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ARTICLE

## The role of NMDA and mGluR5 receptors in calcium mobilization and neurotoxicity of homocysteine in trigeminal and cortical neurons and glial cells

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**Abstract**

Recent studies suggested contribution of homocysteine (HCY) to neurodegenerative disorders and migraine. However, HCY effect in the nociceptive system is essentially unknown. To explore the mechanism of HCY action, we studied short- and long-term effects of this amino acid on rat peripheral and central neurons. HCY induced intracellular Ca<sup>2+</sup> transients in cultured trigeminal neurons and satellite glial cells (SGC), which were blocked by the NMDA antagonist AP-5 in neurons, but not in SGCs. In contrast, 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP), the metabotropic mGluR5 (metabotropic glutamate receptor 5 subtype) antagonist, preferentially inhibited Ca<sup>2+</sup> transients in SGCs. Prolonged application of HCY induced apoptotic cell death of both kinds of trigeminal cells. The apoptosis was blocked by AP-5 or by the mGluR5 antagonist

MTEP. Likewise, in cortical neurons, HCY-induced cell death was inhibited by AP-5 or MTEP. Imaging with 2',7'-dichlorodihydrofluorescein diacetate or mitochondrial dye Rhodamine-123 as well as thiobarbituric acid reactive substances assay did not reveal involvement of oxidative stress in the action of HCY. Thus, elevation of intracellular Ca<sup>2+</sup> by HCY in neurons is mediated by NMDA and mGluR5 receptors while SGC are activated through the mGluR5 subtype. Long-term neurotoxic effects in peripheral and central neurons involved both receptor types. Our data suggest glutamatergic mechanisms of HCY-induced sensitization and apoptosis of trigeminal nociceptors.

**Keywords:** homocysteine, mGluR5, neurodegeneration, NMDA receptor, pain sensitization, trigeminal ganglion.

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Homocysteine (2-amino-4-sulfanylbutanoic acid, HCY), a sulfur-containing amino acid, has been implicated in various cardiovascular and neurodegenerative disorders, such as Alzheimer's and Parkinson's disease (Kuhn *et al.* 2001; Kruman *et al.* 2002; Sachdev 2005) as well as amyotrophic

lateral sclerosis (Zoccolella *et al.* 2010). Hyperhomocysteinemia, a condition with excessive level of HCY in plasma (Shi *et al.* 2003) is often associated with the C677T

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*Abbreviations used:* CSD, cortical spreading depression; DCF, 2',7'-dichlorodihydrofluorescein diacetate; FVA, fluorescent viability assay; HCY, homocysteine; MDA, malondialdehyde; mGluR5, metabotropic glutamate receptor 5 subtype; MTEP, 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine; MTHFR, 5'-10'-methylene tetrahydrofolate reductase; PKC, protein kinase C; ROS, reactive oxygen species; SGC, satellite glial cells; TBARS, thiobarbituric acid reactive substances; TG, trigeminal ganglion;  $\alpha,\beta$ -meATP,  $\alpha,\beta$ -methyleneATP;  $\Delta\psi_m$ , mitochondrial transmembrane potential.

polymorphism of the 5'-10'-methylenetetrahydrofolate reductase gene. Thus, recent data suggest that this polymorphism could be implicated in the pathogenesis of migraine with aura (Moschiano *et al.* 2008; Lea *et al.* 2009; Oterino *et al.* 2010). While the role and neurotoxic mechanisms of HCY in neurodegenerative diseases have been intensely studied (Sachdev 2005), the pro-nociceptive and toxic action of HCY in the trigeminal nociceptive system is still unclear.

Migraine is a complex disorder which involves activation of the meningeal trigeminovascular system and central neuronal circuitries (Messlinger 2009; Pietrobon and Moskowitz 2013). It is widely accepted that migraine with aura is associated with cortical spreading depression (CSD), a slowly propagating neuronal depolarization (Moskowitz 2007; Takano *et al.* 2007), and we recently showed that CSD is associated with the induction of oxidative stress within the trigeminal nociceptive system (Shatillo *et al.* 2013). Enhanced brain excitability in migraine with aura is usually explained by the excess of extracellular glutamate (Pietrobon and Moskowitz 2013). Glutamate can operate via ionotropic and metabotropic receptors leading to neuronal depolarization and potentially to neurotoxicity. Glutamate-activated NMDA receptors are the most important players in the generation of CSD (Ayata and Moskowitz 2006; Peeters *et al.* 2007; Chauvel *et al.* 2012). While NMDA receptors are traditionally associated with neurons, recent findings are consistent with potential expression of these glutamate receptors also in some types of glial cells (Parpura and Verkhratsky 2013). Out of several metabotropic glutamate receptor 5 subtype, (mGluR5) subtype is the most abundant in glial cells (Parpura and Verkhratsky 2013). Recent reports also pointed to the essential role of peripheral NMDA receptors in chronic pain (Zhou and Sheng 2013). Thus, peripherally administrated selective NMDA antagonists suppressed inflammatory pain induced by formalin injection (Carlton 2001). Consistent with this, several studies revealed the expression of NMDA and mGluR5 receptors in the peripheral nervous system, including sensory trigeminal ganglion (TG) cells (Turman *et al.* 2002; Lee and Ro 2007). Since HCY can interact with the glutamate binding site of NMDA receptors, this receptor type could be an important target for the action of elevated HCY (Kim and Pae 1996; Lipton *et al.* 1997; Ganapathy *et al.* 2011; Poddar and Paul 2013). Other studies revealed that HCY can act also on group I metabotropic glutamate receptors (Shi *et al.* 2003; Ziemińska *et al.* 2003; Beard *et al.* 2012; Yeganeh *et al.* 2013). In addition, it is possible that mGluR receptors can promote the activation of NMDA receptors via a protein kinase C dependent phosphorylation (Yu *et al.* 1997; Alagarsamy *et al.* 2002; Sylantsev *et al.* 2013). Such co-expression and crosstalk of two receptors types in trigeminal ganglion cells open a range of possibilities to explain pro-nociceptive mechanisms in migraine.

Hyperactivation of NMDA receptors is often associated with oxidative stress (Tenneti *et al.* 1998; Reyes *et al.* 2012). In line with this, neuronal death induced by HCY is associated with oxidative stress in some experimental models (Kim and Pae 1996; Jara-Prado *et al.* 2003; Matte *et al.* 2004; Sibrian-Vazquez *et al.* 2010). On the other hand, in endothelial cells, HCY shows reductive rather than oxidative effect (Outinen *et al.* 1998) and there are several reports of the antioxidant effects of HCY (Perna *et al.* 2003; Loureiro *et al.* 2010). Thus, the issue of pro- or antioxidant action of HCY in the nociceptive system remains unclear and represents one target of this study.

In this study, we analyzed long- and short-term action of high HCY concentrations on trigeminal ganglion cells and on cortical neurons, using fluorescent viability assays, live-cell imaging and biochemical assays. We show that HCY induced  $Ca^{2+}$  transients in neurons via NMDA and mGluR5 receptors, while in glial cells the short-term effects of HCY are mediated by metabotropic glutamate receptors. We also found that the crosstalk between NMDA and mGluR5 receptors determines the neurotoxic effect of HCY to both neurons and glial cells.

## Materials and methods

### Material and reagents

All procedures using animals were in accordance with recommendations of the Federation for Laboratory Animal Science Associations and approved by the local Institutional Animal Care and Use Committees. All reagents required for the tissue culture were obtained from Invitrogen (Carlsbad, CA, USA) while reagents for experiments and fluorescent dyes were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Wistar rats were obtained from the Animal Facilities of the University of Eastern Finland (UEF) or Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences (IEPhB RAS). The protocol for the care, handling and use of animals followed ARRIVE guidelines and European Communities Council Directive of November 24, 1986, 86/609/EEC and was approved by the local Animal Care and Use Committees of the UEF and IEPhB RAS. Experiments were designed to minimize the number of animals used in research.

### Primary culture of rat trigeminal sensory neurons

TG cultures were prepared as described previously (Malin *et al.* 2007). Male Wistar rats at 10–12 postnatal days were killed by CO<sub>2</sub> inhalation. Trigeminal ganglions were isolated and enzymatically dissociated. Instead of trypsin to dissociate the cells we used 3% collagenase for 20 min. Cells were cultured in F12 medium at 37°C, 5% CO<sub>2</sub> for 48 h prior to experimental treatment.

### Primary culture of rat cortex

Primary cortical cultures were prepared as described earlier (Mironova *et al.* 2007; Sibarov *et al.* 2012). Wistar rats 16 days of gestation were killed by CO<sub>2</sub> inhalation. Fetuses (10–15) were removed, and their cerebral cortices were isolated, enzymatically

dissociated, and used for preparing primary neuronal cultures. Cells were cultivated in Dulbecco's modified Eagle's medium/F-12 medium and used for experiments after 7–15 days in culture (Mironova *et al.* 2007).

### Quantitation of cell viability

For analysis of HCY long-term action, cultured cells were incubated for 5 or 24 h in control condition (culture medium) or in culture medium containing 100  $\mu\text{M}$  or 500  $\mu\text{M}$  D,L-HCY (notably, only L-form of HCY is biologically active) in combinations with 50  $\mu\text{M}$  D,L-AP-5 (D-form is the effective enantiomer for NMDA receptor) or 10  $\mu\text{M}$  3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP). Growth culture medium for TG culture contains 100  $\mu\text{M}$  glycine (Ham's F-12 Nutrient Mix; Gibco, Carlsbad, CA, USA), whereas the cortex culture growth medium contains 250  $\mu\text{M}$  glycine (Dulbecco's modified Eagle's medium/F-12, Gibco). Thus, all our media contained glycine which was critical for activation of NMDA receptors (Johnson and Ascher 1992).

Cell viability was measured by fluorescent viability assay described in Mironova *et al.* (2007) and Sibarov *et al.* (2012). Cells were treated with 0.001% acridine orange for 30 s in basic solution (152 mM NaCl, 2.5 mM KCl, 10 mM glucose, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES, pH adjusted to 7.4 using NaOH). After complete washout of contaminating acridine orange, cells were exposed to 0.002% ethidium bromide for 30 s in basic solution. This procedure was applied immediately before each measurement.

Fluorescence images were captured using Leica SP5 MF scanning confocal microscope (Leica Microsystems Inc., Bannockburn, IL, USA) or Olympus FV1000 confocal microscope (Tokyo, Japan). For two-channel imaging, the emitted fluorescence was acquired at 500 to 560 nm (green region of spectrum for acridine orange) and above 600 nm (red region of spectrum for ethidium bromide). Single focal plane images from both channels were merged and analyzed with standard Leica LAS AF Software (Leica Microsystems, Inc.) and ImageJ software using custom written plug-in (Sibarov *et al.* 2012). On the resulting image, noncolocalized green and red pixels were attributed to live and necrotic neurons, respectively. Yellow–orange pixels with colocalized green and red fluorescence were attributed to the nuclei of apoptotic neurons.

Morphological criteria were used to visually differentiate neurons from satellite glial cells (SGCs) in TG culture as described in Ceruti *et al.* (2008), see (Fig. 3a). Briefly, neurons have soma exceeding 10  $\mu\text{m}$ , whereas SGCs are essentially smaller cells (Figs 1a and 2a).

In primary cortical culture the glial cells were flat and located below the level of neuronal bodies, thus allowing us to pick the confocal plane crossing only neuronal soma excluding glial cells fluorescence from the captured images.

### Calcium imaging

Primary trigeminal cells (DIV2) and primary cortex neurons (DIV7–10) were rinsed with basic solution followed by loading with 2  $\mu\text{M}$  Fluo-3 acetoxymethyl ester at 21–22°C for 60 min (in basic solution). After 20-min post-incubation in basic solution, dishes were transferred to TILL Photonics imaging system (TILL Photonics GmbH, Munich, Germany) and constantly perfused with basic solution at a flow rate of 1.2 mL/min. The setup was equipped by

fast perfusion system (Rapid Solution Changer RSC-200, BioLogic Science Instruments, Grenoble, France), which allowed rapid application of various compounds. Cells were viewed via Olympus IX-70 (Tokyo, Japan) microscope with specific filter using 10x objective and with the wavelength 488 nm. Images were collected using CCD camera (SensiCam, PCO imaging, Kelheim, Germany) at sampling frequency set to 2 fps. Cells were stimulated with 100  $\mu\text{M}$  HCY together with the co-agonist glycine (100  $\mu\text{M}$ ). Cells were further characterized by their responsiveness to a brief applications of  $\alpha,\beta$ -methylene ATP, capsaicin and high potassium (50 mM KCl with compensated osmolality) as a marker for neurons.

### Imaging of reactive oxygen species

Primary cultures from trigeminal ganglia were rinsed with basic solution and loaded with 10  $\mu\text{M}$  2',7'-dichlorodihydrofluorescein diacetate (DCF) for 45 min. Then dishes were moved to the same imaging system that was used for calcium imaging and fluorescent cells were viewed with 10x objective. Fluorescence was excited with 488 nm laser and observed in green spectral region. Sampling frequency was set to 0.067 fps for reactive oxygen species (ROS) imaging. After baseline collection (2 min), cells were treated with 500  $\mu\text{M}$  HCY (without glycine) or 20  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 6 min.

### Imaging of mitochondrial transmembrane potential

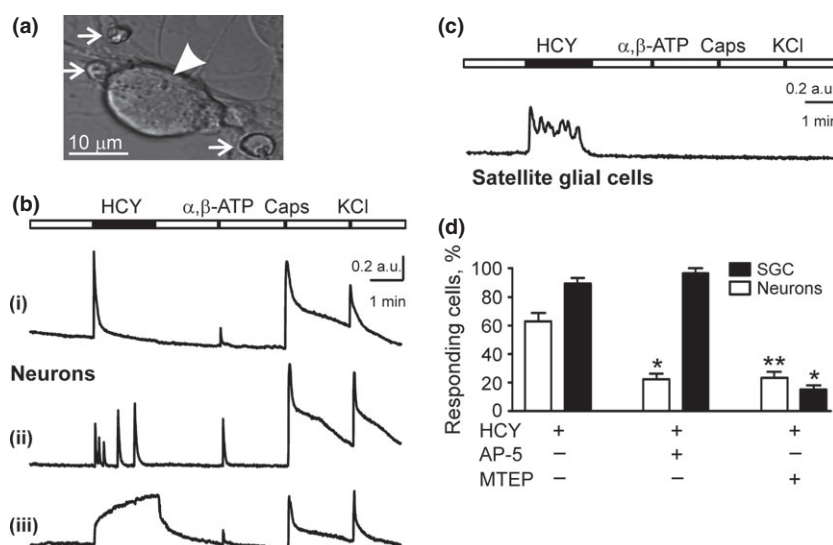
Primary trigeminal cultures were loaded with 5  $\mu\text{M}$  Rhodamine 123 for 30 min in basic solution. Fluorescent cells were viewed with 10  $\times$  objective and the wavelengths 480 and 514 nm were used for excitation and emission, respectively. Sampling frequency was set to 0.03 fps for mitochondrial transmembrane potential ( $\Delta\psi_m$ ). HCY (100  $\mu\text{M}$ ) was added together with 100  $\mu\text{M}$  glycine for 6 min. To test the functional state of mitochondria, the oxidative phosphorylation inhibitor, 4  $\mu\text{M}$  FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) was applied at the end (Duchen 2012).

### TBARS assays

As a marker of oxidative stress malondialdehyde (MDA) concentration was measured using thiobarbituric acid reactive substances (TBARS) assay (Feldman 2004) with slight modifications. A total of 48 h after seeding TG cells were treated for 5 h by 500  $\mu\text{M}$  HCY (in the presence of glycine in medium). After washing with phosphate-buffered saline, cells were collected with ice-cold ristocetin-induced platelet agglutination buffer pH 7.2, sonicated for 5 min in ice and centrifuged at 197  $g$  for 5 min. A total of 100  $\mu\text{L}$  supernatant or MDA standard (0–100  $\mu\text{M}$  1,1,3,3-tetramethoxypropane) was added to 250  $\mu\text{L}$  0.67% thiobarbituric acid in 1 M acetic acid and incubated at 95°C for 1 h. Samples were centrifuged at 1230  $g$  for 15 min at 4°C. Spectrophotometric fluorescence of the supernatant was determined on Victor-2 Wallac 1420 multi-label reader (Wallac Oy, Turku, Finland) with the narrow band excitation filter 485 nm and the maximum of emission at 535 nm. The quantification was made using standard calibration curve. The concentration of MDA was expressed as  $\mu\text{mol}/\text{mg}$  protein.

### Statistical analysis

Imaging data and TBARS data were analyzed using Mann–Whitney U-test and two tailed *t*-test, respectively. The results are expressed as



**Fig. 1** Intracellular  $\text{Ca}^{2+}$  responses induced by short homocysteine (HCY) application to rat primary trigeminal ganglion (TG) cells. (a) Image of trigeminal neurons and satellite glial cells (SGC) in primary culture. Neuron is indicated with arrow head while SGCs are marked with arrows. (b) In neurons loaded with Fluo-3AM HCY induced three types of calcium responses: fast (i), oscillatory (ii) and sustained (iii) responses. (c) In SGC HCY induced oscillatory responses. Application of 100  $\mu\text{M}$  HCY for 2 min followed by 2 s long applications of 10  $\mu\text{M}$   $\alpha$ ,

$\beta$ -meATP ( $\alpha,\beta$ -ATP), 200 nM capsaicin (Caps), and 50 mM KCl as a marker for neurons (application intervals are marked with black bars). (d) Histograms showing percentage of neurons (white columns) and SGCs (black columns) responding to 100  $\mu\text{M}$  HCY alone or to combination of HCY with either 50  $\mu\text{M}$  AP-5 or 10  $\mu\text{M}$  3-((2-Methyl-4-thiazolyl)ethyl)pyridine (MTEP). Mean  $\pm$  SEM ( $n = 4$  experiments, 437 SGCs and 349 neurons). \* $p < 0.05$ ; \*\* $p < 0.01$  by Mann-Whitney test.

mean of data  $\pm$  SEM unless otherwise stated. The level of statistical significance was set to  $p < 0.05$ .

## Results

### Intracellular calcium transients induced by HCY in trigeminal ganglion cells

To study the fast effects of HCY in the peripheral nociceptive system, we characterized the action of this amino acid on the level of intracellular  $\text{Ca}^{2+}$  in neurons and SGCs isolated from trigeminal ganglion. Fig. 1b–d shows that the application of 100  $\mu\text{M}$  HCY with 100  $\mu\text{M}$  glycine to TG culture induced  $\text{Ca}^{2+}$  transients both in neurons and in SGCs. Since only neurons respond to KCl (Simonetti *et al.* 2006), we applied 50 mM KCl to distinguish them from SGC. Interestingly, in neurons, a variable shape of HCY-induced  $\text{Ca}^{2+}$  responses was observed, which could be divided into three types: (i) fast transient (32.4%), (ii) oscillatory signals (44.4%), and (iii) slow sustained (23.2%; 108 neurons in total, Fig. 1b). In contrast, HCY induced only oscillatory  $\text{Ca}^{2+}$  signals in SGCs (224 cells in total, Fig. 1c). To identify the phenotype of HCY-responsive nociceptive neurons we applied agonists of the two main pain transducing receptors expressed in TG neurons: P2X3 and TRPV1 (Simonetti *et al.* 2006). A total of 47% of HCY-responding neurons also responded to  $\alpha,\beta$ -meATP and capsaicin indicating functional co-expression of

P2X3 and TRPV1 receptors, respectively. Comparable fractions of neurons (14%) responded only to either  $\alpha,\beta$ -meATP or capsaicin, while 25% of HCY-positive neurons did not respond to either of these 2 agonists.

It had been previously shown that HCY could activate glutamate NMDA (Lipton *et al.* 1997) and group I metabotropic glutamate receptors (Shi *et al.* 2003; Ziemińska *et al.* 2003; Beard *et al.* 2012; Yeganeh *et al.* 2013). Therefore, in order to understand the molecular mechanisms of HCY induced  $\text{Ca}^{2+}$  transients we tested the effect of the selective NMDA antagonist AP-5. Because it had been shown that mGluR5 receptors were abundantly expressed in glial cells (Papura and Verkhratsky 2013) we also tested the selective mGluR5 antagonist MTEP on HCY responses. We found that HCY-mediated  $\text{Ca}^{2+}$  responses in neurons were largely blocked by AP-5: percentage of neurons responding to HCY decreased from  $63.0 \pm 5.8\%$  ( $n = 6$ ; 85 neurons) in control to  $22.3 \pm 4.0\%$  in the presence of AP-5 ( $n = 6$ ;  $p < 0.05$ ; 75 neurons; Fig. 1d). The mGluR5 antagonist MTEP also decreased this number to  $21.4 \pm 4.0\%$  ( $n = 6$ ;  $p < 0.01$ ; 189 neurons). Interestingly, AP-5 did not affect HCY-induced  $\text{Ca}^{2+}$  responses in SGCs ( $89.3 \pm 3.8\%$  before and  $95.3 \pm 3.6\%$  after AP-5;  $n = 6$ ;  $p > 0.05$ ; 148 and 72 SGCs). However, in SGCs, MTEP (10  $\mu\text{M}$ ) strongly decreased the number of responding cells to  $15.2 \pm 2.9\%$  ( $n = 5$ ;  $p < 0.05$ ; 217 SGCs).

The involvement of NMDA receptors in HCY induced  $\text{Ca}^{2+}$  responses in neurons was further supported by the ability of the glutamate co-agonist glycine to enhance their responsiveness to HCY. Thus, in the presence of 100  $\mu\text{M}$  glycine, HCY induced  $\text{Ca}^{2+}$  responses in  $63.0 \pm 5.8\%$  of neurons ( $n = 6$ ; 85 neurons), whereas in the absence of the co-agonist HCY was effective only in  $40.6 \pm 5.5\%$  of neurons ( $n = 5$ ;  $p < 0.05$ ; 122 neurons). Consistent with this lack of functional NMDA receptors in SGCs, glycine did not

affect the responses of this cell type. Thus,  $89.3 \pm 3.8\%$  of SGCs responded to HCY in the presence of glycine ( $n = 6$ ; 148 SGCs) while  $92.6 \pm 5.0\%$  of SGCs to the application of HCY without glycine ( $n = 5$ ; 76 SGCs;  $p > 0.05$ ).

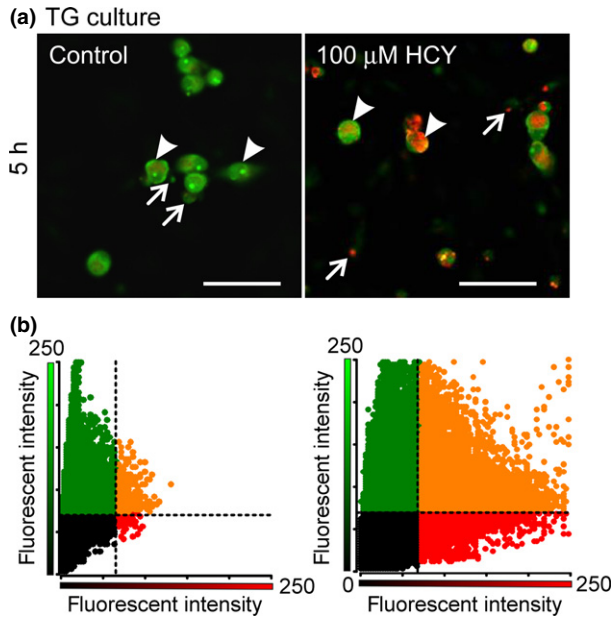
These data are consistent with involvement of both ionotropic NMDA and metabotropic mGluR5 receptors in HCY induced  $\text{Ca}^{2+}$  transients in neurons and the leading role of mGluR5 receptors in the activation of glial cells.

**Neurotoxic effects of HCY in TG cells**

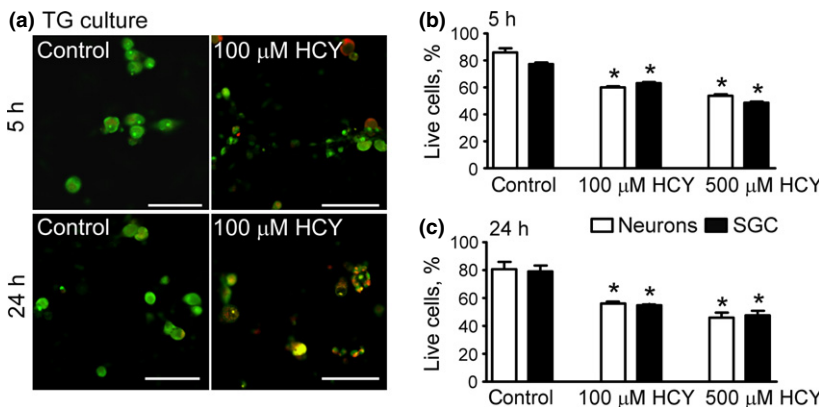
Sustained increase of intracellular  $\text{Ca}^{2+}$  in trigeminal cells can potentially lead either to  $\text{Ca}^{2+}$  dependent nociceptive sensitization or to cell death. To address the long-term toxic effects of HCY on TG cells, we used the double labeling method suggested by Mironova *et al.* (2007), which allows the quantitation of the different cell populations: live, apoptotic, and necrotic cells. Fig. 2 shows that the main effect of 100  $\mu\text{M}$  HCY after 5 h exposure was apoptosis in a significant fraction of the cells with a relatively minor effect on cell necrosis: from 0.64% in control to 5.1% after HCY treatment.

Figure 3 shows that the long-term (5 or 24 h) incubation of cultured TG cells with 100  $\mu\text{M}$  or 500  $\mu\text{M}$  HCY significantly diminished the number of live neurons and SGCs. In control conditions without HCY, after 5 h, the percentage of live neurons was for  $85.7 \pm 3.2\%$  ( $n = 4$ ; 221 neurons) whereas for SGCs it was  $77.2 \pm 1.2\%$  ( $n = 4$ ; 528 SGCs). These results are consistent with data obtained by others (Simonetti *et al.* 2006). The application of 100  $\mu\text{M}$  HCY for 5 h reduced the percentage of live neurons to  $60.0 \pm 0.8\%$  ( $n = 4$ ;  $p < 0.05$ ; 186 neurons) and SGCs to  $63.2 \pm 0.8\%$  ( $n = 4$ ;  $p < 0.05$ ; 144 SGCs). Exposure to 500  $\mu\text{M}$  HCY for 5 h reduced the percentage of live neurons to  $54.0 \pm 0.9\%$  ( $n = 4$ ;  $p < 0.05$ ; 237 neurons) and percentage of live SGCs to  $48.5 \pm 1.0\%$  ( $n = 4$ ;  $p < 0.05$ ; 110 SGCs; Fig. 3b). Extension of the treatment to 24 h only slightly decreased the fraction of living cells (Fig. 3c).

To test the role of different glutamate receptors in HCY toxic effects, we used the selective antagonists of NMDA



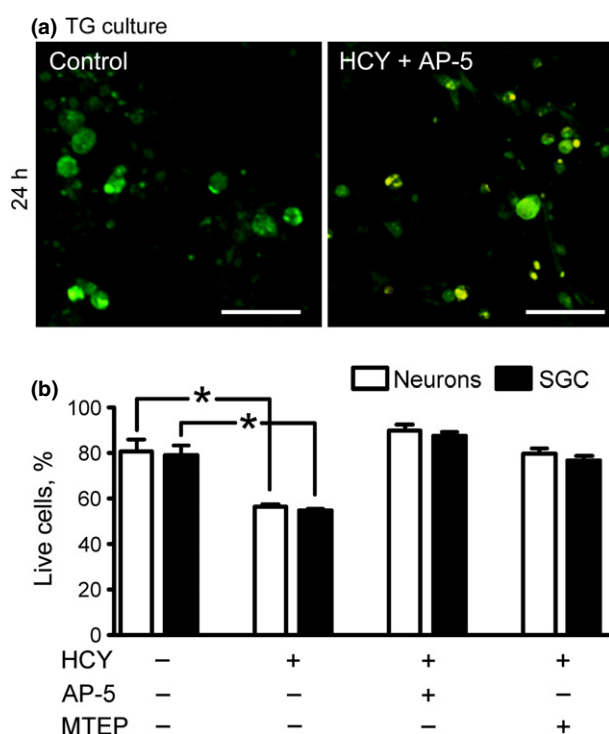
**Fig. 2** Homocysteine (HCY) induced neurodegeneration in rat trigeminal ganglion (TG) cells. (a) An overlay of confocal images taken in green and red spectra of TG cultures in control or after 5 h exposure to 100  $\mu\text{M}$  HCY. Neurons is indicated with arrow head while satellite glial cells (SGCs) are marked with arrows. Scale bar 100  $\mu\text{m}$ . (b) Correlation plots for the images presented in (a) indicating live (green), necrotic (red) and apoptotic (orange) cells. Notice the specific increase in the number of orange pixels in the presence of HCY indicating apoptosis as the main effect of HCY. Dashed lines indicate thresholds to separate visible fluorescence from dark pixels.



**Fig. 3** Dose- and time-dependence of homocysteine (HCY) neurotoxicity in rat trigeminal cells. (a) An overlay of confocal images of TG cells in green and red spectra after 5 and 24 h exposure to 100  $\mu\text{M}$  HCY. Scale bar 100  $\mu\text{m}$ . (b and c) Histograms showing percentage of live neurons (white columns) and satellite glial cells (SGCs) (black columns) in control and after exposure to 100  $\mu\text{M}$  or to 500  $\mu\text{M}$  HCY for 5 hours (b) or 24 h (c). Mean  $\pm$  SEM ( $n = 4$ ). \* $p < 0.05$  by Mann-Whitney U-test.

receptors AP-5 and mGluR5 antagonist MTEP with a similar approach as done to study  $\text{Ca}^{2+}$  responses. 50  $\mu\text{M}$  AP-5 applied for 24 h completely removed the neurotoxic effect of 100  $\mu\text{M}$  HCY (Fig. 4). Thus, in the presence of AP-5 the percentage of neurons surviving exposure to HCY increased to  $89.8 \pm 2.6\%$  ( $n = 5$ ;  $p < 0.05$ ; 311 neurons) while the fraction of live SGCs increased up to  $87.6 \pm 1.4\%$  ( $n = 5$ ;  $p < 0.05$ ; 237 SGCs; Fig. 4b). Furthermore, to test the contribution of metabotropic glutamate receptors in HCY neurotoxicity, we tested cells viability in the presence of MTEP. As presented in Fig. 4, neurotoxicity evoked by 100  $\mu\text{M}$  HCY was significantly reduced by 10  $\mu\text{M}$  MTEP both in neurons ( $79.6 \pm 2.4\%$ ;  $n = 5$ ;  $p < 0.05$ ; 325 neurons) and in SGCs ( $76.8 \pm 2.0\%$ ;  $n = 5$ ;  $p < 0.05$ ; 771 SGCs; Fig. 4b).

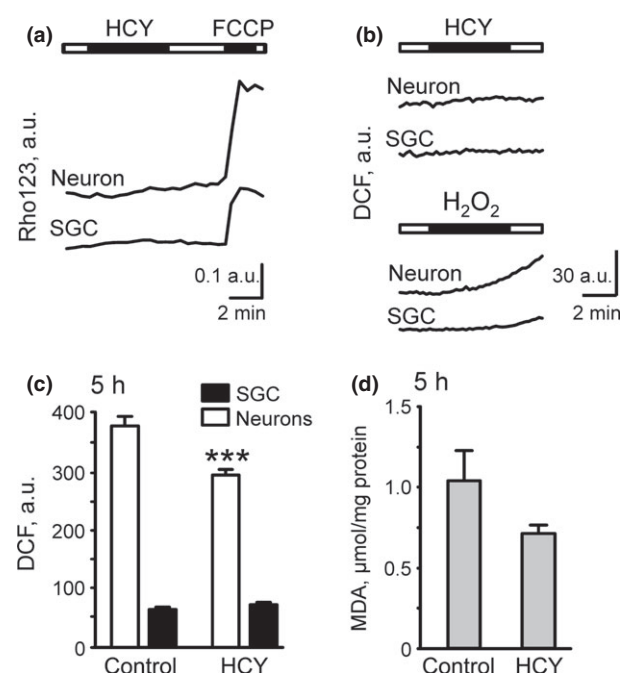
These data indicated that the neurotoxicity of HCY is mediated both by ionotropic and metabotropic receptors which is consistent with the intimate interactions between these glutamate-triggered signaling cascades (Nanou *et al.* 2009).



**Fig. 4** Prevention of homocysteine (HCY) neurotoxicity in trigeminal ganglion (TG) culture with antagonists of NMDA and mGluR5 receptors. (a) An overlay of confocal images of TG cells after 24 h exposure to 100  $\mu\text{M}$  HCY alone or in combination with 50  $\mu\text{M}$  AP-5. Scale bar 100  $\mu\text{m}$ . (b) Histograms showing percentage of live neurons (white columns) and satellite glial cells (SGCs) (black columns) in control and after exposure to 100  $\mu\text{M}$  HCY alone or in combination with 50  $\mu\text{M}$  AP-5 or 10  $\mu\text{M}$  3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP). Mean  $\pm$  SEM ( $n = 4-5$ ). \* $p < 0.05$  by Mann-Whitney U-test.

### Testing redox-dependence of HCY action

As presented in the Introduction, one of the highly debated issues is whether HCY is able to induce ROS which can then mediate its neurotoxic effects. To test potential induction of ROS in the nociceptive system we first studied the ability of HCY to induce acute oxidative stress. First, we analyzed the action of HCY (together with glycine) on  $\Delta\psi_m$  after a 6 min application (Fig. 5a). HCY did not induce significant changes in  $\Delta\psi_m$ . In sharp contrast, the mitochondrial oxidative phosphorylation inhibitor FCCP, as expected (Duchen 2012) largely increased Rhodamine 123 fluorescence ( $n = 4$ , 47 neurons, 25 SGCs, Fig. 5a). These data suggest that HCY did not induce loss of  $\Delta\psi_m$ . Next we tested the ability of HCY to induce ROS in these cells. Fig. 5b shows that the acute application of 500  $\mu\text{M}$  HCY for 6 min did not increase the fluorescence of the ROS-sensitive dye DCF. Rise of fluorescence in this time window, however,



**Fig. 5** Testing redox-dependence of homocysteine (HCY) action in trigeminal ganglion (TG) cells. (a) Fluorescence of the mitochondrial dye Rhodamine 123 in neurons and satellite glial cells (SGC) exposed to 100  $\mu\text{M}$  HCY and 4  $\mu\text{M}$  FCCP. (b) Fluorescence of the reactive oxygen species (ROS) sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (DCF) in neurons or SGCs exposed to 500  $\mu\text{M}$  HCY or 20  $\mu\text{M}$   $\text{H}_2\text{O}_2$  as a control. (c) Quantitative analysis of DCF fluorescence in neurons and SGCs in control condition and after 5 h exposure in 500  $\mu\text{M}$  HCY containing culture medium. Mean  $\pm$  SEM ( $n = 4$  separate experiments, 157 SGCs and 328 neurons). \*\*\* $p < 0.0001$  by Mann-Whitney U-test. (d) Histograms showing the level of lipid peroxidation [in terms of malondialdehyde (MDA) concentration] in HCY-treated TG cells. Notice that 500  $\mu\text{M}$  HCY did not change the lipid peroxidation of TG cells ( $n = 3$ ;  $p > 0.05$  by paired  $t$ -test).

was observed following application of a relatively low (20  $\mu\text{M}$ ) concentration of  $\text{H}_2\text{O}_2$  demonstrating that DCF was highly sensitive to ROS in this model ( $n = 5$ , 97 neurons, 35 SGCs, Fig. 5b).

Although HCY did not induce acute oxidative stress, it is possible that upon longer exposure, this agent can trigger signaling cascades leading to the delayed generation of ROS, promoting HCY neurotoxicity. To address this issue, TG cells were exposed to 500  $\mu\text{M}$  HCY (together with glycine) for 5 h and the basal level of DCF fluorescence was measured. Fig. 5c shows that HCY significantly reduced the level of DCF fluorescence, in neurons ( $n = 4$ ;  $p < 0.0001$ ; 170 neurons), but not in SGCs ( $n = 4$ ;  $p > 0.05$ ; 72 SGCs) suggesting that in these conditions HCY did not show pro-oxidant properties.

As an independent approach, induction of oxidative stress by HCY was finally tested with the TBARS assay, commonly used to evaluate the level of lipid peroxidation. Fig. 5d shows that there was no difference in MDA concentrations in TG cells after 5 h exposure to 500  $\mu\text{M}$  HCY ( $n = 3$ ;  $p > 0.05$ ) consistent with the data obtained with DCF. To sum up, our data suggests that in these conditions HCY demonstrates anti-oxidant rather than pro-oxidant properties.

#### Short- and long-term effects of HCY in central neurons

To compare the action of HCY in central versus peripheral neurons we used a mixed culture of cortical neurons and glia. In cortical neurons short-term applications of HCY induced  $\text{Ca}^{2+}$  responses similar to those observed in TG neurons (Fig. 6a). Glial cells (which did not react to  $\text{K}^+$ ) responded to HCY with the oscillatory type  $\text{Ca}^{2+}$  transients (Fig. 6a). AP-5 (50  $\mu\text{M}$ ) significantly reduced the number of neurons responding to 100  $\mu\text{M}$  HCY from  $80.5 \pm 2.9\%$  down to  $39.4 \pm 5.4\%$  ( $n = 4$ ;  $p < 0.05$ ; 157 neurons). In sharp contrast, in glial cells the number of  $\text{Ca}^{2+}$  responding cells remained unchanged ( $78.2 \pm 8.6\%$ ;  $n = 4$ ,  $p > 0.05$ ; 125 glial cells). As in the case of TG cells, 10  $\mu\text{M}$  MTEP significantly reduced HCY responsiveness both in neurons and in glial cells. Thus, the number of responding neurons was reduced from  $80.5 \pm 2.9\%$  down to  $39.5 \pm 5.7\%$  ( $n = 3$ ;  $p < 0.05$ ; 64 neurons) and glial cells from  $67.4 \pm 7.4\%$  to  $30.9 \pm 5.8\%$  ( $n = 3$ ;  $p < 0.05$ ; 91 glial cells; Fig. 6b).

On primary cortical cells, the long-term action of HCY also resulted in neurotoxic effects (Fig. 6c). For cortical primary culture, viability data are provided only for neurons (for details, see Material and Methods). The number of live cells in control condition (without 100  $\mu\text{M}$  HCY) after 24 h was  $76.2 \pm 0.8\%$  for neurons ( $n = 5$ ;  $p < 0.05$ , Fig. 6d) consistent with data obtained by others (Mironova *et al.* 2007). After 24 h incubation of cortical cultures with 100  $\mu\text{M}$  HCY there were  $50.4 \pm 2.9\%$  of live neurons ( $n = 5$ ;  $p < 0.05$ ; Fig. 6d). With 500  $\mu\text{M}$  of HCY this

number was reduced to  $25.6 \pm 2.6\%$  ( $n = 3$ ;  $p < 0.01$ ; Fig. 6d). Interestingly, as in the case of TG neurons, either AP-5 or MTEP prevented the toxic effect of HCY on cortical cells (Fig. 6d). Thus, the number of live cells increased to  $63.3 \pm 2.2\%$  ( $n = 5$ ;  $p > 0.05$ ) in the case of AP-5 and  $61.8 \pm 2.1\%$  of control in case of MTEP ( $n = 5$ ;  $p > 0.05$ ).

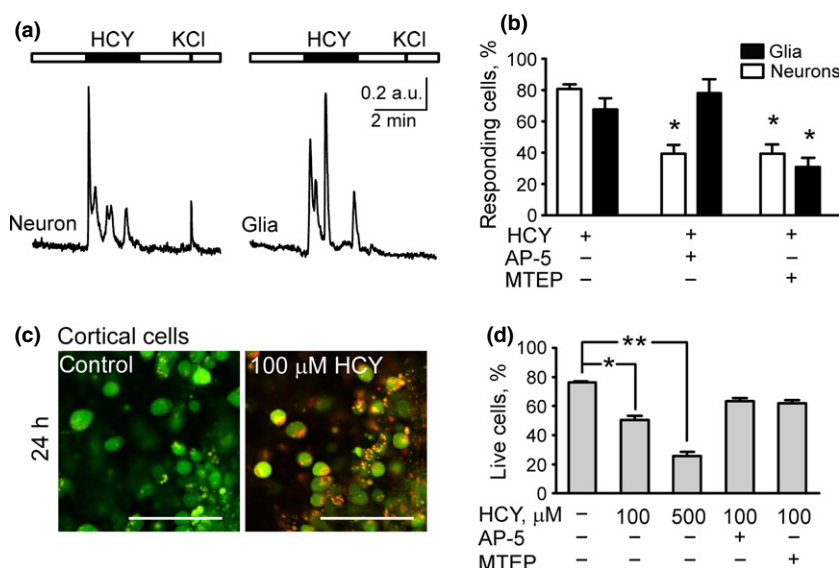
Taken together, these results are consistent with a key role for NMDA and mGluR5 receptors in the toxic effects of HCY on cortical neurons and glial cells.

## Discussion

The main finding of this study is that in sensory neurons HCY operates via co-activation of NMDA and mGluR5 receptors to induce calcium transients and promote delayed cell death. The short-term effects of HCY could be associated with pain sensitization, while longer exposure to this endogenous redox active amino acid resulted in apoptotic cell death. Notably, neurotoxicity was enhanced when both neurons and glia were co-activated suggesting an intense crosstalk between the two cell types. Consistent with this, we found that the survival of glial cells could be controlled by neurons in NMDA receptor-dependent manner. Similar results were obtained on cortical neurons providing a rationale for neurodegenerative changes observed recently in migraine patients.

#### Homocysteine is involved in migraine and neurodegeneration

Recent findings of micro-lesions in migraine patients with aura (Lakhan *et al.* 2013) raised the issue of the role of diffusible messengers which could mediate this type of neurodegeneration. Although glutamate was recognized as the trigger of enhanced excitability at least in familial hemiplegic migraine (Pietrobon and Moskowitz 2013) the life of this amino acid is limited because of the high activity of glutamate uptake by glial cells. HCY emerged recently as a redox active agent which concentration is enhanced in the brain of migraineurs, especially migraine with aura (Isobe and Terayama 2010). Several other reports confirmed the potential role of HCY in migraine (Lea *et al.* 2009; Oterino *et al.* 2010) although there is still controversy on this issue (Hering-Hanit *et al.* 2001). Recent data indicated an important contribution of neuron-glia crosstalk in many chronic pain states (Ceruti *et al.* 2008; Davies *et al.* 2010; Durham and Garrett 2010; Gu *et al.* 2010; Jasmin *et al.* 2010; Suadicani *et al.* 2010). Notably, homocysteic acid, the close endogenous analog of HCY, could be released from glial cells by glutamate activation of respective ionotropic and metabotropic receptors (Benz *et al.* 2004). Also, it has been shown that homocysteic acid is a strongest NMDA agonist compared with other HCY-related substances (Shi *et al.* 2003).



**Fig. 6**  $\text{Ca}^{2+}$  responses and neurotoxicity of homocysteine (HCY) in rat cortical primary culture. (a)  $\text{Ca}^{2+}$  responses induced by 100  $\mu\text{M}$  HCY applied for 2 min to neurons and glial cells loaded with Fluo-3 AM. Application of 50 mM KCl was used to differentiate neurons from glial cells (application intervals are marked with black bars). (b) Histograms showing percentage of neurons (white columns) and glial cells (black columns) responding to 100  $\mu\text{M}$  HCY alone or in the presence of HCY combination with 10  $\mu\text{M}$  3-((2-Methyl-4-thiazolyl)ethynyl)pyridine

### Short-term effects of homocysteine

In this study, within the nociceptive system, including peripheral sensory trigeminal and central cortical neurons, we focused on two types of HCY actions: fast (early) and delayed. Our observation that HCY elicited fast large calcium transients not only in trigeminal neurons but also in surrounding satellite glial cells is consistent with previous studies demonstrating that HCY induced intracellular  $\text{Ca}^{2+}$  signals in cerebellar granule cells, neural crest cells and vascular smooth muscle cells (Mujumdar *et al.* 2000; Ziemińska *et al.* 2003; Heidenreich and Brauer 2008). Furthermore, our findings showed the presence of functional glutamate receptors in both trigeminal neurons and satellites. This is in line with the known ability of HCY to act as an agonist of glutamate binding site of NMDA receptor (Lipton *et al.* 1997). Furthermore, we show that  $\text{Ca}^{2+}$  mobilization in neurons by HCY is mediated via group I mGluR5 subtype. Unlike neurons, HCY-induced  $\text{Ca}^{2+}$  transients in SGC were insensitive to the NMDA antagonist AP-5. Enhanced intracellular  $\text{Ca}^{2+}$  elevation in sensory neurons may have several long-term outcomes. For instance, we showed previously that activation of CaMKII (a known target of intracellular  $\text{Ca}^{2+}$ ) is involved in the sensitization of trigeminal neurons (Giniatullin *et al.* 2008). On the other hand, long-lasting  $\text{Ca}^{2+}$  elevation can also activate classical neurodegenerative pathways (Choi 1995;

MTEP). Mean  $\pm$  SEM ( $n = 3$  experiments, 162 neurons and 184 glial cells). (c) An overlay of confocal images of cortical cultures taken in green and red spectra in control or after 24 h exposure to 100  $\mu\text{M}$  HCY. Scale bar 100  $\mu\text{m}$ . (d) Histograms showing percentage of live cortical cells in control and after 24 h exposure to 100  $\mu\text{M}$  or 500  $\mu\text{M}$  HCY alone or to the combination of HCY with 50  $\mu\text{M}$  AP-5 or 10  $\mu\text{M}$  MTEP. ( $n = 3$ –5 experiments with automatic counting with Image J). Mean  $\pm$  SEM \* $p < 0.05$ ; \*\* $p < 0.01$  by Mann–Whitney U-test.

Stout *et al.* 1998; Tenneti *et al.* 1998) finally leading to neuronal cell death.

### Homocysteine promotes cells death

It is suggested that HCY could be implicated in several neurodegenerative disorders like Alzheimer's disease and Parkinson's disease (Kuhn *et al.* 2001; Kruman *et al.* 2002). We show that high level of HCY could induce neurodegeneration also within the nociceptive system. Using a novel imaging assay we showed that HCY neurotoxicity in sensory system induced mainly apoptosis, with a minimal contribution of necrosis. What could be the mechanism of pro-apoptotic action of HCY? Like in short time action, we demonstrate that neuronal survival could be largely improved by treatment with NMDA and mGluR5 antagonists. Interestingly, despite the absence of evidence for expression of NMDA receptors in satellite cells, NMDA antagonist also promoted their survival, suggesting that the survival of glial cells is controlled by cross-talk with neurons expressing functional NMDA receptors. In other words, apoptosis of satellites could be secondary to long-lasting activation of neurons via NMDA receptors. Recent studies showed the contribution of MAPK to HCY induced death of embryonic cortical neurons mediated by NMDA receptors (Poddar and Paul 2013). Other studies suggested the role of HCY oxidative stress in neuronal death (Kim and Pae 1996;



Sibrian-Vazquez *et al.* 2010). It is well known that the hyperactivation of NMDA receptors is associated with oxidative stress (Tenneti *et al.* 1998; Reyes *et al.* 2012). In line with this, it has been shown in cultured hippocampal neurons that HCY (250  $\mu$ M) exposure leads to poly-ADP-ribose polymerase activation and NAD depletion that precedes mitochondrial oxidative stress and apoptosis (Kruman *et al.* 2000). HCY increases lipid peroxidation, a marker of oxidative stress, in rat brain synaptosomes (Jara-Prado *et al.* 2003) as well as in rat parietal, cingulate and prefrontal cortices (Matte *et al.* 2004). However, our testing of classical markers of oxidative stress (such as MDA) after HCY application to nociceptive neurons did not reveal significant changes in lipid peroxidation. Consistent with this, Rhodamine 123 and DCF imaging did not show essential changes in the  $\Delta\psi_m$  or ROS level suggesting little disturbance of the redox state of trigeminal cells. Moreover, HCY reduced DCF fluorescence in neurons suggesting a reducing, rather than a pro-oxidative effect of HCY. The latter is not surprising, if we take into account the chemical nature of HCY which possesses the SH-groups and ability to serve as precursor of cysteine/glutathione pathway (Lu 2009). Consistent with this, in endothelial cells, HCY can lead to reductive rather than oxidative stress (Outinen *et al.* 1998). In some other models, anti-oxidative and protective effects of HCY have been reported (Perna *et al.* 2003; Loureiro *et al.* 2010). In addition, HCY prevents H<sub>2</sub>O<sub>2</sub>-induced expression of HSP70 elevation which is linked to oxidative stress response (Outinen *et al.* 1998). Interestingly, in primary cortical astrocytes and in acutely prepared cerebellar neurons, HCY induced ROS production without affecting cellular viability (Loureiro *et al.* 2010; Sergeeva *et al.* 2010).

### Cell and receptors crosstalk

Many neurodegenerative diseases and chronic pain states are based on neuroglial interactions (Tsuda *et al.* 2013). In the trigeminal ganglia, increased signaling between neuronal cell bodies and satellite glia cells play a supporting factor for chronic pain (Ceruti *et al.* 2008; Davies *et al.* 2010; Durham and Garrett 2010; Suadicani *et al.* 2010). Similar to cortical neurons, trigeminal cells express NR1, NR2A and NR2B subunits of NMDA receptor (Abushik *et al.* 2013), while out of various group I metabotropic glutamate receptors the mGluR5 subtype is mostly expressed (Lee and Ro 2007). It is well known that in the CNS the activation of mGluR1/5 usually enhance neuronal excitability via the PLC-IP<sub>3</sub> pathways and Ca<sup>2+</sup>-dependent activation of protein kinase C (Nanou *et al.* 2009). Moreover, there is a membrane delimited interaction between group I metabotropic receptors and NMDA channels (Matta *et al.* 2011; Sylantsev *et al.* 2013). This, in turn, leads to protein kinase C-dependent facilitation of NMDA currents (Zhang *et al.* 1996; Lea *et al.* 2002).

Our data indicate that in glial cells fast Ca<sup>2+</sup> responses are also mediated by mGluR5 receptors. Interestingly, apoptosis of glial cells was prevented by NMDA antagonists, suggesting an intense crosstalk between neurons and neighboring satellites during prolonged action of HCY. Our hypothesis is that the activation of mGluR5 receptors was insufficient to trigger cell death pathways alone, unless this takes place in the presence of hyperactive neurons in an NMDA receptor-dependent manner.

### Conclusion

In summary, we report complex NMDA and mGluR5 dependent actions of the redox active amino acid HCY on intracellular Ca<sup>2+</sup> mobilization and survival of nociceptive peripheral and central neurons, which further highlights the contribution of this endogenous compound to migraine pathophysiology, suggesting therapeutic benefits from normalized levels of HCY in affected subjects.

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