-H concentrations between AD and control tissues and reported a three-fold increase in pNF-H in AD tissue without increase in total NF-H levels or change in soluble (pin1) levels (140). The pin-1 enzyme may be a mediator for phosphorylation and methylation and thereby play a role in the formation of NFT (140). The pin-1 enzyme, one of the peptidyl-prolyl isomerases catalyzes the isomerisation of the peptide bond in proteins, thereby regulating their biological functions which include protein assembly, folding, intracellular transport, intracellular signalling, transcription, cell cycle progression and apoptosis and protects neurons under in vitro conditions.

The significant reverse association between plasma folate and brain pNF-H levels remains unsolved and calls for further investigation. It has been reported that folate may have a protective effect on glutamate toxicity. If glutamate toxicity can lead to accumulation of pNF-H in neuronal cells (140) this could suggest that a higher folate concentration might be capable of reducing pNF-H by decreasing the stimulation of the glutamate receptor mediated by HCY.

In the current study HHCY and thereby perturbance in methylation was not related to the marker of inflammation TNF-α, in cerebral tissue. TNF-α concentrations measured in rat cerebral tissue were similar when comparing control diet group with homocystine enriched and methionine enriched diet group. This suggests that inflammation does not function as an important neurotoxic modulator in HHCY.

Our study results were in accordance with a study (3) with a small patient collective in which no significant difference in TNF-α concentrations in serum or CSF between AD patients and controls were found. In addition there was also no correlation in either subject group between the concentrations of these inflammatory mediators in serum or CSF, and the change in cognitive status or the progression of the atrophy of the medial temporal lobe. There seems to

be regional differences, reflected by concentrations of TNF-α in AD patients being significantly lower in the frontal cortex (32%, $p = 0.024$), the superior temporal gyrus (57%, $p = 0.021$), and the entorhinal cortex (49%, $p = 0.009$) compared with non-AD subjects. The fact that decreased cerebral concentrations of TNF-α are located in areas showing neuropathology in AD may indicate a dysregulation of the inflammatory process in AD. The large overlap between concentrations of TNF-α in AD patients and other subjects does not support the use of inflammatory mediators as predictive parameters for AD, also considering the poor relation to clinical signs of AD.

VII Conclusions

- Homocystine and methionine enriched diets increased plasma tHcy by more than 10-fold and effectively induced experimental HHCY in rats.
- Homocystine and methionine diets led to a reduced methylation status reflected by SAH increase and SAM/SAH decrease in two different brain regions (frontal cortex and brain stem).
- Plasma concentrations of folate and vitamin B_{12} significantly correlated to brain SAM/SAH ratio. Better B-vitamin status is directly related to better brain methylation status.
- Homocystine and methionine diets caused P-tau accumulation both to a similar extent, in frontal cortex of rat brain.
- P-tau accumulation in the frontal cortex is positively correlated to plasma tHcy and negatively correlated to plasma folate concentration.
- P-tau accumulation could not be explained by changes in PP2A activity or PP2A protein level but by folate status.
- Requirements and turnover of methyl groups are different according to brain region and may reflect different regional biochemical pathways.
- Concentrations of pNF-H in frontal cortex did not differ according to diet.
- Folate status was related to increased pNF-H in brain tissues from rats, especially those subjected to HHCY diet.
- Concentrations of TNF-α in frontal cortex did not differ according to diet, so HCY neurotoxicity is not explained by inflammation in our study.

Micronutrient deficiency is often a problem in the elderly population. The role of HHCY and B-vitamin deficiency has been repeatedly demonstrated as a risk factor and prognostic factor for the development of dementia and other neurodegenerative diseases. Hence, enhancing status of methylation is promising when trying to counter metabolic changes related to the development of dementia. The experimental model inducing HHCY cannot be directly extrapolated to human beings without further research. Low serum folate is very common in elderly people and folate supplementation may form an early pathway to protect against dementia. Future studies should test in vivo the role of micronutrients like B-vitamins

especially folate and SAM in preventing or reversing P-tau accumulation in brain and/or the effect on pNF-H. Strategies aiming at arresting or reversing phosphorylation of tau or enhancing its dephosphorylation should consider long-term folate supplementation.

Concentrations of tHcy that we achieved in our study were above the limits usually found in the general population and the exposure time was relatively short. Nevertheless, the development of dementia takes several decades to develop and even mild to moderate disturbances in the methyl group metabolism might be of importance. Future studies also might test the effect of mild or moderate HHCY over longer periods of time on P-tau accumulation and pNF-H.

VIII Reference List

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