Genetic Evidence for Adenylyl Cyclase 1 as a Target for Preventing Neuronal Excitotoxicity Mediated by N-Methyl-D-aspartate Receptors*

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The excessive activation of N-methyl-d-aspartate (NMDA) receptors by glutamate results in neuronal excitotoxicity. cAMP is a key second messenger and contributes to NMDA receptor-dependent synaptic plasticity. Adenylyl cyclases 1 (AC1) and 8 (AC8) are the two major calcium-stimulated ACs in the central nervous system. Previous studies demonstrate AC1 and AC8 play important roles in synaptic plasticity, memory, and persistent pain. However, little is known about the possible roles of these two ACs in glutamate-induced neuronal excitotoxicity. Here, we report that genetic deletion of AC1 significantly attenuated neuronal death induced by glutamate in primary cultures of cortical neurons, whereas AC8 deletion did not produce a significant effect. AC1, but not AC8, contributes to intracellular cAMP production following NMDA receptor activation by glutamate in cultured cortical neurons. AC1 is involved in the dynamic modulation of cAMP-response element-binding protein activity in neuronal excitotoxicity. To explore the possible roles of AC1 in cell death in vivo, we studied neuronal excitotoxicity induced by an intracortical injection of NMDA. Cortical lesions induced by NMDA were significantly reduced in AC1 but not in AC8 knock-out mice. Our findings provide direct evidence that AC1 plays an important role in neuronal excitotoxicity and may serve as a therapeutic target for preventing excitotoxicity in stroke and neurodegenerative diseases.

Neuronal death in stroke and other neurodegenerative diseases is mainly mediated by excitotoxicity. The central role of glutamate receptors in mediating excitotoxic neuronal death has been well established (1–5). Injury to the central nervous system triggers an abnormal release of glutamate and other excitatory amino acids that contributes significantly to the neurological outcome (5–8). The released glutamate causes an excessive activation of the NMDA2 receptors, leading to calcium overload in neurons and subsequent neuronal death (1, 3, 6, 9). The type of cell death in neuronal excitotoxicity seems to depend on the nature of the injury. Necrosis occurs after an acute insult, whereas apoptotic cell death is involved in propagation of the secondary injury (10–12). Neuronal apoptosis induced by activation of NMDA receptors has been postulated to underlie the loss of neurons in many central nervous system disorders (13, 14). Despite the initial promise of NMDA receptor antagonists and calcium channel blockers for the reduction of excitotoxic damage, clinical trials with these agents had to be abandoned because of toxic side-effects (15–17). The intervention of signaling events, which are downstream in excitotoxic cascade, might provide new strategies for the treatment of neuronal excitotoxicity.

The cAMP signaling pathway has been shown to act downstream of NMDA receptor activation in synaptic plasticity (18–22). The effect of the cAMP signaling pathway on cell survival depends on cell type and the specific type of cellular stress and may be influenced by cross-talk with other signaling pathways. The paradoxical roles of cAMP in neuronal death have been reported. cAMP has been shown to protect against cell death in cerebellar granule cells and promote the survival of rat sympathetic and sensory neurons in culture (23–25). In contrast, cAMP has also been shown to inhibit some forms of neurotoxin-mediated neuronal survival after serum deprivation in cultured cortical neurons (26). In addition, NMDA-induced neuronal death can be prevented through the inhibition of cAMP accumulation by cannabinoids (27). One possible explanation for the dual effects of cAMP on neuronal death is that different subtypes of adenylyl cyclases (ACs) may be involved in the process. ACs convert ATP to cAMP and initiate the cAMP signaling pathway. Two AC isoforms, AC1 and AC8, which are directly stimulated by calcium and calmodulin, act as the downstream effectors of NMDA receptors and couple its activation to the cAMP signaling pathway; previous studies demonstrated that AC1 and AC8 play important roles in synaptic plasticity, memory, and persistent pain (18, 22, 28). More recent studies have shown that calcium-stimulated ACs modulate ethanol-induced neurodegeneration and double knock-out (DKO) of AC1 and -8 in mice enhances ethanol-induced neurodegeneration.

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2 The abbreviations used are: NMDA, N-methyl-D-aspartate; AC, adenylyl cyclase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; AP-5, 6,7-D-amino-5-phosphonovaleric acid; CRE, cAMP-response element; CREB, cAMP-responsive element-binding protein; pCREB, phosphorylated CREB; KO, knock-out; DKO, double knock-out; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; 5-HT7 receptor, 5-hydroxytryptamine receptor type 7.
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ion in the brains of neonatal mice (29). However, the levels of physiological cell death, death after hypoxia/ischemia, and excitotoxic cell death are not increased in the brains of AC1 and AC8 mice lacking AC1 or AC8 and wild-type mice were generated and maintained as previously described (19, 28, 31). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Toronto, in accordance with the guidelines of the Canadian Council on Animal Care. Cortical neurons were prepared from postnatal day 0 mice. The cerebral cortex of mice were dissected, minced, and trypsinized for 15 min using 0.125% trypsin (Invitrogen). The role of AC1 in neuronal excitotoxicity is isoform-specific, because deletion of AC8 has no such effect. AC1 contributes to the time-dependent changes of CREB activity during neuronal excitotoxicity. This novel role of AC1 further indicates that the cAMP signaling pathway is involved in neuronal excitotoxicity caused by glutamate. AC1 plays an important role in regulating neuronal response to excitotoxicity mediated by NMDA receptors and may serve as a novel target for preventing neuronal excitotoxicity in stroke and neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Animals and Primary Culture of Cortical Neurons—Mutant mice lacking AC1 or AC8 and wild-type mice were generated and maintained as previously described (19, 28, 31). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Toronto, in accordance with the guidelines of the Canadian Council on Animal Care. Cortical neurons were prepared from postnatal day 0 mice. The cerebral cortex of mice were dissected, minced, and trypsinized for 15 min using 0.125% trypsin (Invitrogen). Cells were seeded at the density of 3–4 × 10^6 cells/cm² onto either 24-well plates containing glass coverslips (Fisher Scientific), 60-mm dishes, or 96-well plates precoated with 50 μg/ml poly-D-lysine (Sigma) in water, and grown in Neurobasal-A medium (Invitrogen) supplemented with B27 and 2 mM Glutamine, 105 cells/cm² onto 12-mm-round glass coverslips precoated with poly-D-lysine. Apoptotic neurons were quantified by counting TUNEL-positive cell bodies, and results are expressed as percentages of TUNEL-positive cells.

Cell Viability Assay—Cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bro-mide) assay. Briefly, MTT (Sigma, 5 mg/ml in PBS) was added to culture medium of neurons cultured on 96-well plates at the final concentration of 0.25 mg/ml, and the plate was incubated for 4 h in a humidified atmosphere. After the incubation, the cells were solubilized in 70% isopropanol acidified with 0.1 N HCl. The absorbance of the samples was measured at a wavelength of 595 nm. The extent of MTT conversion in cells exposed to glutamate is expressed as a percentage of control.

TUNEL Staining—Cortical neurons were plated onto 12-mm-round glass coverslips precoated with poly-D-lysine. Apoptotic neurons were detected using the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI) according to the protocol of manufacturer. Cells were washed with PBS, fixed with 4% parafomaldehyde for 30 min, and rinsed three times with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS and then treated with 0.3% H₂O₂ for 30 min to eliminate endogenous peroxidases. The DNA end-labeling reaction was performed by incubation with rTdT reaction mix for 60 min at 37 °C. Cells were then rinsed in PBS and incubated with streptavidin-horseradish peroxidase for 30 min at 37 °C. After rinsing, the labeling was visualized using diaminobenzidine. The apoptotic neurons were quantified by counting TUNEL-positive cell bodies, and results are expressed as percentages of TUNEL-positive cells.

Caspase-3 Activity Assay—The activity of caspase-3 was measured using the Caspase Apoptosis Detection Kit (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s instructions. In brief, cells (1 × 10⁶) were pelleted by centrifugation, washed two times with PBS, and incubated in 500 μl of lysis buffer on ice for 10 min, and then 1× reaction buffer and 10 μl of DEVD-7-amino-4-trifluoromethyl coumarin substrates were added to lysis buffer. The reaction mixtures were incubated at 37 °C for 60 min. The fluorescence of free 7-amino-4-trifluoromethyl coumarin, generated as a result of cleavage of the DEVD-7-amino-4-trifluoromethyl coumarin bond, was monitored continuously with fluorescence microplate reader at excitation and emission wavelengths of 400 and 505 nm, respectively.

Western Blot Analysis—The cultured cortical neurons were harvested and homogenized in lysis buffer (10 mM Na₂HPO₄, pH 7.2, 150 mM NaCl, 1 mM EDTA, 2 mM Na₂VO₃, 20 mM NaF, 0.1% SDS) containing protease inhibitor mixture (Sigma). Brain samples were homogenized in the same lysis buffer containing 0.5% SDS and protease inhibitor mixture. Protein was quantified by Bradford assay, and electrophoresis of equal amounts of total protein was performed on SDS-PAGE gels. Separated proteins were transferred to polyvinylidene fluoride membranes at 4 °C for Western blot analysis. Membranes were incubated with the primary antibody overnight at 4 °C. The primary antibodies used are the rabbit polyclonal anti-NR1, anti-NR2A, anti-NR2B (1:2000, Chemicon, Temecula, CA), anti-phospho-CREB (Ser-133), or anti-CREB (1:1000, Cell Signaling, Beverly, MA). The membranes were incubated with the appropriate horseradish peroxidase-coupled secondary antibody diluted at 1:3000 for 1 h followed by enhanced chemiluminescence detection of the proteins with Western lightning chemiluminescence reagent plus (PerkinElmer Life Sciences) according to the manufacturer’s instructions. To verify equal loading, membranes were also probed with anti-actin antibody (Sigma). The density of immunoblots was measured using National Institutes of Health ImageJ software.

Electrophysiology—Cultured cortical neurons were bath-perfused with Mg²⁺-free extracellular solution containing 140 mM NaCl, 2.5 mM KCl, 25 mM HEPES, 20 mM glucose, 1.3 mM CaCl₂, 0.0003 mM tetrodotoxin, and 0.01 mM glycine (pH 7.4 with NaOH, 320–335 mmol/kg) on the stage of an Olympus CKX41 inverted microscope. The recording pipettes (3–5 MΩ) were filled with solution containing 145 mM potassium gluconate, 5 mM NaCl, 1 mM MgCl₂, 0.2 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM Na₃-GTP (pH 7.2 with KOH, 290–300 mmol/kg). The cell membrane potential was held at −70 mV, and NMDA (10 μM) was applied for 3 s every 45 s to minimize the current amplitude decrease due to receptor desensitization. Pharmacological agents were applied with Mg²⁺-free extracellular solution by a constant local gravity-fed perfusion from a quartz glass pipette (inner diameter, 300 μm) connected
to a manifold with <1 μl dead space (ALA Scientific Instruments, Inc., Westbury, NY). NR2B subunit antagonist Ro25-6981 was purchased from Tocris Cookson Inc. (Ellisville, MO). Recorded currents were filtered at 5 kHz, digitized at 10 kHz, and stored in a personal computer for display and analysis with an Axopatch 200B amplifier, DigiData 1200 series interface, and the pClamp 8 software suite (Molecular Devices, Sunnyvale, CA).

cAMP Assay—Cortical neurons were plated in 96-well tissue culture dishes. After treatment with glutamate, cells were harvested and lysed in 0.1 M HCl. Direct cAMP measurements were performed using the direct cAMP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer’s protocol. Phosphodiesterase was inhibited by the addition of 1 mM 3-isobutyl-1-methylxanthine (Sigma) to cultures for 30 min.

CRE Luciferase Assay—Transcriptional activities of CREB were determined by CRE luciferase assay. Cultured cortical neurons were co-transfected with the pGL3-CRE-firefly luciferase and pGL3-CMV-Renilla luciferase constructs using Lipo-2000 reagent (Invitrogen) 48 h before the experiment. Luciferase activity was assayed in the cell lysate by using the dual-luciferase reporter assay system (Promega) according to the manufacturer’s protocol. Luciferase activities of the CRE-luciferase vector were normalized based on Renilla luciferase activity of the co-transfected vector.

NMDA Microinjection into Cerebral Cortex—Male mice (22–28 g) were used. The mice were anesthetized with isoflurane and placed in a stereotaxic apparatus fitted with a mouse adaptor that positioned the skull horizontally between bregma and lambda. The dura overlying the cortex was exposed. NMDA (30 nmol in 300 nl of sterile 0.1 M PBS, pH 7.4) was injected into the cerebral cortex of mice at the right side at the following coordinates: 2 mm posterior to bregma, 3 mm lateral from the midline, and at the depth of 0.8 mm from the skull surface. The injection was performed at the rate of 50 nl/min using a 30-gauge beveled needle with cannula tubing connected to a Hamilton syringe, which was mounted on a microinjection pump. The needle was left in place for 15 min before being slowly withdrawn. Mice were deeply anesthe-
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Data Analysis—Results are presented as mean ± S.E. Statistical comparisons between experimental groups were performed with Student t test or one-way analysis of variance. In all cases, p < 0.05 was considered statistically significant.

RESULTS

Genetic Deletion of AC1 Protects against Neuronal Death Induced by Glutamate—First, the effect of genetic deletion of AC1 or AC8 on glutamate-induced cell death was evaluated by measuring cell viability in cultured cortical neurons after treatment with glutamate. By MTT assay, we first checked the cell viability in cultured cortical neurons from wild-type mice at different time points during the 24 h after treatment with 100 μM or 250 μM glutamate. As shown in Fig. 1A, we found that the cell viability was reduced in a time-dependent manner and most of the cell death occurred during the first 8 h after treatment with glutamate. Then, we further compared the cell viability in cultured cortical neurons from wild-type and genetic knock-out mice at the time point of 8 h after treatment with 100 μM or 250 μM glutamate. It was found that the reduction of cell viability was significantly attenuated in cortical neurons from AC1 KO (p < 0.01, n = 9 wells from three experiments, Fig. 1B). However, there was no difference in cell viability between AC8 KO and wild-type mice (p > 0.05, compared with wild-type, n = 9 wells from three experiments, Fig. 1B). These data indicate that genetic deletion of AC1 can attenuate neuronal excitotoxicity induced by glutamate in primary culture of cortical neurons.

Previous studies have documented that apoptosis is implicated in excitotoxic neuronal death as an apoptosis-necrosis continuum (32). We then characterized apoptotic cell death following excitotoxicity in neuronal culture. The neuronal apoptosis was assessed by TUNEL staining, a low level of apoptosis (~5%) could be found in cultured cortical neurons of either wild-type, AC1 or AC8 KO mice at the baseline level. At the time point of 8 h after treatment with 100 μM or 250 μM glutamate, the cell apoptosis was increased in cultured cortical neurons and the increase of TUNEL-positive cells in cortical neurons from AC1 KO mice was prevented compared with wild-type mice (p < 0.05, n = 6 experiments, Fig. 1, C and D). In contrast, there is no significant difference between AC8 KO and wild-type mice (p > 0.05, compared with wild-type, n = 6 experiments, Fig. 1, C and D). These results indicate that genetic deletion of AC1 can protect cultured cortical neurons from apoptosis induced by glutamate and suggest that AC1 may play an important role in glutamate-induced neuronal apoptosis during neuronal excitotoxicity.

Activation of caspase-3 is a hallmark of apoptotic cell death and precedes changes in nuclear morphology (33, 34). To further confirm that glutamate could induce apoptotic neuronal death in our model system, we measured caspase-3 activity at various time points in extracts from cortical neuron cultures during the 8 h after treatment with 250 μM glutamate. Excitotoxic stimulation with glutamate increased caspase-3 activity in a time-dependent manner, and the increase of caspase-3 activity was blocked in cultured cortical neurons from AC1 KO mice compared with wild-type mice (p < 0.01, n = 6 experiments, Fig. 1E). However, no difference was observed between the cultured cortical neurons from AC8 KO and wild-type mice (p >

tized with isoflurane and perfused with 4% paraformaldehyde in 0.1 M PBS 24 h after injection. Coronal sections were serially cut into a cryostat and stained with thionin for determination of lesion volumes. For caspase-3 assay, mice were killed after being anesthetized with isoflurane inhalation. The cerebral cortex of the injected side were collected and homogenized in the cell lysis buffer.

FIGURE 2. Neuronal excitotoxicity induced by NMDA was prevented in cultured cortical neurons from AC1 KO mice. A, the NMDA receptor antagonist AP-5 blocked glutamate-induced excitotoxicity in cultured cortical neurons from wide-type, AC1, and AC8 KO mice. Cultures were pretreated with either AP-5 (100 μM) for 10 min prior to treatment with 250 μM glutamate in the presence of AP-5. Neuronal viability was qualified by MTT assay at 8 h after treatment with glutamate. *, p < 0.05; **, p < 0.01 compared with glutamate alone. Data are presented as mean ± S.E., n = 9 wells. B, the reduction of cell viability caused by NMDA was prevented in cultured cortical neurons from AC1 KO mice. Cultures were treated with 25 μM, 100 μM, and 250 μM NMDA. Neuronal viability was qualified by MTT assay at 8 h after treatment with NMDA. *, p < 0.01, compared with wild-type cultures. Data are presented as mean ± S.E., n = 9 wells.
KO mice compared with wild-type mice ($p < 0.01, n = 9$ wells, Fig. 2B). However, there was no difference in cell viability between AC8 KO and wild-type mice ($p > 0.05$, compared with wild-type, $n = 9$ wells, Fig. 2B). These findings further confirm that genetic deletion of AC1 can prevent neuronal excitotoxicity mediated by NMDA receptors.

**NMDA Receptors in Cultured Cortical Neurons of AC1 or AC8 KO Mice**—NMDA receptors are heteromers composed of two NR1 subunits and two or three NR2 subunits. Among the four NR2 subunits (A–D), NR2A and NR2B subunits predominate in the forebrain (39–41). We further investigated the expression profile of NMDA receptor subunits in cultured cortical neurons by Western blot and found that there are no significant differences in the expression level of NR1, NR2A, and NR2B subunits between wild-type, AC1, and AC8 KO mice (Fig. 3, A and B). These data indicate that genetic deletion of AC1 or AC8 has not altered the expression of NMDA receptor subunits in cultured cortical neurons. Next, we used whole cell patch recordings to test whether NR2A or NR2B subunit-mediated currents were affected by genetic deletion of AC1 or AC8 in cultured cortical neurons. The contribution of NR2A or NR2B subunits containing NMDA receptor-mediated currents to the total amount of NMDA receptor-mediated currents was determined by application of the selective inhibitor of NR2A subunit, NVP-AAM077 (41, 42), or the selective inhibitor of NR2B subunit, Ro25-6981 (43, 44). In cortical neurons from wild-type mice, NMDA (10 μM)-induced current was largely blocked by NVP-AAM077 (0.4 μM) (84.7 ± 3.1% of control, $n = 5$ neurons, Fig. 3, C and D), whereas it was only slightly inhibited by Ro25-6981 (0.5 μM) (16.7 ± 4.0%, $n = 4$ neurons, Fig. 3, C and D). There was no significant difference in either NVP-AAM077-sensitive (NR2A component) or Ro25-6981-sensitive (NR2B component) current in cultured AC1 KO cortical neurons (NR2A, 81.7 ± 2.6%, $p > 0.05$, compared with wild-type, $n = 5$ neurons; NR2B, 15.7 ± 2.9%, $p > 0.05$, compared with wild-type, $n = 4$ neurons, Fig. 3, C and D). Similar results were obtained from AC8 KO cultured neurons (NR2A, 84.5 ± 1.6%, $p > 0.05$, compared with wild-type, $n = 5$ neurons; NR2B, 15.3 ± 4.1%, $p > 0.05$, compared with wild-type, $n = 5$ neurons, Fig. 3, C and D). Therefore, the protective effect of genetic deletion of AC1 against neuronal excitotoxicity is likely due to the decrease in NMDA receptor-mediated currents contributed by NR2B subunits.
excitotoxicity does not simply result from inherent defects in NMDA receptors caused by the deletion of AC1.

AC1 Mediates Intracellular cAMP Increase—The activation of NMDA receptors by glutamate can elicit a Ca\(^{2+}\)/H11001-dependent increase in cAMP in the CA1 area of the hippocampus (45, 46). Here, we found that the intracellular cAMP levels were elevated at 0.5 h after treatment with 250 \(\mu M\) glutamate, and pretreatment with the NMDA receptor antagonist AP-5 (100 \(\mu M\)) blocked the elevation of intracellular cAMP levels induced by glutamate (\(p < 0.01\), compared with glutamate treatment, \(n = 6\) wells, Fig. 4A). The results indicate that the elevation of intracellular cAMP levels induced by glutamate is mediated by NMDA receptors. The intracellular levels of cAMP were further measured in cultured cortical neurons at various time points during the 8 h after treatment with 250 \(\mu M\) glutamate. Treatment with glutamate caused a detectable increase in levels of cAMP in cultured cortical neurons of both AC8 KO and wild-type mice, and there was no significant difference between AC8 KO and wild-type (\(p > 0.05\), \(n = 6\) wells, Fig. 4B). In contrast, the increase in intracellular cAMP levels was significantly reduced in cultured cortical neurons of AC1 KO mice compared with wild-type mice, and this effect can be seen at the time points from 0.5 to 8 h (\(p < 0.01\), compared with wild-type, \(n = 6\) wells, Fig. 4B). To make sure that the difference in intracellular cAMP levels was not a result of cell death induced by glutamate, we tested the cell viability using MTT assay at the time points from 2 to 8 h after treatment with 250 \(\mu M\) glutamate. We found no difference in cell viability between different genotypes during the first 2 h after glutamate treatment (Fig. 4C). Because genetic deletion of AC1, but not AC8, can significantly decrease glutamate-induced cAMP production, it can be concluded that AC1, but not AC8, contributes to the elevation of intracellular cAMP levels elicited by NMDA receptor activation in cultured cortical neurons.

5-HT\(_7\) Receptor Activation Does Not Induce Cell Death in Neuronal Culture—It has been reported that 5-HT\(_7\) receptors are positively coupled to adenylyl cyclases, and the activation of 5-HT\(_7\) receptors can stimulate adenylyl cyclases by increasing intracellular Ca\(^{2+}\) (47). To further explore the role of cAMP in neuronal excitotoxicity, we next tested the effect of a 5-HT\(_7\) receptor agonist AS 19 (48, 49) in cultured cortical neurons. We found that AS 19 could significantly increase the cAMP accumulation in cultured cortical neurons at the concentration of 1 \(\mu M\) and 10 \(\mu M\) (Fig. 5A). The increase in intracellular cAMP from wild-type mice were pretreated with AP-5 (100 \(\mu M\)) for 10 min prior to treatment with 250 \(\mu M\) glutamate in the presence of AP-5. The intracellular cAMP was qualified at 0.5 h after treatment with glutamate. Data are presented as mean ± S.E., *\(p < 0.01\), compared with the control or compared between glutamate treatment and AP-5 pretreatment, \(n = 6\) wells. B, the intracellular cAMP levels in cultured cortical neurons from wild-type, AC1 KO, and AC8 KO mice after treatment with glutamate. The cAMP levels were analyzed at different time points during the 8 h after treatment with 250 \(\mu M\) glutamate. There is no difference in the basal levels of intracellular cAMP between wild-type, AC1, and AC8 KO neuronal culture. Data are presented as mean ± S.E., *\(p < 0.01\), compared with the wild-type cells, \(n = 6\) wells. C, the time-dependent neuronal excitotoxicity induced by glutamate in cultured cortical neurons. Cortical neurons were treated with 250 \(\mu M\) glutamate. Cell viability was measured by MTT assay at the different time points during the 8 h after treatment with glutamate. Data are presented as mean ± S.E., *\(p < 0.01\), compared with the wild-type neurons, \(n = 9\) wells from three experiments.
levels was significantly blocked in cultured cortical neurons of AC1 KO mice (p < 0.01, compared with wild-type, n = 6 wells, Fig. 5B). In contrast, there was no significant difference between AC8 KO and wild type (p > 0.05, n = 6 wells, Fig. 5B). The results indicate that AC1, but not AC8, contributes to cAMP production stimulated by the activation of 5-HT7 receptors and acts as a downstream effector of 5-HT7 receptors. However, no cell death was caused by AS 19 at the concentration of both 1 μM and 10 μM as detected by MTT assay (Fig. 5C). These data suggest stimulation of AC1 cannot cause cell death by itself, and the role of AC1 in neuronal excitotoxicity is specifically related to NMDA receptors.

**Time-dependent Changes in CREB Activity**—The cAMP-responsive element binding protein (CREB) is a transcription factor that plays an important role in neuronal survival (50–52). There are several signaling pathways that lead to activation of CREB, phosphorylated CREB (pCREB) can be used as a marker for activation of adenylyl cyclases in the central nervous system (28, 53). It has been reported that glutamate exposure can cause the rapid and transient enhancement of CREB phosphorylation in neurons (50, 52, 54). To examine the possible relationship between calcium-stimulated ACs and the activity of CREB in glutamate-induced neuronal excitotoxicity, we investigated the pCREB (Ser-133) levels in cultured cortical neurons before and after treatment with glutamate by Western blot. As shown in Fig. 6 (A and B), there was no difference in the basal pCREB levels in cultured cortical neurons between wild-type and AC1 KO or AC8 KO mice. Consistent with previous studies, we also found that the pCREB levels underwent the time-dependent changes in neuronal excitotoxicity. The pCREB levels were transiently increased at 0.25 h after treatment with 250 μM glutamate in cultured cortical neurons of both wild-type and AC1 KO or AC8 KO mice. The increase of pCREB levels was partially blocked in AC1 KO culture. This result indicates that AC1 is involved in phosphorylation of CREB due to NMDA receptor activation. Then, the level of pCREB decreased rapidly in a time-dependent manner in cultures from both wild-type and AC8 KO mice, whereas the decrease in pCREB levels was blocked in AC1 KO culture (p < 0.01 or p < 0.05, n = 5 experiments, Fig. 6, C and D). It suggests that AC1 might also be involved in the dephosphorylation of CREB caused by neuronal excitotoxicity. These findings were further confirmed by determination of the CREB-mediated gene transcriptional activities using a dual luciferase reporter system (Fig. 6E). The difference in pCREB levels and CREB-mediated transcriptional activity between AC1 KO and wild-type cortical neurons is not likely caused by cell death, because no difference in excitotoxicity induced by glutamate can be seen at the time point of 2 h as shown in Fig. 5C.
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A

WT AC1<sup>-/-</sup> AC8<sup>-/-</sup>
pCREB CREB

B

Wild-type AC1<sup>-/-</sup> AC8<sup>-/-</sup>

Relative pCREB levels

C

Wild-type 250 μM glutamate

pCREB CREB AC1<sup>-/-</sup> CREB AC8<sup>-/-</sup> pCREB CREB

260 μM glutamate

0 0.25 1 2 4 8 (h)

D

Relative pCREB levels

E

Relative pCREB activity

FIGURE 6. Dynamic changes in CREB activity in neuronal excitotoxicity induced by glutamate. A, representative Western blot of basal pCREB levels in cultured cortical neurons from wild-type (WT), AC1 KO, and AC8 KO mice. B, quantified data for pCREB levels by Western blot in cultured cortical neurons of wild-type, AC1 KO, and AC8 KO mice. There was no significant difference in the basal pCREB levels in cultured cortical neurons between wild-type, AC1 KO, and AC8 KO mice, p > 0.05. Data were normalized to expression level of wild-type mice and are presented as mean ± S.E., n = 5 for each group. C, representative Western blot of pCREB levels in cultured cortical neurons treated with glutamate. Cultures from wild-type (WT), AC1 KO, and AC8 KO mice were treated with 250 μM glutamate, and the pCREB levels were detected at different time points during the 8 h after glutamate treatment. D, quantified data for pCREB levels in cultured cortical neurons treated with glutamate by Western blot. The pCREB levels were transiently increased at 0.25 h after glutamate treatment, and the increase in pCREB levels was partially blocked in AC1 KO neurons; p < 0.01, compared with the wild-type cultures. The pCREB levels in cultured cortical neurons treated with glutamate decreased rapidly from the time point of 1 h, and the decrease in pCREB levels was blocked in AC1 KO neurons; p < 0.01, compared with the wild-type cultures. Data were normalized to expression level of control and presented as mean ± S.E., n = 6 wells for each group. E, the CREB-mediated transcriptional activity in cultured cortical neurons treated with glutamate. Cortical neurons were co-transfected with the CRE-firefly luciferase and CMV-Renilla luciferase constructs 48 h before treatment with 250 μM glutamate. The pCREB activities were transiently increased at 0.25 h after glutamate treatment, and the increase in pCREB activities was partially blocked in AC1 KO neurons; p < 0.01, compared with the wild-type cultures. The pCREB activities in cultured cortical neurons treated with glutamate decreased rapidly from the time point of 1 h, and the decrease in pCREB activities was blocked in AC1 KO neurons; p < 0.01, compared with the wild-type cultures. Data were normalized to expression level of control and presented as mean ± S.E., n = 6 wells for each group.

4C. Taken together, these results show that AC1 contributes to the modulation of CREB activity in neuronal excitotoxicity.

AC1 Is Involved in the Neuronal Excitotoxicity Induced by Intracortical Injection of NMDA—To confirm the role AC1 in neuronal excitotoxicity mediated by NMDA receptors, an in vivo model of neuronal excitotoxicity was produced by intracortical injection of NMDA in whole mice. We first investigated the expression profiles of NMDA receptors in cerebral cortex by Western blot and found that there was no difference in the expression of NR1, NR2A, and NR2B subunits in the cerebral cortex between wild-type, AC1 KO, and AC8 KO mice (Fig. 7, A and B). In the in vivo model of neuronal excitotoxicity induced by intracortical injection of NMDA, we found that cortical lesions were significantly reduced in AC1 KO mice, compared with wild-type mice (p < 0.01, n = 6 mice per group, Fig. 7, C and D). There was no significant difference between AC8 KO and wild-type mice (p > 0.05, n = 6 mice, Fig. 7, A, C, and D). The caspase-3 activity in cerebral cortex of the injected side was also reduced in AC1 KO mice compared with wild-type mice (p < 0.01, n = 5 mice per group, Fig. 7E). These results indicate that AC1, but not AC8, is involved in the neuronal excitotoxicity mediated by NMDA receptors.

DISCUSSION

Based on the deficits in neuronal function in AC1 KO mice and the importance of the cAMP signaling pathway in cell survival, we hypothesized that calcium-stimulated ACs might mediate the neuronal response to excitotoxicity mediated by NMDA receptors. In this study, the roles of AC1 and AC8 in NMDA receptor-mediated neuronal excitotoxicity were investigated in cultured cortical neurons and in models in vivo produced by intracortical injection of NMDA in wild-type and AC1 or AC8 KO mice. We demonstrate that genetic deletion of AC1 significantly attenuated neuronal cell death induced by activation of NMDA receptors. In contrast, genetic deletion of AC8 had no such protective effect. These findings suggest that AC1 plays an important role in modulating neuronal response to excitotoxicity and may serve as a novel target for treatment of neuronal excitotoxicity in stroke and neurodegenerative diseases.
the two major AC isoforms primarily stimulated by calcium and calmodulin (20). Activation of NMDA receptors has been postulated to result in activation of the calcium-stimulated ACs, because both direct application of NMDA to hippocampal slices and electrical stimulation that activates NMDA receptors result in calcium-dependent cAMP elevation (45, 46). The deficits displayed by AC1 and -8 DKO mice in NMDA-dependent phenomena have also led to the suggestion that the activation of the NMDA receptors is coupled to activation of the calcium-stimulated ACs (19). In the present study, we found that glutamate administration could lead to elevation of intracellular cAMP in cultured cortical neurons of both wild-type and AC8 KO mice but not in neurons from AC1 KO mice. This difference was unlikely caused by the difference in NMDA receptors themselves, because no differences were observed in the expression level and composition of NMDA receptor in the cultured cortical neurons between wild-type, AC1, or AC8 KO mice as shown by Western blot and electrophysiological recordings. Our previous data have shown that NMDA receptor-dependent long-term potentiation is the major form of long-term plasticity in the anterior cingulate cortex, and AC1 is essential for the induction of long-term potentiation in anterior cingulate cortex neurons, whereas AC8 subunit partially contributes to forskolin-induced potentiation (22). Taken together, we propose that AC1 is the major calcium sensor for NMDA receptors, because one obvious consequence of NMDA receptor activation is the increase in intracellular calcium concentration. AC1 is directly stimulated by Ca\(^{2+}\) and calmodulin in vivo with half-maximal stimulation at 150 to 200 nM free Ca\(^{2+}\), a concentration just above resting free Ca\(^{2+}\) in neurons. AC8 is also stimulated by calmodulin, but its calcium sensitivity is 5-fold lower than AC1 (half-maximal activation by free Ca\(^{2+}\) is 800 nM for AC8) (20, 55, 56). Thus, compared with AC8, AC1 is the major downstream effector of NMDA receptor activation during neuronal excitotoxicity. One possible explanation for the difference between AC1 and AC8 in the modulation of neuronal response to glutamate-induced excitotoxicity may lie in the fact that AC1 is more sensitive to calcium than AC8. Among the 10 AC isoforms, AC1 is one of the neuron-specific isoforms (57). Therefore, the isofrom

FIGURE 7. AC1 deletion protects against neuronal excitotoxicity induced by intracortical injection of NMDA. A, representative Western blot of NR1, NR2A, and NR2B subunits in cerebral cortex from wild-type (WT), AC1 KO, and AC8 KO mice. B, quantified data for expression levels of NR1, NR2A, and NR2B subunits by Western blot in cerebral cortex from wild-type, AC1 KO, and AC8 KO mice. There was no significant difference in the expression of NMDA receptor subunits in cultured cortical neurons between wild-type, AC1 KO, and AC8 KO mice. There was no significant difference in the expression of NMDA receptor subunits in cultured cortical neurons between wild-type, AC1 KO, and AC8 KO mice. C, representative micrographs of cortical lesions in wild-type (left panel), AC1 KO (middle panel), and AC8 KO (right panel) mice as shown by thionin staining. Scale bars: upper panel, 500 μm; bottom panel, 200 μm. D, determination of cortical lesions induced by intracortical injection of NMDA. Brain sections of 24-h survival time after injection were stained by thionin. Cortical lesion volumes were measured by Scion Image program and normalized to the wild-type mice. Data are presented as mean ± S.E., n = 6 mice. E, caspase-3 assay in neuronal excitotoxicity induced by intracortical injection of NMDA. Caspase-3 assay was performed in cerebral cortices of the injected side at 24 h after injection and normalized to the wild-type mice. Data are presented as mean ± S.E., *p < 0.01, compared with the wild-type mice, n = 5 mice.
specific roles of AC1 in neuronal excitotoxicity make it an ideal target for treatment of this pathological state.

Two forms of cell death, necrosis and apoptosis, are implicated in excitotoxic neuronal death as an apoptosis-necrosis continuum. Glutamate triggers the excessive activation of ionotropic glutamate receptors leading to rapid influx of calcium that culminates in neuron death (2, 58). One well-established consequence of glutamate-induced elevation of calcium within neurons is the activation of a group of cysteine proteases termed caspases. The activation of caspases is essential for the induction of apoptosis in many non-neuronal or neuronal cells (59, 60). Once activated, caspases are responsible for the proteolytic cleavage of several substrates leading to cell death. Accordingly, it has been reported that there is an increase in both caspase-3 expression and activity when cultured neurons are treated with glutamate or NMDA (33, 34, 61–63). We assessed the caspase-3 activity in cultured cortical neurons after glutamate treatment and found that the caspase-3 activity was significantly reduced in the cortical neurons of AC1 KO mice, but not in the cortical neurons from AC8 KO mice, compared with wild-type mice. Similar results were obtained in the in vivo models induced by intracortical injection of NMDA. These findings further support that AC1 contributes to neuronal excitotoxicity.

CREB has been shown to play an important role in neuronal survival (50–52). We found that glutamate can induce a rapid and transient increase in CREB activity in neuronal culture. AC1 is involved in the up-regulation of CREB activity, because the increase in CREB activity was partially blocked in AC1 KO neuronal cultures. However, the decrease in CREB activity was also blocked in AC1 KO neurons. AC1 contributes to dynamic regulation of CREB activity in neuronal excitotoxicity. The CREB activity was regulated by its phosphorylation and dephosphorylation (21, 52, 54). It is possible that stimulation of AC1 by NMDA receptor activation can not only induce phosphorylation of CREB but also activate the signaling pathways that result in dephosphorylation of CREB.

In the present study, we also found that activation of 5-HT7 receptor can induce the cAMP accumulation in cultured neurons, and this effect is primarily mediated by AC1. However, no cell death was induced in this process compared with the control, even though the intracellular cAMP levels were elevated. The explanation for this phenomenon is that AC1-cAMP is only one of the signaling pathways that are activated and involved in cell death following NMDA receptor activation. cAMP cannot cause cell death by itself. AC1 activation is specifically involved in the signaling pathways to induce or promote cell death once it is initiated by NMDA receptor activation. Future experiments are needed to identify signaling pathway by which AC1 is involved in neuronal excitotoxicity.

A recent study using an in vivo excitotoxic model induced by systemic administration of glutamate has not found any significant difference in excitotoxic cell death in brain between AC1 and -8 DKO and wild-type mice. In addition, there was no difference in apoptotic neurodegeneration after hypoxia/ischemia as measured by caspase-3 activity levels in hippocampus ipsilateral to the ligated carotid artery (29). In contrast, we have used both in vitro and in vivo neuronal excitotoxicity models of AC1 or AC8 KO mice in the present study and found that AC1 plays an important role in neuronal excitotoxicity. The differences between these findings may be explained by different models used for induction of cell death. There are two kinds of model in their study: 1) Excitotoxic models. They tested excitotoxic cell death in brain of neonatal mouse after administration of 1.25 g/kg monosodium glutamate. Compared with intracortical injection of NMDA, the excitotoxic cell death might be limited in this condition. In addition, the age of mice is different; they have used neonatal mice, whereas adult mice are used in our study. 2) Hypoxic/ischemic models by carotid artery ligation. The cell death in this model may be complicated with many other factors besides neuronal excitotoxicity.

In summary, we have provided strong evidences that AC1 is important for neuronal excitotoxicity triggered by activation of NMDA receptors. Genetic deletion of AC1, but not AC8, can attenuate neuronal excitotoxicity caused by excessive NMDA receptor activation. Our findings indicate that AC1 plays an isoform-specific role in modulation of NMDA receptor-dependent neuronal excitotoxicity and may become a therapeutic target for preventing neuronal excitotoxicity in stroke and other neurodegenerative diseases.

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REFERENCES
1. Choi, D. W. (1988) Neuron 1, 623–634
2. Sattler, R., and Tymianski, M. (2001) Mol. Neurobiol. 24, 107–129
3. Arundine, M., and Tymianski, M. (2003) Cell Calcium 34, 325–337
4. Hynd, M. R., Scott, H. L., and Dodd, P. R. (2004) Neurochem. Int. 45, 583–595
5. Lee, J. M., Zipfel, G. J., and Choi, D. W. (1999) Nature 399, A7–A14
6. Choi, D. W. (1994) Ann. N. Y. Acad. Sci. 747, 162–171
7. Zeron, M. M., Hansson, O., Chen, N., Wellington, C., Leavitt, B., Brundin, P., Hayden, M. R., and Raymond, L. A. (2002) Neuron 33, 849–860
8. Bal-Price, A., and Brown, G. C. (2001) J. Neurosci. 21, 6480–6491
9. Zipfel, G. J., Lee, J. M., and Choi, D. W. (1999) Neur. Engl. J. Med. 341, 1543–1544
10. Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., and Lipton, S. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7162–7166
11. Liu, X. Z., Xu, X. M., Hu, R., Du, C., Zhang, S. X., McDonald, J. W., Dong, H. X., Wu, Y. J., Fan, G. S., Jaccquin, M. F., Hsu, C. Y., and Choi, D. W. (1997) J. Neurosci. 17, 5395–5406
12. Yakovlev, A. G., Knoblach, S. M., Fan, L., Fox, G. B., Goodnight, R., and Faden, A. I. (1997) J. Neurosci. 17, 7415–7424
13. Yu, S. P., Canzoniero, L. M., and Choi, D. W. (2001) Curr. Opin. Cell Biol. 13, 405–411
14. Wang, Y., Ju, W., Liu, L., Fam, S., D’Souza, S., Taghibiglou, C., Salter, M., and Wang, Y. T. (2004) J. Biol. Chem. 279, 41267–41270
15. Gladstone, D. J., Black, S. E., and Hakim, A. M. (2002) Stroke 33, 2123–2136
16. Ilkonomidou, C., and Turski, L. (2002) Lancet. Neurol. 1, 383–386
17. MacDonald, J. F., Xiong, Z. G., and Jackson, M. F. (2006) Trends Neurosci. 29, 75–81
18. Xia, Z., and Storm, D. R. (1997) Curr. Opin. Neurol. 7, 391–396
19. Wang, S. T., Athos, J., Figueroa, X. A., Pineda, V. V., Schaefer, M. L., Chavkin, C. C., Muglia, L. J., and Storm, D. R. (1999) J. Neurosci. 23, 787–798
20. Wang, H., and Storm, D. R. (2003) Mol. Pharmacol. 63, 463–468
