RESEARCH ARTICLE

Chronic homocysteine exposure causes changes in the intrinsic electrophysiological properties of cultured hippocampal neurons

Christina Schaub · Mischa Uebachs · Heinz Beck · Michael Linnebank

Abstract Homocystinuria is an inborn error of metabolism characterized by plasma homocysteine levels up to 500 µM, premature vascular events and mental retardation. Mild elevations of homocysteine plasma levels up to 25 µM, which are common in the general population, are associated with vascular disease, cognitive impairment and neurodegeneration. Several mechanisms of homocysteine neurotoxicity have been investigated. However, information on putative effects of hyperhomocysteinemia on the electrophysiology of neurons is limited. To screen for such effects, we examined primary cultures of mouse hippocampal neurons with the whole-cell patch-clamp technique. Homocysteine was applied intracellularly (100 µM), or cell cultures were incubated with 100 µM homocysteine for 24 h. Membrane voltage was measured in current-clamp mode, and action potential firing was induced with short and prolonged current injections. Single action potentials induced by short current injections (5 ms) were not altered by acute application or incubation of homocysteine. When we elicited trains of action potentials with prolonged current injections (200 ms), a broadening of action potentials during repetitive firing was observed in control neurons.

This spike broadening was unaltered by acute application of homocysteine. However, it was significantly diminished when incubation with homocysteine was extended to 24 h prior to recording. Furthermore, the number of action potentials elicited by low current injections was reduced after long-term incubation with homocysteine, but not by the acute application. After 24 h of homocysteine incubation, the input resistance was reduced which might have contributed to the observed alterations in membrane excitability. We conclude that homocysteine exposure causes changes in the intrinsic electrophysiological properties of cultured hippocampal neurons as a mechanism of neurological symptoms of hyperhomocysteinemia.

Keywords Homocysteine · Excitability of neurons · Epilepsy

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Introduction

Homocysteine is a sulfur containing, reactive, non-proteinogenic amino acid occurring as ubiquitous intermediate of methionine metabolism. Depending on the cell type, homocysteine can be metabolized within or released from the cell (Mudd et al. 2001). Several enzymes, transporter proteins and transmembrane carriers are involved in methionine–homocysteine metabolism, in which folate, riboflavin, vitamin B6 and vitamin B12 act as co-factors. Several hereditary enzyme defects and vitamin deficiencies can cause severe hyperhomocysteinemia, in which disulfides of homocysteine form homocystine. Because homocystine is renally excreted, this metabolic condition is denominated homocystinuria (Mudd et al. 2001). Neurological symptoms associated with untreated homocystinuria include psychomotor retardation, disturbances

of central nervous system myelination and epilepsy (Mudd et al. 1985). Whereas homocysteine plasma levels in homocystinuria patients can reach 500 μM, mild hyperhomocysteinemia is defined by homocysteine plasma levels above the upper reference range of 10–15 μM up to 25 μM (Stanger et al. 2009). Polymorphic variants of proteins involved in homocysteine metabolism, vitamin deficiencies and renal dysfunction are common reasons of mild hyperhomocysteinemia (Mudd et al. 2001). Numerous studies investigated the relevance of mild hyperhomocysteinemia for central nervous system disorders. Mild hyperhomocysteinemia is associated not only with cerebrovascular disease, but also with mild cognitive impairment, Alzheimer's disease and epilepsy (Stanger et al. 2009). Moreover, the recent VITACOG trial showed a significant reduction in brain atrophy as effect of homocysteine-lowering vitamins in individuals with mild cognitive impairment (Smith et al. 2010). Thus, a causal relationship between homocysteine and neuronal damage may be assumed, but the underlying mechanisms remain speculative. Homocysteine acts as a partial antagonist of the glycine site of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors, but is also an agonist at the glutamate binding site of this receptor. As NMDA receptors are up-regulated in alcohol-overconsuming individuals, because alcohol acts as an antagonist at NMDA receptors, elevated homocysteine plasma levels in actively drinking patients may contribute to seizures in alcohol withdrawal and explain the correlation between homocysteine plasma levels and the risk of withdrawal seizures (Bleich et al. 2006). Further, neuronal calcium influx mediated by effects of glutamatergic hyperexcitation of NMDA receptors is a pathomechanism of Alzheimer's disease and can induce cell death of retinal ganglion cells (Ganapathy et al. 2011). Thus, the rationale of this study is the assumption that homocysteine impacts the electrophysiology of neurons as one underlying mechanism of the association of hyperhomocysteinemia with mild cognitive impairment and dementia (Chan et al. 2008; Stanger et al. 2009; Smith et al. 2010). In the present study, we aimed at elucidating acute and chronic effects of homocysteine on the excitability of primary mouse hippocampal neurons.

Materials and methods

Patch-clamp recordings

Primary cultured neurons were superfused with an extracellular solution containing (in mM) 125 NaCl, 3.5 KCl, 1.25 NaH$_2$PO$_4$, 2 MgSO$_4$, 2 CaCl$_2$, 26 NaHCO$_3$, and 15 D-glucose, osmolality 315 mOsm. Patch pipettes (resistance 3–4 MΩ) were filled with an intracellular solution

containing (in mM) 130 K-gluconate, 20 KCl, 10 HEPES, 0.16 ethylene glycol-bis (2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 2 Mg-adenosine 5′-triphosphate (ATP) and 2 Na$_2$-ATP; pH was titrated to 7.25 with KOH; osmolality was adjusted to 295 mOsm using sucrose.

A liquid junction potential of −15 mV was measured between the intra- and extracellular solutions and corrected so that the data points were shifted in a hyperpolarizing direction relative to the voltage axis. We obtained tight-seal whole-cell recordings with an EPC9 amplifier (HEKA, Lamprecht, Germany). Voltage signals were filtered at 10 kHz (3 dB, four-pole low-pass Bessel filter) and sampled at 20 kHz or more by an interface (ITC16, Instrutech). All experiments were done at room temperature (21–23 °C).

Neuronal primary cell culture

Primary hippocampal neurons were prepared from Wistar rats as described previously (Schoch et al. 2001). Pregnant rats were killed, and the uterus containing the embryos (embryonic days 17–18) was removed. The hippocampus was isolated, and the tissue was washed and then digested with trypsin (0.025 g/ml for 10 min at 37 °C). After several washing steps, the tissue was dissociated by trituration using a plastic tip. The solution was centrifuged at 1,000 rpm for 10 min, and medium was added. After counting, the cells were plated at a density of 40,000/24 well on glass coverslips coated with poly-L-lysine (Sigma-Aldrich). Neurons were cultured at 37 °C in a humidified incubator with 95 % air/5 %CO$_2$ in basal medium eagle (BME) supplemented with 0.5 % glucose, 10 % fetal calf serum (FCS), 2 % B-27 and 0.5 mM L-glutamine (all Invitrogen, Karlsruhe, Germany) for 9–14 days before use.

Dissociated hippocampal neurons were incubated with 100 μM homocysteine (DL-homocysteine, Sigma-Aldrich) for 24 h or were treated with acute intracellular application of 100 μM homocysteine via inclusion into the patch pipette. This concentration is ten times higher than physiological homocysteine plasma concentrations, but commonly observed in inborn errors of homocysteine metabolism, for example, due to deficiency of cystathionine beta-synthase or methylenetetrahydrofolate reductase. Also, severe vitamin B12 deficiency can lead to such plasma concentrations (Stanger et al. 2009).

All neurons used in our experiments had a pyramidal neuron-like morphology with one or two major dendrites emanating from the cell body.

Analysis and statistics

The analysis of the recorded traces was performed by an automated algorithm using the software Igor (Wavemetrics). The activation threshold of action potentials was determined

as the point with the maximal increase in the slope. Parameters extracted from the traces were averaged, and unpaired Student's t tests were performed for statistical comparison (Excel, Microsoft). The analysis was performed automated and blinded to avoid a bias. All data are presented as mean values, error bars represent the SEM. A significance level <0.05 is marked with asterisks throughout all figures. The number of recorded cells ranged from 10 to 20 (see figure legends for details).

Results

We obtained patch-clamp recordings from primary cultures of hippocampal neurons and first assessed the passive membrane properties of these neurons. No significant differences between the resting membrane potential of the cells could be observed when comparing cells cultured under control conditions, following incubation with homocysteine or during acute application of homocysteine (data not shown). We injected small subthreshold depolarizing currents to examine input resistance (Fig. 1a, inset). Fitting the subthreshold current–voltage relation allowed us to determine the input resistance (Fig. 1a). We found that preincubation with homocysteine (24 h, see "Methods") caused a pronounced reduction in the input resistance by 42 % (Fig. 1b, from 529 to 305 MΩ, $n = 10$ and 10, $p < 0.05$). In contrast, acute intracellular application of 100 μM homocysteine via inclusion into the patch pipette did not affect input resistance (Fig. 1c, $n = 17$ and $n = 16$ for control and homocysteine). The apparent differences between the two control groups are due to variability between batches of cell cultures and consistently turned out to be not significant, whereas the comparison between control and application/incubation was always performed within one batch.

We next studied if the properties of action potentials are altered by homocysteine preincubation (Inc) or intracellular application of homocysteine (IC). Individual action potentials were elicited by brief current injections (5 ms, lowermost trace in Fig. 2a) elicited firing of single action potentials (uppermost trace in Fig. 2a). The threshold for generation of an action potential was unaffected by homocysteine, regardless of its mode of application (Fig. 2c), as was the action potential peak voltage (Fig. 2d), the time until the action potential peak was attained (Fig. 2e) and the duration of the action potential at threshold level (Fig. 2f). To examine spike afterpotentials, the depolarizing effects of the current injection were subtracted. To achieve this, we subtracted the voltage trace elicited by just subthreshold current injection (middle trace in Fig. 2a) scaled to the size of the initial depolarization preceding the action potential (dashed line in Fig. 2b). This revealed an after spike hyperpolarization (lowermost trace in Fig. 2b), which was also not altered
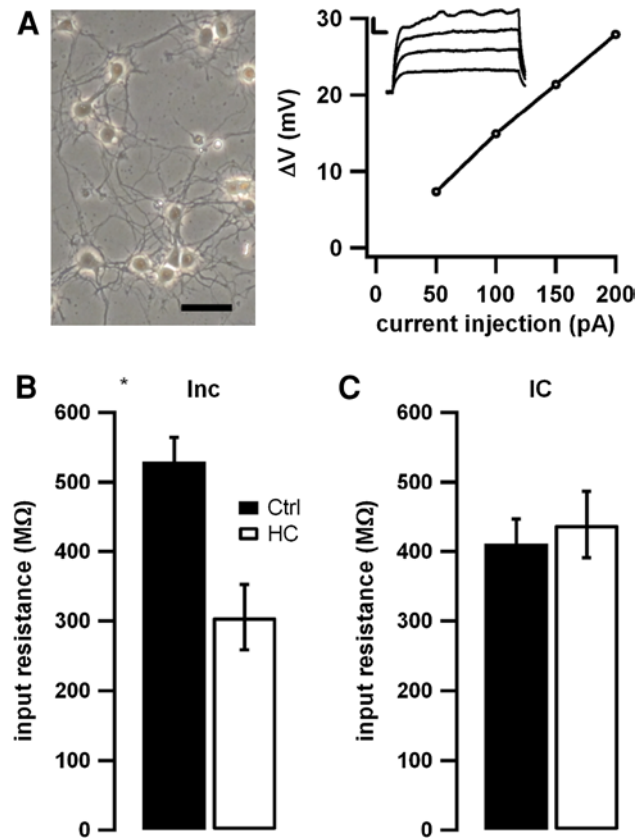
Fig. 1 Homocysteine effects on passive properties of hippocampal neurons. **a** Photomicrograph of a representative neuronal culture on the *left*. *Right panel* shows input resistance calculated according to Ohm's law from the current–voltage relation of passive voltage deflections (see *inset*, *scale bars* indicate 5 mV and 20 ms) elicited by small subthreshold current injections. **b**, **c** Preincubation with homocysteine (Inc, *open bars* in **b**, $n = 10$), but not acute application of homocysteine (IC, *open bars* in **c**, $n = 16$), resulted in a decreased input resistance compared to control cells (*filled bars* in **b**, $n = 10$ and **c**, $n = 17$)

by homocysteine preincubation or intracellular application (Fig. 2g). Thus, it appears that neither application of homocysteine via the patch pipette nor 24-h preincubation with homocysteine modifies the properties of individual action potentials.

We next studied the input–output relation of hippocampal neurons, and if this can be affected by homocysteine. The input–output relation was assessed by injecting current pulses of various amplitudes (200 ms, Fig. 3a, b, lowermost traces) and counting the number of resultant action potentials. We hypothesized that the decrease in input resistance seen in Fig. 1 would render homocysteine preincubated neurons less excitable and reduce action potential output. Indeed, homocysteine preincubated cells generated significantly less action potential output for small current injections (100 and 150 pA, see Fig. 3c; asterisks indicate
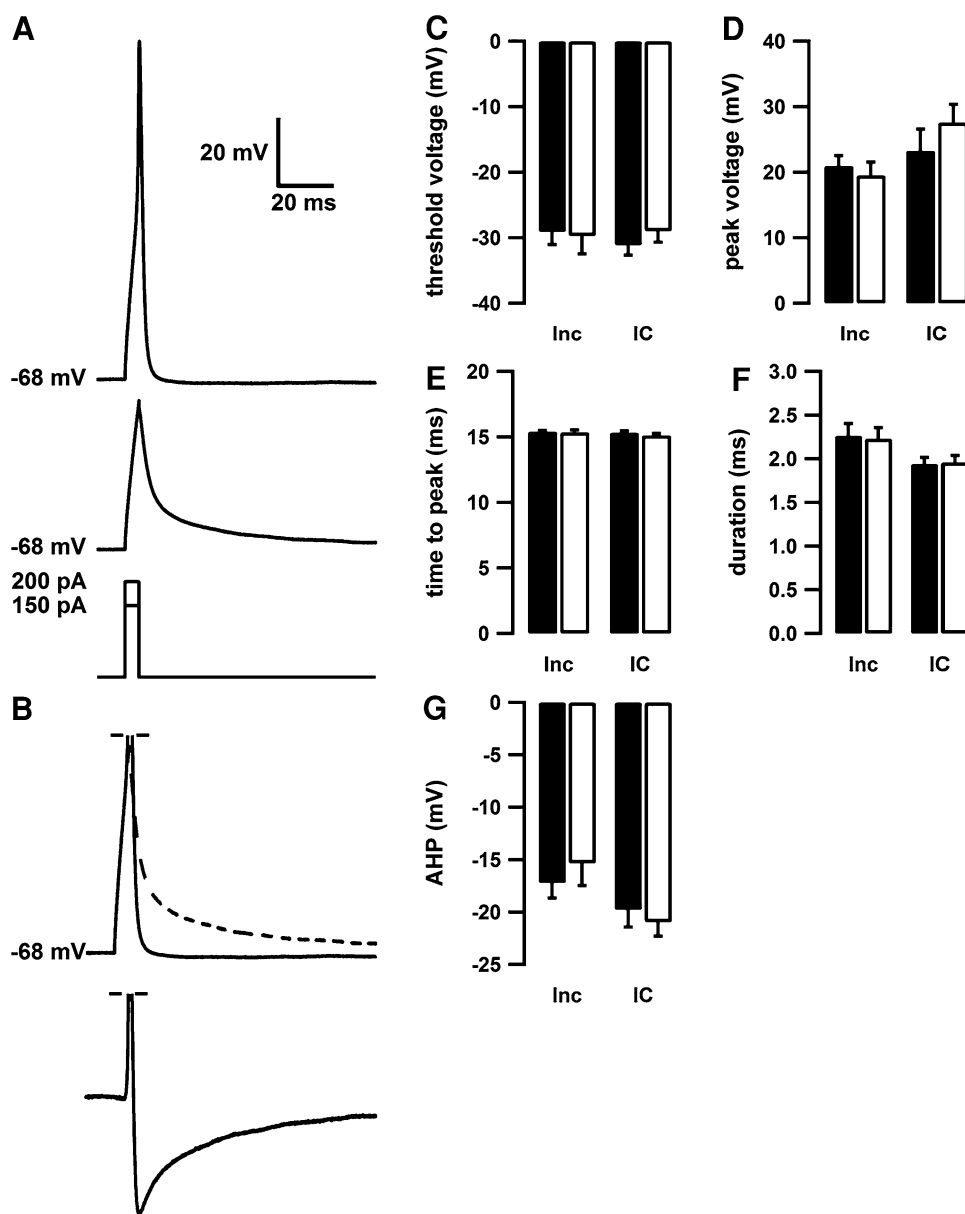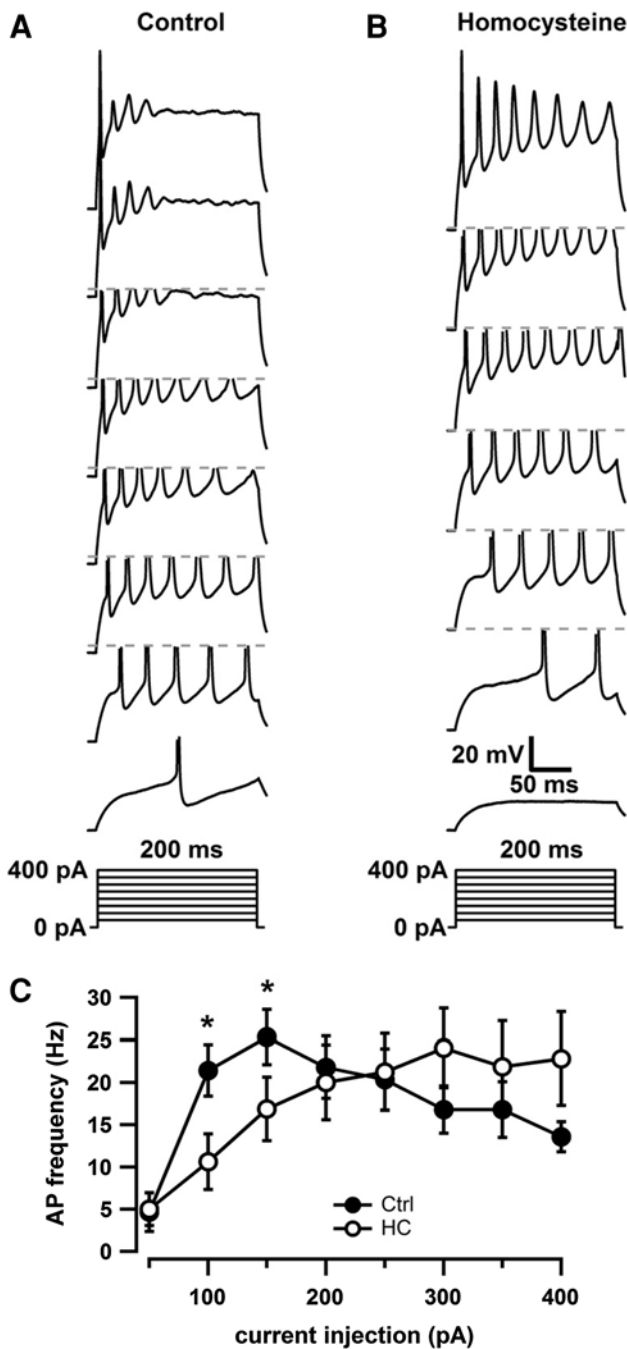
Fig. 2 Homocysteine does not affect the action potential shape. **a** Representative voltage trace of an action potential (*upper trace*) and a passive depolarization (*middle trace*, see lowermost part for current injections). Neither the action potential threshold (**c**), the minimum current injection needed to elicit an action potential (**d**), the peak voltage (**d**), the time to peak (**e**) nor the width of the action potential at threshold level (**f**) is altered following acute intracellular application (*open bars*, IC, *n* = 15) or 24-h incubation (*open bars*, Inc, *n* = 14) with 100 μM homocysteine compared to control cells (*filled bars n* = 16 (IC group) and 13 (Inc group)). **b** To analyze the afterpotentials of the action potential, the voltage trace elicited with a subthreshold current injection (**a** middle trace) was scaled to fit the passive phase of the action potential trace (**a** upper trace). The up-scaled subthreshold trace (*dashed line*) was subtracted from the action potential trace (*full line*) to correct the voltage trace for the artificial voltage deflection due to the experimental setup. The resulting trace (lowermost part) represents the active voltage changes during an action potential and reveals a pronounced after spike hyperpolarization. **g** The AHP was quantified in control cells (*filled bars*) and following intracellular application (IC) or 24-h incubation (Inc) with homocysteine (*open bars*) and was unaffected by homocysteine

$p < 0.05$, $n = 10$ for homocysteine and control groups). Conversely, the application of homocysteine via the patch pipette did not affect the input–output relation ($n = 17$ and $n = 16$ for control and homocysteine, data not shown).

To characterize the shape of the action potentials and their activity-dependent change during action potential trains, we analyzed the first voltage trace that displayed five action potentials. The action potential firing displayed

Action potentials physiologically broaden during a train of action potentials, reflecting the progressive inactivation of voltage-gated ion channels (Fig. 5a). Spike broadening was quantified at the level of the half Ap amplitude (see dashed lines in Fig. 5b_1, b_2). The spike broadening during trains was significantly reduced in cells preincubated 24 h in homocysteine (open circles in Fig. 5c). In contrast to the homocysteine effects on input resistance and frequency adaptation, this loss of spike broadening was also exerted by acute application of homocysteine (open circles in Fig. 5d). These results show that long-term exposure to homocysteine strongly affects neuronal excitability, whereas short-term exposure has only discrete effects.

Discussion

The present study aimed at analyzing electrophysiological effects of homocysteine as a possible mechanism of the association between hyperhomocysteinemia and neurological diseases.

We tested the effects of intra- and extracellular elevations of homocysteine concentrations on primary mouse neurons and observed that the input resistance decreases following long-term homocysteine exposure. This phenomenon results in a decrease in the neuronal input–output relation, rendering the neurons less excitable to lower- and mid-range current injections. Further, we found a significant reduction in both spike broadening and frequency adaptation during repetitive firing following 24-h homocysteine incubation. While a reduction in input resistance may be interpreted as a reduction in excitability, this latter effect is consistent with an increased excitability, as spike accommodation limits the capability of cells to fire high-frequency discharges (Zhou and Hablitz 1996). These results indicate that the effects of homocysteine on neurons elude a simple classification into pro- and anti-excitatory effects.

a frequency adaptation during the train in control conditions (see Fig. 4a). This adaptation turned out to be significant when quantified as increasing interspike intervals for all recorded control cells (as indicates by the arrow in Fig. 4a, see filled circles and asterisks in Fig. 4b, c). Following 24-h preincubation with homocysteine, this frequency adaptation was markedly reduced and no longer significant (open circles in Fig. 4b). This was not the case when homocysteine was introduced via the patch (open circles in Fig. 4c). Neither preincubation nor acute application of homocysteine affected the initial spike interval.

Fig. 4 Homocysteine abolishes the frequency adaptation of repetitive firing. **a** The first action potential train with at least 5 action potentials was analyzed to address the frequency adaptation during the train. Interspike intervals as indicated by the *arrow* were quantified. During the recorded period, a slight but significant increase in the interspike interval was observed (*filled circles* in **b**, $n = 10$ and **c**, $n = 17$, $p < 0.05$, see *asterisks*). Following 24-h preincubation (Inc) with homocysteine, this frequency adaptation was abolished (*open circles* in **b**, $n = 10$, not significant), but it was not affected by acute intracellular application (IC) of homocysteine (**c** *open circles*, $n = 16$)
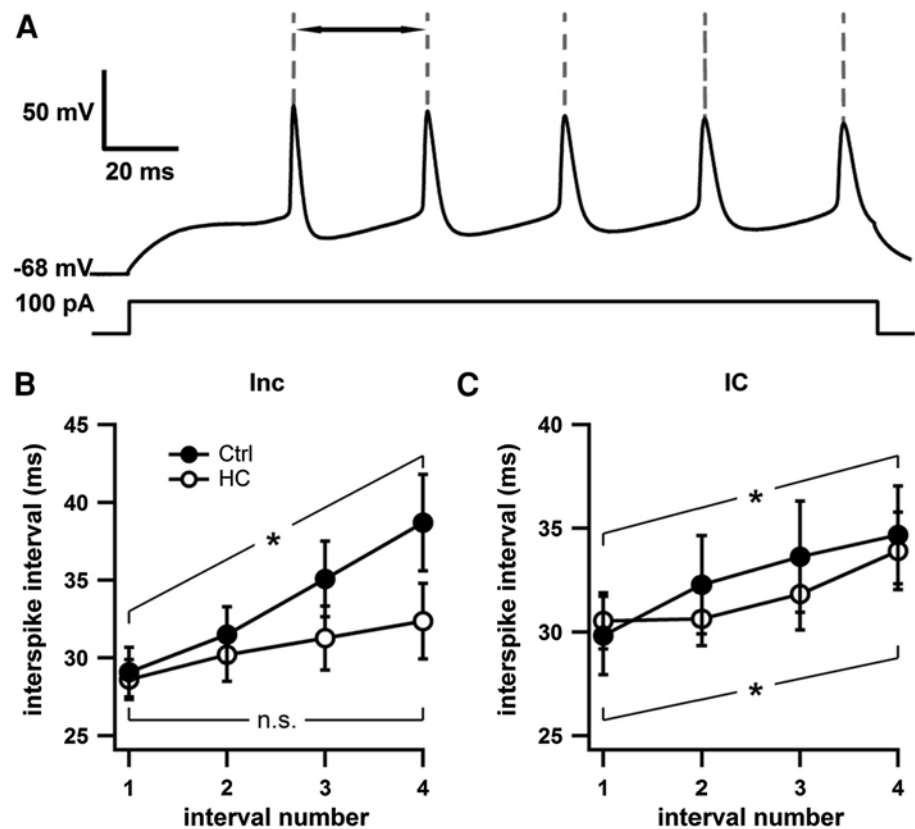

Homocysteine is associated with vascular and neurodegenerative diseases, mechanisms are only poorly understood. This study suggests that interaction of homocysteine with electrophysiological properties of cells may be a relevant mechanism. It remains unclear whether interaction with ion channels or ions themselves is the reason. We have shown that homocysteine and copper interact probably by binding of copper by homocysteine (Linnebank et al. 2006). Thus, for the future, the channels and ions homocysteine is interfering with, should be explored. As limitation, we used an artificial patch-clamp approach with experimental 24-h incubation or intracellular application of homocysteine, whereas toxicity of mild or severe hyperhomocysteinemia occurs over years and decades.

Alterations of biophysical properties of cells may arise from unspecific toxicity or apoptosis, and homocysteine has been reported to induced apoptosis of neurons even at low concentrations (50–250 µM, Ganapathy et al. 2011; Kruman et al. 2000). No degradation of cells was seen, and we selected cells in healthy conditions by visual inspection for our recordings, but we did not quantitatively evaluate the neuronal cultures for cell loss or changes in neuronal morphology. However, the functional changes were quite selective, with the resting membrane potential and most action potential properties being unaffected. We believe this argues against, but does not completely exclude, large toxic effects of homocysteine in our cultures.

As we aimed at analyzing toxicity of hyperhomocysteinemia as a chronic condition in humans, we did not analyze per-acute effects of homocysteine incubation, but incubated cells for 24 h before testing as a compromise. Homocysteine is not taken up by cells, but can be excreted to the extracellular space (Fowler 2005). Thus, concerning intracellular application, we made analyses immediately, as intracellular concentrations after application were unpredictable and could not be monitored.

In patients, available evidence suggests that hyperhomocysteinemia is associated with increased CNS excitability. For instance, hyperhomocysteinemia is a risk factor for epileptic seizures in alcohol-dependent patients under conditions of alcohol withdrawal (Bleich et al. 2006). One explanation for this hyperexcitable phenotype in vivo may be that in intact neuronal networks, the pro-excitatory effects of homocysteine dominate. Alternately, synaptic effects of homocysteine may become relevant. In the case of withdrawal seizures, NMDA receptors, which are inhibited by alcohol, are up-regulated in actively drinking patients. Homocysteine is both an agonist at the glutamate binding site of the NMDA receptor complex and an antagonist at the glycine co-agonist site. This raises the possibility that homocysteine may exert NMDA agonistic actions, in particular, if glycine levels in the CNS are increased (Lipton et al. 1997; Bleich et al. 2006).

Fig. 5 Spike broadening is reduced following incubation with homocysteine. **a** To address the spike broadening during the train, the same traces as in Fig. 3 were analyzed. **b** Action potential width was measured at half width level (*dashed horizontal line*) as depicted for the first (**b₁** as indicated in **a**) and last action potential (**b₂** as indicated in **a**). Width of all action potentials was normalized to the width of the first action potential, averaged for all cells. In control cells, the spikes broadened to the ~1.5 width of the first action potential (*filled circles* in **c**, n = 10 and **d**, n = 17). This broadening was significantly reduced following 24-h incubation (Inc) with homocysteine (**c** *open circles*, n = 10) or acute application (IC) of homocysteine (**d** *open circles*, n = 16)
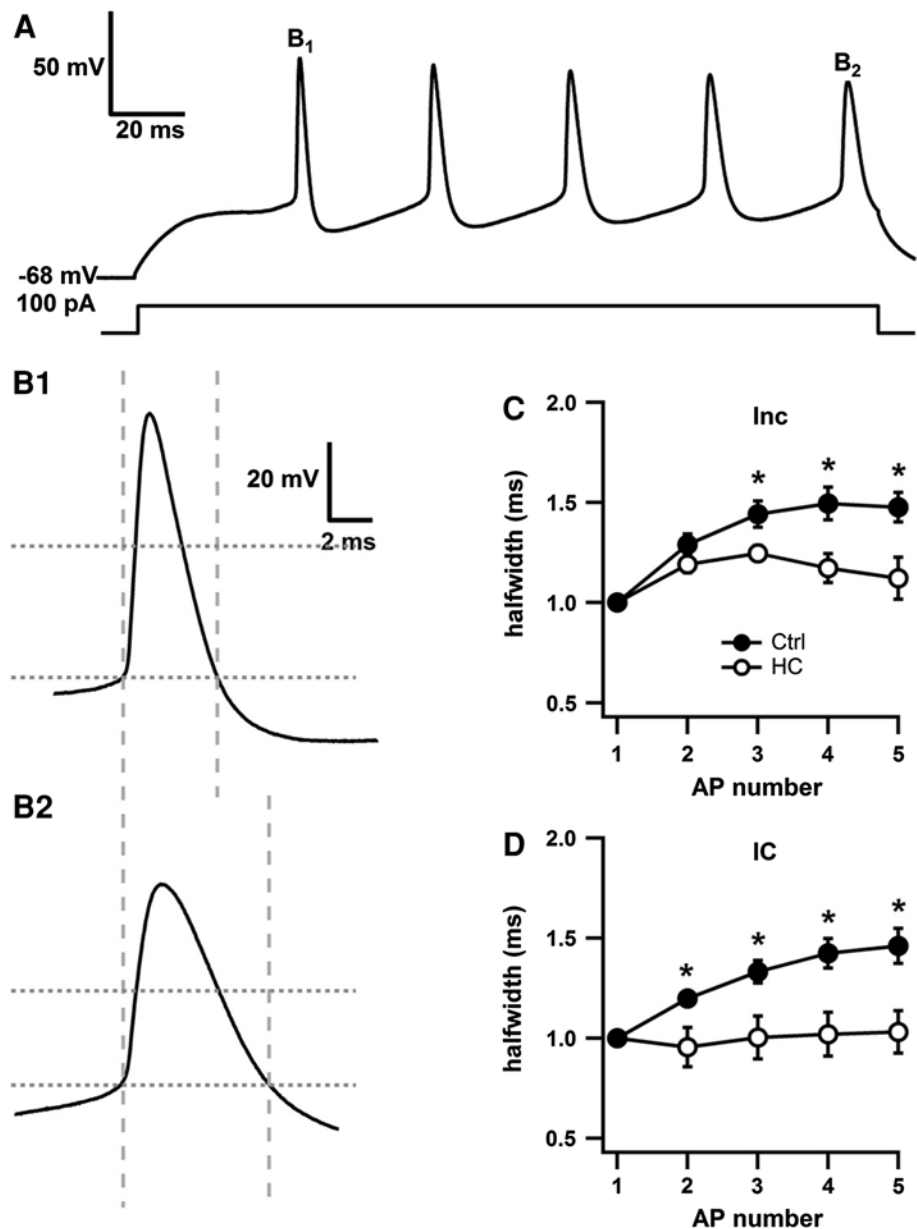
Epilepsy is one of the most common neurological disorders. In most cases, a lifetime therapy with antiepileptic drugs (AED) is necessary. Despite development of a significant number of new AEDs, about 30 % of patients with epilepsy remain refractory to drug treatment (Regesta and Tanganelli 1999). Even though drug resistance is a significant clinical problem, the molecular and cellular mechanisms underlying the clinical phenomenon of drug resistance remain incompletely understood. Most AED are associated with decreased folate or vitamin B12 plasma levels and thus dispose to hyperhomocysteinemia (Linnebank et al. 2011). Hyperhomocysteinemia due to AED long-term treatment may contribute to secondary pharmacoresistance observed in several patients with initial AED response. In addition, inhibitory and excitatory effects of hyperhomocysteinemia may contribute to long-term AED side effects on the central nervous system like fatigue and cognitive impairment in addition to the increased risk for ischemic heart disease (+34 %) and fatal cardiovascular disease (+68 %) in individuals with epilepsy (Gaitatzis et al. 2004).

In conclusion, our data describe heterogeneous electrophysiological effects of homocysteine on neurons, some of which may contribute to neurological impairment and seizures associated with hyperhomocysteinemia.

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