GluR2 knockdown reveals a dissociation between $[Ca^{2+}]_i$ surge and neurotoxicity

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Abstract

Reduction in GluR2 subunit expression and subsequent increases in AMPA receptor mediated $Ca^{2+}$ influx were postulated to exacerbate glutamate neurotoxicity following seizures or global ischemia. To directly test the effects of shifting the GluR1/GluR2 subunit ratio on excitotoxicity, GluR2 antisense deoxyoligonucleotides (AS-ODNs) were applied to dissociated hippocampal cultures for 1–8 days. The GluR1/GluR2 protein ratio was examined immunohistochemically and by Western blotting. $[Ca^{2+}]_i$ concentrations were determined by ratiometric imaging of Fura 2-loaded cells. The cultures were exposed to glutamate, AMPA, NMDA or kainic acid (KA) 3 days after GluR2 knockdown and cell viability was determined 1 day later by MTT reduction assay or Trypan blue exclusion. Although GluR2 AS-ODNs increased the GluR1/GluR2 protein ratio in a time dependent manner, neurons and glia appeared healthy and MTT reduction values were similar to untreated and sense controls. Basal $[Ca^{2+}]_i$ levels were unchanged but $[Ca^{2+}]_i$ was selectively increased by agonist stimulation of AMPA receptors. Unexpectedly, delayed neurotoxicity was attenuated at saturating doses of glutamate while little difference in cell viability was observed at lower doses or with the other excitotoxins at any concentration. Therefore, there was a dissociation between rises in AMPA receptor-mediated $Ca^{2+}$ influx and neurotoxicity despite marked decreases in GluR2 but not GluR1 immunoreactivity. It is proposed that a modification of AMPA receptor stoichiometry that raises agonist-stimulated $Ca^{2+}$ influx during an excitotoxic insult may have eventual neuroprotective effects.

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1. Introduction

Sustained elevation of intracellular $Ca^{2+}$ is considered to be a major cause of neuronal cell death associated with acute or chronic neuropathological disorders. This presumably results from persistent release of glutamate and subsequent over-activation of ionotropic glutamate receptors (Orrenius et al., 1989; Zoghbi et al., 2000). In vitro models of neurotoxicity or neuroprotection, have been powerful in disclosing which cell types are vulnerable and under which conditions neurons and glia are sensitive to excitotoxins such as glutamate, NMDA or non-NMDA agonists (reviewed by Matson and Mark, 1996). In general, neurotoxicity can be mediated through ionotropic glutamate receptors (iGluRs) (Zoghbi et al., 2000) and neuroprotection via metabotropic receptors (mGluRs) (Adamchik and Baskys, 2000; Bruno et al., 2000). For AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid)-type glutamate receptors, four genes have been cloned designated GluR1–GluR4 (Hollmann et al., 1991; Keinänen et al., 1990). It is now known that these receptors exist as $Ca^{2+}$ permeable and $Ca^{2+}$ impermeable channels. Receptors lacking GluR2 subunits are $Ca^{2+}$ permeable (Hollmann et al., 1991; Verdoo et al., 1991). Therefore, a reduction in GluR2 subunit expression was proposed to enhance glutamate excitotoxicity within principal neurons due to increases in cytosolic $Ca^{2+}$ mediated through AMPA receptor homomeric or heteromeric assemblies (e.g. GluR1, GluR3 or GluR4(D) (the GluR2 hypothesis) (Friedman et al., 1994, 1997; Pellegrini-Giampietro et al., 1999). This hypothesis was supported in vivo using the kainic acid (KA) model of delayed neurodegeneration since in adult rats, KA induces status epilepticus and pronounced decreases in GluR2 mRNA (Friedman et al., 1994) and protein in the vulnerable CA3 subregion but prior to cell
loss (Friedman, 1998). Our study of KA-induced seizures in development also supported the GluR2 hypothesis, since at young ages, rat pups that are relatively resistant to CA3 damage do not show decreases in GluR2 expression (Friedman et al., 1997). In addition, intrahippocampal knockdown of the GluR2 subunit in rat pups produced electrographic paroxysmal activity and CA3 damage (Friedman and Velísková, 1998; Friedman and Koudinov, 1999).

Accordingly, GluR2 editing-deficient seizure phenotypes (Brosa et al., 1995; Feldmeyer et al., 1999) develop a post-natal epilepsy and selective CA3 neurodegeneration in immature mice. These studies all suggested that the GluR2 subunit could play a direct role in the pathology. However, the role of the GluR2 subunit in selective neurodegeneration is unresolved. For example, the GluR2 editing-deficient mutant mice (neo/neo) with the highest Ca\(^{2+}\) permeability were lethargic and developmentally retarded but they did not exhibit seizures or neurodegeneration (Feldmeyer et al., 1999). Other genetic manipulations of the GluR2 editing Q/R site show that mutants with 30% GluR2 editing deficiency and two-fold increases in AMPA receptor-mediated Ca\(^{2+}\) influx do not exhibit any degree of hippocampal pathology (Kusk et al., 1998). Furthermore, mature GluR2 knockout mice also do not display seizures nor hippocampal damage (Jia et al., 1996). Recently, Ishara et al. (2003) reported a complimentary study in cortical cultures derived from GluR2 null mutants showing that excitotoxic death does not correlate with Ca\(^{2+}\) permeability. The aim of our in vitro study was to test whether a selective reduction in GluR2 subunit expression by our antisense treatment paradigm could increase Ca\(^{2+}\) permeability through AMPA receptor GluR2 lacking channels (e.g. GluR1/GluR3) and subsequently enhance neurotoxicity by brief excitotoxin application. Dissociated hippocampal cultures were used for knockdown experiments. Ratiometric analysis of [Ca\(^{2+}\)]\_i with the dye indicator Fura 2AM was used to measure changes in Ca\(^{2+}\) concentrations and MTT reduction assay and Trypan blue exclusion tests were used for monitoring delayed glutamate-, AMPA-, NMDA- or KA-induced cell death.

2. Methods

2.1. Tissue culturing

Timed pregnant rats (18–19 days) were sacrificed by cervical dislocation and embryos carefully taken out to a chilled dissecting medium, which consists of oxygenated Leibovitz L15 medium (Biological Industries, Bet Haemek, IL) enriched with 0.6% glucose and gentamycin (Sigma, 20 μg/ml), and freshly added 2 mM glutamax. Prior to plating, cells were counted with a hemocytometer and resuspended to about 0.5 × 10^6 cells per well. An equal volume of this suspension was plated in each well (0.5 ml per well) to control the density (Mytilineou et al., 1999). Each well (15 mm diameter) contained 13 mm poly-L-lysine or poly-ornithine coated coverslips. Cells were left to grow in the incubator at 37 °C, 5% CO\(_2\), for 2 days, then medium was changed to 10% HS in enriched MEM. A mixture of 5-fluoro-2-deoxyuridine (FUDR)/uridine (Sigma, 20 g and 50 g/ml, respectively) was added after the glia was confluent. This treatment prevents overgrowth of glial cells that would otherwise kill the neurons. The next day the medium was changed to 10% HS in enriched MEM. Cultures were supplemented with fresh media every 4 days and conditioned media was collected for later use.

2.2. GluR2 AS-ODN and excitotoxin treatment

Phosphorothioated 21 mers were previously designed (Friedman and Velísková, 1998) to selectively decrease the hippocampal synthesis of the GluR2 subunit: 5’-CCGTGTTTATGGGGACTGATT-3’ base pairs 37-57, near the initiation codon start site. This sequence did not overlap with other mammalian sequences determined by a search of the Genebank/EMBL database. GluR2 sense (S-ODNs) (5’-AATACAGTCCCCCATAAAACCG-3’) or antisense (MS-ODNs), 5’-CACAAACCTTTCATATCGTT-3’ were derived from the antisense sequence. GluR2 antisense deoxyoligonucleotides (AS-ODNs) were dissolved in PBS (0.1 mM, pH 7.4) to a stock solution of 1 mM. To decrease GluR2 subunit synthesis, cultures were treated with 2 μl of the stock solution on days in vitro (DIV) 10-17 followed by 1 μl every other day. Control GluR2 S- and MS-ODNs from the antisense sequence were added at the same concentration to a separate group of sister cultures for comparison. The serum was replaced with a serum-free medium (B-27 Supplement, Gibco BRL) at the time treatment with AS-, S-, or MS-ODNs were initiated. Untreated controls were also changed to the serum-free medium and used to determine baseline cell viability under the same conditions. Four excitotoxins were added to sister cultures. Based on immunocytochemistry studies, the GluR2 AS-ODNs (2 μM initially and 1 μM supplement at 48 h) were added for 3 days. Three to eight cultures were exposed to several doses of glutamate (50, 100, 200, and 500 μM), AMPA (10, 100, and 500 μM), NMDA (10, 50, 100, and 200 μM) or KA (30, 100, and 500 μM) per experiment. Experiments were repeated three to nine times for each toxin and dose after the GluR2 AS-ODN treatment. In order to observe cell survival after glutamate, cultures were treated for 5–9 min at the highest dose (500 μM). To observe significant toxicity at lower doses, AMPA, NMDA, and KA excitotoxins were incubated for 15 min at 37 °C at all of the doses tested.
according to other studies (Weaver et al., 1998). When neurotoxicity to AMPA was measured, the medium contained 50 μM 2-APV to block NMDA channels. When neurotoxicity to NMDA was measured, the incubation medium contained 20 μM DNQX. In two experiments, the same cocktail used to monitor glutamate responses in the calcium imaging experiments described below was also tested in a few cultures (4–6) for comparison. To remove the excitotoxins and reduce baseline toxicity, cultures were washed with previously collected conditioned media three times. Cultures were then returned to the incubator for 24 h with fresh defined media and dissolved sense or antisense probes before performing viability assays.

2.3. Immunocytochemistry

Dissociated cultures plated onto coverslips were processed for GluR1 and GluR2 immunocytochemistry within flat well dishes as described (Friedman, 1998). All antisera are commercially available (Chemicon Int.). Culture wells containing coverslips were washed in cold PBS 1× containing coverslips were washed in cold PBS 1× are commercially available (Chemicon Int.). Culture wells containing coverslips were washed in cold PBS 1× and incubated for 4°C for 40 min. Anti-GluR1 (0.6 μg/ml) or anti-GluR2 (diluted 1:200) were added shaking overnight at 4°C. Coverslips were washed (3 × 10 min each) in PBS (0.01 M, pH 7.4) to remove unbound primary antibodies. Secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-rabbit IgG (H+L, diluted 1:200; Vector Laboratories, Burlingame, CA) were added for 2 h at 25°C. After three washes, ABC solution (Vector Laboratories) was added for 1 h. For visualization, the cultures were reacted with the chromogen, 3,3′-diaminobenzidine tetrahydrochloride (DAB: 10 mg/20 ml and 6 μl hydrogen peroxide). After the DAB reaction, coverslips were removed from the wells with forceps, then dehydrated and mounted onto slides. An upright Biological Nikon Microscope (Nikon Eclipse 800) equipped with a color digital spot camera and connected to a G3 Macintosh computer was used for morphological densitometry analysis with NIH image software and digital imaging.

2.4. Controls

Immunocytochemical assessment of signal specificity was performed on coverslips. Conditions were (1) PBS, followed by goat anti-rabbit or horse anti-mouse secondary antibody; (2) pre-immune mouse or rabbit serum at the same dilution as the primary antibody; or (3) primary antibody, to which had been pre-absorbed with the peptide antigen, followed by secondary antibody. The three control conditions gave no specific labeling.

2.5. Electrophoresis and immunoblotting

To solubilize membranes, cultures were scraped and homogenized in 200 μl of homogenization buffer supplemented with 1% Triton-X 100 and incubated 30 min at 4°C similar to that described previously (Friedman, 1998). After centrifugation (16,000 rpm for 20 min) the supernatant was frozen at −70°C. SDS-PAGE was performed using 5% polyacrylamide mini gels (Laemmli, 1970). Cellular proteins were transferred to nitrocellulose using a tank transfer system with transfer buffer (25 mM Tris, 192 mM glycine, 20% ethanol (v/v) as described previously (Towbin et al., 1979). The blots were blocked with 5% non-fat dry milk in TBS/0.01% sodium azide for 1 h. Polyclonal antibodies to GluR1 (0.5 μg/ml; Upstate Biotechnology) and GluR2 (generous gift of Dr. R. Wenthold) were applied overnight at 4°C. The immunoblots were probed for excess antibody to determine quantitative values for GluR1 and GluR2 antigens. For detection, a chemiluminescent assay (alkaline phosphatase-conjugated goat-anti-rabbit IgG and Immun-Lite125 II detection system, Bio-Rad) was used. Blots were exposed to X-ray film for several exposures and the optical density of the bands were quantified by computer densitometry (NIH Image software) to estimate the amount of receptor subunit protein in the plasma membrane. Densitometric replicate samples were averaged from two gels prepared from two groups of three platings (n = 6).

2.6. Loading of membrane permeable dyes

Fura-2 [Ca2+]i imaging was performed in dissociated hippocampal cultures at 24, 60 and 72 h after knockdown. Coverslips were washed with recording medium and incubated, at room temperature in a shaking bath for 1 h with Fura-2 AM (2.5 μM, Molecular Probes Inc.). Cultures were then washed in the recording medium, and viewed with an inverted Nikon microscope, using oil immersion lens (Nikon CF UV-F 40×, NA = 1.3). The coverslip was placed in a small (0.8 ml) chamber on the stage of the inverted microscope and perfused with the recording medium. Eight to ten fields of untreated, sense and antisense treated cultures were randomly illuminated at alternating wavelengths through narrow bandpass filters of 340 and 380 nm. There were typically two to six cells in a field. Paired images were taken using a cooled CCD camera before and after exposure to glutamate, AMPA, or NMDA at a rate of 0.5 Hz. Off line analysis of selected areas of the image was made with custom-made software (Segal, 1995). Calcium concentrations were derived from standard ratio imaging methods and the equation [Ca2+]i = Kd(Fmax/Fmin) [(R − Rmin)/(Rmax − R)] (Grynkiewicz et al., 1985). Drugs were applied via pressure pipettes placed near the imaged cells. The recording medium contained (in mM): 25 HEPES, 125 NaCl, 5 KCl, 0.5 MgCl2, 3 CaCl2, 30 glucose and 0.5 μM tetrodotoxin [pH 7.4, 330 mMΩ]. AMPA responses were measured in two ways. Responses to glutamate (100 μM) were measured for the detection of AMPA responses when the recording medium contained 50 μM 2-APV, and 3 mM Mg2+ to block NMDA receptors; nifedipine (20 μM) was used to block voltage-gated Ca2+ channels.
channels. When direct responses to AMPA (50 μM in the pressure pipette) were measured, the recording medium contained 3 mM MgCl2, 50 μM 5-APV and 100 μM Cd2+. For comparison responses to NMDA were measured and the recording medium contained 0 MgCl2, 10 μM glycine and 20 μM DNX.

2.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay

The MTT reduction assay was used to determine cell viability of the entire culture (neurons, glia, and fibroblasts) as described (Mytilineou et al., 1999). Briefly, 100 μl of a 5 mg/ml solution of MTT was added to each culture well containing 1 ml of medium. After 3 h of incubation at 37 °C, the medium was carefully aspirated and 1 ml of isopropanol alcohol was added for solubilization. Culture plates were gently rotated on a shaker for 10 min to fully release formazan crystals trapped within viable cells. Absorbency measurements were determined at 570 nm in a microplate reader (Spectramax 250).

2.8. Cell counts

After 24 h, 0.4% Trypan blue was added to estimate the number of dying cells in control and experimental groups. A subpopulation of cells (six fields per dish, 200× magnification for glutamate, NMDA and KA) from GluR2 AS-ODN and control (5 or untreated) cultures were counted by an experimenter blinded to the treatment with an inverted Nikon microscope and a grid lens (25 mm2) under phase-contrast. Unlabeled (viable) cells were also counted within the same field. Numbers of dying and viable cells were averaged to obtain total values. Percentages of dead and viable cells were then calculated for experimental and control groups.

2.9. Statistical analysis

Significant differences between control and experimental groups were determined by two-tailed Student’s t-test. For multiple comparisons one- or two-way ANOVA followed by Fishers least square protection test was used. Significance was set at P < 0.05. MTT baseline cell death from media replacement ranged from 10 to 15% compared to unchanged control cultures fed with serum in the media.

3. Results

3.1. GluR1/GluR2 protein ratio after GluR2 knockdown in vitro

Specific antibodies directed against GluR1 and GluR2 receptor subunits were used in parallel to determine whether selective changes in protein occur after the oligo treatments. Immunocytochemistry in culture showed that exposure of the GluR2 AS-ODNs for 2–3 days was sufficient to significantly reduce GluR2 but not GluR1 protein expression and the neurons looked healthy with normal morphology (Figs. 1 and 4). In S-ODN controls and after 3 days of GluR2 AS-ODN exposure, GluR1 immunoreactive neurons were intense and also healthy with long, mature processes (Fig. 1B–C). These were indistinguishable from control untreated cultures that were changed to defined medium (not illustrated). GluR1 protein levels were also sustained within individual neurons after 8 days of GluR2 AS-ODN treatment (Fig. 1B–C). GluR1 antibodies also marked the glia that appeared healthy and well labeled at all time points (Fig. 1). In contrast, few neurons expressed control levels of GluR2 protein after 3 days of knockdown and glia were faintly labeled (Fig. 2). Light microscopy showed that decreases in GluR2 protein expression were non-uniform and apparent in most fields of the culture (Fig. 1). Optical density decreases in GluR2 somatic immunoreactivity of neurons were first detected at 2 days (control, 168 ± 2.03 versus knockdown, 138 ± 5.18, n = 92 cells, P < 0.0001) (Fig. 2). At 3 days, reductions in GluR2 immunolabeling of total averaged neurons were reduced to 58.5 ± 4.2% of control. After 8 days, optical densitometry showed that immunoreactivity declined to very low levels (to 32 ± 3.47% of control) throughout the entire culture (Figs. 1 and 2). The reduction in GluR2 immunolabeling was not an artifact of the particular antibody used since the same downregulation was observed with three specific GluR2 antibodies (not illustrated).

Western blot analysis of membrane proteins from control and antisense treated cultures was performed to enable densitometric quantification of the GluR1/GluR2 protein ratio at 3 and 8 days after GluR2 knockdown. Equal volumes of split samples containing equal protein concentrations were run on the same acrylamide gel, transferred to nitocellulose membranes and probed with polyclonal GluR1 or GluR2 antibodies. After detection with chemiluminescence, optical densities of the bands were measured and the GluR1/GluR2 ratio for each sample was calculated (Fig. 3 and Table 1). Replicate samples were averaged from two gels prepared from two groups of three platings (n = 6, Table 1). 3 days, the GluR1/GluR2 protein ratio increased. At 8 days, GluR2 protein levels were low and GluR1 was sustained.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GluR1/GluR2 Western analysis 3 and 8 days after GluR2 knockdown</th>
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<tbody>
<tr>
<td></td>
<td>3 days</td>
</tr>
<tr>
<td>Sense</td>
<td>1.128 ± 0.15</td>
</tr>
<tr>
<td>Antisense</td>
<td>1.02 ± 0.14</td>
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<tr>
<td></td>
<td>8 days</td>
</tr>
<tr>
<td>Sense</td>
<td>1.89 ± 0.26</td>
</tr>
<tr>
<td>Antisense</td>
<td>3.88 ± 0.26</td>
</tr>
</tbody>
</table>

Values represent the ratio of optical density measurements for GluR1/GluR2 labeling and are therefore arbitrary numbers. Two experiments were run simultaneously. Replicate samples were averaged from two gels prepared from two groups of three platings (n = 8).

**P < 0.01, Student’s t-test.**
confirming the immunocytochemical observations. Thus, the loss of GluR2 immunoreactivity in culture or in the western proceeded in a time dependent manner and the GluR1/GluR2 ratio steadily increased with increased days of GluR2 AS-ODN exposure (Figs. 1–3).

3.2. Increased AMPA receptor-mediated Ca\textsuperscript{2+} responses after GluR2 knockdown

Intracellular Ca\textsuperscript{2+} imaging was carried out to determine whether a transient decrease in GluR2 synthesis induced

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**Fig. 1.** Digital micrographs of GluR2 immunoreactivity following GluR2 knockdown in vitro. (A, D) GluR1 and GluR2 immunolabeled neurons were intensely stained in all fields of the S-ODN control cultures. Healthy glia cells were also marked by these antibodies (small arrow). (E, F) After 3 days of GluR2 AS-ODN treatment, GluR2 but not GluR1 (B) immunoreactivity was highly (arrow head) or moderately reduced in all fields (large arrow). After 8 days of GluR2 AS-ODN treatment, GluR1 immunopositive neurons as well as glia were intensely stained throughout the soma and processes (small arrow) (C), whereas GluR2 immunoreactivity was nearly absent in neurons and glia were faint or not visible (F). Scale bar = 50 μm.

**Fig. 2.** Quantification of the total GluR1/GluR2 densitomeric ratio over increasing days in culture. Values are expressed as total raw data and represent the mean ± S.E.M. of densitometric measurements (NIH image) of six averaged fields quantified from sense control and antisense treated hippocampal cultures. *P < 0.05; **P < 0.001; †P < 0.0001. One- and two-way ANOVA with post hoc Fischer protected least significant difference (PLSD) test was carried out for statistical analysis.
by GluR2 AS-ODNs would cause a detectable increase in AMPA receptor-mediated Ca\textsuperscript{2+} permeability alone or in response to brief excitotoxic applications. Ca\textsuperscript{2+} image pairs were obtained from control cultures (untreated, S-ODN and MS-ODN) and Ca\textsuperscript{2+} measurements were determined at 24, 60, and 72h following of GluR2AS-ODN treatment. Basal levels of [Ca\textsuperscript{2+}] were unchanged after knockdown relative to S-ODN and MS-ODN and untreated serum-free control cultures at all times examined (Fig. 4). At 24h, no change in AMPA receptor-mediated Ca\textsuperscript{2+} responses to glutamate stimulation was observed (not illustrated, n = 2 experiments). In contrast, at 60–72h, GluR2 knockdown resulted in selective increases in AMPA receptor-mediated Ca\textsuperscript{2+} responses to glutamate in the same neurons in the presence of APV (50μM) and nifedipine (20μM). AS-ODN treated cultures had higher [Ca\textsuperscript{2+}] compared to MS-ODN treated controls at each time point following glutamate application and there were more cells with large responses (12 of 28 cells) than in the control cultures (only 7 of 52 cells) (Figs. 4 and 5A,B).

In another series of experiments following pulse application of AMPA in the presence of Ca\textsuperscript{2+} (100μM), control responses of untreated, S- and MS-ODN treated cultures were small (Baseline [Ca\textsuperscript{2+}]: 77±4.9 nM and after AMPA, 141±20.9 nM, n = 12 cells; 78.5±3.6 nM and during response to AMPA, 159±12 nM, n = 16 cells Figs. 4 and 5C). Similar to glutamate experiments, after GluR2 knockdown, responses to direct application of AMPA were also significantly higher relative to untreated and S-ODN treated cultures (knockdown baseline [Ca\textsuperscript{2+}]: 69.5±2.6 nM and during the response to AMPA [Ca\textsuperscript{2+}], reached 507±22 nM, n = 27 cells, P < 0.01) Figs. 4 and 5C. In contrast, when responses to NMDA were measured, the recording medium contained 0MgCl\textsubscript{2}, 10μM glycine and 20μM nifedipine. (E, F) AMPA responses with glutamate as stimulator were blocked by DNQX. The cell sizes are about 20μm in diameter.

In contrast to AMPA responses, NMDA responses were unchanged after GluR2 knockdown in sister cultures (S-ODN baseline: 74.5±2.6 nM, response to NMDA: 561.4±22, n = 42 cells; and knockdown baseline: 81.4±3.7, response to NMDA: 562.8±29.4, n = 30 cells) (Fig. 5C). Most neurons that showed increased Ca\textsuperscript{2+} responses after knockdown were significantly blocked with DNQX with little or no residual effect mediated by NMDA receptors (Fig. 5D).

Approximately 10–15% of the cells in all groups showed high Ca\textsuperscript{2+} permeability (>125 nM), consistent with the percentage of neurons that express GluR2-lacking AMPA receptor channels in similar hippocampal culture preparations (Pruss et al., 1991). Each experiment was replicated at least three times.

### 3.3. Reduced or unaltered glutamate and KA toxicity in culture after GluR2 knockdown

To determine whether sustained decreases of the GluR2 subunit prior to insult would enhance excitotoxicity, dose responses to glutamate, AMPA, NMDA, and KA were...
determined after brief application and 72 h exposure to the GluR2 AS-ODNs. At this time, GluR2 protein decreased and AMPA-receptor mediated Ca2+ permeability induced by glutamate or AMPA agonist applications increased significantly. Sister cultures that received S- or MS-ODN sequences or media alone were subjected to equal volumes of conditioned agonist-free medium and washes during excitotoxin applications without effect. Glutamate, AMPA, KA, and NMDA produced dose-dependent neurotoxicity responses such that glutamate and NMDA were most potent to produce insults followed by KA then AMPA (Fig. 5). However, triplicate absorbency values of the MTT reduction assay (570 nm), were not significantly altered following GluR2 knockdown. No effect was observed at unsaturating and saturating doses of AMPA, KA, or NMDA in the presence of appropriate antagonists for individual toxins compared with S-ODN treated cultures or serum-free media untreated controls (Fig. 5A–C). Interestingly, a dose–response curve for glutamate showed delayed toxicity was significantly reduced at high but not low doses of glutamate. This was observed as higher viability readings in the MTT reduction assay (Fig. 5B). In addition, counting the number of dying (Trypan blue positive) and viable cells (unlabeled cells, generally large in size) counted in sister cultures after glutamate, NMDA, or KA (each at 100 µM for 15 min 37°C), confirmed the MTT reduction assay observations. For example, after saturating doses of KA or NMDA, the percentage of dying cells was similar in control and antisense groups (percentage of dead cells: S-ODN + KA (500 µM), 68 ± 3.6% versus AS-ODN + KA, 63 ± 2.5%; and S-ODN + NMDA (100 µM), 63.5 ± 2.1 versus AS-ODN versus. 58 ± 2% averaged from six fields per dish, and three separate experiments, and Fig. 5C). Likewise, the percentage of viable (unlabeled) cells was significantly higher after GluR2 knockdown and 100 µM glutamate and but unaltered after moderate doses of KA (100 µM) (Figs. 5D and 6). Toxicity was suppressed by co-treatment with an antagonist of NMDA and voltage gated channels receptors (glutamate + APV and nifedipine) at 24 h (not illustrated).
4. Discussion

4.1. Lack of correlation between Ca\textsuperscript{2+} permeability and neurotoxicity

GluR2 knockdown strategies were used to elucidate ionic mechanisms underlying changes in AMPA receptor subunit composition in delayed neurodegeneration in vitro. Previously, in vivo models of seizure and ischemia induced delayed cell death proposed to be partly due to an excessive rise in intracellular Ca\textsuperscript{2+} via GluR2 lacking receptors (the GluR2 hypothesis). Indeed, selective downregulation of the functional expression of GluR2 subunits after limbic seizures in regions that will subsequently degenerate have been reported (Pollard et al., 1993; Friedman et al., 1994; Grooms et al., 2000). However, contrary to our expectations, the present in vitro study showed that GluR2 AS-ODNs directly exposed to hippocampal cultured neurons produce a time-dependent reduction in GluR2 protein without inducing a generalized neurotoxicity at any of the time points. In addition, delayed cell death was attenuated or unchanged in cultures pre-treated with GluR2 AS-ODNs and subsequently exposed to excitotoxins. This occurred in lieu of marked decreases in GluR2 protein and the increased ability of glutamate or AMPA to stimulate Ca\textsuperscript{2+} influx via AMPA receptors. Although measured increases in AMPA receptor-mediated Ca\textsuperscript{2+} permeability were in seconds and toxicity was measured after many hrs, selective increases in AMPA-receptor mediated Ca\textsuperscript{2+} permeability were achieved with the antisense treatment prior to excitotoxin exposure. This suggests that inducing a shift in the GluR1/GluR2 ratio to generate GluR2 lacking AMPA receptors, does not produce cell death on its own nor enhance glutamate stimulated neurotoxicity.

In keeping with this, the downregulation of the GluR2 subunit was recently not correlated with neurotoxicity in the vulnerable CA1 region of hippocampus of immature rats following hypoxia-induced seizures (Sanchez et al., 2001). In addition, microinfusing GluR2(B)-AS-ODNs directly into the hippocampus of adult rats is insufficient to induce neuronal cell death unless the animals succumb to seizures also suggesting that other factors are responsible for cell death (Friedman and Velísková, 1998; Friedman et al., submitted).

Furthermore, damage to CA1 or other brain regions that are...
highly vulnerable to seizures or hypoxia (e.g. amygdala or limbic cortex) do not co-exist in seizure phenotype GluR2 mutants despite global rises in AMPA-mediated Ca\(^{2+}\) permeability throughout the hippocampal subfields and within extrahippocampal principal neurons (Brusa et al., 1995). In rat pups, infusion of a GluR1 AS-ODN decreases GluR1 expression without effect on pathology or epileptogenesis (Friedman and Koudinov, 1999). Similarly, GluR1 knockdown in our cultures also did not kill cells (not shown) and GluR1 knockout mutants do not exhibit neurodegeneration or neuronal loss (Zamanillo et al., 1999). Therefore, loss of either of the GluR1 or GluR2 subunits is insufficient to induce cell death. Interestingly, in cultured neurons from GluR2 null mice, KA excitotoxicity was increased, but the toxicity paralleled rises in KA-stimulated whole cell currents rather than increases in AMPA receptor-mediated Ca\(^{2+}\) permeability (Ihara et al., 2001). Because removal of extracellular Na\(^+\) was neuroprotective against the excitotoxic injury in a homogeneous population of GluR2 lacking neurons other major ionic currents are implicated in the cell death (Ihara et al., 2001).

4.2. Possible mechanisms of neuroprotection

It is possible that the neuroprotection observed for the higher concentrations of glutamate herein, was due to loss of total AMPA receptor expression and activation of remaining receptors induced by the GluR2 AS-ODNs. However, GluR1 protein expression was not reduced in the cultures at any time point examined and current responses to glutamate were not different from controls (unpublished observation). This suggests that the functional properties of the GluR1 subunit were unaffected and that raised levels in Ca\(^{2+}\) detected by Fura 2AM could be mediated through increased homomeric assemblies or heteromeric assemblies with GluR3 or GluR4. Thus, stable levels of the GluR1 subunit after GluR2 knockdown could lead to a change in AMPA receptor stoichiometry prior to an excitotoxic insult, and have adaptive, protective effects under certain conditions. For example, in slices prepared from rat pups exhibiting seizure behavior after GluR2 hippocampal knockdown, long-term potentiation (LTP) was not maintained whether stimulation was delivered near or distant from the infusion site. Thus, the epileptic tissue appeared to be more tolerant to glutamatergic responses possibly by rises in Ca\(^{2+}\) within the cell body that result in higher excitation of dendrites in accordance with higher CA1 responses measured after knockdown (Friedman and Koudinov, 1999). Another study supporting adaptation mechanisms shows that elicitation of NMDA responses in epileptic tissue is more difficult to achieve due to chronic elevation of basal [Ca\(^{2+}\)]\(_i\) levels (Isokowa, personal communication). Alternatively, since basal [Ca\(^{2+}\)]\(_i\) was not altered until after depolarizing excitotoxins were applied, it is possible that loss of the Na\(^+\) response itself would result in less depolarization due to the knockdown of total AMPA receptors and produce protection at higher doses of glutamate. Another possibility could be due to activation of protective metabolotropic receptors at the high doses (Bruno et al., 2000).

It is thought that acute activation of immediate early genes (IEGs) and heat shock proteins (HSPs) in vulnerable regions after recurrent seizures may be protective against glutamate toxicity (Sonnenberg et al., 1989a,b; Lowenstein et al., 1994). For example, transgene expression of HSP72 attenuates KA and ischemia-induced delayed cell death (Yenari et al., 1998). However, the duration of elevated expression of HSPs is not always specific to vulnerable neurons and the expression appears related to the type of stress stimulus applied (Lowenstein et al., 1994; Planas et al., 1995; Kruengar et al., 1999; Akbar et al., 2001). Expression of IEGs and HSPs is unlikely responsible for the selective neuroprotection observed at high doses of glutamate in our study because there were no differences in basal [Ca\(^{2+}\)]\(_i\) levels with the GluR2 AS-ODN treatment at any of the time points examined. In addition, no reactive gliosis was detected in our cultures with the GluR1 and GluR2 antibodies that also mark astrocytes (Fan et al., 1999). On the other hand, glial cells are thought to be neuroprotective, responsible for clearance of extracellular glutamate. The astrocytic glutamate transporter (GLT-1) appears critical to the survival of neurons and protects against glutamate/NMDA neurotoxicity contingent upon certain conditions (Ahlener et al., 2002). For example, at low doses of glutamate, mixed neuronal/glial cultures did not exhibit cell death unless the GLT-1 was simultaneously suppressed (Kawahara et al., 2002). In contrast, when the Na\(^+\)/K\(^+\)-ATPase was compromised, antagonizing the GLT-1 transporter protected neurons, suggesting that the status of astrocytic GLT-1 transporters can contribute to neurotoxicity (Kawahara et al., 2002). It is therefore, possible that reducing the GluR2 subunit within glial cell types may alter their homeostasis and contribute to the neuroprotective effect observed after knockdown and at high but not low doses of glutamate.

In other in vitro studies, alterations in AMPA receptor expression have been correlated with KA toxicity in several neuronal cell types. In cultured Purkinje and cortical neurons, KA selectively kills neurons expressing Ca\(^{2+}\) permeable AMPA receptors (as detected by KA-induced Co\(^{2+}\) uptake; Broson et al., 1994; Lu et al., 1996). Even though the toxic effect of KA was less than that of glutamate, we still did not observe any difference in the number of dying cells. This might be due to insufficient change in extracellular Ca\(^{2+}\) (Choi, 1985) or rapid desensitization of AMPA receptors (Westbrook, 1994). Lack of an intact neuronal circuitry appears critical because surviving neurons develop epileptiform activities and long-term elevations in [Ca\(^{2+}\)]\(_i\) homeostasis post-glutamate application (Sun et al., 2001). Accordingly, Broson et al. (1999) showed that substantial increases in [Ca\(^{2+}\)]\(_i\), can occur even in neurons that contain high levels of GluR2 subunits when KA stimulation is prolonged.

Since KA receptors have many of the same stoichiometric characteristics as AMPA receptors, it is also possible...
that Ca²⁺ permissive AMPA receptors after KA receptor function to preferentially influence neuronal death. This could explain why CA3 neurons specifically become prematurely vulnerable in editing mutants or in rat pups after GluR2 knockdown in an otherwise resistant brain or why their demise is augmented by KA-induced seizures after knockdown at adult ages. In addition, excess release of zinc at mossy fiber synapses associated with the sustained release of L-glutamate contributes to the excitotoxicity, preferentially via voltage-gated Ca²⁺ channels (Choi and Koh, 1998). Furthermore, dantrolene, an antispastic drug, prevents Ca²⁺ release from intracellular Ca²⁺ stores and can almost completely prevent delayed degeneration of CA1 and CA3 neurons after KA-induced status epilepticus (Berg et al., 1995). Therefore, excess release from intracellular stores may be important for delayed neurodegeneration rather than increased Ca²⁺ influx. Rises in intracellular Ca²⁺ can also be neuroprotective as prolonged depolarization under various circumstances can prevent cell death (Franklin and Johnson, 1992). In keeping with this, thapsigargin, an inhibitor of [Ca²⁺]i sequestration, causes a sustained rise of intracellular Ca²⁺ concentration, which results in partial protection against cell death of sympathetic neurons induced by deprivation of nerve growth factor (NGF) (Lampe et al., 1995). It seems that Ca²⁺ can stimulate certain intracellular cascades that can be either neurotoxic or neuroprotective. However, it is still not certain whether the amount of glutamate-evoked Ca²⁺ influx or the actual route of increased intracellular Ca²⁺ is responsible for neuronal injury (Tymianski and Tator, 1996). Thus, other critical factors such as single channel Na⁺ conductance properties, maturation of anatomical excitatory connections, receptor kinetics, or allosteric modulation likely contribute to neurotoxicity rather than differential Ca²⁺ permeability though AMPA receptors.

In conclusion, antisense approaches in vitro and in vivo models do not support the original assumption that formation of Ca²⁺ permeable AMPA receptors, due to selective downregulation of the GluR2 subunit, induces neuronal cell death. Future antisense studies may elucidate the relevance of increasing Ca²⁺ to a certain beneficial level as a means of neuroprotection possibly by controlling the route of elevation.

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