Role of N-Methyl-D-aspartate Receptors in the Neuroprotective Activation of Extracellular Signal-regulated Kinase 1/2 by Cisplatin*

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Neurons are exposed to damaging stimuli that can trigger cell death and subsequently cause serious neurological disorders. Therefore, it is important to define defense mechanisms that can be activated in response to damage to reduce neuronal loss. Here we report that cisplatin (CPDD), a neurotoxic anticancer drug that damages DNA, triggered apoptosis and activated the extracellular signal-regulated kinase 1/2 pathway in cultured rat cortical neurons. Inhibition of ERK1/2 activation using either pharmacological inhibitors or a dominant-negative mutant of the ERK1/2 activator, mitogen-activated protein kinase kinase 1, increased the toxicity of CPDD. Interestingly, N-methyl-D-aspartate (NMDA) receptor (NMDAR) antagonists reduced the ERK1/2 activation and exacerbated apoptosis in CPDD-treated neurons. Pre-treatment with CPDD increased ERK1/2 activation triggered by exogenous NMDA, suggesting that CPDD augmented NMDAR responsiveness. CPDD-enhanced response of NMDAR and CPDD-mediated ERK1/2 activation were both decreased by inhibition of poly(ADP-ribose) polymerase (PARP). Interestingly, PARP activation did not produce ATP depletion, suggesting involvement of a non-energetic mechanism in NMDAR regulation by PARP. Finally, CPDD toxicity was reduced by brain-derived neurotrophic factor, and this protection required ERK1/2. In summary, our data identify a novel compensatory circuit in central nervous system neurons that couples the DNA injury, through PARP and NMDAR, to the defensive ERK1/2 activation.

The central nervous system is exposed to damaging stimuli that may trigger neuronal death and cause serious neurological diseases (1). However, most neurons survive minor damages with which they are challenged during the life span of the organism. Therefore, one can expect the existence of defense mechanisms that help neurons to survive initial insult and resume proper functions after damage. Neurons receive multiple signals inhibiting cell death (2). For example, neuronal survival during development is promoted by neurotrophins and neurotransmitters. The effects of these agents on survival are mediated through several signaling molecules, including extracellular signal-regulated kinase 1/2 (ERK1/2) and phoshpatidyl-dioinositol 3-kinase (1, 2). Consequently, survival signaling pathways are good candidates to contribute to the defense mechanisms in injured neurons.

Glutamate is an important neurotransmitter that promotes survival. It acts through several types of receptors, including two families of ionotropic receptors, AMPA and NMDA (3). NMDA receptor (NMDAR) is required for neuronal survival during development (4, 5). On the other hand, excessive activation of NMDAR signaling produces excitotoxicity (6). Therefore, NMDAR inhibitors are used to improve the outcome of several neurological diseases (7). In addition, it has been recently proposed that NMDAR antagonists may be active against malignant tumors and that their combination with anticancer chemotherapy would be a valuable therapeutic approach (8).

It is intriguing that neurons, which are postmitotic cells, demonstrate high vulnerability to DNA damage (9, 10). Often, genotoxic anticancer agents including cisplatin (CPDD) produce neurological side effects that limit their usage against central nervous system tumors (11, 12). DNA damage may also be an important trigger of neuron loss in common neurodegenerative diseases (13).

DNA damage activates both the reparative response and death signaling (14). For example, DNA damage may mobilize poly(ADP-ribose) polymerase (PARP), which ribosylates target proteins to activate DNA repair (15). The substrate of PARP is a highly energetic molecule, NAD+. In consequence, PARP activation may deplete cellular energy stores, resulting in neuronal membrane depolarization with enhanced NMDAR signaling and, finally, necrotic cell death (15).

Genotoxic-induced neuronal death can be suppressed by

* This work was supported by State Committee for Scientific Research (KBN, Poland) Grants 6P04A00421 (to M. H.) and 6P05A00321 (to J. A.) and by National Institutes of Health Grant P20-RR15576 (to M. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ERK1/2, extracellular signal-regulated kinase 1/2; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; KA, kynurenic acid; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; CPDD, cis-diaminodichloroplatinum; PARP, poly(ADP-ribose) polymerase; MKK1/2, mitogen-activated protein kinase kinase 1/2; 3-ABA, 3-amino-5-phosphonovaleric acid; NQXK, 2,3-dioxo-6-nitro-7-sulfamoylbenzo-[f]quinoxaline; CNQX, cyano-nitroquinoxaline; TPDD, trans-diaminodichloroplatinum; BAPTA/AM, 1,2-bis(2-aminoethyl)-hoethane-N,N,N’,N’-tetraacetic acid tetrakis(acetoxymethyl ester); 3,4,5-dimethoxyxanthol-2-01,2,5-diphenyltetrazolium bromide; Me6SO, dimethyl sulfoxide; Z-VAD-fmk, benzoyloxycarbonyl-VAD-fluoromethyl ketone; HA, hemagglutinin; MK-801, dizocilpine.
Materials—The following plasmids have been described elsewhere: pON260 (19), HA-tagged expression vectors for wild type, constitutive active MKK1 (ΔN3-S218E/S222D), and dominant-negative MKK1 (K97M) (20). A polyclonal anti-phospho-ERK2/1 antibody (anti-AC-TIVE™ mitogen-activated protein kinase polyclonal antibody) was purchased from Promega; anti-ERK2 antibody was obtained from Santa Cruz Biotechnology; monoclonal anti-ERK1/2 antibody was obtained from Cell Signaling; polyclonal antibody to β-galactosidase was obtained from 5 Prime, Inc. (Boulder, CO), and anti-HA monoclonal antibody (12CA5) was obtained from Roche Applied Science. Polyclonal antibody to poly(ADP)ribose and all secondary antibodies were obtained from Calbiochem. PD98059 and LY294002 were purchased from Calbiochem. 3-aminobenzenamide (ABA), 6(S)-phentanthridine (PHEN), BDNF, cycloheximide, dizocilpine (MK-801), 3-ABA, PHEN, and transplatin (TPDD) were obtained from Sigma. BAPTA/AM was purchased from Molecular Probes.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Cortical neurons were prepared from newborn rats (Sprague-Dawley) and kept in basal medium Eagle supplemented with 10% heat-inactivated bovine calf serum (HyClone), as described (17). Recombinant forms of MKK1 were expressed in Rat-1 fibroblasts as described (18). pON260 plasmid DNA, which contains an expression cassette for β-galactosidase, was obtained from 5 Prime, Inc. (Boulder, CO), and anti-HA antibody was obtained from Santa Cruz Biotechnology. Western blot analysis with anti-poly(ADP-ribose) and all secondary antibodies were obtained from Santa Cruz Biotechnology.

Drug Treatment—Cells were treated with CPDD or TPDD. These drugs were dissolved in dimethyl sulfoxide (Me2SO). The final concentration of Me2SO in the media was 0.2%. PD98059, SL327, NBQX, CNQX, 3-ABA, PHEN, and BAPTA/AM were also dissolved in Me2SO. When cultures were co-treated with CPDD and one of these drugs, the final concentration of Me2SO was below 0.4%. BDNF was diluted in phosphate-buffered saline containing 0.1% bovine serum albumin before addition to the cells. SL327 or PD98059 was added 30 min before CPDD in co-treatment experiments.

Quantitation of Apoptosis by Nuclear Morphological Changes—To visualize nuclear morphology, cells were fixed in 4% paraformaldehyde and stained with 2.5 μg/ml of the DNA dye Hoechst 33258 (Sigma) (17). Apoptosis was quantitated by scoring the percentage of cells with condensed or fragmented nuclei. Adhesion of nuclear morphology, cells were examined by fluorescence microscopy. Apoptotic cells were identified by their characteristic appearance and scored as described in the text. Cell death significantly increased at 24 and 48 h after CPDD treatment. CPDD induced more apoptosis at 24 h than 48 h after CPDD treatment. CPDD induced more apoptosis at 5 μM (p < 0.01). Averages of duplicate determinations from at least three independent experiments are shown. Bars indicate S.E.

Caspase Assay—Caspase assay was performed with a colorimetric caspase assay kit purchased from Promega. For each measurement, protein lysate from 1 × 10⁶ cells was used. To reveal the caspase-dependent activity, each sample was incubated with the caspase substrate in the absence or presence of 10 μM Z-VAD-fmk, a specific caspase inhibitor.

Western Analysis and Immunostaining—Western blot analysis with anti-phospho-ERK1/2 or anti-ERK1/2 antibodies was performed as described (17). Briefly, 10 μg of total protein was used in each lane. In addition, blots were re-probed with antibodies specific for total ERK1/2 or ERK2 to ensure equal protein loading of the blots. Quantification of phospho-ERK1/2 was performed by densitometric analysis and normalized against total ERK1/2. Western blot analysis with anti-poly(ADP-ribose) antibody was performed using 4 μg of nuclear proteins per sample.
Fig. 2. CPDD activates ERK1/2.
Neurons were treated as indicated in each panel. ERK1/2 pathway activity was determined by Western blotting using an antibody specific for phosphorylated ERK1/2. Blots were re-probed with an antibody specific for total ERK1/2. Phospho-ERK1/2 (pERK1/2) levels are relative to controls. Data in B and C represent averages of three independent experiments. Bars indicate S.E. A and B, kinetics of ERK1/2 activation after treatment with 10 μg/ml CPDD. C and D, dose response of ERK1/2 activation by CPDD at 24 h. E, TPDD, a non-DNA damage-inducing isomer of CPDD, fails to increase ERK1/2 activity. Neurons were treated for 24 h with either TPDD or CPDD at 10 μg/ml. F, cycloheximide (CHX) does not reduce ERK1/2 activation by CPDD. Neurons were treated for 24 h with cycloheximide (1 μg/ml) and CPDD (10 μg/ml) as indicated. Numbers under the blots in E and F are relative pERK1/2 levels. Results shown in E and F were replicated in three independent experiments.

sample. Nuclear proteins were extracted as described (22). Transfected cells were detected by immunostaining with an antibody against β-galactosidase and Texas Red-conjugated goat antibody to rabbit immunoglobulin. Cells transfected with the HA epitope-tagged constructs were also immunostained with an antibody to HA followed by fluorescein-conjugated goat antibody to mouse immunoglobulin.

Determination of Glutamate and Glycine Concentration—Amino acids were extracted from culture media with 0.6 N perchloric acid, followed by centrifugation and neutralization with potassium hydroxide. The analysis was performed as described (23).

NAD+ and ATP Assays—To extract nucleotides, 2 × 10^6 cells were treated with 3.5% perchloric acid as described (24). NAD+ level was measured in a reaction catalyzed by alcohol dehydrogenase as described (25). ATP measurement was performed according to the two-step procedure of Williamson and Corkey (26).

Statistical Analysis—Statistical analysis of the data was performed by using one- or two-way analysis of variance followed by post hoc tests.

RESULTS

CPDD-induced Apoptosis in Cortical Neurons—To test the hypothesis that DNA damage can activate the defensive ERK1/2 pathway, we treated cortical neurons with CPDD. CPDD applied for 48 h reduced neuronal survival (Fig. 1A). Interestingly, cells exposed to 10 μg/ml CPDD showed higher survival rates than cells treated with 5 μg/ml (56.4 versus 28%, Fig. 1A). Neurons treated with CPDD showed an apoptotic pattern of DNA fragmentation (Fig. 1B) and activation of pro-apoptotic caspases (Fig. 1C). In addition, cells dying in response to CPDD displayed morphological features of apoptosis including fragmentation and condensation of nuclear chromatin (Fig. 1, D–J). 10 μg/ml CPDD induced significantly less apoptosis than 5 μg/ml (24 h, 28 versus 51.2%, respectively; p < 0.01) (Fig. 1J). A translation inhibitor, cycloheximide, protected against CPDD-induced death (Fig. 1K), suggesting that protein synthesis may be involved in this process. This finding is also consistent with the apoptotic character of CPDD-induced death in cortical neurons. In addition, TPDD, an isomer of CPDD that is unable to induce DNA strand breaks (27), did not produce neuronal apoptosis (Fig. 1L), indicating that CPDD-induced neuronal apoptosis is triggered by DNA damage.

Activation of ERK1/2 Pathway in CPDD-treated Cortical Neurons—Phosphorylation of ERK1/2 residues Thr183 and Tyr185 (position numbers as in human ERK2) by MKK1/2 controls ERK1/2 activation (28). Therefore, we determined the activity of the ERK1/2 pathway by immunoblotting for phosphorylated ERK1/2 (Fig. 2). In cells exposed to 10 μg/ml CPDD, activation peaked at 24 h after treatment (Fig. 2, A and B). At that time point, the extent of CPDD-mediated activation of the ERK1/2 pathway was directly proportional to the concentration of CPDD (Fig. 2C and D), with the maximum stimulation by 10 μg/ml (5.9-fold above controls). Maximal ERK1/2 activation by 10 μg/ml CPDD correlated with the decreased toxicity of CPDD at 10 μg/ml, as compared with 5 μg/ml (Fig. 1). This finding suggests ERK1/2 involvement in the defensive reaction to damage. Importantly, TPDD (10 μg/ml) did not increase ERK1/2 activity (Fig. 2E). ERK1/2 activation by CPDD was not affected by cycloheximide (1 μg/ml) (Fig. 2F). These data suggest that the ERK1/2 response is triggered by DNA damage and does not involve protein synthesis.

CPDD-mediated Activation of ERK1/2 Supports Neuronal Survival—Pharmacological inhibitors of the ERK1/2 pathway, PD98059 or SL327, effectively abolished ERK1/2 activation by CPDD (Fig. 3, A and D). Therefore, we used these compounds to determine the effect of the ERK1/2 response on CPDD-induced cell death. Consistent with our previous observations (17), neither SL327 (50 μM) nor PD98059 (40 μM) significantly affect basal apoptosis in cortical neurons (Fig. 3, B and E). By 24 h, cells exposed to either 5 or 10 μg/ml CPDD showed a significant increase of apoptosis upon co-treatment with SL327 (28.3% at 10 μg/ml CPDD versus 57.8% at SL327 + 10 μg/ml of CPDD; p < 0.001) (Fig. 3B). Interestingly, the CPDD concentration dependence of the SL327 effect on apoptosis correlated with the concentration dependence of CPDD-mediated ERK1/2 activation (Fig. 2, C and D). PD98059 also increased apoptosis in-
produced by CPDD (Fig. 3E, p < 0.001). In addition, SL327 (50 μM) or PD98059 (40 μM) further reduced neuronal viability after CPDD treatment (Fig. 3, C and F). To complement the pharmacological approach, we studied the effects of a dominant-negative mutant form of the ERK1/2 activator, MKK1 (MKK1dn) (20), on CPDD-induced apoptosis. Cortical neurons were transfected with expression plasmids for either wild type (wt) or dominant-negative (dn) mutant form of MKK1. The empty expression vector for MKK1 (pCEP4, Vector) was used as an additional control. Forty-eight hours after transfection, neurons were treated with either 10 μg/ml CPDD or vehicle (0.2% Me2SO). After 24-h exposure to the drug, cells were fixed and immunostained to detect transfected gene products. In addition, cell nuclei were counterstained with Hoechst 33258 to detect apoptosis. G and H, representative photomicrographs depicting a transfected neuron that co-expresses β-galactosidase (β-gal) and either the wild type (wt) or dominant-negative (dn) mutant form of MKK1. The empty expression vector for MKK1 (pCEP4, Vector) was used as an additional control. Forty-eight hours after transfection, neurons were treated for 24 h with either vehicle (0.2% Me2SO) or CPDD (10 μg/ml). After 24-h exposure to the drug, cells were fixed and immunostained to detect transfected gene products. In addition, cell nuclei were counterstained with Hoechst 33258 to detect apoptosis. G and H, representative photomicrographs depicting a transfected neuron that co-expresses β-galactosidase (β-gal) and either the wild type (wt) or dominant-negative (dn) mutant form of MKK1. The empty expression vector for MKK1 (pCEP4, Vector) was used as an additional control. Forty-eight hours after transfection, neurons were treated for 24 h with either vehicle (0.2% Me2SO) or CPDD (10 μg/ml). In vehicle-treated cells, apoptosis was unaffected by any of the transfected plasmids (average of 15.5%, Fig. 3I). In contrast, CPDD caused apoptosis in more cells expressing MKK1dn (46.5% versus 26% in MKK1wt-transfected neurons; p < 0.001, Fig. 3I). Therefore, inhibition of ERK1/2 increased apoptotic cell death induced by CPDD.

Finally, we studied the effects of PD98059 on CPDD-mediated caspase activation, a marker of apoptotic cell death. 5 but not 10 μg/ml CPDD significantly activated caspases at 12 h (1.9-fold above control, Fig. 3J). When PD98059 was combined with 10 μg/ml CPDD, caspase activation occurred (1.7-fold
above control (Fig. 3J). This result confirms that ERK1/2 activation by CPDD suppresses neuronal apoptosis.

CPDD Activates ERK1/2 through NMDA Receptors—ERK1/2 can be activated in cortical neurons by the glutamate-triggered influx of Ca^{2+} ions through an open NMDAR channel (29). Because moderate activity of NMDA is implicated in anti-apoptotic signaling (5, 30), we tested for NMDAR involvement in the protective ERK1/2 activation by CPDD.

The CPDD-mediated increase of ERK1/2 activity (6.1-fold above controls) was reduced by the NMDAR antagonists kynurenic acid plus MgCl_{2}, MK-801, or APV (1.4-, 0.5-, or 0.6-fold above controls, respectively; Fig. 4A). Also, co-treatment with the cytosolic Ca^{2+} chelator BAPTA/AM significantly reduced ERK1/2 activation by CPDD (7.1- versus 3.7-fold above controls; Fig. 4A). Furthermore, NMDA receptor antagonists increased apoptosis induced by 24-h treatment with CPDD (30.3% at 10 μg/ml of CPDD versus 51.4% at 10 μg/ml CPDD plus 10 μM MK-801; p < 0.01) (Fig. 4, B and C). Inhibitors of AMPA/KΑ receptor antagonists (CNQX or NBQX), did not affect the ERK1/2 response to CPDD (Fig. 4D). Collectively, the data suggest that the defensive ERK1/2 activation by CPDD is mediated through NMDAR.

CPDD Increases NMDAR Signaling—NMDAR can be activated by increased concentrations of its ligands, glutamate or NMDA (Fig. 5A). If increased concentrations of NMDAR ligands are responsible for CPDD activation of ERK1/2, one would expect that conditioned media from cells that are exposed to CPDD would produce ERK1/2 activation in untreated neurons. However, ERK1/2 was not activated by conditioned media collected from cells treated with 10 μM CPDD (Fig. 5B). Consistently, media concentrations of the NMDAR ligand, glutamate, and its co-ligand, glycine (3), did not significantly increase after CPDD treatment (Fig. 5C). Therefore, it seems unlikely that activation of NMDAR by CPDD is caused by an elevated release of the NMDAR ligands.

An alternative possibility is that CPDD increases the neuronal responses to basal levels of NMDAR stimulation. Indeed, we found that neurons that were pretreated with 5 μg/ml CPDD for 24 h demonstrated increased ERK1/2 responses to NMDA (Fig. 5, D and E). In cells that were not exposed to CPDD, a 5-min treatment with NMDA evoked a concentration-dependent ERK1/2 activation that appeared at 20 μM (6.1-fold above control) and declined at 50 or 100 μM (3.6- or 2.3-fold above control, respectively; Fig. 5, D and E). Neurons that were pretreated with 5 μg/ml CPDD for 24 h responded with significantly enhanced ERK1/2 activation (p < 0.01) which was present at 10 μM NMDA (5.3-fold above control), reached maximal levels at 50 μM (7.6-fold above control), and slightly declined at 100 μM (4.9-fold above control; Fig. 5, D and E). Therefore, it appears that CPDD increases the threshold of NMDAR stimulation that is required to activate ERK1/2 and inhibits desensitization of the ERK1/2 response following more intense NMDAR stimulation. These data indicate that CPDD enhances intracellular signaling by NMDAR.

PARP Activity Contributes to CPDD-mediated ERK1/2 Activation—ERK1/2 activation apparently was caused by CPDD-induced DNA strand breaks (Fig. 2E) and was dependent upon NMDAR (Figs. 4 and 5). Thus, augmentation of NMDAR signaling in CPDD-treated neurons may be secondary to DNA damage. It has been proposed that a DNA damage-response enzyme, PARP, may regulate NMDAR in neurons (15). Therefore, we have evaluated the possibility that the CPDD-mediated increase in NMDAR signaling is mediated by PARP. Indeed, we observed increased PARP activity after CPDD treatment, indicated by the elevated polyribosylation of neuronal nuclear proteins (Fig. 6A). Also, cellular NAD+ content that is reduced during PARP activation (31) significantly decreased after CPDD (Fig. 6B). The NAD+ decrease preceded the CPDD-induced reduction of neuronal survival (Fig. 6C). These data indicate that CPDD activates PARP.

To evaluate whether PARP activation can contribute to the protective ERK1/2 activation by CPDD, we studied the effects of the PARP inhibitors, 3-ABA (5 mM) or PHEN (50 μM), on CPDD-induced ERK1/2 activation or cell death. 3-ABA or...
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PHEN reduced ERK1/2 activation in response to CPDD (Fig. 6D) and increased neuronal apoptosis triggered by CPDD (Fig. 6E). Neuronal apoptosis in basal conditions was not affected by either 3-ABA or PHEN (data not shown). Also, the CPDD-mediated reduction in neuronal survival was enhanced in the presence of 3-ABA (Fig. 6F). Therefore, our data suggest that PARP contributes to protective ERK1/2 activation by CPDD.

To further support the idea that PARP activation contributes to the increased responsiveness of NMDAR in CPDD-treated neurons, we evaluated the effects of 3-ABA on ERK1/2 activation by NMDA in cells that were pretreated with CPDD (5 μg/ml). Interestingly, neurons pretreated with CPDD in the presence of 3-ABA did not show enhanced ERK1/2 signaling after stimulation with NMDA (Fig. 6G). PARP inhibition abolished ERK1/2 activation at 10 μM NMDA (5.9-fold versus 0.7-fold above control; Fig. 6G), decreased the activation level at 20 μM (10.2-fold versus 1.8-fold above control; Fig. 6G), and increased desensitization of ERK1/2 response at 100 μM NMDA (7.4-versus 1.3-fold above control; Fig. 6G). These data suggest that PARP enhances NMDAR-mediated ERK1/2 activation in CPDD-treated neurons.

The PARP-mediated increase in NMDAR signaling has been suggested to result from ATP depletion after increased NAD⁺ re-synthesis (31). However, we did not find a significant reduction of ATP levels until at least 12 h after CPDD addition (Fig. 6H). A moderate decrease of ATP content occurred at 24 h (79.1% of control values; p < 0.001). This alteration correlates with 82.4% cell survival found at 24 h after 10 μg/ml CPDD treatment (Fig. 6C). Therefore, the reduced ATP content most likely reflects cell loss rather than the energetic deprivation of living neurons. In summary, these data suggest that PARP regulates NMDAR-mediated ERK1/2 activation through an ATP depletion-independent mechanism.

**BDNF Reduces CPDD Toxicity by Activation of ERK1/2 Pathway**—A question can be raised whether neuroprotective agents that activate ERK1/2 would be able to reduce CPDD-mediated cell death. A 6-h treatment with 10 ng/ml BDNF activated ERK1/2 pathway in the absence or presence of CPDD (5 μg/ml) (Fig. 7A). BDNF also decreased CPDD-induced apoptosis from 48.5 to 22.0% (Fig. 7B, p < 0.001). This effect was abolished by 50 μM SL327 indicating that ERK1/2 is required for BDNF-mediated protection of CPDD-treated neurons.

To determine whether ERK1/2 activation is sufficient for BDNF-mediated protection, we used a constitutively active mutant form of the ERK1/2 activator, MKK1 (MKK1ca) (20). Cortical neurons were transfected with either MKK1ca or empty cloning vector, pCEP4. Forty-eight hours after transfection, neurons were treated for 24 h with 5 μg/ml CPDD. Neurons receiving MKK1ca were protected against CPDD-induced apoptosis (50.3% in pCEP4 versus 19.0% in MKK1ca-transfected cells; p < 0.001) (Fig. 7C). These results suggest that activation of ERK1/2 is both required and sufficient for BDNF to reduce CPDD-induced apoptosis in neurons.

**DISCUSSION**

In this study, we tested the possibility that ERK1/2 is activated by DNA damage to support survival of stressed neurons. Indeed, we observed that CPDD-induced apoptosis in rat primary cortical neurons was accompanied by anti-apoptotic ERK1/2 activation. We also identified PARP and NMDAR as
mediators of CPDD-induced ERK1/2 response (Fig. 8). Finally, we showed that ERK1/2 activation was both necessary and sufficient for BDNF-mediated protection against CPDD-induced apoptosis.

In our hands, CPDD induced cortical neuron apoptosis. CPDD was also shown to induce apoptosis in peripheral nervous system neurons of sensory and auditory systems (32, 33). Apoptotic death of these neuronal populations has been suggested as a mechanism of peripheral neurotoxicities of CPDD. Similarly, CPDD-induced cortical neuron apoptosis may underlie the robust central nervous system neurotoxicity observed after local delivery of CPDD to treat intracranial tumors (12).

CPDD activated ERK1/2 by increasing NMDAR signaling. CPDD increased NMDAR sensitivity to low concentrations of NMDA and also inhibited desensitization of ERK1/2 response after intense receptor stimulation. Furthermore, our results indicate that the mechanism of enhanced NMDAR signaling involves PARP activation. It has been suggested that the PARP-mediated increase of NMDAR signaling results from ATP depletion after increased NAD+ resynthesis (15, 31). However, we did not find a significant reduction of ATP levels in neurons that showed robust activation of ERK1/2. Consequently, the possible mechanisms of PARP-mediated NMDAR regulation in CPDD-treated cells may include posttranscriptional modifications, differential expression of NMDAR subunits, depolarization of the membrane through activity of other glutamate receptors, and, finally, enhanced coupling of NMDAR to ERK1/2.

In hippocampus, enhancement of the NMDAR response is produced by the primary increase of AMPA/KA receptor activity (3). This mechanism is unlikely to explain increased NMDAR signaling in CPDD-treated neurons because AMPA/KA receptor blockers did not affect ERK1/2 activation by CPDD. The alternative mechanism producing enhanced NMDAR signaling during development is increased expression of NMDAR subunits (34). However, we found no CPDD-induced increases in expression of the two NMDAR subunits, NR1 or NR2B, whose expression is detectable in cultured cortical neurons. Therefore, it remains to be resolved which mechanism contributes to CPDD/PARP-mediated regulation of NMDAR.

It is well established that excessive stimulation of NMDARs results in excitotoxic neuronal death (6). However, the toxic abilities of NMDARs are not directly proportional to their ac-

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2 A. Gozdz and M. Hetman, unpublished data.
including cancer cells, embryonic mouse tissue, and rat hippocampal neurons (37–39). For instance, in a rat global ischemia model, CA1 neuron loss was enhanced by the PARP inhibitor 3-ABA (38). On the other hand, other reports showed that intense PARP activation depletes cellular energy stores and induces necrosis (15). The discrepancies between observations suggesting that PARP mediated protection and those indicating that deleterious PARP effects can be attributed to the differences in the intensity of PARP activation. For example, PARP activation after CPDD resulted in a 20% reduction of NAD+ levels at 12 h after treatment and undetectable changes in ATP levels. Similarly, protective PARP activation by global ischemia did not significantly reduce NAD+ levels (38). In contrast, deleterious PARP activation after treatment with an alkylating drug, N-methyl-N’-nitro-N-nitrosoguanidine, decreased NAD+ levels by at least 80% within 60 min (40). Therefore, PARP activation by CPDD may be insufficient to produce pro-necrotic energy depletion. In conclusion, data presented here suggest that, as in the case of NMDAR signaling, moderate PARP activation also promotes neuronal survival.

Defensive activation of ERK1/2 by CPDD is not a unique stress-activated compensatory response in neurons. In fact, it has been revealed that NF-kB activated by various forms of stress promotes neurons from death (41). Interestingly, Gonzalez-Zulueta et al. (42) reported that transient ischemic stimulation activated ERK1/2 by NMDAR. Inhibition of this signaling increased sensitivity to a subsequent ischemic insult. Therefore, ERK1/2 activation by NMDAR may be used by neurons to resist various forms of injury.

In summary, CPDD-induced genotoxic stress activated an anti-apoptotic ERK1/2 response. This effect was mediated by PARP, which enhanced NMDAR signaling in CPDD-treated neurons. Therefore, our results identify a novel compensatory circuit to defend central nervous system neurons against genotoxic apoptosis. This defensive pathway couples DNA damage through PARP and NMDAR to ERK1/2 activation. Moreover, our data suggest that PARP regulates NMDAR signaling using a novel, ATP decline-independent mechanism. Our data also indicate the possibility of potentially toxic interactions between clinically used NMDAR antagonists, including ketamine or memantine, and genotoxic therapies that target tumors.

Acknowledgments—We thank Drs. Scott Whittomere, Theo Hagg, Richard Benton, Jennifer Glick, and Jane Cavanaugh for critical reading of this manuscript.

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