Neurons Efficiently Repair Glutamate-induced Oxidative DNA Damage by a Process Involving CREB-mediated Up-regulation of Apurinic Endonuclease 1

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Jenq-Lin Yang‡§, Takashi Tadokoro‡, Guido Keijzers§, Mark P. Mattson‡,§, and Vilhelm A. Bohr‡,§

From the Laboratories of‡ Molecular Gerontology and §Neurosciences, National Institute on Aging Intramural Research Program, Baltimore, Maryland 21224

Glutamate, the major excitatory neurotransmitter in the brain, activates receptors coupled to membrane depolarization and Ca\(^{2+}\) influx that mediates functional responses of neurons including processes such as learning and memory. Here we show that reversible nuclear oxidative DNA damage occurs in cerebral cortical neurons in response to transient glutamate receptor activation using non-toxic physiological levels of glutamate. This DNA damage was prevented by intracellular Ca\(^{2+}\) chelation using non-toxic physiological levels of glutamate. Glutamate receptor activation, nor is it known if and how neurons might respond to such glutamate-induced DNA damage. Ca\(^{2+}\) channels. Ca\(^{2+}\) then activates kinases such as Ca\(^{2+}\)/calmodulin-dependent kinases (CaMK) and mitogen-activated protein kinases, resulting in activation of transcription factors such as cAMP-response element-binding protein (CREB), which mediate long lasting changes in neuronal structure and function (2–5). Glutamate receptor activation also stimulates an increase in mitochondrial respiration (electron transport) to generate the ATP necessary to drive the activity of ion-motive ATPases that restore ion gradients across cellular membranes (6). Mitochondrial Ca\(^{2+}\) uptake and increased mitochondrial respiration can result in production of the damaging free radical superoxide (7, 8), as well as mitochondrial membrane permeability changes that trigger cell death, a process called excitotoxicity (9, 10).

Damage to DNA in neurons occurs early during excitotoxicity (11, 12) and may be a pivotal event in cell death because selective inhibition or knockdown of the DNA damage response proteins p53 (13, 14) and PARP-1 (15, 16) can prevent glutamate-induced neuronal death. Ca\(^{2+}\) and mitochondrial-derived superoxide are believed to play key roles in glutamate-induced DNA damage and cell death because PARP-1 activation is mediated by Ca\(^{2+}\) and mitochondrial reactive oxygen species (17), and because mitochondrial Mn-SOD and exogenous antioxidants protect neurons against excitotoxicity (18–20). However, it is not known if oxidative lesions to nuclear DNA occur in response to non-pathological subtoxic levels of glutamate receptor activation, nor is it known if and how neurons might respond to such glutamate-induced DNA damage.

Base excision repair (BER) is the primary DNA repair pathway for removal of small base modifications such as alkylation, deamination, and oxidation (21, 22). This process occurs both in the nucleus and in mitochondria. By far most information on the molecular mechanisms of DNA damage and repair comes from studies of non-neuronal cells, and the extent of DNA damage and repair in neurons under physiological and pathological conditions is largely unknown (23). Several BER enzymes, including DNA polymerase β, DNA glycosylases, and

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\(^4\) The abbreviations used are: CREB, cAMP-response element-binding protein; BER, base excision repair; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, tetraacetoxymethyl ester; CsA, cyclosporin A; MnTMPyP, manganese(III)-5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine chloride tetrakis(methochloride); FPG, formamidopyrimidine DNA glycosylase; PTP, permeability transition pore; OGG1, 8-oxoguanine DNA-glycosylase 1; NEL1, nei endonuclease VIII-like 1; UDG, uracil DNA glycosylase; CaMK, Ca\(^{2+}\)/calmodulin-dependent protein kinase; APE1, apurinic endonuclease 1; PARP-1, poly(ADP-ribose) polymerase 1; KD, knockdown.

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\(^{†}\) Present address: Center for Health Aging, University of Copenhagen, Copenhagen, Denmark.

\(^{‡}\) Both authors contributed equally to this work.

\(^{§}\) To whom correspondence should be addressed: 251 Bayview Blvd., Ste. 100, Baltimore, MD 21224-8626. Fax: 410-535-8157; E-mail: bohrv@grc.nia.nih.gov.

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apurinic/apyrimidinic endonuclease 1 (APE1) are expressed in brain cells (24–28), but their roles in repair of oxidative DNA lesions in neurons are relatively poorly characterized.

APE1 is a multifunctional protein that plays an essential role in the DNA BER pathway in response to oxidative DNA damage. APE1 is also a redox effector factor-1, a reductive activator that responds to several transcription factors including AP-1, p53, NF-κB, HIF-1α, and PAX5 (29–31). APE1 also interacts with the calcium-responsive element (nCaRE) and is a represor of the parathyroid hormone gene promoter and its own promoter (32, 33). Earlier studies reported that APE1 mRNA and protein are induced under oxidative stress (32, 34, 35).

In the present study we show that transient activation of glutamate receptors in cerebral cortical neurons results in oxidative DNA damage that is efficiently repaired. Glutamate-induced DNA damage results from Ca²⁺-mediated mitochondrial superoxide production, and is repaired in a process involving CREB-mediated up-regulation of APE1 expression. Protein levels and activities of common oxidative lesion removing glycosylases and DNA polymerase β were not affected by glutamate. Our results suggest that APE1 is the key enzyme involved in this glutamate-induced DNA repair response.

EXPERIMENTAL PROCEDURES

Cerebral Cortical Cell Cultures and Experimental Treatments— Cultures of primary cortical neurons were prepared from 18-day-old embryos of Sprague-Dawley rats using methods described previously (36). Cells were plated in 60-mm diameter plastic dishes on a polyethyleneimine substrate in minimum essential medium with Earle's salts supplemented 10% with heat-activated fetal bovine serum and containing 1 mM glutamine, 1 mM pyruvate, 20 mM KCl, and 26 mM sodium bicarbonate (pH 7.2). Following cell attachment (3–4 h, post-plating), the plating medium was replaced with culture maintenance medium (Neurobasal medium containing B-27 supplements (Invitrogen), 1 mM HEPES, 2 mM L-glutamine, and 0.001% gentamycin sulfate). All experiments were performed in 8- to 10-day-old cultures in which >95% of the cells were neurons. Cells that had been in culture for 7–9 days were treated with 20 μM glutamate for 10 min at 37 °C, washed twice with fresh Neurobasal medium, incubated for various periods in culture maintenance medium, and then harvested for biochemical assays.

BAPTA-AM (1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetracetoxymethyl ester) (AnaSpec), cyclosporin A (CsA) (Sigma), MnTMPyP (manganese(III)-5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine chloride tetramethyl ester (methochloride) (Sigma), and KN-93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) (Calbiochem) were prepared as concentrated stocks in Neurobasal medium. The cortical neurons were pretreated with BAPTA-AM for 30 min, or with CsA, MnTMPyP, and KN-93 for 1 h, prior to exposure to glutamate (BAPTA-AM, CsA, and MnTMPyP were also present in the post-glutamate incubation medium). KN-93 was eliminated with glutamate after treatment.

Alkali Single-cell Gel Electrophoresis (Comet Assay)—These methods were similar to those described previously (37). Cultures were washed with cold PBS to remove dead cells and debris. Cells (~1 million per culture) were harvested in 1 ml of PBS and gently trituated. A 10-μl aliquot of the cells (~10,000 cells) was mixed with 75 μl of pre-warmed 0.5% low melting point agarose and the cells were spread onto an agarose-coated glass slide. A coverslip was added to the slide and the slide was placed in an ice-chilled aluminum tray for 5 min. The coverslip was removed, another 75 μl of low melting point agarose was added, a coverslip was added, and the slide was placed in the chilled aluminum tray for 5 min. The coverslip was removed and the slide incubated in lysis buffer (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, and 1% Triton X-100) for at least 4 h (or overnight). The slides were then washed (three 10 min washes) with neutralization buffer (0.4 mM Tris, pH 7.4), followed by a 10-min incubation with REC buffer (10 mM HEPES-KOH, 100 mM KCl, 10 mM EDTA, 0.1 mg/ml BSA, pH 7.4). Each slide was then incubated with 8 units (in 100 μl volume) of formamidopyrimidine DNA glycosylase (FPG) (New England Biolabs) at 37 °C for 1 h. The FPG-treated slides were rinsed with Alkali buffer (300 mM NaOH and 1 mM EDTA; pH 12.1) for 20 min to denature DNA. Electrophoresis was then performed at 25 volts for 15 min, and the slides were dehydrated in 100% ethanol for 5 min, and then stained with ethidium bromide (10 ng/ml).

DNA Repair Enzyme Incision Activity Assays—The cultured neurons were rinsed with PBS, scraped into 1 ml of PBS, and pelleted by centrifugation. Cells were then either extracted immediately or stored at −80 °C for future use. Cells were extracted by re-suspending in buffer I (10 mM Tris-HCl, 200 mM KCl, pH 7.8) and adding an equal volume of buffer II (10 mM Tris-HCl, 600 mM KCl, 2 mM EDTA, 40% (v/v) glycerol, 0.2% (v/v) Nonidet P-40, 2 mM dithiothreitol (DTT) 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1× protease inhibitor mixture (Roche Applied Science; pH 7.8)). The lysate was briefly sonicated to completely disrupt cell and nuclear membranes. A 16,000 × g centrifugation at 4 °C for 10 min was performed to remove cellular debris and DNA. The cell extract was dialyzed overnight with buffer III (25 mM HEPES-KOH, 100 mM KCl, 12 mM MgCl₂, 1 mM EDTA 17% glycerol, 1 mM DTT, pH 8.0) at 4 °C. A brief centrifugation was employed to remove precipitation after dialysis. The amounts of total protein used for incision activity assays for each enzyme were as follows: 2.5 ng for APE1, 2 μg for UDG, 6 μg for OGG1 and NEIL1. For polymerase β gap filling we used 0.5 μg of total protein. The procedures for incision assays were described previously (27, 28).

Quantitative Real Time PCR—Approximately 1 million cells were lysed in 1 ml of TRIzol™ (Invitrogen), 300 μl of chloroform (Sigma) was added and the solution was vortexed for 30 s. The tube was centrifuged at 8,000 × g for 30 min at room temperature. The upper aqueous layer was transferred and mixed with an equal volume of 70% ethyl alcohol. A RNeasy purifica-
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Figure 1. Glutamate induces reversible oxidative DNA damage in cerebral cortical neurons. A and B, cortical cultures were exposed to 20 μM glutamate for 10 min, then, after 6 or 24 h the cells were harvested and comet assays were performed in the presence of no enzyme, FPG, or T4 endonuclease. Panel A shows representative images of nuclear DNA. First row of panel A is the control group, without glutamate treatment, pre-treated with FPG or T4 endo. The second and third rows of panel A are 6 and 24 h after glutamate treatment, respectively. Panel B shows the results of quantitative measurements of DNA damage (Olive Tail Moment). C, cellular ATP levels at the indicated time points after a 10-min exposure to 20 μM glutamate; values for glutamate-treated cultures are normalized to the value for untreated control cultures. Values for treated cultures are expressed as a percentage of the value for untreated control cultures (100%) (mean ± S.D.; *, p < 0.05; **, p < 0.01; ***, p < 0.001).

RESULTS

Glutamate-induced Oxidative DNA Damage Is Repaired Efficiently in Cortical Neurons—Previous studies have shown that glutamate exerts dose-dependent effects on neurons in cell culture and in vivo, with low/transient doses promoting cell survival and modifying dendritic architecture and synaptic plasticity, whereas high/sustained doses cause cell death (3, 9, 38, 39). There is a large body of literature describing the consequences of toxic doses of glutamate; whereas much less is known about the effects of lower, physiological doses. In preliminary experiments we found that when rat cortical neurons were exposed to a relatively low concentration of glutamate (20 μM) for a 10-min period in culture, there was no significant cell death during the subsequent 24-h culture period (data not shown).

FPG is a DNA glycosylase with AP-lyase activity; it specifically recognizes and removes oxidized bases from DNA including 8-oxoguanine, 8-oxoadenine, formamidopyrimidine (FAPY)-guanine, FAPY-adenine, 5-hydroxyecytosine, 5-hydroxyaminouracil, thymine glycol, 5-(N-methyl-5-oxo)uracil, and uracil glycol. This N-glycosylase activity is important because there is an increasing body of literature showing that S-adenosylmethionine (SAM) dependent DNA methyltransferases (DNMT) use a similar mechanism to methylate DNA. This process is called DNA methylation and can be used to silence a gene, a process that is important in development and for regulating gene expression. FPG can also remove oxidized bases from DNA such as 8-oxoguanine, 8-oxoadenine, formamidopyrimidine (FAPY)-guanine, FAPY-adenine, 5-hydroxyecytosine, 5-hydroxyaminouracil, thymine glycol, 5-(N-methyl-5-oxo)uracil, and uracil glycol. This N-glycosylase activity is important because there is an increasing body of literature showing that S-adenosylmethionine (SAM) dependent DNA methyltransferases (DNMT) use a similar mechanism to methylate DNA. This process is called DNA methylation and can be used to silence a gene, a process that is important in development and for regulating gene expression.

ThermoScientific Open Biosystems. The scrambled control shRNA (5'-CTTACAGTTAAGTCCGCTC-6271-CCGGAGGAGGAGCGACCTTA-ACCTTAGG-3') was obtained from Addgene. All shRNAs were incorporated into the plKO.1 vector. HEK 293T cells were transfected with shRNA, packaging, and envelope plasmids simultaneously using FuGENE 6 (Roche Applied Science) to produce lentiviral particles. The 3-day in vitro rat cortical neurons were infected with lentivirus using procedures described in the Addgene plasmid 10878 protocol.

Immunobots—Cultured neurons were extracted in RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1× protease inhibitor mixture (Roche) and 50 mM Tris; pH 8.0) and the total protein concentration of cell extracts was determined using a BCA™ protein assay kit (Pierce). Thirty micrograms of total protein from each sample was applied for immunoblotting. Precast 12% SDS-polyacrylamide gels and PVDF membrane filter paper were purchased from Invitrogen. The washing buffer was 0.1% Tween 20 in Tris-buffered saline (20 mM Tris and 150 mM NaCl; pH 7.4) and the blocking buffer was 5% skim milk (Bio-Rad) in washing buffer. The dilution factors for the primary antibodies were: APE1 (Santa Cruz), 1:500; UDG (Santa Cruz), 1:200; and pCREB (Santa Cruz), 1:200; and pCREB (Santa Cruz), 1:500. The secondary antibodies (Vector Laboratories) were diluted 1:10,000.

Statistics—Statistical comparisons were made using a two-tail Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). All values shown in graphs are the mean ± S.D. of determinations in at least 3 separate experiments.
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and 5-hydroxyuracil. Comet assays demonstrated significant nuclear DNA fragmentation in cortical neurons at 6 h, but not at 24 h after exposure to glutamate (Fig. 1, A and B). As a control, we assayed for another lesion, pyrimidine dimers, that are normally introduced by UV light and are detected by the T4 endo V glycosylase. As expected, neurons that had been exposed to glutamate did not exhibit pyrimidine dimers at 6 or 24 h as indicated by lack of a comet tail in samples treated with this glycosylase (Fig. 1, A and B). These results indicate that glutamate specifically produces oxidative lesions in the nuclear DNA. Because DNA repair enzyme activities require ATP, and excessive glutamate receptor activation can result in ATP depletion (40), we measured cellular ATP levels in neurons that had or had not been exposed to 20 μM glutamate. During the first 20–30 min of glutamate exposure the ATP level was reduced by ~40%; ATP levels subsequently returned to baseline levels 6 h after exposure to glutamate suggesting that the neurons maintain ATP in amounts sufficient to support DNA repair enzyme activities (Fig. 1C).

Glutamate-induced DNA Damage Is Mediated by Ca^{2+} and Mitochondrial Superoxide Radicals—Ca^{2+} influx through NMDA receptors and voltage-gated Ca^{2+} channels mediates physiological responses to glutamate including cell survival and synaptic plasticity, but Ca^{2+} also mediates excitotoxic cellular damage (9). To determine whether Ca^{2+} is involved in glutamate-induced oxidative DNA damage, we treated neurons with the intracellular Ca^{2+} chelator BAPTA-AM, exposed them to glutamate, and then quantified the oxidative DNA damage. BAPTA-AM treatment completely prevented glutamate-induced oxidative DNA damage (Fig. 2, A and B). No DNA fragmentation was observed in glutamate-treated neurons when FPG was not applied in the comet assay (Fig. 2B), indicating that glutamate did not induce nonspecific endonuclease activity.

Glutamate-induced Ca^{2+} influx can elicit several changes in mitochondria including membrane depolarization, superoxide production, and increased permeability of mitochondrial membranes (41–43). To determine whether mitochondrial superoxide anion radicals were involved in glutamate-induced DNA damage, we pretreated neurons with the mitochondrial superoxide dismutase mimetic agent MnTMPyP and then exposed them to 20 μM glutamate for 10 min. Oxidative DNA damage was reduced to about 50% at 1 and 6 h after exposure to glutamate in MnTMPyP-treated neurons compared with neurons not treated with MnTMPyP (Fig. 2C), suggesting a key role for mitochondrial superoxide in formation of the DNA damage. It was recently reported that bursts of mitochondrial superoxide...
Increased in Response to Glutamate Receptor Activation
damage formation.
though considerable progress has been made in identifying
vented glutamate-induced DNA damage (Fig. 2)
that inhibition of the PTP with cyclosporin A completely pre-
and primary cultured hippocampal neurons (44). We found
drial permeability transition pores (PTP) in cardiac myocytes
 generation are triggered by transient openings of mitochon-
drial permeability transition pores (PTP) in cardiac myocytes and primary cultured hippocampal neurons (44). We found
that inhibition of the PTP with cyclosporin A completely pre-
vented glutamate-induced DNA damage (Fig. 2D), consistent
with a role for PTP-mediated superoxide production in DNA
damage formation.
APE1 Expression and AP Site Incision Activity Are Selectively Increased in Response to Glutamate Receptor Activation—Although considerable progress has been made in identifying
protein and mRNA in control and glutamate-stimulated neu-
rons and found that glutamate induced a statistically significant
increase in the amount of APE1 protein (by 6 h) (Fig. 4
involving Ca2+
pathway up-regulated in response
to glutamate treatment. The activities of OGG1
and NEIL1 were not changed after glutamate treatment.
The enzymatic activities and protein levels of uracil DNA glycosylase (UDG) (supplemental Fig. S1) and poly-
merase β (supplemental Fig. S2) were also unaffected by glutamate, although there was a trend toward increased polymerase β activity and protein level. The protein levels of OGG1 (supplemental Fig. S3) and ligase III (supplemental Fig. S4) were examined by immunoblotting and were also not affected by glu-
tamate. In contrast, the incision activity of APE1 was significantly increased within 1 h after stimula-
tion with glutamate, and remained elevated through 6 h, and then returned to baseline by 24 h (Fig. 4A). These results suggested that APE1 is the only protein in the BER pathway up-regulated in response to glutamate-induced oxidative DNA
lesions.

Evidence that Glutamate Induces APE1 Expression via a Pathway Involving Ca2+/Calmodulin-dependent Kinase and CREB—We next measured relative levels of APE1

FIGURE 3. Incision activity of OGG1 and NEIL1 are not significantly changed after glutamate treatment. The 8-oxoguanine and 5-hydroxycytosine lesions are mainly produced by oxidation and predominately removed by OGG1 and NEIL1, respectively. The results of biochemical and immunoblotting assays for incision activities of OGG1 (A) and NEIL1 (B) (upper panels; *, 32P-labeled 5′-end; the filled circle marks the 8-OxoG and 5-OHC lesion sites) were not affected by a 10-min pulse glutamate treatment. Values for treated cultures are expressed as a percentage of the value for untreated control cultures (100%).

A.
B.
C.

FIGURE 4. APE1 DNA incisional activity, and mRNA and protein levels, are elevated in cortical neurons in response to glutamate. Cortical cultures were exposed to glutamate for the indicated time periods and then harvested for analyses of APE1 DNA incision activity (A), APE1 protein levels (B), and APE1 mRNA levels (C). A, an example of an APE1 DNA incision assay (upper; *, 32P-labeled 5′-end; the filled circle marks the abasic site lesion) and measurements obtained using this assay (graph). B, an example of an APE1 protein immunoblot (upper) and the results of densitometric analysis of blots (graph). C, relative levels of APE1 mRNA as determined by quantitative RT-PCR analysis. Values for treated cultures are expressed as a percentage of the value for untreated control cultures (100%) (mean ± S.D.; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
consensus sequence (35, 48), consistent with the possibility that glutamate receptor activation induces CREB-mediated APE1 expression. Indeed, we found that levels of activated CREB (phospho-CREB) increased rapidly (within 15 min with a peak increase at 30 min) in neurons in response to glutamate receptor stimulation (Fig. 5A). Thus, CREB was activated many hours in advance of the glutamate-induced increase in APE1 levels (Fig. 4B). Because Ca^{2+}/calmodulin-dependent protein kinases (CaMK) mediate CREB activation in response to glutamate receptor stimulation, we asked whether inhibition of CaMK would prevent glutamate-induced APE1 expression. To this end, we pretreated neurons with the CaMK inhibitor KN-93 and then exposed them to glutamate. Phosphorylation of CREB in response to glutamate was significantly inhibited starting from 15 min until 24 h in neurons pretreated with KN-93 (Fig. 5B). Moreover, glutamate failed to increase APE1 levels in neurons treated with KN-93; APE1 levels remained unchanged during the first 3 h of exposure to glutamate, then decreased by 6 h (Fig. 5C).

We also employed lentiviral shRNA to knockdown CREB (CREB KD) (Fig. 6A) and found that the phosphor-CREB and APE1 protein levels were very low in CREB KD neurons (data not shown). The results from the comet assay showed that the repair of oxidative DNA lesions was deficient in CREB KD neurons compared with control neurons expressing scrambled shRNA. 24 h after exposure to glutamate (Fig. 6, B and C) CREB KD clearly inhibited BER activity causing unrepaired damage and accumulation of lesions. These results suggested that CREB KD reduces BER activity via up-regulation of APE expression. We then knocked down APE1 (APE1 KD) by lentiviral shRNA (Fig. 7A) and examined the BER repair efficiency in glutamate pulse-treated neurons by the comet assay (Fig. 7B). Quantification of the comet tails showed that APE1 KD neurons accumulated oxidative DNA damage (Fig. 7C) after 24 h. The BER activity was, as expected, very low due to lack of APE1. The results suggest that glutamate regulates APE1 expression by activation of CaMK, which stimulates CREB-mediated expression of APE1.

**DISCUSSION**

Our findings reveal that nuclear DNA in neurons is oxidatively modified in response to transient moderate activation of glutamate receptors. This is a novel observation that links synaptic transmission to DNA damage and its processing.
We detected an up-regulation of APE1 in response to glutamate receptor activation, which is likely involved in the repair of the oxidative DNA lesions. Extensive unrepaired nuclear DNA damage occurs in neurons during the process of excitotoxic cell death (11–16). In contrast, we found that a subtoxic transient glutamate exposure induces DNA damage evident for up to 6 h and is completely repaired by 24 h. We found that MnTMPyP prevents DNA damage caused by glutamate, suggesting that mitochondrial superoxide plays a pivotal role in the reversible DNA damage caused by physiological levels of glutamate receptor activation. The abilities of cyclosporin A and BAPTA-AM to prevent glutamate-induced DNA damage suggest a scenario in which glutamate induces Ca\(^{2+}\) influx resulting in opening of the mitochondrial membrane PTP and superoxide generation (Fig. 8). Previous studies have shown that elevated intracellular Ca\(^{2+}\) levels can induce mitochondrial superoxide production (41, 49) and that oxidative stress can trigger opening of the PTP (50). However, PTP opening can stimulate superoxide production in isolated mitochondria (51), and recent findings suggest that spontaneous bursts of mitochondrial superoxide occur in response to transient openings of mitochondrial PTP in excitable cells (44). Our findings are consistent with PTP-mediated superoxide production causing reversible DNA damage induced by transient activation of glutamate receptors.

The increased APE1 expression and DNA incision activity in response to glutamate receptor activation is, to our knowledge, the first evidence that a neurotransmitter can stimulate DNA repair. Although it increased the APE1 activity, glutamate did not affect the repair activity levels of UDG, OGG1, and NEIL1, and also did not significantly affect the gap-filling activity level of polymerase \(\beta\). APE1 is the major eukaryotic apurinic/apyrimidinic endonuclease involved in the repair of oxidative DNA lesions (52). APE1 is also an essential BER enzyme that plays a key role in the DNA repair process in both nuclei and mitochondria. Studies of cancer cells have shown that APE1 expression is increased in response to oxidative stress (53, 54), but is decreased by

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**FIGURE 6.** Depletion of CREB using RNA interference technology abolishes the ability of neurons to repair DNA damaged as a result of glutamate receptor activation. A, Western blot showing that CREB levels are greatly reduced in neurons expressing shRNA directed against the CREB mRNA compared with neurons expressing a scrambled control shRNA. B, images of nuclear DNA from neurons expressing CREB shRNA or scrambled control shRNA at 6 or 24 h after a 10-min exposure to 20 \(\mu\)M glutamate. C, results of quantitative measurements of DNA damage demonstrated that neurons expressing scrambled control shRNA recovered from damage, whereas oxidative lesions in neurons expressing CREB shRNA were not repaired within 24 h (mean \(\pm\) S.D.; ***, \(p < 0.001\)).

**FIGURE 7.** Depletion of APE1 using RNA interference technology results in accumulation of glutamate-induced oxidative DNA damage. A, Western blot showing that APE1 levels are greatly reduced in neurons expressing shRNA directed against the APE1 mRNA compared with neurons expressing a scrambled control shRNA. B, images of nuclear DNA from neurons expressing APE1 shRNA or scrambled control shRNA at 6 or 24 h after a 10-min exposure to 20 \(\mu\)M glutamate. C, results of quantitative analysis of DNA damage demonstrated that neurons expressing scrambled control shRNA were recovered from damage, whereas oxidative lesions in neurons expressing APE1 shRNA were not repaired within 24 h (mean \(\pm\) S.D.; ***, \(p < 0.001\)).
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enzymes, including XRCC1, polymerase β, and ligase III have also been suggested to play neuroprotective roles (58, 59).

Glutamate receptor-mediated Ca\(^{2+}\) influx regulates major structural and functional changes in neurons including dendrite outgrowth/retraction and synaptic plasticity (1, 3). We found that a transient moderate stimulation of the glutamate receptors induced Ca\(^{2+}\)-mediated oxidative DNA damage. This damage was repaired in cortical neurons, suggesting the possibility that DNA damage occurs in response to physiological activation of glutamate receptors. Superoxide is generated during normal spontaneous glutamate-mediated synaptic activity and, indeed, data suggest that superoxide plays an important role in synaptic plasticity and in learning and memory (41, 60). Moreover, some evidence suggests a key role for mitochondrial PTP opening in synaptic plasticity (61). It is therefore possible that oxidative DNA damage occurs in response to the increased excitatory synaptic transmission involved in learning and memory processes. Our findings suggest that physiological levels of glutamate receptor activation can induce APE1 expression via a CaMK- and CREB-mediated pathway to elevate BER incision activity. It has been demonstrated that transcriptional activation of the APE1 gene occurs in response to oxidative stress (34, 35). Another group reported that Ca\(^{2+}\) influx through NMDA receptors or calcium channels leads to multiple downstream signaling pathways, one of them involving the Ca\(^{2+}\)-dependent phosphorylation of the transcription factor CREB (2). CaMKs mediate CREB phosphorylation to regulate downstream gene expression in hippocampal neurons (48, 49). Knockdown of CREB clearly affected BER efficiency and caused increased accumulation of nuclear oxidative DNA damage. This effect of CREB deficiency resulted from impaired up-regulation of APE1 expression. Taken together with our findings, the available information suggests the APE1 expression is regulated via a Ca\(^{2+}\)-mediated CaMK-CREB signaling pathway.

Our findings suggest that normal amounts of glutamate receptor stimulation may elevate oxidative DNA repair capability in neurons thereby preventing the accumulation of potentially harmful or lethal amounts of DNA damage. Compromised DNA repair mechanisms could therefore render neurons vulnerable to glutamate receptor-mediated dysfunction and death. Indeed, patients with genetic defects in DNA repair often exhibit major neurodegenerative phenotypes (63) and DNA damage occurs in neurodegenerative disorders such as Alzheimer disease in which overactivation of glutamate receptors is implicated (15, 27, 62, 64). A better understanding of the signaling pathways that enhance DNA repair may therefore lead to novel approaches for protecting neurons against injury and neurodegenerative disorders.

REFERENCES

1. Bliss, T. V., and Collingridge, G. L. (1993) Nature 361, 31–39
2. Ghosh, A., Ginty, D. D., Bading, H., and Greenberg, M. E. (1994) J. Neurobiol. 25, 294–303
3. Mattson, M. P., Dou, P., and Kater, S. B. (1988) J. Neurosci. 8, 2087–2100
4. Soderling, T. R., Tan, S. E., McGlade-McCulloh, E., Yamamoto, H., and Fukunaga, K. (1994) J. Neurobiol. 25, 304–311
5. Wei, F., Vadakkan, K. L., Toyoda, H., Wu, L. J., Zhao, M. G., Xu, H., Shum, F. W., Jia, Y. H., and Zhuo, M. (2006) J. Neurosci. 26, 851–861
6. Yadava, N., and Nicholls, D. G. (2007) J. Neurosci. 27, 7310–7317
7. Chinpoulos, C., Tretter, L., Roza, A., and Adam-Vizi, V. (2000) J. Neurosci. 20, 2094–2103
8. Sengpiel, F., Preis, E., Kriegstein, J., and Prehn, J. H. (1998) Eur. J. Neurosci. 10, 1903–1910
9. Mattson, M. P. (2003) Neuronomolecular. Med. 3, 65–94
10. Reynolds, I. J. (1999) Ann. N.Y. Acad. Sci. 893, 33–41
11. Didier, M., Bursztajn, S., Adamiec, E., Passani, L., Nixon, R. A., Coyle, J. T., Wei, J. Y., and Berman, S. A. (1996) J. Neurosci. 16, 2238–2250
12. Gwag, B. J., Koh, J. Y., DeMaro, J. A., Ying, H. S., Jacquin, M., and Choi, D. W. (1997) Neuroscience 77, 393–401
13. Culmsee, C., Zhu, X., Yu, Q. S., Chan, S. L., Camandola, S., Guo, Z., Greig, N. H., and Mattson, M. P. (2001) J. Neurochem. 77, 220–228
14. Miller, F. D., Poznai, C. D., and Walsh, G. S. (2000) Cell Death Differ. 7, 880–888
15. Kruman, I. L., Culmsee, C., Chan, S. L., Kruman, Y., Guo, Z., Penix, L., and Mattson, M. P. (2000) J. Neurosci. 20, 6920–6926
16. Mandir, A. S., Poitras, M. F., Berliner, A. R., Herr ing, W. J., Guastella, D. B., Feldman, A., Poirier, G. G., Wang, Z. Q., Dawson, T. M., and Dawson, V. L. (2000) J. Neurosci. 20, 8005–8011
17. Duan, Y., Gross, R. A., and Sheu, S. S. (2007) J. Physiol. 585, 741–758
18. Keller, J. N., Kindy, M. S., Holtsberg, F. W., St. Clair, D. K., Yen, H. C., Germeyer, A., Steiner, S. M., Bruce-Keller, A. J., Hutchins, J. B., and Mattson, M. P. (1998) J. Neurosci. 18, 687–697
19. Li, Y., Copin, J. C., Reola, I. F., Calaguì, B., Gobbel, G. T., Chen, S. F., Sato, E., Epstein, C. J., and Chan, P. H. (1998) Brain Res. 814, 164–170
20. Uz, T., Giusti, P., Franceschini, D., Kharlamos, A., and Manev, H. (1996) Neuroscience 73, 631–636
21. Baute, J., and Depicker, A. (2008) Crit. Rev. Biochem. Mol. Biol. 43, 239–276
22. Wilson, D. M., 3rd, and Bohr, V. A. (2007) DNA Repair 6, 544–559
23. Englander, E. W. (2008) Mech. Ageing Dev. 129, 475–482
24. Imam, S. Z., Karahalil, B., Hogue, B. A., Souza-Pinto, N. C., and Bohr, V. A. (2006) Neurobiol. Aging 27, 1129–1136
25. Raffoul, J. J., Cabelof, D. C., Nakamura, J., Meira, L. B., Friedberg, E. C., and Heydari, A. R. (2004) J. Biol. Chem. 279, 18425–18433
26. Wei, W., and Englander, E. W. (2008) J. Neurochem. 107, 734–744
27. Weissman, L., Jo, D. G., Sersenlen, M. M., de Souza-Pinto, N. C., Markesbery, W. R., Mattson, M. P., and Bohr, V. A. (2007) Nucleic Acids Res. 35, 5545–5555
28. Weissman, L., de Souza-Pinto, N. C., Mattson, M. P., and Bohr, V. A. (2008) Neurobiol. Aging 30, 2080–2081
29. Evans, A. R., Limp-Foster, M., and Kelley, M. R. (2000) Mutat. Res. 461, 83–108
30. Tell, G., Damante, G., Caldwell, D., and Kelley, M. R. (2005) Antioxid.
Glutamate Induces DNA Damage Repaired by APE1