Sequential Involvement of Distinct Glutamate Receptors in Domoic Acid-Induced Neurotoxicity in Rat Mixed Cortical Cultures: Effect of Multiple Dose/Duration Paradigms, Chronological Age, and Repeated Exposure

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Domoic acid (DOM) is a marine toxin produced by algal species that causes amnesic shellfish poisoning (ASP), a condition characterized by severe memory impairment and gastrointestinal and sensorimotor disturbances (Clayton et al., 1999; Perl et al., 1990; Wright et al., 1990). In severe cases of ASP, neurological symptoms develop within 48 h and may include seizures and even death. In experimental animals DOM has been shown to produce learning/memory deficits (Petrie et al., 1992). When transferred through the food chain, DOM causes accidental poisoning of humans (Perl et al., 1990; Teitelbaum et al., 1990) and marine animals (Gulland et al., 2002; Lefebvre et al., 1999; Scholin et al., 2000). DOM, a structural analogue of the excitatory neurotransmitter glutamate, activates glutamate receptors (Hampson et al., 1992; Hampson and Manalo, 1998; Verdoorn and Dingerline, 1988) and produces excitatory responses throughout the mammalian central nervous system (Biscoe et al., 1976; Bureau et al., 1999; Stewart et al., 1990; Yool et al., 1992) as well as hippocampal neurodegeneration by binding to 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionylate (AMPA)–type (AMPARs) and kainate (KA)-type GluRs (KARs) (Larm et al., 1997; Stewart et al., 1990).

DOM is highly potent at AMPARs and KARs (Doucette et al., 2003; Larm et al., 1997; Stewart et al., 1990), probably because of high-affinity binding (Hampson et al., 1992; Hampson and Manalo, 1998) and nondesensitizing responses at AMPARs (Carcache et al., 2003; Stewart et al., 1990) leading to persistent ionic disturbances, swelling, and neurodegeneration (Attwell et al., 1993). In addition, DOM lacks a high-affinity uptake system (Suzuki and Hierlihy, 1993) and...
can cause secondary release of nitric oxide (NO) and endogenous glutamate, further exacerbating neuronal death (Berman and Murray, 1997; Brown and Nijjar, 1995; Chandrasekaran et al., 2004; Dakshinamurti et al., 1993).

Calcium influx evoked by DOM, which is correlated with DOM-induced neuronal death, is sensitive to both NMDAR and non-NMDAR antagonists (Berman et al., 2002; Xi et al., 1997), suggesting involvement of NMDARs and Ca$^{2+}$-permeable AMPARs in DOM toxicity (Fischer et al., 2002; Hollmann et al., 1991; Jensen et al., 2001). Previous studies have shown that application of either AMPA/KAR or NMDAR antagonists confer significant protection against DOM (Berman and Murray, 1997; Jensen et al., 1998; Larm et al., 1997; Tasker and Strain, 1998), but it is not clear what the sequential involvement of each receptor subtype is, or whether mGluRs, which can either protect or exacerbate neuronal death (Conn and Pin, 1997; Koh et al., 1991; Strasser et al., 1998), contribute to DOM excitotoxicity. Moreover, it is unclear what the time course of DOM-induced neuronal death is and whether it is dependent on exposure duration and/or concentration.

Other factors such as advancing age have been correlated with increased severity of ASP (Auer, 1991; Chandrasekaran et al., 2004; Perl et al., 1990) and increased DOM-induced dysfunction in experimental animals (Dakshinamurti et al., 1993), while other studies indicate increased susceptibility of neonates to DOM toxicity (Xi et al., 1997). Because whole-animal studies are confounded by age-related changes in physiological parameters affecting DOM pharmacokinetics (Mayer, 2000; Xi et al., 1997), we used a barrierless preparation to compare DOM toxicity at different times in vitro and related differences to developmental changes in iGluR expression. We used cerebral cortical cultures, since they display excitotoxic responses earlier during neuronal maturation than CGCs (Frandsen and Schousboe, 1990).

In this study different dose/duration paradigms of DOM treatment were used in order to provide insight into the complex mechanisms underlying DOM neurotoxicity. Our results show critical involvement of iGluRs, but not mGluRs, in DOM-induced neuronal death, especially during the first 2 h after DOM exposure, with AMPA/KARs playing a more prominent role earlier than NMDARs following DOM exposure and higher doses of DOM completing the neuronal death cascade more rapidly. We found a high correlation between DOM potency and subunit-specific iGluR expression during neuronal maturation in vitro. Finally, we explored the effect of prior DOM exposure on the effects of a subsequent exposure and found that toxicity was intensified. In combination, these data indicate a short critical window for neuroprotection by iGluR antagonists if applied following DOM exposure and implicate multiple factors influencing DOM toxicity, including dose, age, and prior history of DOM exposure. A preliminary subset of these data has been published previously (Qiu and Currás-Collazo, 2002).

### MATERIALS AND METHODS

#### Mixed cortical cultures. Animal care and use procedures were conducted in accordance with Guide and Care for the Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and were approved by the Institutional Animal Care and Use Committee of University of California, Riverside. We chose to study cortical cultures containing glia, since DOM has access to both neurons and glia in vivo, and astrocytes contain targets of DOM action such as glutamate transporters and AMPARs (Burnashev et al., 1992; Patneau et al., 1994) and may contribute to enhanced nitric oxide (NO) synthase activity after DOM exposure (Chandrasekaran et al., 2004). Moreover, astrocytes have been shown to both potentiate (Bal-Pierce and Brown, 2001; Dugan et al., 1995) and protect against excitotoxicity (Rothstein et al., 1996). Mixed cortical cultures were prepared similarly to that described previously (Curras et al., 1991) using embryonic pugs obtained from pregnant Holtzman rats (Harlan, Chicago, IL) on embryonic day (ED) 16–18. Briefly, the fetal brains were dissected and collected in ice-cold Hank’s balanced salt solution (HBSS) under sterile conditions. Cerebral cortices including hippocampus were isolated and minced, and enzymatically digested in HBSS solution at 37°C for 30 min. Subsequently, the tissue was triturated in filter-sterilized Minimum Essential Medium (MEM) until completely dissociated into individual cells. The cells were then diluted with fortified MEM and plated on poly-D-lysine (0.1 mg/ml)-coated culture plates or 12-mm glass cover slips at a density of 4 × 10⁶ cells/cm². Cortical cultures were grown in a humidified Chamber maintained at 36.5°C containing air/CO₂ (95/5%). At day in vitro (DIV) 5 and every 3 days thereafter, culture medium was replaced by 50% with serum-free MEM. Cytosine arabinoside (Ara-C, 10 μM) was added at DIV 5 to reduce glial proliferation.

#### Cell culture medium and chemicals. HBSS and MEM solutions were purchased from Invitrogen. The MEM was fortified with 10% heat-inactivated fetal bovine serum (FBS, Atlantic Biologicals), 5% horse serum (GIBCO BRL), 1% (V/V) antibiotic and antifungic MIXTURE (GIBCO BRL), gentamycin sulfate (1 mg/ml), and sodium pyruvate (1 mg/ml), pH 7.22. Glutamate receptor ligands including domoic acid, D-2-amino-5-phosphonopentanoate (D-AP5), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), 1-aminoindan-1,5-dicarboxylic acid (AIDA), (S)-alpha-methyl-4-carboxyphenylglycine (MCPG), and (S)-2-amino-2-(15,25)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) were purchased from Tocris Cookson. All other chemicals were obtained from Sigma.

#### Treatment of cultures. Unless stated otherwise, cultures at DIV 11 were used, and experiments were run at 37°C in a CO₂ incubator. Prior to each experiment, the volume of culture medium in each well was calibrated and reset to original volume. Receptor ligands were applied using MEM as the diluent. After DOM or antagonist drug treatments, conditioned medium from unexposed sister cultures was used to washout the drug-containing medium. When developmental susceptibility tests were carried out, cultures were challenged with DOM (1 μM–1000 μM) at DIV 3, 5, 8, and 11, and neuronal viability was measured. In addition, various equitopotent exposure paradigms that produced ~50% neuronal death at DIV 11 were employed to study neuronal susceptibility with time in vitro and protection offered by different antagonists. In experiments examining the effects of multiple DOM exposure, cultures were pretreated with a subtoxic dose of DOM (1 μM DOM) on DIV 8 for 48 h and subsequently treated on DIV 11 with one of the above-mentioned DOM exposure paradigms, and neuronal survival was measured 24 h later. Neuronal survival in these treated cultures was compared with that in DIV 11 sister cultures not receiving 1 μM DOM pretreatment on DIV 8.

#### Assessment of neuronal cell death. Cultures at DIV 3–11 were treated with DOM (0.1–1000 μM) for varying durations (10 min–24 h) with or without antagonist, and cell survival was measured immediately after treatment or after washout. Cell viability was measured using an MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay, and absorbance values were normalized to values obtained from control sister cultures treated with medium replacement or antagonists alone in the absence of DOM. Neuronal death values
obtained using MTT assay were found to correspond proportionately with the number of dead or damaged neurons identified using the trypan blue dye exclusion test in response to DOM exposure. Neuronal counts and morphology were assessed, and digital pictures were taken using a Nikon Coolpix 990 camera under phase-contrast microscopy (Nikon TMS-F).

Cell survival was converted to percent of maximum neuronal death assessed 24 h after cultures were exposed to 10 mM NMDA for 2 h and subsequently washed with conditioned medium from unexposed sister cultures. NMDA applied for 24 h produces no injury in neocortical astrocyte cultures, since astrocytes do not contain functional NMDA receptors (David et al., 1996). Mixed cortical cultures show normal astrocyte morphology when grown on cover slips, treated in this manner with NMDA, and fixed at 24 h (data not shown). In some experiments, the effect of DOM on cell viability was measured at 2 h after treatment. In experiments comparing DOM efficacy across time in culture, percent neuronal cell death at each time point examined was calculated by normalizing death obtained at each DIV to maximum cell death (produced by 1 mM/24 h DOM at DIV 11). These values represented DOM efficacy and were compared to maximum cell death produced by NMDA (10 μM/2 h and measured 24 h later) at each DIV.

Maximal neuronal death and EC50 values from concentration-response curves for DOM and other GluR ligands were determined using nonlinear regression analysis of the dose-response relationship (Y = 100/(1 + 10^{logEC50-X} * Hill Slope)), where Y and X represent the percentage of neuronal death and the logarithm of DOM concentration, respectively) using Prism software (GraphPad Software Inc., San Diego, CA). Based on initial concentration-response curves for 24-h, 2-h, and 10-min exposures (and cell survival measured at 24 h after the start of treatment), three equipotent EC50 paradigms were established in this study representing long, moderate, and brief exposure durations (3 μM for 24 h, 10 μM for 2 h, and 50 μM for 10 min; see the result sections).

Application of glutamate receptor antagonists. We tested an array of glutamate receptor antagonists, including those for the ionotropic receptor classes: NBQX (an AMPAR antagonist at 2 μM and a nonselective AMPAR/KAR antagonist at 10 μM), D-AP5 (an NMDA receptor antagonist; 10 and 100 μM), and a nonselective mGluR antagonist MCPG (500 μM; Bushell et al., 1996; Hayashi et al., 1994); AIDA (1 mM), a potent and selective antagonist for group I mGluRs (Strasser et al., 1998), and LY341495 (1 μM), a selective group II/III mGluR antagonist at this dose (Howson and Jane, 2003; Kingston et al., 1998). All drugs were dissolved in MEM and administered either 15 min before or at various times after the start of DOM application and in the continued presence of DOM.

Immunocytochemical analysis. Standard immunocytochemical and Western blot methods were used to examine glutamate receptor expression profiles at different times in vitro. For immunocytochemistry, mixed cultures grown on glass cover slips were fixed with 4% paraformaldehyde for 2 h. After washes with cold 0.01 M phosphate buffered saline (PBS), the cover slips were incubated in 0.05% H2O2 to block endogenous peroxidase activity. They were subsequently incubated with polyclonal antibodies (all purchased from Chemicon) against the NMDAR subunit NR1 (1:100, 1 μg/ml), or AMPAR subunit GluR1 (1:100, 1 μg/ml), or KAR subunit GluR5 (1:100, 1 μg/ml) or GluR6/7 (1:100, 1 μg/ml) for 2 h at room temperature and later washed three times with PBS. When double-label immunocytochemistry was used to determine glial and neuronal cell content, antibodies for a neuron-specific DNA-binding protein, neuronal nuclear antigen, NeuN (Mullen et al., 1992; anti-NeuN monoclonal antibody; Chemicon, 1:200, 0.5 μg/ml), and for GFAP; glial fibrillary acidic protein (rabbit anti-rat GFAP antibody; Chemicon, 1:500, 0.4 μg/ml), were applied concurrently. The coverslips were then incubated in synthetic HRP-conjugated anti-rabbit or anti-mouse polypeptides (DakoCytochrome) for 1 h, and antigens were visualized by adding diaminobenzidine (DAB) chromagen solution (0.1 mg/ml) with or without nickel sulfate intensification. Methodological controls consisted of slides not receiving the primary antibodies but receiving incubation with the secondary antibodies and with DAB. Other controls received only DAB. Neither control group showed background or cell-specific staining. Immunoreactivity was visualized under bright field optics with an Axioptoph microscope (Nikon), and the numbers of GFAP-positive astroglia and NeuN-positive neuronal nuclei were counted to determine the glia/neuron ratio. Glia and neurons could be distinguished because the NeuN antibody only stains the somata of neurons, while GFAP stain was distributed throughout multiple astrocytic processes. The use of Ara-C inhibited glial proliferation and allowed individual astrocytes to be identified and counted. Average counts were calculated from values obtained from three experiments. For each experiment, four fields in each of four cover slips were analyzed.

For Western blot analysis, cultures on 12-well plates were dislodged from the wells and sonicated in 150 μl protease inhibitor cocktail solution containing 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 100 μM leupeptin, 1 μM pepstatin, 10 μg/ml aprotinin, 10 μg/ml bacitracin, and 100 μM phenylmethylsulfonyl fluoride. Protein content in homogenates of each sample was determined using a bicinchoninic acid assay (Pierce Biotechnology). Aliquots of samples were then mixed with an equal volume of 2× loading buffer (10 mM Tris-OH, pH 6.8, 10% BME, 0.1% bromophenylblue, 10% SDS, and 40% glycerol), and 5 μg of total protein were resolve in 10% SDS-polyacrylamide gels and later transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore). The membranes were then incubated overnight at 4°C in a blocking solution containing 5% nonfat dry milk and 1% bovine serum albumin. Primary and secondary antibodies were applied in a way similar to that described above for the immunocytochemistry procedure. The integrated optical density (IOD) of immunoreactive bands detected using enhanced chemiluminescence detection (Amersham) was quantified using ImagePro V4.5 software (Media Cybernetics) and was normalized to α-tubulin IOD values in the same sample to compensate for loading errors.

Statistical Analysis. Statistical differences were tested at p < 0.05 using Student’s t-test for two-group comparison, or one-way followed by Student-Neuman-Keuls or Tukey’s test for multiple-group comparisons. When two-way ANOVA analysis was conducted, a post hoc Bonferroni test was used for multiple comparisons. A Spearman bivariate linear model was used for testing correlation between GluR expression and excitotoxicity.

RESULTS

Characteristics of Mixed Cortical Cultures

Neurons gradually developed their mature morphology with increasing time in vitro, as manifested by the multipolar morphology and intricate neurite networks associated with phase-bright cell bodies. When results of immunocytochemical experiments were analyzed, the majority of neurons (>85%) at DIV 11 expressed NR1, GluR1, and GluR6/7, which are subunits for NMDA, AMPA, and KA receptors, respectively (Fig. 1). Immunoblotting data also indicated expression of GluR2/3 and GluR5 in our cultures (Fig. 8B). Using antibodies that are specific for either NeuN or GFAP proteins, and serve as specific markers for neurons or astrocytes, respectively. We found that cells constituted 13.3 ± 7.1% of total cell numbers in DIV 11 cultures (Fig. 1). This neuronal/glial ratio was kept consistent throughout the plates and throughout the cultures by treatment with Ara-C. Methodological controls receiving no primary antibody showed little or no immunoreactivity (data not shown).

Microscopic observation revealed virtually complete neuronal death in DIV 11 cultures exposed to 10 mM NMDA for 2 h (n = 5 experiments; data not shown). DOM (100 μM, 24 h)
produced maximal neuronal death (100.2 ± 5.9% of control, n = 6) similar to results shown by others using murine cortical cultures (Larm et al., 1997). Exposure of cultures to 50 μM/10 min DOM produced swelling in the majority of neurons examined. The swelling was prominent between 10 and 30 minutes after DOM washout but was reversed in the majority of neurons by 2 h after washout (Fig. 2, n = 4 experiments).

**Potency and Efficacy of DOM Relative to Other Excitatory Amino Acids (EAAs)**

Concentration-response curves were generated by measuring neuronal death responses to 24-h exposures of different DOM doses (0.1 μM to 1000 μM) and compared to those measured in DOM-naive cultures at DIV 11. Our results show that DOM induces neuronal death in a concentration-dependent manner, with an EC₅₀ of 4.2 μM when applied for 24 h (Fig. 3A). This EC₅₀ is similar to DOM affinity at [³H]-AMPA binding sites in rat brain membranes (IC₅₀ of 2 μM; Hampson and Manalo, 1998). To compare the potency of DOM with that of other known prototypic EAAs, the dose-response profiles of NMDA, kainate, and glutamate were also examined. All agonists reduced neuronal survival dose dependently (Fig. 3A). EC₅₀ values obtained for kainate, NMDA, and glutamate were 15.6 ± 6.2, 25.8 ± 7.1, and 36.4 ± 15.2 μM, respectively (n = 3–4), giving a rank order of potency of DOM > KA > NMDA ≈ glutamate (p < 0.05). Therefore, DOM was the most potent EAA measured using our cultured cortical neuron preparation, consistent with previous reports using in vivo and in vitro methods (Doucette et al., 2003; Koh and Choi, 1988; Larm et al., 1997). When exposed for 24 h, all EAAs examined produced maximum responses when compared to neuronal death produced by 10 mM/2 h NMDA. Neuronal death was 100.2 ± 5.9, 101.3 ± 5.2, 103.3 ± 4.5, and 99.4 ± 7.4% of control for DOM, kainate, NMDA, and glutamate, respectively (n = 6).

**Effect of Concentration and Duration on DOM Toxicity**

We next tested the effects of different combinations of DOM concentration and exposure duration. Mixed cortical cultures were challenged with 3, 5, 10, or 50 μM DOM for a variety of exposure durations (10 min, 30 min, 1 h, or 2 h), after which DOM was washed out and the culture medium replaced with conditioned medium from unexposed sister cultures. In all cases neuronal death was measured at 24 h following the beginning of exposure. A two-way ANOVA analysis showed significant effects of both DOM exposure time (F₄,₁₂₆ = 66.2, p < 0.001) and DOM concentration (F₃,₁₂₆ = 102.3, p < 0.001), suggesting that DOM-induced neuronal death is determined by...
both concentration and duration of DOM exposure. Figure 3B shows that, in general, neuronal survival decreased proportionately as a function of exposure time. However, DOM applied at the 50 μM concentration produced disproportionately greater neuronal death than the lower concentrations at every exposure time except that of 24 h. In the latter case, neuronal death was linearly related to DOM concentration. For example, after a 10-min exposure 50 μM DOM produced marked neuronal death of 47.4 ± 5.1% as compared to 2.8 ± 2.2, 4.6 ± 1.8, and 6.7 ± 2.3% triggered by slightly lower concentrations: 3, 5, and 10 μM, respectively. By 1 h of treatment, 50 μM DOM produced near maximal neuronal death, and longer exposures did not increase neuronal death further. Post hoc Bonferoni analysis indicated no statistical difference in neuronal death produced by 50 μM DOM treatment for 60 min (81.6 ± 6.8%), 2 h (85.9 ± 5.6%), and 24 h (89.5 ± 8.5%; p > 0.05, n = 6), suggesting that strong activation of iGluR receptors by high concentrations of DOM triggers near maximal neuronal death after shorter activation of iGluRs than that required for low doses. In contrast, 10 μM DOM required 24 h of exposure to produce greater than 50% neuronal death.

**Time Dependency of DOM-Induced Neuronal Death**

In order to examine the time course of neuronal death after different concentrations of DOM exposure, mixed cortical cultures were exposed to different doses of DOM for 2 h, and neuronal death was measured either immediately or 22 h post-DOM washout. A two-way ANOVA revealed time and concentration effects that were statistically significant (F = 13.6, df = 1, p < 0.001 for time effects; and F = 122.5, df = 5, p < 0.001 for concentration effects). The effect of exposure duration at different concentrations of DOM can be seen in Figure 3C. A significant outcome of our studies was the finding that low concentrations of DOM produced more neuronal death if this was measured 22 h after the washout of DOM than if measured immediately after DOM treatment (post hoc Bonferoni, p < 0.05), while higher concentrations of DOM (20–100 μM) produced equivalent degrees of neuronal death when measured at these two time points (p > 0.05). Accordingly, we observed that DOM potency is significantly greater in the 2 h DOM + 22 h wash (10.7 ± 4.2 μM) than in the 2 h DOM group (19.6 ± 5.9 μM; n = 5, t = 2.75; p = 0.0251).

In combination, these data suggest that low concentrations of DOM can reach their maximum efficacy even with weak activation of AMPAR/KARs if secondary events having delayed onset are allowed to occur. By producing stronger activation of AMPAR/KARs, the higher doses of DOM (50 and 100 μM) can result in maximal cell death by 2 h after exposure.

**Protective Effects of iGluR Antagonists against DOM-Induced Neuronal Death**

To evaluate the contribution of iGluRs targeted by different doses of DOM in cortical neurons we examined the effects of
NBQX, an AMPA/KAR receptor antagonist (Sheardown et al., 1990), and D-AP5, an NMDA receptor antagonist (Davies and Watkins, 1982). The antagonists were applied prior to and concurrently with increasing concentrations of DOM applied for 24 h. Both antagonists acted in a dose-dependent manner to reduce DOM-induced neuronal death. Figure 4 shows that both NBQX and D-AP5 shifted the DOM dose-response curve to the right and decreased the maximum response to DOM as expected for competitive antagonism. At maximal concentrations, NBQX (10 μM) and D-AP5 (100 μM) each produced nearly complete suppression of neuronal death evoked by low to moderate concentrations (1–5 μM) of DOM. NBQX proved relatively more neuroprotective than D-AP5 with increasing DOM concentration. At high concentrations of DOM (50 and 100 μM), however, neither NBQX nor combined application of both antagonists could provide full protection, as expected for competitive antagonism.

Temporal Involvement of AMPA/KAR and NMDARs in DOM Toxicity

To address the temporal involvement of both AMPA/KA and NMDA receptors in DOM toxicity, we applied the above-tested iGluR antagonists with variable time delay and compared the effects on DOM-induced neuronal death. Each antagonist was applied alone or in combination with the other 15 min before or 30 min after the start of an EC₅₀ DOM exposure (10 μM/2 h, measured at 24 h). Our results, shown in Figure 5, revealed significant protection against DOM toxicity after preapplication of either 10 μM NBQX, 100 μM D-AP5, or both (#, Fig. 5), with preapplication of both antagonists affording the best protection against DOM exposure (102.6 ± 5.2% of total neuronal survival). In comparison, preapplication of D-AP5 (100 μM) provided significant less protection (77.4 ± 3.9%) when compared with combined effects of NBQX and D-AP5 (n = 6, p < 0.001, Student’s t-test).

For NBQX, significantly better protection was seen when it was preapplied 15 min before the start of DOM treatment than when application was delayed 30 min post-DOM (97.1 ± 5.1 vs. 64.0 ± 4.2% of control neuronal survival, respectively, n = 6, p < 0.001, **Fig. 5). NBQX was ineffective against DOM-induced cell death if application of the antagonist was delayed for 1 h after the start of DOM exposure. In comparison, application of D-AP5 provided significant protection when given either before DOM (77.4 ± 3.9%), 30 min after DOM (73.6 ± 4.7%), or 1 h after DOM (64.3 ± 5.1% of control, n = 6, p > 0.05). When given 30 min after DOM, combined application of NBQX and D-AP5 markedly improved protection against DOM-evoked neuronal death (94.5 ± 4.9% neuronal survival) as compared to that offered by either antagonist separately (73.6 ± 4.7% for D-AP5, p < 0.05, and 64.0 ± 4.2% for NBQX, p < 0.01). This protection

FIG. 4. Protective effects of iGluRs antagonists against DOM neurotoxicity. Successively increasing doses of DOM were applied to DIV 11 cultures for 24 h in the presence or absence of D-AP5 or NBQX, and neuronal death was measured immediately after. Neuronal death values were normalized to those measured in response to 10 mM NMDA for 2 h and measured 22 h later. DOM dose-response curves were shifted to the right and showed reduced maximum responses when DOM was applied after preapplication and in the continued presence of either antagonist. D-AP5 treatment was somewhat less effective than NBQX. Combined treatment with NBQX (10 μM) and D-AP5 (100 μM) offered slightly better neuroprotection than NBQX alone when using high doses of DOM. Data represent means ± Sem of six experiments, with 6–8 replicates per experiment.
could be partially achieved even if concurrent antagonist treatment was delayed for 1 h after the start of DOM exposure. The additional protection by D-AP5 over that provided by NBQX (Figs. 4 and 5) points to a synergistic involvement of both NMDA receptors and AMPA/KAR 30 min after DOM exposure. Importantly, no protection was seen if either antagonist, alone or in combination, was applied 2 h after DOM application and continued to present during the following 22 h period.

Effect of Different DOM Dose/Exposure Paradigms

We next determined what dose-duration combinations yielded 50% DOM responses when measuring neuronal death 24 h after the start of exposure. We established three EC_{50} exposure paradigms, which represent weak/prolonged exposure (3 μM/24 h), moderate concentration and duration exposure (10 μM/2 h), and strong/brief exposure (50 μM/10 min). Figure 6 shows that these DOM exposure conditions were equieffective and resulted in 58.2 ± 6.1, 54.5 ± 6.4, and 46.9 ± 4.6% neuronal death when measured at 24 h, respectively.

Next, we examined whether NMDARs and AMPA/KARs contributed to toxicity produced by these EC_{50} paradigms. In each case D-AP5 (100 μM) or NBQX (10 μM) was applied 30 min after the start of DOM exposure, when both NBQX and D-AP5 offered significant protection against 10 μM/2h DOM (see Figs. 5 and 6). Figure 6 demonstrates that D-AP5 and NBQX each significantly rescued neurons from DOM-induced death. When using a brief, strong DOM challenge (50 μM/10 min), 100 μM D-AP5 (87.5 ± 5.2% of control) provided better protection than 10 μM NBQX (59.5 ± 4.4% of control, n = 5, p < 0.01). In comparison, NBQX rescued more neurons relative to D-AP5 during the 3 μM/24 h exposure conditions (97.4 ± 5.2 and 84.6 ± 5.8%, respectively, n = 5, p < 0.01). It is important to note, however, that the protection offered by D-AP5 against the three regimens was similar, suggesting a similar contribution and timing of NMDAR activity to DOM toxicity with low and high doses of DOM. The reduced AMPA/KAR involvement in response to high doses of DOM may represent a quicker transition to NMDAR activation or may be related instead to the absence of DOM at the binding sites, since the high dose was applied briefly and was washed out before the application of NBQX.

Lack of Contribution of Metabotropic Glutamate Receptors to DOM Toxicity

Since DOM has been shown to result in elevated levels of extracellular glutamate and aspartate in other culture preparations (Berman and Murray, 1997), and this may indirectly activate NMDA receptors in our cultures, we explored whether DOM recruits mGluR activity in producing excitotoxicity. Therefore, we examined the effects of several mGluR antagonists using the DOM exposure paradigms established above. The group I selective mGluR antagonist, aminoindan-1,5-dicarboxylic acid (AIDA) is neuroprotective against glutamate-induced excitotoxicity (Strasser et al., 1998). We tested the possible involvement of Group I, Group II, and Group III mGluRs using AIDA (1mM), MCPG (500 μM), and LY341495 (10 μM). None of these drugs significantly altered neuronal death induced by DOM, nor did they alter neuronal death by themselves (Fig. 7, p > 0.05 for each drug tested). Similar results were obtained with 10 μM LY341495, a non-selective mGluR antagonist at this dose. Therefore, it is unlikely that mGluRs, activated by DOM-induced secondary glutamate release, play an important role in DOM neurotoxicity under the in vitro conditions we tested.

Increased Neuronal Susceptibility to DOM during In Vitro Development: Correlation with iGluR Expression

Previous studies have shown that the susceptibility of central neurons to DOM neurotoxicity is age related in whole animals (Auer, 1991; Kerr et al., 2002; Mayer, 2000; Xi et al., 1997; Xi and Ramsdell, 1996), although the reasons for this are unclear. Using murine cortical cultures, Jensen et al. (1999) reported that 20 μM DOM (6 h exposure) produced ~10% more cell death at DIV 12 than at DIV 8. We have extended these findings by determining the developmental regulation of DOM potency and efficacy and correlating this to NMDAR, AMPAR, and KARs subunit expression. In Figure 8A neuronal survival is expressed as a percent of maximum cell death produced by 24-h exposure to 100 μM or 1 mM DOM at each DIV time point.
metabolotropic mGluR antagonists do not protect against DOM-induced neurotoxicity. Cultures were treated with either 3 μM/24 h, 10 μM/2 h, or 50 μM/10 min DOM, washed at corresponding times, and neuronal death measured at 24 h post DOM treatment. mGluR ligands were applied 15 min before and continuously during DOM exposure and after washout until neuronal survival was measured at 24 h. mGluR antagonists AIDA (1 mM), MCPG (500 μM), and LY341495 (10 μM) produced no significant effects on neurotoxicity induced by various DOM exposure paradigms (p > 0.05, one-way ANOVA). Application of these mGluRs antagonists alone (control) for 24 h did not affect neuronal survival (p > 0.05, one-way ANOVA).

Figure 7. Metabotropic GluR antagonists do not protect against DOM-induced neurotoxicity. Cultures were treated with either 3 μM/24 h, 10 μM/2 h, or 50 μM/10 min DOM, washed at corresponding times, and neuronal death measured at 24 h post DOM treatment. mGluR ligands were applied 15 min before and continuously during DOM exposure and after washout until neuronal survival was measured at 24 h. mGluR antagonists AIDA (1 mM), MCPG (500 μM), and LY341495 (10 μM) produced no significant effects on neurotoxicity induced by various DOM exposure paradigms (p > 0.05, one-way ANOVA). Application of these mGluRs antagonists alone (control) for 24 h did not affect neuronal survival (p > 0.05, one-way ANOVA).

measured. DOM dose-response curves at DIV 3, DIV5, DIV8, and DIV 11 were constructed using a 24-h exposure paradigm (data not shown); calculated EC50 values are shown above bars in Figure 8A. Our results indicate that the potency of DOM increases gradually with successively increasing time in vitro until DIV 8 and then stabilizes; the calculated EC50 values were 40.8 ± 13.1, 33.2 ± 12.5, 4.93 ± 3.37, and 4.17 ± 2.11 μM for DIV 3, 5, 8, and 11, respectively (n = 3–5). In addition, DOM efficacy was assessed using three different dose/exposure duration paradigms and compared to that of a saturating NMDA exposure (10 μM/2 h). Using a 24-h exposure paradigm, DOM-induced cell death was maximal at all DIV time points examined, ranging from 96.6 ± 7.4% (at DIV 3) to 103.7 ± 9.9% (at DIV 11). NMDA acted similarly to DOM, in that it produced 100% neuronal death, except on DIV 3 when NMDA produced only 72% neuronal death.

A chronological increase in DOM efficacy was also observed for each of the three EC50 exposure paradigms (F3,97 = 34.5, p < 0.001 for time effects, two-way ANOVA). However, by DIV 8 mixed cortical cultures reach a maximum response to DOM regardless of the dosing paradigm used. For example, neuronal death achieved with 3 μM/24 h, 10 μM/2 h, and 50 μM/10 min DOM was 43.1 ± 4.6, 46.7 ± 4.1, and 43.1 ± 3.7% of maximum neuronal death observed at DIV 8 and 48.9 ± 3.8, 54.2 ± 3.2, and 51.6 ± 3.5% of maximum neuronal death observed at DIV 11, respectively. Therefore, no significant increase in DOM-induced maximum cell death and DOM potency could be detected between DIV 8 and 11 as could be detected between DIV 5 and 8 (p < 0.005).

Figure 8B displays representative Western blots for NMDA, AMPA, and KA receptor subunits for homogenates of cultures grown for different times in vitro. NR1 and all AMPAR and KAR subunits examined were detected except GluR4, as expected (Dai et al., 2002), and showed time-dependent increases in expression, reaching plateau levels around DIV 8. More strikingly, NR1 and GluR2/3 immunoreactive bands show dramatically elevated protein levels between DIV 5 and 8. OD values for NMDAR NR1 and GluR 2/3 AMPARs at which DOM produces nondesensitizing currents (Carcaiche et al., 2003; Stern-Bach et al., 1998) were 0.00, 0.14, 0.78, 0.93, and 0.27, 0.36, 2.74 and 3.63 at DIV 3, 5, 8, and 11, respectively. The IODs for the AMPAR GluR1 and KAR GluR5, at which DOM possesses very high affinity and produces nondesensitizing currents (Crawford et al., 1999; Hampson et al., 1992; Sciancalepore et al., 1990; Sommer et al., 1992), were also markedly increased at these times but to a lesser degree: 0.21, 0.54, 1.67, and 1.99, respectively (GluR1), and 0.07, 0.19, 0.58, and 0.64, respectively (GluR5).

Figure 8C shows that neuronal susceptibility to DOM (1/EC50) is highly and significantly correlated with increased mean expression of total iGluRs (includes NR1, GluR1, GluR2/3, GluR5, GluR6/7) during maturation (r2 = 0.996). The dramatic increase in DOM potency between DIV 5 and DIV 8 was also particularly well described by upregulated NR1 (r2 = 0.957), GluR2/3 (r2 = 0.923), and GluR6/7 levels (r2 = 0.973). Regression analysis results for the relationship between DOM potency and either total iGluR, NR1, GluR2/3, or GluR6/7 values were significant (p < 0.01). Western analysis indicated incremental expression of NeuN and GFAP in our cultures with time in vitro to a maximum level at DIV 8, suggesting near maximal maturation of cultures at this time (Fig. 8B). Developmental studies have also shown a delay in high-level NeuN expression in neocortical neurons, which has been ascribed to the state of differentiation or level of functioning of the neuron (Mullen et al., 1992). These neuronal and glial markers, therefore, indicate increasing culture maturation with time.

Effects of Repeat Exposure to DOM

In order to examine the potential for increased risk to different DOM exposure paradigms following a previous prolonged, low-dose exposure, we exposed cultures to 1 μM DOM for 48 h (starting at DIV 8), washed them, subjected them to a second treatment of DOM, and measured neuronal death 24 h after the start of DOM application (at DIV 11). Figure 9 demonstrates that low-dose pretreatment did not produce significant neuronal death on its own when measured 24 h after washout (at DIV 11; 95.6 ± 5.3% of control neuronal survival). Preconditioned cortical cultures responded more vigorously to
a subsequent DOM test treatment. For example, neuronal
survival values in cultures with or without DOM pretreatment
were 31.3 ± 3.6 and 48.7 ± 4.2%, respectively, for a 3
mM/24 h exposure and 35.2 ± 4.7 and 51.7 ± 3.9%, respectively, for
a 100 κM/2 h exposure. The effect of preconditioning was
statistically significant for both exposure paradigms (**
p < 0.01, Fig. 9). In contrast, preexposure to prolonged, low-dose
DOM treatment did not augment cell death triggered by the
50 κM/10 min paradigm.

**DISCUSSION**

Temporal Involvement of iGluRs in DOM-Induced
Excitotoxicity

Like glutamate neurotoxicity (Hartley et al., 1990; Prehn
et al., 1995), DOM toxicity involves both NMDA and non-
NMDA-type iGluRs as reported previously for cultured
cerebellar granule cells (CGCs; Berman and Murray, 1997)
and murine neocortical neurons (Jensen et al., 1999).
NBQX completely rescued neurons if applied concurrently
with DOM, but was 33% less effective if application was
delayed by 30 min, pointing to an early phase mediated by
AMPA/KARs after binding of DOM (Hampson and Manalo,
1998). Concurrently, DOM produced swelling that has been
shown in CGCs to be dependent on AMPA/KAR activity
(Berman and Murray, 1997). In contrast to NBQX, the
NMDAR antagonist DAP5 was still neuroprotective at 1 h
post-DOM.

NMDAR-mediated toxicity, therefore, occurs with greater
delay (30–60 min later) and is observed even after removal of
the toxin, suggesting that it occurs indirectly, presumably via
elevations in extracellular glutamate subsequent to AMPA/
KAR-mediated depolarization. In CGCs extracellular glutama-
tate (and aspartate) is elevated within minutes after DOM
exposure as a result of cell swelling and reversed glutamate
uptake (Berman and Murray, 1997). The early phase may
synergize with delayed NMDAR-mediated activity by pro-
viding depolarization-elicited removal of the Mg2+ block from

**FIG. 8.** Correlation between DOM-induced neuronal death and iGluR subunit protein levels with increasing neuronal maturation. (A) Cultures at different
times in vitro received either 3 mM/24 h, 10 mM/2 h, or 50 mM/10 min DOM, and neuronal death was assessed 24 h after the start of DOM. DOM-induced cell death
was normalized to neuronal death produced by 10 mM/2 h NMDA and measured 24 h after the start of DOM treatment (set at 100%). Maximum neuronal death at
each DIV is expressed as a percent of death produced by 100 mM DOM applied for 24 h at DIV 11. The open bar represents maximum neuronal death produced
NMDA. Numbers above the bars are EC50 values (μM) derived from DOM dose-response curves constructed using 24-h exposure data at each DIV time (data not
shown). For all DOM dose-duration paradigms, DOM efficacy and potency increases with increasing DIV up through DIV 8 and then stabilizes. (B) Western blots
show the developmental profiles of the principal NMDAR subunit NR1, AMPAR subunits GluR1, GluR2/3, and GluR4, and the KAR subunits GluR5 and GluR6/7.
Protein levels of all subunits (except GluR4, which was below the level of detection) increased with in vitro chronological age, especially through DIV 8. A similar
profile was detected for the neuronal and glial markers NeuN and GFAP, respectively. (C) IODs of iGluR immunoreactive bands in (B) were converted to percent of
that measured at DIV 11, pooled (n = 3), and plotted with 1/EC50 against time in vitro. Increased DOM potency is significantly and positively correlated with total
iGluR expression during development (r² = 0.996).
the receptor-channel (Mayer and Westbrook, 1987). Two hours after DOM exposure, iGluR antagonists do not afford significant neuronal protection, indicating a “critical window” for neuronal rescue exists after DOM. A summarizing figure concerning differential roles of GluRs and potential excitotoxicity mechanisms of DOM is presented in Fig. 10.

Effect of Different Exposure Paradigms

Each of the exposure paradigms, including 50 μM/10 min DOM, were equieffective, providing approximately 50% DOM-induced neuronal death. Results of experiments employing a 30-min delay in the application of iGluR antagonists indicated that low concentrations of DOM (3 μM/24 h and 10 μM/2 h) produced as much if not more toxicity mediated via AMPA/KARs activation as compared to 50 μM/10 μM DOM during this early period (Fig. 6). The reduced protection provided by delayed NBQX treatment against high concentrations of DOM (50 μM) may indicate a quicker progression from early to the late phase of DOM toxicity, evidenced by sensitivity to D-AP5 but not NBQX, since delayed NMDAR blockade protected equally well against DOM toxicity in each of the three exposure paradigms (82–88%). The similar efficacy of NMDAR blockade in all paradigms and the significant protection afforded by NMDA receptor blockade in vivo could be exploited in DOM poisoning accidents (Tasker and Strain, 1998).

In combination, these data indicate that early onset AMPAR/KAR activation occurs within the first 10 min after DOM application and is able in all three paradigms to trigger the secondary processes leading to NMDAR activation. NMDAR activation probably begins soon after DOM application, since glutamate and aspartate release reaches ~80% of maximum within the first 10 min in cultured CGCs (Berman and Murray, 1997), but may trigger maximum damage if continued for 30–60 min. At 2 h post-DOM, AMPAR and NMDAR activation no longer contribute to the ensuing DOM toxicity, which may result from NO-induced neurodegeneration (Chandrasekaran et al., 2004).

Timecourse of DOM Excitotoxicity

A novel finding is that the timecourse of DOM excitotoxicity is dependent on DOM concentration, with high concentrations producing complete neuronal death within just 2 h after exposure. At low doses the time course of cell death was more prolonged, and maximum cell death was not achieved until many hours later. Therefore, the potency of DOM increases with time of exposure (except at high doses), like that reported for NMDA and kainate (Koh and Choi, 1988). Rapid DOM toxicity that correlated with rapid swelling after high doses implicates acute processes related to iGluR activation, since iGluR antagonists rescue neurons only during this critical 2-h window post-DOM (Fig. 5). In support of this, glutamate induces neuronal swelling, intracellular Ca2+ deregulation, and neuronal death within 2 h (Tymianski et al., 1993). The slower effect of low DOM concentrations (3, 5, and 10 μM) may involve apoptosis, since excitatory amino acids can trigger either necrotic or apoptotic processes, depending on the intensity of the insult. The greater efficacy of DOM after brief application resembles more the effects of NMDA than kainate (Koh and Choi, 1988). In combination with the prominent NMDAR-mediated component of DOM (but not kainate toxicity; Jensen et al., 1999) and the similar requirement for NMDARs seen with low and high DOM concentrations (see Fig. 6), these data suggest that rapid DOM toxicity may involve expedited activation of downstream signaling pathways associated with NMDARs.

Lack of Significant Contribution of mGluRs to DOM Excitotoxicity

DOM does not bind directly to mGluRs (Hampson et al., 1992; Hampson and Manalo, 1998), but may activate mGluRs indirectly through release of endogenous EAs. In our study, none of the mGluR antagonists changed DOM-induced neuronal death in any exposure paradigm. In contrast, similar concentrations of AIDA protect against NMDA receptor-mediated excitotoxicity in pure neuronal cortical cultures (Strasser et al., 1998).

Since Group I mGluRs produce presynaptic vesicular release of glutamate (Cartmell et al., 2000), and DOM-induced reversal of glutamate uptake (Berman and Murray, 1997) is likely to depend on glia, the inclusion of a moderate astrocytic component in our cortical cultures may mask a potential contribution
by Group I mGluRs. Reduction of glutamate-induced EAA release by blocking Group I mGluRs, therefore, may not change effective levels of extracellular glutamate in the presence of DOM. Alternatively, receptors activated by DOM-triggered adenosine that inhibit exocytic glutamate release (Berman and Murray, 1997) may compensate for release triggered by glutamate activation of Group I mGluRs. Presynaptic autoreceptors of inhibitory Group II and III mGluRs (Cartmell et al., 2000) do not appear to limit DOM toxic effects, and this cannot be attributed to lack of mGluR expression, since cortical cultures express Group I, II, and III mGluRs (Heck et al., 1997; Janssens and Lesage, 2001).

Developmental Susceptibility to DOM Excitotoxicity: Correlation with iGluR Expression

Saturating concentrations of DOM, if given for 24 h, induce maximal neuronal loss at all DIV examined. Using any of the three submaximal DOM treatment paradigms, we observed an increase in excitotoxicity with increasing time in vitro until DIV 8, as reported previously (Choi et al., 1987; Jensen et al., 1999). Regression analysis indicated that DOM potency is positively correlated with total iGluR expression (Figs. 8A and 8C). In particular, levels of the obligatory NMDAR subunit, NR1, are dramatically elevated between DIV 5 and 8, when DOM potency increases significantly. Previous studies have reported increased expression and function of NMDARs with time in vitro (Cheng et al., 1999; Hoffmann et al., 2000; Sinor et al., 1997), which could intensify NMDA receptor-mediated responses leading to augmented DOM potency.

Low to robust expression of some AMPAR/KAR subunit members (GluR1, GluR 2/3, and GluR 6/7) could be detected at DIV 3 when a 24-h exposure to DOM (100 μM–1 mM) produced maximal cell death (Fig. 8A). Large increases in expression were most notable for the AMPAR subunits GluR1 and GluR 2/3 as well as the KAR subunits GluR5 and GluR6/7, subunits shown to be predominantly expressed by cerebral cortex (Dai et al., 2002; Sommer et al., 1992). GluR2-containing AMPARs have been implicated in AMPAR-mediated excitotoxicity that increases during development (Janssens and Lesage, 2001) and may partially explain the developmental regulation of DOM potency. An alternative explanation is that DOM’s enhanced effects during maturation may be due to activation of more Ca2+-permeable AMPARs or AMPAR isoforms generating non- or slowly desensitizing excitatory currents (Hampson and Manalo, 1998; Jensen et al., 1998; Patneau et al., 1994; Sommer et al., 1992; Stern-Bach et al., 1998). Increased functional expression of KARs, which has been implicated in DOM toxicity (Jensen et al., 1999), may also contribute. Moreover, increased glutamate
uptake activity at later times in vitro (Gailet et al., 2001) may provide proportionately more capacity for “reversed glutamate uptake” (glutamate release) and increase the efficacy of DOM.

In line with our findings, whole-animal studies report that neonates are highly susceptible to DOM exposure (Xi et al., 1997) and that DOM potency is two-fold greater in the first week of postnatal development (Doucette et al., 2003). In contrast to whole-animal studies that are confounded by developmental changes in the blood–brain barrier and detoxifying mechanisms (Mayer, 2000; Xi et al., 1997), our results using a barrierless in vitro system indicate that intrinsic neuronal properties such as iGluR expression/function may be critical in determining the efficacy and potency of DOM.

Increased Susceptibility to Subsequent DOM Exposure after Prior Preconditioning

Another significant finding derived from this study is that a 48-h preconditioning to a sublethal dose of DOM increases the susceptibility of neurons to subsequent treatment with 3 μM/2 h DOM. DOM toxicity, therefore, is dependent on prior history of DOM exposure, a property that is not well appreciated today, and may constitute a significant risk factor for ASP poisoning. Potential mechanisms for increased susceptibility after preconditioning could be due to the fact that either cortical neurons are unable to mount a tolerance response, or cortical neurons in culture (as opposed to in vivo conditions) lack tolerance induction mechanisms. Our results differ from those of Kerr et al. (2002), who reported tolerance to the acute electrophysiological effects of DOM in hippocampus. However, the exposure times were much shorter, and neuronal death was not assessed in that study. A caveat, however, should be noted that the preconditioning stimuli used in this study differ from the stimuli used in other excitotoxicity studies, so that neurons may have been rendered fragile by the strength of the preconditioning stimulus. Our rationale in choosing a relatively longer DOM preconditioning duration was that longer exposure might reduce glutamate receptor expression and, therefore, lead to subsequent tolerance to DOM. Surprisingly, the latter proved not to be the case. Because the preconditioning concentration and duration might still produce sublethal effects on cultured cortical neurons, care should be taken in extrapolating these results to other excitotoxicity studies, especially when other confounding factors are present, such as neuronal developmental stages (this study) or animal age (Hesp et al., 2004). Interestingly, preconditioning did not alter the response to a subsequent high dose of DOM (50 μM/10 min). Increased DOM toxicity after repeat exposure is consistent with results from an in vivo study showing greater reductions in glial and neuronal enzyme activity associated with glutamate homeostasis or to increased Ca$^{2+}$ influx in rats previously exposed in utero (Dakshinamurti et al., 1993; also see Clayton et al., 1999; Peng et al., 1997). While these parameters, as well as increased extracellular glutamate levels, can change within 4 h after DOM treatment, a time frame comparable to our preconditioning period, our second treatment with DOM occurred after washout of preconditioned medium, ruling out increased glutamate background as a factor.

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DOMOIC ACID-INDUCED NEUROTOXICITY


