Highlights File

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- Neuronal effects of HCy were at concentrations above the clinically-relevant range.
- HCy actions at clinical concentrations were vascular.
- Clinical and preclinical data support HCy as a mediator for VCID.

Homocysteine, hyperhomocysteinemia and vascular contributions to cognitive impairment and dementia (VCID)

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Abstract

Homocysteine is produced physiologically in all cells, and is present in plasma of healthy individuals (plasma [HCy]: 3-10μM). While rare genetic mutations (*CBS, MTHFR*) cause severe hyperhomocysteinemia ([HCy]: 100-200μM), mild-moderate hyperhomocysteinemia ([HCy]: 10-100μM) is common in older people, and is an independent risk factor for stroke and cognitive impairment. As B-vitamin supplementation (B6, B12 and folate) has well-validated homocysteine-lowering efficacy, this may be a readily-modifiable risk factor in vascular contributions to cognitive impairment and dementia (VCID).

Here we review the biochemical and cellular actions of HCy related to VCID. Neuronal actions of HCy were at concentrations above the clinically-relevant range. Effects of HCy <100 μM were primarily vascular, including myocyte proliferation, vessel wall fibrosis, impaired nitric oxide signalling, superoxide generation and pro-coagulant actions. HCy-lowering clinical trials relevant to VCID are discussed. Extensive clinical and preclinical data support Hcy as a mediator for VCID. In our view further trails of combined B-vitamin supplementation are called for, incorporating lessons from previous trails and from recent experimental work. To maximise likelihood of treatment effect, a future trial should: supply a high-dose, combination supplement (B6, B12 and folate); target the at-risk age range; target cohorts with low baseline B-vitamin status.

Contents

1. Introduction

1.1 Homocysteine and vascular contributions to cognitive impairment and dementia (VCID).

Brain vascular lesions contribute to dementing illness in the form of vascular dementia, vascular factors exacerbating Alzheimer's disease (AD), and other cognitive impairment states where diagnostic criteria for dementia are unmet [81,87]. This considerable burden of dementia-related illness is covered by the concept of vascular contributions to cognitive impairment and dementia (VCID) [35,87,107]. The most common cause of VCID is thought to be a common brain vascular pathology called cerebral small vessel disease (SVD) [28,82,87,92].

Homocysteine (HCy) is a thiol-containing non-essential amino acid (Table 1) that is produced in all cells, as a product of normal folate and methionine metabolism. Elevated plasma homocysteine is referred to as hyperhomocysteinemia (HHCy). HHCy is a robust and independent risk factor for stroke [44,48,61,97,98] and for cognitive impairment [101,105] and is associated with pathologically-confirmed AD [16]. HHCy is associated with an increased rate of hippocampal atrophy [16] and with accelerated cognitive decline in AD patients [88] and is now accepted as a risk factor for AD [6]. Plasma HCy concentration is strongly associated with hippocampal atrophy, white matter lesions and lacunar infarcts in cross-sectional studies [32,61,117,123]. The association with hippocampal atrophy appears to be independent of amyloid pathology [14]. White matter lesions reflect vascular damage [92] and it is therefore notable that white matter changes are associated with HHCy [47,49,64,94] and with low vitamin B_{12} levels [21] and low folate [51,100]. Evidence supporting a causal relationship between HHCy and cerebrovascular injury includes: i) independent, graded association of HHCy with stroke in prospective and retrospective clinical studies [48,61]; ii)

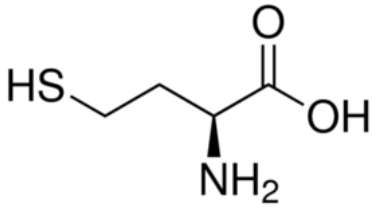
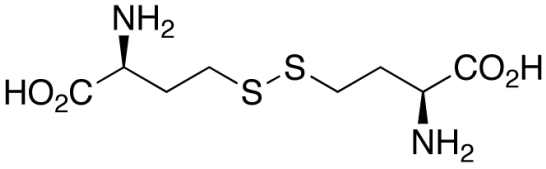
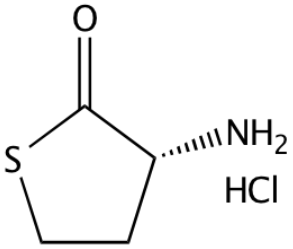
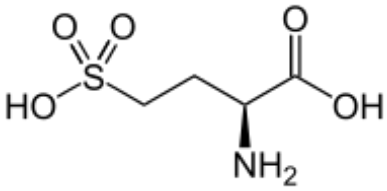
genetic association studies of genes regulating HCy metabolism, using Mendelian randomisation [9]; iii) HHCy-induced lesions in experimental animals [4,68,108,111]. Dietary supplementation with the appropriate B-vitamins (B_6, B_{12} and folate) clearly ameliorates HHCy. Thus HHCy may be a modifiable risk factor in VCID.

1.2 Biochemistry of homocysteine.

Rare genetic mutations in folate and methionine metabolizing enzymes (*CBS, MTHFR*) produce severe HHCy (plasma HCy in excess of 100 µM; up to ~500 µM). Plasma HCy can also be elevated by smoking, aging, deficiency in folate or vitamin B_{12}, or renal failure. Clinical mild-moderate HHCy is common, especially in older people. Mild-moderate HHCy is various defined, with lower limits ranging from 10-15 µM and upper limits 30-100 µM.

Plasma HCy is derived from cellular export via cysteine and folate transporters [50,56] and circulating HCy levels are maintained at relatively low levels by ongoing enzymatic conversion to either methionine or cysteine (see Figure 1). Plasma total HCy concentration in healthy individuals is typically in the range 5-10 µmoles/litre (µM). Previous detailed reviews are available on HCy biochemistry [52,115]. For the purposes of this review (including data from biochemical assays, cell cultures, experimental animals and clinical populations) we have defined mild-moderate HHCy by homocysteine concentrations in the range 10-100 µM, and severe HHCy as >100 µM.

Table 1. Homocysteine and related molecular species

Molecule	**Structure**	**Formation**
Homocysteine, HCy		Formed in normal cell metabolism of folate and methionine
Homocystine		Disulphide bonded dimerization of HCy
Homocysteine thiolactone (shown as hydrochloride)		Condensation of the SH and carboxylic acid groups of HCy
Homocysteic acid, HCA (-SO_3H dissociates to form homocysteate anion, -SO_3^-)		Uncertain, possibly oxidation of HCy

Abbreviations. HCy, homocysteine. HCA, homocysteic acid.

1.3 Homocysteine and cerebral small vessel disease (SVD).

SVD is a vessel pathology that affects small penetrating arteries (up to 400 μm outer diameter) in deep subcortical brain regions, primarily the basal ganglia and deep white matter [31,77]. It is characterised neuropathologically by fibrous, hyaline thickening of vessel wall, loss of myocytes, and relative preservation of endothelium [40,90]. SVD is common in brains of older people, and is a prevalent cause of microinfarcts and lacunes, lacunar stroke, and diffuse white matter lesions ("leukoaraiosis") [87,90,92]. Further, neuroimaging [28,114,116] and neuropathology [30,31,104] data suggest that SVD is also the most common cause of VCID[87,90,92]. While increasing age and hypertension are risk factors for SVD, the pathogenesis of SVD is not well understood [34,90].

Multiple studies have shown that mild-moderate HHCy is a risk factor for SVD [32,44,45,61,61,62,117,123]. In case-control studies HHCy was positively-associated with SVD severity, with highest plasma total HCy concentration (plasma [HCy]; average 20 μM) in patients with confluent leukoaraiosis on MRI scans, indicative of more severe SVD [44,61]. The association between SVD and HCy was markedly attenuated after controlling for circulating markers of endothelial dysfunction, suggesting a possible endothelial mechanism for the HHCy-SVD association [44].

2. Cellular actions of HCy

Here we review the actions of HCy at the molecular and cellular level with particular reference to brain cells, specifically neurones and vascular cells. Several reported actions occur at HCy concentrations below 100 μM, within the range of mild-moderate clinical

HHCy, thus direct actions of HCy in vivo on neurones, vascular endothelial cells and VSMC in vivo appear likely.

2.1 Effects of HCy on brain neurones

We carried out a systematic review of concentration dependence of the actions of HCy in brain cells (results shown in Table 2). We searched PubMed (on 12th June 2015) for articles in English containing the terms: (brain OR cerebr*) AND (homocyst*) AND (potency OR half-maximal OR IC50 OR EC50 OR ED50 OR affinity). This yielded 109 results. Abstracts were viewed by two independent reviewers (NY, AHH) and 41 were discarded by consensus (not relevant, no quantitative data, or not primary data source). Of the 68 retained, on viewing the full text data were extracted from all where quantitative data were given on the potency of homocysteine actions (Table 2) [2,3,25,27,37,38,46,54,75]. Quantitative data were also extracted on the actions of HCA (see Supplementary table S1).

Table 2. Effects of HCy on brain neurones and glial cells

Effects observed	Effective concentration	Cell or tissue	Species	Reference
Neurite loss and size reduction	>100 μM	Mesencephalic tegmental neurons	Rat	Heider et al. (2004) [46]
Inhibition of neural network activity	400 μM	Embryonic neocortical neurons	Rat	Gortz et al. (2004) [37]

Lipid peroxidation	100 - 1000 μM	Synaptosomes	Rat	Jara-Parado et al. (2003)[54]
Inhibition of glutamine uptake	2 mM	Cerebral neuronal non-synaptic mitochondria	Rat	Albrecht et al. (2000)[2]
PAG inhibition	2 mM	Cerebral neuronal non-synaptic mitochondria	Rat	Albrecht et al. (2000)[2]
L-glutamate transporter inhibition	1000 μM	Synaptosomes, from pre-frontal cortex	Mouse	Griffiths et al. (1989)[38]
$GABA_A$ receptor binding	K_i(apparent) : >10 mM	Synaptic membranes	Cow	Egubta & Griffiths (1987) [25]
Inhibition of high affinity GABA uptake	IC_{50} >5 mM	Synaptosomes [whole brain]	Rat	Allen et al. (1986) [3]
Inhibition of high affinity taurine uptake	IC_{50}: 2.8 mM	Synaptosomes [whole brain]	Rat	Allen et al. (1986)[3]
Inhibition of high affinity taurine	IC_{50}: 4.8 mM	Astrocytes from cerebral cortex	Rat	Allen et al. (1986)[3]

uptake				
Inhibition of MMP-2 activity	IC_{50}: 727 μM	Brain tissue homogenate	Pig	Emara & Cheung (2006)[27]
Inhibition of MMP-9 activity	IC_{50}: 2.35 mM	Brain tissue homogenate	Pig	Emara & Cheung (2006)[27]
Inhibition of kynurenic acid production	IC_{50}: 6.4 mM	Cortical neurons	Rat	Luchowska et al. (2005)[75]

Abbreviations. MMP, matrix metalloproteinase; PAG, Phosphate-activated glutaminase.

Neurotoxicity and morphological changes. Incubation of mesencephalic tegmental neurons from rats with HCy (100μM) for 48 hours produced morphological changes in the neurites of the tyrosine hydroxylase positive neurons (assumed to be dopaminergic)[46]. Fewer and shorter neurites were observed, although there were no significant effects on cell numbers *[46]*. This suggests that HCy exhibits a mild toxicity towards these dopaminergic neurons at a concentration of 100μM. In vivo injection of HCy into the left ventricle produced dose-dependent neuronal loss [124], with marked neuronal loss. These effects may be mediated by the NMDA and metabotropic glutamate receptors, as co-administration with antagonists of these receptors attenuated the neurotoxic effects of HCy [124].

Neural network activity suppression. HCY reduced neural network activity in spontaneously active embryonic rat cortical neurons (as measured by basal spontaneous spike rate). The IC_{50} for HCy (401μM) was above the range for severe HHCy, so the authors concluded that these

effects were not clinically relevant. Homocysteate, by contrast, had an IC_{50} of 1.3μM. Homocysteate is often found to be elevated in patients with HHCy [37] and HCA may be a clinically relevant species.

Lipid peroxidation. HCy (100-1000μM) increased lipid peroxidation in rat brain synaptosomes in a concentration-dependent manner [54]. This action was prevented by an NMDA receptor antagonist [54] supporting a glutamate receptor dependent pathway for HCy-mediated neuronal damage.

Several other cellular actions of HCy are reported, with effective concentrations in the millimolar range, (Table 2). These are unlikely to be relevant to clinical HHCy.

Homocysteate and Homocysteic acid. The physiological metabolic pathways of HCA (see Table 1 for structure) are relatively little known. There is evidence that HCy may be oxidised to form HCA within brain and other tissues [37]. Homocysteate has neurotoxic effects, independent of HCy [37,46]. These appear to be due to NMDA and metabotropic glutamate receptor-dependent excitotoxicity (see Supplementary table S1)[26,36,93,96]. HCA is clearly more potent than HCy in some cellular actions [37,38,46]. While HCA/homocysteate may participate in some of the cytotoxic processes observed in HHCy, native HCA levels appear to be low.

2.2 Effects of HCy on vascular cells

Cell damaging effects of HCy on blood vessels have been suggested for many years. Mild-moderate HHCy could directly cause cell damage in vivo, or could represent a secondary

event or biomarker of a pathological process. The salient cellular actions of HCy at clinically relevant concentrations are listed in Table 3 and are discussed below.

The effects reported at extracellular HCy concentrations below 100 µM suggest that direct HCy cytotoxicity may be relevant in vivo. We note with caution that there is little information on the clinically relevant range of intracellular HCy concentrations (in physiological conditions, approximately 100 µM). Amino acid transporters are present in vascular smooth muscle cells and endothelial cells, with high affinity for HCy (K_M 40-100 µM)[56].

Table 3. HCy effects in vascular cells

	Effective concentration	Cell type	Species	References
In vascular smooth muscle cells				
Increased cell proliferation	50 -100 µM	Thoracic aortic VSMC	Chick	Dalton et al. 1997 [17]
Increased protein kinase C activity	3 -100 µM	Thoracic and abdominal aorta VSMC	Chick	Dalton et al. 1997 [17]
Increased ERK2 activity	0.1-100 µM, EC_{50} 0.5 µM	Thoracic and abdominal aorta VSMC	Chick	Brown et al. 1998[7]
Increased CTGF release, Collagen I	50-500 µM	Umbilical vein VSMC	Human	Liu et al. 2008[73] Majors

and fibronectin synthesis				et al. 1997[76]
Lysyl oxidase inhibition (by HCy lactone)	5 – 50 µM, K_i 20 µM	Whole aorta	Calf	Liu et al. 1997 [72]
In endothelial cells				
Reduced cell proliferation	10 – 50 µM, EC_{50} 10-20 µM	HUVEC	Human	Wang et al. 1997[119]
Downregulation of cyclin A	50 µM	HUVEC	Human	Jamaluddin et al. 2007 [53]
Inhibition of endothelial NOS	50 – 200 µM	Thoracic aorta endothelial cells	Human & Mouse	Jiang et al. 2005[57]
Inhibition of endothelial-dependent vasodilatation	10 – 100 µM, EC_{50} 10 µM	Coronary artery endothelial cells	Pig	Chen et al. 2002 [11]
Increased superoxide formation	30 -1000 µM	Aortic endothelial cells	Pig	Lang et al. 2000 [67]
Increased release of MMP-9, loss of Integrin-1	12 - 40 µM, EC_{50} 20 µM	Cerebral microvessel endothelial cells	Mouse	Shastry & Tyagi 2004[103]
Reduced binding	10 µM	Aortic	Pig	Nishinaga et al.

of anti-thrombin III		endothelial cells		1992 [86]
Other actions				
Increased MCP-1, IL-6 release	10 -1000 µM, EC_{50} 30 µM	Aortic adventitial fibroblasts	Rat	Liu et al. 2012[74]
Cell transformation of fibroblasts to myo-fibroblasts	10-100 µM	Aortic adventitial fibroblasts	Rat	Liu et al. 2012[74]
Reduced platelet-fibrinogen binding	1 - 10 µM	Whole blood	Human	Riba et al. 2004[95]
Reduced ApoA-fibrin binding	8 - 2000 µM, EC_{50} ~ 100 µM	Fresh plasma	Human	Harpel et al. 1992 [43]
Increased MCP-1, IL-8 release from blood monocytes	10 -1000 µM, EC_{50} 10-30 µM	Whole blood	Human	Zeng et al. 2003 [125]

Abbreviations. ApoA, apolipoprotein-A. CTGF, connective tissue-derived growth factor. ERK2, Extracellular signal-regulated kinase. HUVEC, human umbilical vein endothelial cells. IL-6, -8, interleukin-6, -8. MCP-1, monocyte chemotactic protein-1. MMP-9, matrix metalloproteinase-9. NOS, nitric oxide synthase. VSMC, vascular smooth muscle cells.

2.2.1 Vascular smooth muscle cells

Cell proliferation. Moderate concentrations of HCy (10-100 μM) enhanced proliferation of vascular smooth muscle cells (VSMC) in primary cultures [17,76]. In VSMC cultured from embryonic thoracic aorta, acute HCy-induced cell proliferation was associated with induction of c-fos and c-myb, and translocation of protein kinase C (PKC) to the plasma membrane [17]. In agreement with a PKC-dependent mechanism, HCy concentrations as low as 3 μM (range 3-100 μM) stimulated diacylglycerol synthesis in these cells [17]. The growth-related MAP kinase ERK2 was also rapidly activated by very low concentrations of HCy (EC_{50} 0.5 μM) in aortic VSMC cultured from chick embryos [7]. How this action relates to normal adult physiology (with ambient plasma HCy well above this concentration) is unclear.

Mitogenic actions of acute HCy treatment were seen in myocyte cultures derived from aorta of adult animals, but only as higher concentrations (500-1000 μM)[112,113]. These agree with earlier in vivo studies in nonhuman primates undergoing chronic intravascular infusion with HCy [42]. In these animals steady state plasma HCy of 160 μM produced intimal lesions with endothelial cell loss and myointimal cell proliferation, resembling arteriosclerosis [42]. The cell proliferative effect of HCy is clearly not a generalized response, as HCy suppresses cell proliferation in endothelial cells, (see 2.2.2 below). HCy may also influence cell fate. HCy (10-100 μM) treatment of adventitial fibroblasts cultured from rat aorta stimulated transformation to myofibroblast-like phenotype (expression of the myocyte-associated proteins smooth muscle actin and SM22α) [74].

Fibrosis and collagenosis. HCy treatment potently increased collagen synthesis in smooth muscle cells in vitro (over a concentration range 50 - 500 μM) [76]. In accord with a pro-fibrotic action, treatment of human VSMC cultures with a moderate concentration of HCy (50 μM) increased expression of the pro-fibrotic growth factor CTGF, collagen type I, and

fibronectin [73]. In accord with effects of HCy on vessel fibrosis, HCy lactone (see Table 1) irreversibly inhibited lysyl oxidase (K_i 21 μM) [72]. This enzyme catalyses cross-linking between elastin and fibrillar collagen, and is essential for growth and repair of connective tissue matrices.

2.2.2 Endothelial effects of homocysteine

Mild-moderate HHCy is associated with endothelial dysfunction in humans [10,44,109], experimental primates[71], and rodents [18,24,59,68,108,122].

Inhibition of cell proliferation. HCy suppresses cell proliferation in endothelial cells, contrary to its proliferative action in vascular myocytes. In preparations of human aortic endothelial cells (HAEC), pig aortic endothelial cells and HUVEC, tritiated thymidine incorporation was inhibited dose-dependently by modest HCy concentrations (10 to 50 μM; 24hr exposure) [119]. The half-maximal concentration for L-HCy was approximately 10 μM in these various endothelial preparations, with D,L-homocysteine showing roughly 2-fold lower potency, and L-cysteine without effect, indicating a specific action of L-HCy [119]. HCy-treated HAEC had greatly increased levels of s adenosyl homocysteine (SAH; which is a potent inhibitor of methyl transferase enzyme activity) and reduced carboxyl methylation of p21-ras. Inhibition of endothelial proliferation has also been reported at higher HCy concentrations (100 to 1000 μM) [112].

At these high concentrations, caspase-dependent cell death is seen (500 - 1000 μM) [69]. This pro-apoptotic action of high HCy concentration was abrogated by the nitric oxide donor SNAP, the antioxidant alpha-tocopherol, or by an antioxidant mixture of superoxide

dismutase and catalase [69]. High concentrations of HCy (>200 μM) produced endoplasmic reticulum (ER) stress in endothelial cell cultures [89].

Inhibition of endothelial nitric oxide synthase (eNOS). In cultures of aortic endothelial cells from adult mice, moderate concentrations of HCy (range 5 - 200 μM) inhibited eNOS activity, evidenced by depressed L-citrulline and nitrite formation [57]. This accords with in vivo data showing lower eNOS activity in *Cbs* null mice relative to wildtype mice, and intermediate eNOS activity in $Cbs^{+/-}$ heterozygotes [57]. Similarly, in coronary artery rings from adult pigs application of HCy (10 - 100 μM) reduced endothelium-dependent vasodilation [11]. Endothelium-independent vasodilation (in response to the NO donor nitroprusside) was unaffected, supporting an endothelial action for HCy. Downregulation of eNOS expression was also seen in human aortic endothelial cells but only at higher HCy concentration (200 μM)[57]. By way of caution, the endothelial preparations in most of these studies came from large arteries, and may not entirely reflect microvascular signalling.

Gene induction effects. Screening for gene induction in endothelial cells following HCy exposure revealed several candidates, [65,102]. In microvascular endothelial cells derived from mouse brain, treatment with moderate concentrations of HCy (up to 40 μM, 24 hours) increased activity of the matrix metalloproteinase MMP-9 concentration-dependently, while growth factor synthesis decreased (VEGF, FGFα, leptin) [103]. Over the same HCy concentration range, shedding of β1 integrin from the endothelial cell surface was also seen[103]. Such effects in intact blood vessels could undermine the extracellular matrix integrity in the intimal layers around endothelial cells, and contribute to the anti-proliferative effects of HCy in endothelial cells.

2.2.3 Homocysteine actions on blood components

Acute exposure to physiological concentrations of HCy (less than 10 µM) increased the affinity and degree of specific binding for fibrin to Lp(a), a complex that includes Apolipoprotein-A and shares structural homology with plasminogen [43]. HCy increased binding by plasmin-modified fibrin (20 fold) and by native fibrin (4 fold). This may be a thiol-dependent action as other small thiol compounds (cysteine, GSH) had a similar effect, whereas sulphur containing compounds that lack the free -SH group including methionine and SAH had no effect. Low HCy (10 µM) also greatly reduced binding of anti-thrombin III to endothelial cells derived from porcine aorta [86].

The pro-coagulant effect of modest levels of HHCy was evident in patients with peripheral occlusive arterial disease (diagnosed as intermittent claudication). In isolated platelets taken from patients with higher plasma HCy (average 19 µM) the inhibitory effect of a nitric oxide donor (GSNO, 1 µM) on platelet-fibrinogen binding was 5-fold lower than that of subjects with normal HCy (average 11 µM)[95]. In HUVEC cultures, binding of tissue plasminogen activator (tPA) to the cell surface was inhibited by high concentrations of HCy (up to 1.5 mM) [41].

3. Experimental animal models relevant to elevated homocysteine

Induction of HHCy in an animal model can be achieved via genetic manipulation or diet. Here we outline animal models that have shown HHCy, sufficient to cause cognitive deficits and vascular adverse events in the brain. Here we discuss transgenic mouse strains with mutations in the genes that underlie severe HHCy in humans (*CBS*, *MTHFR*), and animals

with diet-induced HHCy. Further information on animal models is given in previous reviews [19,58,110].

3.1 Cystathionine beta synthase (CBS) converts HCy to cystathionine, which is then converted to cysteine, during normal biochemical processing. In humans, deficiencies in CBS result in severe HHCy and increased risk of thrombosis, and are the most common cause of hereditary HHCy. While homozygous *Cbs* knockout mice [121] have a neonatal lethal phenotype, inducible *Tg-hCBS* mice avoid this problem [39]. In *Tg-hCBS* mice, human *CBS* is under the control of a zinc-inducible promoter. During pregnancy and lactation drinking water is supplemented with Zn^{2+} to rescue the neonatal lethal phenotype[120]. At weaning, the zinc supplementation is withdrawn and the progeny develop HHCy (plasma HCy 170 µM, relative to 5 µM in control animals). Another CBS transgenic model reproduces the I278T mutation, which is the second most common allele found in *CBS*-deficient patients[66]. *Tg-I278T Cbs*$^{-/-}$ mice have less than 3% wildtype CBS activity and develop HHCy (plasma HCy 290 µM). *Tg-I278T Cbs*$^{-/-}$ mice also exhibit facial alopecia, osteoporosis, ER stress in the liver and kidney, and reduced mean survival, *Tg-hCBS* mice do not show any of these phenotypic changes. Although thrombosis is the most common symptom of CBS deficiencies in humans, neither mouse model exhibits any thrombotic or vascular defects.

Cbs$^{+/-}$ heterozygous mice have a 50% lower CBS activity compared to wildtype mice, and develop mild HHCy on normal diet (plasma HCy 6-8 µM) [4,59,121], augmented by high methionine diet for 8-20 weeks (plasma HCy ~20 µM)[4]. *Cbs*$^{+/-}$ heterozygote mice exhibit substantial vascular dysfunction. They have thickened arteries, including cerebral arteries, with evidence of endothelial damage [4,59]. Cerebral arteriolar walls are 25% thicker in *Cbs*$^{+/-}$ heterozygote mice, relative to WT controls [4]. This was accompanied by mild hypertension and blood-brain barrier dysfunction [4]. This vasomotor dysfunction is primarily based on endothelial dysfunction, and is largely attributed to redox imbalance and

decreased bioavailability of NO. Compared to *Cbs* null mice, *Cbs*$^{+/-}$ mice display a milder HHCy and may be a more useful model to study hyperhomocysteinemic effects on the vasculature.

3.2 Methylenetetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme in conversion of HCy to methionine. In humans, several *MTHFR* polymorphisms produce HHCy and neurological conditions, including progressive demyelinating neuropathy and cognitive impairment. *MTHFR* knockout mice [13] exhibit elevated plasma HCy . Similar to the *Cbs* mouse model, *MTHFR*$^{+/-}$ heterozygotes exhibit mild HHCy (plasma HCy ~20 μM, relative to 3-5 μM in wildtype littermates)[80] and appear outwardly healthy, while *MTHFR*$^{-/-}$ homozygotes (plasma HCy 31-33 μM, relative to 3 μM in WT animals) survive poorly and present with motor and gait abnormalities within 5 weeks after birth. *MTHFR*$^{+/-}$ mice exhibit some loss of function in cerebral vessels [85] and high intramural collagen in peripheral arteries [85]. *MTHFR*$^{-/-}$ homozygotes also present with abnormal lipid deposition in the aorta and disruption of the laminar structure of the cerebellum (though with no obvious changes in the cerebral cortex or cerebrum).

3.3 Dietary induction of HHCy in mice and rats can be achieved through either a reduction in vitamins B6, B9 and B12, or by diets supplemented with HCy or methionine [4,18,68,108,111]. Vascular dysfunction has been reported in primates (*Cynomolgus* monkeys) following diet-induced mild-moderate HHCy (plasma HCy 11 μM, relative to 4 μM on normal diet) [70,71]. (Earlier studies in baboons employed direct infusion of HCy [42]). Responses of resistance vessels to endothelium-dependent vasodilators were markedly impaired in HHCy monkeys, and anticoagulant thrombomodulin activity in the aorta decreased by 34 % [71].

Vitamins B6, B9 and B12 act as essential cofactors for the conversion of HCy to methionine or cysteine, combined with enrichment in dietary methionine, causes an increase in conversion to HCy. In 6-month-old rats fed a folate-deficient diet for 8 weeks, increased plasma HCy (increased from 6 µM to 19 µM) was accompanied by ultrastructural changes to cerebral capillaries, endothelial damage, swelling of pericytes, basement membrane thickening and fibrosis [63]. Rats fed a diet high in HCy for 5 or 15 months (plasma HCy 26 µM at both time-points, relative to 10 µM in controls) both showed cognitive impairments, decreased acetylcholine in the brain and cortical micro-hemorrhages[91]. In wild-type mice fed a B-vitamin deficient diet, cognitive deficits resulted from relatively-brief (10 week) diet-derived mild HHCy (plasma HCy 35 µM, relative to 5 µM on normal diet) [111]. The authors found a decline in spatial learning (water maze task) accompanied by rarefaction of hippocampal blood vessels and of microglia [111]. Aged Tg2576 mice on HCy-elevating diet (plasma HCy 27 µM, relative to 5 µM in controls) also showed deficits in spatial reference memory, but not working memory tasks (delayed non-match to position)[5].

Plasma HCy levels were also increased in 3-month-old mice that were fed a diet deficient in vitamins B6, B9 and B12 and also enriched in methionine for 3 months [108]. These mice exhibited cognitive impairments on the two-day radial arm water maze, cerebral microhemorrhages and increased MMP2 and MMP9 activity. This dietary model results in plasma HCy in the range 70-90 µM [108]. While these concentrations are higher than most human subjects classified under mild-moderate HHCy, this level is classified as moderate HHCy in mice [29].

4. Overview of clinical interventional trials relevant to stroke and dementia

If the hypothesis that HHCy causes cognitive decline is correct, then lowering plasma HCy concentrations (e.g. by B vitamin supplementation) should slow cognitive decline and possibly prevent dementia. Multiple clinical trials have tested B vitamin administration (folic acid, vitamins B_6 and B_{12}) with a cognitive endpoint. There have been three meta-analyses of these intervention trials [15,33,118].

4.1 Meta-analyses of clinical trials.

Three meta-analyses [15,33,118] will be considered here, with respect to the following important features related to trial design [78].

i) The hypothesis being tested. If the hypothesis is that treatment slows cognitive decline, then the placebo group must exhibit cognitive decline. Unless the placebo group declines, the trial can only show whether the treatment enhances or worsens cognition.

ii) Age range of the test cohort. If cognitive decline is the outcome, the age of the subjects should be in the range where cognitive decline occurs.

iii) Trial duration. Is this sufficient to observe cognitive decline? In cognitively healthy elderly people, MMSE declines by only about 0.1 points per year on average.

iv) The instrument used to assess a treatment effect. Is the instrument sensitive enough to detect change over the period of the trial? The instrument may display ceiling or floor effects, which limit the detectable effect size. Points (ii), (iii) and (iv) will together dictate whether the trial has sufficient power to detect a real effect.

v) Is the intervention appropriate? For B vitamins, the daily doses should be sufficient to lower plasma HCy by at least 20%.

vi) Is the group likely to respond to intervention? A B-vitamin supplement will produce no effect if the participants already have adequate B vitamin status. Such trials should

be limited either to subjects with high plasma HCy or insufficient B vitamin function. Alternatively, subgroup analyses should be performed to stratify for baseline values of B vitamins and plasma HCy.

The majority of the 26 trials analysed in the three meta-analyses [15,33,118] did not fulfil these requirements. Furthermore, by combining data from different trials (e.g. by using standardised mean differences) the meta-analyses have neutralised some of these requirements, even though they were fulfilled in individual trials. These three meta-analyses [15,33,118] have recently been appraised by others [78] and are outlined below.

In one analysis 7 of the 9 trials were of short duration (≤ 12 months; the two shortest lasted 1 month) with an overall median duration of 6 months [118]. In the trial with the largest number of participants (n=910), which accordingly was given a strong weighting, the doses of folic acid (0.2 mg) and vitamin B12 (1 μg) were too low to influence plasma HCy. In a trial where the doses of vitamins were adequate [79], there was no significant cognitive decline in the placebo group (mean MMSE 29.17 at baseline, 29.32 after 2 y). Thus, the conclusion that folic acid has no effect on the prevention of age-related cognitive decline [118] may be false. The authors concluded that longer trials of folate supplementation are needed, with larger cohorts and with cognitive decline and dementia as outcomes [118].

A larger meta-analysis combined 19 trials (n= 5,398 participants) [33] of which 8 were in the previous analysis [118]. In this analysis, only 6 of the trials (n=785 participants) were performed on subjects with cognitive impairment and the results were reported separately for this group and for participants without cognitive impairment. This study concluded "It remains to be established whether chronic treatment with B-vitamins is associated with better

cognitive outcomes, or if their use could reduce the risk of dementia in later life" [33]. This meta-analysis also suffered from lack of statistical power to detect differences associated with small effect sizes (<0.2) between the supplemented and placebo groups. Another limitation is that the analysis combined data from 62 different cognitive tests, by converting the individual test scores to standard deviation scores, in order to compute standardised mean differences. Thus, there was considerable clinical heterogeneity in the combined data. In order to analyse the data, the authors assumed zero correlation between the assessments at baseline and study endpoint. This may be a serious limitation, as baseline cognitive test performance is a well-known predictor of later test performance. Thus, in any analysis of cognitive decline it is imperative to use a model that controls for the baseline value (which is not possible with the widely-used standardised mean difference method). A further limitation of this method is that it depends upon the *t* test and so the analysis cannot be adjusted for important co-variates (e.g. gender and education) that are known to influence cognitive change.

A third meta-analysis[15] aimed to test the effect of lowering plasma HCy on 'cognitive ageing' (though this was not defined). The analysis considered 11 trials (n=22,000) of which 5 had been included in one or both of the previous two studies, and concluded "B-vitamin supplementation caused HCy-lowering but was without significant effect on cognitive function or cognitive aging"[15]. Trials on subjects with a prior diagnosis of cognitive impairment or dementia were specifically excluded[15]. Furthermore, in most subjects in the included trials, cognitive decline could not be measured since baseline global cognitive measures were not available for 76% of subjects. It is therefore unclear what can be concluded from such trials, apart from a lack of a cognitive enhancing (or a harmful) effect of B vitamins.

4.2 The FACIT and WAFACS trials

Two of the trials (n=2,825) that were included in the above meta-analyses did report cognitive scores at baseline. Both these trials showed significant effects of B vitamins on cognitive change in those subjects with high plasma HCy [23], or with poor B vitamin status [60]. The FACIT trial (n=818; mean age 60 y) recruited people from the Netherlands with plasma HCy was between 13 and 26 μM, but with adequate vitamin B12 status[23]. Folic acid (0.8 mg/day) was administered for 3 y. At the end of the trial plasma HCy concentration was 26% lower than in the placebo group. The trial assessed several cognitive domains and found that performance on sensorimotor speed, information processing speed, and complex speed had all declined in the placebo group after 3 y but that the decline was slowed by folic acid treatment. There was an improvement in memory performance in the placebo group (likely due to learning effect) but a greater degree of improvement occurred in the folic acid group. Global cognitive performance also improved more in the folic acid group. Notably, there was a larger beneficial effect of folic acid on information processing speed in those whose base-line plasma HCy was above the median than in those below the median. The authors estimated that folic acid treatment conferred cognitive performance of someone 4.7 y younger for memory, 1.7 y younger for sensorimotor speed, 2.1 y younger for information processing speed, and 1.5 y younger for global cognitive function. Overall, this trial was consistent with the view that lowering plasma HCy slows decline in those cognitive domains that are sensitive to ageing[23].

In the US-based WAFACS trial (n=5442, all female, age >40y) a daily combination of B vitamins (2.5 mg folate, 50 mg B6, 1 mg B12) was tested for secondary prevention of cardiovascular disease [60]. Cognitive testing was carried out in a substudy (n=2009,

age>65y). While cognitive change from baseline did not differ between the vitamin-treated and placebo groups in the cohort as a whole, there was a significant protective effect in those participants with low baseline dietary intake of folate [60].

4.3 The VITACOG trial

VITACOG was designed to test whether lowering plasma HCy would slow the accelerated rate of brain atrophy that occurs in people with MCI [106]. Volunteers (n = 168) with MCI were randomized to placebo or a daily combination of folic acid (0.8 mg), vitamin B12 (0.5 mg) and vitamin B6 (20 mg) for two years. Volumetric MRI scans were performed at the start and end of the trial. B vitamin treatment led to a highly significant slowing in the rate of global brain atrophy (average 30%). The effect of B vitamin treatment depended on baseline plasma HCy, with a 53% slowing of atrophy in those in the top quartile (> 13 μM)[106].

A further analysis of the MRI scans from VITACOG subjects revealed strong effects of B vitamin treatment on regional brain atrophy in MCI [22]. There was 7-fold less regional brain atrophy in the B vitamin group compared with the placebo group, though this effect was significant only in people with plasma HCy above the median. The gray matter regions protected by B vitamin treatment included: structures of the medial temporal lobe, the precuneus, angular gyrus and supramarginal gyrus, all regions known to be vulnerable to the AD disease process. It was concluded that the B vitamin treatment had slowed the atrophy of AD-related regions of the brain, in people with raised plasma HCy [22].

VITACOG was not powered to detect any effect of homocysteine-lowering on cognition. Nevertheless, a pre-specified analysis stratifying for plasma HCy showed that in people with

baseline plasma HCy above the median (11.3 μM), cognitive decline was virtually prevented in the following domains: episodic memory, semantic memory and global cognition (MMSE) [20].

4.4 Importance of initial homocysteine concentration.

The VITACOG trial implies a threshold effect of plasma HCy on biological outcomes such as brain atrophy and cognition. Slowing of brain atrophy and of cognitive decline occurred only in people with plasma HCy > 11 μM, while improvement in clinical measures was only found with plasma HCy > 13 μM. These findings are consistent with previous observations. For example, there is a concentration-related (> 10 μM) association of plasma HCy with incidence of dementia. In the OPTIMA study, only concentrations above 11 μM were associated with an increased rate of atrophy of the medial temporal lobe[16]. At concentrations > 10 μM there was a concentration-dependent increase in the rate of cognitive decline in AD patients under 75 y [88]. If the threshold for effects of plasma HCy is about 10-11 μM that could explain why some studies, for example in countries that already employ mandatory folic acid fortification, do not find associations of plasma HCy with cognitive or brain outcomes. Thus, an 18-month trial of high-dose B vitamins in patients with AD in the USA (baseline plasma HCy 9.16 μM) did not find an overall effect of treatment on cognitive decline[1]. It should be noted that those patients with mild AD did show a protective effect of B vitamins after 15 months, which may indicate that timing of HCy-lowering intervention is critical.

4.5 Interactions with other risk factors.

Retrospective analysis of the VITACOG data revealed an interaction with omega-3 fatty acids. It emerged that the protective effect of B vitamin treatment on both brain atrophy and

cognitive decline in MCI only occurred in those participants with a good status of long-chain omega-3 fatty acids [55]. We also observed that the beneficial effect of B vitamins on brain atrophy was absent in subjects taking aspirin [106]. These results emphasize the importance of considering risk factor interactions. This may be a reason why some trials of B vitamins have failed, if the treated population were taking aspirin regularly, as in many trials included in the meta-analysis by Clarke et al.[15], or had a poor omega-3 fatty acid status. Further trials are needed to test B vitamins in combination with omega-3 fatty acids, in people not taking regular aspirin.

4.6 Future trials of B vitamin supplementation for HHCy lowering

In our view further trails of combined B vitamin supplementation are called for. These should incorporate the lessons from previous trails, and from recent experimental work. In order to maximise likelihood of treatment effect, future trials should aim to target individuals who are in the at-risk age range, but not demented. Cohorts with inadequate baseline B vitamin status should be targeted, and a full combination supplement (B6, B12 and folate) supplied at high dose. With the benefit of extant trial data, accurate power analyses should be possible, to allow conservative design in terms of sufficient cohort size and treatment duration.

A possible scenario could be a government-sponsored scheme within areas of social deprivation, where long-term vitamin inadequacy is prevalent. Participants would be randomised to a highly-flavoured tablet formulation, such as a vitamin C preparation, without B vitamins (placebo) or with a substantial dose of B_6/B_{12}/folate (treatment). Recruitment would be offered to all older persons in a public setting. Recruitment, registration and supply would be at a routine public forum, such as weekly pension collection, or GP drop-in clinics for cardiovascular monitoring.

For a future trial in VCID, suitable endpoints may be MRI-derived measures of disease progression [99]. Such surrogate biomarkers may be of utility in trials for VCID for two main reasons. First, a large proportion of individuals who exhibit VCID and VCID biomarkers will not convert to dementia. Second, MRI-based endpoints will permit a test of efficacy in a smaller cohort than would be required to detect a difference in cognitive decline.

4.7 Preclinical studies lowering Hcy levels.

While no animal studies have been done to lower Hcy levels and test cognition or neuropathology, some animal studies have been done with the aim of studying cardiovascular effects of Hcy lowering. One study used the CBS-/- genetic model, induced hypercholesterolemia, and then targeted CBS expression using a viral vector to restore low Hcy levels. Using this approach, the investigators found that the restoration of low Hcy levels improved infarct healing and attenuated remodelling after a myocardial infarction [83]. The same group used the same genetic approach in a model of pressure overload-induced cardiomyopathy and found that Hcy lowering reduced mortality and lowered oxidative stress [84]. A few studies have examined the effects of various B-vitamins, the most common being folic acid, on learning and memory, however, these studies did not examine homocysteine levels [8,12].

5. Conclusions

The molecular targets of HCy that are relevant to VCID are not fully defined. While numerous biochemical and cellular actions have been reported, on inspection most resulted from HCy concentrations above the clinically relevant range of 10-100 μM. Reported actions

of HCy below 100 μM were primarily vascular. These included: PKC activation and proliferation of vascular myocytes; vessel wall fibrosis and collagenosis; reduced proliferation and suppression of nitric oxide signalling in endothelial cells; heightened superoxide formation, and release of inflammatory mediators (MCP-1, IL-6, IL-8, MMP9); various pro-coagulant actions on different blood components (fibrinogen, ApoA, antithrombin-III). Mouse models of HHcy and the extensive body of epidemiology data indicate a strong relationship between HHcy and cognition. Further, it is clear from the mouse studies that HHcy can influence AD pathology, a further consideration for clinical studies. Future studies are clearly required to fully characterize and identify the key molecular pathways linking Hcy to VCID.

At least 19 clinical trials relevant to VCID have tested HCy-lowering interventions. Many were compromised by the challenges of performing a cognitive clinical trial (trial duration, statistical power, cohort age, B vitamin status). Three trials supported a beneficial effect (FACIT, WAFACS, VITACOG). The question as to whether or not lowering plasma HCy slows cognitive decline requires further well-designed trials. The extensive clinical and preclinical data strongly support Hcy as a key mediator for VCID. The challenge is now to design clinical studies that fully address this link. Preclinical animal model studies will be essential for the identification of the appropriate time-points when Hcy effects can be manipulated and reversed.

Figure Legend

Figure 1. Biochemical Pathways linking homocysteine and B vitamins.

Methionine is converted to homocysteine by methylation and subsequent hydrolysis. Homocysteine is then either cycled back to methionine via the folate cycle, catalysed by methylenetetrahydrofolate reductase (MTHFR) and the essential cofactor vitamin B_{12}. Alternatively homocysteine can be further metabolized to cysteine via cystathionine beta synthase (CBS) and the essential cofactor vitamin B_6. Homocysteine conversion to cysteine occurs primarily in the liver. All other reactions are ubiquitous. For further details on HCy biochemistry, see [52,115]. SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine.

References

[1] P.S. Aisen, L.S. Schneider, M. Sano, R. az-Arrastia, C.H. van Dyck, M.F. Weiner, T. Bottiglieri, S. Jin, K.T. Stokes, R.G. Thomas, L.J. Thal, High-dose B vitamin supplementation and cognitive decline in Alzheimer disease: a randomized controlled trial, JAMA 300 (2008) pp. 1774-1783.

[2] J. Albrecht, M. Dolinska, W. Hilgier, A.W. Lipkowski, J. Nowacki, Modulation of glutamine uptake and phosphate-activated glutaminase activity in rat brain mitochondria by amino acids and their synthetic analogues, Neurochem. Int. 36 (2000) pp. 341-347.

[3] I.C. Allen, A. Schousboe, R. Griffiths, Effect of L-homocysteine and derivatives on the high-affinity uptake of taurine and GABA into synaptosomes and cultured neurons and astrocytes, Neurochem. Res. 11 (1986) pp. 1487-1496.

[4] G.L. Baumbach, C.D. Sigmund, T. Bottiglieri, S.R. Lentz, Structure of cerebral arterioles in cystathionine beta-synthase-deficient mice, Circ. Res. 91 (2002) pp. 931-937.

[5] A. Bernardo, M. McCord, A.M. Troen, J.D. Allison, M.P. McDonald, Impaired spatial memory in APP-overexpressing mice on a homocysteinemia-inducing diet, Neurobiol. Aging 28 (2007) pp. 1195-1205.

[6] M.A. Beydoun, H.A. Beydoun, A.A. Gamaldo, A. Teel, A.B. Zonderman, Y. Wang, Epidemiologic studies of modifiable factors associated with cognition and dementia: systematic review and meta-analysis, BMC. Public Health 14 (2014) p.643.

[7] J.C. Brown, T.H. Rosenquist, D.T. Monaghan, ERK2 activation by homocysteine in vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 251 (1998) pp. 669-676.

[8] J. Budni, A.D. Zomkowski, D. Engel, D.B. Santos, A.A. dos Santos, M. Moretti, S.S. Valvassori, F. Ornell, J. Quevedo, M. Farina, A.L. Rodrigues, Folic acid prevents depressive-like behavior and hippocampal antioxidant imbalance induced by restraint stress in mice, Exp. Neurol. 240 (2013) pp. 112-121.

[9] J.P. Casas, L.E. Bautista, L. Smeeth, P. Sharma, A.D. Hingorani, Homocysteine and stroke: evidence on a causal link from mendelian randomisation, Lancet 365 (2005) pp. 224-232.

[10] J.C. Chambers, P.M. Ueland, O.A. Obeid, J. Wrigley, H. Refsum, J.S. Kooner, Improved vascular endothelial function after oral B vitamins: An effect mediated through reduced concentrations of free plasma homocysteine, Circulation 102 (2000) pp. 2479-2483.

[11] C. Chen, B.S. Conklin, Z. Ren, D.S. Zhong, Homocysteine decreases endothelium-dependent vasorelaxation in porcine arteries, J. Surg. Res. 102 (2002) pp. 22-30.

[12] T.F. Chen, R.F. Huang, S.E. Lin, J.F. Lu, M.C. Tang, M.J. Chiu, Folic Acid potentiates the effect of memantine on spatial learning and neuronal protection in an Alzheimer's disease transgenic model, J. Alzheimers. Dis. 20 (2010) pp. 607-615.

[13] Z. Chen, A.C. Karaplis, S.L. Ackerman, I.P. Pogribny, S. Melnyk, S. Lussier-Cacan, M.F. Chen, A. Pai, S.W. John, R.S. Smith, T. Bottiglieri, P. Bagley, J. Selhub, M.A. Rudnicki, S.J. James, R. Rozen, Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition, Hum. Mol. Genet. 10 (2001) pp. 433-443.

[14] Y.M. Choe, B.K. Sohn, H.J. Choi, M.S. Byun, E.H. Seo, J.Y. Han, Y.K. Kim, E.J. Yoon, J.M. Lee, J. Park, J.I. Woo, D.Y. Lee, Association of homocysteine with hippocampal volume independent of cerebral amyloid and vascular burden, Neurobiol. Aging 35 (2014) pp. 1519-1525.

[15] R. Clarke, D. Bennett, S. Parish, S. Lewington, M. Skeaff, S.J. Eussen, C. Lewerin, D.J. Stott, J. Armitage, G.J. Hankey, E. Lonn, J.D. Spence, P. Galan, L.C. de Groot, J. Halsey, A.D. Dangour, R. Collins, F. Grodstein, Effects of homocysteine lowering with B vitamins on cognitive aging: meta-analysis of 11 trials with cognitive data on 22,000 individuals, Am. J. Clin. Nutr. 100 (2014) pp. 657-666.

[16] R. Clarke, A.D. Smith, K.A. Jobst, H. Refsum, L. Sutton, P.M. Ueland, Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease, Arch. Neurol. 55 (1998) pp. 1449-1455.

[17] M.L. Dalton, P.F. Gadson, Jr., R.W. Wrenn, T.H. Rosenquist, Homocysteine signal cascade: production of phospholipids, activation of protein kinase C, and the induction of c-fos and c-myb in smooth muscle cells, FASEB J. 11 (1997) pp. 703-711.

[18] S. Dayal, T. Bottiglieri, E. Arning, N. Maeda, M.R. Malinow, C.D. Sigmund, D.D. Heistad, F.M. Faraci, S.R. Lentz, Endothelial dysfunction and elevation of S-adenosylhomocysteine in cystathionine beta-synthase-deficient mice, Circ. Res. 88 (2001) pp. 1203-1209.

[19] S. Dayal and S.R. Lentz, Murine models of hyperhomocysteinemia and their vascular phenotypes, Arterioscler. Thromb. Vasc. Biol. 28 (2008) pp. 1596-1605.

[20] C.A. de Jager, A. Oulhaj, R. Jacoby, H. Refsum, A.D. Smith, Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial, Int. J. Geriatr. Psychiatry 27 (2012) pp. 592-600.

[21] L.M. de Lau, J.B. van Meurs, A.G. Uitterlinden, A.D. Smith, H. Refsum, C. Johnston, M.M. Breteler, Genetic variation in homocysteine metabolism, cognition, and white matter lesions, Neurobiol. Aging 31 (2010) pp. 2020-2022.

[22] G. Douaud, H. Refsum, C.A. de Jager, R. Jacoby, T.E. Nichols, S.M. Smith, A.D. Smith, Preventing Alzheimer's disease-related gray matter atrophy by B-vitamin treatment, Proc. Natl. Acad. Sci. U. S. A 110 (2013) pp. 9523-9528.

[23] J. Durga, M.P. van Boxtel, E.G. Schouten, F.J. Kok, J. Jolles, M.B. Katan, P. Verhoef, Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomised, double blind, controlled trial, Lancet 369 (2007) pp. 208-216.

[24] R.T. Eberhardt, M.A. Forgione, A. Cap, J.A. Leopold, M.A. Rudd, M. Trolliet, S. Heydrick, R. Stark, E.S. Klings, N.I. Moldovan, M. Yaghoubi, P.J. Goldschmidt-Clermont, H.W. Farber, R. Cohen, J. Loscalzo, Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia, J. Clin. Invest 106 (2000) pp. 483-491.

[25] J.O. Egbuta and R. Griffiths, Binding of [3H]muscimol to calf cerebrocortical synaptic membranes and the effects of sulphur-containing convulsant and non-convulsant compounds, Neurochem. Res. 12 (1987) pp. 589-595.

[26] S. Eimerl and M. Schramm, Acute glutamate toxicity in cultured cerebellar granule cells: agonist potency, effects of pH, Zn2+ and the potentiation by serum albumin, Brain Res. 560 (1991) pp. 282-290.

[27] M. Emara and P.Y. Cheung, Inhibition of sulfur compounds and antioxidants on MMP-2 and -9 at the activity level found during neonatal hypoxia-reoxygenation, Eur. J. Pharmacol. 544 (2006) pp. 168-173.

[28] T. Erkinjuntti, A. Kurz, S. Gauthier, R. Bullock, S. Lilienfeld, C.V. Damaraju, Efficacy of galantamine in probable vascular dementia and Alzheimer's disease combined with cerebrovascular disease: a randomised trial, Lancet 359 (2002) pp. 1283-1290.

[29] S. Ernest, A. Hosack, W.E. O'Brien, D.S. Rosenblatt, J.H. Nadeau, Homocysteine levels in A/J and C57BL/6J mice: genetic, diet, gender, and parental effects, Physiol Genomics 21 (2005) pp. 404-410.

[30] M.M. Esiri, G.K. Wilcock, J.H. Morris, Neuropathological assessment of the lesions of significance in vascular dementia, J Neurol. Neurosurg. Psychiatry 63 (1997) pp. 749-753.

[31] M.S. Fernando, J.E. Simpson, F. Matthews, C. Brayne, C.E. Lewis, R. Barber, R.N. Kalaria, G. Forster, F. Esteves, S.B. Wharton, P.J. Shaw, J.T. O'Brien, P.G. Ince, White matter lesions in an unselected cohort of the elderly: molecular pathology suggests origin from chronic hypoperfusion injury, Stroke 37 (2006) pp. 1391-1398.

[32] M.J. Firbank, S.K. Narayan, B.K. Saxby, G.A. Ford, J.T. O'Brien, Homocysteine is associated with hippocampal and white matter atrophy in older subjects with mild hypertension, Int. Psychogeriatr. (2010) pp. 1-8.

[33] A.H. Ford and O.P. Almeida, Effect of homocysteine lowering treatment on cognitive function: a systematic review and meta-analysis of randomized controlled trials, J. Alzheimers. Dis. 29 (2012) pp. 133-149.

[34] M.O. Giwa, J. Williams, K. Elderfield, N.S. Jiwa, L.R. Bridges, R.N. Kalaria, H.S. Markus, M.M. Esiri, A.H. Hainsworth, Neuropathologic evidence of endothelial changes in cerebral small vessel disease, Neurology 78 (2012) pp. 167-174.

[35] P.B. Gorelick, A. Scuteri, S.E. Black, C. DeCarli, S.M. Greenberg, C. Iadecola, L.J. Launer, S. Laurent, O.L. Lopez, D. Nyenhuis, R.C. Petersen, J.A. Schneider, C. Tzourio, D.K. Arnett, D.A. Bennett, H.C. Chui, R.T. Higashida, R. Lindquist, P.M. Nilsson, G.C. Roman, F.W. Sellke, S. Seshadri, Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the american heart association/american stroke association, Stroke 42 (2011) pp. 2672-2713.

[36] A. Gorman and R. Griffiths, Sulphur-containing excitatory amino acid-stimulated inositol phosphate formation in primary cultures of cerebellar granule cells is mediated predominantly by N-methyl-D-aspartate receptors, Neuroscience 59 (1994) pp. 299-308.

[37] P. Gortz, A. Hoinkes, W. Fleischer, F. Otto, B. Schwahn, U. Wendel, M. Siebler, Implications for hyperhomocysteinemia: not homocysteine but its oxidized forms strongly inhibit neuronal network activity, J. Neurol. Sci. 218 (2004) pp. 109-114.

[38] R. Griffiths, A. Grieve, J. Dunlop, I. Damgaard, H. Fosmark, A. Schousboe, Inhibition by excitatory sulphur amino acids of the high-affinity L-glutamate transporter in synaptosomes and in primary cultures of cortical astrocytes and cerebellar neurons, Neurochem. Res. 14 (1989) pp. 333-343.

[39] S. Gupta, J. Kuhnisch, A. Mustafa, S. Lhotak, A. Schlachterman, M.J. Slifker, A. Klein-Szanto, K.A. High, R.C. Austin, W.D. Kruger, Mouse models of cystathionine beta-synthase deficiency reveal significant threshold effects of hyperhomocysteinemia, FASEB J. 23 (2009) pp. 883-893.

[40] A.H. Hainsworth, A.T. Oommen, L.R. Bridges, Endothelial cells and human cerebral small vessel disease, Brain Pathol. 25 (2015) pp. 44-50.

[41] K.A. Hajjar, Homocysteine-induced modulation of tissue plasminogen activator binding to its endothelial cell membrane receptor, J. Clin. Invest 91 (1993) pp. 2873-2879.

[42] L.A. Harker, J.M. Harlan, R. Ross, Effect of sulfinpyrazone on homocysteine-induced endothelial injury and arteriosclerosis in baboons, Circ. Res. 53 (1983) pp. 731-739.

[43] P.C. Harpel, V.T. Chang, W. Borth, Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: a potential biochemical link

between thrombosis, atherogenesis, and sulfhydryl compound metabolism, Proc. Natl. Acad. Sci. U. S. A 89 (1992) pp. 10193-10197.

[44] A. Hassan, B.J. Hunt, M. O'Sullivan, R. Bell, R. D'Souza, S. Jeffery, J.M. Bamford, H.S. Markus, Homocysteine is a risk factor for cerebral small vessel disease, acting via endothelial dysfunction, Brain 127 (2004) pp. 212-219.

[45] A. Hassan, B.J. Hunt, M. O'Sullivan, K. Parmar, J.M. Bamford, D. Briley, M.M. Brown, D.J. Thomas, H.S. Markus, Markers of endothelial dysfunction in lacunar infarction and ischaemic leukoaraiosis, Brain 126 (2003) pp. 424-432.

[46] I. Heider, V. Lehmensiek, T. Lenk, T. Muller, A. Storch, Dopaminergic neurotoxicity of homocysteine and its derivatives in primary mesencephalic cultures, J. Neural Transm. Suppl (2004) pp. 1-13.

[47] E. Hogervorst, H.M. Ribeiro, A. Molyneux, M. Budge, A.D. Smith, Plasma homocysteine levels, cerebrovascular risk factors, and cerebral white matter changes (leukoaraiosis) in patients with Alzheimer disease, Arch. Neurol. 59 (2002) pp. 787-793.

[48] Homocysteine Studies Collaboration, Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis, JAMA 288 (2002) pp. 2015-2022.

[49] B. Hooshmand, T. Polvikoski, M. Kivipelto, M. Tanskanen, L. Myllykangas, T. Erkinjuntti, M. Makela, M. Oinas, A. Paetau, P. Scheltens, E.C. van Straaten, R. Sulkava, A. Solomon, Plasma homocysteine, Alzheimer and cerebrovascular pathology: a population-based autopsy study, Brain 136 (2013) pp. 2707-2716.

[50] B. Hultberg, Extracellular concentration of homocysteine in human cell lines is influenced by specific inhibitors of cyst(e)ine transport, Clin. Chem. Lab Med. 42 (2004) pp. 378-383.

[51] D.V. Iosifescu, G.I. Papakostas, I.K. Lyoo, H.K. Lee, P.F. Renshaw, J.E. Alpert, A. Nierenberg, M. Fava, Brain MRI white matter hyperintensities and one-carbon cycle metabolism in non-geriatric outpatients with major depressive disorder (Part I), Psychiatry Res. 140 (2005) pp. 291-299.

[52] D.W. Jacobsen, Homocysteine and vitamins in cardiovascular disease, Clin. Chem. 44 (1998) pp. 1833-1843.

[53] M.D. Jamaluddin, I. Chen, F. Yang, X. Jiang, M. Jan, X. Liu, A.I. Schafer, W. Durante, X. Yang, H. Wang, Homocysteine inhibits endothelial cell growth via DNA hypomethylation of the cyclin A gene, Blood 110 (2007) pp. 3648-3655.

[54] A. Jara-Prado, A. Ortega-Vazquez, L. Martinez-Ruano, C. Rios, A. Santamaria, Homocysteine-induced brain lipid peroxidation: effects of NMDA receptor blockade, antioxidant treatment, and nitric oxide synthase inhibition, Neurotox. Res. 5 (2003) pp. 237-243.

[55] F. Jerneren, A.K. Elshorbagy, A. Oulhaj, S.M. Smith, H. Refsum, A.D. Smith, Brain atrophy in cognitively impaired elderly: the importance of long-chain omega-3 fatty acids and B vitamin status in a randomized controlled trial, Am. J. Clin. Nutr. 102 (2015) pp. 215-221.

[56] X. Jiang, F. Yang, E. Brailoiu, H. Jakubowski, N.J. Dun, A.I. Schafer, X. Yang, W. Durante, H. Wang, Differential regulation of homocysteine transport in vascular endothelial and smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 27 (2007) pp. 1976-1983.

[57] X. Jiang, F. Yang, H. Tan, D. Liao, R.M. Bryan, Jr., J.K. Randhawa, R.E. Rumbaut, W. Durante, A.I. Schafer, X. Yang, H. Wang, Hyperhomocystinemia impairs endothelial function and eNOS activity via PKC activation, Arterioscler. Thromb. Vasc. Biol. 25 (2005) pp. 2515-2521.

[58] N.S. Jiwa, P. Garrard, A.H. Hainsworth, Experimental models of vascular dementia and vascular cognitive impairment. A systematic review, J. Neurochem. 115 (2010) pp. 814-828.

[59] A.F. Kamath, A.K. Chauhan, J. Kisucka, V.S. Dole, J. Loscalzo, D.E. Handy, D.D. Wagner, Elevated levels of homocysteine compromise blood-brain barrier integrity in mice, Blood 107 (2006) pp. 591-593.

[60] J.H. Kang, N. Cook, J. Manson, J.E. Buring, C.M. Albert, F. Grodstein, A trial of B vitamins and cognitive function among women at high risk of cardiovascular disease, Am. J. Clin. Nutr. 88 (2008) pp. 1602-1610.

[61] U. Khan, C. Crossley, L. Kalra, A. Rudd, C.D. Wolfe, P. Collinson, H.S. Markus, Homocysteine and its relationship to stroke subtypes in a UK black population: the south London ethnicity and stroke study, Stroke 39 (2008) pp. 2943-2949.

[62] U. Khan, A. Hassan, P. Vallance, H.S. Markus, Asymmetric dimethylarginine in cerebral small vessel disease, Stroke 38 (2007) pp. 411-413.

[63] J.M. Kim, H. Lee, N. Chang, Hyperhomocysteinemia due to short-term folate deprivation is related to electron microscopic changes in the rat brain, J. Nutr. 132 (2002) pp. 3418-3421.

[64] R.P. Kloppenborg, M.I. Geerlings, F.L. Visseren, W.P. Mali, M. Vermeulen, Y. van der Graaf, P.J. Nederkoorn, Homocysteine and progression of generalized small-vessel disease: the SMART-MR Study, Neurology 82 (2014) pp. 777-783.

[65] K. Kokame, H. Kato, T. Miyata, Homocysteine-respondent genes in vascular endothelial cells identified by differential display analysis. GRP78/BiP and novel genes, J. Biol. Chem. 271 (1996) pp. 29659-29665.

[66] J.P. Kraus, M. Janosik, V. Kozich, R. Mandell, V. Shih, M.P. Sperandeo, G. Sebastio, F.R. de, G. Andria, L.A. Kluijtmans, H. Blom, G.H. Boers, R.B. Gordon, P. Kamoun, M.Y. Tsai, W.D. Kruger, H.G. Koch, T. Ohura, M. Gaustadnes,

Cystathionine beta-synthase mutations in homocystinuria, Hum. Mutat. 13 (1999) pp. 362-375.

[67] D. Lang, M.B. Kredan, S.J. Moat, S.A. Hussain, C.A. Powell, M.F. Bellamy, H.J. Powers, M.J. Lewis, Homocysteine-induced inhibition of endothelium-dependent relaxation in rabbit aorta: role for superoxide anions, Arterioscler. Thromb. Vasc. Biol. 20 (2000) pp. 422-427.

[68] H. Lee, J.M. Kim, H.J. Kim, I. Lee, N. Chang, Folic acid supplementation can reduce the endothelial damage in rat brain microvasculature due to hyperhomocysteinemia, J. Nutr. 135 (2005) pp. 544-548.

[69] S.J. Lee, K.M. Kim, S. Namkoong, C.K. Kim, Y.C. Kang, H. Lee, K.S. Ha, J.A. Han, H.T. Chung, Y.G. Kwon, Y.M. Kim, Nitric oxide inhibition of homocysteine-induced human endothelial cell apoptosis by down-regulation of p53-dependent Noxa expression through the formation of S-nitrosohomocysteine, J. Biol. Chem. 280 (2005) pp. 5781-5788.

[70] S.R. Lentz, M.R. Malinow, D.J. Piegors, M. Bhopatkar-Teredesai, F.M. Faraci, D.D. Heistad, Consequences of hyperhomocyst(e)inemia on vascular function in atherosclerotic monkeys, Arterioscler. Thromb. Vasc. Biol. 17 (1997) pp. 2930-2934.

[71] S.R. Lentz, C.G. Sobey, D.J. Piegors, M.Y. Bhopatkar, F.M. Faraci, M.R. Malinow, D.D. Heistad, Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia, J. Clin. Invest 98 (1996) pp. 24-29.

[72] G. Liu, K. Nellaiappan, H.M. Kagan, Irreversible inhibition of lysyl oxidase by homocysteine thiolactone and its selenium and oxygen analogues. Implications for homocystinuria, J. Biol. Chem. 272 (1997) pp. 32370-32377.

[73] X. Liu, F. Luo, J. Li, W. Wu, L. Li, H. Chen, Homocysteine induces connective tissue growth factor expression in vascular smooth muscle cells, J. Thromb. Haemost. 6 (2008) pp. 184-192.

[74] Z. Liu, H. Luo, L. Zhang, Y. Huang, B. Liu, K. Ma, J. Feng, J. Xie, J. Zheng, J. Hu, S. Zhan, Y. Zhu, Q. Xu, W. Kong, X. Wang, Hyperhomocysteinemia exaggerates adventitial inflammation and angiotensin II-induced abdominal aortic aneurysm in mice, Circ. Res. 111 (2012) pp. 1261-1273.

[75] E. Luchowska, P. Luchowski, R. Paczek, A. Ziembowicz, T. Kocki, W.A. Turski, M. Wielosz, J. Lazarewicz, E.M. Urbanska, Dual effect of DL-homocysteine and S-adenosylhomocysteine on brain synthesis of the glutamate receptor antagonist, kynurenic acid, J. Neurosci. Res. 79 (2005) pp. 375-382.

[76] A. Majors, L.A. Ehrhart, E.H. Pezacka, Homocysteine as a risk factor for vascular disease. Enhanced collagen production and accumulation by smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 17 (1997) pp. 2074-2081.

[77] H.S. Markus, Genes, endothelial function and cerebral small vessel disease in man, Exp. Physiol 93 (2008) pp. 121-127.

[78] A. McCaddon and J.W. Miller, Assessing the association between homocysteine and cognition: reflections on Bradford Hill, meta-analyses, and causality, Nutr. Rev. (2015).

[79] J.A. McMahon, T.J. Green, C.M. Skeaff, R.G. Knight, J.I. Mann, S.M. Williams, A controlled trial of homocysteine lowering and cognitive performance, N. Engl. J. Med. 354 (2006) pp. 2764-2772.

[80] L.G. Mikael, X.L. Wang, Q. Wu, H. Jiang, K.N. Maclean, R. Rozen, Hyperhomocysteinemia is associated with hypertriglyceridemia in mice with methylenetetrahydrofolate reductase deficiency, Mol. Genet. Metab 98 (2009) pp. 187-194.

[81] P. Moorhouse and K. Rockwood, Vascular cognitive impairment: current concepts and clinical developments, Lancet Neurol. 7 (2008) pp. 246-255.

[82] MRC-CFAS, Pathological correlates of late-onset dementia in a multicentre, community-based population in England and Wales. Neuropathology Group of the Medical Research Council Cognitive Function and Ageing Study (MRC CFAS), Lancet 357 (2001) pp. 169-175.

[83] I. Muthuramu, F. Jacobs, N. Singh, S.C. Gordts, G.B. De, Selective homocysteine lowering gene transfer improves infarct healing, attenuates remodelling, and enhances diastolic function after myocardial infarction in mice, PLoS. One. 8 (2013) p.e63710.

[84] I. Muthuramu, N. Singh, R. Amin, E. Nefyodova, M. Debasse, H. Van, I, F. Jacobs, G.B. De, Selective homocysteine-lowering gene transfer attenuates pressure overload-induced cardiomyopathy via reduced oxidative stress, J. Mol. Med. (Berl) 93 (2015) pp. 609-618.

[85] M.F. Neves, D. Endemann, F. Amiri, A. Virdis, Q. Pu, R. Rozen, E.L. Schiffrin, Small artery mechanics in hyperhomocysteinemic mice: effects of angiotensin II, J. Hypertens. 22 (2004) pp. 959-966.

[86] M. Nishinaga, T. Ozawa, K. Shimada, Homocysteine, a thrombogenic agent, suppresses anticoagulant heparan sulfate expression in cultured porcine aortic endothelial cells, J. Clin. Invest 92 (1993) pp. 1381-1386.

[87] J.T. O'Brien, T. Erkinjuntti, B. Reisberg, G. Roman, T. Sawada, L. Pantoni, J.V. Bowler, C. Ballard, C. DeCarli, P.B. Gorelick, K. Rockwood, A. Burns, S. Gauthier, S.T. DeKosky, Vascular cognitive impairment, Lancet Neurol. 2 (2003) pp. 89-98.

[88] A. Oulhaj, H. Refsum, H. Beaumont, J. Williams, E. King, R. Jacoby, A.D. Smith, Homocysteine as a predictor of cognitive decline in Alzheimer's disease, Int. J. Geriatr. Psychiatry 25 (2010) pp. 82-90.

[89] P.A. Outinen, S.K. Sood, S.I. Pfeifer, S. Pamidi, T.J. Podor, J. Li, J.I. Weitz, R.C. Austin, Homocysteine-induced endoplasmic reticulum stress and growth arrest leads to specific changes in gene expression in human vascular endothelial cells, Blood 94 (1999) pp. 959-967.

[90] L. Pantoni, Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges, Lancet Neurol. 9 (2010) pp. 689-701.

[91] M. Pirchl, C. Ullrich, C. Humpel, Differential effects of short- and long-term hyperhomocysteinaemia on cholinergic neurons, spatial memory and microbleedings in vivo in rats, Eur. J. Neurosci. 32 (2010) pp. 1516-1527.

[92] N.D. Prins and P. Scheltens, White matter hyperintensities, cognitive impairment and dementia: an update, Nat. Rev. Neurol. 11 (2015) pp. 157-165.

[93] L.M. Pullan, J.W. Olney, M.T. Price, R.P. Compton, W.F. Hood, J. Michel, J.B. Monahan, Excitatory amino acid receptor potency and subclass specificity of sulfur-containing amino acids, J. Neurochem. 49 (1987) pp. 1301-1307.

[94] P. Rajagopalan, X. Hua, A.W. Toga, C.R. Jack, Jr., M.W. Weiner, P.M. Thompson, Homocysteine effects on brain volumes mapped in 732 elderly individuals, Neuroreport 22 (2011) pp. 391-395.

[95] R. Riba, A. Nicolaou, M. Troxler, S. Homer-Vaniasinkam, K.M. Naseem, Altered platelet reactivity in peripheral vascular disease complicated with elevated plasma homocysteine levels, Atherosclerosis 175 (2004) pp. 69-75.

[96] Y. Sagara, K. Ishige, C. Tsai, P. Maher, Tyrphostins protect neuronal cells from oxidative stress, J. Biol. Chem. 277 (2002) pp. 36204-36215.

[97] G. Saposnik, The role of vitamin B in stroke prevention: a journey from observational studies to clinical trials and critique of the VITAmins TO Prevent Stroke (VITATOPS), Stroke 42 (2011) pp. 838-842.

[98] G. Saposnik, J.G. Ray, P. Sheridan, M. McQueen, E. Lonn, Homocysteine-lowering therapy and stroke risk, severity, and disability: additional findings from the HOPE 2 trial, Stroke 40 (2009) pp. 1365-1372.

[99] R. Schmidt, S. Seiler, M. Loitfelder, Longitudinal change of small-vessel disease-related brain abnormalities, J. Cereb. Blood Flow Metab (2015).

[100] T.M. Scott, K.L. Tucker, A. Bhadelia, B. Benjamin, S. Patz, R. Bhadelia, E. Liebson, L.L. Price, J. Griffith, I. Rosenberg, M.F. Folstein, Homocysteine and B vitamins relate to brain volume and white-matter changes in geriatric patients with psychiatric disorders, Am. J. Geriatr. Psychiatry 12 (2004) pp. 631-638.

[101] S. Seshadri, A. Beiser, J. Selhub, P.F. Jacques, I.H. Rosenberg, R.B. D'Agostino, P.W. Wilson, P.A. Wolf, Plasma homocysteine as a risk factor for dementia and Alzheimer's disease, N. Engl. J. Med. 346 (2002) pp. 476-483.

[102] S. Shastry, N. Tyagi, M.R. Hayden, S.C. Tyagi, Proteomic analysis of homocysteine inhibition of microvascular endothelial cell angiogenesis, Cell Mol. Biol. (Noisy. -Le-Grand) 50 (2004) pp. 931-937.

[103] S. Shastry and S.C. Tyagi, Homocysteine induces metalloproteinase and shedding of beta-1 integrin in microvessel endothelial cells, J. Cell Biochem. 93 (2004) pp. 207-213.

[104] A. Smallwood, A. Oulhaj, C. Joachim, S. Christie, C. Sloan, A.D. Smith, M. Esiri, Cerebral subcortical small vessel disease and its relation to cognition in elderly subjects: a pathological study in the Oxford Project to Investigate Memory and Ageing (OPTIMA) cohort, Neuropathol. Appl. Neurobiol. 38 (2012) pp. 337-343.

[105] A.D. Smith, The worldwide challenge of the dementias: a role for B vitamins and homocysteine?, Food Nutr. Bull. 29 (2008) p.S143-S172.

[106] A.D. Smith, S.M. Smith, C.A. de Jager, P. Whitbread, C. Johnston, G. Agacinski, A. Oulhaj, K.M. Bradley, R. Jacoby, H. Refsum, Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial, PLoS. One. 5 (2010) p.e12244.

[107] H.M. Snyder, R.A. Corriveau, S. Craft, J.E. Faber, S.M. Greenberg, D. Knopman, B.T. Lamb, T.J. Montine, M. Nedergaard, C.B. Schaffer, J.A. Schneider, C. Wellington, D.M. Wilcock, G.J. Zipfel, B. Zlokovic, L.J. Bain, F. Bosetti, Z.S. Galis, W. Koroshetz, M.C. Carrillo, Vascular contributions to cognitive impairment and dementia including Alzheimer's disease, Alzheimers. Dement. (2014) pp. S1552-5260(14)02862-3.

[108] T.L. Sudduth, D.K. Powell, C.D. Smith, A. Greenstein, D.M. Wilcock, Induction of hyperhomocysteinemia models vascular dementia by induction of cerebral microhemorrhages and neuroinflammation, J. Cereb. Blood Flow Metab 33 (2013) pp. 708-715.

[109] A. Tawakol, T. Omland, M. Gerhard, J.T. Wu, M.A. Creager, Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans, Circulation 95 (1997) pp. 1119-1121.

[110] A.M. Troen, The central nervous system in animal models of hyperhomocysteinemia, Prog. Neuropsychopharmacol. Biol. Psychiatry 29 (2005) pp. 1140-1151.

[111] A.M. Troen, M. Shea-Budgell, B. Shukitt-Hale, D.E. Smith, J. Selhub, I.H. Rosenberg, B-vitamin deficiency causes hyperhomocysteinemia and vascular cognitive impairment in mice, Proc. Natl. Acad. Sci. U. S. A 105 (2008) pp. 12474-12479.

[112] J.C. Tsai, M.A. Perrella, M. Yoshizumi, C.M. Hsieh, E. Haber, R. Schlegel, M.E. Lee, Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis, Proc. Natl. Acad. Sci. U. S. A 91 (1994) pp. 6369-6373.

[113] J.C. Tsai, H. Wang, M.A. Perrella, M. Yoshizumi, N.E. Sibinga, L.C. Tan, E. Haber, T.H. Chang, R. Schlegel, M.E. Lee, Induction of cyclin A gene expression by homocysteine in vascular smooth muscle cells, J. Clin. Invest 97 (1996) pp. 146-153.

[114] E.J. van Dijk, N.D. Prins, H.A. Vrooman, A. Hofman, P.J. Koudstaal, M.M. Breteler, Progression of cerebral small vessel disease in relation to risk factors and cognitive consequences: Rotterdam Scan study, Stroke 39 (2008) pp. 2712-2719.

[115] S. Veeranki and S.C. Tyagi, Defective homocysteine metabolism: potential implications for skeletal muscle malfunction, Int. J. Mol. Sci. 14 (2013) pp. 15074-15091.

[116] A. Verdelho, S. Madureira, C. Moleiro, J.M. Ferro, C.O. Santos, T. Erkinjuntti, L. Pantoni, F. Fazekas, M. Visser, G. Waldemar, A. Wallin, M. Hennerici, D. Inzitari, White matter changes and diabetes predict cognitive decline in the elderly: the LADIS study, Neurology 75 (2010) pp. 160-167.

[117] S.E. Vermeer, E.J. van Dijk, P.J. Koudstaal, M. Oudkerk, A. Hofman, R. Clarke, M.M. Breteler, Homocysteine, silent brain infarcts, and white matter lesions: The Rotterdam Scan Study, Ann. Neurol. 51 (2002) pp. 285-289.

[118] D.S. Wald, A. Kasturiratne, M. Simmonds, Effect of folic acid, with or without other B vitamins, on cognitive decline: meta-analysis of randomized trials, Am. J. Med. 123 (2010) pp. 522-527.

[119] H. Wang, M. Yoshizumi, K. Lai, J.C. Tsai, M.A. Perrella, E. Haber, M.E. Lee, Inhibition of growth and p21ras methylation in vascular endothelial cells by homocysteine but not cysteine, J. Biol. Chem. 272 (1997) pp. 25380-25385.

[120] L. Wang, K.H. Jhee, X. Hua, P.M. DiBello, D.W. Jacobsen, W.D. Kruger, Modulation of cystathionine beta-synthase level regulates total serum homocysteine in mice, Circ. Res. 94 (2004) pp. 1318-1324.

[121] M. Watanabe, J. Osada, Y. Aratani, K. Kluckman, R. Reddick, M.R. Malinow, N. Maeda, Mice deficient in cystathionine beta-synthase: animal models for mild and severe homocyst(e)inemia, Proc. Natl. Acad. Sci. U. S. A 92 (1995) pp. 1585-1589.

[122] N. Weiss, S. Heydrick, Y.Y. Zhang, C. Bierl, A. Cap, J. Loscalzo, Cellular redox state and endothelial dysfunction in mildly hyperhomocysteinemic cystathionine beta-synthase-deficient mice, Arterioscler. Thromb. Vasc. Biol. 22 (2002) pp. 34-41.

[123] C.B. Wright, M.C. Paik, T.R. Brown, S.P. Stabler, R.H. Allen, R.L. Sacco, C. DeCarli, Total homocysteine is associated with white matter hyperintensity volume: the Northern Manhattan Study, Stroke 36 (2005) pp. 1207-1211.

[124] F. Yeganeh, F. Nikbakht, S. Bahmanpour, K. Rastegar, R. Namavar, Neuroprotective effects of NMDA and group I metabotropic glutamate receptor antagonists against neurodegeneration induced by homocysteine in rat hippocampus: in vivo study, J. Mol. Neurosci. 50 (2013) pp. 551-557.

[125] X. Zeng, J. Dai, D.G. Remick, X. Wang, Homocysteine mediated expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human monocytes, Circ. Res. 93 (2003) pp. 311-320.

Figure

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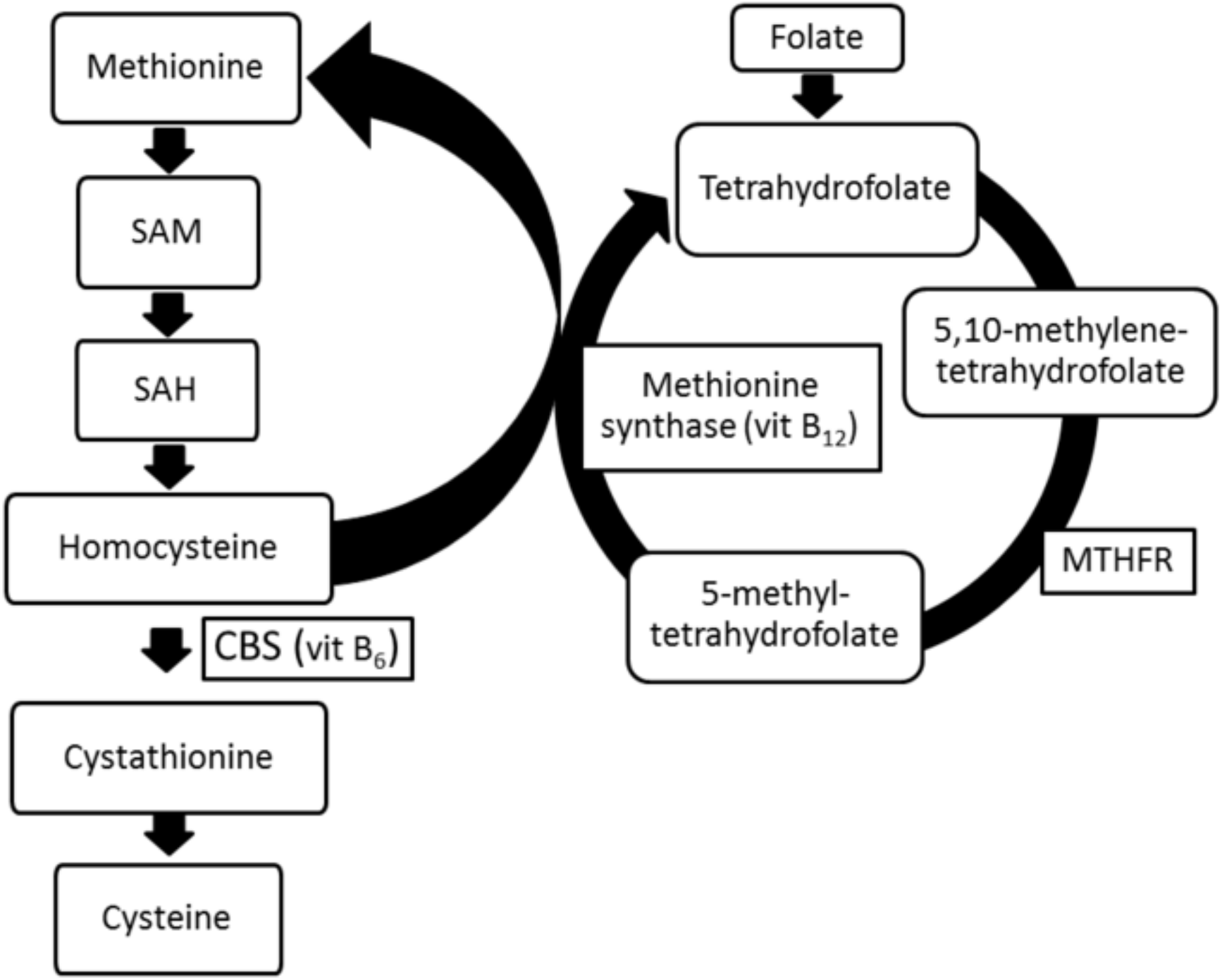

Hainsworth et al. BBA review "HHCy and VCID" Table S1

Effects of Homocysteic acid (or homocysteate) on brain cells

Effects observed	Effective concentration	Species	Reference
Inhibition of low affinity PMB-TBOA [blocks high affinity excitatory NT transporters] insensitive D-aspartate uptake in astrocytes	5mM	Rat	(Holten et al., 2008)
Neurite loss and size reduction	>100μm after 48 h	Rat	(Heider, Lehmensiek, Lenk, Muller, & Storch, 2004)
KYNA and KAT activity inhibition	IC50 *KYNA*= 3.93mM *KAT*= 5.0mM	Rat	(Kocki et al., 2003)
Inhibition of neural network activity (spontaneous spike rate)	IC50 1.3μM	Rat	(Görtz et al., 2004)
Hippocampal (CA1) & cortical culture toxicity (death) w/ short and long incubation	<u>Short incubation</u> 50μM (cort. tox) 100μM (hippo. tox) <u>Long incubation (48hrs)</u> All tox at 5mM	Rat	(Flott-Rahmel et al., 1998)
Excitatory currents in granular and purkinje cells	50μM	Chicken	(Kataoka, 1996)
Agonism of mGluR4a currents and inhibition of lateral perforant pathway neurons	EC50 for inhibition of mGluR4a inhibition of cAMP: 49μM IC50 for inhibition of lateral perforant pathway depolarisation [electrode stimulated]: 400μM	Hamster	(Johansen et al., 1995)
IP3 formation + NMDA agonism In cerebellar granule cells	EC50: 117μM	Cell culture	(Gorman & Griffiths, 1994)
Release of D-aspartate (exogenous) & Activation of excitatory NTR in cerebellar granule cells	500 μM	Cell culture	(Dunlop & Grieve, 1992)
NMDA and AMPA steady state	EC50	Cell	(Curras &

inward current production	NMDA: 13μM AMPA: 430μM	culture	Dingledine, 1991)
Neuronal death	50μM	Cell culture	(Eimerl & Schramm, 1991)
Cell death Neurotoxicity	1000μM	Cell culture	(Murphy, Schnaar, Coyle, & Johns, 1990)
Glycine antagonism at NMDA	100μM	Rat	(Mcdonald, 1990)
Agonism of NMDA coupled to [3H]GABA release in striatum . Measured via increases in GABA conc over 3min depolarisation period	EC50: 36μM	Mouse	(Weiss, 1990)
NA release from hippocampal neurones	2mmol/L	Rat	(Wu, Vezzani, & Samanin, 1989)
High affinity L-Glu transporter inhibition in synaptosomes, neurons and astrocytes (from mice)	Synaptosomes: 5000μM, Neurons: 2500μM Astrocytes: 1250μM	Mouse	(R Griffiths et al., 1989)
Neurotoxicity & Neuronal death	ED50 40μM	Mouse	(Kim, Koh, & Choi, 1987)
Inhibition of [(3)H]l-glutamate uptake in astrocytes	1mM	Rat & mouse	(Balcar, Schousboe, Spoerri, & Wolff, 1987)
Displacement GABA-mimetic [3H]muscimol from specific, high-affinity sites	Ki(apparent) 4800 microM	Cow	(Egbuta & Griffiths, 1987)
1. Receptor binding assays: NMDA, AMPA & katinate 2. Sodium flux through NMDA 3. Excitotoxicty in retinas on histopath eval	1. NMDA= 3.3μM, Katinate=110μM, AMPA=20μM 2. Potency: 1.4μM 3. Lowest effective dose: 200μM	Rat & Chicken	(Pullan et al., 1987)
Inhibition of high affinity taurine uptake in to astrocytes	IC50: 1.5mM	Rat	(Allen, Schousboe, & Griffiths, 1986)
Displacement at excitatory amino acid receptors	Ki (μM) D,L-HCA: 45.9 D-HCA: 64.2 L-HCA: 38.7	Snail & rat	(Pin, Bockaert, & Recasens, 1986)

Induction of Glutamate binding sites in hippocampus	0.1-10mM	Rat	(Kessler, Baudry, Cummins, Way, & Lynch, 1966)
Cell death- glioma C6 cells	EC50: 2mM	Cell culture	(Kato, Higashida, Higuchi, Hatakenaka, & Negishi, 1984)
Na^+ efflux from hippocampal slices	EC50: 50μM	Rat	(Baudry, Kramer, Recasens, Lynch, & December, 1983)
Competitive inhibition of [3H]muscimol in synaptic membranes	Ki value=1.96 mM for free receptor Ki =13 mM for receptor-muscimol complex	Cow	(Roger Griffiths et al., 1983)
Depolarisation	500μM	Guinea pig	(Yamamoto & Sawada, 1982)
Competitive binding to rat brain membranes (displacement of Glutamic acid)	IC50: 2.0μM	Rat	(Biziere, Thompson, & Coyle, 1980)

References

Allen, I., Schousboe, A., & Griffiths, R. (1986). Effect of L-Homocysteine and derivatives on the high affinity uptake of taurine and GABA into synaptosomes an cultured neurons and astrocytes. *Neurochemical Research*, *11*(11), 1487–1496.

Balcar, V., Schousboe, a, Spoerri, P., & Wolff, J. (1987). Differences between substrate specificities of l-glutamate uptake by neurons and glia, studied in cell lines and primary cultures. *Neurochemistry International*, *10*(2), 213–217. doi:10.1016/0197-0186(87)90130-6

Baudry, M., Kramer, K., Recasens, M., Lynch, C., & December, R. (1983). Classification and Properties of Acidic Hippocampus Using Amino Acid Receptors in Hippocampus. II. Biochemical Studies a Sodium Efflux Assay. *Molecular Pharmacology*, *24*, 222–228.

Biziere, K., Thompson, H., & Coyle, J. T. (1980). Characterisation of specific, high-affinity binding sites for L-glutamic acid in rat brain membranes. *Brain Research*, *183*, 421–433.

Curras, C., & Dingledine, R. (1991). Selectivity of Amino Acid Transmitters Acting at N-Methyl-D-aspartate and Amino-3-hydroxy-5-methyl-4-isoxazolepropionate Receptors. *Molecular Pharmacology*, *41*, 520–526.

Dunlop, J., & Grieve, A. (1992). Sulphur-containing excitatory amino acid-evoked release from cultured granule cells : the role of glutamate receptor activation coupled to reversal of the acidic amino acid plasma membrane, *50*(I), 107–115.

Egbuta, J. O., & Griffiths, R. (1987). Binding of [3H]muscimol to calf cerebrocortical synaptic membranes and the effects of sulphur-containing convulsant and non-convulsant compounds. *Neurochemical Research*, *12*(7), 589–595. doi:10.1007/BF00971006

Eimerl, S., & Schramm, M. (1991). Acute glutamate toxicity in cultured cerebellar granule cells " agonist potency , effects of pH , Zn 2 + and the potentiation by serum albumin, *0*(972), 282–290.

Flott-Rahmel, B., Schürmann, M., Schluff, P., Fingerhut, R., Mußhoff, U., Fowler, B., & Ullrich, K. (1998). Homocysteic and homocysteine sulphinic acid exhibit excitotoxicity in organotypic cultures from rat brain. *European Journal of Pediatrics*, *157*(S2), S112–S117. doi:10.1007/PL00014291

Gorman, a., & Griffiths, R. (1994). Sulphur-containing excitatory amino acid-stimulated inositol phosphate formation in primary cultures of cerebellar granule cells is mediated predominantly by N-methyl-d-aspartate receptors. *Neuroscience*, *59*(2), 299–308. doi:10.1016/0306-4522(94)90598-3

Görtz, P., Hoinkes, A., Fleischer, W., Otto, F., Schwahn, B., Wendel, U., & Siebler, M. (2004). Implications for hyperhomocysteinemia: not homocysteine but its oxidized forms strongly inhibit neuronal network activity. *Journal of the Neurological Sciences*, *218*(1-2), 109–14. doi:10.1016/j.jns.2003.11.009

Griffiths, R., Grieve, A., Dunlop, J., Damgaard, I., Fosmark, H., & Schousboe, A. (1989). Inhibition by excitatory sulphur amino acids of the high affinity L-glutamate transporter in synaptosomes and in primary cultures of cortical astrocytes in cerebellar neurons. *Neurochemical Research*, *14*(4), 333–343.

Griffiths, R., Williams, D. C., Neill, C. O., Dewhurst, I. C., Ekuwem, C. E., & Sinclair, C. D. (1983). Synergistic inhibition of [3H]muscimol binding to calf-brain synaptic membranes in the presence of L-homocysteine and pyridoxal 5’-phosphate, *478*, 467–478.

Heider, I., Lehmensiek, V., Lenk, T., Muller, T., & Storch, A. (2004). Dopaminergic neurotoxicity of homocysteine and its derivatives in primary mesencephalic cultures. *Journal of Neural Transmission [Supplementa]*, *68*, 1–13.

Holten, A. T., Talgøy, H. A., Danbolt, N. C., Christian, D. N., Shimamoto, K., Gundersen, V., & Vidar, G. (2008). Low-affinity excitatory amino acid uptake in hippocampal astrocytes: a possible role of Na+/dicarboxylate cotransporters. *Glia*, *56*(9), 990–7. doi:10.1002/glia.20672

Johansen, P., Chase, L., Sinor, A., Koerner, J., Johnson, R., & Robinson, M. (1995). Type 4a Metabotropic Glutamate Receptor : Identification of New Potent Agonists and Differentiation from the L-(+) -2-Receptor in the Lateral Perforant Pathway in Rats. *Molecular Pharmacology*, *48*, 140–149.

Kataoka, Y. (1996). Of Known Neurotrmsmitters , Glutamate is the Most Likely to be Released From Chick Cochlear Hair Cells, *76*(3).

Kato, S., Higashida, H., Higuchi, Y., Hatakenaka, S., & Negishi, K. (1984). Sensitive and insensitive states of cultured glioma cells to glutamate damage. *Brain Research*, *303*(2), 365–373. doi:10.1016/0006-8993(84)91222-8

Kessler, M., Baudry, M., Cummins, J. T., Way, S., & Lynch, G. (1966). Induction of Glutamate Binding Sites in Hippocampal Membranes by Transient Exposure to High Concentrations of Glutamate or Glutamate Analogs, *6*(February).

Kim, J. P., Koh, J., & Choi, D. W. (1987). l-Homocysteate is a potent neurotoxin on cultured cortical neurons. *Brain Research*, *437*(1), 103–110. doi:10.1016/0006-8993(87)91531-9

Kocki, T., Luchowski, P., Luchowska, E., Wielosz, M., Turski, W. a., & Urbanska, E. M. (2003). l-Cysteine sulphinate, endogenous sulphur-containing amino acid, inhibits rat brain kynurenic acid production via selective interference with kynurenine aminotransferase II. *Neuroscience Letters*, *346*(1-2), 97–100. doi:10.1016/S0304-3940(03)00579-2

Mcdonald, J. W. (1990). AND REGIONAL DISTRIBUTION OF STRYCHNINE-INSENSITIVE [3H] GLYCINE BINDING SITES IN RAT BRAIN BY QUANTITATIVE RECEPTOR, *35*(3), 653–668.

Murphy, H., Schnaar, L., Coyle, T., & Johns, T. (1990). Immature cortical neurons are uniquely sensitive to glutamate toxicty by inhibition of cysteine uptake. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *4*, 1624–1633.

Pin, J., Bockaert, J., & Recasens, M. (1986). The Binding of Acidic Amino Acids to Snail , Helix aspersa , Periesophagic Ring Membranes Reveals a Single High-Affinity Glutamate / Kainate Site, *366*, 290–299.

Pullan, L. M., Olney, J. W., Price, M. T., Compton, R. P., Hood, W. F., Michel, J., & Monahan, J. B. (1987). Excitatory Amino Acid Receptor Potency and Subclass Specificity of Sulfur-Containing Amino Acids. *Journal of*

Neurochemistry, *49*(4), 1301–1307. doi:10.1111/j.1471-4159.1987.tb10024.x

Weiss, S. (1990). Pharmacological Properties of the N-Methyl-D-Aspartate Receptor System Coupled to the Evoked Release of y-[3H] Aminobutyric Acid from Striatal Neurons in Primary Culture. *Journal of Pharmacology and Experimental Therapeutics*, *252*(§), 380–386.

Wu, H.-Q., Vezzani, A., & Samanin, R. (1989). Stimulation of [3H]norepinephrine release from hippocampal slices by excitatory amino acids. *Acta Pharmacologica Sinica*, *10*(3), 211–215.

Yamamoto, C., & Sawada, S. (1982). Sensitivity of hippocampal neurons to glutamic acid and its analogues. *Brain Research*, *235*(2), 358–362. doi:10.1016/0006-8993(82)91014-9