Astrocytes Regulate N-Methyl-D-aspartate Receptor Subunit Composition Increasing Neuronal Sensitivity to Excitotoxicity*

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Received for publication, February 26, 2001, and in revised form, April 2, 2001
Published, JBC Papers in Press, April 16, 2001, DOI 10.1074/jbc.M101740200

We have examined the dependence of rat cerebellar granule neurons (CGNs) for protection against glutamate toxicity. Under co-culture conditions, rat CGNs require astrocytes to protect against glutamate. The CGNs become more sensitive to glutamate toxicity in co-culture than when grown in cultures with only low numbers of astrocytes. If the protection of the astrocytes was withdrawn or blocked, this sensitivity led to neuronal death. Differing changes in NMDA receptor subunit subtype composition were noted depending on the conditions in which the CGNs were grown. Suppression of individual NMDA subunit subtypes by oligonucleotide knockdown resulted in inhibition of toxicity. This result implies that astrocytes regulate the expression of NMDA receptor subunit subtypes which influence neuronal sensitivity to glutamate toxicity.

Astrocytes are the support cells for neurons in the brain. The way in which astrocytes protect neurons from various insults is under continued investigation. In the cerebellum where glutamate is a major neurotransmitter (1), the astrocyte population provides effective protection against the excitotoxicity of glutamate (2). Astrocytes clear the glutamate released by neurons via active uptake through glutamate-transporting proteins such as GLAST and GLT-1 (3–6). Without rapid clearance there is the possibility that glutamate will have a toxic effect mediated through binding to NMDA receptors (7). The presence of astrocytes in neuron-rich cultures has been shown to reduce the toxic potency of glutamate (8, 9). When glutamate is not cleared, levels of glutamate can easily rise activating NMDA receptors, causing increased calcium entry, internal oxidative stress, mitochondrial dysfunction, and eventually apoptosis (7, 10–13). Therefore, understanding what regulates glutamate uptake by astrocytes is essential.

There is now strong evidence that factors from neurons alter the level of expression of the astrocyte glutamate transporters (14–16). This can result in an increased clearance of glutamate via these transporters (17, 18). Thus, factors released by neurons signal to astrocytes the presence of neurons and induce an alteration of astrocyte protein expression that results in increased protection of neurons by astrocytes against glutamate toxicity. We have identified vasoactive intestinal peptide as one such factor released by neurons (18). It has been shown that substances such as vasoactive intestinal peptide not only alter the effectiveness of glutamate transport by astrocytes but stimulate the release of protective factors, which also help neurons resist the excitotoxicity of substances such as glutamate and NMDA (19, 20). In understanding the mechanism by which glutamate toxicity is prevented in the normal brain, there is clearly a need to understand not just how glutamate uptake by astrocytes is altered by the presence of neurons but also how neuronal sensitivity to glutamate is regulated by the presence of astrocytes other than their removal of glutamate by active uptake.

Previous studies have shown that mouse cerebellar neurons become dependent on astrocytes for protection from glutamate toxicity (17, 21). Cerebellar neurons co-cultured with astrocytes show an increased sensitivity to the toxicity of glutamate as compared with cerebellar neurons not co-cultured. This effect is not dependent on the regional origin of the astrocytes used for co-culture (17). Region-specific effects of astrocytes have been suggested to be contact-mediated (22), and the changes to glutamate sensitivity were related to diffusible factors, some of which have been defined (18). In the presence of astrocytes, this increased sensitivity to the toxic effects of glutamate is normally not noticeable because of the survival promoting effects of astrocytes such as clearance of glutamate and release of protective factors. Increased sensitivity to glutamate toxicity is only of consequence if the protective effectiveness of astrocytes is compromised. We have found several ways this compromise can be caused, which lead to glutamate-induced neuronal death: 1) physical removal of astrocytes, 2) addition of substances that inhibit astrocytic glutamate uptake (21), 3) substances that activate astrocytes (e.g. transforming growth factor-β) (17), or 4) inactivation of protective factors such as interleukin-6 (21). This increased sensitivity to glutamate is induced in neurons by a factor released by astrocytes. Neurons treated with conditioned medium (NAM)1 from astrocytes exposed to medium from neuronal cultures showed decreased survival. This decreased survival was a result of glutamate toxicity, but it was not caused by increased glutamate concentration (21). Thus, some additional factor released by astrocytes into NAM makes neurons more sensitive to glutamate toxicity. Release of this factor is enhanced by the presence of neurons themselves or by conditioned medium from neurons (17). Clearly, identification of this factor and its mechanism of action are of great importance because of their possible role in regulating neuronal sensitivity to excitotoxic death.

* This work was supported in part by a grant from the Royal Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: NAM, neuron-conditioned astrocyte medium; CGN, cerebellar granule neuron; AM, astrocyte conditioned medium; PDC, 1-trans-pyrrolidine-2,4-dicarboxylic acid; PCR, polymerase chain reaction; RT, reverse transcription; GPAP, glial fibrillary acidic protein; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDGA, nordihydroguaiaretic acid; NDADM, neuron-dependent astrocyte-derived maturation factor; NR, NMDA receptor.
Astrocytes and Glutamate Neurotoxicity

In the present report, we have examined regulation of neuronal (CGN) sensitivity to glutamate. We have used the rat system and confirmed that this displays the same characteristics as the mouse system. We have identified changes in NMDA receptors subunit subtype composition that parallel increased sensitivity to glutamate toxicity. Oligonucleotide knockdown of expression of these subunit subtypes inhibits glutamate toxicity markedly. Oligonucleotide therapy based on these observations may provide an effective way to combat excitotoxic neuron death in a variety of diseases.

**Experimental Procedures**

Unless specified chemicals and pharmacological agents were from Sigma. The rats used were Wistar from Harlan. 1-trans-Pyrrolidine-2,4-dicarboxylic acid (TDC) was from Torr.

**Glial Culture**—Mixed glial cultures were prepared from dissociating cerebral cortices of newborn rats. 4–5 cortices were trypsinized in 0.05% trypsin (Sigma) and plated in a 75-cm² culture flask (Falcon) in Dulbecco’s minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Sigma) and 1% antibiotic solution (penicillin/streptomycin, Life Technologies, Inc.). Cultures were maintained at 37 °C with 5% CO₂ for 14 days until glial cultures were confluent.

Astrocytes were isolated and characterized following standard methods as described previously (23). Micrograins were dislodged into the medium and discarded. Type 1 astrocytes were purified by taking the remaining adhesive cells and trypsinizing. The cells were preplated for 30 min, rinsing with applying the trypsinized astrocytes to the cultures. The medium was then collected and plated for 2 h at 10⁶ cells/well into 24-well trays, after which the medium was replaced to remove less adhering contaminating cells. Purified astrocytes were maintained under the conditions described for mixed glial cultures. Purity of cultures was determined as described previously (21, 23). Astrocyte cultures were not used unless purity was close to 100%. Astrocyte cultures were stained for glial fibrillary acidic protein (GFAP) using a rabbit polyclonal antibody (Dako) and detected with a fluorescein-conjugated secondary antibody type 2 (NR2b) subtypes. The primers for PCR were chosen to ensure identical amounts of protein were loaded onto gels. After electrophoresis, the protein was transferred to a nylon membrane (Immobilon, Millipore) with a semi-dry blotter (Bio-Rad). Detection of specific antigens was carried out using a chemiluminescence detection system (ECL, Amersham Pharmacia Biotech). Stained cells were examined using a Leitz fluorescence microscope. Conditioned media were prepared from type 1 astrocyte cultures in which the cells had been plated at 10⁶ cells/well. Cells were kept in culture for 24 h before addition of fresh medium. Media were collected after 2 days of exposure of the cells to the medium and other agents, and the conditioned media were used without freezing by direct application to neuronal cultures. NAM was prepared by exposing the astrocytes for 2 days to neuromonially conditioned medium.

**Neuronal Cell Culture**—Preparation of cerebellar cells from seven or 8 day old mice (P7–8) was as described previously (21). Briefly, the cerebella were dissected in Hanks' salt solution (Life Technologies, Inc.) containing 0.5% trypsin and plated at 1–2 × 10⁶ cells/cm² in 24-well trays (Falcon) coated with poly-γ-lysine (50 μg/ml, Sigma). Cultures were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum and 1% antibiotics (penicillin, streptomycin). Cultures were maintained at 37 °C with 7% CO₂.

Neuronal cells were stained in cerebellar cell cultures with anti-neuronal nucleus mouse (Chemicon), and detection with a fluorescein-conjugated anti-mouse IgG antibody.

A neuronal conditioned medium was prepared by taking cerebellar cells that had been in culture for 2 days and exposing them to fresh medium. The conditioned medium was collected after 2 days and applied directly to astrocyte cultures without freezing or storage. This medium was centrifuged to remove cellular particles.

**Co-culture**—For co-culture experiments, cerebellar cells were plated as normal in 24-well trays. Astrocytes and/or microglia were plated in tissue culture inserts (Falcon) with 3.0-μm pores. Pharmacological agents were added to both wells. The volume of the dual well system was maintained at 1 ml. Cerebellar cells were maintained in culture for 1 day before addition of astrocytes. For co-culture experiments, astrocytes were plated in inserts at 10⁵ cells/insert. Treatment with agents followed 2 days of co-culture and continued for 4 days afterward. The medium was exchanged 2 days later, and the pharmacological agents re-applied with the fresh medium. Before assaying cerebellar cell survival, the inserts were removed and 3,4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT, Sigma) assays carried out on the cerebellar cells. Fractionation of NAM was performed using Microcon spin concentrators (Amersham Pharmacia Biotech). NAM was passed sequentially through a series of concentrator filters with molecular mass cut-offs of 5, 10, 30, and 50 kDa. The filtrated was collected, and the retained material was reconstituted to the same volume to pass through the next filter. This produced fractions with solutes approximately <3, 3–10, 10–30, 30–50, and >50 kDa.

**Survival Assay**—The assay used for testing the survival of cerebellar cell cultures was as described previously (24) and was based on the conversion of MTT to a formazan product by respiring cells. MTT was diluted to 200 μM in Hanks’ solution (Life Technologies, Inc.) and added to cultures for 1 h at 37 °C. The MTT formazan product was released from cells by addition of dimethyl sulfoxide (Sigma) and measured at 570 nm in an Unicam Helios spectrophotometer (ATI Unicam). Relative survival in comparison to untreated controls could then be determined.

**PCR Analysis**—Total RNA was extracted from cultured astrocytes grown under various conditions using a kit (Qiagen). The cerebral cultures were either 1) untreated, 2) treated with astrocyte condition medium, 3) exposed to NAM for 2 days, or 4) co-cultured with cerebellar neurons. The RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Equivalent amounts of cDNA were used for PCR analysis of NMDA receptor type 1 (NR1) and NMDA receptor type 2 (NR2) subtypes. The method used was as previously described (25, 26). For the NR1 subtypes NR1a and NR1b were detected using a single PCR reaction using the primers CTCCCCACCTGCAGGCTT (forward) and GTATGTGTCATTGGCCG (reverse) where the product for NR1b is the larger of the two products. The NR2 subunit primers were first amplified with the primers GGGTGTCCTGGCATCGACATCC (forward) and GACACGAAAGGCCCCACAC (reverse). The product is then digested with one of three restriction enzymes, which can distinguish the different subtypes Bpm1 (NR2a), Bfa1 (NR2b), or Scal (NR2c). The primers for PCR were generated in house by the PNAC Facility. PCR for the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene was carried out using the specific primers (sense, 5’-GGGAGGGCTGCGTGGTACGGCCTG-3’; antisense, 5’-CGACGGGACACATTGGGGGTAGG-3’) to ensure equivalence of the samples. Additionally, RT-PCR was used to assess the level of 5-lipoxygenase expression. The primers for this analysis were 5’-TGGAACCCGCCCCTCTTTTGGAG3’ (senses) and 5’-AAAGCCCCATGCTACCTTTGGA-3’. Densitometric analysis of the bands was carried out using NIH Image.

**Western Blot Analysis**—Protein was extracted from CGNs and astrocytes by homogenization in an extraction buffer (20 mM Tris acetate, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM pyrophosphate, 1 mM Triton X-100, 1 mM sodium orthovanadate, 0.1% β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). After 20 min of incubation at 4 °C, the homogenates were centrifuged at 14,000 rpm for 10 min. The supernatant was taken as the extract. A protein determination was carried out using a bicinchoninic acid (BCA) protein assay kit (Sigma) to ensure identical amounts of protein were loaded onto gels. After boiling, extracts were electrophoresed on a 10% acrylamide gel with SDS in a Bio-Rad Mini-Protean 2 system. After electrophoresis, the protein was transferred to a nylon membrane (Immobilon, Millipore) with a semi-dry blotter (Bio-Rad). Detection of specific antigens was carried out following blocking the membrane with 5% milk powder in Tris-buffered saline (pH7.4). Primary antibodies included anti-NMDA receptor type 1 (NR1, mouse, Affinity Bioreagents), anti-NMDA receptor type 2 (NR2, mouse, Affinity Bioreagents), anti-synaptophysin (mouse, Sigma), anti-GFAP rabbit, Dako), and anti-α-tubulin (mouse, Sigma). Detection was via binding of an appropriate HRP-conjugated secondary antibody and detection using the ECL chemiluminescence reagent (Amersham Pharmacia Biotech) and exposure to x-ray film (Eastman Kodak Co.).

**Oligonucleotide Knockdown**—Antisense oligonucleotides specific to NR1 and NR2 subtypes were prepared as described by others (27): NMDA NR1, 5’-CCCGCGCTTGGCTGCTTCTAC (NR1b, CTCCACGACCCTTCTTCGCTTCTAC (NR1b), CTCCACGACCCTTCTTCGCTTCTAC (NR1b), CTCCACGACCCTTCTTCGCTTCTAC (NR1b), CTCCACGACCCTTCTTCGCTTCTAC (NR1b), CTCCACGACCCTTCTTCGCTTCTAC (NR1b), CTCCACGACCCTTCTTCGCTTCTAC (NR1b), CTCCACGACCCTTCTTCGCTTCTAC (NR1b), 5’-AAAAGCCAGTCGTACTTTGAA-3’. The products for PCR were generated in house by the PNAC Facility. PCR for the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene was carried out using the specific primers (sense, 5’-GGGAGGGCTGCGTGGTACGGCCTG-3’; antisense, 5’-CGACGGGACACATTGGGGGTAGG-3’) to ensure equivalence of the samples. Additionally, RT-PCR was used to assess the level of 5-lipoxygenase expression. The primer for this analysis was 5’-TGGAACCCGCCCCTCTTTTGGAG3’ (senses) and 5’-AAAGCCCCATGCTACCTTTGGA-3’. Densitometric analysis of the bands was carried out using NIH Image.

**Results**

**Astrocytes Influence Glutamate Neurotoxicity**—For the experiments to be described here, CGN from 7–8-day-old rats were prepared identically but treated four different ways. (a) control cultures were untreated, (b) cultures were co-cultured with astrocytes placed in removable inserts, (c) cultures were
exposed to astrocyte conditioned medium (AM), and (d) cultures were exposed to NAM. Unless stated AM and NAM were applied at 20% of the total volume of medium. For some experiments neuronal conditioned medium was used to treat CGN cultures, but as reported previously (21) this medium had no effect on the survival of CGNs (data not shown). Cultures grown under the four conditions for 4 days were evaluated using immunostaining to detect neurons and astrocytes. All cultures contained neurons and astrocytes (Fig. 1) but treatment of cultures with NAM resulted in an apparent reduction in neuronal number. Using Hoechst staining, it was apparent that only cultures treated with NAM contained cells with fragmented or shrunken nuclei beyond those of controls (control 563, co-culture 553, AM 534, NAM 5174, average cells per field).

The survival of the cultures was also determined using an MTT assay. Only NAM reduced the survival of CGN cultures after 4 days of treatment (Fig. 2A). NAM was also applied to CGN cultures at increasing concentrations and was also applied to CGNs co-cultured with astrocytes. NAM but not AM killed CGN cultures in a dose-dependent manner (Fig. 2B). Co-culture of CGN cultures with astrocytes inhibited NAM toxicity. NAM toxicity could also be inhibited with MK801 (Fig. 2A). The glutamate transport inhibitor PDC was applied to CGN cultures alone or co-cultured with astrocytes for 4 days. PDC had no effect on CGNs not co-cultured but was toxic to CGNs co-cultured with astrocytes (Fig. 2C). This effect could also be inhibited with MK801 (data not shown). These results suggest that NAM makes neurons more sensitive to glutamate toxicity and that co-culture protects CGNs from glutamate toxicity by active clearance of glutamate. These results are similar to those from work investigating the toxicity of NAM to mouse CGNs (21). This implies that the system previously investigated in the mouse and this system in the rat are interchangeable.

Direct deprivation of CGNs from astrocytic protection against glutamate can be achieved in our co-culture system by growing CGNs with astrocytes in co-culture for 4 days and then withdrawing the astrocytes in the co-culture insert. Although this method does not remove all the astrocytes in the culture, it does diminish them considerably. CGNs treated in this manner were treated with increasing concentrations of glutamate or NMDA. Both glutamate and NMDA were more toxic to CGNs removed from co-culture than to those that had not experienced co-culture or to those maintained in co-culture (Fig. 3). These results suggest that co-culture with astrocytes causes specific
changes in neurons that make them more sensitive to glutamate and NMDA toxicity.

Astrocytes were exposed to conditioned medium from neurons grown in serum-free medium. Serum-free AM was also produced (data not shown). The serum-free AM or NAM were applied to CGNs. Neither medium produced in this manner was toxic to CGNs. However, when NMDA was applied in parallel, it was far more toxic to CGNs exposed to serum-free NAM. This toxicity was fully blocked by MK801, suggesting that the toxicity is mediated through the NMDA receptor (Fig. 3 C). The implication of this result is that NAM alters NMDA receptor response to NMDA or glutamate. Serum-free NAM was produced and heated to 75 °C to denature proteins that might have been secreted by astrocytes. NAM produced this way had no effect on the toxicity of NMDA (Fig. 3 C). NAM was also fractionated according to molecular weight of its solutes. These fractions were tested for toxicity on CGNs. The majority of the toxicity was associated with the 10–30-kDa fraction. These results imply that the factor from astrocytes in NAM making CGNs more susceptible to the toxicity of NMDA or glutamate is heat-sensitive and thus likely to be a protein of 10–30 kDa.

**NMDA Receptor Subunits—** We investigated whether our four culture conditions alter the expression of NMDA receptors subunits (NR1, NR2) using Western blotting. As controls synaptophysin and tubulin were examined. Co-culture of CGNs with astrocytes did not alter the levels of any of these proteins as compared with untreated CGNs (Fig. 4). Treatment with AM enhanced the expression of synaptophysin dramatically. This suggests that AM greatly enhances the formation of synaptic structures in cultures of CGNs. Furthermore, levels of NR1 and NR2 were also elevated by AM treatment. In comparison tubulin was not significantly elevated (n = 4, Student’s t test on densitometric analysis of blots, p > 0.05). Interestingly, NAM did not show the same effect as AM. Although NAM produced a slight elevation of synaptophysin, there was no increase in the level of NR1or NR2. The implication of this is that some factor additionally released by astrocytes into NAM and not into AM suppressed the action of AM or a neuronal factor suppressed astrocytic release of a factor that would otherwise stimulate expression of synaptophysin and NMDA receptors.

As it was difficult to ascertain accurately by Western blotting the proportion of each subtype of NR1 and NR2 expressed by CGNs grown under the four conditions, quantitative PCR was used to examine the expression of receptor subunit subtypes. The analysis is shown in Fig. 5, and the quantitation shown in Table I. Analysis of NR1 again shows an increased total level of NR1 subtypes in AM-treated CGNs with a lesser effect on NAM-treated cultures. Additionally, both co-cultured CGNs

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Withdrawal of astrocytic protection makes neurons more sensitive to the toxicity of glutamate and NMDA. CGNs were co-cultured with astrocytes for 4 days; after this time, the inserts containing the astrocytes were discarded and glutamate (A) or NMDA (B) added at various concentrations. In parallel, cultures of CGNs that had not been co-cultured were also treated with glutamate or NMDA. After 2 days survival was determined using an MTT assay. Shown are CGNs not co-cultured (C) and CGNs withdrawn from co-culture (●). C, control CGN cultures were maintained in serum-free conditions. Some cultures were treated with NAM that had been prepared using serum-free medium. Other cultures were treated with heat-inactivated serum-free NAM. Additionally, cultures were either treated with 50 μM NMDA (gray bars) or 50 μM NMDA and 50 μM MK801 (black bars) or neither (open bars). At the end of 4 days of treatment, survival was determined using an MTT assay. D, toxicity of NAM after partition of solutes according to molecular size. NAM was passed through a series of filters with molecular mass cut-offs of 3, 10, 30, and 50 kDa. The filtered material was collected, and the retained material was reconstituted to the same volume to pass through the next filter. This produced fractions with solutes approximately <3, 3–10, 10–30, 30–50, and >50 kDa. These fractions were then tested for toxicity on CGNs as compared with unfiltered NAM. Shown are the mean and S.E. for four experiments with three determinations each.

![Figure 4](http://www.jbc.org/)

**FIG. 4.** Changes in NMDA receptor subunits. Extracts from CGNs grown as controls (C), in co-culture (Co), in the presence of AM or NAM were prepared and run on a 7% acrylamide SDS-PAGE gel. Following Western blot, immunodetection of synaptophysin, tubulin, NR1, and NR2 using specific monoclonal antibodies was carried out.
and NAM-treated CGNs showed a higher proportion of NR1b subunit than controls of AM-treated cultures. Analysis of NR2 showed little change in total levels of NR2 under any condition. However, NAM-treated astrocytes showed virtually no NR2b, implying a higher level of NR2a and NR2c present in NAM-treated CGNs. Co-culture of CGNs with astrocytes resulted in an increase in the amount of NR2c. The implication is that NAM might have the effect of elevating the percentage of NR2a and NR1b subunits while co-culture elevates NR2c and NR1b.

**Oligonucleotide Knockdown and Neurotoxicity**—In order to determine if different NMDA subunit subtype composition influences the susceptibility of CGNs to glutamate toxicity in our model, we tested the effect of oligonucleotides specific for each of the five subunit subtypes on NAM toxicity and compared this to the effects of the oligonucleotides on CGNs exposed to AM (Fig. 6). The oligonucleotides had no effect on CGN survival in the presence of AM. The anti-NR1b oligonucleotide decreased NAM toxicity the most. The anti-NR2a oligonucleotide decreased NAM toxicity whereas anti-NR2c enhanced toxicity, suggesting that the two subunits have antagonistic roles in terms of sensitivity to NAM toxicity. These results suggest that the changes in some of the NMDA receptor subunit types (NR1b and NR2a) we observed in NAM-treated cultures can be directly related to their increased sensitivity to NAM toxicity. As NAM toxicity is related to either glutamate or NMDA, changes in the subunits caused by NAM may regulate sensitivity to glutamate.

**Death Signal from NMDA Receptors**—We investigated the effect of NAM in terms of inducing cell death through the NMDA receptor. Nordihydroguaiaretic acid (NDGA) is known to inhibit toxicity mediated through NMDA receptors. Treatment with NDGA but not indomethacin blocked the toxicity of NAM to CGNs (Fig. 7A). These results suggest that NAM might modify NMDA receptors in a way that initiates synthesis of arachidonic acid when high glutamate is present. To investigate this further, we measured the levels of lipoxygenase in CGNs grown under various conditions. Increased lipoxygenase mRNA was detected using RT-PCR in cultures of CGNs exposed to NAM or co-cultured. The greatest effect was seen with NAM. NAM produced without serum had a weaker effect (Fig. 7B). These results suggest that regulation of the arachidonic acid synthesis pathway is involved in the toxicity induced by NAM.

**DISCUSSION**

In this article, we have investigated two model systems in parallel. The first, co-culture of astrocytes and CGNs, represents a complete model of the effect of astrocytes on neurons in terms of their interaction to modulate effects of glutamate. The second involves use of conditioned medium from astrocytes, which have themselves been pre-treated with conditioned medium from neurons. The effects of this conditioned medium (NAM) show similarities to that of co-culture in that NAM sensitizes CGNs to the toxicity of glutamate. CGNs withdrawn from co-culture with astrocytes are also more sensitive to glutamate and NMDA toxicity. With this system we were then able to dissect changes in the composition of NMDA receptors.
that underlie this change in sensitivity to glutamate toxicity.

Of particular interest in the current work was the analysis of changes in NR1 and NR2 subtype. This was our main interest in using the rat system for this analysis because of the broader knowledge basis available. In particular it has been suggested that the subunit NR1b is associated with increased sensitivity to glutamate toxicity (28, 29). In our experiments, we observed that both co-culture with astrocytes and treatment with NAM resulted in an increase in NR1b. Anti-NR1b oligonucleotide inhibited NAM toxicity, implying that this subunit is involved in the increased toxicity of NAM. As CGNs are protected by astrocytic clearance of glutamate when co-cultured, this would explain why the expression of this subunit in co-cultured CGNs does not result in increased cell death. Despite its role in sensitivity to glutamate, the NR1b isoform has been shown to be necessary for normal regeneration in certain systems such as the retina (30).

During normal granule cell development, there is a known progression in the expression of the NR2 subunits subtypes. Early in development, the predominant subtype is NR2B but as development progresses there is a switch to NR2A and then an increase in the levels of NR2C (31, 32). Change in the subunit composition of NMDA receptors alters electrophysiological responses of CGNs (33, 34). Co-cultured CGNs demonstrated a NR2 subtype profile more like that of a more mature GN. The

CGNs in NAM showed an increase in type NR2A, but this seemed to be associated with increased sensitivity to NAM whereas NR2C appeared to be protective. Therefore, increased sensitivity to glutamate in the NAM-treated CGNs might represent the inadequacies of a transition phase in development.

These findings regarding NMDA subunits are important with relation to treatment to prevent excitotoxic damage in vivo. The suppression of certain subtypes of NMDA subunits using antisense oligonucleotides might represent a possible effective way to diminish damage by glutamate or analogues in many diseases. Alternatively, targeting astrocytes to alter release of substances that modulate NMDA receptor composition might be beneficial. However, it would first be important to determine the precise nature of the molecules released and the mechanisms governing them.

These findings also continue our investigation into the interaction of astrocytes and neurons in terms of regulating and preventing glutamate toxicity. We have previously suggested (17, 18, 21) that astrocytes release a factor upon stimulation by neurons (i.e. released into NAM) that causes neurons to change their sensitivity to glutamate. We have shown in this work that this change in sensitivity is mediated by changes induced in NMDA receptor composition. We have also observed that astrocyte conditioned medium altered the level of synaptophysin. This effect appears to be inhibited if the astrocytes producing the conditioned medium were first exposed to medium from neurons (see Fig. 4). This was possibly because a factor released by neurons inhibited this effect of astrocytes via their conditioned medium. This change in synaptophysin was also not observed in co-culture. The changes in NMDA receptors do not follow this pattern; therefore, what we observe is the stimulation of production of an astrocytic factor rather than an inhibition of one. This factor regulating NMDA receptor composition we tentatively describe as neuron-dependent astrocyte-derived maturation factor (NDADM).

NDADM is itself not toxic, as serum-free NAM does not induce cell death but increases CGN sensitivity to NAM. One of the results of the effect of NDADM or another factor released into NAM is the alteration in the activity of lipooxygenase. This enzyme is involved in the metabolism of arachidonic acid. Arachidonic acid is released in response to NMDA receptor stimulation (35). Low levels of arachidonic acid are beneficial to neurons but at high levels are toxic as they generate oxidized metabolites (36). We found that NDGA, which inhibits lipooxygenase activity, inhibited NAM toxicity, which supports the idea that NAM toxicity is mediated via arachidonic acid as others have found (37).

Our findings provide further evidence that regulation of NMDA receptor subunit expression modulates death mediated through NMDA receptors and that controlling this expression is important for understanding the mechanism of excitotoxicity. The continued emergence of astrocytes as a key regulator of neuronal survival now indicates that, in addition to protecting directly by clearance of glutamate, they also modulate neuronal sensitivity to glutamate. The doorway is now open for the discovery of a new class of modulating factors released by astrocytes, the control of which might be the key to preventing excitotoxicity.

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