α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionate Receptor Subunit Composition and cAMP-response Element-binding Protein Regulate Oligodendrocyte Excitotoxicity*

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Developing oligodendrocytes (OLs) are highly vulnerable to glutamate excitotoxicity. Although OL excitotoxicity is mainly mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (AMPARs) and is Ca2+-dependent, the molecular basis for AMPAR-mediated Ca2+ influx in OLs remains largely unclear. Ca2+ permeability of AMPARs is inversely correlated with the abundance of the AMPAR subunit glutamate receptor 2 (GluR2). Here we report that GluR2-containing and GluR2-lacking AMPARs are co-expressed in individual OLs and that a subset of AMPARs on each OL are Ca2+-permeable and mediate OL excitotoxicity. Virus-mediated overexpression of GluR2 reduces OL excitotoxicity, whereas expression of its unedited form GluR2(Q) enhances the excitotoxicity. These findings indicate that GluR2 critically controls OL excitotoxicity. During OL excitotoxicity, the transcriptional factor cAMP-response element-binding protein (CREB) is transiently phosphorylated and subsequently down-regulated. Virus-mediated expression of a constitutively active form of CREB, both in cultured OLs in vitro and in developing cerebral white matter in vivo, up-regulates GluR2, inhibits Ca2+ permeability, and protects OLs from excitotoxicity. Overall, these data suggest that targeting GluR2-lacking AMPARs or CREB may be a useful strategy for treating nervous system disorders associated with OL excitotoxicity.

Emerging evidence indicates that oligodendrocytes (OLs) share with neurons a high vulnerability to excitotoxic injury. Unlike neuronal excitotoxicity that is mediated predominantly by glutamate receptors (GluRs) of the N-methyl-D-aspartate (NMDA) type, excitotoxicity in developing OLs is mainly mediated by non-NMDA-type GluRs (9, 10). Furthermore, non-NMDA receptor expression on OLs is developmentally regulated (6, 8), and developing OLs (OL precursors, pre-OLs) are more sensitive to excitotoxic injury than mature OLs (6–8). Although previous studies demonstrate that pre-OL excitotoxicity in vivo and in vitro is mainly mediated by the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (AMPARs) (6–8) and is Ca2+-dependent (7), the molecular basis for AMPAR-mediated Ca2+ permeability in pre-OLs remains largely unclear. AMPARs are multimeric proteins formed by various combinations of GluR1–4 subunits (11). Ca2+ permeability of AMPARs is determined by receptor composition. In particular, the GluR2 subunit critically regulates Ca2+ permeability of AMPARs; AMPARs containing GluR2 have very limited Ca2+ permeability compared with GluR2-lacking channels in various systems (12, 13). However, the specific role of GluR2 in pre-OL excitotoxicity remains unknown.

Excitotoxic signals and Ca2+ influx can lead to activation of cAMP-response element-dependent transcription (14). Activation of the transcription factor cAMP-response element-binding protein (CREB) is involved in cell survival-related gene expression (14). However, it is unknown whether CREB can be a survival factor in AMPAR-mediated excitotoxic injury to pre-OLs.

In this study, we first report that individual pre-OLs express both GluR2-containing and GluR2-lacking AMPARs, and that pre-OL excitotoxicity is mediated by the subset of AMPARs that are GluR2-lacking and Ca2+-permeable. We next demonstrate a novel effect of excitotoxicity resulting in transient phosphorylation and subsequent down-regulation of CREB in pre-OLs. Furthermore, virus-mediated expression of either edited GluR2 or constitutively active CREB in pre-OLs increases GluR2 levels, blocks Ca2+ permeability, and protects the cells from excitotoxicity. In addition, CREB regulates GluR2 expression in pre-OLs in vitro and in vivo. Our data suggest that GluR-
mediated Ca\(^{2+}\) permeability in pre-OLs is a result of the differential assembly of GluR subunits that produce different discretely functioning channels within a single cell, and that targeting GluR2-lacking AMPARs or CREB may be a useful strategy for treating nervous system disorders associated with pre-OL excitotoxicity.

Elucidation of the mechanisms by which excitotoxicity develops in pre-OLs is important for improved understanding of the pathogenesis of cerebral white matter disorders. Previous studies have shown a critical role of GluR2 in excitotoxicity in neurons as well as in non-neural cells with expressed recombinant GluRs. This study indicates that GluR2-lacking AMPARs and CREB regulate Ca\(^{2+}\) permeability and pre-OL excitotoxicity, and provides new insights into excitotoxic mechanisms of pre-OL injury. Another interesting and novel finding from this study is the CREB regulation of GluR2 expression. Expression of constitutively active CREB in cultured pre-OLs or in the developing cerebral white matter results in up-regulation of GluR2 and inhibition of Ca\(^{2+}\) permeability. The data illustrate a new mechanism regulating Ca\(^{2+}\) permeability in pre-OLs and in the cerebral white matter.

**MATERIALS AND METHODS**

**Cell Culture**—Highly enriched pre-OLs were obtained from newborn Sprague-Dawley rat brains as described previously (7, 8, 15). In brief, forebrains were dissected; the meninges were removed, and the brains were dissociated by gentle trituration. The cell suspension was filtered through a 70-μm nylon cell strainer (Falcon), and the cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, sodium pyruvate (1 mM), penicillin (50 IU/ml), and streptomycin (50 μg/ml). The mixed glial cells from two brains were plated in a 75-cm\(^2\) flask pre-coated with poly-L-lysine (100 μg/ml) and the cells were cultured in Dulbecco’s modified Eagle’s medium containing D-glucose (25 mM), L-glutamine (4 mM), sodium pyruvate (1 mM), human apotransferrin (50 μg/ml), bovine pancreatic insulin (5 μg/ml), sodium selenium (30 nm), hydrocortisone (10 nm), d-biotin (10 nm), bovine serum albumin (1 mg/ml), recombinant human platelet-derived growth factor-AA (10 ng/ml), and basic fibroblast growth factor (10 ng/ml) for 2 days with fresh medium change every 2 days. All experiments were performed with cultures maintained in the chemically defined medium without the supplemental growth factors (platelet-derived growth factor + basic fibroblast growth factor). Cultures were routinely characterized by immunocytochemical detection of the expression of developmental stage-specific OL markers, A2B5 (progenitors), O4 (later-stage precursors), O1 (immature OLs), and myelin basic protein (MBP) (mature OLs). A representative pre-OL culture had the following composition: 95% A2B5+, 90% O4+, 4% O1+, and 1% MBP+. All cultures contained less than 2% of glial fibrillary acidic protein (GFAP)-positive astrocytes and essentially nondetectable CD11+ microglia.

**Oxygen-glucose Deprivation (OGD)—** Cultures were switched to the same medium that was deoxygenated and lacked glucose (Invitrogen) and transferred to an anaerobic chamber filled with 95% N\(_2\) plus 5% CO\(_2\) at 37 °C (7, 15). Pharmacological agents were applied 10 min before exposure of the cells to OGD for 2 h. Cell death was assessed 24 h later, as described previously (7, 15).

**Immunocytochemistry—** After fixation with 4% paraformaldehyde and blocking nonspecific binding with 2% bovine serum albumin, cells were incubated for 1 h with a primary antibody, washed three times with phosphate-buffered saline, and incubated for 30 min with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgM (1:100) for A2B5, O4, or O1, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100) for MBP, GFAP, or anti-GluRs. For the staining of intracellular antigens (MBP and GFAP), cells were first permeabilized with 0.1% Triton X-100 for 30 min. Cells were mounted in Fluoromount G (Southern Scientific Associates, Inc.) and viewed with a Zeiss epifluorescence microscope.

**Cobalt (Co\(^{2+}\)) Staining—** Co\(^{2+}\) uptake evoked by AMPA plus the AMPAR desensitization blocker cyclothiazide (CTZ) was used to assess AMPAR-mediated Ca\(^{2+}\) permeability (16). Cultures were exposed to 5 mM CoCl\(_2\) in uptake buffer (139 mM sucrose, 57.5 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 1 mM CaCl\(_2\), 12 mM glucose, and 10 mM HEPES, pH 7.4) stimulated with AMPA (100 μM) + CTZ (50 μM) for 20 min at room temperature. Cultures were then washed twice in uptake buffer containing EDTA (2 mM) to remove extracellular Co\(^{2+}\). The intracellular Co\(^{2+}\) was precipitated with 0.55% (NH\(_4\))\(_2\)S in uptake buffer for 10 min. Cultures were then washed with three changes of uptake buffer and fixed with 4% paraformaldehyde for 30 min, and the Co\(^{2+}\) staining was enhanced by a silver intensification with 0.066% AgNO\(_3\) in development buffer (292 mM sucrose, 15.5 mM hydroquinone, and 42 mM citric acid) (16). The incubation was performed at 55 °C, and the solution was changed at 15-min intervals for 45 min. The reaction was terminated by rinsing in warm development buffer. In experiments where the AMPAR blocker 6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione (NBQX) was used, NBQX (50 μM) was added in uptake buffer 10 min before adding AMPA and CTZ. In some experiments, cells were then permeabilized and immunocytochemically labeled with the OL precursor marker A2B5 and/or anti-GluRs.

**GluR2 Immunodepletion and Western Blots—** To deplete GluR2 from pre-OL lysates, immunoprecipitation was performed (17). A highly specific anti-GluR2 antibody (2 μg; Chemicon) was incubated with 10 μl of packed resin of protein A-agarose (Amersham Biosciences) for 4 h. The resin was then washed and mixed overnight with 90 μl of pre-OL lysates. The mixture was centrifuged briefly, and the unbound fraction was collected. The same volumes of the unbound fractions, as well as the untreated lysates that served as controls, were electrophoresed on 10% SDS-polyacrylamide gels and transferred to
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polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk for 1 h and then incubated with the primary antibody against GluR1/2/3/4 (anti-GluR1, 2, and 4, Chemicon; anti-GluR3, Zymed Laboratories Inc.; specificity, no cross-reaction of each antibody to other GluR subunits) overnight at 4 °C, followed by a horseradish peroxidase-conjugated secondary antibody (1:20,000). Immunoreactive bands were visualized using enhanced chemiluminescence according to the instructions from the manufacturer (Pierce).

Infection of Cultured Cells with Recombinant Virus—Vector construction for herpes simplex virus (HSV) carrying GluRs or LacZ has been described elsewhere (18). GluR or LacZ cDNAs were inserted into the HSVprpUC ampiclon, packaged with helper 5dl1.2, purified on a sucrose gradient, pelleted, and resuspended in 10% sucrose. Average titer of the virus stocks was 2.0 × 10⁷ infectious units/ml, and transgene expression was regulated by the HSV constitutively active promoter IE 4/5. Adenovirus carrying VP16-CREB, a fusion protein between HSV VP16 (amino acids 363–490) and CREB (amino acids 88–341), was constructed and produced as described previously (19). The final concentration for adenoviral infection was 10¹⁰ infectious units/ml. Pre-OLs were exposed to HSV in regular culture medium for 4 h or adenovirus for 30 min. Following a complete change of the medium, the cultures were maintained for an additional 24 h before any experiments were performed. The viral vector preparations used at the specific concentrations in this study had diminished toxicity, because they contained no detectable wild-type virus, were purified of cellular debris, and had increased ratios of amplicon (virus particles that carry the transgene) to helper virus (virus particles necessary to package amplicon).

Stereotactic Intracerebral Injections—To investigate whether CREB might regulate GluR2 expression in vivo, we performed stereotactic, intracerebral injections of the virus carrying constitutively active VP16-CREB into the pericallosal white matter of developing Long-Evans rats at P6 using our protocol described previously (6). In brief, by the use of aseptic surgical technique, a scalp incision was made in the skull surface, and a burr hole was placed above the desired injection location. The rat was placed in a stereotactic apparatus, and a pulled-glass micropipette attached to a nanoinjector (Drummond Scientific) was lowered with a micromanipulator to the pericallosal white matter, 1 mm lateral to the midline and 1 mm posterior to the periglomerular layer. Virus particles were infused with a volume of 0.5 μl at 0.1 μl/min. After injection, the scalp wound was sutured, and the pup recovered on a heated blanket to maintain temperature at 33–34 °C and then was returned to its dam. Rats were sacrificed 24 h after intracerebral injection. Coronal brain slices (300 μm) from decapitated P7 rat pups were collected in oxygen-rich rat artificial cerebral spinal fluid at 4 °C, stabilized, and then maintained at 22 °C. Slices were then used to perform Co²⁺ staining and immunocytochemistry in situ using methods as described above for cultured cells.

Data Analysis—All experiments were performed in triplicate, and all data represent the mean ± S.E. values of at least three independent experiments. Statistical differences were assessed by one-way analysis of variance with Tukey post hoc analysis for multiple comparisons. Student’s t test was used when only two independent groups were compared. Statistical significance was determined at p < 0.05.

RESULTS

AMPA Heterogeneity in pre-OLs: Co-existence of GluR2-containing and GluR2-lacking AMPARs on a Single Cell—The GluR2 subunit dictates the Ca²⁺ permeability of AMPARs (11–13). Although we and others have reported previously the presence of functional Ca²⁺-permeable AMPARs on pre-OLs (6–8), it was not known whether this was a property of all or just a fraction of AMPARs. We first examined GluR2 expression on pre-OLs, as GluR2-lacking AMPARs are associated with Ca²⁺ permeability. Interestingly, we found that each individual pre-OL, as identified by the precursor marker A2B5, expresses GluR2 (Fig. 1A). As pre-OLs are highly vulnerable to Ca²⁺-mediated excitotoxic injury, we determined whether GluR2-lacking AMPARs might also be present on the same cells expressing GluR2-containing AMPARs. We performed Co²⁺ staining on pre-OLs stimulated with AMPA (100 μM) plus the AMPAR desensitization blocker CTZ (50 μM) for 20 min. Co²⁺ is a divalent ion that enters the cell via Ca²⁺-permeable AMPARs and can be used to assay for GluR2-lacking AMPARs (16). Strikingly, we found each individual pre-OL was positive for Co²⁺ staining (Fig. 1A). Furthermore, the AMPAR antagonist NBQX (50 μM) blocked Co²⁺ staining (Fig. 1A). NBQX rather than more selective channel blockers such as Joro spider toxin was used to block the Co²⁺ uptake, because channel blockers allowed fractional Ca²⁺ entry and thus failed to fully block divalent permeability (7). These results indicate that individual pre-OLs co-express GluR2-lacking and GluR2-containing AMPARs. To corroborate this finding with an alternative biochemical approach, immunoblotting of pre-OL total cell lysates versus the cell lysates after GluR2 immunodepletion with a highly specific anti-GluR2 antibody was performed (Fig. 1B). The presence of GluR2-free AMPARs was demonstrated by the detection of GluR1/3/4 subunits in the GluR2-immunodepleted cell lysates in which GluR2 levels were virtually nondetectable (Fig. 1B). Taken together, these data suggest that differential assembly of receptor subunits in individual pre-OLs results in both Ca²⁺-permeable and Ca²⁺-impermeable AMPARs within a single cell.

GluR2-lacking AMPARs Mediate Pre-OL Excitotoxicity—To determine whether pre-OL excitotoxicity was specifically mediated by Ca²⁺-permeable AMPARs lacking GluR2, we examined the effect of virus-mediated GluR2 overexpression on Ca²⁺ permeability of AMPARs and on OGD-induced pre-OL excitotoxicity. Unlike other AMPAR subunits, GluR2 undergoes RNA editing that alters a genetically coded glutamine into an arginine located in the pore region of the molecule (11–13). The Glu/Arg change makes AMPARs that contain the GluR2 subunit virtually impermeable to Ca²⁺ (11–13). Thus, a selective increase in edited GluR2 could have important functional consequences. We infected pre-OLs with a recombinant herpes simplex virus (HSV) vector encoding edited GluR2 (HSV-GluR2). For comparison, we also infected sister cultures with viral vectors encoding GluR1 (HSV-GluR1), which served as a receptor subunit control, LacZ (HSV-LacZ), which encodes β-galactosidase and served as a control protein, or the unedited
form of GluR2 (HSV-GluR2(Q)), which forms channels that retain permeability to Ca\(^{2+}\). These HSV vectors mediated transgene expression in ~80% of the cells, as determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining of the HSV-LacZ-infected cultures (data not shown). Although GluR2 was overexpressed in ~80% of the cells (Fig. 2A), in the HSV-GluR2-infected cultures the expression of GluR1 (Fig. 2A) and other AMPAR subunits (data not shown) was not changed, indicating that the viral gene transfer did not affect expression of other GluR subunits.

To examine the effect of virus-mediated GluR2 overexpression on Ca\(^{2+}\) permeability of AMPARs, we performed Co\(^{2+}\) staining on the infected cultures stimulated by AMPA (100 μM) plus CTZ (50 μM), with nonstimulated cultures as the basal condition (Fig. 2B). Expression of HSV-GluR2 virtually abolished AMPA-evoked Co\(^{2+}\) uptake compared with that of the control vector HSV-LacZ or the receptor subunit control HSV-GluR1 (Fig. 2B).

We also examined the effect of HSV-GluRs on OGD (2 h)-induced pre-OL death, which we have shown previously to occur by AMPAR-mediated toxicity (7, 15). HSV-LacZ was found to be slightly toxic as compared with the nonviral control (Fig. 2C). Expression of HSV-GluR2 markedly decreased OGD-induced toxicity, whereas replacing endogenous GluR2 with its unedited form, GluR2(Q), which converts GluR2-containing Ca\(^{2+}\)-impermeable AMPARs to Ca\(^{2+}\)-permeable receptors, increased pre-OL vulnerability to OGD (p < 0.001). Expression of HSV-GluR1 also significantly decreased cell survival in the OGD paradigm from 37.9 ± 4.95% to 15.2 ± 3.45% (p < 0.01). Because GluR1 is Ca\(^{2+}\)-permeable, increased GluR1 expression on the cells by HSV gene transfer is expected to enhance Ca\(^{2+}\) permeability and cell death. Taken together, these results indicate that expression of the GluR2 subunit critically regulates pre-OL excitotoxicity.

CREB Is Transiently Phosphorylated and Subsequently Down-regulated during Pre-OL Excitotoxicity—Excitotoxic signals and Ca\(^{2+}\) influx have been linked to activation of the nuclear factor CREB (14, 20). CREB phosphorylation at Ser-133 is necessary for its transcriptional activating function (14). As CREB is expressed in cells of the OL lineage (21, 21), we examined whether CREB Ser-133 phosphorylation was regulated by the AMPAR antagonist kainate or OGD in pre-OLs. Pre-OLs were treated with kainate or exposed to OGD and harvested at selected time points before cell death was apparent. Cell extracts were then immunoblotted with a phospho-specific antibody (anti-p-CREB) that recognizes the 43-kDa CREB protein only when the Ser-133 amino acid residue is phosphorylated (14, 20). Kainate (100 μM) (Fig. 3A) or OGD (2 h) (Fig. 3B) elicited initially a transient phosphorylation of the nuclear factor, which then declined toward basal levels. The AMPAR antagonist NBQX prevented kainate-induced CREB Ser-133 phosphorylation (Fig. 3A). Immunoblot analysis with an anti-CREB antibody recognizing both the phosphorylated and unphosphorylated forms of CREB showed that total CREB protein levels are then down-regulated following its transient Ser-133 phosphorylation in response to kainate (Fig. 3A) or OGD (Fig. 3B). NBQX prevented kainate-induced down-regulation of total CREB (Fig. 3A). Taken together, excitotoxic signals cause transient phosphorylation and subsequent down-regulation of the nuclear factor CREB in pre-OLs.
Expression of a Constitutively Active Form of CREB Protects Pre-OLs from Excitotoxicity—To examine whether CREB could be a survival factor in AMPAR-mediated pre-OL excitotoxicity, we expressed VP16-CREB, a constitutively active form of CREB, in pre-OLs. The VP16-CREB chimeric cDNA was inserted into an adenovirus delivery vector that also contained the green fluorescence protein (GFP) cDNA (19). The efficiency of the transgene expression was ~90%, as shown by the GFP expression (Fig. 4A). Expression of the VP16-CREB fusion protein was confirmed by Western blotting with anti-VP16 and antibodies against total CREB or p-CREB (Fig. 4B). We next examined the vulnerability of pre-OLs infected with virus with or without the VP16-CREB to excitotoxicity. Cells expressing VP16-CREB showed a marked resistance to kainate (100 μM) or OGD (2 h)-induced cell death compared with respective control without VP16-CREB (p < 0.001) (Fig. 4C). These results indicate that persistent activation of CREB in pre-OLs can overcome excitotoxicity.

DISCUSSION

Developing OLs are highly vulnerable to excitotoxic injury. We have shown previously that non-NMDA GluR expression is developmentally regulated on OLs in vivo and in vitro and that these receptors mainly mediate OL excitotoxicity (6–8). In this study, we report that AMPARs are heterogeneous in individual pre-OLs and that these cells co-express GluR2-lacking and GluR2-containing receptors. A critical role of Ca2+-impermeable GluR2-lacking AMPARs in pre-OL excitotoxicity is supported by the demonstration that virus-mediated overexpression of

Expression of VP16-CREB Up-regulates GluR2 on Pre-OLs and Blocks Ca2+ Permeability—CREB has more than 100 target genes (14, 20), many of which may play a role in regulation of GluR2 and Ca2+ permeability of AMPARs (11, 22). We examined whether the protective effects of constitutively active VP16-CREB against pre-OLs excitotoxicity could be related to the regulation of GluR2 levels. Immunoblotting showed that GluR2 was selectively up-regulated by ~3.5-fold in the VP16-CREB-infected culture compared with control, whereas the GluR4 level was unchanged (Fig. 5A). Furthermore, pre-OLs expressing VP16-CREB were resistant to Co2+ uptake stimulated with AMPA (100 μM) plus CTZ (50 μM), indicating the AMPARs are Ca2+-impermeable in the VP16-CREB-infected culture (Fig. 5B). Thus, these data indicate that expression of VP16-CREB up-regulates GluR2 on pre-OLs and blocks Ca2+ permeability.

Virus-mediated Expression of VP16-CREB in Vivo Up-regulates GluR2 Expression and Blocks Co2+ Uptake in Situ in Developing White Matter—To investigate whether CREB might regulate GluR2 expression in vivo, we performed intracerebral injections of the virus carrying constitutively active VP16-CREB into the pericallosal white matter of developing Long-Evans rats at P6 using our protocol published previously (6). The expression of the constitutively active CREB was shown by the GFP expression in the pericallosal white matter (Fig. 6A). Many but not all GFP-positive cells were co-localized with the pre-OL marker O4 (Fig. 6A), whereas others appeared to be astrocytes. Immunocytochemistry showed that GluR2 expression was up-regulated in the VP16-CREB-infected white matter compared with control, whereas GluR4 expression was unchanged (Fig. 6B). Furthermore, VP16-CREB-infected white matter slices were resistant to AMPAR-mediated Co2+ uptake stimulated with AMPA (100 μM) plus CTZ (50 μM) compared with control white matter slices (Fig. 6B). Thus, these data indicate that expression of VP16-CREB up-regulates GluR2 expression and blocks Ca2+ permeability in situ in the developing cerebral white matter.
edited GluR2 in pre-OLs confers resistance to excitotoxicity, whereas expression of unedited GluR2(Q), which converts GluR2-containing AMPARs to Ca\(^{2+}\)-permeable receptors, enhances pre-OL vulnerability to excitotoxicity. Furthermore, excitotoxic signals cause transient phosphorylation of CREB and subsequent down-regulation of the protein, and expression of a constitutively active form of CREB both in vitro and in vivo up-regulates GluR2 levels, blocks Ca\(^{2+}\) permeability, and protects pre-OLs from excitotoxicity. Thus, this study provides new insights into excitotoxic mechanisms of pre-OL injury by showing that overexpression of edited GluR2 or a constitutively active form of CREB is protective against pre-OL excitotoxicity and that CREB regulates GluR2 expression in pre-OLs both in vitro and in vivo.

Although the notion that AMPA/kainate receptors but not NMDA receptors mediate OL death has become dogma, three recent reports documented NMDA subtype GluRs in OLs in vivo (23–25). Developing and mature OLs express NMDA receptors on cellular processes, whereas AMPA/kainate receptors are mostly localized on somas. Overstimulation of AMPA/kainate receptors leads to OL death, whereas overstimulation of NMDA receptors leads to loss of cellular processes. Thus, OLs express both NMDA and AMPA/kainate receptors with distinct compartmentalization of these receptor subtypes. These studies support the fact that AMPA/kainate receptors play a critical role in OL death, and also highlight the notion that NMDA receptors might play an important role in OL damage by inducing cellular process loss (23–25). Focusing on AMPA/kainate receptor-mediated cell death may miss more subtle and perhaps important changes in NMDA receptor-mediated damage to OL processes. Obviously, the long term effects of over-stimulation of NMDA receptors on OLs deserve additional study. In addition, although we have previously shown that AMPAR-preferring antagonists are significantly more protective against OL excitotoxicity than antagonists relatively selective for kainate receptors (7), OLs do also express high levels of kainate receptors (9, 10), and activation of kainate receptors can lead to excitotoxicity when AMPARs are blocked (26, 27). Thus, the role of kainate receptors in OL injury should also not be overlooked. Nevertheless, this study focuses on studying the role of Ca\(^{2+}\)-permeable AMPARs in pre-OL excitotoxicity and suggests that these receptors are both necessary and sufficient in mediating pre-OL injury.

**Heterogeneity of AMPARs on Individual Pre-OLs**—AMPARs are assembled from receptor subunits GluR1–4 and exhibit enormous diversity in their functional properties depending upon receptor composition (11, 22, 28). In this study, we showed that pre-OLs, although demonstrating AMPAR-mediated Ca\(^{2+}\) uptake, also express GluR2, suggesting that GluR2-containing and GluR2-lacking AMPARs co-exist in individual pre-OLs. Previous data suggest that subunit composition of AMPARs does not necessarily adhere to a fixed stoichiometry (29), thus allowing for differential assembly of subunits within a single cell.

**FIGURE 4.** Expression of constitutively active VP16-CREB protects pre-OLs from excitotoxicity. A, nuclear staining and GFP fluorescence image of pre-OLs expressing VP16-CREB. B, immunoblots of the control or VP16-CREB-infected culture with anti-VP16, anti-CREB, or anti-p-CREB. C, protective effect of VP16-CREB expression in pre-OLs against kainate (100 \(\mu\)M) or OGD (2 h)-induced cell death at 24 h. ***, \(p < 0.001\) versus corresponding control.

**FIGURE 5.** Expression of constitutively active VP16-CREB up-regulates GluR2 on pre-OLs and inhibits Ca\(^{2+}\) permeability. A, immunoblots and densitometric quantifications showing GluR2, GluR4, and actin levels in the control versus VP16-CREB expressing culture. B, Ca\(^{2+}\) staining of the control versus VP16-CREB expressing cells stimulated with AMPA + CTZ.
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Despite the presence of relatively high levels of GluR2 on pre-OLs, it is still possible that GluR2 subunits may be simply unassembled into functional AMPARs. Numerous previous studies have documented the trafficking of GluR2 to the cell surface. Furthermore, we have shown previously that functional GluR2-containing, Ca\(^{2+}\)-impermeable AMPARs are indeed expressed on the cell surface of pre-OLs (7, 8). We have demonstrated the cell-surface expression of GluR2 in pre-OLs by a cell-surface protein biotinylation experiment (7), and we have shown that the I–V relationship by whole-cell patch clamping recording on pre-OLs exhibits a linear or moderate outward rectification in response to kainate stimulation, indicating that edited GluR2 receptors are included in the heteromeric AMPARs in the surface of these cells (8).

Taken together, despite the expression of GluR2-containing AMPARs on pre-OLs, a subset of GluR2-lacking AMPARs is also expressed on the same cells, confers Ca\(^{2+}\)-impermeability, and mediates pre-OL excitotoxicity. Thus, the heterogeneity of AMPARs in a single cell accounts for AMPAR-mediated Ca\(^{2+}\) permeability in pre-OLs and vulnerability of pre-OLs to excitotoxicity. Differential assembly of GluRs appears to be a common phenomenon and may play important roles in many physiological and pathological processes (30). The differential assembly of GluRs may be modified by diseases affecting OL survival to alter Ca\(^{2+}\) permeability of the receptors, and thus may have important clinical implications in these disorders.

Role of Ca\(^{2+}\)-permeable AMPARs in Pre-OL Excitotoxicity—Our results indicate that only a subset of AMPARs on individual pre-OLs are GluR2-lacking and Ca\(^{2+}\)-permeable and mediate pre-OL excitotoxicity. Targeting GluR2-lacking AMPARs, while still permitting GluR2-containing AMPARs to fulfill their physiological roles, may be an appealing possibility for treating pre-OL excitotoxicity. We thus propose that targeting Ca\(^{2+}\)-permeable AMPARs selectively, leaving Ca\(^{2+}\)-impermeable AMPARs unaffected, may have a more favorable profile of adverse effects and may represent a useful therapeutic strategy in treating excitotoxic pre-OL injury seen in human cerebral white matter disorders.

Role of CREB in Pre-OL Excitotoxicity—Our results indicate that the transcription factor CREB is a nuclear target for signaling pathways initiated by AMPAR activation in pre-OLs, consistent with the observations in many other systems (14, 20). The initial transient phosphorylation of CREB in response to excitotoxic signals in pre-OLs is similar to the neuronal response to excitotoxic and other toxic stimuli, perhaps representing the initiation of common stress signaling pathways (14, 20). Our data further show that CREB is subsequently down-regulated during pre-OL excitotoxicity, and that expression of a version of the CREB gene that is always activated can protect the vulnerable pre-OLs from excitotoxic injury, consistent with the demonstration that persistent CREB activation is involved in cell survival-related gene expression (14, 20). Furthermore, we found that expression of VP16-CREB selectively up-regulates GluR2 relative to the other AMPAR subunit GluR4 on pre-OLs both in vitro and in vivo. GluR2 confers Ca\(^{2+}\) impermeability to AMPARs, and thus may at least in part account for the protective effects of constitutively active VP16-CREB.
against pre-OL excitotoxicity. Over 100 genes have been reported to be directly regulated by CREB or related proteins (14, 20). Of these, different repertoires of gene expression have been implicated in regulating Ca2+ permeability and cell survival (14, 20). Although further studies are needed to fully address the mechanisms by which CREB controls GluR2 gene expression, our study establishes that CREB is a survival factor in AMPAR-mediated excitotoxic injury to pre-OLs, and the protective effects of constitutively active VP16-CREB against pre-OLs excitotoxicity are related to the up-regulation of GluR2 on the cells.

Overall, our study reveals the heterogeneity of AMPARs on OLs, establishes a critical role of GluR2 and CREB in OL excitotoxicity, demonstrates the CREB regulation of GluR2 expression in vitro and in vivo, and suggests that targeting GluR2-lacking AMPARs or CREB may be a useful approach for treating disorders associated with OL excitotoxicity. The vulnerability of pre-OLs to excitotoxicity may at least in part account for cerebral white matter injury following hypoxia-ischemia. Hypoxic-ischemic injury to the brain leads to devastating neurological consequences, and the pattern of injury is highly age-dependent. In term infants, the pattern of injury predominantly involves cerebral cortex with characteristic neuronal injury, but in premature infants, the pattern selectively involves white matter with prominent OL injury, a disorder termed periventricular leukomalacia (31, 32). Pre-OLs are the principal cell type that is injured in periventricular leukomalacia, which is the leading cause of cerebral palsy in premature infants (31, 32). OL excitotoxicity may also be involved in the pathogenesis of demyelinating diseases, such as multiple sclerosis (33, 34), and in white matter injury in stroke (35, 36). Our study provides new insights into the mechanisms of OL injury and potential therapeutic approaches for such disorders.

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REFERENCES

1. Yoshioka, A., Hardy, M., Younkin, D. P., Grinspan, J. B., Stern, J. L., and Pleasure, D. (1995) J. Neurochem. 64, 2442–2448
2. Yoshioka, A., Bacskai, B., and Pleasure, D. (1996) J. Neurosci. Res. 46, 427–438
3. Matute, C., Sanchez-Gomez, M. V., Martinez-Millan, L., and Miledi, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8830–8835
4. McDonald, J. W., Althomsons, S. P., Krzysztof, L. H., Choi, D. W., and Goldberg, M. P. (1998) Nat. Med. 4, 291–297
5. Fern, R., and Moller, T. (2000) J. Neurosci. 20, 34–42
6. Follett, P. L., Rosenberg, P. A., Volpe, J. J., and Jensen, F. E. (2000) J. Neurosci. 20, 9235–9241
7. Deng, W., Rosenberg, P. A., Volpe, J. J., and Jensen, F. E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 6801–6806
8. Rosenberg, P. A., Dai, W., Gan, X. D., Ali, S., Fu, J., Back, A. S., Sanchez, R. M., Segal, M. M., Follett, P. L., Jensen, F. E., and Volpe, J. J. (2003) J. Neurosci. Res. 71, 237–245
9. Patneau, D. K., Wright, P. W., and Wisden, W. (1994) Neuron 12, 357–371
10. Gallo, V., and Ghiani, C. A. (2000) Trends Pharmacol. Sci. 21, 252–258
11. Hollmann, M., and Heinemann, S. (1994) Annu. Rev. Neurosci. 17, 31–108
12. Burns, N., Monyer, H., Seeburg, P. H., and Sakmann, B. (1992) Neuron 8, 189–198
13. Jonas, P., and Burns, N. (1995) Neuron 15, 987–990
14. Lonze, B. E., and Ginty, D. D. (2002) Neuron 35, 605–623
15. Deng, W., Wang, H., Rosenberg, P. A., Volpe, J. J., and Jensen, F. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7751–7756
16. Sanchez, R. M., Koh, S., Rio, C., Wang, C., Lamperti, E. D., Sharma, D., Corfas, G., and Jensen, F. E. (2001) J. Neurosci. 21, 8154–8163
17. Wenthold, R. J., Petralia, R. S., Blahos, J., and Niedzielski, A. S. (1996) J. Neurosci. 16, 1982–1989
18. Neve, R. L., Howe, J. R., Hong, S., and Kalb, R. G. (1997) Neuroscience 79, 435–447
19. Barco, A., Alarcon, J. M., and Kandel, E. R. (2002) Cell 108, 689–703
20. Shaywitz, A. J., and Greenberg, M. E. (1999) Annu. Rev. Biochem. 68, 821–861
21. Pende, M., Fisher, T. L., Simpson, P. B., Russell, J. T., Blenis, J., and Gallo, V. (1997) J. Neurosci. 17, 1291–1301
22. Dingleline, R., Borges, K., Bowie, D., and Traynied, S. F. (1998) Pharmaco. Rev. 51, 7–61
23. Karadottir, R., Cavelier, P., Bergeisen, L. H., and Attwell, D. (2005) Nature 438, 1162–1166
24. Saltz, M. G., and Fern, R. (2005) Nature 438, 1167–1171
25. Micu, I., Jiang, Q., Coderre, E., Ridsdale, A., Zhang, L., Woulfe, J., Yin, X., Trapp, B. D., McRory, J. E., Rehak, R., Zamponi, G. W., Wang, W., and Stys, P. K. (2006) Nature 439, 988–992
26. Alberdi, E., Sanchez-Gomez, M. V., Marino, A., and Matute, C. (2002) Neurobiol. Dis. 9, 234–243
27. Sanchez-Gomez, M. V., and Matute, C. (1999) Neurobiol. Dis. 6, 475–485
28. Ozawa, S., Kamiya, H., and Tsuzuki, K. (1998) Prog. Neurobiol. 54, 581–618
29. Washburn, M. S., Numberger, M., Zhang, S., and Dingledine, R. (1997) J. Neurosci. 17, 9393–9406
30. Rubio, M. E., and Wenthold, R. J. (1997) Pediatr. Res. 41, 112–130
31. Volpe, J. J. (2000) Neurology of the Newborn, 4th Ed., pp. 217–276, W. B. Saunders Co., Philadelphia
32. Volpe, J. J. (2003) Pediatr. 112, 176–180
33. Pitt, D., Werner, P., and Raine, C. S. (2000) Nat. Med. 6, 67–70
34. Matute, C., Alberdi, E., Domercq, M., Perez-Cerda, F., Perez-Samartin, A., and Sanchez-Gomez, M. V. (2001) Trends Neurosci. 24, 224–230
35. Stys, P. K. (2004) Curr. Mol. Med. (Hilversum) 4, 113–130
36. Liu, Y., Silverstein, F. S., Skoff, R., and Barks, J. D. (2002) Pediatr. Res. 51, 25–33