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Homocysteic and homocysteine sulphinic acid exhibit excitotoxicity in organotypic cultures from rat brain

Abstract The excitotoxic action of homocysteine and related sulphur-containing metabolites was investigated in organotpyic cultures derived from rat brain cortex and hippocampus by inhibition experiments using antagonists selective for different glutamate receptor subtypes. In addition the direct interaction of these metabolites with glutamate receptors expressed in frog oocytes was tested by conventional two electrode voltage clamp techniques.

Conclusion Neurodegeneration and epilepsy observed in homocystinuria may be mediated by L-homocysteic and L-homocysteine sulphinic acid. Both metabolites exhibit excitotoxic potency by interaction with different glutamate receptor subtypes.

Key words Homocystinuria · Excitotoxicity · Organotypic cultures · *Xenopus* oocytes

Abbreviations *APV* amino-5-phosphonovaleric acid · *CNQX* 6-cyano-7-nitroquinoxaline-2,3-dione · *HCys* homocysteine · *HCA* homocysteic acid · *HCSA* homocysteine sulphinic acid · *MK801* dibenzo-cyclo-heptenimine · *NMDA* N-methyl-D-aspartate

Introduction

Neurological complications in patients suffering from homocystinuria due to cystathionine-β-synthase deficiency include mental retardation, epileptic seizures, dystonia and other pyramidal signs. These symptoms are generally considered to be the consequence of vascular lesions [30]. However several clinical observations argue against this theory, at least in some of these patients: (1) most patients with mental retardation exhibit no focal neurological symptoms; (2) MRI of the brain has revealed no alterations in patients with severe mental retardation and dystonia; (3) neuropathological studies showed no brain infarction in patients with dystonia; and (4) diet may improve the mental status of patients [24].

Homocysteine (HCys) and related metabolites are thought to be excitotoxic. The concept of excitotoxicity describes the potential neuronal damage due to excessive activation of glutamate receptors [3, 20, 26, 27, 33, 37].

Seizures induced by intraperitoneal application of HCys in mice and rats can be inhibited by non-(NMDA) N-methyl-D-aspartate glutamate receptor antagonists [1, 7, 8, 13, 18]. Wuerthele et al. [47] described a similar activation of central neurons by HCys and glutamate. L-HCys has been shown to inhibit glutamate decarboxylase with an in vitro Ki of ~ 1 mM [40]. In addition homocysteic acid (L-HCA) and homocysteine sulphinic acid (L-HCSA) may be candidate neurotoxins as they have been identified as potent glutamate analogues and neurotransmitters acting on NMDA and non-NMDA receptors [5, 6, 11, 19, 22, 33, 35, 38, 42, 44, 45]. L-HCA and L-HCSA are elevated in the urine of patients with homocystinuria [2, 32]; however the concentration of these substances in the CSF as well as brain tissue of patients is unknown.

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Besides direct activation of excitatory amino acid receptors an increase in excitation may be mediated by reduction of inhibitory input and/or inhibition of transmitter inactivation (e.g. glutamate reuptake). Following HCys administration reduced cortical γ-aminobutyric acid concentrations were found in the rat and L-HCA and L-HCSA are potent agonists of the glutamate uptake system [1, 4, 16].

Thus several data indicate that the neurological symptoms in homocystinuria might be a consequence of excitotoxic mechanisms.

Organotypic brain tissue cultures are a suitable model to assess candidate neurotoxins [25, 43]. Using this approach, Vornov et al. [43] described typical lesion patterns for different glutamate receptor agonists in hippocampal slice cultures. In cosntrast to dissociated cell cultures, organotypic cultures provide a more physiological system, as e.g. different nerve cells with physiological cell contacts and transmitter inactivation are present. To elucidate the excitotoxic potential of metabolites which may accumulate in the CNS in homocystinuria we tested the neurotoxic effects of L-HCys, L-methionine and related metabolites (L-homocystine, S-adenosyl-L-homocysteine, L-HCA, L-HCSA) in hippocampal and cortical organotypic cultures from rat brain. In addition the effects of these substances on glutamate receptor-mediated membrane currents were investigated in frog oocytes expressing different glutamate receptor subtypes.

Methods

Organotypic cultures

Hippocampal and cortical slices were prepared from rat brain (6 days postnatal) [15]. The tissue was cut into 350 µm thick slices with a McIlwain tissue chopper and incubated in Minimum Essential Medium (MEM) for 30 min at 4°C. The slices were cultivated on membranes [39] (Nunc, 0.02 µm) which were inserted into 6-well culture dishes. The culture medium consisted of 50% MEM, 25% Hanks balanced salt solution and 25% horse serum including 2 mM glutamine and 0.044% $NaHCO_3$, pH 7.3 (38°C, 5% CO_2) [14].

Short-term incubation experiments with glutamate receptor agonists were performed in Hepes buffered salt solution (NaCl 143.4 mM, Hepes 5 mM, KCl 5.4 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1.2 mM, $CaCl_2$ 2 mM, D-Glucose 10 mM) [43] containing either glutamate, kainate (100 µM), or NMDA plus glycine (100 µM + 10 µM). After 30 min incubation with agonists, cells were transferred to serum-free medium (75% MEM, 25% BME, and 2 mM glutamine) for a 24-h recovery period. Short-term incubation with L-HCA, L-HCSA, DL-HCys (prepared by basic hydrolysis of its thiolactone), L-homocystine, L-Met and S-adenosyl-L-HCys (10 µM–5 mM) was performed in the same way.

For long-term incubation over 7 days, DL-HCys, L-HCys, L-homocystine, L-Met and S-adenosyl-L-HCys (1–5 mM) were applied within the culture medium on the 1st day of cultivation. The medium was changed every day.

Application of antagonists

For protection experiments during short-term incubation, cultures were pretrated with the non-competitive NMDA-receptor antagonist MK801 (dibenzo-cycloheptenimine) (20 µM, RBI) and the non-NMDA-receptor and glycine site antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 µm, RBI) for 20 min prior to co-incubation with the respective agonists/metabolites. In long-term experiments antagonists were added to the metabolite-containing culture medium.

Examination of cell damage

Nuclei of degenerating cells were visualized by propidium iodide (Sigma) staining [43]. Cell cultures were incubated at room temperature for 3 min in serum-free medium containing propidium iodide (6.5 µm). The staining solution was then replaced by dye-free medium and the cultures observed using a fluorescence microscope (Axioscope, ZEISS).

Enzyme activities of methionine metabolism in cultures and tissue

Enzyme activities of cystathionine β-synthase [9], methylenetetrahydrofolate reductase [10], and methionine synthase [10] were examined in organotypic hippocampal cultures as well as in uncultured brain tissue. For enzyme analysis the cultures were scraped off the membranes. Cystathionine β-synthase (CBS) activities (nmol/h/mg protein, presence of 1 mmol pyridoxal phosphate) were as follows: (1) cultured brain tissue 43.4, n = 1; (2) uncultured brain tissue 28.5, n = 1; (3) fibroblasts 15.2, n = 20.

Methylenetetrahydrofolate reductase activities (nmol/h/mg protein, FAD present) were: (1) cultured brain tissue 9.2, n = 1; (2) uncultured brain tissue 11.3, n = 1; (3) fibroblasts 8.5, n = 32. Methionine synthase activities (nmol/h/mg protein, methylcobalamin present) were as follows; (1) cultured brain tissue 1.9, n = 1; (2) uncultured brain tissue 3.9, n = 1; (3) fibroblasts 9.1, n = 14. Cystathione-β-synthase activity in organotypic cultures was relatively high compared with uncultured brain tissue, whereas the activity of methionine synthase is relatively low in cultured brain tissue as compared to fibroblasts.

Electrophysiological investigations

To investigate if L-HCA, L-HCSA, DL-HCys, L-HCys, L-Met and S-adenosyl-L-HCys exhibit any influence on glutamate receptors, oocytes of *Xenopus laevis* were injected with mRNA isolated from neocortical tissue of rat brain [31]. Expression of glutamate receptors was ensured by electrophysiological measurements in buffer supplemented with either NMDA plus glycine (100 µM + 10 µM) or AMPA (100 µM) or kainate (100 µM). The effects of L-HCA, L-HCSA, DL-HCys, L-HCys, L-Met and S-adenosyl-L-HCys were tested by adding these substances (50 µM–1 mM in the presence of 10 µM glycine) to the medium in the absence or presence of the respective glutamate receptor agonists. L-HCys was not tested as it was insoluble in the oocyte buffer. Membrane currents were measured using the conventional two electrode voltage clamp technique. The holding potential was –70 mV.

Results

Short-term incubation (30 min) of hippocampal slice cultures with glutamate receptor agonists (glutamate, kainate, NMDA) induced similar lesion patterns to those described for hippocampal roller cultures [42] (Fig. 1). Cell damage was visualized by propidium iodide staining which labels the nuclei of degenerating cells. Cortical cultures also exhibited massive cell death after treatment with the same agonists (Fig. 1). These effects were prevented by incubation with the respective receptor antagonists (MK801, 20 µM; CNQX, 10 µM) indicating that glutamate sensitive neurons were present in the culture. Short-term incu-

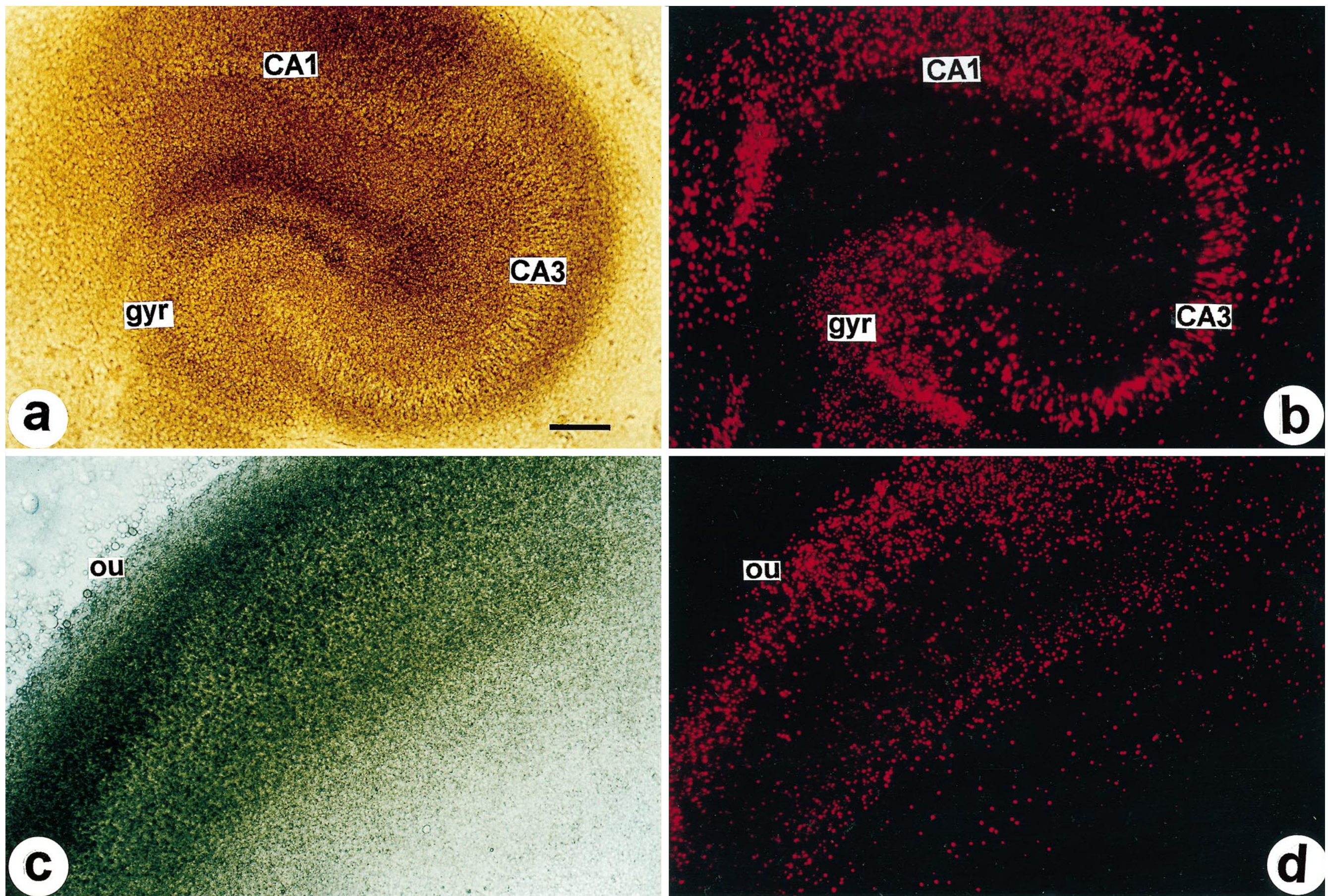

Fig. 1 Cell death visualized by propidium iodide fluorescence in organotypic slice cultures after incubation with glutamate receptor agonists. **a** Hippocampus (NMDA, 100 µM), phase contrast. **b** Same as in a, fluorescence. **c** Cortex (NMDA, 100 µM), phase contrast. **d** Same as in c, fluorescence. (*CA1* region CA1 of the pyramidal cell layer, *CA3* region CA3 of the pyramidal cell layer, *gyr* gyrus dentatus, *ou* outer border of the cortical culture). Bar in A: 200 µM for **a–d**

bation (30 min) of hippocampal and cortical slices with L-Met, S-adenosyl-L-HCys, DL-HCys, L-HCys, and L-homocystine (10 µM–5 mM) did not lead to significant cell death.

In contrast short-term incubation with L-HCA induced massive neuronal death in both culture types. In hippocampal cultures cell death was restricted to the CA1 region when L-HCA was applied at a concentration of 100 µM (Fig. 2 a) spreading over all CA regions including the dentate gyrus with increasing concentrations. Toxicity in cortical cultures (Fig. 2 b) appeared with 50 µM L-HCA. Cell death in both cultures was almost completely prevented by the NMDA receptor antagonist MK801, indicating that L-HCA was acting predominantly on NMDA receptors. Similar effects could be observed with cysteine-S-sulphate being less effective as L-HCA (neurotoxicity: 1 mM for hippocampal and 100 µM for cortical cultures (Fig. 2 c, d). L-HCSA was toxic at a concentration of 1 mM to both hippocampal and cortical cultures (Fig. 2 e, f). Neurotoxicity was clearly attenuated but not completely prevented by CNQX. Simultaneous application of MK801 further reduced the toxic effect.

In contrast to short-term experiments, long-term incubation (7 days) of hippocampal and cortical cultures with L-homocystine, L-HCys, DL-HCys and S-adenosyl-L-HCys (5 mM respectively) resulted in cell death starting after 48 h. Cell death was not restricted to separated areas of the cultures and could not be attenuated by application of glutamate receptor antagonists. L-Met was not toxic even at the highest concentration (5 mM).

Electrophysiological data of frog oocytes expressing glutamate receptors showed that incubation with L-HCA induced membrane currents comparable to those elicited by NMDA (Fig. 3). These membrane currents could be completely blocked by application of the compatible NMDA-receptor antagonist amino-5-phosphonovaleric acid (APV) or by addition of Mg^{2+} which blocks the NMDA receptor channel [28]. Membrane currents induced by L-HCSA were different and characterized by large oscillations (Fig. 3). These membrane currents were attenuated by CNQX but neither by APV nor by addition of Mg^{2+} indicating that non-NMDA receptors had been activated. The results correspond to those found for organotypic cultures. The residual oscillations found in the presence of L-HCSA and CNQX indicates that other glutamate (G-protein coupled) receptors had been additionally activated. L-Met, S-adenosyl-L-HCys, DL-HCys and L-HCys did not cause changes in oocyte membrane current. Activation of

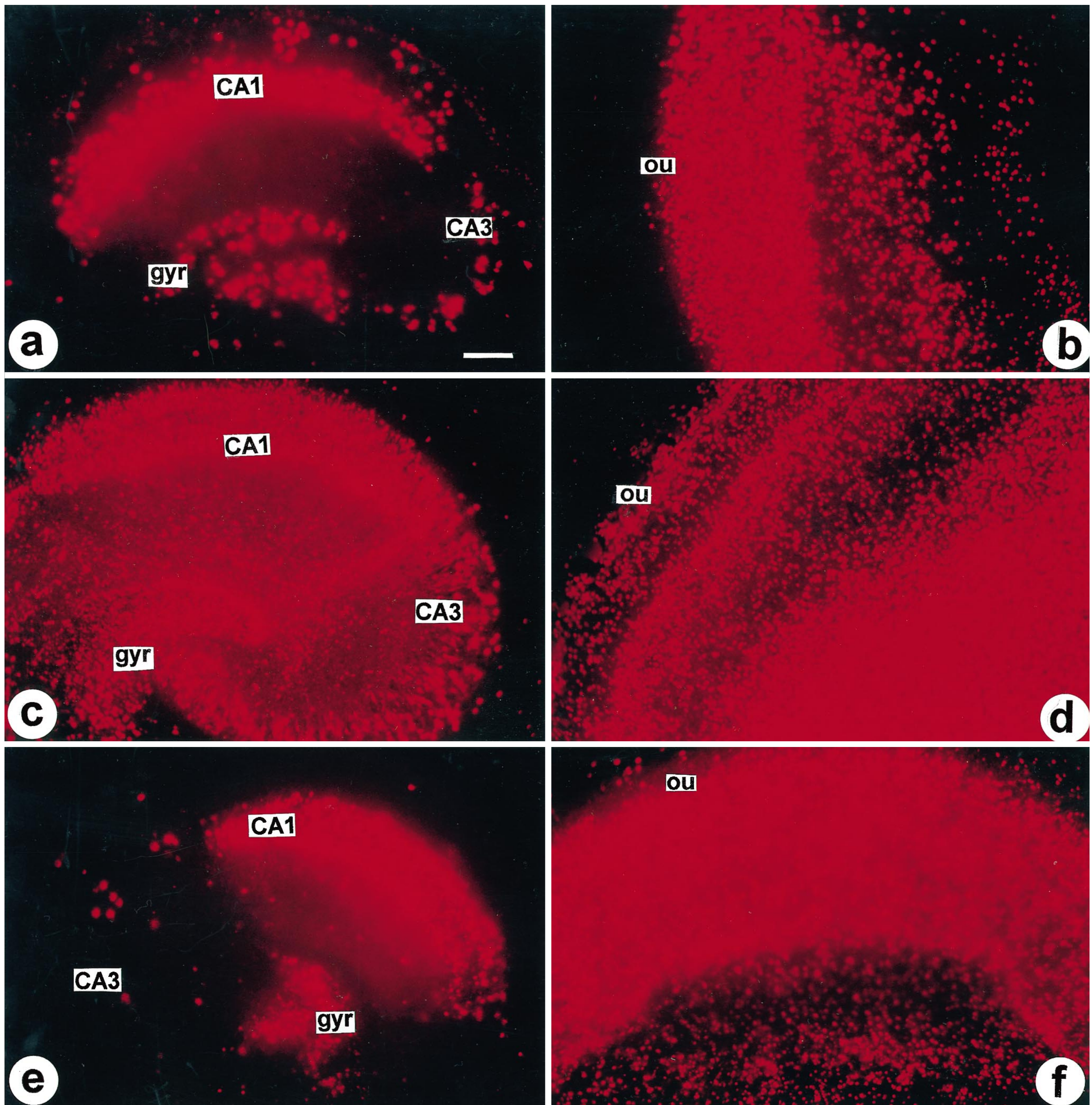

Fig. 2 Cell death visualized by propidium iodide fluorescence in organotypic slice cultures after incubation with metabolites. **a** Hippocampus (L-HCA, 100 µM). **b** Cortex (L-HCA, 500 µM). **c** Hippocampus (1 mM L-cysteine-S-sulphate). **d** Cortex (1 mM L-cysteine-S-sulphate). **e** Hippocampus (1 mM L-HCSA). **f** Cortex (1 mM L-HCSA). (*CA1* region CA1 of the pyramidal cell layer, *CA3* region CA3 of the pyramidal cell layer, *gyr* gyrus dentatus, *ou* outer border of the cortical culture). Bar in A: 200 µm for **a–f**

glutamate receptors by cysteine-S-sulphate could completely be inhibited by APV whereas the non-NMDA receptor antagonist MK801 prevented cell death in culture experiments by only ~ 80%.

Discussion

Our findings from cell culture and oocytes experiments indicate that excitotoxic mechanisms could play a role in the development of neurological signs in patients with homocystinuria. L-HCA and L-HCSA were potent agonists of glutamate receptors interacting with different glutamate receptor subtypes. Our results in organotypic cell cultures principally confirm earlier findings which showed that L-HCA interacts with NMDA receptors and L-HCSA with non-NMDA receptors [12, 21, 34, 26]. In contrast, Lee et al. [23], and Pullan et al. [26] reported on interaction of both L-HCA and L-HCSA with non-NMDA and

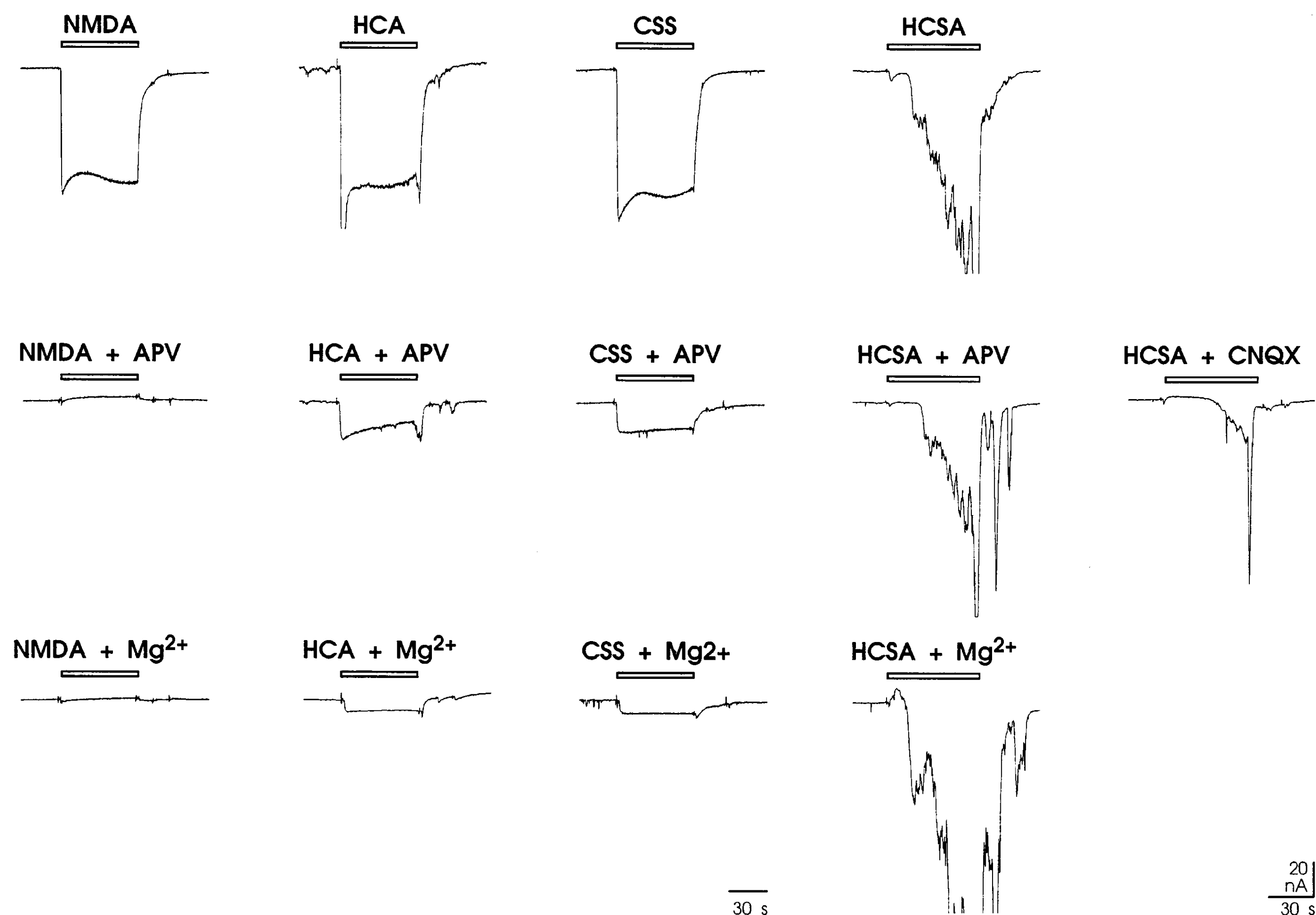

Fig. 3 Effects of L-HCA (100 µM), L-cysteine-S-sulphate (100 µM) and L-HCSA (25 µM) on membrane currents of *Xenopus* oocytes and inhibition by glutamate receptor antagonists APV (100 µM) CNQX (100 µM) and Mg^{2+} (0.8 mM). Administration is marked by *horizontal bars*. Recordings 3–5 days after injection of mRNA from rat neocortex. Holding potential: –70 mV. Inward current: downward deflection

NMDA receptors. The oscillation phenomena in oocyte experiments suggest the L-HCSA in addition may act on G-protein coupled receptors (e.g. metabotropic glutamate receptors). It could be speculated that *in vivo* concentrations of L-HCA and L-HCSA leading to neuronal damage may be lower since diffusion problems inherent to the cell culture model do not exist *in vivo*. L-HCA and L-HCSA have been proposed as neurotransmitters of the rat and human brain. However the exact physiological function remains unclear as most recently, cortical and cerebellar glia cells were described to store the substances [5, 9, 17, 41].

Very high concentrations (5 mM) of L-HCys resulted in neuronal death which could not be prevented by administration of glutamate receptor antagonists. Thus other non-glutamate receptor mediated mechanisms may lead to neuronal damage in homocystinuria. As HCys-induced seizures in neonatal rats could be prevented by glutamate receptor antagonists we speculate that these seizures are consequent to oxidation of HCys to L-HCA and/or L-HCSA (see introduction).

In conclusion these results give evidence that excitotoxic mechanisms may play a role in the development of neurological signs in homocystinuria. The high vulnerability of hippocampal pyramidal neurons to the metabolites tested is in accordance with morphological findings in patients described by White et al. [46].

We hope that these preliminary data will initiate the analysis of relevant metabolites in CSF during follow up of patients.

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