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Short communication

Bi-directional alterations of LTP after acute homocysteine exposure

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ABSTRACT

Homocysteine (HCY) is a known risk factor for neuronal diseases. We here report that HCY (10–1000 μM) interfered bi-directionally with long-term potentiation (LTP) in hippocampal slices, causing an impairment at concentrations <100 μM, and enhancement ≥500 μM. By comparison, NMDA unidirectionally reduced LTP, whereas L-cysteine led to facilitated LTP. Such HCY-induced alterations in neuronal communication may contribute to cognitive failure in dementia.

Diet and nutrition are acknowledged to be major contributors to neurodegenerative disease [4,18–20]. Metabolism of dietary protein via methylation reactions involves several B vitamin-dependent pathways and may contribute to the risk of developing adult onset vascular disease and late onset dementia. Homocysteine (HCY), a non-essential sulphur-containing amino acid, is formed as an intermediate of the methionine cycle, and its production is regulated by availability of B vitamins and interactions with the folic acid cycle. Developmentally, HCY has been suggested as the cause of spina bifida [27] and ageing-related elevations in plasma levels of HCY have been linked to Alzheimer's disease (AD) and vascular dementia: in the elderly, age related dietary vitamin deficiencies are common, exacerbating degenerative processes. Indeed, epidemiological and longitudinal studies propose a causal link between HCY and cognitive impairment [22] and suggest an action through cardiovascular as well as direct neurotoxic mechanisms [25].

HCY is produced from methionine by the action of two intermediates: S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH); once produced, HCY can either be re-methylated to methionine in a B12-dependent reaction or converted to cysteine by the enzyme cystathionine B-synthase (CBS) in a B6-dependent reaction. HCY remethylation is an important source of methyl groups in the brain and is necessary for reactions such as synthesis and degradation of neurotransmitters, membrane phospholipids and DNA methylation [26]. Hyperhomocysteinemia, caused by mutations or dietary imbalances disrupts the methionine and folate cycles and result in chronically elevated plasma HCY levels ranging from moderate (5–15 μM) to severe (>100 μM) [13]. HCY's potential neuro- and vaso-toxic actions are clearly implicated by the human condition of severe CBS deficiency which results in mental retardation, cerebral atrophy and seizures [21]. Accordingly, homozygous mice deficient in CBS are severely retarded and have a short life expectancy whereas heterozygous animals are viable but present with twice the normal plasma HCY levels [29]. Electrophysiological studies on the latter have confirmed enhanced long-term potentiation (LTP) *in vitro* [7] suggesting that HCY affects synaptic plasticity. This was also supported by studies from our group, in which chronic exposure to HCY *in vivo* altered basic synaptic transmission and LTP *ex vivo* [1,3] bi-directionally dependent on the length of treatment. *In vitro* studies in neuronal cell cultures reported that HCY may induce DNA damage, oxidative stress, apoptosis as well as abnormal metabolic activity (reviewed in [18]). Additionally, HCY may act as a glutamate receptor agonist [6,17], linking it to excitotoxicity. Homocysteic acid (HCA), an oxidative product of HCY, may also function as an excitatory neurotransmitter acting on NMDA receptors [5,15].

Since our previous *ex vivo* studies [1,3] did not allow to differentiate between putative direct vs. indirect (extra-hippocampal) causes for altered hippocampal plasticity, we here set out to characterise HCY acute effects on synaptic transmission and plasticity in the hippocampal slice preparation, and compared this with the action of L-cysteine and NMDA.

Hippocampal slices were prepared from male hooded Lister rats aged 6–8 weeks as described previously (e.g. [3]); all experimental

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procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986. Briefly, animals were terminally anaesthetised using the inhalant anaesthetic Halothane and the brain quickly removed and placed in ice-cold, gassed (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF; composition (in mM): 129.5 NaCl, 1.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.5 KH_2PO_4, 25 $NaHCO_3$ and 10 glucose (pH 7.4)). Slices of 400 μm were prepared using a McIllwain tissue chopper and transferred to pre-warmed (32 °C), gassed aCSF for at least 1 h prior to commencement of experiments.

Electrophysiological recording in slices were conducted by placing monopolar stimulation electrodes (WPI, 0.5 MΩ) into the Schaffer collateral / commissural fibres and a borosilicate glass aCSF-filled recording electrode (Harvard, 3–7 MΩ) in the CA1 pyramidal cell body layer for recordings of population spikes (PSs). Extracellular recording of PSs represents the systems-relevant output of summed action potentials in the vicinity of the recording electrode. This robust output signal is widely used as a measurement of changes in synaptic transmission and plasticity at hippocampal synapses [8,23,28], and was used in our previous ex vivo studies [1,3].

Initially, input–output (IO) relationships were produced by stepwise increases of voltage until saturation of the signal was reached (5–40 V; 100 μs stimulation duration), and subsequent LTP experiments were conducted at 50% of this maximum. A second set of IO measurements were performed at 60 min post-tetanus to ascertain whether LTP had indeed caused a genuine potentiation over the full IO range.

Baseline recordings were conducted for at least 10 min before HCY was applied via the perfusion line (5–8 ml/min). Slices were discarded if signals varied by more than 10% and control LTP experiments were conducted at regular intervals to ascertain stable and reproducible recording conditions. HCY was applied for 10 min (flanking the tetanus, 5 min pre- and 5 min post-tetanus); to induce LTP, a 100 Hz tetanus protocol (3 x 100 Hz stimuli, 1 second each) was applied and post tetanus values recorded for at least 1 h.

For multiple comparisons of IO data, 2-way repeated measure analyses of variance (RM ANOVA) were conducted. IO data matched within the same experiment were analysed as stimulus vs. post-tetanus. Additional paired t tests were conducted at 50 and 100% of the stimulation maximum. IO data were normalised to the pre-tetanus maximal response for pre- and post-tetanus comparison.

For multiple comparisons of LTP data, 2-way RM ANOVA (stimulus vs. time) followed by post-hoc *t*-tests (with Bonferroni correction) were performed on post-tetanus time-points only and comparisons made between control and HCY groups.

Hippocampal slice experiments assessed the action of acutely applied HCY on synaptic transmission and on hippocampal LTP over a wide concentration range. For clarity, data are split for low micromolar concentrations (<100 μM; Fig. 1A), and higher concentrations up to 1 mM (Fig. 1B).

Control LTP ($n = 14$) displayed a stable post-tetanus PS amplitude (~160%), which was affected by HCY in a non-linear, dose-dependent manner: at 10 μM, HCY caused no change in LTP compared to controls ($n = 6$, $P > 0.05$), but at 50 μM ($n = 9$) and 80 μM ($n = 5$) HCY, a considerably reduced potentiation was observed; this reduction was maintained for 60 min of recording (Fig. 1A) and was highly significant for both concentrations (F's > 7; P's < 0.01) with potentiation reaching 143% and 131% at 60 min post-tetanus, respectively.

Comparison of amplitudes for pre- and post-LTP IO curves at low HCY concentrations (Fig. 2A–C) revealed a significant enhancement of the signal over the whole testing range in controls after LTP. For HCY groups, all concentrations tested showed a significant effect of potentiation over the whole IO range (10 μM ($F_{(1, 75)} = 25.4$; $P < 0.01$), 50 μM ($F_{(14, 105)} = 3.0$; $P < 0.01$: interaction, $F_{(1, 105)} = 104.8$; $P < 0.01$) and at 80 μM ($F_{(1, 60)} = 39.1$; $P < 0.01$).

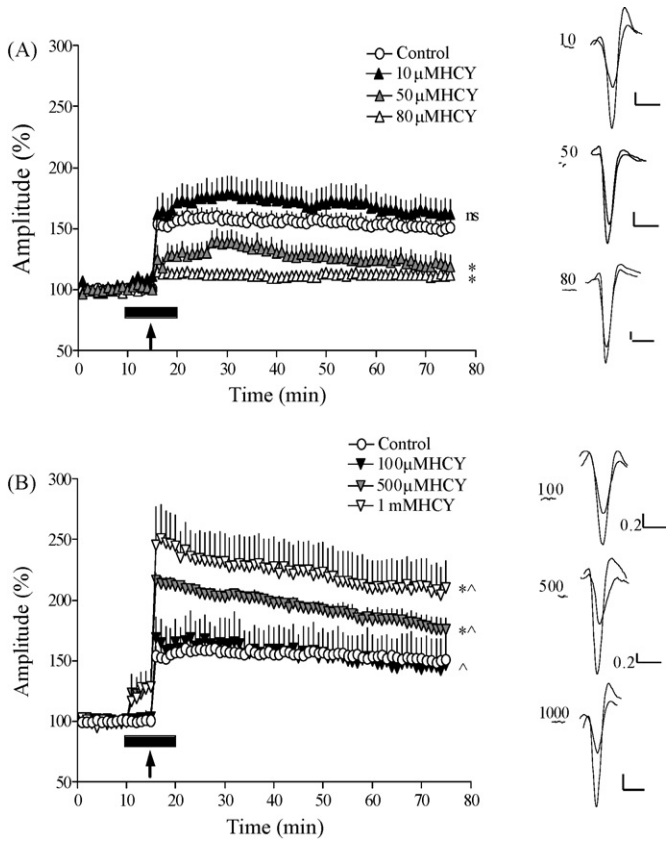

Fig. 1. HCY affects LTP in bi-directional manner dependent on dose. Time courses of population spike amplitudes in percent of baseline. Superimposed sample traces for baseline and 60 min post-tetanus are displayed for all experimental groups; scale bars indicate 0.5 mV (y) and 2.5 ms (x) unless otherwise indicated. Bars show duration of drug application and arrows mark onset of tetanisation. (A) HCY (50 and 80 μM) significantly *reduced* hippocampal CA1 LTP. (B) LTP was *enhanced* by high concentrations of HCY whereas an intermediate concentration (100 μM) had no effect on the potentiation relative to control.

Notably, LTP data for 100 μM HCY ($n = 6$) showed that spike amplitudes were not different to control levels (see Fig. 1B, $P > 0.05$); however, LTP was significantly enhanced relative to controls for 500 μM (~200%, $n = 7$, $F_{(59, 1121)} = 7.3$; $P < 0.0001$) and 1 mM (~250%, $n = 6$, $F_{(1, 1062)} = 13.6$; $P < 0.01$). These high concentrations slightly enhanced baseline amplitudes during the 5 min pre-tetanus application of both 500 μM and 1 mM HCY, indicative of enhanced excitation. After correction of the baseline effect (i.e. baseline in HCY is set as 100%), facilitated LTP was still observed for 1 mM ($F_{(59,1062)} = 5.2$; $P < 0.0001$) but not for 500 μM HCY, yet the latter still revealed a significant interaction ($P < 0.0001$, data not shown).

Comparison of baseline and post-LTP IOs for 100 μM HCY (see Fig. 2 D) revealed that amplitudes were significantly increased at the higher range of voltages (25–40 V, $F_{(1, 60)} = 36.82$; $P < 0.01$) but not in the IOs range < 25 V. Amplitudes were significantly enhanced in comparison to baseline over the whole range of the IO for both 500 μM (Fig. 2 E; $F_{(1,75)} = 42.11$; $P < 0.01$) and 1 mM (Fig. 2 F; $F_{(1, 105)} = 87.19$; $P < 0.01$) HCY.

To ascertain whether the pharmacological profile obtained for HCY corresponded with its suggested action as an NMDA receptor agonist [17], the next set of experiments examined L-cysteine and NMDA for comparison. Initial pilot studies have established concentrations of NMDA which were devoid of baseline effects, to match HCY's lack of baseline effect in the lower micromolar range and to avoid excitotoxicity associated with strong NMDA receptor stimulation. It was found that NMDA potently affected synaptic transmission at concentrations greater than 1 μM.