

Short communication

Homocysteine affects the excitability of cultured thalamic neurons to a minor extentKatarina Ondacova¹ , Matus Tomko¹  and Lubica Lacinova¹ ¹ Centre of Biosciences, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia

Abstract. Homocysteine is a naturally occurring amino acid that serves as an intermediate in the metabolism of methionine. It is involved in several physiological as well as pathological processes. Hyperhomocysteinemia is a condition characterized by elevated plasma homocysteine levels. It is accompanied by multiple neurological pathologies, including epilepsy. Excitability of thalamic neurons plays a substantial role in the development of epileptic seizures. We used a primary culture of thalamic neurons to investigate changes in excitability following acute and chronic exposure to elevated homocysteine concentrations. Homocysteine did not affect input resistance, but at high concentration, it hyperpolarized the resting membrane potential. Action potential firing activated by depolarizing current pulses was not affected, neither was altered intracellular calcium concentration. Spontaneous activity was enhanced by both acute and chronic application of homocysteine.

Key words: Hyperhomocysteinemia — Thalamic excitability — Primary neuronal culture — Action potential

Homocysteine (Hcy) is a naturally occurring amino acid that serves as an intermediate in the metabolism of methionine (Finkelstein and Martin 2000). It is involved in several physiological as well as pathological processes. Physiologically, it provides the methyl donor for methylation reactions in the organism (Chiang et al. 1996) and provides the cysteine for the synthesis of glutathione (Anderson 1998). Elevated Hcy levels in blood caused by altered methionine metabolism result in a clinical condition called Hyperhomocysteinemia (HHcy) (Tanaka et al. 2023). While normal blood Hcy concentration is between 5 and 14 μM , levels of 15–30 μM correspond to mild HHcy, and more than 100 μM to severe HHcy (Gonzalez-Lamuno et al. 2023). Clinical symptoms of HHcy are diverse, including vascular symptoms, ophthalmological conditions, musculoskeletal abnormalities, and neuropsychiatric symptoms. Neurological conditions related to elevated Hcy concentration include cognitive deficits,

Alzheimer's disease, mood disorders such as depression, and epileptic seizures (Kripps et al. 2022).

Multiple mechanisms were shown to contribute to the neuropsychiatric disorders. Elevated levels of Hcy may promote the production of reactive oxygen species, leading to oxidative stress and neuronal cell damage (Karababa et al. 2017). Altered synthesis and metabolism of neurotransmitters like norepinephrine, serotonin, and dopamine may contribute to the development of depression and anxiety (Esnafoğlu and Özturan 2020; Hu et al. 2024). Aberrant DNA methylation alters the expression of various proteins involved in synaptic plasticity and neuronal signaling (Esnafoğlu and Özturan 2020; Fuentes-Claramonte et al. 2024). Increased activation of the excitatory neurotransmitter NMDA by high concentrations of Hcy was demonstrated (Lipton et al. 1997; Poddar and Paul 2009; Bolton et al. 2013; Deep et al. 2024).

Many pathologies, including HHcy-related neuropathologies, are characterized by altered cellular excitability, suggesting dysfunction of voltage-dependent ion channels (VDIC). The possible direct modulation of VDCI by Hcy has rarely been investigated. Only a few studies demonstrating such effects are available. Tjiattas et al. (2004) showed that 250 μM

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Hcy applied for 2 hours increased the L-type calcium current in chicken caudate nucleus neurons. Zou et al. (2015) demonstrated increased voltage-dependent sodium current in caudate nucleus neurons from rats exposed to 100 μM Hcy for 12 hours. In the recombinant system, 300 μM Hcy applied for 24 hours facilitated expression of the $\text{Ca}_v3.2$ channel (Gaifullina et al. 2019).

One of the neuropathologies related to HHcy is epilepsy (Kripps et al. 2022), and the excitability of thalamic neurons plays an essential role in its development. It was suggested that increased $\text{Ca}_v3.2$ channel activity contributes critically to the development of epileptic discharges (Gomora et al. 2001; Vitko et al. 2007). Therefore, we investigated the effect of elevated Hcy levels on primary cultures of thalamic neurons. We have used a similar strategy to our previous work, in which we showed that the excitability of primary cultured hippocampal neurons from rats exposed to Hcy for up to 14 days was moderately increased. In contrast, a 24-hour exposure had only a minimal effect (Filipova et al. 2025).

For experiments, we have used Wistar rats obtained from the Department of Toxicology and Laboratory Animals Breeding, Centre of Experimental Medicine, Slovak Academy of Sciences, Dobra Voda, Slovak Republic. Animals were mated after an acclimatization period, and brains from neonatal offspring were used to prepare neuronal cultures.

Primary cultures of neonatal rat thalamic neurons were generated from postnatal day 0 (P0) rats. Brains ($n = 6$ pups of both sexes per preparation) were dissected in ice-cold Hank's balanced salt solution (HBSS; Gibco, USA) supplemented with 1% antibiotic-antimycotic (Sigma-Aldrich, Germany) and 0.3 M HEPES (Sigma-Aldrich, Germany), under a stereomicroscope (Optika Microscopes, Italy), following the procedure of Reichova et al. (2021). Thalamic tissues were enzymatically dissociated for 20 min at 37°C in a solution containing HBSS, 2 mg/ml papain (Sigma-Aldrich, Germany), 0.1% trypsin (Gibco, USA), and 0.1 mg/ml DNase I (Roche, Germany). The resulting cell suspension was resuspended and plated onto poly-D-lysine-coated (10 $\mu\text{g}/\text{ml}$; Sigma-Aldrich, Germany) 24-well plates containing coverslips (Sarstedt, Germany) and 96-well plates. Cells were seeded at a density of 2×10^5 cells/ml in RPMI medium (Gibco, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Germany). After 3 hours of incubation at 37°C in 5% CO_2 , the plating medium was replaced with selective growth medium (Neurobasal A; Gibco, USA) supplemented with 1% antibiotic-antimycotic (Sigma-Aldrich, Germany), 1% glutamine (Sigma-Aldrich, Germany), and 2% B27 supplement (Gibco, USA). This time point was designated as day *in vitro* 1 (DIV1). At DIV5, 50% of the medium was replaced with fresh growth medium containing 10 μM cytosine β -D-arabinofuranoside (Sigma-Aldrich, Germany). Thalamic neurons were maintained for 11–13

days before electrophysiological recordings and intracellular calcium measurements.

Hcy was applied to thalamic cultures either acutely or chronically. Acute Hcy treatments (50 μM , 100 μM , and 300 μM) were administered 24 hours before measurements. Chronic Hcy exposure (30 μM , 50 μM , and 100 μM) was initiated on day 1 *in vitro* and maintained throughout the entire culture period. One-quarter of the cultures served as untreated controls, while the remaining three-quarters were exposed to Hcy at the indicated concentrations and timing.

Whole-cell current-clamp recordings were performed using the patch-clamp technique. Signals were acquired with a HEKA EPC10 amplifier (HEKA Electronics, Lambrecht, Germany) controlled by PatchMaster v2x73.3 software (HEKA Electronics, Lambrecht/Pfalz, Germany). Action potential (AP) parameters were extracted from recorded traces using custom scripts based on the open-source Electrophysiological Feature Extraction Library (eFEL; <https://github.com/BlueBrain/eFEL>).

Patch pipettes were pulled from borosilicate glass and exhibited a resistance of 3–4 M Ω . The extracellular solution for primary thalamic cultures contained (in mM): 130 NaCl, 3 KCl, 10 HEPES, 2 CaCl_2 , 1 MgCl_2 , and 10 D-glucose; pH was adjusted to 7.4 with NaOH. The intracellular solution contained (in mM): 120 K-gluconate, 20 KCl, 2 MgCl_2 , 2 Na_2ATP , 0.25 Na_2GTP , and 10 HEPES; pH was adjusted to 7.4 with KOH (all reagents from Sigma-Aldrich, Germany). The intracellular solution osmolarity was 290–300 mOsm/l (Osmomat 030, Gonotec, Germany). The extracellular solution osmolarity was adjusted with sucrose to a final value 2–3 mOsm/l lower than that of the intracellular solution. The resting membrane potential (V_{rest}) of each neuron was recorded immediately after achieving whole-cell access and switching to current-clamp mode. For all subsequent measurements, the membrane potential was maintained at -70 mV. Input resistance (R_{inp}) was measured using a series of 250-ms hyperpolarizing current steps (increments of -25 pA). The membrane potential was determined at the plateau of each step, and R_{inp} was calculated from the voltage-current relationship using Ohm's law. AP trains were elicited with a series of 300-ms depolarizing pulses, each with a $+50$ -pA increment. Spontaneous activity was recorded for 5 min at each neuron's intrinsic membrane potential.

For intracellular calcium (Ca_i) measurements, cells were incubated in 1 $\times$ Hank's Balanced Salt Solution (HBSS) containing (in mM): 137 NaCl, 5.4 KCl, 0.5 $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.4 $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.44 KH_2PO_4 , 0.34 $\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$, 1.3 CaCl_2 , 4.2 NaHCO_3 , and 5.5 D-glucose. Before dye loading, cells were washed three times with 1 $\times$ HBSS supplemented with 1 mg/ml BSA. Fura-2 AM (Sigma-Aldrich, 47989) was prepared in 1 $\times$ HBSS+BSA and applied at a final concentration of 4 nM/ml. Cells were incubated with the dye for 1 h at 37°C in 5% CO_2 . Following incubation,

cells were washed three times with 1× HBSS without BSA and equilibrated in 1×HBSS for 30 min at 37°C in 5% CO₂ before imaging.

Calcium signals were recorded using a Synergy H4 Multi-Mode Microplate Reader (BioTek, USA) with excitation wavelengths of 340 nm and 380 nm and an emission

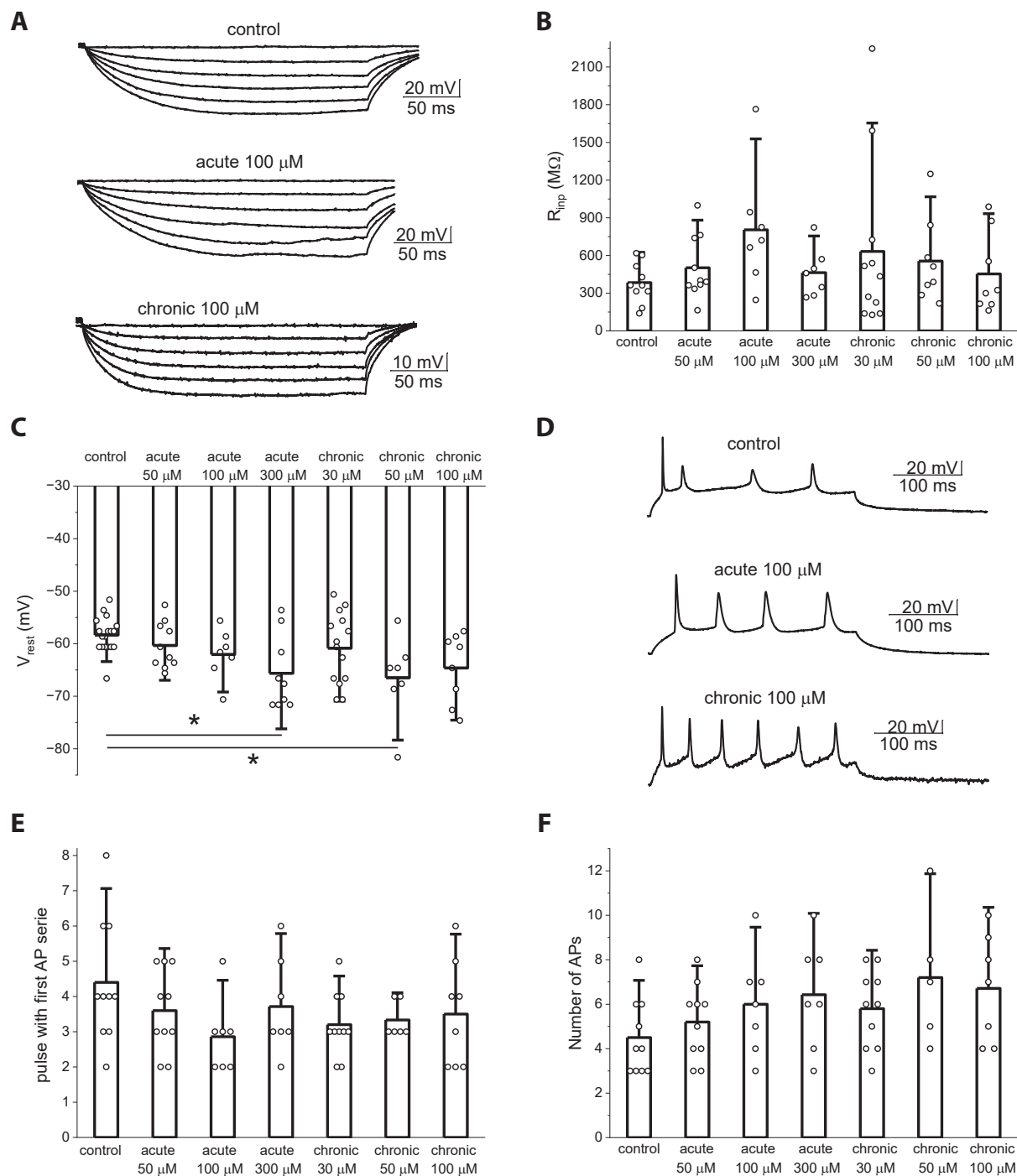


Figure 1. Passive membrane properties and parameters of firing AP series. **A.** Examples of voltage recordings used for calculation of R_{inp} . **B.** Average values of R_{inp} . **C.** V_{rest} measured at the experimental conditions as marked. * $p < 0.05$. **D.** Examples of voltage recordings of an AP series activated by the second suprathreshold depolarizing current pulse. **E.** Average number of the depolarizing current pulse that activated the first AP series. **F.** Average number of AP in series activated by the second suprathreshold depolarizing current pulse. Experimental conditions are marked at each trace recording or at each data column. 5–18 neurons were tested at individual experimental conditions and protocols.

wavelength of 510 nm. Four wells *per* experimental condition and/or isolation were individually analyzed. Ca_i dynamics was measured in real time at a population level, focusing on sustained or slowly developing Ca^{2+} changes. All calcium-imaging experiments were performed on day 13 *in vitro*.

Electrophysiology data were analyzed using FitMaster v2x73.3 (HEKA Electronics, Lambrecht/Pfalz, Germany) and Origin 2022 Academic (OriginLab, Northampton, MA, USA). All data are presented as a mean $\pm$ SD. The GraphPad Instat 3.1 (GraphPad Software Inc., U.S.A.) was used for

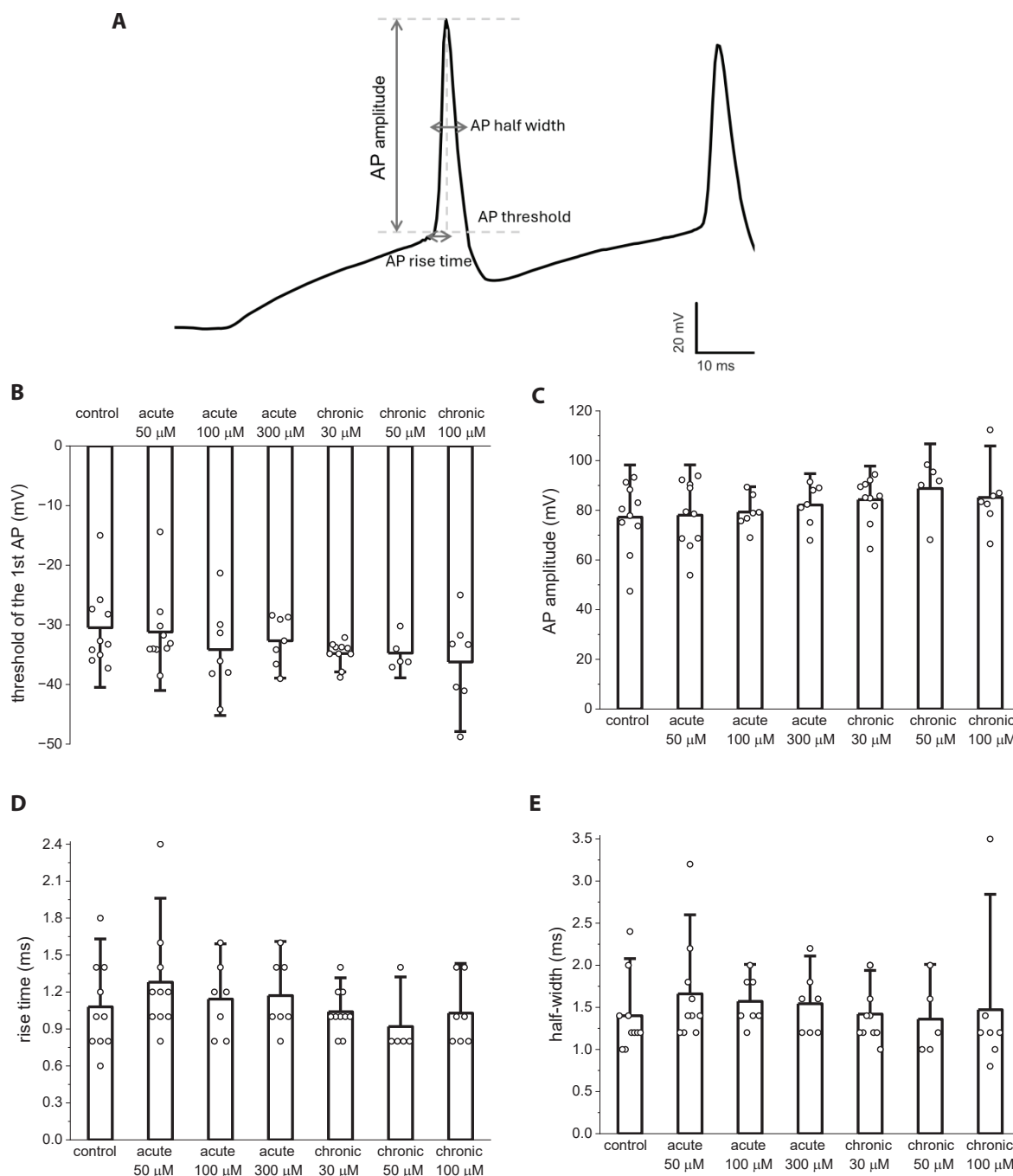


Figure 2. Parameters of the first AP in series activated by the second suprathreshold depolarizing current pulse. **A.** An example of a voltage trace illustrating the analyzed parameters. **B.** Average value of voltage threshold. **C.** Average value of AP amplitudes. **D.** Average rise time of an AP. **E.** Average half-width of an AP. Experimental conditions are marked at each data column. 5–10 neurons were tested at individual experimental conditions.

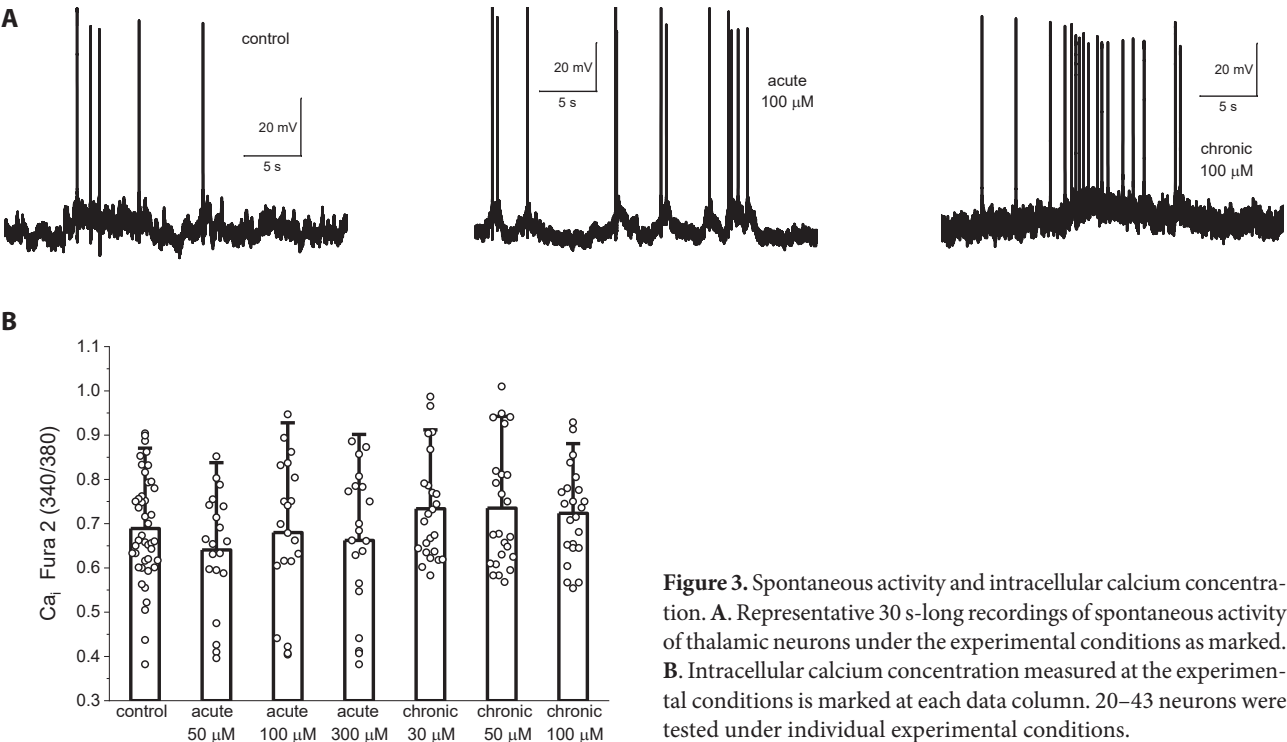


Figure 3. Spontaneous activity and intracellular calcium concentration. **A.** Representative 30 s-long recordings of spontaneous activity of thalamic neurons under the experimental conditions as marked. **B.** Intracellular calcium concentration measured at the experimental conditions is marked at each data column. 20–43 neurons were tested under individual experimental conditions.

statistical analysis of experimental data. The significance of differences across multiple datasets was tested using One-way ANOVA, followed by the Tukey post hoc test for normally distributed data, or the Kruskal-Wallis test if at least one dataset was not normally distributed. Probability less than 0.05 was considered significant.

Input resistance was not significantly altered by any Hcy concentration (Fig. 1A, B). As R_{inp} reflects VDIC expression, this finding is consistent with our previous report (Filipova et al. 2025) in hippocampal cells, in which we did not detect either R_{inp} or VDIC gene transcription. The resting membrane potential was significantly hyperpolarized by high concentrations of Hcy (Fig. 1C). The relationship between the value of V_{rest} and excitability is complex. Hyperpolarization by 5–10 mV suppressed the excitability of hippocampal CA1 neurons (Lauritzen et al. 2000; Nawreen et al. 2021). On the other hand, hyperpolarization of the V_{rest} in entorhinal–hippocampal slices from $HCN1^{-/-}$ mice potentiated neuronal excitability (Huang et al. 2009). Therefore, this observation should be interpreted in the context of the

specific experimental object and experimental conditions. Only minimal I_h current was reported in thalamic slices on the third postnatal day (Kanyshkova et al. 2009). We did not observe a hump characteristic of I_h activation during hyperpolarizing current pulses (Fig. 1A). Therefore, under our experimental conditions, hyperpolarization of V_{rest} may facilitate neuronal excitability.

Prolonged depolarizing current pulses with increasing amplitude activate the firing of multiple APs. As a series of APs, we considered 3 consecutive APs or more. The ability of neurons to fire action potential series can be characterized by the number of depolarizing current pulses needed to activate the first APs series (Fig. 1E) and the number of APs in a series activated by the second suprathreshold pulse (Fig. 1F). Apparent decrease of the first value and increase of the second value with an increasing Hcy concentration were not statistically significant (Fig. 1E,F). More detailed insight into an AP activation by the depolarizing current pulses is offering an analysis of the waveform of the first AP in the second suprathreshold series, as illustrated in the Figure 2A. Volt-

Table 1. Parameters of spontaneous activity of thalamic neurons

	control	acute		chronic		
		50 μM	100 μM	30 μM	50 μM	100 μM
% of spontaneously active neurons	57	50	75	63	75	80
Number of tested neurons	7	2	4	8	4	5

age threshold for the AP firing (Fig. 2B), the AP amplitude (Fig. 2C), the AP rise time (Fig. 2D), and the AP half-width (Fig. 2E) were not significantly affected by any Hcy concentration and/or application time. In our previous report on hippocampal neurons, we observed moderate facilitation of AP firing, manifested as an increase in AP numbers upon application of 300 μ M Hcy and a shortening of the AP rise time upon chronic Hcy application (Filipova et al. 2025). VDIC might have mediated such an effect; nevertheless, in thalamic neurons, it was not observable.

After 10–11 DIV, cultured neurons form neuronal networks and become spontaneously active. Examples of 30-second-long recordings out of a total recording time of 5 min are shown in Figure 3A. The percentage of cells exhibiting spontaneous activity increased with both Hcy concentration and exposure duration (Table 1). A moderate increase in spontaneous activity is consistent with our observation in hippocampal neurons (Filipova et al. 2025). Spontaneous activity in primary cultured neurons involves an activation of glutamate NMDA receptors (She and Craig 2011). Increased glutamate receptor activity evoked by an increased Hcy concentration was shown (Deep et al. 2019; Jindal et al. 2019; Deep et al. 2024). Increased glutamate receptor activity increases Ca_i (Deep et al., 2019). In our model, intracellular calcium concentration was not significantly altered under any experimental conditions (Fig. 3 B). Ca^{2+} dynamics was measured in a well at a population level. We may speculate that the increase in a number of spontaneously active neurons in one well was not sufficient to translate into a statistically significant enhancement of calcium transients.

Lack of significant effect of Hcy on Ca_i is in line with our report on hippocampal neurons (Filipova et al. 2025), but in contrast with the report of Deep et al. (Deep et al. 2019). Deep and coauthors attributed the Ca_i increase to an increased activity of NMDA receptors. Our observation of increased spontaneous activity (Table 1) supports increased NMDA receptor activity in our model; however, this increase was moderate and did not translate into an increase in Ca_i . Also, Deep et al. used a different primary neuronal culture established from rat embryonic cortical neurons. GluN2A and GluN2B subunits are dominant NMDA subunits in the cortex, including the hippocampus (Zhou et al. 2013; Kellermayer et al. 2018). In contrast, GluN2B and GluN2D subunits are dominant in thalamic neurons (Topolski et al. 2025). Preferential interaction of Hcy with GluN2A is commonly considered (Deep et al. 2019). However, the possible involvement of GluN2B was also suggested (Sibarov et al. 2020). Therefore, a different composition of NMDA receptors in hippocampal versus thalamic neurons may contribute to the higher sensitivity of hippocampal neurons to Hcy. Effects on thalamic neurons may be secondary, resulting from cortical damage and consequent network disruptions.

We concluded that elevated Hcy levels have only a minor impact on the excitability of thalamic neurons in primary culture. The involvement of the thalamus in neuropathologies related to homocysteinemia may depend on interactions with other brain regions and should be investigated across the whole brain. This and our previous (Filipova et al. 2025) studies suggest that in a primary neuronal culture model, increased Hcy concentration is unlikely to affect voltage-dependent ion channels, and the observed minor to moderate effects may be due to enhanced glutamate receptor activity.

Ethics approval. All experimental procedures were approved by the State Veterinary and Food Administration of the Slovak Republic, with permit No. 6604/2022, issued on July 26th, 2022. Animal handling followed the Guide for the Care and Use of Laboratory Animals (N.R.C., 1996) and the European Communities Council Directive 2010/63/EU of September 22, 2010.

Availability of data and materials. All data generated or analyzed during this study are included in this published article or are available from the corresponding author upon reasonable request.

Funding. VEGA 2/0081/22 grant to LL.

Authors' contributions. KO performed electrophysiological recordings and analyzed and interpreted the results. MT analyzed and interpreted the results and prepared figures. LL designed and supervised the study, analyzed and interpreted the results, prepared the figures, and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements. We thank Emilia Kocurova for technical assistance.

Conflict of interests. The authors claim that there is no conflict of interest.

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<https://doi.org/10.1007/s12031-015-0616-4>
- Received: November 11, 2025
Final version accepted: December 12, 2025