

# Multiple Aspects of Homocysteine Neurotoxicity: Glutamate Excitotoxicity, Kinase Hyperactivation and DNA Damage

Pei I. Ho,<sup>1,2</sup> Daniela Ortiz,<sup>1</sup> Eugene Rogers,<sup>1,2,3</sup> and Thomas B. Shea<sup>1,2,4\*</sup>

<sup>1</sup>Center for Cellular Neurobiology and Neurodegeneration Research, University of Massachusetts, Lowell, Massachusetts

<sup>2</sup>Department of Biochemistry, University of Massachusetts, Lowell, Massachusetts

<sup>3</sup>Department of Health and Clinical Sciences, University of Massachusetts, Lowell, Massachusetts

<sup>4</sup>Department of Biological Sciences, University of Massachusetts, Lowell, Massachusetts

Homocysteine (HC) is a neurotoxic amino acid that accumulates in several neurological disorders including Alzheimer's disease (AD). We examined the consequences of treatment of cultured murine cortical neurons with HC. Homocysteine-induced increases in cytosolic calcium, reactive oxygen species, phospho-tau immunoreactivity and externalized phosphatidyl serine (indicative of apoptosis). Homocysteine-induced calcium influx through NMDA channel activation, which stimulated glutamate excitotoxicity, as evidenced by treatment with antagonists of the NMDA channel and metabotropic glutamate receptors, respectively. The NMDA channel antagonist MK-801 reduced tau phosphorylation but not apoptosis after HC treatment, suggesting that HC-mediated apoptosis was not due to calcium influx. Apoptosis after HC treatment was reduced by co-treatment with 3-aminobenzamide (3ab), an inhibitor of poly-ADP-ribose polymerase (PARP), consistent with previous reports that ATP depletion by PARP-mediated repair of DNA strand breakage mediated HC-induced apoptosis. Treatment with 3ab did not reduce tau phosphorylation, however, therefore hyperphosphorylation of tau may not contribute to HC-induced apoptosis under these conditions. Inhibition of mitogen-activated protein kinase by co-treatment with the kinase inhibitor PD98059 inhibited tau phosphorylation but not apoptosis after HC treatment. HC accumulation reduces cellular levels of S-adenosyl methionine (SAM); co-treatment with SAM reduced apoptosis, suggesting that inhibition of critical methylation reactions may mediate HC-induced apoptosis. These findings indicate that HC compromises neuronal homeostasis by multiple, divergent routes.

© 2002 Wiley-Liss, Inc.

**Key words:** homocysteine; calcium influx; excitotoxicity; oxidative stress; tau phosphorylation; Alzheimer's disease

Homocysteine (HC), a nonprotein amino acid reversibly formed and secreted during metabolism, is a po-

tent neurotoxin. The deleterious influences of homocysteinemia have long been known in cardiovascular disease (Sobra, 1996). More recently, evidence has accumulated that elevated levels of HC are correlated with multiple neurological disorders, including age-related dementias and Alzheimer's disease (Gottfries et al., 1998; Seshadri et al., 2002). Moderate elevations of HC occur in 20–30% of the entire elderly population (Allen et al., 1998) and are even more prominent in the psychogeriatric population (Nilsson et al., 1994; Nilsson-Ehle, 1998). Plasma HC has been reported to be elevated in some (Joosten et al., 1997; Clarke et al., 1998; Seshadri et al., 2002), but not all (Fekkes et al., 1998) confirmed cases of AD and represents an early marker of cognitive impairment in the elderly (Lehmann et al., 1999); however, sufficient evidence to indicate causality is lacking.

The full range of causes for HC accumulation in dementia remains unclear. Intracellular HC levels are normally controlled in part by remethylation back to methionine (Kreis and Goddenow, 1987; Hultberg et al., 1995); however, the activity of the enzymes responsible for remethylation (methionine synthase and 5,10-methylenetetrahydrofolate reductase) are decreased in dementia (Trolin et al., 1995). Notably, the activity of these enzymes are dependent upon vitamins B12 and folate (Fiskerstrand et al., 1997; Jakubowski, 1997; Allen et al., 1998). Folate and

Abbreviations used: A $\beta$ ,  $\beta$ -amyloid; AIDA, 1-aminoindin-1,5-dicarboxylic acid; 3ab, 3-aminobenzamide; CPPG, S-4-carboxylphenylglycine; DMSO, dimethyl sulfoxide; DFCD, dichlorofluorescein diacetate; HC, homocysteine; NMDA, N-methyl-D-aspartate; PARP, poly-ADP-ribose polymerase; PS, phosphatidyl serine.

Contract grant sponsor: Office of Collaborative Research, University of Massachusetts.

\*Correspondence to: Thomas B. Shea, Center for Cellular Neurobiology and Neurodegeneration Research, University of Massachusetts, Lowell, MA 01854. E-mail: Thomas\_Shea@uml.edu

Received 22 March 2002; Revised 19 June 2002; Accepted 1 July, 2002

B12 deficiencies are common among psychogeriatric populations (Nilsson et al., 1994; Nilsson-Ehle, 1998), and can contribute to reversible dementia (Nilsson-Ehle, 1998; Pietrzik and Bronstrup, 1998). Dietary supplementation of folate is sufficient to modulate HC levels under normal conditions (Lucock et al., 1996). B12 deficiency correlates with AD and senile dementia of the Alzheimer type (Regland et al., 1988, 1992; Levitt and Karlinsky, 1992; McCaddon and Kelly, 1992; Clarke et al., 1998; Wynn and Wynn, 1998a, b). In addition, B12 deficiency also compromises the ability to utilize intracellular folate (Allen et al., 1998).

Homocysteine is toxic to human and murine neuronal cells in vitro (Kruman et al., 2000), in part through overstimulation of NMDA receptors and resultant calcium influx (Lipton et al., 1997; Kim, 1999; Kruman et al., 2000). Homocysteine potentiates glutamate excitotoxicity (Kruman et al., 2000) and  $\beta$ -amyloid ( $A\beta$ ) toxicity (Ho et al., 2001; White et al., 2001). Homocysteine has also been reported to induce apoptosis by damaging DNA (Kruman et al., 2000). We further examine the mechanisms by which HC induces neurotoxicity in culture.

## MATERIALS AND METHODS

### Cell Culture and Treatment

Cortical neurons were harvested from Day 16 embryonic mice and cultured on poly-L-lysine in DMEM/F12 containing B27 supplements (Gibco, Grand Island, NY) in the absence of serum (Ekinici et al., 1999). Cells were utilized 3–21 days after plating, during which time they established polarity and elaborated axons (e.g., Ekinici et al., 1999). Cultures were then treated for 30 min to 4 hr with 10–250  $\mu$ M HC, 500  $\mu$ M glutamate or 10  $\mu$ M  $\beta$ -amyloid<sub>25–35</sub> ( $A\beta$ ) in the presence and absence of the NMDA channel antagonist MK-801 (1  $\mu$ M; Gray and Patel, 1995), the L voltage-sensitive channel antagonist nimopridine (1  $\mu$ M; Ueda et al., 1997), antagonists of the Group I (1-aminoindin-1,5-dicarboxylic acid; AIDA; 1  $\mu$ M) and Group II and III (1-4-carboxylphenylglycine; CPPG; 1  $\mu$ M) metabotropic glutamate receptors, and the competitive antagonist of non-NMDA glutamate receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 1  $\mu$ M; Ozawa et al., 1998), the inhibitor of MEK-1 (PD98059; Pang et al., 1995), the poly-ADP-ribose polymerase (PARP) inhibitor 3ab (3-aminobenzamide; 5 mM; Kruman et al., 2000) or *s*-adenosyl methionine (SAM; 100  $\mu$ M).  $A\beta$ <sub>25–35</sub> peptides were dissolved initially in a small volume of DMSO, and diluted to a final stock concentration of 200  $\mu$ M in serum-free medium and aggregated overnight at 37°C before use (Ekinici et al., 1999). All supplies were from Sigma (St. Louis, MO) unless otherwise specified. Additional control cultures were treated with 0.1% DMSO, the final concentration utilized in experiments involving  $A\beta$ , which, consistent with our previous studies (Ekinici et al., 1999), did not affect any of the parameters examined. Treated cultures and untreated controls were then processed as described below. All experiments were carried out at least twice.

### Monitoring of Intracellular Calcium Concentrations

Intracellular calcium concentration was monitored as described previously (Ekinici et al., 1999) by incubation with

Fluo-3 (acetoxymethyl ester; Molecular Probes Inc., Eugene, OR) for 30 min by examination of multiple cultures under fluorescein optics.

### Monitoring of Intracellular Peroxide Concentrations

To monitor production of reactive oxygen species (ROS), cultures were treated with 2'-7' dichlorofluorescein diacetate (DCFDA; Kodak) for 20 min, rinsed with serum-free medium, and intracellular peroxide levels were measured for individual cells in multiple cultures under fluorescein optics as described previously (Ekinici et al., 1999).

### Monitoring of Tau Phosphorylation

Cultures were fixed for 15 min with 4% paraformaldehyde in 0.1 M phosphate buffer and immunostained by sequential reaction with a mouse monoclonal antibody (PHF-1; generous gift of Dr. Peter Davies) raised against tau from paired helical filaments from AD brains, followed by rhodamine-conjugated goat anti-mouse IgG and visualization under epifluorescent illumination. Additional controls, which yielded only background fluorescence, included substitution of non-immune murine IgG for PHF-1, or omission of primary antibody.

### Externalization of PS as a Measurement of Apoptosis

$A\beta$ -induced neurodegeneration in culture is accompanied by markers of apoptosis (Forloni et al., 1993). We therefore monitored levels of externalized phosphatidyl serine (PS), the accumulation of which provides an index of apoptosis (Rimon et al., 1997). Our previous analyses demonstrated that externalization of PS correlated precisely with  $A\beta$ -induced neurodegeneration as monitored by the commercial "Live/Dead" assay (Molecular Probes; Ekinici and Shea, 2000). Cultures treated with 10  $\mu$ M  $A\beta$  for 8 hr, along with untreated controls, were rinsed with PBS and incubated with 10  $\mu$ l/ml merocyanine (Upstate Biotech, Lake Placid, NY), followed by examination under rhodamine optics as described previously (Ekinici et al., 1999; Ekinici and Shea, 2000).

### Densitometric Analyses

For all of the above fluorometric assays, 50–100 cells in at least five microscopic fields selected randomly were scored for fluorescent intensity using NIH Image analysis software. Images were captured under epifluorescent and phase-contrast illumination using a DAGE CCL-72 cooled CCD camera via a Scion LG-3 frame grabber operated by NIH Image and stored as PICT files on a Macintosh Power PC 7100AV. Representative background areas devoid of cells were similarly analyzed and subtracted from cell values to yield net densitometric values. All fields for each individual assay were illuminated, captured, and processed at identical intensity. Corresponding phase-contrast images were obtained. Cell aggregates were excluded from the assays; only individual cells were quantified. Values were exported to Excel for statistical analyses via Student's *t*-test and to StatView for one-way ANOVA with Fisher's PLSD.

## RESULTS

### HC Induces Neuropathology

Treatment with cortical neurons with HC over 4 hr induced progressive increases in calcium influx, ROS,

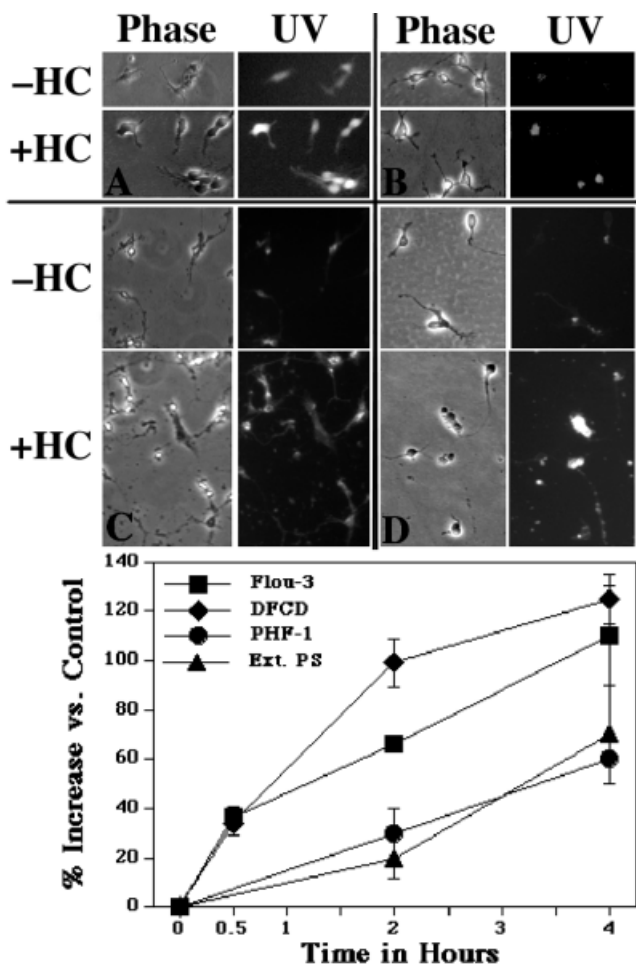


Fig. 1. Effect of HC on cultured cortical neurons. Micrographs present phase-contrast and corresponding UV images of representative cortical neurons before and after 2-hr treatment with 250  $\mu$ M HC followed by Fluo-3 (A), DFCD (B), PHF-1 (C) or merocyanine (D) as described in Materials and Methods. The accompanying graph presents the mean ( $\pm$ SEM) derived from densitometric analyses of 10–50 neurons from  $\geq 2$  cultures each from  $\geq 2$  separate experiments after treatment of 30 min to 4 hr. Values are expressed as a percentage increase over that observed at  $t = 0$  to facilitate presentation on the same axes. Note that HC treatment induced a progressive increase in all parameters. Comparison of densitometric values for each parameter at 4 hr differed statistically ( $P < 0.05$ ; Student's  $t$ -test) from their respective values at Time 0.

PHF-1 immunoreactivity, and externalization of PS (Fig. 1). The influence of HC on calcium and ROS was more rapid than on tau phosphorylation and apoptosis (Fig. 1). In addition to this time-dependent response, the extent of calcium influx was also proportional to HC concentration (Table I). Because HC is reported to induce calcium influx via NMDA channels (Kim, 1999; Kruman et al., 2000; Ho et al., 2001a), we treated additional cultures with the NMDA channel antagonist MK-801, which prevented the HC-induced increase in cytosolic calcium (Fig. 2). By contrast, nimopridine, an antagonist of the L voltage-sensitive calcium channel, did not prevent the HC-

TABLE I. HC Induces a Dose-Dependent Increase in Cytosolic Calcium\*

HC ( $\mu$ M)	Mean	SEM	% Increase
0	1.65	0.08	—
10	1.95	0.13	18
100	2.68	0.23	62
250	3.28	0.16	98

\*Cultures were assayed for cytosolic calcium levels via Fluo-3 before or 2 hr after treatment with 10–250  $\mu$ M HC. Values represent the mean ( $\pm$  SEM) derived from densitometric analyses of 10–50 neurons from  $\geq 2$  cultures each from  $\geq 2$  separate experiments. Values following HC treatment at all concentrations tested differed statistically from untreated cultures ( $P < 0.05$ ; Student's  $t$ -test).

mediated increase in cytosolic calcium (Fig. 2). MK-801 treatment under these conditions was likely to be specific for NMDA channels because: 1) it prevented calcium influx in neurons treated with glutamate (Fig. 2), which has also been reported to stimulate NMDA channels and induce tau phosphorylation (Mattson, 1990); and 2) did not prevent A $\beta$ -induced calcium influx (Fig. 2), consistent with previous reports that A $\beta$  induces calcium influx via the L voltage-sensitive calcium channel rather than the NMDA channel (Ueda et al., 1997; Ekinici et al., 1999; Silei et al., 1999). NMDA receptors are present at relatively low levels within 2–3 days of culture (e.g., Compagnone and Mellon, 1998) but increase during continued culture (Jensen et al., 1998). If HC mediates neurotoxicity via stimulation of NMDA channels, neurotoxicity should increase during continued culture; therefore, we treated neurons with HC for 2 hr after 3, 7, 14, and 21 days in culture. Homocysteine treatment resulted in progressive increases in cytosolic calcium, ROS, tau phosphorylation and externalization of PS between Days 3–7 in culture, with leveling off of these increases between Days 7–21 (Fig. 2). Moreover, the increase in cytosolic calcium was prevented at all time points by co-treatment with MK-801 (Fig. 2). These data are consistent with HC-induced stimulation of NMDA channels (see also Ho et al., 2001a).

Because generation of ROS, increased phosphorylation of tau, and apoptosis are downstream consequences of calcium influx after treatment with multiple agents, e.g., as calcium ionophore, A $\beta$ , and glutamate (Shea et al., 1997; Mattson, 1990), we examined whether or not prevention of HC-induced calcium influx would also prevent these additional neurotoxic consequences of HC treatment. MK-801 also prevented the HC-mediated increase in PHF-1 immunoreactivity (Fig. 3) whereas nimopridine did not prevent either response. MK-801 attenuated but did not completely prevent the HC-induced increase in ROS or apoptosis, suggesting that HC may induce ROS and apoptosis by mechanism(s) in addition to calcium influx via the NMDA channel (discussed in more depth below). Nimopridine did not attenuate the HC-induced increase in apoptosis, indicating that activity of the L voltage-sensitive calcium channel did not mediate apoptosis after HC treatment.

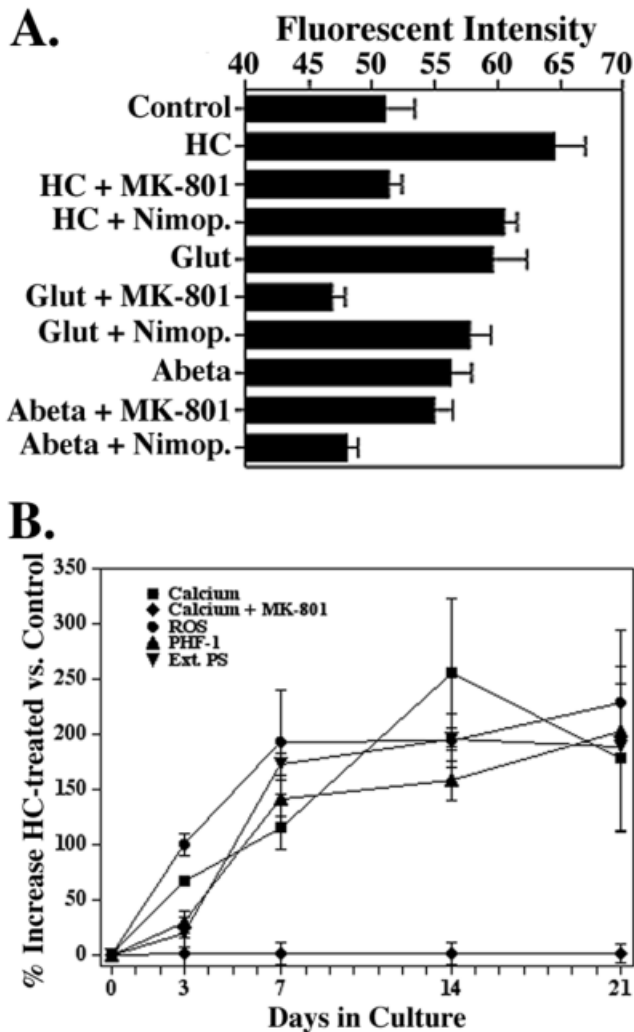


Fig. 2. HC induces calcium influx via the NMDA channel. **A:** Cortical neurons were treated with 250  $\mu$ M HC, 10  $\mu$ M A $\beta$  or 500  $\mu$ M glutamate for 2 hr in the presence and absence of 1  $\mu$ M MK-801 or 1  $\mu$ M nimopridine, then analyzed for cytosolic calcium via Fluo-3. Note that MK-801 blocked the increase in cytosolic calcium induced by HC and glutamate ( $P < 0.001$ , ANOVA, for HC or glutamate in presence of MK-801 vs. HC or glutamate alone); nimopridine did not significantly prevent this increase. By contrast nimopridine, but not MK-801, blocked the increase induced by A $\beta$  ( $P < 0.05$  for A $\beta$  in presence of nimopridine vs. A $\beta$  alone). **B:** Cortical neurons were treated for 2 hr with 250  $\mu$ M HC after 3, 7, 14 and 21 days in culture. Values represent the mean densitometric intensity  $\pm$  SEM. Note that HC treatment resulted in progressive increases in cytosolic calcium, ROS, tau phosphorylation and externalization of PS between Days 3–7 in culture, and that these parameters leveled off between Days 7–21. Note further that co-treatment with MK-801 prevented the increase in cytosolic calcium at all time points.

### HC Induces Glutamate Excitotoxicity

Because NMDA channel activation can promote glutamate release and resultant glutamate excitotoxicity, HC-induced NMDA channel activation might ultimately

lead to neuronal degeneration via glutamate excitotoxicity (e.g., Kruman et al., 2000). If this is the case, HC-induced calcium influx should be curtailed by antagonists of one or more of the metabotropic glutamate receptors that are stimulated after release of glutamate, because these receptors when activated foster calcium influx through their associated calcium channels (for review, see Ozawa et al., 1998). We therefore treated additional cultures with broad-spectrum antagonists of these receptors at the same time as HC treatment. When antagonists of Class I-III, non-NMDA metabotropic glutamate receptors were added to cultures during HC treatment, we observed a marked inhibition of calcium influx (Fig. 4). Taken together with the above data implicating the NMDA receptor in HC-mediated calcium influx, these data suggest that initial influx of calcium through NMDA channels fosters glutamate release, leading to activation of metabotropic glutamate receptors and secondary calcium influx.

### MAP Kinase Contributes to HC-Induced Tau Phosphorylation

Prevention of the HC-induced increase in PHF-1 immunoreactivity by the calcium channel antagonist MK-801 (Fig. 3) indicates a role for calcium influx in HC-induced tau phosphorylation. Because MAP kinase can contribute to increased PHF-1 immunoreactivity after calcium influx (Ekinici et al., 1999), and MAP kinase has been reported to be activated after HC treatment (Shi et al., 1999) and also as a consequence of NMDA channel activation (Jiang et al., 2000; Sato et al., 2001), we also tested the potential role of MAP kinase activation in tau phosphorylation. Treatment with the MEK1 inhibitor PD98059 inhibited HC-induced tau phosphorylation (Fig. 5), indicating that MAP kinase contributes to tau phosphorylation after HC treatment.

By contrast, PD98059 did not significantly reduce the increase in apoptosis that accompanied HC treatment (Fig. 5), suggesting that MAP kinase did not mediate HC-induced apoptosis. Furthermore, this suggested that increased tau phosphorylation at the PHF-1 epitope did not contribute significantly to the increase in apoptosis accompanying HC treatment, at least in these short-term (e.g., 2-hr) analyses.

### HC Induces DNA Damage

Previous studies have indicated that HC neurotoxicity is due in part to DNA damage (Kruman et al., 2000). We examined whether this was the case in our cortical cultures. The DNA repair enzyme PARP undergoes a two-fold increase in activity after HC treatment and the resultant increase in PARP activity depletes neuronal energy reserves to the extent that it promotes apoptosis in cultured neurons (Kruman et al., 2000). Co-treatment with the PARP inhibitor 3ab (Kruman et al., 2000) diminished HC-induced apoptosis (Fig. 6), indicating that increased DNA breakage after HC treatment contributed to apoptosis. By contrast, 3ab did not inhibit HC-induced calcium influx and only partially reduced ROS after HC treatment (Fig. 6). HC-induced PARP activation has been suggested to contribute to neuronal oxidative stress, and a

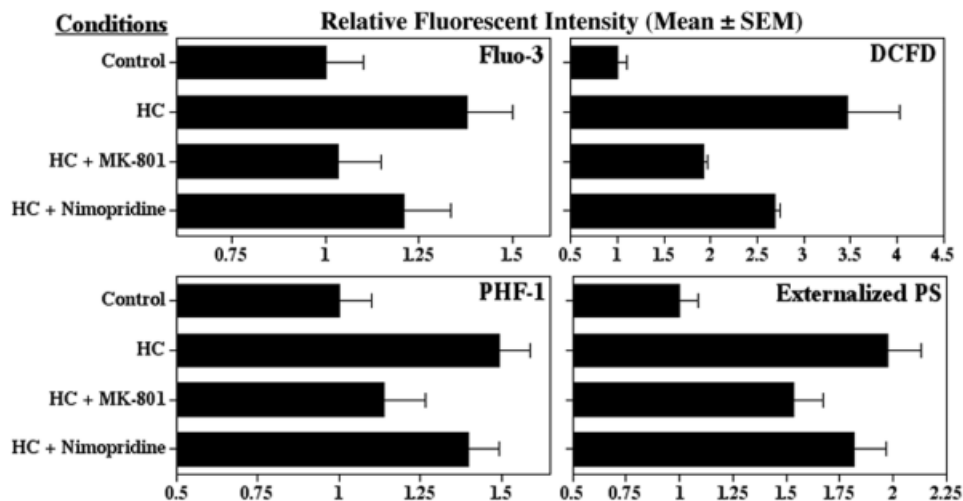


Fig. 3. The NMDA channel antagonist MK-801 alleviates HC-induced calcium influx and tau phosphorylation, but only partially reduced ROS and apoptosis. Cortical neurons were treated with 250  $\mu$ M HC for 2 hr in the presence and absence of 1  $\mu$ M MK-801 and analyzed for cytosolic calcium, ROS, PHF-1 immunoreactivity and externalized PS as described in Materials and Methods. Values are presented as the relative increase (mean  $\pm$  SEM) in PHF-1 immunofluorescent intensity over that observed in untreated neurons, where densitometric values for untreated cultures were defined as 1. As shown above in Figures 1 and 2, HC statistically increased all of these parameters ( $P < 0.001$ , one-way ANOVA). Co-treatment with MK-801

prevented the HC-induced increase in cytosolic calcium and PHF-1 immunoreactivity ( $P < 0.001$ , one-way ANOVA with Fischer's PLSD). MK-801 also attenuated the HC-induced increase in ROS or apoptosis; notably, whereas values after MK-801 treatment differed statistically different from controls ( $P < 0.0001$ ), values after MK-801 treatment did not differ statistically from those receiving HC ( $P < 0.1282$  and  $P < 0.1056$ , controls vs. HC + MK-801, respectively, one-way ANOVA with Fischer's PLSD), suggesting that additional factors beyond interaction with the NMDA channel may influence HC-induced generation of ROS and apoptosis. Nimopridine did not inhibit HC-induced increases in any of these parameters.

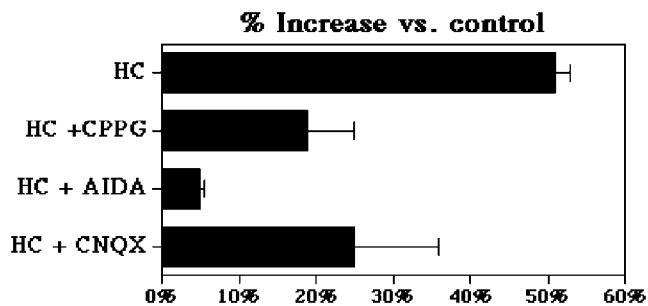


Fig. 4. HC induces glutamate excitotoxicity. Cortical neurons were treated with 250  $\mu$ M HC for 2 hr in the presence and absence of AIDA, CPPF and CNQX (1  $\mu$ M each), then analyzed for cytosolic calcium as described in Materials and Methods. Values are presented as the % increase (mean  $\pm$  SEM) in Fluo-3 over that observed in untreated neurons. All of these inhibitors statistically reduced the HC-induced increase in cytosolic calcium ( $P < 0.05$ ; Student's *t*-test).

reduction in HC-induced ROS by co-treatment with 3ab was also noted by Kruman et al. (2000); although HC-induced calcium influx promotes ROS (see also Ho et al., 2001a), HC may also induce ROS via PARP activation, independent of calcium influx. Taken together with the above data indicating that prevention of calcium influx by MK-801 did not completely prevent apoptosis, these data suggest that calcium influx by HC contributes to glutamate

toxicity and tau phosphorylation but may not contribute significantly to apoptosis under these conditions.

#### Depletion of SAM Contributes to HC-Induced Apoptosis

One potential mechanism by which HC could foster increased DNA damage is by inhibition of cellular levels of SAM (Cantoni, 1986). SAM mediates multiple transmethylation reactions including those required for methylation of DNA (Wu and Santi, 1987; Wainfan et al., 1989; Mudd and Cantoni, 1991). In addition, inhibition of transmethylation by HC has been shown to promote apoptosis (Endresen et al., 1994, 1996). To test this possibility and attempt to alleviate HC neurotoxicity, we added 100  $\mu$ M SAM along with HC and monitored apoptosis. SAM attenuated the HC-induced increase in apoptosis by approximately 50% (Fig. 7). These data are consistent with the possibility that impaired transmethylation of DNA played a role in induction of apoptosis by HC. Notably, however, this does not exclude the possibility that other critical transmethylation events are involved in induction of neuronal apoptosis by HC.

#### DISCUSSION

These findings of the present study confirm and extend earlier studies documenting neurotoxicity of HC in culture (Flott-Rahmel et al., 1998; Parsons et al., 1998; Kruman et al., 2000; Ho et al., 2001a; White et al., 2001) by demonstrating that HC can impart multiple neuro-

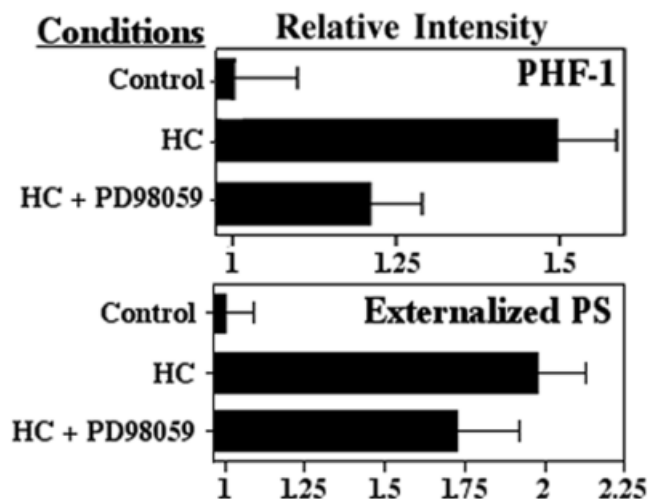


Fig. 5. Effect of kinase inhibitors on HC-induced tau phosphorylation and apoptosis. Cortical neurons were treated with 250  $\mu$ M HC for 2 hr in the presence and absence of 10  $\mu$ M PD98059, then assayed for PHF-1 immunoreactivity as an index of tau phosphorylation and externalized PS as an index of apoptosis. Values represent the relative increase (mean  $\pm$  SEM) in densitometric values obtained for treated cultures versus untreated controls, with control values defined as 1. As shown above in Figure 1, HC significantly increased both PHF-1 immunoreactivity and increased externalized PS ( $P < 0.001$ , one-way ANOVA for HC treated cultures vs. untreated controls). PD98059 significantly diminished the HC-induced increase in PHF-1 immunoreactivity ( $P < 0.001$ , HC + PD98059 vs. HC alone), but did not inhibit the HC-induced externalization of PS ( $P < 0.232$ ; HC + PD98059 vs. HC alone; one-way ANOVA with Fischer's PLSD).

pathological effects, including cytosolic accumulation of calcium, induction of oxidative stress, hyperphosphorylation of tau, and apoptosis, all of which are characteristic of affected neurons in AD (for review, see Holscher, 1998). Prevention of HC-induced calcium influx by co-treatment with the NMDA channel blocker MK-801 is consistent with previous reports that HC is an agonist for NMDA channels (Kim, 1999). These findings, and those of earlier reports (MacDonald and Wojtowicz, 1980; Kim, 1999; Kruman et al., 2000) suggests that HC may exert deleterious effects on neurons by acting as an excitotoxin, or by rendering neurons hypersensitive to excitotoxicity. In this regard, we demonstrate in this study that the initial calcium influx induced by HC in turn fosters glutamate excitotoxicity, because the full extent of HC-induced calcium influx was attenuated by antagonists of non-NMDA glutamate receptors.

Inhibition of calcium influx by co-treatment with MK-801 prevented tau phosphorylation, but did not affect HC-induced apoptosis, and inhibited some but not all ROS generated in response to HC treatment. Similarly, we demonstrated that MAP kinase, known to be stimulated by NMDA channels, mediated HC-induced tau phosphorylation but was not involved in apoptosis. Finally, 3ab inhibited apoptosis and partially reduced ROS but did not curtail HC-induced calcium influx. These data

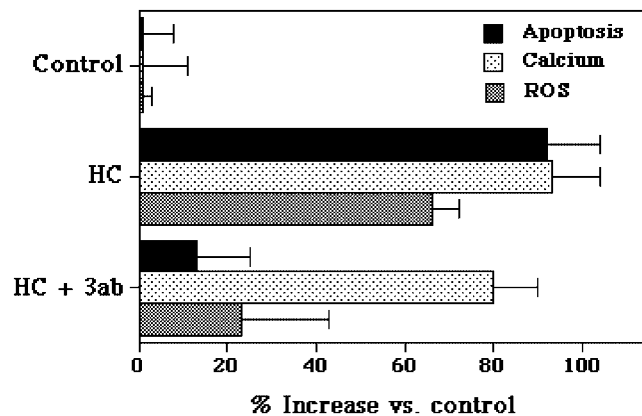


Fig. 6. The PARP inhibitor 3ab inhibited HC-induced apoptosis and ROS. Cortical neurons were treated for 2 hr with 250  $\mu$ M HC with and without 5 mM 3ab, then probed for cytosolic calcium (Fluo-3), ROS (DFCD) and apoptosis (meracyanine) as described in Materials and Methods. Values represent the % increase (mean  $\pm$  SEM), where control values were defined as 1, derived from densitometric analyses of 10–50 cells from two cultures under each condition from two separate, independent experiments. As shown in Figure 1, HC statistically increased cytosolic calcium, ROS and apoptosis ( $P < 0.001$ ; one-way ANOVA). Note that 3ab attenuated HC-induced ROS and apoptosis ( $P < 0.001$ , HC vs. HC + 3ab), but did not prevent the increase in cytosolic calcium ( $P < 0.133$ ; HC vs. HC + 3ab, one-way ANOVA with Fischer's PLSD).

indicate that HC induces divergent neurotoxic effects on cultured neurons. Although calcium influx can induce ROS in these neurons (e.g., Ekinici et al., 1999), it has also been suggested that HC induces oxidative stress by a mechanism in addition to that resulting from calcium influx alone (Kim and Pae, 1996; Kruman et al., 2000). The findings of Kruman et al. (2000) indicated that HC-induced DNA damage depletes neuronal NAP<sup>+</sup> and ATP due to PARP activation, and provided evidence that this effect preceded, and may be causal to, HC-oxidative stress. These findings were corroborated herein, because inhibition of PARP reduced oxidative stress without inhibiting calcium influx. Accordingly, generation of ROS after HC treatment may be derived in part from calcium influx and in part from depletion of cellular energy reserves in attempts to repair DNA. The pivotal role of ROS in mediating HC toxicity has been demonstrated by prevention of HC toxicity by superoxide dismutase and catalase (Kim and Pae, 1996) and by the glutathione precursor *N*-acetyl cysteine (Ho et al., 2001a).

The failure of inhibition of PARP to prevent the HC-induced increase in PHF-1 immunoreactivity can be interpreted to indicate that tau phosphorylation does not contribute to HC-induced apoptosis in these short-term analyses. A similar conclusion was reached after A $\beta$  treatment of these cortical neurons, in which ROS generation but not tau phosphorylation correlated with apoptosis (Ekinici and Shea, 2000) and after induction of osmotic stress in cultured human neuroblastoma, where inhibition of increased tau phosphorylation did not attenuate apo-

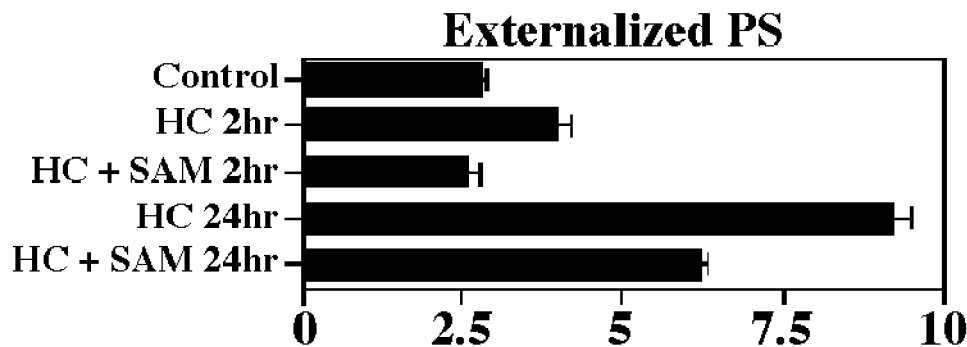


Fig. 7. SAM attenuates HC-induced apoptosis and ROS but not calcium influx. Cortical neurons were treated for 2 hr with 250  $\mu$ M HC with and without 100  $\mu$ M SAM, after which cultures were monitored for externalized PS using merocyanine staining as an indication of apoptosis as described in Materials and Methods. Values represent the mean ( $\pm$ SEM) in arbitrary densitometric units derived from densitometric analyses of 10–50 neurons from two cultures under

each condition from two separate, independent experiments. HC progressively induced externalization of PS; values for HC-treated cultures differed statistically from untreated controls at 2 and 24 hr ( $P < 0.001$ , one-way ANOVA), indicating a progressive increase in apoptosis. Addition of SAM completely prevented PS externalization at 2 hr and reduced it by  $\approx 50\%$  at 24 hr ( $P < 0.05$ , one-way ANOVA with Fisher's PLSD).

ptosis (Stoothoff and Johnson, 2001). Importantly, none of these short-term culture studies measured the impact of paired helical filament formation on apoptosis.

The full range of causes of AD remains unclear, but has been considered to be multifactorial (Holscher, 1998). Clinical studies implicate homocysteinemia in neurodegeneration, including that related to AD (Joosten et al., 1997; Clarke et al., 1998; Fekkes et al., 1998; Gottfries et al., 1998; Lehmann et al., 1999). Cell culture studies directed toward elucidating neuropathology in AD have reported increases in cytosolic calcium, increased generation of ROS, increased activity of calcium-dependent kinases, increased tau phosphorylation and ultimate apoptosis (Ekinici et al., 1999). Our studies demonstrate that HC induced all of these phenomena in cultured neurons. These findings, along with previous studies from our laboratory (Ho et al., 2001a) and others (Kruman et al., 2000) are consistent with the possibility that key metabolic deficiencies, including abnormal accumulation of excitotoxic amino acids such as HC, may contribute to neuronal compromise and promote neurodegeneration in AD. They further extend the clinical association between hyperhomocysteinemia and neurodegeneration by demonstrating that HC can induce neurotoxicity directly, apart, or moreover in addition to potentially detrimental effects on cerebral vasculature. Notably, HC potentiates A $\beta$  neurotoxicity (Ho et al., 2001a; White et al., 2001) and glutamate toxicity (Kruman et al., 2000), leaving open the possibility that combined exposure to levels of HC, A $\beta$  and glutamate that are themselves relatively benign could lead to neurodegeneration. The results presented here suggest that therapeutic approaches to alleviate the neurotoxic effects of HC should encompass diverse approaches including quenching of ROS, replenishment of energy reserves, and maintenance of transmethylation.

#### ACKNOWLEDGMENT

We thank J. Lyons-Weiler for advice on statistical analyses.

#### REFERENCES

- Allen RH, Stabler SP, Lindenbaum J. 1998. relevance of vitamins, homocystein and other metabolites in neuropsychiatric disorders. *Eur J Pediatr* 157(Suppl):122–126.
- Cantoni GL. 1986. The centrality of S-adenosylhomocysteine in the regulation of the biological utilization of S-adenosylmethionine. In: Borchardt C, Ueland PM, editors. *Biological methylation and drug design: experimental and clinical roles of SAM*. Clifton: Humana Press Inc. p 227–237.
- Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. 1998. Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease. *Arch Neurol* 55:1449–1455.
- Compagnone NA, Mellon SH. 1998. Dehydroepiandrosterone: a potential signaling molecule for neocortical organization during development. *Proc Natl Acad Sci USA* 95:4678–4683.
- Ekinici FJ, Shea TB. 2000. Hyperphosphorylation of tau does not correlate with neurodegeneration in cortical neurons in culture. *J Alz Dis* 2:7–12.
- Ekinici FJ, Malik KM, Shea TB. 1999. Beta-amyloid induces calcium influx and neurodegeneration by MAP kinase-mediated activation of the L voltage-sensitive calcium channel. *J Biol Chem* 274:30322–30327.
- Endresen PC, Loennechen T, Kildalsen H, Aarbakke J. 1996. Apoptosis and transmethylation metabolites in HL-60 cells. *J Pharmacol Exp Ther* 278:1318–1324.
- Endresen PC, Prytz PS, Lysne S, Aarbakke J. 1994. Homocysteine increases the relative number of apoptotic cells and reduces the relative number of apoptotic bodies in HL-60 cells treated with 3-deazaadenosine. *J Pharmacol Exp Ther* 269:1245–1253.
- Fekkes D, van der Cammen TJ, van Loon CP, Verschoor C, van Harskamp F, de Koning I, Schudel WJ, Peplinkhuizen L. 1998. Abnormal amino acid metabolism in patients with early stage Alzheimer's dementia. *J Neural Transm* 105:287–294.
- Fiskerstrand T, Ueland PM, Refsum H. 1997. Folate depletion induced by methotrexate affects methionine synthase activity and its susceptibility to inactivation by nitrous oxide. *J Pharmacol Exp Ther* 282:1305–1311.

- Flott-Rahmel B, Schurmann M, Schluff P, Fingerhut R, MuBhoff U, Fowler B, Ullrich K. 1998. Homocysteic and homocysteine sulphinic acid exhibit excitotoxicity in organotypic cultures from rat brain. *Eur J Pediatr* 157(Suppl):112–117.
- Forloni G, Chiesa R, Smiroldo S, Verga L, Salmona M, Tagliavini F, Angeretti N. 1993. Apoptosis-mediated neurotoxicity induced by chronic application of  $\beta$  amyloid fragment 25–35. *Neuroreport* 4:523–526.
- Gottfries CG, Lehmann W, Reglan B. 1998. Early diagnosis of cognitive impairment in the elderly with the focus on Alzheimer's disease. *J Neural Transm* 105:773–786.
- Gray CW, Patel AJ. 1995. Neurodegeneration mediated by glutamate and beta-amyloid peptide: a comparison and possible interaction. *Brain Res* 691:169–179.
- Ho P, Collins SC, Dhitavat S, Ortiz D, Ashline D, Rogers E, Shea TB. 2001a. Homocysteine potentiates  $\beta$ -amyloid neurotoxicity: role of oxidative stress. *J Neurochem* 78:249–253.
- Holscher C. 1998. Possible causes of Alzheimer's disease: amyloid fragments, free radicals, and calcium homeostasis. *Neurobiol Dis* 5:129–141.
- Hultberg B, Andersson A, Isaksson A. 1995. Metabolism of homocysteine, its relation to the other cellular thiols and its mechanism of cell damage in a cell culture line (human histiocytic cell line U-937. *Biochim Biophys Acta* 1269:6–12.
- Jakubowski H. 1997. Metabolism of homocysteine thiolactone in human cell cultures: possible mechanism for pathological consequences of elevated homocysteine levels. *J Biol Chem* 272:1935–1942.
- Jensen JB, Schousboe A, Pickering DS. 1998. AMPA receptor mediated excitotoxicity in neocortical neurons is developmentally regulated and dependent upon receptor desensitization. *Neurochem Int* 32:505–513.
- Jiang Q, Gu Z, Zhang G, Jing G. 2000. *N*-methyl-D-aspartate receptor activation results in regulation of extracellular signal-related kinases by protein kinases and phosphatases in glutamate-induced neuronal apoptotic-like death. *Brain Res* 887:285–292.
- Joosten E, Lesaffre E, Riezler R, Ghekiere V, Dereymaeker L, Pelemans W, Dejaeger E. 1997. Is metabolic evidence for vitamin B-12 and folate deficiency more frequent in elderly patients with Alzheimer's disease? *J Gerontol A Biol Sci Med Sci* 52:76–79.
- Kim WK. 1999. S-nitrosation ameliorates homocysteine-induced neurotoxicity and calcium responses in primary culture of rat cortical neurons. *Neurosci Lett* 265:99–102.
- Kim WK, Pae YS. 1996. Involvement of *N*-methyl-D-aspartate receptor and free radical in homocysteine-mediated toxicity on rat cerebellar granule cells in culture. *Neurosci Lett* 216:117–120.
- Kreis W, Goodenow M. 1987. Methionine requirement and replacement by homocysteine in tissue cultures of selected rodent and human malignant and normal cells. *Cancer Res* 38:2259–2262.
- Kruman II, Culmsee C, Chan SL, Kruman Y, Guo Z, Penix L, Mattson MP. 2000. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. *J Neurosci* 20:6920–6926.
- Lehmann M, Gottfries CG, Regland B. 1999. Identification of cognitive impairment in the elderly: homocysteine is an early marker. *Dement Geriatr Cogn Disord* 10:12–20.
- Levitt AJ, Karlinsky H. 1992. Folate, vitamin B12 and cognitive impairment in patients with Alzheimer's disease. *Acta Psychiatr Scand* 86:301–305.
- Lipton SA, Kim W-K, Choi Y-B, Kumar S, D'Emilia DM, Rayudu PV, Arnelle DR, Stamler JS. 1997. Neurotoxicity associated with dual actions of homocysteine at the *N*-methyl-D-aspartate receptor. *Proc Natl Acad Sci USA* 94:5923–5928.
- Lucock MD, Daskalakis IG, Wild J, Anderson A, Schorah CJ, Lean MEJ, Levene MI. 1996. The influence of dietary folate and methionine on the metabolic disposition of endotoxic homocysteine. *Biochem Mol Med* 59:104–111.
- MacDonald JF, Wojtowicz JM. 1980. Two conductance mechanisms activated by applications of L-glutamic, L-aspartic, DL-homocysteic, *N*-methyl-D-aspartic, and DL-kainic acids to cultured mammalian central neurones. *Can J Physiol Pharmacol* 58:1393–1397.
- Mattson MP. 1990. Antigenic changes similar to those seen in neurofibrillary tangles are elicited by glutamate and Ca<sup>2+</sup> influx in cultured hippocampal neurons. *Neuron* 4:105–117.
- McCaddon A, Kelly CL. 1992. Familial Alzheimer's disease and vitamin B12 deficiency. *Age Ageing* 23:334–337.
- Mudd SH, Cantoni GL. 1964. Biological transmethylation methyl-group neogenesis and other one carbon metabolic reactions dependent upon tetrahydrofolic acid. In: Florkin M, Stot EH, editors. *Comprehensive biochemistry*, Vol 15. Amsterdam: Elsevier. p 147.
- Nilsson K, Gustafson L, Faldt R, Anderson A, Hultberg B. 1994. Plasma homocysteine in relation to serum cobalamin and blood folate in a psychogeriatric population. *Eur J Clin Invest* 24:600–606.
- Nilsson-Ehle H. 1998. Age-related changes in cobalamin (vitamin B12) handling: implications for therapy. *Drugs Aging* 12:277–292.
- Ozawa S, Kamiya H, Tsuzuki K. 1998. Glutamate receptors in the central nervous system. *Prog Neurobiol* 54:581–618.
- Pang L, Sawada T, Decker SJ, Saltiel AR. 1995. Inhibition of MAP kinase: kinase blocks the differentiation of PC-12 cells induced by nerve growth factor *J Biol Chem* 270:13585–13588.
- Parsons RB, Waring RH, Ramsden DB, Williams AC. 1998. In vitro effect of the cysteine metabolites homocysteic acid, homocysteine and cysteic acid upon human neuronal cell lines. *Neurotoxicology* 19:599–603.
- Pietrzik K, Bronstrup A. 1998. Vitamins B12, B6 and folate as determinants of homocysteine concentration in the healthy population. *Eur J Pediatr* 157(Suppl):135–138.
- Regland B, Abrahamsson L, Blennow K, Gottfries CG, Wallin A. 1992. Vitamin B12 in CSF: reduced CSF/serum B12 ratio in demented men. *Acta Neurol Scand* 85:276–281.
- Regland B, Gottfries CG, Oreland L, Svennerholm L. 1988. Low B12 levels related to high activity of platelet MAO in patients with dementia disorders: a retrospective study. *Acta Psychiatr Scand* 78:451–457.
- Rimon G, Bazenet CE, Philpott KL, Rubin LL. 1997. Increased surface phosphatidylserine is an early marker of neuronal apoptosis. *J Neurosci Res* 48:563–570.
- Sato M, Suzuki K, Shigetada Nakanishi S. 2001. NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. *J Neurosci* 21:3797–3805.
- Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, Wilson PWF, Wolf PA. 2002. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N Engl J Med* 346:476–483.
- Shea TB, Parabhakar S, Ekinci FJ. 1997a. Beta-amyloid and ionophore-mediated calcium influx evoke neurodegeneration by distinct intracellular pathways: differential involvement of the calpain/protein kinase C system. *J Neurosci Res* 49:759–768.
- Shi SS, Day RM, Halpner AD, Blumberg JB, Suzuki YJ. 1999. Homocysteine and  $\alpha$ -lipoic acid regulate p44/42 MAP kinase phosphorylation in NIH3T3 cells. *Antioxid Redox Signal* 1:123–128.
- Silei V, Fabrizi C, Venturini G, Salmona M, Bugiani O, Tagliavini F, Lauro GM. 1999. Activation of microglial cells by PrP and  $\beta$ -amyloid fragments raises intracellular calcium through L-type voltage sensitive calcium channels. *Brain Res* 818:168–170.
- Sobra J. 1996. Hyperhomocysteinemia. *Cas Lek Cesk* 135:266–269.
- Stoothoff WH, Johnson GVW. 2001. Hyperosmotic stress-induced apoptosis and tau phosphorylation in human neuroblastoma cells. *Neurosci Res* 65:573–582.

- Trolin CG, Regland B, Orelund L. 1995. Decreased methionine adenosyltransferase activity in erythrocytes of patients with dementia disorders. *Eur Neuropsychopharmacol* 5:107–114.
- Ueda K, Shinohara S, Yagami T, Asakura K, Kawasaki K. 1997. Amyloid  $\beta$  protein potentiates  $\text{Ca}^{2+}$  influx through L-type voltage-sensitive  $\text{Ca}^{2+}$  channels: a possible involvement of free radicals. *J Neurochem* 68:265–271.
- Wainfan E, Dixik M, Stender M, Christman JK. 1989. Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl deficient diets. *Cancer Res* 49:4094–4097.
- White AR, Huang X, Jobling MF, Barrow CH, Beyreuther K, Masters CL, Bush AI, Cappai R. 2001. Homocysteine potentiates copper- and amyloid  $\beta$  peptide-mediated toxicity in primary cultures: possible risk factors in the Alzheimer's-type neurodegenerative pathways. *J Neurochem* 76:1509–1520.
- Wu JC, Santi DV. 1987. Kinetic and catalytic mechanism of Hha. *J Biol Chem* 262:4778–4786.
- Wynn M, Wynn A. 1998a. The danger of B12 deficiency in the elderly. *Nutr Health* 12:215–226.
- Wynn M, Wynn A. 1998b. Fortification of grain products with folate: should Britain follow the American example? *Nutr Health* 12:147–161.