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Role of GluN2A NMDA receptor in homocysteine-induced prostaglandin E2 release from neurons

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Abstract

Hyperhomocysteinemia or systemic elevation of homocysteine is a metabolic condition that has been linked to multiple neurological disorders where inflammation plays an important role in the progression of the disease. However, it is unclear whether hyperhomocysteinemia contributes to disease pathology by inducing an inflammatory response. The current study investigates whether exposure of primary cultures from rat and mice cortical neurons to high levels of homocysteine induces the expression and release of the proinflammatory prostanoid, Prostaglandin E2 (PGE2). Using enzymatic assays and immunoblot analysis we show concurrent increase in the activity of cytosolic phospholipase A2 (cPLA2) and level of cyclooxygenase-2 (COX2), two enzymes involved in PGE2 biosynthesis. The findings also show an increase in PGE2 release from neurons. Pharmacological inhibition of GluN2A-containing NMDAR (GluN2A-NMDAR) with NVP-AAM077 significantly reduces homocysteine-induced cPLA2 activity, COX2 expression, and subsequent PGE2 release. Whereas, inhibition of GluN2B-containing NMDAR (GluN2A-NMDAR) with Ro 25–6981 has no effect. Complementary studies in neuron cultures obtained from wild type and GluN2A knockout mice show that genetic deletion of GluN2A subunit of NMDAR attenuates homocysteine-induced neuronal increase in cPLA2 activity, COX2 expression, and PGE2 release. Pharmacological studies further establish the role of both extracellular-regulated kinase/mitogen-activated protein kinase and p38 MAPK in homocysteine-GluN2A NMDAR-dependent activation of cPLA2-COX2-PGE2 pathway. Collectively, these findings reveal a novel role of GluN2A-NMDAR in facilitating homocysteine-induced proinflammatory response in neurons.

Keywords

COX2; cPLA2; GluN2A NMDA receptor; homocysteine; NFκB; PGE2

Homocysteine is a sulfur-containing amino acid that is formed as an intermediate of the methionine cycle. Elevated levels of total plasma homocysteine, also known as hyperhomocysteinemia, is a metabolic condition that is considered to be a risk factor for

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All experiments were conducted in compliance with the ARRIVE guidelines.

several neurological disorders (Sharma *et al*. 2015; Seshadri *et al*. 2002; Zoccolella *et al*. 2006; Hankey and Eikelboom 2001; Zoccolella *et al*. 2010; Ansari *et al*. 2014). In both *in vitro* and *in vivo* models of neurological disorders, prolonged exposure to elevated levels of homocysteine has been shown to enhance neuronal vulnerability to injury. *In vitro* studies using primary neuronal cultures have shown that exposure to elevated levels of homocysteine potentiates MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), amyloid β-peptide, excitotoxic or oxidative stress induced injury (Duan *et al*. 2002; Kruman *et al*. 2002; Kruman *et al*. 2000). *In vivo* studies using an animal model of Parkinson's disorder has shown that predisposition to hyperhomocysteinemia desensitize dopaminergic neurons to degeneration and enhance motor dysfunction (Duan *et al*. 2002). Also, in an animal model of Alzheimer's disease, hyperhomocysteinemia has been shown to increase neuronal death in the hippocampus (Kruman *et al*. 2002).

It is evident from earlier studies that homocysteine is an agonist of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, and prolonged exposure of neurons to high levels of homocysteine leads to NMDAR-mediated cell death (Lipton *et al*. 1997; Kruman *et al*. 2000; Kruman *et al*. 2002; Mattson and Shea 2003; Jara-Prado *et al*. 2003; Poddar and Paul 2009; Poddar and Paul 2013; Poddar *et al*. 2017). The tetrameric NMDAR channel is comprised of two essential GluNl subunits and two regulatory GluN2 subunits (GluN2A/B/C/D). In the adult forebrain, GluN1/GluN2A containing NMDARs (GluN2A-NMDAR) and GluNl/GluN2B containing NMDARs (GluN2B-NMDAR) are the two most predominant functional NMDARs (Dingledine *et al*. 1999). In earlier studies, we have shown that GluN2A-NMDAR plays a key role in homocysteine-induced neuro-toxicity and exacerbation of ischemic brain damage (Poddar and Paul 2009; Poddar and Paul 2013; Jindal *et al*. 2019). These findings show that the intracellular neurotoxic signaling cascade downstream of homocysteine-GluN2A-NMDAR stimulation involves Ca^{2+}-dependent sustained activation of extracellular-regulated kinase/mitogen-activated protein kinase (ERK MAPK). We also observed sustained activation of the stress-activated p38 MAPK, which is downstream of and dependent on ERK MAPK activation (Poddar and Paul 2009; Poddar and Paul 2013). This unique crosstalk involves ERK MAPK-dependent internalization of the Ca^{2+}-impermeable GluA2-subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) resulting in increased Ca^{2+} influx through the GluA2-lacking AMPAR, which leads to p38 MAPK activation (Poddar *et al*. 2017). The increased p38 MAPK activation subsequently leads to caspase-3-dependent neuronal cell death (Poddar and Paul 2013).

In addition to its role in neurotoxicity, elevated levels of homocysteine has also been implicated in inducing inflammatory response in the brain (da Cunha *et al*. 2010; Scherer *et al*. 2014). Prior studies have indicated that acute (1–12 h) or chronic administration (30 days) of high levels of homocysteine significantly increases the levels of the proinflammatory prostanoid prostaglandin E2 (PGE2), cytokines and chemokines in the cerebral cortex and hippocampus (da Cunha *et al*. 2010; da Cunha *et al*. 2012). These findings raise the possibility that pre-disposition to hyperhomocysteinemia could enhance the inflammatory response in the brain during neurological disorders. However, the precise role of neurons in homocysteine-induced increase in inflammatory response in the brain is yet to be determined. To address this issue, in the current study we exposed primary cortical

neuron cultures with a level of homocysteine (50 μM) that is typically observed in individuals with moderate hyperhomocysteinemia, to evaluate its effect on the release of the proinflammatory prostanoid PGE2 (Refsum *et al.* 1998). Our findings demonstrate for the first time that homocysteine induces an increase in PGE2 release from neurons and establish the role of GluN2A-NMDAR in mediating this inflammatory response. The intracellular signaling cascade downstream of homocysteine-GluN2A-NMDAR stimulation involves activation of ERK and p38 MAPKs as well as cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX2), two rate limiting enzymes involved in the biosynthesis of PGE2 (Takemiya *et al.* 2007; Bazan *et al.* 2002; Minghetti 2007).

Material and methods

Animals

Female adult Sprague-Dawley pregnant rats (RRID:RGD_737903) were purchased from Envigo (Livermore, CA, USA) for establishing primary neuron cultures. GluN2A NMDAR Knockout (GluN2A-KO) mice (RRID:IMSR_RBRC01813) were obtained from Dr Andrew Holmes, NIH/NIAAA (Sakimura *et al.* 1995; Brigman *et al.* 2008), and bred at the animal facility of University of New Mexico. No custom-made materials were generated for this study using these mice. Heterozygous breeding pairs were maintained in humidity and temperature controlled environment (20 ± 1°C) under standard cage density conditions with one female and one male per cage. The animals had access to food and water *ad libitum.* The offspring were genotyped using the following primers sets: (1) forward primer 5 ‘GCCTGCTTGCCGAATATCATGGTGGAAAAT3 ‘ and reverse primer 5 ‘CCCGTTAGCCCGTTGAGTCACCCCT3 ‘; (2) forward primer 5 ‘ TCTGGGGCCTGGTCTTCAACAATTCTGTGC3 ‘ and reverse primer 5’ATTCTTTGATAAATATGCAATGTATGGGG G3’ (Sakimura *et al.* 1995; Brigman *et al.* 2008). The PCR reaction was performed using the following conditions: 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 62°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 7 min using GoTaq green master mix (Cat # M7122; Promega Corporation, Madison, WI, USA). Adult wild type (WT) male and female mice as well as adult GluN2A-KO male and female mice were mated to generate timed pregnant female mice. The males were removed from the females after 24 h period, which was considered gestational day 1. Pregnancy was verified by the presence of vaginal plugs and/or weight gain. The study was conducted in strict accordance with the recommendations in the ‘Guide for the Care and Use of Laboratory Animals’ of the National Institutes of Health and complies with the ARRIVE guidelines. Institutional Animal Care and Use Committee of University of New Mexico, Health Sciences Center approved all animal procedures used in the current study. The Animal Welfare Assurance number is D16–00228 (A3350–01) and USDA registration number is 85-R-0014. The study was not pre-registered. There was no randomization of animals for these studies, and rat or mouse embryos were obtained and sacrificed from an individual timed pregnant female for preparing primary neuron cultures. No blinding was performed during the preparation of primary neuron cultures or during the course of experiments. No sample size calculation was performed and there were no differences in sample sizes for each experiment between the beginning and end of the study.

Materials and reagents

Reagents used for establishing primary neuronal cultures (Dulbecco's Modified Eagle's Medium/F12, DMEM/F12, Cat #11330–032; Modified Eagle's Medium, MEM, Cat #12360–038; Fetal bovine serum, Cat #10082–147; Hanks Balanced Salt solution, HBSS, Cat #24020–117; antibiotic/antimycotic mixture, Cat #15240–062) were obtained from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). L-homocysteine thiolactone (Cat #H6503), cytosine D-arabinofuranoside (Cat #C1768), N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonicd acid (TES; Cat #T1375), glycine (Cat #3570), and Hoechst 33342 (Cat #14533) were purchased from Millipore Sigma (St. Louis, MO, USA). Anti-COX2 polyclonal antibody (RRID:AB_2085144) was obtained from Abcam (Cambridge, UK), anti β-tubulin monoclonal antibody (RRID:AB_2241191) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA), and anti-rabbit (RRID:AB_2099233) and anti-mouse (RRID:AB_330924) horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). All antibodies were purchased commercially and the data validating the antibody is available from the company. Bicinchoninic acid (BCA) protein estimation kit (Cat #23225) and West Pico supersignal chemiluminescence reagent (Cat #34580) were purchased from Pierce (Rockland, IL, USA). Selective pharmacological inhibitors were obtained as follows: DL-2-Amino-5-phosphopentanoic acid (DL-AP5; Cat #A5282), MK-801 hydrogen maleate (MK801, Cat #475878), PD98059 (Cat #513001), SB203580 (Cat #559398), and NVP-AAM077 (Cat #504528) were obtained from Millipore Sigma (St. Louis, MO, USA). Bengamide B (Cat #5273) was purchased from R & D systems (Minneapolis, MN, USA) and CAY10404 (Cat #70210) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). PGE2 enzyme-linked immunosorbent assay (ELISA) kit (Cat #K051-H1) was purchased from Arbor assays (Ann Arbor, MI, USA) and cPLA2 activity assay kit (Cat #765021) was obtained from Cayman chemicals (Ann Arbor, MI, USA).

Neuron culture, L-Homocysteine preparation and stimulation

Embryos obtained from pregnant female Sprague-Dawley rats (16–17 day gestation) or WT and GluN2A KO mice (15–16 day gestation) were used to establish primary neuronal cultures, as described earlier (Poddar and Paul 2009; Poddar and Paul 2013). Briefly, the pregnant females were sacrificed using inhaled CO_2, based on American Veterinary Medical Association guidelines for euthanasia in animals and death was confirmed by absence of heartbeat. Embryos were removed post-mortem and the cortex was dissected out. Brains from embryos obtained from a single pregnant dam were pooled and single cell suspension was made using fire-polished pasteur pipettes, plated on poly-D-lysine coated 60 mm culture dishes (BD BioCoat plates; Cat # 356468 from Fisher Scientific) for 7 min. Following removal of non-adhering cells the neurons were grown in DMEM supplemented with 5% fetal bovine serum and antibiotic/antimycotic mixture for 3 days following which they were treated with 1 μM cytosine arabinoside at DIV 3 for 24 h to prevent glial expansion. The neurons were thereafter maintained in MEM containing 5% fetal bovine serum and antibiotic/antimycotic mixture for 12–14 days prior to treatment with L-homocysteine.

L-homocysteine (200 mM stock) was prepared by alkali hydrolysis of L-homocysteine thiolactone hydrochloride followed by neutralization with 2N HCL and maintained in 0.02 mM of TES buffer pH 7.4 (Poddar *et al.* 2001). For receptor stimulation neurons were treated with freshly prepared 50 μM of L-homocysteine in HBSS (Poddar and Paul 2009; Poddar and Paul 2013; Poddar *et al.* 2017) containing 50 μM of glycine (Lipton *et al.* 1997). The concentration of L-homocysteine (50 μM) used for these experiments is based on our previous dose-response study (Poddar and Paul 2009) and is typically observed in individuals with moderate hyperhomocysteinemia. In a subset of experiments pharmacological inhibitors (MK801, NVP-AAM007, PD98059, SB203580, Bengamide or CAY10404, each prepared according to respective manufacturer's instructions or purchased in solution) were added 10 min prior to treatment with L-homocysteine. Cells were processed for either immunoblotting or cPLA2 activity assay. The culture media were processed for measurement of PGE2 levels.

Immunoblotting

Rat and mice neuronal cultures were washed with PBS (pH 7.4), containing sodium pyrophosphate and sodium vanadate as phosphatase inhibitors, and harvested in SDS sample buffer (Laemmli 1970). Equal protein from total cell lysates, estimated using BCA kit, was resolved by SDS-PAGE (7.5%) followed by western blotting on PVDF membranes. Immunoblot analysis was performed by first blocking with 5% non-fat dry milk for 1 h at (25°C) followed by incubation with either anti-COX2 (1 : 1000) or anti-IB (1 : 1000) antibodies as described in each experiment (Poddar and Paul 2009). The blots were washed and incubated with horseradish peroxidase conjugated secondary antibodies were used according (1 : 2000). Signals from immune complexes in the blots were developed using West Pico supersignal chemiluminescence reagents and then captured on X-ray films. Densitometric analysis of the images was performed using the NIH Image J software.

Measurement of cPLA2 activity and PGE2 levels

For measurement of cPLA2 activity, cell lysates were harvested in ice-cold Tris-buffered saline (pH 7.4) containing phosphatase inhibitor, sonicated 3 times with 5 s bursts and placed on ice (2 min) between each burst of sonication. The lysed cell suspensions were centrifuged at 10,600 *g* (10 min) and the supernatant was collected in another tube. Equal amounts of protein from the supernatant were processed for cPLA2 activity assay according to the manufacturer's protocol. For measurement of PGE2 levels released from neurons, culture medium was collected from each experimental plate and centrifuged at 200 *g* for 5 min to remove cellular debris. Equal volume (100 μL) of the supernatant from each sample was used to determine PGE2 level using the PGE2 ELISA kit according to the manufacturer's instructions.

Statistical analysis

Statistical analysis and comparison was performed using GraphPad Prism (version 5a) software. One-way analysis of variance (ANOVA, Bonferroni's multiple comparison test) were analyzed and differences were considered significant when $p < 0.05$. Assessment of data normality and test for determining outliers were not performed for the datasets. Experiments

were performed from independent cell culture preparations and the number of independent cell culture preparation (n) for each experiment is included in the Figure legends.

Results

Homocysteine induced increase in neuronal cPLA2 activity, COX2 protein level and PGE2 release

In initial studies rat neuronal cultures were treated with L-homocysteine (50 μM) for varying time periods (0, 1, 2, 4 h) to examine the temporal profile of cPLA2 activity in neurons. Figure 1a shows that treatment with homocysteine results in significant increase in cPLA2 activity over time with a maximum increase by 4 h, when compared to untreated control. We next treated neuron cultures with L-homocysteine (50 μM) for the specified time periods (0, 1, 2, 4 h) and analyzed the cell lysates by immunoblotting with anti COX2 antibody. The representative immunoblot and the corresponding bar diagram show a significant increase in COX2 protein level by 2 h of stimulation with homocysteine that remain elevated throughout the rest of the time studied (Fig. 1b). Immunoblot analysis with β-tubulin confirms that equal amount of total protein was analyzed in each case. The culture media obtained from the same samples were also analyzed to estimate the amount of PGE2 released from the neurons following treatment with homocysteine. Figure 1c shows a significant increase in PGE2 level within 2 h of homocysteine exposure that increases further at 4 h after treatment.

Homocysteine-induced cPLA2 activity, COX2 protein level and PGE2 release is dependent on GluN2A-NMDAR stimulation

To determine the role of NMDARs in modulating cPLA2 activity following exposure to homocysteine, neurons were treated with L-homocysteine (50 μM, 4 h) in the presence of the NMDAR inhibitor MK801 (10 μM) (Poddar and Paul 2009). Figure 2a shows that homocysteine-mediated increase in cPLA2 activity is blocked in the presence of MK801. To delineate the role of GluN2A- and GluN2B-NMDAR in homocysteine-induced increase in cPLA2 activity, neurons were treated with L-homocysteine (50 μM, 4 h) in the presence of NVP-AAM077 (30 nM) (Martel *et al*. 2009) or Ro 25–6981 (1 μM) (Soria *et al*. 2014), selective inhibitors of GluN2A- and GluN2B-NMDAR, respectively (Auberson *et al*. 2002; Fischer *et al*. 1997; Mutel *et al*. 1998). Figure 2b shows that co-incubation with NVP-AAM077 inhibits homocysteine-mediated increase in cPLA2 activity. In contrast, treatment with Ro 25–6981 fails to attenuate homocysteine-mediated increase in cPLA2 activity (Fig. 2c). To further confirm the role of GluN2A-NMDAR in homocysteine-mediated increase in cPLA2 activity, neuronal cultures obtained from WT and GluN2A-KO mice were subjected to L-homocysteine treatment for 4 h. Figure 2d shows that following homocysteine treatment cPLA2 activity increase significantly in the neuron cultures obtained from WT mice, while cPLA2 activity remain unchanged in the neuron cultures obtained from GluN2A-KO mice.

To determine the role of NMDARs in homocysteine-induced increase in COX2 expression, cell lysates from neuronal cultures treated with L-homocysteine (50 μM, 4 h) in the presence of MK801 were subjected to immunoblot analysis with anti-COX2 antibody. The results show that co-incubation with MK801 blocks homocysteine induced COX2 protein

expression (Fig. 3a). Subsequent studies investigated the effect of GluN2A-NMDAR (NVP-AAM077, 30 nM) or GluN2B-NMDAR (Ro 25–6981, 1 μM) inhibition on the homocysteine-NMDAR induced increase in COX2 protein level. As shown in Fig. 3b treatment with NVP-AAM007 blocks homo cysteine-induced increase in COX2 protein expression, while treatment with Ro 25–6981 fails to ameliorate homocysteine-induced increase in COX2 protein level. Consistent with these observations studies in neuron cultures obtained from WT and GluN2A-KO mice shows that exposure to L-homocysteine (50 μM, 4 h) leads to increase in COX2 protein level in WT mice cultures, while it fails to augment COX2 protein expression in GluN2A-KO mice cultures (Fig. 3c).

To evaluate whether the GluN2A-NMDAR stimulation also play a role in homocysteine-dependent PGE2 release, culture media from neurons treated with L-homocysteine (50 μM, 4 h) in the absence or presence of MK801, NVP-AAM077 or Ro 25–6981 were analyzed for PGE2 level. The results show that homocysteine-induced increase in PGE2 release is significantly reduced in the presence of MK801 or NVP-AAM007 (Fig. 4a and 4). However, treatment with Ro 25–6981 fails to alter homocysteine-induced increase in PGE2 level (Fig. 4c). Additional studies in neuronal cultures from WT and GluN2A-KO mice show that exposure to homocysteine (50 μM, 4 h) significantly increases PGE2 release from WT mice cultures, while it fails to induce PGE2 release from cultures obtained from GluN2A-KO mice (Fig. 4d). These findings in conjunction with the observations in Figures 2 and 3 indicate that homocysteine-induced up-regulation of GluN2A-NMDARs plays a key role in cPLA2 activation, COX2 expression and PGE2 release.

Homocysteine-GluN2A-NMDAR-induced cPLA2 activation and COX2 expression involves crosstalk between ERK and p38 MAPK

To test the hypothesis that homocysteine-GluN2A-NMDAR induced crosstalk between ERK and p38 MAPK (Poddar and Paul 2013; Poddar *et al.* 2017) plays a role in the increase in cPLA2 activity, COX2 protein levels and subsequent PGE2 release, we evaluated the effect of pharmacological inhibition of ERK and p38 MAPK. For these experiments neurons were treated with L-homocysteine (50 μM, 4 h) in the presence of selective inhibitors for ERK MAPK phosphorylation (PD98059, 15 μM; Fig. 5) (Poddar and Paul 2009) or p38 MAPK phosphorylation (SB203580, 5 μM; Fig. 6) (Poddar and Paul 2013). Assessment of cPLA2 activity in cell lysates shows that treatment with PD98059 during exposure to homocysteine significantly attenuates homocysteine-mediated increase in cPLA2 activity (Fig. 5a). Immunoblot analysis of cell lysates with anti-COX2 antibody show that homocysteine-induced COX2 protein expression is significantly reduced in the presence of PD98059 (Fig. 5b). PGE2 level in the culture medium also decreases significantly following exposure to homocysteine in the presence of PD98059 (Fig. 5c). Inhibition of p38 MAPK with SB203580 also shows significant reduction in homocysteine-mediated increase in cPLA2 activity (Fig. 6a), COX2 protein levels (Fig. 6b) and PGE2 release (Fig. 6c).

Homocysteine-induced COX2 expression involves p38 MAPK mediated activation of NFκB

Earlier studies indicated that both ERK and p38 MAPKs are involved in NF-B mediated regulation of inflammatory mediators in different cell types (Kaltschmidt *et al.* 2002; Pikarsky *et al.* 2004; Kim *et al.* 2008; Huang *et al.* 2001; Kang *et al.* 2006; Guo *et al.* 2013).

To clarify the role NFκB in homocysteine-GluN2A NMDAR induced activation of the COX2/PGE2 signaling pathway and the role of MAPKs in this process, we next evaluated the effect of homocysteine on IB degradation, a seminal step in NF-B activation (Karin and Ben-Neriah 2000). For these experiments neurons were treated with L-homocysteine (50 μM, 4 h) in the presence of ERK or p38 MAPK inhibitor. Immunoblot analysis of cell lysates with anti-IB antibody shows that treatment with homocysteine (50 μM, 4 h) alone led to significant decrease in the cellular level of IB, indicating increased IB degradation and NFκB activation (Fig. 7a, lane 2). Whereas, exposure to homocysteine in the presence of ERK or p38 MAPK inhibitor effectively blocks homocysteine induced degradation of IB (Fig. 7a, lanes 3–4). In additional studies neurons were incubated with homocysteine (50 μM, 4 h) in the presence of Bengamide B (500 nM) (Johnson *et al.* 2012), a potent inhibitor of NF-B activation. Immunoblot analysis shows that co-incubation with Bengamide B attenuates homocysteine-induced increase in COX2 protein level. Quantitative determination of PGE2 release in the culture medium obtained from the same experiment shows significant decrease in PGE2 level following exposure to homocysteine in the presence of Bengamide B (Fig. 7c). To further confirm that the release of PGE2 is a consequence of homocysteine-induced increase in COX2 protein expression, neurons were treated with L-homocysteine (50 μM, 4 h) in the presence of CAY10404 (100 nM) (Karim *et al.* 2005), a selective inhibitor of COX2 (Habeeb *et al.* 2001). Figure 7d shows that homocysteine-induced PGE2 release is significantly reduced in the presence of CAY10404.

DISCUSSION

A key finding of the current study is that exposure of neurons to high concentrations of homocysteine triggers an inflammatory response, which involves concomitant up-regulation of cPLA2 activity and COX2 expression resulting in increased PGE2 release. The findings also highlight the role of GluN2A-NMDAR in mediating homocysteine-induced neuronal PGE2 release.

The biosynthesis of PGE2 is initiated by the enzyme cPLA2, which catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid (Farooqui *et al.* 1997; Farooqui and Horrocks 2006; Ong *et al.* 2010). Arachidonic acid is then metabolized by cyclooxygenases (COX) to generate the intermediate prostaglandin precursor Prostaglandin H2, which serves as the substrate for the synthesis of biologically active prostaglandins (PGE2, PGD2, PGF2α, PGI2) by specific prostaglandin synthases (Ueno *et al.* 2005; Smith *et al.* 2000). Cyclooxygenases exist in two distinct isoforms referred to as COX1 and COX2 (Vane *et al.* 1998). COX1 is expressed constitutively in most cells under normal conditions and is the dominant source of prostaglandins that promotes housekeeping functions (Kirkby *et al.* 2012; Terlain *et al.* 1995; Griswold and Adams 1996; Seibert *et al.* 1997). In contrast, COX2 is an inducible enzyme that is expressed in response to a variety of stimuli, ranging from growth factors to cytokines and appears to be the primary COX controlling pro-inflammatory PGE2 synthesis (Seibert *et al.* 1995; Terlain *et al.* 1995; Seibert *et al.* 1997; Kirkby *et al.* 2016). Since elevated expression and activity of COX2 is a key element in the pathophysiology of several inflammatory disorders its regulation differs between cell types. A particular contribution of the current study is the identification of the novel role of GluN2A-NMDARs in mediating increased cPLA2 activity and COX2 expression in neurons.

The above finding is especially important in the field of NMDAR signaling as growing evidence indicate that the subunit composition of NMDARs is a critical determinant of whether NMDAR-mediated signaling has beneficial or detrimental effects in neurons. GluN2A-NMDAR stimulation has been primarily implicated in synaptic plasticity and promoting cell survival whereas, GluN2B-NMDAR stimulation has been shown to result in long-term depression and is detrimental to neurons (Foster *et al*. 2010; Liu *et al*. 2004; Massey *et al*. 2004; Chen *et al*. 2008; Hardingham and Bading 2003; Lynch and Guttmann 2002; Liu *et al*. 2007). In contrast to this general notion, our findings now show that homocysteine-induced GluN2A-NMDAR stimulation can trigger a pro-inflammatory response involving neuronal PGE2 release. The role of GluN2A-NMDAR in neuronal PGE2 release is not only based on studies using a selective pharmacological inhibitor against GluN2A-NMDAR but also by genetic deletion of GluN2A-subunit in neurons. The inability of a GluN2B-NMDAR inhibitor to attenuate the activation of cPLA2/COX2 signaling pathway and PGE2 release further confirms the selective role of GluN2A-NMDARs in mediating the detrimental effects of homocysteine in neurons. In addition to our findings, an earlier study has also addressed the detrimental role of GluN2A-NMDAR signaling in neurons in response to a different stimulus (Zhou *et al*. 2013). Their findings indicate that GluN2A-NMDAR stimulation has a small but significant role in promoting excitotoxic cell death when compared with GluN2B-NMDAR stimulation. Together these findings modify the current perception that GluN2A-NMDAR mediated signaling in neurons exclusively enhances plasticity and survival promoting genes.

It has been reported previously that phosphorylation of cPLA2 at Ser^{505} by either ERK or p38 MAPK augments cPLA2 activity and the specific MAPK involved in this process depends on the type of stimuli (Lin *et al*. 1993; Kramer *et al*. 1996). Emerging evidence also show that depending on the stimuli and cell type, ERK or p38 MAPK can enhance the transcription and/or stability of COX2 & mRNA resulting in increased protein levels (Ridley *et al*. 1998; Bartlett *et al*. 1999; Dean *et al*. 1999; Lasa *et al*. 2000; Svensson *et al*. 2003; Rockwell and Kaminski 2004; Park and Kwon 2011). Our findings now show that inhibition of either ERK or p38 MAPK can attenuate homocysteine-GluN2A NMDAR mediated enhanced cPLA2 activity and COX2 protein level. Since homocysteine-induced p38 MAPK activation is downstream of and dependent on ERK MAPK activation (Poddar and Paul 2013; Poddar *et al*. 2017), it would suggest that sequential activation of ERK and p38 MAPK is essential for activation of cPLA2/COX2 signaling pathway in neurons, following exposure to homocysteine. These findings reveal an important additional mechanism of regulation of the cPLA2/COX2 pathway that involves concerted effort of both ERK and p38 MAPK. Consistent with this interpretation we also observe that inhibition of either ERK or p38 MAPK blocks the degradation of IB that is known to result in activation of NFκB signaling cascade, a major stress response pathway for COX2 gene expression (Karin *et al*. 2004; Kaltschmidt *et al*. 2002; Tak and Firestein 2001). Our findings also show that pharmacological inhibition of NFκB attenuates homocysteine induced increase in COX2 protein level and PGE2 release. Together these findings demonstrate that NFκB activation plays an intermediary role in ERK and p38 MAPK-dependent COX2 expression, and provides a molecular basis for homocysteine-GluN2A NMDAR mediated release of PGE2 from neurons.

Excessive or persistent release of PGE2 in the brain has been associated with microglial activation, which is a major source for the production of proinflammatory cytokines and matrix metalloproteinases. (Wang *et al*. 2007; Smith *et al*. 2000; Candelario-Jalil *et al*. 2007; Quan *et al*. 2013; Yenari *et al*. 2010; Nakamura 2002; Raivich *et al*. 1999). This in turn could lead to BBB disruption and peripheral immune cell infiltration resulting in inflammatory response that has been associated with the progression of both acute and chronic neurological disorders. As such, from the current study it could be postulated that a neurological insult under hyperhomocysteinemic condition could contribute to the inflammatory milieu of the brain through induction of PGE2 release from neurons to accelerate cellular injury. The findings provide a valuable basis for further evaluation in future studies whether predisposition to hyperhomocysteinemia could be a determinant of the severity of the inflammatory response and pathological outcome in neurological disorders.

Acknowledgments and conflict of interest disclosure

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Abbreviations used

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
COX2	cyclooxygenase-2
cPLA2	cytosolic phospholipase A2
ERK MAPK	extracellular-regulated kinase/mitogen-activated protein kinase
GluN2A-KO	GluN2A subunit knockout mice
NFκB	nuclear factor kappa B
NMDAR	N-methyl-D-aspartate receptor
PGE2	prostaglandin E2

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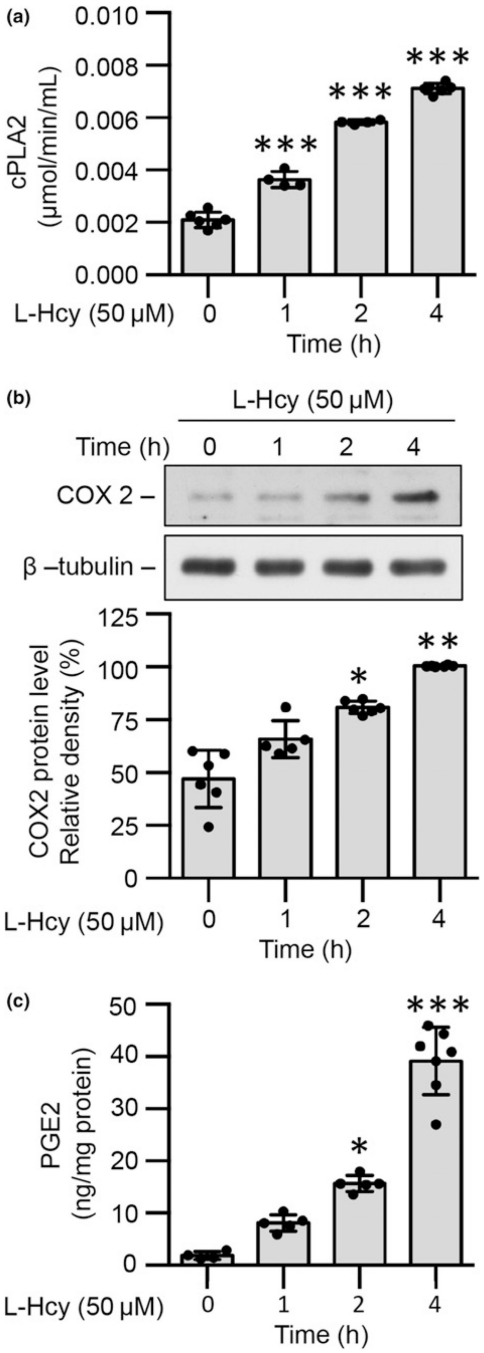

Fig. 1.

Homocysteine induces neuronal cPLA2 activation, COX2 expression and PGE2 release. (a–c) Neuronal cultures from rat embryonic brain were treated with 50 μM L-homocysteine (L-Hcy) for the specified times. (a) Cell lysates with equal amount of protein from each sample was analyzed for cPLA2 activity using enzymatic assay, (b) Equal protein from each sample was analyzed by immunoblot analysis using anti-COX2 (upper panel) and β–tubulin (lower panel) antibodies. COX2 protein level in each sample was quantified using computer-assisted densitometry and Image J analysis. (c) Equal amounts of culture media from each

sample was analyzed for PGE2 levels using ELISA. Values are expressed as mean ± SD (number of independent cell culture experiments = 4–7). *$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$ from 0 h of homocysteine treatment.

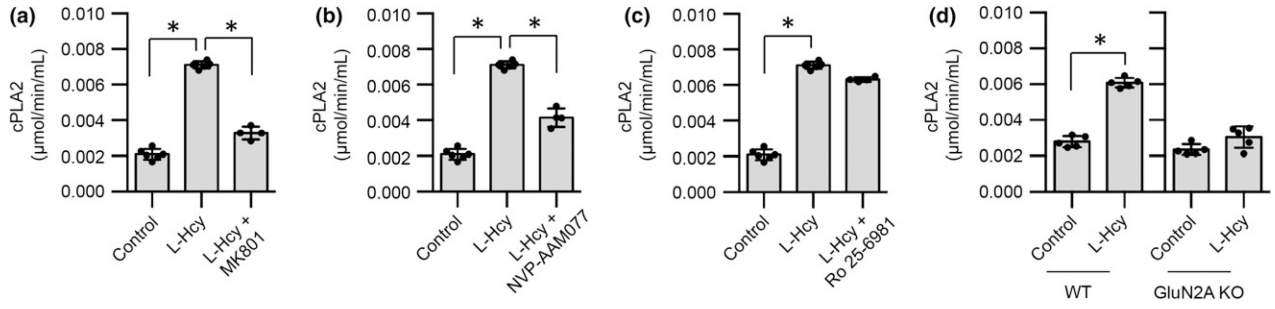

Fig. 2.

Role of GluN2A-NMDAR in homocysteine-induced cPLA2 activation in neurons. (a–c) Rat neuronal cultures were treated with 50 μM L-homocysteine (L-Hcy) for 4 h in the absence or presence of (a) MK801 (10 μM), (b) NVP-AAM077 (30 nM) or (c) Ro 25–6981 (1 μM). (d) WT and GluN2A-KO mice neuronal cultures were treated with 50 μM L-Hcy for 4 h. (a–d) Equal amounts of protein from each sample were analyzed for cPLA2 activity using enzymatic assay. Values are expressed as mean ± SD (number of independent cell culture experiments = 4–6). *$p < 0.0001$ from 4 h homocysteine treatment.

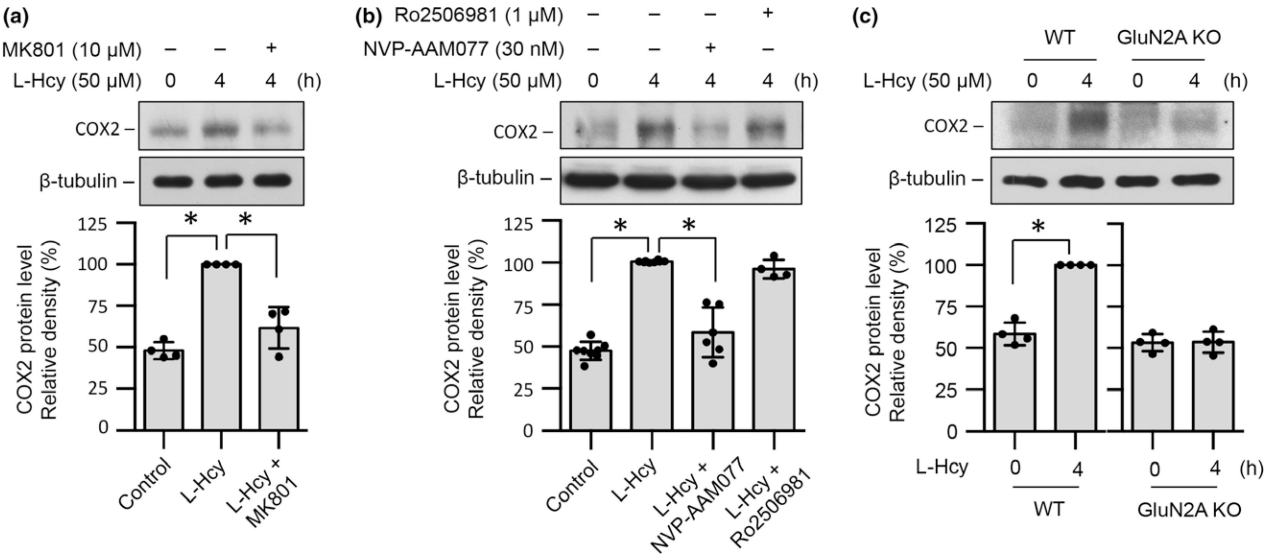

Fig. 3.

Role of GluN2A-NMDAR in homocysteine-mediated increase in COX2 protein level in neurons. (a and b) Rat neuronal cultures were treated with 50 μM L-homocysteine (L-Hcy) for 4 h in the absence or presence of (a) MK801 (10 μM) or (b) NVP-AAM077 (30 nM) and Ro 25–6981 (1 μM). (c) WT and GluN2A-KO mice neuronal cultures were treated with 50 μM L-Hcy for 4 h. (a–c) Cell lysates were analyzed by immunoblotting with anti-COX2 (upper panels) and β–tubulin (lower panels) antibodies. COX2 protein levels were quantified using computer-assisted densitometry and Image J analysis. Values are mean ± SD (number of independent cell culture experiments = 4–6). *$p < 0.001$ from 4 h homocysteine treatment.

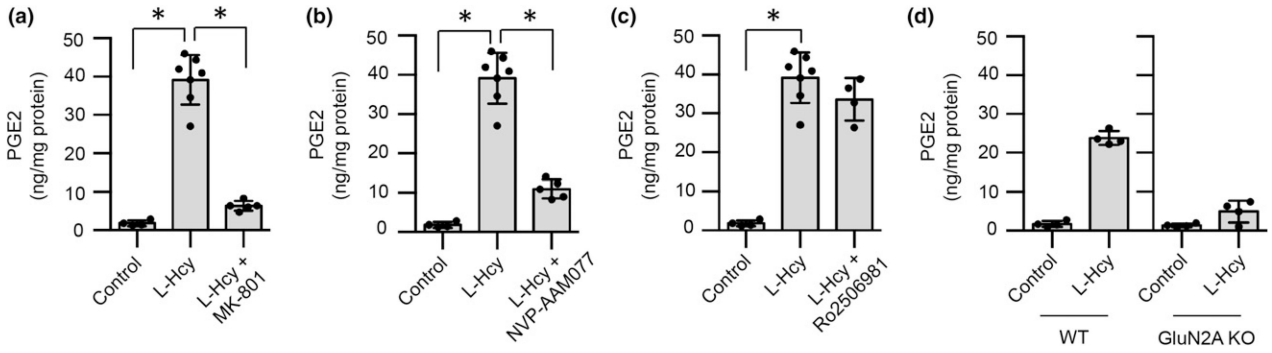

Fig. 4.

Role of GluN2A-NMDAR in homocysteine-induced PGE2 release from neurons. (a–c) Rat neuronal cultures were treated with 50 μM L-homocysteine (L-Hcy) for 4 h in the absence or presence of (a) DL-AP5 (200 μM), (b) NVP-AAM077 (30 nM) or (c) Ro2506981 (1 μM). (d) WT and GluN2A-KO mice neuronal cultures were treated with 50 μM L-Hcy for 4 h. (a–d) Equal amounts of culture media from each sample were analyzed for PGE2 levels using ELISA. Values are represented as mean ± SD (number of independent cell culture experiments = 4–7). *$p < 0.0001$ from 4 h homocysteine treatment.

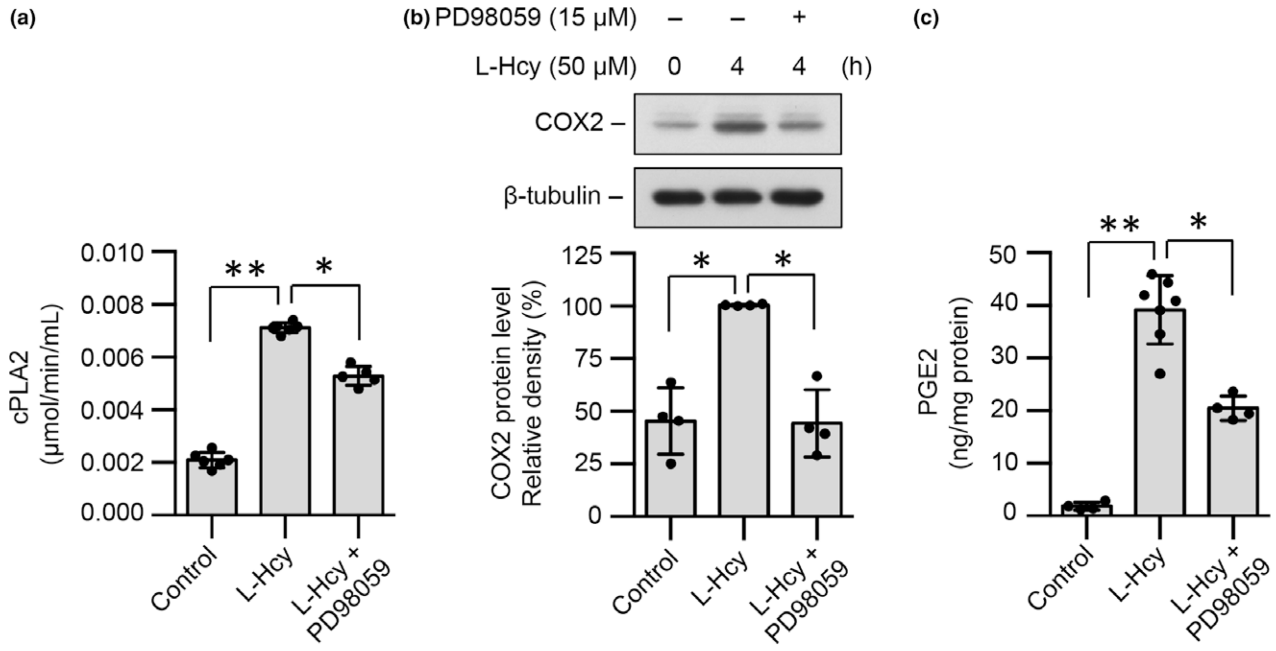

Fig. 5.

Role of extracellular regulated-kinase/mitogen-activated protein kinase in homocysteine-GluN2A-NMDAR-dependent activation of cPLA2-COX2-PGE2 pathway in neurons. (a–c) Rat neuronal cultures were treated with 50 μM L-homocysteine (L-Hcy) for 4 h in the absence or presence of PD98059 (15 μM). (a) Equal amounts of protein from cell lysates was analyzed for cPLA2 activity using enzymatic assay. (b) Immunoblot analysis of equal protein from neuronal lysates using anti-COX2 (upper panel) and β–tubulin (lower panel) antibodies. COX2 protein levels were quantified using computer-assisted densitometry and Image J analysis. (c) Equal amounts of culture media from each sample were analyzed for PGE2 levels using ELISA. Values are represented as mean ± SD (number of independent cell culture experiments = 4–7). *$p < 0.05$ and **$p < 0.0001$ from 4 h homocysteine treatment.

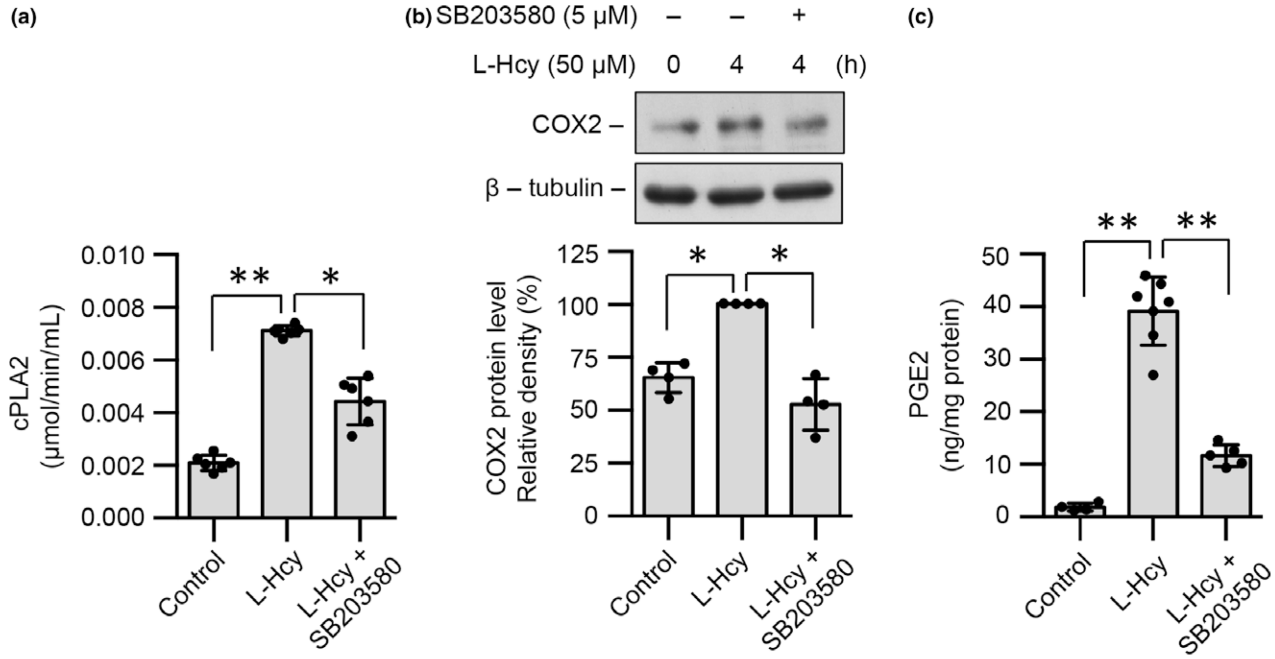

Fig. 6.

Role of p38 MAPK in homocysteine-GluN2A-NMDAR-dependent activation of cPLA2-COX2-PGE2 pathway in neurons. (a–c) Rat neuronal cultures were treated with 50 μM L-homocysteine (L-Hcy) for 4 h in the absence or presence of SB203580 (5 μM). (a) Equal amounts of protein from cell lysates was analyzed for cPLA2 activity using enzymatic assay. (b) Immunoblot analysis of equal protein from neuronal lysates using anti-COX2 (upper panel) and β–tubulin (lower panel) antibodies. COX2 protein levels were quantified using computer-assisted densitometry and Image J analysis. (c) Equal amounts of culture media from each sample were analyzed for PGE2 levels using ELISA. Values are represented as mean ± SD (number of independent cell culture experiments = 4–7). *$p < 0.05$ and **$p < 0.0001$ from 4 h homocysteine treatment.

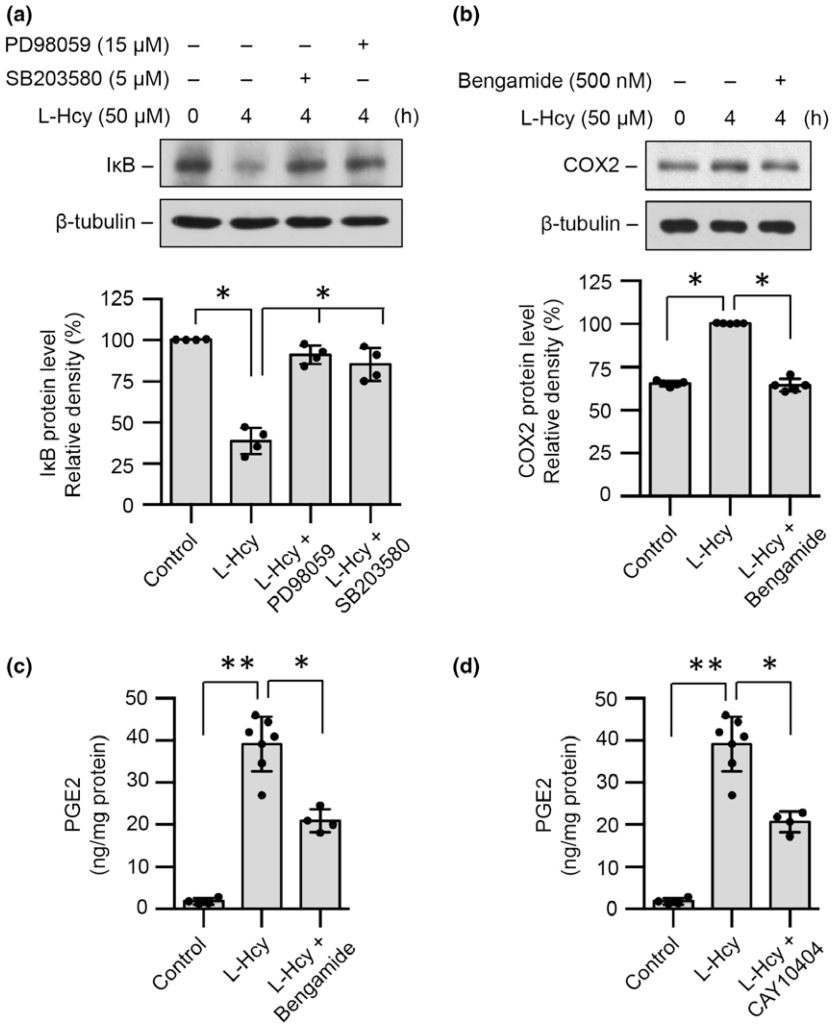

Fig. 7.

NFκB regulates ERK-p38 MAPK-dependent neuronal PGE2 release. (a–d) Rat neuronal cultures were treated with 50 μM L-homocysteine (L-Hcy) for 4 h in the absence or presence of (a) PD98059 (15 μM) or SB203580 (5 μM), (b and c) Bengamide B (500 nM), or (d) CAY10404 (100μM). (a and b) Immunoblot analysis of equal protein from neuronal lysates with (a) anti-IB (upper panel) and β–tubulin (lower panel) antibodies; or (b) anti-COX2 (upper panel) and β–tubulin (lower panel) antibodies. IB and COX2 protein levels were quantified using computer-assisted densitometry and Image J analysis. (c and d) Equal amounts of culture media from each sample were analyzed for PGE2 levels using ELISA. Values are represented as mean ± SD (number of independent cell culture experiments = 4–7). *$p < 0.001$ and **$p < 0.0001$ from 4 h homocysteine treatment.