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Role of AMPA receptors in homocysteine-NMDAR induced crosstalk between ERK and p38 MAP kinase

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Abstract

Homocysteine, a metabolite of the methionine cycle has been reported to play a role in neurotoxicity through activation of NMDAR-mediated signaling pathway. The proposed mechanisms associated with homocysteine-NMDAR induced neurotoxicity involve a unique signaling pathway that triggers a crosstalk between ERK and p38 MAPKs, where activation of p38 MAPK is downstream of and dependent on ERK MAPK. However, the molecular basis of the ERK MAPK mediated p38 MAPK activation is not understood. The current study investigates whether AMPARs play a role in facilitating the ERK MAPK mediated p38 MAPK activation. Using surface biotinylation and immunoblotting approaches we show that treatment with homocysteine leads to a decrease in surface expression of GluA2-AMPA subunit in neurons, but has no effect on the surface expression of GluA1-AMPA subunit. Inhibition of NMDAR activation with D-AP5 or ERK MAPK phosphorylation with PD98059 attenuates homocysteine-induced decrease in surface expression of GluA2-AMPA subunit. The decrease in surface expression of GluA2-AMPA subunit is associated with p38 MAPK phosphorylation, which is inhibited by NASPM, a selective antagonist of GluA2-lacking Ca²⁺-permeable AMPARs. These results suggest that homocysteine-NMDAR mediated ERK MAPK phosphorylation leads to a decrease in surface expression of GluA2-AMPA subunit resulting in Ca²⁺ influx through the GluA2-lacking Ca²⁺-permeable AMPARs and p38 MAPK phosphorylation. Cell death assays further show that inhibition of AMPAR activity with NBQX/CNQX or GluA2-lacking Ca²⁺-permeable AMPAR activity with NASPM attenuates homocysteine-induced neurotoxicity. We have identified an important mechanism involved in homocysteine-induced neurotoxicity that highlights the intermediary role of GluA2-lacking Ca²⁺-permeable AMPARs in the crosstalk between ERK and p38 MAPKs.

Graphical abstract

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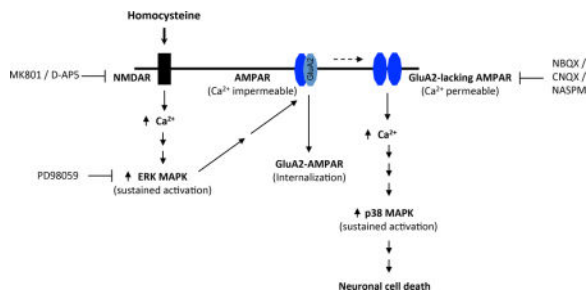
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Homocysteine-induced neurotoxicity involves NMDAR-mediated activation of a unique crosstalk between ERK and p38 MAPKs. We report here that ERK MAPK activation leads to decrease in GluA2-AMPA surface expression causing increased Ca^{2+} influx through GluA2-lacking Ca^{2+} -permeable AMPARs resulting in p38 MAPK phosphorylation. The findings reveal an important intermediary role of GluA2-lacking AMPARs in homocysteine-NMDAR mediated crosstalk between ERK and p38 MAPKs.



Keywords

Homocysteine; NMDA receptors; GluA2-AMPA receptors; ERK MAPK; p38 MAPK; neurotoxicity

INTRODUCTION

Hyperhomocysteinemia is a common metabolic disorder that is characterized by systemic elevation of homocysteine, a thiol containing amino acid formed as a metabolite of the methionine cycle. Hyperhomocysteinemia has been recognized as an independent risk factor for multiple neurodegenerative disorders (Obeid & Herrmann 2006, Seshadri *et al.* 2002, Sacco *et al.* 1998, Zoccollella *et al.* 2006, Miller 1999). It has been shown that elevated levels of homocysteine can induce neuronal injury and subsequent brain damage by stimulation of glutamate receptors (Duan *et al.* 2002, Kruman *et al.* 2002, Kruman *et al.* 2000). *In vitro* studies in cultured neurons further established the role of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors in mediating homocysteine-induced neuronal cell death (Lipton *et al.* 1997, Kruman *et al.* 2000, Kruman *et al.* 2002, Mattson & Shea 2003, Jara-Prado *et al.* 2003, Poddar & Paul 2009, Poddar & Paul 2013). However, the intracellular signaling pathways associated with homocysteine-NMDA receptor (NMDAR) induced neuronal cell death is still not well understood.

Earlier studies have shown that Ca^{2+} influx following NMDAR stimulation with glutamate is biphasic in nature (Paul & Connor 2010, Hyrc *et al.* 1997, Cheng *et al.* 1999). A rapid low level initial increase through NR2A-containing NMDARs is followed by a delayed and larger increase that is NR2B-NMDAR dependent. Prior studies have also shown that Ca^{2+} influx following NMDAR stimulation with homocysteine is substantially smaller than that observed with glutamate (Kruman *et al.* 2000), which raises the possibility that low level Ca^{2+} influx in response to homocysteine treatment is mediated through NR2A-containing NMDARs. Consistent with this view, our earlier findings demonstrated that homocysteine-induced NMDAR stimulation leads to sustained phosphorylation of the extracellular signal-

regulated kinase/mitogen activated protein kinase (ERK MAPK) and inhibition of NR2B-NMDARs with selective antagonists Ro-256981 or Ifenprodil failed to attenuate homocysteine-induced ERK MAPK phosphorylation. The study also showed that the active ERK MAPK plays a critical role homocysteine-NMDAR mediated neurotoxicity (Poddar & Paul 2009). These findings strongly indicate that homocysteine-NMDA receptor induced neurotoxicity involves a unique signaling pathway that requires stimulation of NR2A containing NMDARs. In a more recent study, we further demonstrated that homocysteine-mediated NMDAR stimulation leads to activation of the stress-activated kinase p38 MAPK, which is dependent on and downstream of ERK MAPK activity (Poddar & Paul 2013). Induction of p38 MAPK activation in turn leads to activation of caspase-3 resulting in neuronal apoptosis (Poddar & Paul 2013). The findings highlight a novel interplay between ERK and p38 MAPKs in response to homocysteine-mediated NMDAR stimulation and provide the first evidence that a crosstalk between the two MAPKs is essential for neuronal cell death.

The present study sought to determine the role of AMPA (α -Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors (AMPA) in the crosstalk between ERK and p38 MAPKs. Our findings show that activation of ERK MAPK in response to homocysteine treatment leads to decrease in surface expression of the GluA2-AMPA subunit. The removal of GluA2 subunit from cell surface is associated with increased influx of Ca^{2+} through the GluA2-lacking Ca^{2+} -permeable AMPARs resulting in p38 MAPK phosphorylation that causes neuronal cell death.

EXPERIMENTAL PROCEDURES

Materials and reagents

All reagents required for primary cortical neuron cultures were obtained from Invitrogen (Carlsbad, CA, USA). L-homocysteine thiolactone, cytosine D-arabinofuranoside, glycine and Hoechst 33342 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-ERK1/2 (Thr202/Tyr204) monoclonal antibody (p-ERK, RRID:AB_331768), anti-phospho-p38 (Thr180/Tyr182) polyclonal antibody (p-p38, RRID:AB_331762), anti-p38 polyclonal antibody (RRID:AB_330731), anti-GluA2 antibody (RRID:AB_2650557), anti-rabbit (RRID:AB_2099233) and anti-mouse (RRID:AB_330924) horse-radish-peroxidase conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-ERK2 polyclonal antibody (ERK, RRID:AB_2141292) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GluA1 antibody (RRID:AB_2113602) and anti-phospho-GluA2-Ser880 (p-GluA2, RRID:AB_880227) were obtained from Abcam (Cambridge, MA, USA). Anti-rabbit-Alexa 488-conjugated antibody (RRID:AB_143165) was obtained from Molecular Probes (Eugene, OR, USA) and anti-mouse-Cy3 conjugated antibody (RRID:AB_2338690) was purchased from Jackson ImmunoResearch laboratories (West Grove, PA USA). West Pico supersignal chemiluminescence reagents, EZ-link NHS-PEG4-Biotin and NeutrAvidin agarose was obtained from Pierce (Rockland, IL, USA). Complete protease inhibitor cocktail was obtained from Roche (Nutley, NJ, USA). Selective pharmacological inhibitors were obtained as follows: MK801 hydrogen maleate and 6-cyano-7-nitroquinoxaline-2, 3, -dione (CNQX)

from Sigma-Aldrich (St. Louis, MO, USA), 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide (NBQX) and 1-Naphthyl acetyl spermine trihydrochloride (NASPM) from Tocris, (Bristol, UK), D-(–)-2-Amino-5-phosphopentanoic acid (D-AP5) and PD98059 were obtained from EMD biosciences (Billerica, MA, USA). For establishing primary neuron cultures, Sprague-Dawley (RRID:RGD_737903) female pregnant rats (gestation day 15) were purchased from Harlan Laboratories (Livermore, CA, USA). Institutional Animal Care and Use Committee of University of New Mexico, Health Sciences Center approved all animal procedures used in the current study.

Neuron culture, L-Homocysteine preparation and stimulation

Primary cortical neuronal cultures were established from 16–17 day old rat embryos as described earlier (Poddar & Paul 2009, Poddar & Paul 2013), and the cells were maintained in culture for 12–14 days prior to experiment. L-homocysteine was prepared by alkali hydrolysis of L-homocysteine thiolactone hydrochloride followed by neutralization, and maintained in 0.02 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer pH 7.4 (Poddar *et al.* 2001). For receptor stimulation neurons were treated with freshly prepared 50 μ M of L-homocysteine in Hank's balanced salt solution (Poddar & Paul 2009, Poddar & Paul 2013) containing 50 μ M of glycine (Lipton *et al.* 1997). Cells were processed for immunoblotting and biotinylation studies after 4 h of homocysteine treatment or for cell death assay after 18 h of treatment. Initial studies evaluating the effect of tetrodotoxin (0.5 μ M) showed that L-homocysteine induced changes in ERK and p38 MAPK phosphorylation were insensitive to tetrodotoxin treatment (data not shown). For some studies, selective antagonists D-AP5, MK801, NBQX, CNQX, NASPM or PD98059 were added either 30 min or 3.5 h after the onset of L-homocysteine treatment (Poddar & Paul 2013) to assess the effect of both long-term and short-term inhibition of NMDAR, AMPAR and GluA2-lacking Ca²⁺ permeable AMPAR activity as well as ERK MAPK phosphorylation on GluA2-AMPA subunit surface expression as well as ERK and p38 MAPK phosphorylation. Cell lysates were analyzed by immunoblotting, biotinylation or cell death assay as specified in each experiment.

Subcellular fractionation

Subcellular fractionation of cultured neurons, treated with L-homocysteine (4 h) was performed as described previously (Dunah & Standaert 2001) with minor modifications. Briefly, neurons were washed twice with ice-cold TEVP buffer (10 mM Tris-HCl, 5 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, and 1 mM EGTA, pH 7.4) following treatment with homocysteine and then homogenized on ice in TEVP buffer containing 320 mM sucrose. The cell homogenate was centrifuged at 4°C for 10 min at 800 × g to remove the nuclei and large debris. The supernatant (S1) was collected into a separate tube and further centrifuged at 4°C for 15 min at 9,200 × g to obtain a pellet that was comprised of the crude synaptosomal fraction (P2). The resulting supernatant (S2) was removed and centrifuged again at 4°C for 2 h at 1,65,000 × g to obtain the final cytosolic fraction (S3). The pellet obtained (P3) consisted of the light membrane/microsome-enriched fraction. At each step, small amounts of the supernatants (S1, S2 and S3) were removed for immunoblot analysis.

To avoid possible crossover contamination, all the pellets (P1, P2 and P3) were rinsed once with cold TEVP buffer before immunoblot analysis.

Surface Biotinylation assay

Neurons were treated with L-homocysteine for 4 h in the presence or absence of pharmacological inhibitors as specified in each experiment. The inhibitors were added either 30 min or 3.5 h after the onset of homocysteine treatment. At the end of the treatment the cells were rinsed twice in ice-cold phosphate-buffered saline, pH 7.4 (PBS). Cell surface protein biotinylation and isolation of biotinylated proteins were performed using EZ-link NHS-PEG4-Biotin according to manufacturer's protocol. Briefly, neurons were incubated on ice with PBS containing 500 µg/ml EZ-link NHS-PEG4-Biotin for 20 min. Following incubation, the unreacted biotinylation reagent was removed by washing the cells three times with an ice-cold buffer containing 20 mM Tris-HCl and 150 mM NaCl (pH 7.4). Cells were then harvested with lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton-X 100, 0.5% Sodium deoxycholate, 50 mM NaF; 10 mM Na₄P₂O₇; 1 mM Na₃VO₄ and complete protease inhibitor cocktail. Homogenates were centrifuged at 14,000 × g for 15 min at 4°C. Equal amounts of protein from the resulting supernatants were incubated overnight with NeutrAvidin agarose (50 µL) to isolate the biotinylated proteins. The purified biotinylated proteins were analyzed by immunoblotting as specified in each experiment. The amount of total protein of interest present in each of the input lysate was determined by immunoblot analysis of equal proteins from the input lysates.

Immunoblotting

Equal protein from total cell lysates and subcellular fractions as well as the purified biotinylated proteins were resolved in 8% SDS-PAGE, and subjected to immunoblot analysis with antibodies as described in each experiment. All primary antibodies (anti-p-ERK, -ERK, -p-p38, -p38, -GluA1, -GluA2 and -p-GluA2-ser880) and secondary-horse radish peroxidase conjugated antibody concentrations were used according to recommendations made by the manufacturer. Signals from immune complexes were developed using West Pico supersignal chemiluminescence reagents and then captured on X-ray films. Densitometric analysis of the images was performed using the Image J software.

Cell death assay

Neuronal cultures grown on 2-well culture slides were treated with L-homocysteine in the presence or absence of selective inhibitors for 18 h. Cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) and stained with Hoechst 33342 dye as previously described (Poddar & Paul 2009). The percentage of pyknotic cells was assessed using fluorescent microscopy after counting a total of 1,500 nuclei for each set of experiments (n = 4).

Statistical analysis

Statistical analysis and comparison was performed using One-way analysis of variance (ANOVA, Bonferroni's multiple comparison test) and differences were considered significant when $p < 0.05$.

RESULTS

Differential regulation of ERK and p38 MAPK by AMPAR

Our previous studies showed that homocysteine-NMDAR induced phosphorylation of both ERK and p38 MAPK in neurons follows a two-tier pattern (Poddar & Paul 2013). A rapid initial increase by 2.5 min is followed by a delayed larger increase by 4 h and the delayed increase in ERK and p38 MAPKs phosphorylation is primarily responsible for homocysteine-NMDAR dependent neurotoxicity. To examine the role of AMPARs in regulating the delayed increase in the phosphorylation of ERK and p38 MAPKs, neuronal cultures were treated with L-homocysteine (50 μ M) for 4 h in the presence or absence of NMDA or AMPAR antagonists. The NMDAR antagonists, D-AP5 (100 μ M) and MK801 (5 μ M) or the AMPAR antagonists, NBQX (10 μ M) and CNQX (50 μ M) were added either 30 min (long-term inhibition) or 3.5 h (short-term inhibition) after the onset of homocysteine treatment. Immunoblot analysis was performed with anti-p-ERK or -p-p38 MAPK antibody. As previously reported (Poddar & Paul 2013), phosphorylation of both ERK and p38 MAPKs increased significantly following treatment with homocysteine (Fig. 1A–D). Application of D-AP5 or MK801, either 30 min or 3.5 h after the onset of homocysteine treatment blocked homocysteine-induced phosphorylation of both ERK MAPK (Fig. 1A) and p38 MAPK (Fig. 1B). However, treatment with NBQX or CNQX had no effect on ERK MAPK phosphorylation (Fig. 1C), regardless of the time of application of the inhibitors, but they effectively blocked p38 MAPK phosphorylation (Fig. 1D). This indicates a role of AMPARs in regulating homocysteine-induced p38 MAPK phosphorylation. The findings also raise the possibility that AMPAR activity play an intermediary role in regulating the cross talk between ERK MAPK and p38 MAPK.

To examine the role of AMPARs in homocysteine-induced neuronal cell death, neurons were incubated with L-homocysteine (50 μ M) in the absence or presence of NBQX (10 μ M) or CNQX (50 μ M) and cell viability was assessed 18 h later by Hoechst DNA staining (Fig. 2). As previously demonstrated (Poddar & Paul 2009, Poddar & Paul 2013), treatment with L-homocysteine alone significantly increased neuronal cell death (17.39% \pm 1.35 for control vs. 44.65% \pm 1.6 for homocysteine). Application of NBQX or CNQX during homocysteine treatment led to a significant reduction in neuronal cell death (20.82% \pm 1.29 for NBQX and 18.98% \pm 1.81 for CNQX), confirming a role of AMPARs in homocysteine-induced neuronal cell death.

Homocysteine reduces cell surface expression of GluA2-AMPA subunit

AMPA receptors at excitatory synapses in the forebrain consist predominantly of GluA1 and GluA2 subunits with low levels of GluA3 and GluA4, and alteration in surface expression of any subunit can dramatically alter AMPAR activity and function (Sprengel 2006, Malinow & Malenka 2002, Brecht & Nicoll 2003, Geiger *et al.* 1995, Sans *et al.* 2003, Tsuzuki *et al.* 2001). To explore the possible role of homocysteine in altering cell surface expression of GluA1 and GluA2 AMPAR subunits, neurons were treated with or without L-homocysteine (50 μ M) for 4 h followed by biotin labeling of cell surface proteins and affinity purification of the biotinylated proteins. Immunoblot analysis of the purified biotinylated proteins, using GluA1 and GluA2 antibodies showed that L-homocysteine treatment had no effect on the

cell surface expression of GluA1-AMPA subunit (Fig. 3A, upper panel). However, the cell surface expression of GluA2-AMPA subunit decreased significantly in the presence of L-homocysteine (Fig. 3B, upper panel). Immunoblot analysis of the total GluA1 and GluA2 protein levels in the input lysate showed no alteration in the expression of either GluA1 or GluA2 with homocysteine treatment (Fig. 3A, B, lower panel). Taken together, these findings indicate that L-homocysteine plays a role in the loss of surface expression GluA2-AMPA subunit. Such decreases in GluA2 subunit are known to increase AMPAR Ca^{2+} permeability and has profound consequences on intracellular signaling (Hanley 2014).

Earlier studies have provided strong evidence that loss of surface expression of GluA2-AMPA subunit is associated with phosphorylation of serine880 (ser880) at the C-terminus of GluA2 (Perez *et al.* 2001, Matsuda *et al.* 1999, Chung *et al.* 2000, Chung *et al.* 2003, Iwakura *et al.* 2001, Kim *et al.* 2001, Xia *et al.* 2000, Lin & Huganir 2007). These findings also indicate that phosphorylation of ser880 facilitates the internalization of GluA2 as well as inhibits the recycling of GluA2, thus retaining GluA2 intracellularly. To evaluate whether the decrease in surface expression of GluA2 following exposure to L-homocysteine also involves phosphorylation of ser880, in subsequent experiments neurons treated with L-homocysteine (50 μM , 4 h) were processed for immunoblot analysis with anti-p-ser880 antibody. Consistent with the earlier findings, a significant increase in the phosphorylation of GluA2-AMPA subunit at Ser880 was observed following treatment with L-homocysteine (Figure 3C), suggesting a causal link between the loss of surface expression of GluA2-AMPA subunit and its phosphorylation.

Role of NMDARs in homocysteine-induced surface expression of GluA2-AMPA subunit

To determine the role of NMDARs in the homocysteine-mediated decrease in surface expression of GluA2-AMPA subunit, neurons were treated with L-homocysteine (50 μM , 4 h) in the absence or presence of D-AP5 (100 μM) added either 30 min or 3.5 h after the onset of homocysteine treatment. Surface expression of GluA2-AMPA subunit was evaluated by immunoblot analysis following biotinylation and purification of cell surface proteins. Figure 4A (upper panel) shows that application of the inhibitor either 30 min or 3.5h after the onset of homocysteine treatment attenuated homocysteine-dependent loss in surface expression of GluA2-AMPA subunit. Analysis of the GluA2 total protein levels in the input lysates shows that the expression level of GluA2 did not alter with NMDAR inhibition. To examine the effect of NMDAR inhibition on phosphorylation of GluA2-AMPA subunit at ser880, neurons were treated with L-homocysteine (50 μM , 4 h) in the presence of D-AP5, added either 30 min or 3.5h after the onset of L-homocysteine treatment. Immunoblot analysis (Fig. 4B) shows that the homocysteine-induced phosphorylation of GluA2 at ser880 was inhibited in the presence of D-AP5, irrespective of the time of its application. The findings indicate a role of NMDARs in homocysteine-induced loss of surface expression GluA2-AMPA subunit and its phosphorylation at Ser880.

Role of ERK MAPK in regulating homocysteine-induced surface expression of GluA2-AMPA subunit

To test the possibility that homocysteine-NMDAR mediated phosphorylation of ERK MAPK plays a role in GluA2-AMPA subunit internalization, initial studies examined the intra-cellular localization of phosphorylated ERK MAPK. Neurons were treated with L-homocysteine (50 μ M) for 30 min or 4 h and then analyzed by immunocytochemistry using p-ERK (red) and ERK (green) antibodies. Figure 5A shows that within 30 min of homocysteine exposure, phosphorylated ERK MAPK was predominantly localized in the extranuclear region, where it remained sequestered for at least 4 h after the onset of treatment. To examine the subcellular localization of the extra-nuclear phosphorylated ERK MAPK, neurons treated with L-homocysteine (50 μ M, 4 h) were subjected to biochemical fractionation (see Methods) and immunoblot analysis. Figure 5B (upper panel) shows that in the homocysteine treated samples, the supernatant (S1 fraction), obtained following the removal of nuclear material was highly enriched in phosphorylated ERK MAPK. Figure 5C shows that in the homocysteine treated samples, phosphorylated ERK MAPK was present in both the supernatant (S2 fraction) and the crude synaptosomal pellet (P2) obtained from the S1 fraction, although it was present at higher levels in the S2 fraction. High-speed centrifugation of the S2 fraction resulted in the separation of the cytoplasmic fraction (S3) and the light membrane/microsomal enriched fraction (P3). Immunoblot analysis of the S3 and P3 fractions showed that phosphorylated ERK MAPK was highly enriched in the cytoplasmic fraction (Fig. 5C, upper panel). Analysis of marker proteins for each sub-cellular compartment (Fig. 5C, panels 3 and 4) further confirmed that synaptophysin, a synaptic vesicle membrane protein was highly enriched in the crude synaptosomal fraction (P2), while calnexin, an integral membrane protein found in endoplasmic reticulum was highly enriched in the light membrane fraction (P3).

The sub-cellular localization of phosphorylated ERK MAPK in the synaptosomal fraction (P2) suggests that ERK MAPK signaling could play a role in regulating homocysteine-dependent loss of surface expression of GluA2-AMPA subunit. To test this possibility in subsequent studies, neurons were treated with L-homocysteine (50 μ M, 4 h) in the absence or presence of PD98059 (15 μ M), a pharmacological inhibitor of ERK MAPK. The inhibitor was added either 30 min or 3.5h after the onset of homocysteine treatment. Cells were then processed for biotinylation and purification of surface proteins followed by immunoblot analysis with anti-GluA2 antibody. As shown in Figure 6A (upper panel), PD98059 attenuated homocysteine-induced loss in surface expression of GluA2-AMPA subunit, irrespective of the time of application of the inhibitor. Total level of GluA2 remained unaltered by this treatment (Fig. 6A, lower panel). Figure 6B shows that inhibition of ERK MAPK with PD98059, added either 30 min or 3.5h after the onset of homocysteine treatment, blocked the phosphorylation of ser880 in GluA2-AMPA subunit. In addition, homocysteine-induced phosphorylation of p38 MAPK was also blocked following both long-term (3.5h) and short-term (30 min) inhibition of ERK MAPK (Fig. 6C), which is consistent with earlier findings (Poddar & Paul 2013). Thus, it appears that phosphorylated ERK MAPK regulates both the surface expression of GluA2-subunit of AMPARs and phosphorylation of p38 MAPK.

Inhibition of GluA2-lacking AMPAR activity reduces homocysteine-induced p38 MAPK phosphorylation and neuronal cell death

Our earlier studies have shown that homocysteine-induced p38 MAPK phosphorylation and subsequent neuronal cell death are Ca^{2+} dependent (Poddar & Paul 2013). Since AMPARs lacking GluA2 subunit are Ca^{2+} -permeable (Hollmann *et al.* 1991, Burnashev *et al.* 1992, Geiger *et al.* 1995, Swanson *et al.* 1997), it raises the possibility that Ca^{2+} influx through GluA2-lacking AMPARs may play a role in p38 MAPK phosphorylation. To examine the contribution of GluA2 lacking AMPAR activity in facilitating homocysteine-mediated p38 MAPK phosphorylation, neurons were treated with L-homocysteine (50 μM , 4h) in the absence or presence of NASPM (100 μM), a selective antagonist of GluA2-lacking Ca^{2+} permeable AMPARs (Koike *et al.* 1997, Russell *et al.* 2016, Gerace *et al.* 2014, Oguro *et al.* 1999, Dong *et al.* 2015, Conrad *et al.* 2008, Noh *et al.* 2005). The inhibitor was added either 30 min or 3.5 h after the onset of homocysteine treatment. Immunoblot analysis with anti-p-p38 antibody shows that both long-term (3.5h) and short-term (30 min) inhibition of GluA2-lacking Ca^{2+} -permeable AMPAR activity with NASPM blocked homocysteine-induced phosphorylation of p38 MAPK (Fig 7A). However, homocysteine-mediated phosphorylation of ERK MAPK remained unaltered in the presence of NASPM, irrespective of the time of its application (Fig 7B). To further clarify the role of the GluA2 lacking Ca^{2+} -permeable AMPARs in homocysteine-mediated neuronal cell death, neurons were treated with homocysteine in the absence or presence of NASPM for 18 h and analyzed by Hoechst DNA staining. Figure 7C shows that treatment with NASPM significantly reduced homocysteine-induced neuronal cell death ($44.65\% \pm 1.6$ for homocysteine vs. $23.07\% \pm 2.45$ for NASPM). The findings establish the role of Ca^{2+} permeable AMPAR activity in homocysteine-induced p38 MAPK phosphorylation and subsequent cell death.

DISCUSSION

The key finding of the current study is that active ERK MAPK, localized in the extranuclear compartments plays a role in homocysteine-NMDAR dependent decrease in surface expression of the GluA2-AMPA subunit. Ca^{2+} influx through GluA2-lacking, Ca^{2+} -permeable AMPARs lead to phosphorylation and activation of p38 MAPK resulting in neuronal cell death. The findings demonstrate for the first time that AMPAR activity plays a role in homocysteine-induced neurotoxicity by facilitating the crosstalk between ERK and p38 MAPKs.

AMPA receptors are the principal mediators of fast synaptic neurotransmission at majority of the excitatory synapses in the central nervous system (Dingledine *et al.* 1999, Traynelis *et al.* 2010). AMPARs are tetrameric assemblies of GluA1-GluA4 subunits, and the vast majority of the AMPARs at the adult forebrain synapses are heterotetramers of GluA2 and another subunit (Dingledine *et al.* 1999, Tsuzuki *et al.* 2001, Geiger *et al.* 1995, Loschmann *et al.* 2004). Majority of GluA2 subunit in the adult brain contains an arginine residue in the pore lining membrane domain at position 607 in place of the genomically encoded glutamine, owing to RNA editing, which renders functional AMPARs impermeable to Ca^{2+} (Sommer *et al.* 1991, Hume *et al.* 1991, Hollmann & Heinemann 1994, Man 2011). In the adult brain, both transient and prolonged alteration in the level of GluA2-AMPA at the synapses in

response to neuronal activity modulates Ca^{2+} entry into neurons and has profound implications on neuronal communication, long-term potentiation and homeostatic plasticity (Greger & Esteban 2007, Beattie *et al.* 2000, Hollmann *et al.* 1991, Sutton *et al.* 2006, Man 2011, Hou *et al.* 2008). AMPAR subunit profile also changes in some brain areas during the critical period of postnatal development with the recruitment of GluA2-lacking Ca^{2+} -permeable AMPARs (Stubblefield & Benke 2010, Kumar *et al.* 2002, Henley & Wilkinson 2016, Pellegrini-Giampietro *et al.* 1992a), a process that is thought to be important for glutamatergic synapse maturation and experience-dependent strengthening of neural connectivity (Isaac *et al.* 2007, Liu & Savtchouk 2012, Liu & Cull-Candy 2000, Ho *et al.* 2007, Takahashi *et al.* 2003, Shi *et al.* 1999, Louros *et al.* 2014). However, such increase in GluA2-lacking Ca^{2+} -permeable AMPARs during development may also increase the vulnerability of the immature brain to hypoxia-induced injury and seizure (Talos *et al.* 2006a, Talos *et al.* 2006b, Hagberg *et al.* 1994, Koh *et al.* 2004). As such, neurons tightly regulate the function of this subunit through a number of processes at the level of gene expression, RNA editing, recycling and trafficking to ensure normal brain function (Hou *et al.* 2008, Tanaka *et al.* 2000).

In addition to its role in synaptic plasticity and adaptation during development and in the adult brain GluA2-lacking, Ca^{2+} permeable AMPARs also appears to be involved in neurological disorders and diseases associated with excitotoxicity (Liu & Zukin 2007, Pellegrini-Giampietro *et al.* 1997). Considerable evidence indicates that delayed neurodegeneration following global ischemia and limbic seizures is triggered by enhanced AMPAR-elicited Ca^{2+} influx in vulnerable hippocampal neurons (Gorter *et al.* 1997, Opitz *et al.* 2000, Pellegrini-Giampietro *et al.* 1992b, Noh *et al.* 2005, Friedman *et al.* 1994, Huang *et al.* 2002, Pollard *et al.* 1993). The Ca^{2+} permeability of AMPAR under these pathological conditions involves down regulation of GluA2 mRNA and subunit expression resulting in a long lasting switch from GluA2-containing to GluA2-lacking functional AMPARs (Grooms *et al.* 2000, Park *et al.* 2009). Studies using cultured neurons exposed to glutamate or subjected to oxygen glucose deprivation further showed that internalization of GluA2 subunit along with increased trafficking of GluA2-lacking AMPAR to the plasma membrane could lead to enhanced Ca^{2+} accumulation resulting in excitotoxic cell death (Bose *et al.* 2012, Vikman *et al.* 2008). Additional studies in the hippocampus demonstrated that expression of long-term depression also involves internalization of GluA2-subunit of AMPARs, which is triggered by NMDAR activation (Beattie *et al.* 2000). NMDAR activation has also been associated with increased prevalence of GluA2-lacking AMPARs in the hypothalamus under hypertensive conditions and is involved in neuronal hyperactivity (Li *et al.* 2012). Persistent inflammation and nerve injury also increases GluA2-lacking AMPAR prevalence in the spinal dorsal horn and contributes to nociceptive hypersensitivity and neuropathic pain (Galan *et al.* 2004, Li *et al.* 2012, Vikman *et al.* 2008, Park *et al.* 2009). Such increased prevalence of GluA2-lacking AMPARs in the spinal cord is also associated with NMDAR activation. Consistent with these earlier findings, the present study now demonstrates that homocysteine-induced signaling through NMDAR is critical in the phenotypic switch from predominantly GluA2-containing to GluA2-lacking AMPARs in neurons and ERK MAPK plays an intermediary role in this phenomenon. The causal link between the decrease in surface expression of GluA2-AMPA subunit and neuronal cell

death suggests that Ca²⁺-influx through the GluA2-lacking, Ca²⁺-permeable AMPARs contributes to homocysteine-induced neurotoxicity. The ability of NASPM, which selectively inhibits GluA2-lacking AMPAR activity, to attenuate homocysteine-induced cell death further confirms the role of these receptors in mediating the neurotoxic effects of homocysteine.

The intracellular mechanisms associated with activity dependent modulation of AMPAR trafficking are still poorly understood. Multiple studies have examined the role of Ras and Ras-related protein (Rap), two members of the Ras superfamily of GTPases in AMPAR dependent synaptic plasticity. The findings showed that Ras mediates activity-induced synaptic enhancement by facilitating synaptic insertion of GluA1 or GluA4 subunits, while Rap mediates activity induced synaptic depression by removing synaptic GluA2 or GluA3 subunits (Zhu *et al.* 2002). The downstream molecule(s) that relay Ras and Rap signaling includes ERK MAPK and p38 MAPK. While ERK MAPK activation is involved in synaptic insertion of AMPAR subunits, p38 MAPK is thought to play a role in removal of AMPARs from the synapses (Zhu *et al.* 2002, Iwakura *et al.* 2001, Derkach *et al.* 2007). A key finding of our study is that homocysteine-NMDAR mediated sustained activation and extranuclear retention of the active ERK MAPK promotes removal of surface GluA2-AMPA subunits, resulting in the prevalence of GluA2-lacking Ca²⁺-permeable AMPARs that facilitates Ca²⁺ influx and cell death. Such sequestration of active ERK MAPK in extranuclear compartments has also been reported to be a mediator of gamma-interferon induced cell death where cytosolic ERK promotes the pro-apoptotic function of the Death associated protein kinase by phosphorylation (Xia *et al.* 2000). The opposing role of ERK MAPK on AMPAR trafficking under physiological and pathological conditions suggest that depending on the nature of the upstream signaling, the activation of ERK MAPK could trigger a unique sequence of events causing differential regulation of AMPAR subunits. Identification of the downstream effectors of ERK MAPK in future studies may further our understanding of the function of ERK MAPK in regulating AMPAR activity in neurological disorders.

Although the importance of Ca²⁺-signaling through GluA2 lacking, Ca²⁺-permeable AMPARs is well recognized (Hollmann *et al.* 1991, Burnashev *et al.* 1992, Geiger *et al.* 1995, Swanson *et al.* 1997), relatively little is known about its downstream signaling targets. Our results now show that homocysteine dependent decrease in cell surface expression of GluA2-AMPA subunit is associated with p38 MAPK phosphorylation, suggesting that Ca²⁺ entry through GluA2-lacking Ca²⁺-permeable AMPARs triggers the activation of a signaling cascade necessary for p38 MAPK phosphorylation. Consistent with this interpretation, selective inhibition of GluA2-lacking AMPAR activity with NASPM blocks the homocysteine-dependent p38 MAPK phosphorylation. Whether such phosphorylation of p38 MAPK in the presence of GluA2-lacking AMPAR, as observed in our study, is unique to the homocysteine signaling pathway or could be triggered by stimulating GluA2-lacking AMPARs through other excitatory signals remains to be investigated.

In conclusion, the present study provides mechanistic insight into the signaling cascade underlying homocysteine-NMDAR mediated neurotoxicity. The findings highlight the role of ERK MAPK in regulating homocysteine-NMDAR mediated trafficking of GluA2-AMPA subunit. In addition, the study also provides evidence for a role of GluA2 lacking

Ca²⁺-permeable AMPARs in the crosstalk between ERK and p38 MAPKs. These findings demonstrating the effect of homocysteine in an *ex vivo* neuron culture model provides the molecular basis for evaluating the mechanisms identified here in animal models of hyperhomocysteinemia. Based on the findings subsequent studies will assess whether this unique signaling pathway triggered by homocysteine acts in conjunction with other deleterious cascades to enhance the pathology of neurological disorders under hyperhomocysteinemic conditions.

Acknowledgments

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Abbreviations

AMPAR	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
NMDAR	N-methyl-D-aspartate receptors
ERK MAPK	Extracellular-signal regulated kinase/mitogen activated protein kinase

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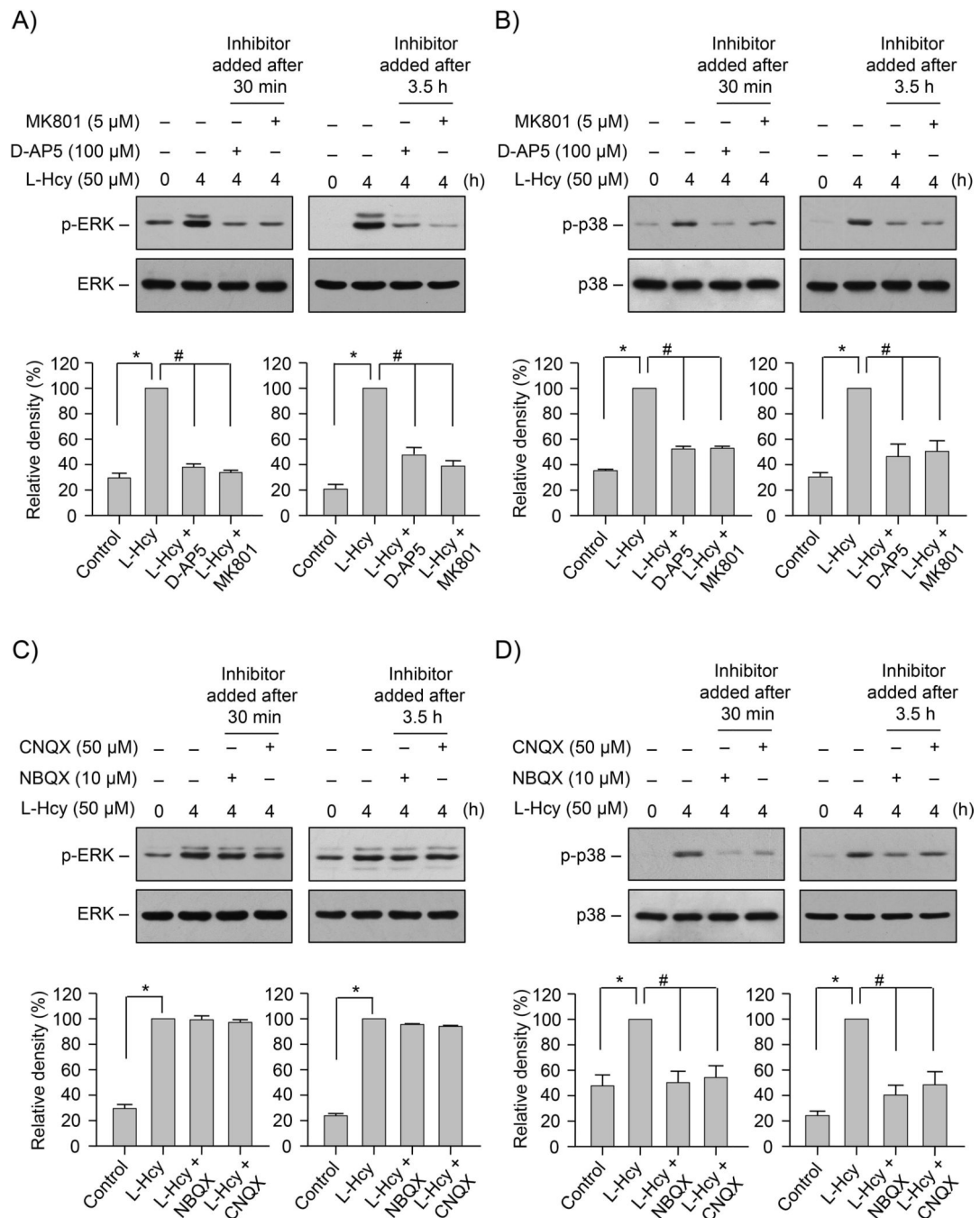


FIGURE 1. AMPAR activity regulates homocysteine-induced p38 MAPK phosphorylation and neuronal cell death

(A – D) Neuronal cultures were treated with 50 μ M L-homocysteine (L-Hcy) for 4 h. (A, B) NMDAR antagonist D-AP5 (100 μ M) and MK801 (5 μ M) or (C, D) AMPAR antagonist NBQX (10 μ M) and CNQX (50 μ M) were added 30 min or 3.5 h after the onset of L-homocysteine treatment. Extracts with equal amounts of protein from each sample were analyzed with (A, C) anti-phospho-pERK1/2 (p-ERK) or anti-ERK antibody; and (B, D) anti-phospho-p38 (p-p38) or anti-p38 antibody. (A-D) Phosphorylated ERK and p38 MAPKs were quantified using computer-assisted densitometry and Image J analysis. Bar

diagrams represent mean \pm SEM (n = 4). *p < 0.001 from 0 min time point or control; #p < 0.001 from 4 h L-homocysteine treatment.

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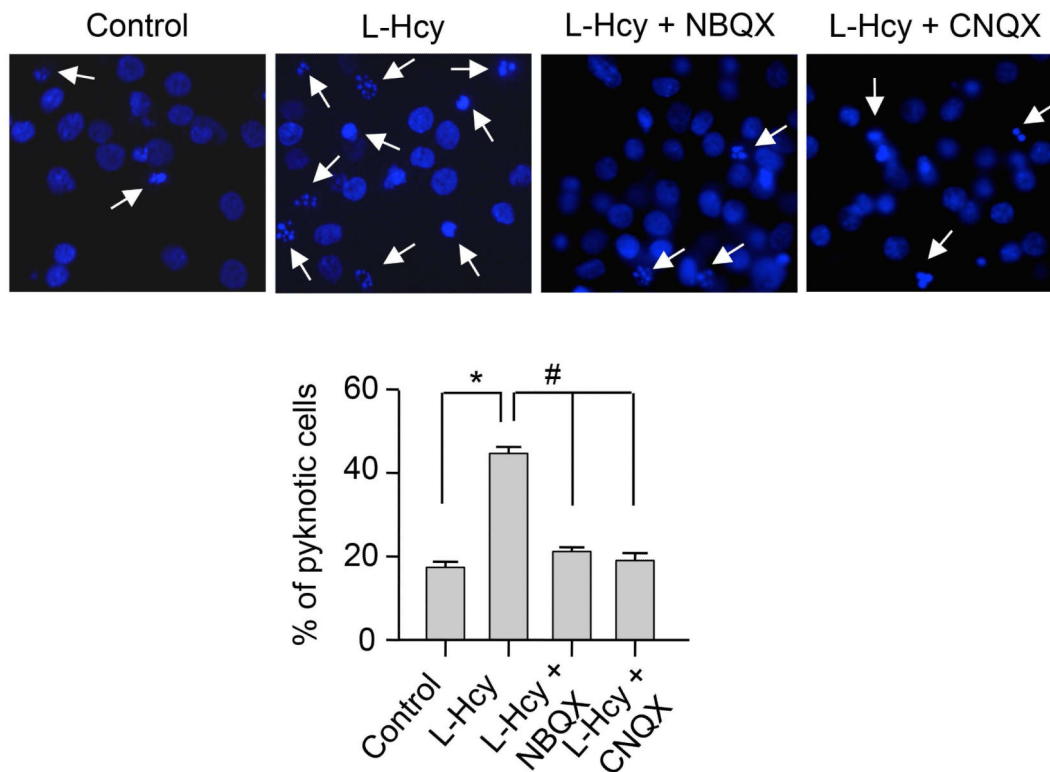


FIGURE 2. Role of AMPAR activity in homocysteine-induced neuronal cell death
 Neuronal cultures were treated with L-homocysteine (50 μ M, 18 h) in the absence or presence of NBQX (10 μ M) or CNQX (50 μ M) that were added 30 min after the onset of L-homocysteine treatment and then stained with Hoechst 33342, 18 h later. Representative photomicrographs show pyknotic DNA stained with Hoechst 33342 (indicated with arrows). Percentage of neurons with pyknotic nuclei is represented as mean \pm SEM (n = 1500 cells/condition from 4 experiments). *p < 0.001 from control; #p < 0.05 from 18 h homocysteine treatment.

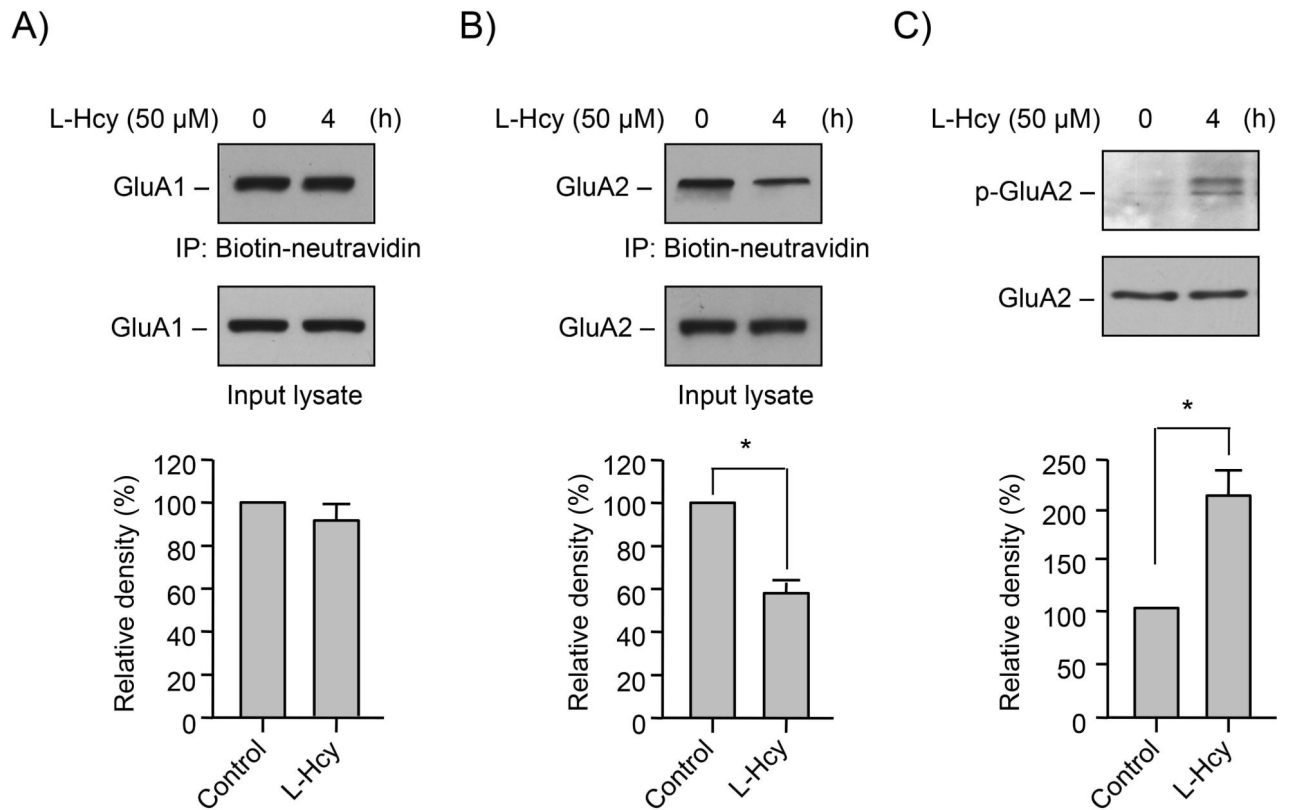


FIGURE 3. Homocysteine regulates surface expression of GluA2-AMPA subunit
 (A–C) Neuron cultures were treated with 50 μ M of L-homocysteine (L-Hcy) for 4 h. (A, B) L-homocysteine treated cells were subjected to biotinylation of surface proteins followed by immunoblot analysis of the purified biotinylated proteins using (A) anti-GluA1 (upper panel) or (B) anti-GluA2 (upper panel) antibody. Equal amounts of protein from total cell extracts (input lysate) were also analyzed by immunoblotting using (A) anti-GluA1 (lower panel) or (B) anti-GluA2 (lower panel) antibody. (C) An equal amount of protein from total cell lysates was assessed for GluA2 phosphorylation at Ser880 by immunoblot analysis using anti-phospho-GluA2-S880 antibody (p-GluA2; upper panel). Total GluA2 was also analyzed to indicate equal protein loading (lower panel). (A–C) Quantitative analysis of GluA1 and GluA2 surface expression as well as GluA2 phosphorylation at Ser880 is represented as mean \pm SEM (n = 4). *p < 0.001 from untreated control.

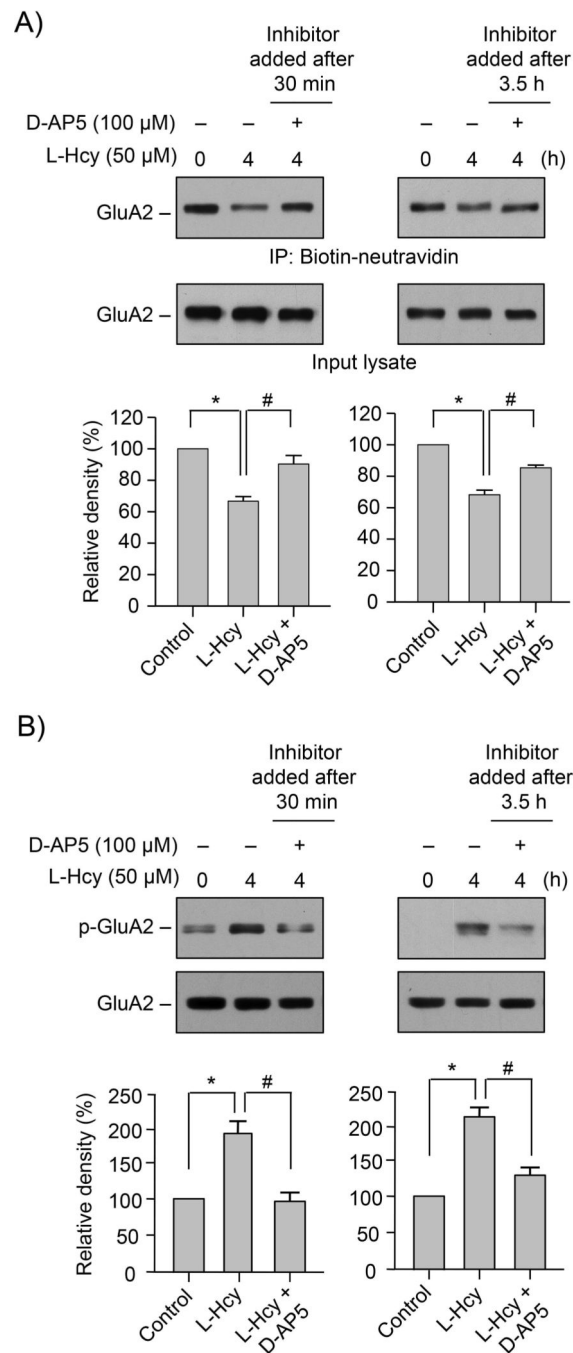


FIGURE 4. Homocysteine-induced alteration in GluA2-AMPA surface expression is regulated by NMDAR

(A, B) Neuron cultures were exposed to 50 μ M of L-homocysteine (L-Hcy) for 4 h and D-AP5 was added either 30 min or 3.5 h after the onset of L-homocysteine treatment. (A) Biotinylated surface proteins were analyzed by immunoblot analysis using anti-GluA2 antibody (upper panel). Total GluA2 in the input lysates was also analyzed by immunoblotting with anti-GluA2 antibody (lower panel). (B) Neuronal lysates were analyzed by immunoblotting with anti-p-GluA2 antibody (upper panel) and blots were re-probed with anti-GluA2 antibody (lower panel). (A, B) Quantitative analysis of GluA2

surface expression and phosphorylation at Ser880 is represented as mean \pm SEM (n=4). *p < 0.05 from 0 min time point or control; #p < 0.05 from 4 h homocysteine treatment.

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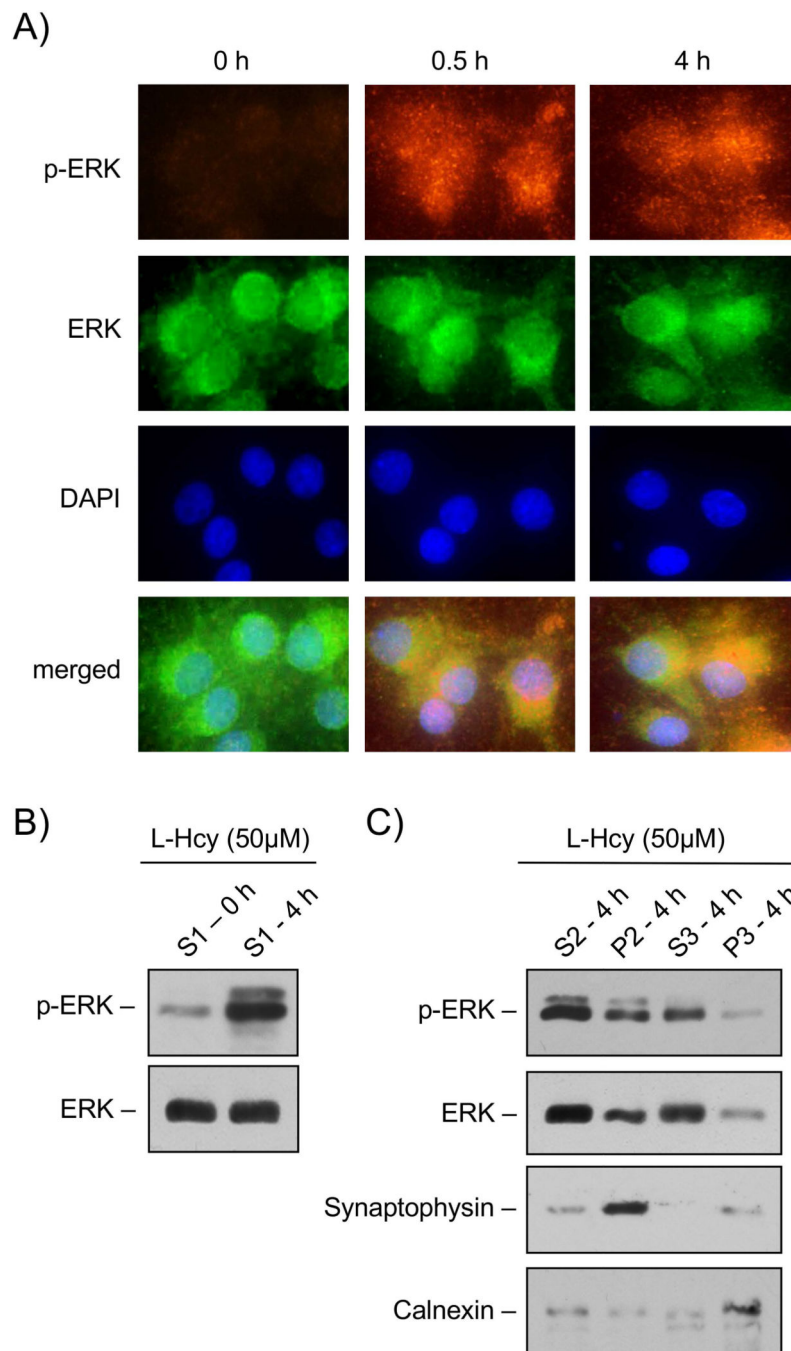


FIGURE 5. Subcellular localization of phosphorylated ERK MAPK in neurons treated with homocysteine

(A) Immunocytochemical analysis illustrating the cytoplasmic and nuclear distribution of p-ERK (red) and ERK (green) MAPK in neurons following 50 μ M of L-homocysteine (L-Hcy) treatment for 0 min, 30 min (0.5 h) or 4 h. The cell nuclei were counterstained with DAPI (blue). (B) Immunoblot analysis of the S1 fraction (supernatant following centrifugation of cell homogenate at 1,000 \times g) obtained from neurons treated with L-homocysteine (50 μ M) for 0 or 4 h using anti-p-ERK (upper panel) or anti-ERK (lower panel) antibody. (C) Subcellular distribution of phosphorylated ERK MAPK in neurons treated with L-homocysteine

(50 μ M, 4 h) was analyzed by immunoblot analysis of biochemical fractions with anti-p-ERK (panel 1) or anti-ERK (panel 2) antibody. Purity of the fractions was also evaluated by immunoblot analysis with anti-synaptophysin (panel 3) and anti-calnexin (panel 4) antibodies. The biochemical fractions evaluated are S2 (supernatant following centrifugation of S1 fraction at $10,000 \times g$), P2 (pellet comprising of crude synaptosomal membrane proteins following centrifugation of S1 fraction at $10,000 \times g$), S3 (final cytosolic fraction obtained following centrifugation of S2 fraction at $1,65,000 \times g$) and P3 (pellet comprising mainly of light membrane proteins obtained following centrifugation of S2 fraction at $1,65,000 \times g$).

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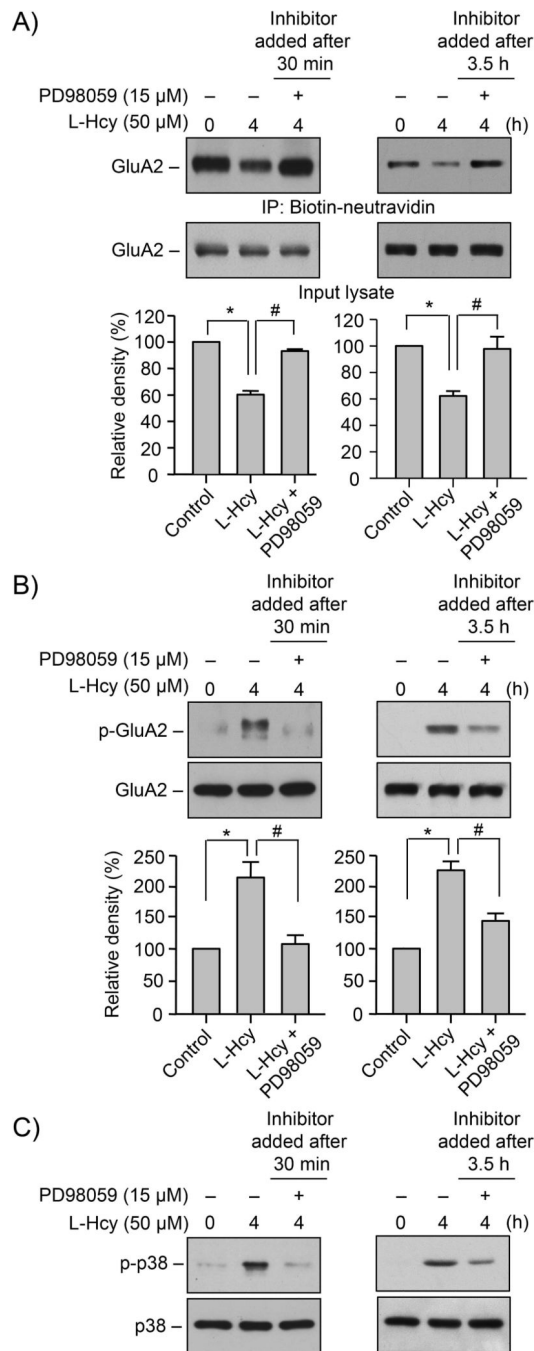


FIGURE 6. Homocysteine-NMDAR induced alteration in GluA2-AMPA surface expression is regulated by ERK MAPK

(A–C) Neuron cultures were exposed to 50 μ M of L-homocysteine (L-Hcy) for 4 h and PD98059 was added either 30 min or 3.5 h after the onset of L-Hcy treatment. (A) Biotinylated surface proteins were processed for immunoblot analysis with anti-GluA2 antibody (upper panel). The total GluA2 in the input lysates was also analyzed using anti-GluA2 antibody (lower panel). (B, C) Equal amount of protein from total cell extracts was analyzed by immunoblotting using (B) anti-p-GluA2 or (C) anti-p-p38 antibody (upper panels). The blots were re-probed with (B) anti-GluA2 or (C) anti-p38 antibody (lower

panels). (A, B) Quantitative analysis of GluA2 surface expression and phosphorylation at Ser880 is represented as mean \pm SEM (n = 4). *p < 0.05 from 0 min time point or control; #p < 0.05 from 4 h homocysteine treatment.

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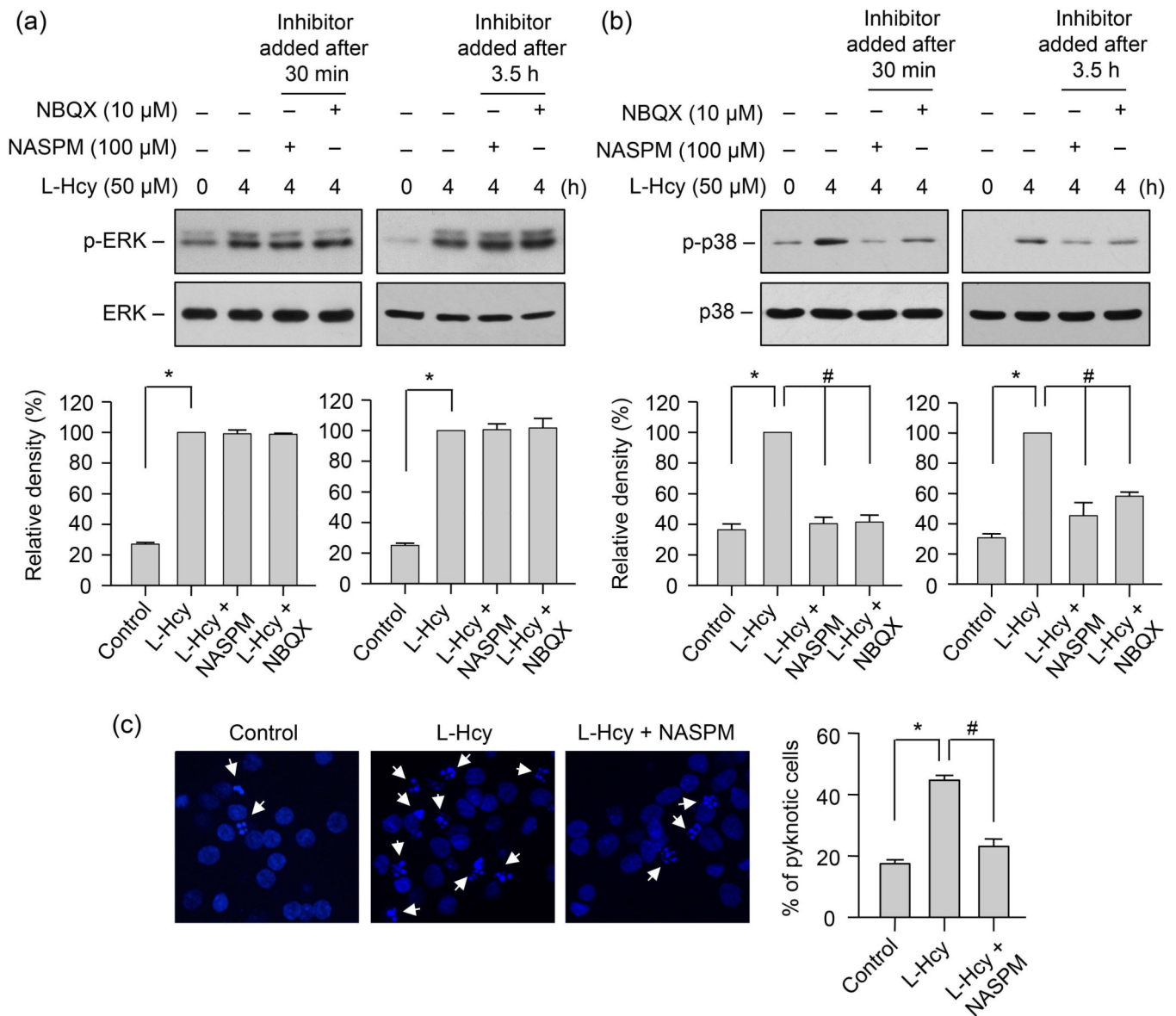


FIGURE 7. Role of GluA2-lacking AMPAR activity in homocysteine-mediated phosphorylation of p38 MAPK and subsequent neuronal death

(A, B) Neuronal cultures were treated with 50 μ M of L-homocysteine (L-Hcy) for 4 h and NASPM (100 μ M) or NBQX (10 μ M) was added 30 min or 3.5 h after the onset of homocysteine treatment. Phosphorylation of ERK MAPK and p38 MAPKs was analyzed using (A) anti-p-ERK antibody or (B) anti-p-p38 antibody (upper panels). Total pERK MAPK or p38 MAPK was analyzed by reprobing the blots with either (A) anti-ERK or (B) anti-p38 antibody (lower panels). Quantitative analysis of phosphorylated ERK and p38 MAPKs is represented as mean \pm SEM ($n = 4$). * $p < 0.001$ from 0 min time point or control; # $p < 0.001$ from 4 h homocysteine treatment. (C) Neurons were treated with L-homocysteine (50 μ M, 18 h) in the absence or presence of NASPM (100 μ M, added 30 min after the onset of L-homocysteine treatment) and then stained with Hoechst 33342, 18 h later. Representative photomicrographs show pyknotic DNA stained with Hoechst 33342

(indicated with arrows). Percentage of neurons with pyknotic nuclei is represented as mean \pm SEM (n = 1500 cells/condition from 4 experiments). *p < 0.001 from control; #p < 0.05 from 18 h homocysteine treatment.