GluN2A-NMDA receptor–mediated sustained Ca\(^{2+}\) influx leads to homocysteine-induced neuronal cell death

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Homocysteine, a metabolite of the methionine cycle, is a known agonist of N-methyl-D-aspartate receptor (NMDAR), a glutamate receptor subtype and is involved in NMDAR-mediated neurotoxicity. Our previous findings have shown that homocysteine-induced, NMDAR-mediated neurotoxicity is facilitated by a sustained increase in phosphorylation and activation of extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK MAPK). In the current study, we investigated the role GluN1/GluN2A-containing functional NMDAR (GluN2A-NMDAR) and GluN1/GluN2B-containing functional NMDAR (GluN2B-NMDAR) in homocysteine-induced neurotoxicity. Our findings revealed that exposing primary cortical neuronal cultures to homocysteine leads to a sustained low-level increase in intracellular Ca\(^{2+}\). We also showed that pharmacological inhibition of GluN2A-NMDAR or genetic deletion of the GluN2A subunit attenuates homocysteine-induced increase in intracellular Ca\(^{2+}\). Our results further established the role of GluN2A-NMDAR in homocysteine-mediated sustained ERK MAPK phosphorylation and neuronal cell death. Of note, the preferential role of GluN2A-NMDAR in homocysteine-induced neurotoxicity was distinctly different from glutamate-NMDAR–induced excitotoxic cell death that involves overactivation of GluN2B-NMDAR and is independent of ERK MAPK activation. These findings indicate a critical role of GluN2A-NMDAR–mediated signaling in homocysteine-induced neurotoxicity.

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2 The abbreviations used are: NMDAR, N-methyl-D-aspartate receptor; NMDA, N-methyl-D-aspartate; ERK MAPK, extracellular signal-regulated kinase/mitogen-activated protein kinase; KO, knockout; Ca\(^{2+}\), intracellular Ca\(^{2+}\) concentration; α-AP5, α-2-amino-5-phosphopentoic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; mGluR, metabotropic glutamate receptor; NVP-AAM077, (S)-1-(4-bromo-phenyl)-ethylaminol-(2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-yl)-methyl] phosphonic acid; AM, acetoxymethyl ester; ANOVA, analysis of variance; l-Hcy, l-homocysteine.

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the extent and duration of the intracellular Ca\(^{2+}\) increase in primary cortical neuronal cultures following exposure to homocysteine. The study further evaluated the role of GluN2A-NMDAR in homocysteine-induced ERK MAPK phosphorylation and neuronal cell death.

**Results**

**Homocysteine induces sustained increase in intracellular Ca\(^{2+}\) level in cortical neurons**

To examine the changes in intracellular Ca\(^{2+}\) dynamics, rat neuronal cultures were loaded with Fura2 acetoxymethyl ester (Fura2-AM) and treated with L-homocysteine (50 \(\mu M\) ; 60 min). The frames in Fig. 1A show the Ca\(^{2+}\) responses (in false-color maps) in a representative group of cells treated with homocysteine. The temporal profile of Fura2 fluorescence ratio measured in the soma of 20 individual neurons (Fig. 1B) and their mean data (Fig. 1C) illustrated a slow and progressive increase in both the Fura2 fluorescence ratio and [Ca\(^{2+}\)]i over time with a maximum increase of ~7.2-fold by 60 min when compared with corresponding control cells (Fig. 1D). In contrast, neurons treated with the NMDAR agonist glutamate (50 \(\mu M\); 10 min) showed a rapid change in Fura2 fluorescence ratio and a ~21-fold increase in [Ca\(^{2+}\)]i within 2.5 min, which then declined progressively with time (Fig. 1, E and F). Such rapid but transient increase in [Ca\(^{2+}\)]i, when challenged with glutamate is also in agreement with earlier reports (33–36). In neurons where the soma and its main dendritic process (out to at least 40 \(\mu M\)) were identifiable, we further analyzed the temporal profile of [Ca\(^{2+}\)]i, increase in the processes following exposure to homocysteine. The findings showed a slow and progressive increase in both the Fura2 fluorescence ratio and [Ca\(^{2+}\)]i, in the processes over time (Fig. 1, G and H). A comparison of the progressive change in [Ca\(^{2+}\)]i between somata and processes showed a similar profile (Fig. 1, I and J).

**Homocysteine-induced increase in intracellular Ca\(^{2+}\) level depends on GluN2A-containing NMDAR**

To determine the primary source of [Ca\(^{2+}\)]i increase, neurons were exposed to L-homocysteine (50 \(\mu M\); 60 min) in the presence of the Ca\(^{2+}\) chelator EGTA. Fig. 2, A–D, show that homocysteine-mediated increases in Fura2 fluorescence ratio and [Ca\(^{2+}\)]i level were blocked in the presence of EGTA in both somata and processes, suggesting that homocysteine-mediated [Ca\(^{2+}\)]i increase is derived from extracellular sources. To examine the role of NMDARs in homocysteine-mediated Ca\(^{2+}\) influx, neurons were treated with L-homocysteine (50 \(\mu M\); 60 min) in the presence of NMDAR inhibitor DL-2-amino-5-phosphopentoanoic acid (DL-AP5). Fig. 2, E–H, show that DL-AP5 attenuated homocysteine-induced increases in Fura2 fluorescence ratio and [Ca\(^{2+}\)]i in both the somata and the processes. To evaluate the role of GluN2A- and GluN2B-containing NMDARs in homocysteine-induced Ca\(^{2+}\) influx, rat neuronal cultures were incubated with L-homocysteine (50 \(\mu M\); 60 min) in the presence of selective antagonists for GluN2A subunit ((\(R\))-\((S\))-1-(4-bromo-phenyl)-ethylamino)-(2,3-dioxo-1,2,3,4-tetrahydroquinoloxin-5-yl)-methylphosphonic acid (NVP-AAM077)) or GluN2B subunit (Ro 25-6981). Fig. 3, A–D, show that coincubation with NVP-AAM077 attenuated the homocysteine-induced increase in Fura2 fluorescence ratio and [Ca\(^{2+}\)]i, in both the somata and the processes. In contrast, exposure to Ro 25-6981 failed to attenuate homocysteine-induced [Ca\(^{2+}\)]i increase in either the somata or processes (Fig. 3, E–H). To establish more directly the role of GluN2A-NMDAR in homocysteine-induced [Ca\(^{2+}\)]i, neuronal cultures obtained from wildtype (WT) and GluN2A-KO mice were treated with L-homocysteine (50 \(\mu M\); 60 min). Fig. 4, A–C, G, and H, show progressive increases in Fura2 fluorescence ratio and [Ca\(^{2+}\)]i in both the somata and the processes of neurons from WT mice as compared with control cells. However, treatment of neurons from GluN2A-KO mice with homocysteine failed to increase the Fura2 fluorescence ratio or [Ca\(^{2+}\)]i in the somata and the processes (Fig. 4, D–F, I, and J) when compared with control cells. In contrast, treatment with glutamate (50 \(\mu M\)) led to rapid increases in Fura2 fluorescence ratio and [Ca\(^{2+}\)]i, in neurons from both WT and GluN2A-KO mice (Fig. 5, A–E). Additional studies evaluating the protein expression of GluN1, GluN2A, and GluN2B subunits in neuronal lysates from WT and GluN2A-KO mice showed no change in the protein level of GluN1 subunit (Fig. 5F). The findings also confirmed the loss of expression of GluN2A subunit in the neurons from GluN2A-KO mice. However, neuronal GluN2B subunit expression went up substantially in the GluN2A-KO mice.

**Homocysteine-induced stimulation of GluN2A-containing NMDAR leads to sustained ERK MAPK phosphorylation and neuronal death**

Our earlier studies showed that a sustained increase in ERK MAPK phosphorylation plays a crucial role in promoting homocysteine-NMDAR–induced neuronal cell death (17). To evaluate the role of GluN2A-NMDAR in homocysteine-induced ERK MAPK phosphorylation, rat neuronal cultures were treated with L-homocysteine (50 \(\mu M\)) for 30 or 60 min in the presence of DL-AP5 (NMDAR inhibitor) or NVP-AAM077 (GluN2A inhibitor). Fig. 6, A and B, show that pharmacological inhibition with either DL-AP5 or NVP-AAM077 attenuated homocysteine-induced ERK MAPK phosphorylation at both time points. To further confirm the role of GluN2A-NMDAR in homocysteine-induced ERK MAPK phosphorylation, neuronal cultures from WT and GluN2A-KO mice were treated with L-homocysteine (50 \(\mu M\)) for 30 or 60 min. Fig. 6C shows that treatment with homocysteine led to a sustained increase in ERK MAPK phosphorylation in neurons obtained from WT mice, whereas it failed to induce ERK MAPK phosphorylation in neurons obtained from GluN2A-KO mice. Subsequent studies evaluated the effect of glutamate (50 \(\mu M\)) on the temporal profile of ERK MAPK phosphorylation (5, 30, or 60 min) in rat neuron cultures. As shown in Fig. 6D, treatment with glutamate led to a rapid but transient increase in ERK MAPK phosphorylation within 5 min of stimulation, which returned to basal level by 30 min. Treatment with DL-AP5 blocked, whereas treatment with NVP-AAM077 failed, to ameliorate the glutamate-induced transient increase in ERK MAPK phosphorylation at.

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5 min (Fig. 6E), indicating that GluN2A-NMDAR does not play a role in glutamate-induced ERK MAPK phosphorylation. Consistent with this interpretation, studies in neuron cultures from WT and GluN2A-KO mice showed that exposure to glutamate (50 µM) led to a rapid but transient increase in ERK MAPK phosphorylation in both WT and GluN2A-KO mouse cultures (Fig. 6F).
To evaluate the role of GluN2A-NMDARs in homocysteine-induced neuronal cell death, rat neuronal cultures were treated with l-homocysteine (50 µM; 18 h) in the presence of NVP-AAM077. Cell death was assessed by Hoechst DNA staining, an early indicator of apoptosis (37). The representative photomicrographs and quantitative analysis of pyknotic nuclei showed a significant increase in cell death following exposure to homocysteine (Fig. 7A), which is consistent with our earlier findings (16). Pharmacological treatment with NVP-AAM077 significantly reduced homocysteine-induced neuronal death (Fig. 7A). To assess the role of ERK MAPK activation in homocysteine-GluN2A-NMDAR–induced neurotoxicity, in
subsequent studies neurons were treated with L-homocysteine (50 μM; 18 h) in the presence of the ERK MAPK inhibitor PD98059 (15 μM). As shown in Fig. 7A, ERK MAPK inhibition also attenuated homocysteine-induced neurotoxicity. Additional studies in neuron cultures obtained from WT and GluN2A-KO mice showed that exposure to homocysteine (50 μM) significantly increased neuronal death in cultures obtained from WT mice, whereas it failed to induce neurotoxicity in cultures obtained from GluN2A-KO mice (Fig. 7B). The findings also showed that homocysteine-induced neurotoxicity

Figure 4. Knockdown of GluN2A subunit blocks homocysteine-induced Ca²⁺ influx. A and D, individual responses in soma of 16–18 neurons obtained from WT (A) or GluN2A-KO (D) mice showing the range of increase in Fura2 fluorescence ratio over time following exposure to L-Hcy (50 μM). B, C, E, and F, temporal profile of increase in Fura2 fluorescence ratio (mean ± S.E.) and [Ca²⁺]i (mean ± S.D.) in the somata of L-Hcy–treated and control cells from WT (B and C) and GluN2A-KO (E and F) mice. For [Ca²⁺]i, in WT mice (n = 16–18), two-way ANOVA shows significant group difference (F₁,12₉ = 123S; p < 0.0001). Post hoc analysis shows p < 0.0001 (****) between L-Hcy–treated group and control group at the given time point. For [Ca²⁺]i in GluN2A-KO mice (n = 16), two-way ANOVA shows no significant group difference (F₁,12₀ = 2.6₆₁; p = 0.1₀5₅). G–J, temporal profile of increase in Fura2 fluorescence ratio (mean ± S.E.) and [Ca²⁺]i (mean ± S.D.) in dendrites of L-Hcy–treated and control cells from WT (G and H) and GluN2A-KO (I and J) mice. For [Ca²⁺]i, in WT mice (n = 8–10), two-way ANOVA shows significant group difference (F₁,₆₄ = 6₀₃.₆; p < 0.0001). Post hoc analysis shows p < 0.0001 (****) between L-Hcy–treated group and control group at the given time point. For [Ca²⁺]i, in GluN2A-KO mice (n = 7–10), two-way ANOVA shows no significant group difference (F₁,₆₀ = 3.₉₇₅; p = 0.0₅₀₇). Error bars represent S.D. or S.E.
observed in neurons from WT mice was significantly reduced in the presence of ERK MAPK inhibitor (Fig. 7B). In contrast, glutamate (50 μM)-induced neuronal cell death in rat neuron cultures remained unaffected by pharmacological inhibition of either GluN2A-NMDAR or ERK MAPK (Fig. 7C). A comparison of the neurotoxic effects of glutamate in neuron cultures obtained from WT and GluN2A-KO mice further showed that deletion of the GluN2A subunit of NMDAR failed to reduce the neurotoxic effects of glutamate (Fig. 7D). Pharmacological inhibition of ERK MAPK also failed to reduce glutamate-induced neurotoxicity in neurons from either WT or GluN2A-KO mice (Fig. 7D).

Discussion

Ca²⁺ is an important intracellular messenger that regulates multiple neuronal functions, including cellular growth, membrane excitability, and synaptic activity. As such, the intracellular Ca²⁺ level in neurons is tightly regulated to ensure efficient control on downstream signaling cascades involved in maintaining cellular physiology (38). The cellular mechanisms that help maintain Ca²⁺ homeostasis include the transmembrane Ca²⁺ gradient, route of Ca²⁺ entry, and presence of various Ca²⁺ buffering and extrusion systems (39, 40). Any changes in these homeostatic control mechanisms under a pathological condition leads to an aberrant increase in the intracellular Ca²⁺ level. Evidences now indicate that overactivation of NMDARs in neurodegenerative disorders involving excessive release of glutamate may lead to an aberrant increase in the [Ca²⁺], level (41–44). However, the extent of Ca²⁺ increase varies depending on the severity of the stimuli and NMDAR subunit composition. A moderate and transient influx of Ca²⁺ has been coupled to GluN2A-NMDAR, whereas rapid, large Ca²⁺ overload is associated with GluN2B-NMDAR (41, 45–47). Additional studies using low-affinity Ca²⁺ indicators have also shown that glutamate-mediated intracellular Ca²⁺ increases occur in two phases, a rapid but low level of initial increase followed by a delayed, larger increase (47–49). The larger, delayed increase in [Ca²⁺], is blocked in the presence of selective antagonists of GluN2B-NMDAR, indicating that the initial smaller Ca²⁺ influx depends on GluN2A-NMDAR (47).

A particular contribution of our study is the identification of the exclusive role of GluN2A-NMDAR in homocysteine-induced Ca²⁺ influx. The sole role of GluN2A-NMDAR in the homocysteine-induced low level of Ca²⁺ influx is not only confirmed using a selective pharmacological inhibitor for GluN2A-NMDAR but also by genetic deletion of GluN2A subunit of NMDAR. The inability of homocysteine to induce [Ca²⁺], increase in neurons obtained from GluN2A-KO mice, despite the higher expression of the GluN2B subunit of NMDAR, further emphasizes the contribution of GluN2A-NMDARs in homocysteine-induced [Ca²⁺], influx. In this context, a recent study utilizing HEK 293T cells expressing recombinant GluN1/2A and GluN1/2B NMDARs showed that GluN1/2B NMDARs undergo rapid desensitization upon exposure to homocysteine, whereas GluN1/2A receptors do not exhibit desensitization (50).

The distinctly different contributions of GluN2A- and GluN2B-containing NMDARs in homocysteine- and glutamate-mediated [Ca²⁺], influx suggest differential roles of these subunits in the regulation of intracellular signaling cascades. This interpretation is substantiated by our findings that homocysteine-me-
diated NMDAR stimulation leads to a sustained increase in ERK MAPK phosphorylation, which is completely blocked by pharmacological inhibition of GluN2A-NMDAR or genetic deletion of GluN2A subunit. Whereas, in the glutamate treatment paradigm, the increase in ERK MAPK phosphorylation is found to be rapid but transient, and it remains unaffected by pharmacological inhibition or genetic deletion of GluN2A-NMDAR. Our findings also show that this difference in ERK MAPK signaling by the two NMDAR agonists has a considerably different effect on the neurotoxic profile. Pharmacological inhibition of GluN2A-NMDAR or genetic deletion of GluN2A subunit as well as pharmacological inhibition of ERK MAPK activity attenuates homocysteine-induced neuronal death, whereas they fail to attenuate glutamate-induced neuronal cell death. The findings highlight the role of sustained ERK MAPK activation in GluN2A-NMDAR–mediated neurotoxicity. Our earlier studies have shown that sustained ERK MAPK activation leads to a decrease in surface expression of the GluA2 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, resulting in Ca²⁺ influx through GluA2-lacking Ca²⁺-permeable AMPA receptors, which leads to p38 MAPK activation (18). This novel interplay between ERK and p38 MAPK results in caspase-3–dependent neuronal cell death (17). Whether this detrimental signaling cascade downstream of GluN2A-NMDAR stimulation, observed in our studies, is unique to the homocysteine signaling pathway or could also be triggered by other extracellular stimuli remains to be investigated. The transient increase in ERK MAPK phosphorylation following exposure to glutamate and its inability to attenuate glutamate-induced neurotoxicity further indicate that transient ERK MAPK activation has different consequences as compared with sustained activation (51, 52).

The present study reveals an important functional consequence of GluN2A-NMDAR activation, which is in contrast to the prevailing theory that mainly emphasizes the function of GluN2A-NMDAR in promoting neuronal survival and growth (20, 53–55). Several in vitro and in vivo studies on excitotoxic and ischemic neuronal injury have demonstrated that selective

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**Figure 6. Homocysteine-induced ERK MAPK phosphorylation is mediated through GluN2A-NMDAR.** A and B, rat neuron cultures were exposed to L-Hcy (50 μM) for 30 or 60 min in the absence and presence of DL-AP5 (200 μM) (A) or NVP-AAM077 (30 nM) (B). C, neuronal cultures from WT and GluN2A-KO mice were treated with L-Hcy (50 μM) for 30 or 60 min. D, rat neuron cultures were exposed to glutamate (Glu; 50 μM) for 5, 30, or 60 min. E, rat neuronal cultures were exposed to glutamate for 5 min in the presence or absence of DL-AP5 (200 μM) or NVP-AAM077 (30 nM). F, neuronal cultures from WT and GluN2A-KO mice were treated with glutamate (50 μM) for 5, 30, or 60 min. A–G, immunoblot analysis of cell lysates with anti-phospho-ERK (pERK) (top) and anti-ERK (bottom) antibodies. Values are mean ± S.D. (n = 3–7). Significant differences were assessed by one-way ANOVA: *, p < 0.001 from corresponding 0 min; #, p < 0.001 from 30-min L-Hcy treatment; ¶, p < 0.001 from 60-min L-Hcy treatment; and §, p < 0.0001 from 5-min Glu treatment. Error bars represent S.D.
inhibition GluN2A-NMDARs either has no effect or exacerbates neuronal death (20, 27, 56, 57). Also, an elegant study investigating the effect of chimeric constructs of GluN2A/2B subunits expressed in cultured neurons and a knockin mouse model has demonstrated that replacing the C-terminal domain of GluN2B with that of GluN2A lowers the vulnerability of neurons to excitotoxic insult, emphasizing a neuroprotective role of GluN2A-NMDARs (58). However, there are a few studies that have investigated the detrimental role of GluN2A-NMDARs in neurons following ischemic/excitotoxic insult (56, 59, 60), but the interpretation of results obtained from these studies is not straightforward. The in vivo study by Morikawa et al. (56) shows that permanent focal cerebral ischemia in GluN2A-KO mice has no effect on stroke outcome when com-

**Figure 7. Homocysteine-induced neurotoxicity is mediated through GluN2A-NMDAR–dependent ERK MAPK activation.** A, rat neuronal cultures were exposed to L-Hcy (50 μM; 18 h) in the absence and presence of NVP-AAM077 (30 nM) or PD98059 (15 μM). B, neurons from WT and GluN2A-KO mice were exposed to L-Hcy (50 μM; 18 h) in the absence or presence of PD98059 (15 μM). C, rat neuronal cultures were exposed to glutamate (50 μM; 1 h) in the absence and presence of NVP-AAM077 (30 nM) or PD98059 (15 μM) and then maintained in original medium for 17 h. D, neurons from WT and GluN2A-KO mice were exposed to glutamate (50 μM; 1 h) in the absence or presence of PD98059 (15 μM) and then maintained in original medium for 17 h. A–D, representative photomicrographs showing pyknotic DNA stained with Hoechst 33342 (indicated with arrows). The percentage of neurons with pyknotic nuclei is represented as mean ± S.D. (n = 13–38 fields with a total of at least 1500 cells/condition from four experiments). Significant differences were assessed by one-way ANOVA: *, p < 0.001 from corresponding control; and #, p < 0.001 from L-Hcy treatment. Error bars represent S.D.
pared with WT mice, whereas 2 h of focal cerebral ischemia in GluN2A-KO mice leads to significant reduction in infarct size as compared with WT littermates. Similarly, the study by Wang et al. (59) shows involvement of GluN2A-NMDARs in selective degeneration of CA1 hippocampal neurons following stroke only after bilateral vertebral arteries were completely cut and coagulated for 12 h prior to induction of stroke. Furthermore, the in vitro study by Zhou et al. (60) shows that blocking GluN2A-NMDAR leads to partial protection against NMDA-mediated excitotoxicity when the duration and severity of the insult are low. Interestingly, they also demonstrate that blocking GluN2A-NMDAR does not show measurable effects when the duration of the insult is increased from 15 to 30 min or the degree of excitotoxic insult is increased from 50 to 100 μM NMDA. This indicates that blocking GluN2A-NMDAR is not effective in attenuating neuronal death under severe excitotoxic condition. In contrast, significant protection was still achieved by blocking GluN2B-NMDAR at all the above experimental conditions. As such, the issue of whether the GluN2A-NMDAR stimulation influences excitotoxic neuropathology has still remained unresolved. Distinct from these previous studies, our findings provide convincing evidence of intracellular signaling via GluN2A-NMDAR as the primary contributor of homocysteine-NMDAR–induced neurotoxicity.

Although the focus of the present study is on homocysteine-NMDAR–induced neurotoxicity, earlier studies have indicated a possible role of metabotropic glutamate receptors (mGluRs) in homocysteine-induced neurotoxicity (61, 62). However, the involvement of mGluRs in homocysteine-induced neurotoxicity in these studies was observed using very high doses of homocysteine (5–25 mM) typically not observed under pathological conditions (1, 3, 5). In addition, the neurotoxicity observed in these studies does not involve Ca²⁺ influx in neurons but, on the contrary, leads to efflux of Ca²⁺ from neurons. In contrast, our study was carried out with a concentration of homocysteine that is generally observed in individuals predisposed to mild to moderate hyperhomocysteinemia (1, 11, 63–65). We also observed that under this condition the increase in [Ca²⁺], plays a key role in homocysteine-induced neurotoxicity. As such, the neurotoxicity observed in our study could not be attributed to mGluR activation. This interpretation is further supported by our earlier study in neuron cultures demonstrating that mGluR inhibition fails to block homocysteine-induced ERK MAPK activation and subsequent neurotoxicity (16). In addition, an earlier study that evaluated the relative potency of mGluRs 1–5 to bind to 1-homocysteine and its acidic derivatives showed that 1-homocysteine by itself is not an agonist of mGluRs (66).

Together, these findings present the novel concept that homocysteine, by stimulating GluN2A-NMDARs, may promote neurotoxicity through sustained activation of Ca²⁺-dependent ERK MAPK signaling.

**Experimental procedures**

**Materials and reagents**

Pregnant female Sprague-Dawley rats were purchased from Envigo. GluN2A-KO mice were obtained from Dr. Andrew Holmes, National Institute on Alcohol Abuse and Alcoholism (67), and timed pregnant mice were generated at the animal facility of University of New Mexico. The Institutional Animal Care and Use Committee of University of New Mexico, Health Sciences Center approved all animal procedures. 1-Homocysteine thiolactone, glycine, EGTA, and Hoescht 33342 were purchased from Sigma-Aldrich. Fura2-AM and all cell culture reagents were purchased from Invitrogen. Anti-phospho-ERK1/2 (Thr-202/Tyr-204) mAb (pERK) and anti-rabbit and -mouse horseradish peroxidase–conjugated secondary antibodies were purchased from Cell Signaling Technology. Anti-MAPK (ERK) and anti-β-tubulin polyclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-GluN2A rabbit mAb was purchased from Abcam. Anti-GluN1 mAb and anti-GluN2B polyclonal antibody were purchased from Millipore Sigma. Ionomycin, DL-AP5, Ro 25-6981, PD98059, and NVP-AAM077 were obtained from EMD Biosciences.

**Neuron culture, 1-homocysteine preparation, and stimulation**

Embryos obtained from pregnant Sprague-Dawley rats (16–17-day gestation) or WT and GluN2A-KO mice (15–16-day gestation) were used to establish primary cortical neuronal cultures as described earlier (16–18). Neurons were grown on (a) 35-mm culture dishes (MatTek Corp.) coated overnight with poly-D-lysine (50 μg/ml) and laminin (10 μg/ml) for Ca²⁺ imaging studies, (b) poly-D-lysine–coated 60-mm dishes (Corning BioCoat) for biochemical studies, and (c) poly-D-lysine–coated 4-well culture slides (Corning BioCoat) for cell-death assay. Neurons were maintained in culture for 12–13 days before experiments. The cells were treated with freshly prepared 1-homocysteine (50 μM) in Hank’s balanced salt solution containing 50 μM glycine (11, 16–18) for the specified time periods. In a parallel series of experiments, cells were treated with glutamate (50 μM) for the specified time periods. Cells were then processed for live-cell imaging, immunoblotting, or cell-death assay. In some experiments, EGTA, DL-AP5, NVP-AAM077, Ro 25-6981, or PD98059 was added 15 min prior to 1-homocysteine or glutamate treatment.

**Calcium measurements**

[Ca²⁺], in neurons was determined using the fluorescent indicator Fura2-AM. Briefly, neurons were loaded with Fura2 (10 μM) in phenol red–free Hank’s balanced salt solution for 30 min at 37 °C followed by postincubation for 20 min (47). Time-lapse live-cell imaging was performed following stimulation using a Nikon Ti Eclipse inverted microscope equipped with Tokai Hit stage-top incubator maintained at 37 °C and infused with 95% air, 5% CO₂ mixture. Fields of five to eight cells were imaged using a 40× oil immersion objective (Nikon). Fluorescence excitations (340 and 380 nm) were performed using a Sutter LB-LS/30 Lambda xenon arc lamp, and fluorescence emissions (510 nm) were captured using a charge-coupled device (CCD) camera (Photometrics). NIS Elements AR software was used for data acquisition and analysis. Ratiometric data (340/380 nm) from regions of interest were corrected for background and converted into estimates of [Ca²⁺], as described previously (47) using the equation developed earlier (68). Maximum and minimum fluorescence ratios were determined from Fura2-loaded cells treated with ionomycin (5 μM).
in calcium-containing medium (2 mM) or in calcium-free medium with EGTA (0.5 mM).

**Immunoblotting**

Rat and mouse neuron cultures were harvested in SDS sample buffer (69). Equal amounts of protein from the cell lysates were resolved by SDS-PAGE (7.5%) and subjected to immunoblotting procedures as described earlier (16, 17, 23). The blots were analyzed with the specified antibodies according to the manufacturer’s protocol. Densitometric analyses of the images captured on X-ray films were performed using ImageJ software.

**Hoechst DNA staining**

Cortical neuron cultures from rats and mice were treated either with L-homocysteine for 18 h or glutamate for 1 h. Glutamate-treated cells were then maintained in their original medium for another 17 h. For some experiments, neurons were treated with L-homocysteine or glutamate in the presence of NVP-AAM077 or PD98059. The neurons were then fixed and stained with Hoechst 33342 dye as described earlier (16). The percentage of pyknotic nuclei was quantitatively assessed by fluorescence microscopy to determine the extent of neuronal death.

**Statistical analysis**

Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) followed by post hoc analysis using Bonferroni’s multiple comparison test. Differences were considered significant for p < 0.05.

**Author contributions**—S. N. D. and R. P. data curation; S. N. D., S. P., and R. P. formal analysis; S. N. D., S. P., and R. P. validation; S. N. D., S. M., and S. R. investigation; S. N. D. and R. P. writing-original draft; R. P. project administration.

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**GluN2A-NMDA receptor–mediated neurotoxicity**

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