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Excitotoxic neuronal injury in chronic homocysteine neurotoxicity studied *in vitro*: The role of NMDA and group I metabotropic glutamate receptors

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Abstract. Elevated homocysteine is a risk factor in cardiovascular diseases and neurodegeneration. Among the putative mechanisms of homocysteine-evoked neurotoxicity, disturbances in methylation processes and NMDA receptor-mediated excitotoxicity have been suggested. Our previous studies demonstrated that group I metabotropic glutamate receptors along with NMDA receptors participate in acute homocysteine-induced neuronal damage. In this study, using propidium iodide staining, we tested whether the same mechanism may mediate chronic homocysteine neurotoxicity. Our results confirmed that the application of D,L-homocysteine in micromolar concentrations for 3 days induces neurodegeneration in primary cultures of cerebellar granule neurons. Uncompetitive NMDA receptor antagonist MK-801, and mGlu1 or mGlu5 receptor antagonists (LY367385 and MPEP, respectively), given alone provided very limited neuroprotection. However, simultaneous application of the NMDA receptor antagonists MK-801, memantine or amantadine and MPEP almost completely prevented chronic homocysteine neurotoxicity. These findings suggest a novel therapeutic strategy to combat neurodegeneration induced by hyperhomocysteinemia comprising a combination of antagonists of group I metabotropic glutamate receptors and NMDA receptors.

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Key words: amantadine, cerebellar granule cells, excitotoxicity, homocysteine, memantine, neuroprotection

INTRODUCTION

An increased concentration of homocysteine in human blood (hyperhomocysteinemia) is a known risk factor in cardiovascular and neurodegenerative diseases (Clark et al. 1998, Hankey and Eikelboom 1999, Morris 2003). Hyperhomocysteinemia often coincides with stroke, Alzheimer's and Parkinson's diseases, schizophrenia and depression, renal diseases, leukemia and diabetes (Blandini et al. 2001, De Luis et al. 2005, Godfrey et al. 1990, Hultberg et al. 1993, Kishi et al. 2000, 2003, Madonna et al. 2002, Parnetti et al. 2004, Pniewski et al. 2003, Yasui et al. 2000). A strong negative correlation between blood homocysteine (Hcy) level and concentrations of folate and vitamins B6 and B12 in body fluids has also been reported (Bissoli et al. 2002, Guerra-Shinohara et al. 2002). There is a reciprocal causal relationship between hyperhomocysteinemia and perturbations in methylation processes (Selhub 1992, Selhub et al. 1999). These metabolic disturbances have been suggested as important factors in the mechanisms of indirect Hcy neurotoxicity (Mattson and Shea 2003). Other studies have reported that Hcy induces NMDA receptor-mediated excitotoxicity (Kim and Pae 1996, Lipton et al. 1997), while several Hcy derivatives are selective agonists of group I metabotropic glutamate receptors (mGluRs) (Shi et al. 2003). Our previous *in vitro* studies demonstrated the strong neuroprotective effect of a combination of antagonists of NMDA receptors and group I mGluRs in Hcy-induced neurotoxicity (Zieminska et al. 2003, 2006).

In our earlier experiments (Zieminska et al. 2003, 2006) we used primary cultures of rat cerebellar granule cells (CGC) in an acute model of Hcy excitoxicity. The protocol included a short 30 min application of high concentrations of excitotoxin to induce neuronal damage that was evident after 24 h. Such a model was suitable for mechanistic studies, although the concentrations of Hcy used were irrelevant to human blood levels observed in even severe hyperhomocysteinemia. The aim of the present study was to characterize the receptor dependence of chronic Hcy-mediated excitotoxicity in primary cultures of rat CGC. Keeping in mind the prospective use of glutamate receptor antagonists in the treatment of Hcy-induced neurodegeneration, we evaluated the neuroprotective potential of antagonists of group I mGluRs and of high or moderate/low affinity NMDA receptor channel blockers, including memantine and amantadine, drugs used in the symptomatic treatment of Alzheimer's and Parkinson's diseases, respectively.

METHODS

Drugs

D,L-homocysteine (Hcy), 3,5-dimethyl-1-adamantanamine hydrochloride (memantine), 1-amino-adamantane (amantadine), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), propidium iodide (PI) dye and materials for cell culture were purchased from Sigma Chemical Company (St. Louis, MO, USA). (S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) and 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) were obtained from Tocris Neuramin Ltd. (Bristol, UK). All chemicals used were of analytical grade.

Cell cultures

Primary cultures of granule neurons were prepared from cerebella of 7-day-old rats using the method of Schousboe and coauthors (1985) and were cultivated in the slightly modified BME medium containing 25 mM KCl, as described previously (Zieminska et al. 2003, 2006). The collection of cerebella from rat pups was performed in accordance with Polish government regulations concerning experiments on animals (Dz.U.97.111.724) and the European Community Council Directive of 24 November 1986 (86/609/EEC). The procedure was approved by the First Local Ethical Committee in Warsaw. All efforts were made to reduce the number of animals used and to minimize animal suffering.

Chronic neurotoxicity: Induction and evaluation

On the 5th day of culture, aliquots of freshly prepared D,L-homocysteine were added directly to cell growth medium to obtain the desired final micromolar concentrations. Depending on the experiment, the medium also contained 50 μM glycine and/or group I mGluR and NMDA receptor antagonists. Culturing was continued for 72 h under standard conditions. The cells were then fixed with 80% methanol, stained with 5 μg/ml propidium iodide and viable and dead neurons were counted using a Zeiss Axiovert fluorescence microscope by an investigator unaware of the exact

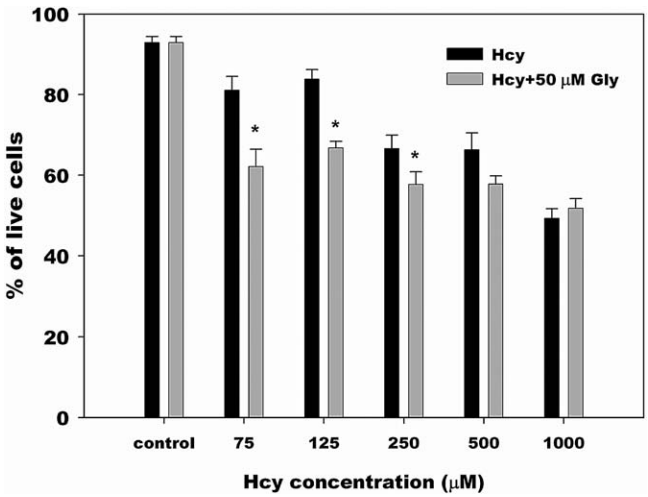

Fig. 1. Concentration-dependent chronic homocysteine (Hcy) neurotoxicity in cultured cerebellar granule cells: potentiation by glycine (Gly). Cells were incubated for 72 h in the presence of Hcy and/or Gly as indicated. Results are means ± SD, (n=6). (*) Means significantly different from the effects of Hcy alone (P<0.05).

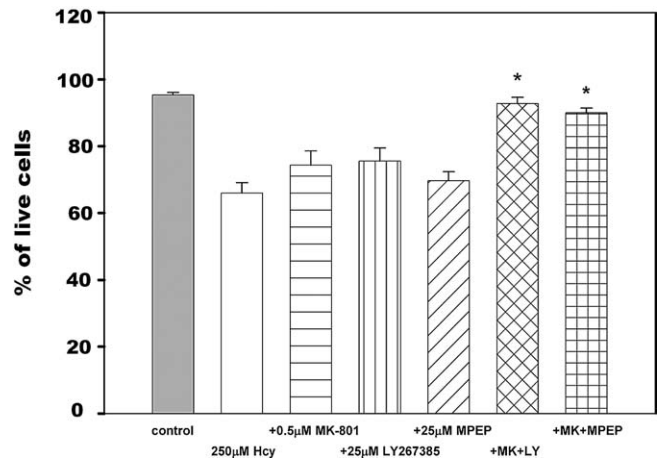

Fig. 2. Role of group I metabotropic glutamate receptors and NMDA receptors in chronic homocysteine-evoked neurotoxicity. Effects of 0.5 μM MK-801, 25 μM LY367385 and 25 μM MPEP on chronic neurotoxicity induced by 72 h incubation with 250 μM D,L-Hcy, were observed in the absence of glycine. Results are means ± SD, (n=6). (*) Means significantly different from effects of 250 μM Hcy (P<0.05).

experimental conditions, as described previously (Zieminska et al. 2003, 2006). Results were expressed as the percentage of live cells.

Data analysis

Experiments were repeated at least 3 times with the same qualitative results and these were presented as means ± standard deviation (SD) from a number of repetitions (n=6).

RESULTS

To characterize the chronic neurotoxicity of Hcy, the effect of supplementing CGC cultures with various concentrations of D,L-Hcy (75–1000 μM) on cell viability were studied. The results presented in Fig. 1 demonstrate a concentration-dependent decrease in viability after chronic (3 day) incubation of neurons with D,L-Hcy at sub-millimolar concentrations. This effect was significantly potentiated by 50 μM glycine, but only at concentrations of D,L-Hcy lower than 250 μM.

In subsequent experiments we tested the hypothesis that, as in the acute model, chronic Hcy neurotoxicity is mediated by the NMDA receptors and by group I mGluRs. In these experiments D,L-Hcy was applied at a concentration of 250 μM. To identify the role of NMDA receptors we used 0.5 μM MK-801, an uncompetitive NMDA receptor antagonist with high affinity for the NMDA channel. The role of group I mGluRs was tested using 25 μM LY367385 and MPEP, which are antagonists of mGlu1 and mGlu5 receptors, respectively. The results presented in Fig. 2 demonstrate that MK-801, LY367385 or MPEP given alone provided only insignificant neuroprotection. However, simultaneous application of MK-801 with either LY367385 or MPEP almost completely prevented Hcy neurotoxicity.

Figure 3 shows the relationship between concentrations of group I mGluR antagonists LY367385 and MPEP, applied in the presence of 0.5 μM MK-801, and the resulting protection against neurotoxicity induced by 3-day exposure of CGC to 250 μM D,L-Hcy. Both LY367385 (2.5–25 μM) and MPEP (5–50 μM) gave concentration-dependent neuroprotection. Under these conditions the neuroprotective potential of LY367385 appeared to be greater than that of MPEP.

Next we compared the neuroprotective potentials in chronic Hcy neurotoxicity of three uncompetitive NMDA receptor antagonists that differ in their affinity for the NMDA channel, applied in combination with 25 μM MPEP. MK-801 was used as a reference high affinity NMDA receptor antagonist and memantine and amantadine, moderate to low affinity NMDA channel blockers. Figure 4 demonstrates that the glutamate receptor antagonists, although neuroprotective,

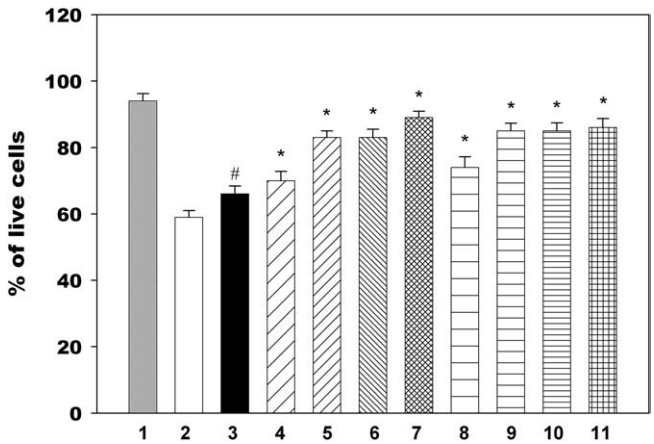

Fig. 3. Effect of different concentrations of antagonists of group I metabotropic glutamate receptors (GI mGluRs): 2.5, 5, 10, and 25 μM LY367385 (bars 4–7, respectively) and 5, 10, 25, and 50 μM MPEP (bars 8–11, respectively) applied in the presence of 0.5 μM MK-801, on neurotoxicity induced by 72 h exposure of cells to 250 μM D,L-Hcy (bar 2). Other bars: (1) control; (3) 250 μM Hcy + 0.5 μM MK-801. Results are means ± SD, (n=6). Means significantly different (P<0.05): (#) 250 μM D,L-Hcy + 0.5 μM MK-801 vs. 250 μM D,L-Hcy; (*) GI mGluRs antagonists + 250 μM D,L-Hcy + 0.5 μM MK-801 vs. 250 μM D,L-Hcy + 0.5 μM MK-801.

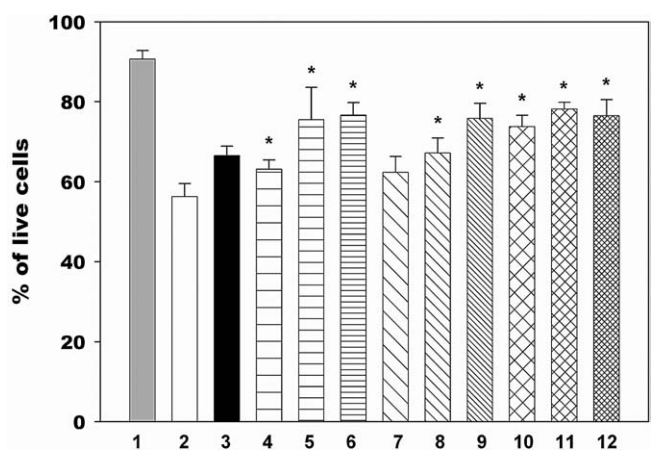

Fig. 4. Effect of different concentrations of antagonists of NMDA receptors, MK-801, memantine and amantadine, on neurotoxicity induced by 72 h exposure of cells to 250 μM D,L-Hcy in the presence of 25 μM MPEP. Bars: (1) control; (2) 250 μM Hcy; (3) 250 μM Hcy + 25 μM MPEP; (4)–(6) Hcy + MPEP + 0.05, 0.1, 0.5 μM MK-801, respectively; (7–9) Hcy + MPEP + 2.5, 5, 10 μM memantine, respectively; (10–12) Hcy + MPEP + 5, 10, 20 μM amantadine, respectively. Results are means ± SD, (n=6). (*) Means significantly different from effects of 250 μM Hcy + 25 μM MPEP (P<0.05).

did not provide the almost complete protection against Hcy neurotoxicity observed using other approaches. The viability of the control neurons (91%), which was reduced to 57% after 3-day exposure to 250 μM Hcy alone, reached 77–78% in the presence of 25 μM MPEP and the highest concentrations of the NMDA receptor antagonists. MK-801, which is the most potent NMDA receptor antagonist with the highest affinity for the NMDA channel, gave the greatest neuroprotection, attaining maximal levels at a concentration of 0.1 μM. Amantadine, a low affinity channel blocker was highly neuroprotective at 0.5 μM, while memantine was still inactive at this concentration. One should bear in mind that in all cases neuroprotection was induced by the combination of MPEP and various NMDA channel blockers. Control experiments demonstrated the lack of neurotoxicity of MK-801, memantine, amantadine, LY367385 and MPEP administered separately to CGC for 3 days at the concentrations used in this study.

DISCUSSION

The results of this study confirmed that growth of primary cultures of CGC for 3 days in the presence of homocysteine at concentrations relevant to severe hyperhomocysteinemia in humans induces neurotoxicity. In addition, the main mechanism of homocysteine-evoked neurotoxicity in this chronic *in vitro* model of excitotoxicity is, as in our previous acute experiments, mediated by both NMDA receptors and group I mGluRs. Finally, our data indicate that the moderate/low affinity uncompetitive NMDA receptors antagonists, memantine and amantadine, applied in combination with group I mGluR antagonists, provide protection against chronic homocysteine neurotoxicity.

This study utilized a chronic model of neurotoxicity, i.e. primary cultures of CGC were cultivated for 3 days in the presence of the neurotoxic substance. However, this experimental model has specific drawbacks. In particular, primary CGC cultures are only useful as models for neurotoxicological studies within a relatively narrow time window, after around 7 days of in vitro growth, i.e. the period between receptor maturation and rapid cell death a few days later (Schousboe et al. 1985). In our experiments Hcy was applied on the 5th day of *in vitro* culture, when maximal cell density was attained (Gallo et al. 1987), and neurotoxicity was evaluated on day 8, after maturation of glutamate

receptors but before the initiation of cell death (Marini et al. 1999).

Another concern is how the nature and concentration of Hcy that is neurotoxic *in vivo* compare with that present in our *in vitro* experiments after its 3-day application to the cultures. It has been estimated that almost 70% of Hcy normally present in human serum (at a concentration of 5–15 μM) is bound to proteins, about 30% is oxidized to the disulfide linked form (homocysteine RSSR), and only 1–2 % represents the free reduced form (homocysteine RSH) (Ueland 1995). The current consensus of opinion is that only free L-homocysteine, i.e. one half of the added D,L-Hcy, is biologically active as a metabolic substrate or the ligand of NMDA receptors (Lipton et al. 1997, Van Aerts et al. 1993, Weiss 2005). We did not measure the level of Hcy or its products in the culture medium during the 72 h incubation with D,L-Hcy. However, Lipton and others (1997) demonstrated that following 6-day incubation of cerebrocortical cultures to which 100 μM D,L-Hcy had been added, this sulfur amino acid was completely metabolized and other excitotoxic sulfur derivatives had not accumulated at concentrations that could interfere with NMDA receptors. Thus, we cannot exclude the possibility that in our experiments a part of the added Hcy could be used up in metabolic processes during the 3-day incubation. On the other hand, minor ($\leq$10%) contamination of the CGC cultures with astrocytes could result in glial Hcy production (Benz et al. 2004, Huang et al. 2005). However, considering the low proportion of astroglia in the CGC cultures, especially in relation to the volume of culture medium, this factor is thought to have been negligible. A considerable proportion of Hcy added to the neuronal cultures may be bound to serum proteins that are present in the culture medium. According to Togawa and coauthors (2000) the formation of disulfide bridges between Hcy and human plasma proteins, a partially oxygen-requiring process dependent on Hcy and protein concentrations, is relatively rapid. In their experiments there was no detectable reduced Hcy (RSH) in the system by 3 h after the application of 0.5 mM Hcy to plasma. According to Jakubowski (2004), the half-life for Hcy in water solutions is about 2 h. Thus, our data concerning the neurotoxic potential of Hcy in vitro during a 3-day exposure are likely to overestimate rather than underestimate the concentrations of Hcy that are present in the incubation medium and which induce detectable neuronal damage.

Traditional explanations of the mechanism of Hcy neurotoxicity point to the key role of disturbances in methylation and re-methylation processes. S-adenosylmethionine accumulated in cells in hyperhomocysteinemia is a very strong competitive antagonist of many transferases (Yudkoff 1999). Their prolonged suppression may inhibit the repair of damaged DNA chains and consequently lead to apoptotic cell death (Duan et al. 2002, Kruman et al. 2000, 2002). It is unclear whether this putative Hcy-evoked DNA damage and oxidative stress selectively causes mutations and damage to nuclear genes, or if it also induces mtDNA mutations, which are thought to be equally important in neurodegenerative disorders (Maruszczak et al. 2006). It is likely that multiple mechanisms contribute to chronic Hcy neurotoxicity. However, the results of the majority of the present chronic exposure experiments, in accordance with earlier acute *in vitro* neurotoxicity data (Zieminska et al. 2003, 2006), appear to exclude practically all mechanisms of Hcy neurotoxicity other than glutamate receptor mediated excitotoxicity. Nevertheless, in some of the chronic experiments, the inhibition of Hcy-induced neuronal death by NMDA receptor and group I mGluR antagonists was strong but incomplete, which may indicate slight participation by mechanisms other than excitotoxicity.

A decade ago, Kim and Pae (1996) proposed that excitotoxicity in Hcy-induced neurodegeneration is mediated specifically by the NMDA receptors. A subsequent investigation by Lipton and colleagues (1997) demonstrated that Hcy is not only an agonist of the glutamate binding site of the NMDA receptor, but it also blocks the glycine binding site in this receptor. In this way Hcy inhibits the activation of the NMDA channel, and an excess of glycine is required to remove Hcy from this modulatory site to achieve maximal activation of the NMDA receptor. To evaluate the maximal NMDA receptor-mediated Hcy neurotoxicity in our chronic experiments we applied 50 μM glycine. The results demonstrated that the potentiation of chronic Hcy neurotoxicity by glycine is relatively weak and can be observed only at D,L-Hcy concentrations that do not exceed 250 μM. Most probably at higher Hcy concentrations, 50 μM glycine cannot compete effectively with Hcy for the glycine binding site. In addition, MK-801, an uncompetitive antagonist of the NMDA receptor with high affinity for the channel when present alone, produced very slight protection

against chronic Hcy neurotoxicity. This result agrees with our previous findings concerning acute Hcy-induced damage to CGC *in vitro*. However, it significantly contradicts the data of Lipton and others (1997), who demonstrated almost complete neuroprotection by MK-801 or memantine in mixed neuronal and glial cerebrocortical cultures exposed to 100 μM D,L-Hcy for 6 days. Possibly, these discrepancies result from differences in the nature of the neuronal cultures and the concentrations of D,L-Hcy used in these studies.

In contrast to the results of Kim and Pae (1996) and Lipton and coauthors (1997), but in agreement with our previous findings (Zieminska et al. 2003, 2006), the present study demonstrated that chronic Hcy neurotoxicity exhibits complex receptor dependence. We observed that chronic Hcy neurotoxicity showed low sensitivity to MK-801 or to group I mGluR antagonists given alone, but it was almost completely prevented by treatment with a combination of NMDA receptor and group I mGluR antagonists. Interestingly, LY367385 and MPEP were equally efficient in inducing the neuroprotective effect. This indicates that both group I metabotropic glutamate receptor isoforms, mGlu1 and mGlu5, are involved in the mechanism of Hcy neurotoxicity.

The exact cellular and molecular mechanisms of Hcy-induced excitotoxicity mediated by the NMDA receptor and group I mGluRs, and particularly the roles of extra- and intracellular calcium in this process remain unclear. Previously, we have shown that acute exposure of CGC cultures to Hcy induces mitochondrial alterations including swelling and cytochrome c release, while calcium influx and transients in these neurons were rather weak (Zieminska et al. 2003, 2006). Moreover, under conditions of acute Hcy neurotoxicity, negligible stimulation of group I mGluR-mediated phosphoinositide hydrolysis was noted (Zieminska et al. 2006). On the other hand, we have also previously demonstrated that Hcy mobilizes intracellular calcium in the rabbit hippocampus *in vivo* and this process is mediated by group I mGluRs (Lazarewicz et al. 2003). Most probably, there are significant differences between Hcy-evoked excitotoxicity and the traditional form of excitotoxic neuronal damage induced by glutamate and NMDA (Ankarcrona et al. 1995, Choi 1985, Kristal and Dubinsky 1997). In addition, our present data do not reveal whether chronic Hcy excitotoxicity in primary CGC cultures represents mainly the necrotic or apoptotic form of neuronal cell death. In spite of this uncertainty over the details of the mechanisms of neurodegeneration induced by increased homocysteine concentrations, our results clearly point to the potential use of NMDA receptor and group I mGluR antagonists in neuroprotective strategies to combat neurodegeneration associated with hyperhomocysteinemia.

The traditional causative treatment of hyperhomocysteinemia, designed to reduce levels of Hcy in human body fluids, involves supplementation of the diet with folic acid and/or vitamins B6 and B12. However, in some cases this treatment is unsuccessful (Scott 2001). Therefore, the symptomatic treatment of Hcy-induced neuronal injury may also be beneficial. Excitotoxicity is an attractive target for neuroprotective efforts because it is implicated directly or indirectly in the pathophysiology of a wide variety of acute and chronic neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and multiple sclerosis (Salinska et al. 2005). These conditions are caused by different specific mechanisms but they may share a final common pathway leading to neuronal injury that involves the overstimulation of glutamate receptors, especially the NMDA subtype (Chen and Lipton 2006). It is generally accepted that acute brain disorders such as stroke, CNS trauma and epilepsy include a component of excitotoxicity. Hyperhomocysteinemia occurs in many acute and chronic CNS disorders and may participate in their pathomechanisms (Mattson and Shea 2003, Molloy and Weir 2001).

A clinically acceptable anti-excitotoxic therapy should be selectively targeted at excessive activation of excitatory amino acid receptors, but it should not interfere with their normal function. Competitive antagonists of the glutamate or glycine sites of the NMDA receptor, or high affinity NMDA channel blockers like MK-801, do not fulfill this criterion because they interfere with normal neuronal activity at doses below those necessary to prevent neurodegeneration (Chen and Lipton 2006). Potentially safe drugs that could indirectly modulate excitatory neurotransmission are ligands of mGluRs (Ritzen et al. 2005). Recently developed allosteric modulators of group I mGluRs, particularly non-competitive mGlu5 receptor antagonists, have shown great neuroprotective potential in *in vitro* and *in vivo* models (Gasparini et al. 2002, Slassi et al. 2005). However, these drugs are still

at the stage of preclinical evaluation. Another tactic promising safe inhibition of excitotoxicity is the use of uncompetitive NMDA receptor antagonists with moderate/low affinity for the NMDA channel that selectively block the effects of high concentrations of extracellular glutamate without interfering with normal neurotransmission (Lipton 2005). Moreover, memantine and amantadine, the main representatives of such antagonists, are already used in the symptomatic treatment of dementia, Alzheimer's and Parkinson's diseases (Danysz et al. 1997).

The results of our *in vitro* study demonstrate the effectiveness in neuroprotection against chronic Hcy-induced neurotoxicity of the mGlu1 and mGlu5 receptor antagonists LY67385 and MPEP, respectively, given in combination with MK-801, memantine or amantadine. Interestingly, our findings indicate that while MK-801 is the most potent NMDA receptor antagonist in protection against chronic Hcy toxicity, amantadine is more effective than memantine. MK-801 is a well characterized high affinity blocker of the NMDA channel, whereas the IC50s of memantine and amantadine have been estimated at 1.4 and 39 μM, respectively (Blanpied et al. 1997). This incongruity between the affinities of the antagonists for the NMDA receptor channel and their neuroprotective potential in Hcy neurotoxicity may be explained by the recently described unique effects of amantadine on NMDA channel gating (Blanpied et al. 2005). Taken together, our results suggest that hyperhomocysteinemia accompanied by neurological complications may be one of the indications for potentially safe combination therapy with a group I mGlu receptor antagonist and moderate/low NMDA receptor antagonists. This proposed treatment regime merits further testing in pre-clinical studies using animal models of hyperhomocysteinemia.

CONCLUSIONS

The findings of the present *in vitro* study indicate that chronic homocysteine-evoked neurodegeneration in primary cerebellar granule cell cultures is due to excitotoxicity mediated by NMDA receptors and both isoforms of group I mGluRs, mGlu1 and mGlu5. This suggests a new therapeutic strategy to manage hyperhomocysteinemia complicated by neurodegeneration that comprises a combination of medium/low affinity NMDA channel blockers and antagonists of group I mGluRs. Surprisingly, our results demonstrate that amantadine, a low affinity NMDA channel blocker, has much greater therapeutic potential in homocysteine-induced neurotoxicity than might be predicted based on measures of affinity for its target.

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