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Expression of mRNAs Encoding Subunits of the *N*-Methyl-D-Aspartate Receptor in Cultured Cortical Neurons

JIE ZHONG, SHARI L. RUSSELL, DOLAN B. PRITCHETT, PERRY B. MOLINOFF, and KEITH WILLIAMS
Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084
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SUMMARY

The expression of mRNAs encoding subunits of the *N*-methyl-D-aspartate (NMDA) receptor was examined in cortical neurons maintained in primary culture. Cultures were prepared from embryonic day 17 rat neocortex. At this developmental age, levels of NR1, NR2A, NR2B, and NR2C mRNA were low or undetectable. Expression of NR1 mRNA increased progressively between days 1 and 21 *in vitro*. The amount of NR2A mRNA did not change between days 1 and 7 but increased between days 7 and 21. In contrast, levels of NR2B mRNA increased between days 1 and 7, with little further change after day 7. The level of NR2B mRNA was ≈4-fold higher than that of NR2A mRNA in 21-day cultures. Using ligand binding assays, the proportion of NMDA receptors having a low affinity for ifenprodil was also found to increase over time in culture. The increase in the expression of receptors having a low affinity for ifenprodil and the increase in NR1 and NR2A mRNAs were reduced or prevented by maintaining cells in medium with a low concentration of serum. The results are consistent with the hypothesis that inclusion of the NR2A subunit in native NMDA receptors is responsible for their low affinity for ifenprodil. Splice variants of NR1 lacking the 5′ (amino-terminal) insert were found to be the predominant forms of NR1 in cultured neurons. Variants containing the 5′ insert represented only a small (≤5%) fraction of total NR1 mRNA, and their proportion was not altered as a function of time in culture. Time-dependent changes in the properties of NMDA receptors and in the expression of subunit mRNA occurring in cultured neurons are similar to changes observed in developing rat brain. Thus, the developmental sequence of NMDA receptor expression that occurs *in vivo* is partially retained in neurons maintained *in vitro*.

Neurons derived from fetal or neonatal rodent brain and maintained in primary culture are widely used as model systems to study the properties and function of NMDA receptors (1, 2). Cultured neurons have also been used extensively to study the pharmacological specificity and mechanisms of excitotoxicity mediated by NMDA receptors (3-5). The expression and regulation of NMDA receptors on cultured neurons can be studied electrophysiologically and by Ca^{2+}-imaging techniques with intact neurons (1, 2) and biochemically using binding assays with ^{125}I-MK-801 with membranes prepared from cultured neurons (6-8). Cultured neurons also provide a system with which to study the expression of NMDA receptor subtypes and of the mRNAs coding for receptor subunits.

We have recently found that the atypical antagonist ifenprodil can discriminate two NMDA receptor subtypes that are differentially expressed during postnatal development (8). In neonatal rat brain, ifenprodil interacts with high affinity (IC_{50} ≈ 0.3 μM) at one population of NMDA receptors. A second population of receptors, with a 300-fold lower affinity for ifenprodil (IC_{50} ≈ 100 μM), is first detected at postnatal day 7 and represents 50% of the NMDA receptors in adult rat forebrain (8). A difference in the inhibitory effects of ifenprodil, mirroring that seen in binding assays, was seen in *Xenopus* oocytes injected with RNA prepared from neonatal and adult rat forebrain. This suggests that the difference may be due to developmentally regulated expression of mRNAs encoding subunits of the NMDA receptor. The delayed developmental expression of NMDA receptors having a low affinity for ifenprodil was also seen in cortical neurons prepared from fetal rat brain and maintained *in vitro* (8). Thus, some of the maturational processes that control the expression of NMDA receptor subtypes *in vivo* may also be operative *in vitro*.

The cDNAs coding for a number of subunits of the NMDA receptor have recently been cloned. These include the NR1, NR2A, NR2B, NR2C, and NR2D genes isolated from rat brain (9-11). The NR1 gene is transcribed as eight alternatively spliced mRNAs characterized by the inclusion of one 5′ and/or two 3′ inserts (12-14). Heteromeric NR1/NR2B receptors expressed in *Xenopus* oocytes have a high affinity for ifenprodil (IC_{50} = 0.34 μM), similar to that seen in neonatal rat brain, whereas NR1/NR2A receptors have a low affinity for ifenprodil

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; FBS, fetal bovine serum; PCR, polymerase chain reaction; GFAP, glial-fibrillary acidic protein; E-17, embryonic day 17; bp, base pair(s).

(IC_{50} = 146 μM), similar to that of the low affinity sites expressed late in development (8, 15). We have proposed that inclusion of the NR2A subunit in native NMDA receptors is responsible for the formation of receptors having a low affinity for ifenprodil (8, 15).

Changes in the expression of mRNAs encoding subunits of the NMDA receptor have been observed in developing mouse brain (16). The ε2 (NR2B) subunit is expressed at high levels in embryonic mouse brain and throughout postnatal development (16). In contrast, expression of mRNA encoding ε1 (NR2A) begins later, appearing during postnatal days 1–7 in mouse forebrain (16). We have examined the developmental expression of mRNAs coding for NMDA receptor subunits and the expression of NMDA receptors having high or low affinities for ifenprodil in cultured cortical neurons. These studies were designed to compare the expression of receptor subunits *in vitro* with that seen *in vivo* and to test the hypothesis that delayed expression of the NR2A subunit in cultured neurons underlies the late expression of NMDA receptors having a low affinity for ifenprodil. Culture conditions that may selectively alter the expression of NMDA receptor subtypes and subunit mRNAs have been identified.

Materials and Methods

Primary culture of cortical neurons. Neuronal/glial co-cultures were prepared from neocortex of embryonic rats at 17 days of gestation (E-17), as described previously (6). Cortical cells were plated in culture medium (Dulbecco's modified Eagle's medium containing 10% FBS, 10% Ham's F-12 nutrient mixture, 50 units/ml penicillin, and 50 μg/ml streptomycin), at a density of 1.0–1.3 × 10^5 cells/cm^2, in 100-mm plastic dishes that had been precoated with poly-D-lysine (20 μg/ml). Cells were maintained at 37° in a humidified atmosphere containing 7% CO_2. Medium was replaced every 2–4 days. Under these conditions, neurons grow in a dispersed monolayer, with only rare clumps, on a layer of dividing glial cells. After 4–6 days in culture, when the glial cell layer had become confluent, cytosine arabinoside (10 μM) was added for 24 hr to prevent further proliferation of non-neuronal cells. NMDA receptors, as studied with ^{125}I-MK-801, appear to be localized exclusively on neurons in these cultures (6, 7).

Binding assays with ^{125}I-MK-801. A well washed membrane fraction was prepared from cultured cells and binding assays with ^{125}I-MK-801 were carried out as described previously (6). Duplicate samples containing ^{125}I-MK-801 (200 pM) and membranes (15–40 μg of protein) were incubated for 3 hr at 32° in assay buffer (20 mM K-HEPES, 1 mM K-EDTA, pH 7.0), in the presence of glutamate, glycine, and spermidine (100 μM each), with various concentrations of ifenprodil as indicated. In the presence of glutamate, glycine, and spermidine (100 μM each), binding of ^{125}I-MK-801 reaches equilibrium within 2–3 hr (6). Nonspecific binding (<10% of total binding) was defined with 10 μM (+)-MK-801. Assays were terminated by rapid filtration (6). Data from inhibition curves were fit to one- or two-site binding isotherms to obtain IC_{50} values and the proportions of low and high affinity ifenprodil sites, as described previously (8).

Isolation of RNA. Total RNA was prepared from cultured cells or E-17 neocortex by a modification of the method of Chomczynski and Sacchi (17). Cells or tissues were homogenized in denaturing solution (4 M guanidinium thiocyanate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate, pH 7.0). After phenol/chloroform extraction, RNA was precipitated at −20° with isopropanol and pelleted by centrifugation (10,000 × *g*, 20 min). After reprecipitation, RNA was washed with 70% ethanol, dried, and resuspended in diethylpyrocarbonate-treated water. The concentration of RNA in each extract was calculated on the basis of absorbance at 260 nm. Samples were stored at −80° until assayed.

Development of probes. The PCR was used to synthesize an NR1 probe, using rat brain cDNA as a template and oligonucleotides designed from the rat NR1 sequence (9). The following primers were used: 5′ primer, nucleotides 115–150; 3′ primer, nucleotides 312–344. The oligonucleotide primers included one to three base substitutions to generate restriction endonuclease recognition sites to facilitate ligation into vectors. PCR products were subcloned into pGEM7Z(+) (pGEM-NR1). Sequencing of the amplified segments demonstrated that the probe was identical to the published NR1 sequence. A probe to detect the presence or absence of the 5′ insert in NR1 was isolated by PCR, using NR1B cDNA (which contains the 5′ insert) as a template and oligonucleotides based on the NR1 sequence (9, 12). The NR1B clone was a gift from Dr. S. Nakanishi (Kyoto University, Kyoto, Japan). The primers for PCR were as follows: 5′ primer, nucleotides 333–362; 3′ primer, nucleotides 714–747 (12). This probe covers the entire length of the 5′ insert together with sequences that flank both sides of the insert (see Results).

Probes for NR2A, NR2B, and NR2C were constructed by subcloning portions of NR2 cDNAs. Plasmid pSP72-NR2A, consisting of a 386-bp segment of NR2A flanked by SP6 and T7 RNA polymerase promoters, was constructed by subcloning the *Bam*HI-*Sac*I fragment of an NR2A clone into the pSP72 vector (Promega). Plasmids pSP72-NR2C and pGEM-NR2B, similarly constructed with pSP72 and pGEM-3Z vectors (Promega), contained a 332-bp *Sal*I-*Stu*I fragment of NR2C and a 259-bp *Sma*I-*Hind*III fragment of NR2B, respectively.

cDNA clones encoding neurofilament protein L (18) and GFAP inserted into pGEM-4 vectors were used to generate ^{32}P-labeled antisense probes for neurofilament and GFAP mRNAs. Riboprobes for neurofilament protein L and GFAP were 0.65 and 0.97 kilobase, respectively. A riboprobe for cyclophilin was generated from a cyclophilin clone (p1B15) consisting of a 0.7-kilobase segment of the coding sequence inserted into pSP65.

Plasmids containing probe sequences were linearized with appropriate restriction endonucleases. For *in vitro* synthesis of ^{32}P-labeled RNA transcripts, linearized plasmid templates (1 μg), RNA polymerases (T7 or SP6; 15–20 units), nucleotides (ATP, GTP, and UTP; 10 mmol of each), transcription buffer, and [^{32}P]CTP (80 μCi) were incubated at 37° for 60 min. Riboprobe was separated from unincorporated nucleotides on a spun G-50 column [Select D G-50 (RF); 5 Prime-3 Prime, Inc.]. Gel electrophoresis and autoradiography were used to verify that full length probes had been synthesized and to verify the purity of the probes.

Solution hybridization/RNase protection assay. RNA samples (5–10 μg) extracted from cultured cells were incubated with ^{32}P-labeled riboprobes (5 × 10^5 cpm) and hybridized overnight at 45°. Samples were then digested with RNase A and RNase T1 (at 37° for 90 min), followed by digestion with proteinase K in 1% sodium dodecyl sulfate and extraction with phenol/chloroform. Samples were precipitated with ethanol and size-fractionated on a urea/acrylamide gel. Quantitative measurement of protected mRNA bands was obtained using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Radioactivity was visualized as a digitized image, and the relative amount of radioactivity in each band was determined (19). The quantity of each species of mRNA was calculated from a standard curve for the ^{32}P-labeled riboprobe. The standard curve was linear over a 100,000-fold range of radioactivity. Results are expressed as attomoles of mRNA/microgram of total RNA.

Materials. (+)-3-^{125}I-MK-801 (specific activity, 2200 Ci/mmol) and [α-^{32}P]CTP (specific activity, 800 Ci/mmol) were purchased from DuPont/New England Nuclear (Boston, MA). (+)-MK-801 was a gift from Merck & Co. Inc. (West Point, PA). L-Glutamate and glycine were purchased from Sigma Chemical Co. (St. Louis, MO). Spermidine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ifenprodil was a gift from Synthélabo Recherche (Bagneux, France). FBS (lot 1111101) was purchased from Hyclone Laboratories Inc. (Logan, UT). Other tissue culture reagents and materials were from sources described previously (6, 7).

Results

***In vitro* development of receptor subunit mRNAs.** Levels of mRNA were quantified using solution hybridization/RNase protection assays. Probes specific for particular NMDA receptor subunits were of different lengths, allowing the separation and detection of NR1, NR2A, NR2B, and NR2C in individual samples analyzed on one lane of an acrylamide gel (Fig. 1). mRNAs encoding NR1, NR2A, NR2B, and NR2C were not detectable or were expressed at low levels in E-17 rat neocortex (Figs. 1 and 2). The level of NR1 mRNA increased between days 1 and 21 *in vitro* (Fig. 2). Expression of NR2A mRNA did not change between days 1 and 7 *in vitro* but increased between days 7 and 21. In contrast, the expression of NR2B mRNA increased between days 1 and 7, with little further change after day 7. In 21-day cultures, the levels of NR1 and NR2B mRNA were 4–5-fold higher than that of NR2A mRNA (Fig. 2). NR2C mRNA was expressed only at very low levels (20–100-fold lower than NR2A or NR2B) in cells cultured for up to 21 days (Fig. 1).

Expression of NMDA receptor subunits was also examined in non-neuronal cells prepared from embryonic rat neocortex. Cells were plated on plastic dishes that had not been coated with poly-lysine, and unattached cells were removed and medium was replaced 1 hr after plating. Cells were harvested after 21 days in culture. These cultures contained few or no neurons and consisted predominantly of glial cells. mRNA encoding GFAP but not neurofilament protein was detected in extracts of non-neuronal cultures (Fig. 3A). Neuronal/glial co-cultures contained both markers (Fig. 3A). mRNAs encoding NR1, NR2A, NR2B, and NR2C were not detected in glial cell cultures (Fig. 3B), suggesting that NMDA receptor subunit mRNAs detected in neuronal/glial co-cultures reflect their exclusive expression in neurons.

In addition to measuring expression of NMDA receptor subunits, we also examined the expression of cyclophilin, neu-

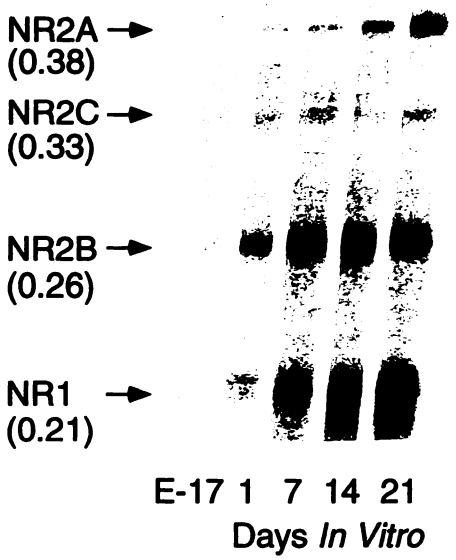

Fig. 1. Autoradiogram of solution hybridization/RNase protection analysis of NMDA receptor subunit mRNA in rat neocortex at E-17 and in cortical neurons cultured for 1–21 days *in vitro*. Ten micrograms of total RNA were hybridized with ^{32}P-labeled riboprobes, and samples were separated on a 5% acrylamide gel and exposed to X-ray film. The sizes (kb) of the protected bands corresponding to NR1, NR2A, NR2B, and NR2C are indicated.

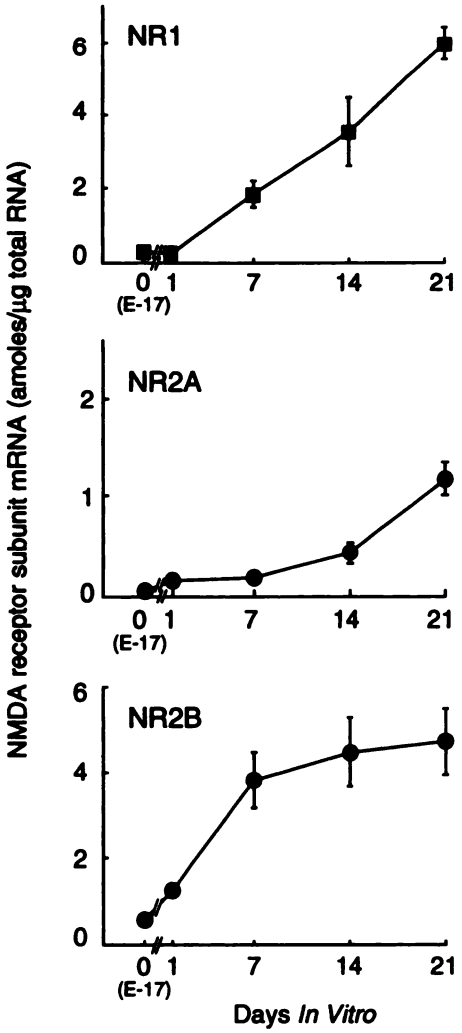

Fig. 2. Expression of subunit mRNAs in embryonic rat neocortex and cultured cortical neurons. Total RNA (5–10 μg) isolated from rat neocortex (E-17) or from cortical cells maintained for 1–21 days *in vitro* was assayed for levels of mRNA. Values are means ± standard errors from six animals (E-17 cortex) or four or five individual batches of cultured cells.

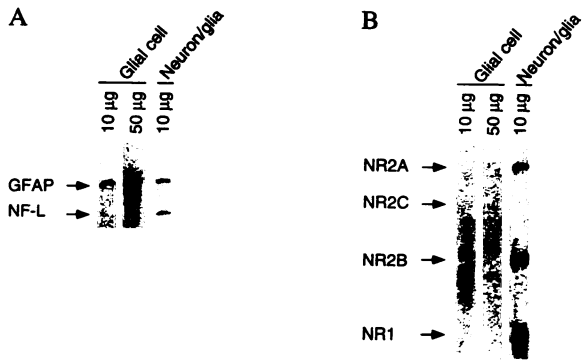

Fig. 3. Lack of NMDA receptor subunit mRNA expression in glial cells. Total RNA extracted from glial cell cultures (10 or 50 μg of RNA) or from neuronal/glial co-cultures (10 μg of RNA) was hybridized with probes for GFAP and neurofilament (*NF-L*) (A) and with probes for NR1 and NR2 subunits (B).

rofilament protein, and GFAP in developing neuron/glia co-cultures (Fig. 4). Expression of cyclophilin mRNA showed little change over time, whereas both neurofilament and GFAP mRNA levels increased with time in culture (Fig. 4). The increase in expression of neurofilament mRNA had a time course similar to that of the expression of NR1 and NR2A mRNA (Figs. 2 and 4). The increase in expression of NR1 and NR2A subunits may thus coincide with maturation of these neurons.

NR1 splice variants. Experiments were carried out to measure the levels of splice variants of NR1 with and without the 5′ insert. We used a solution hybridization/RNase protection assay with a probe that contains the sequence of the 5′ insert (12) and extends to cover flanking sequences on both sides of the insert (Fig. 5). Thus, mRNA that contains the insert yields a single band of protected RNA (Fig. 5, *band I*). In variants that do not contain the insert, two protected bands, corresponding to sequences that flank the insert, are seen (Fig. 5, *bands II* and *III*). Levels of mRNA calculated from the sum of band I plus either band II or band III were equal to levels determined using the pan probe for NR1 (Fig. 5, *band IV*, and see Fig. 6).

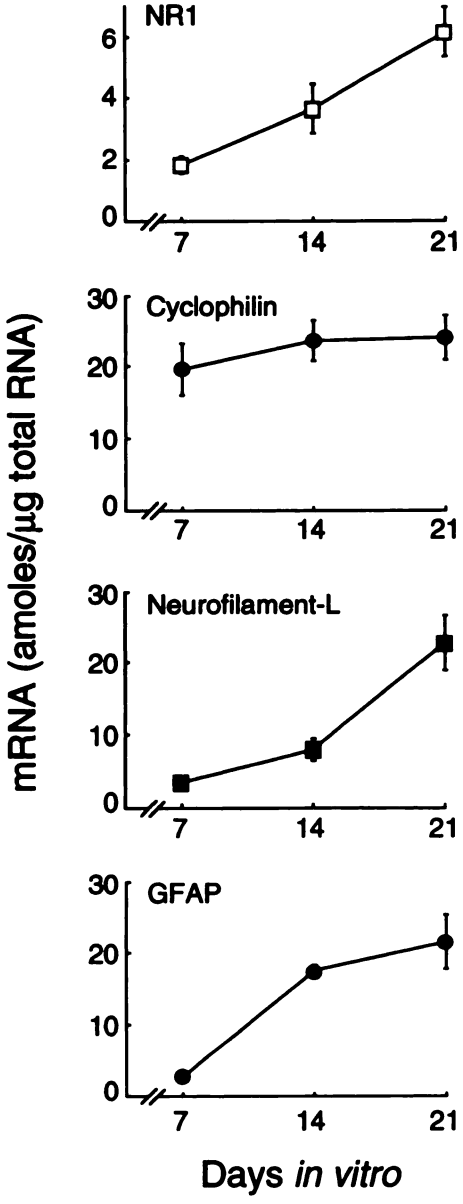

Fig. 4. Expression of mRNAs for NR1, cyclophilin, neurofilament protein L, and GFAP in neuronal/glial co-cultures maintained for 7–21 days *in vitro*. mRNA levels were measured by solution hybridization/RNase protection assays. The NR1, neurofilament, and GFAP probes were analyzed on the same lanes of an acrylamide gel for each sample. Values are means ± standard errors from three or four individual batches of cultured cells.

Variants of NR1 mRNA with and without the 5′ insert were detected in extracts of cultured neurons. The predominant forms of NR1 mRNA were those that do not contain the 5′ insert (Fig. 6). Species of NR1 without the 5′ insert represented about 95% of total NR1 mRNA, and their proportion in cultured neurons remained relatively unchanged up to day 21 *in vitro* (Fig. 6).

Expression of NMDA receptors and effects of serum deprivation. The inhibitory effects of ifenprodil on NMDA receptors change as a function of time in culture (Fig. 7A, *inset*, and see Ref. 8). Ifenprodil inhibited the binding of ^{125}I-MK-801 in a biphasic fashion, with high and low affinity components. In 7-day cultures the low affinity component accounted for 6% of the inhibition, whereas in 21-day cultures it represented 24% of the inhibition (Fig. 7A, *inset*). The time-dependent increase in the proportion of low affinity ifenprodil sites is similar to that seen in rat forebrain during postnatal development (8).

Experiments were carried out to determine whether maintenance of cultured neurons in different media influences the expression of NMDA receptor subtypes. Cells were maintained in media with varying amounts of serum during days 7–21 *in vitro*, the time period during which the proportion of receptors having a low affinity for ifenprodil increases. In cultures maintained in medium with a reduced concentration of FBS (1% or 0%), the proportion of low affinity ifenprodil sites was less than that seen in cells maintained in standard medium (Fig. 7; Table 1). IC_{50} values for the high and low affinity components of inhibition were not significantly altered after growth of cells under different conditions (Table 1). Variablility in the IC_{50} values determined is a result of the small number of low affinity sites, and thus the poor signal to noise ratio for these sites, after serum deprivation (Table 1).

The total number of NMDA receptors, assesed by binding of ^{125}I-MK-801, increased between days 7 and 21 when cells were grown either in the presence or in the absence of serum. The numbers of receptors having high and low affinities for ifenprodil were calculated from the total specific binding of ^{125}I-MK-801 and the proportion of sites with high and low affinity for ifenprodil (Fig. 7B). In neurons maintained in medium containing 1% or 0% FBS, there was no significant difference in the number of receptors having a high affinity for ifenprodil, compared with cells maintained in medium with 10% FBS (Fig. 7B). The number of receptors having a low affinity for ifenprodil did not increase in neurons maintained in medium with reduced serum (Fig. 7B).

Levels of subunit mRNAs were also determined after cells were grown in medium with different concentrations of serum. In cells maintained in medium containing 1% or 0% FBS between days 7 and 21 *in vitro*, levels of NR1 and NR2A mRNA did not increase (Fig. 8). Thus, maintenance of cells in medium with a reduced concentration of serum prevented the time-dependent increase in expression of NR1 and NR2A mRNAs that was seen in cells maintained in 10% FBS. The level of

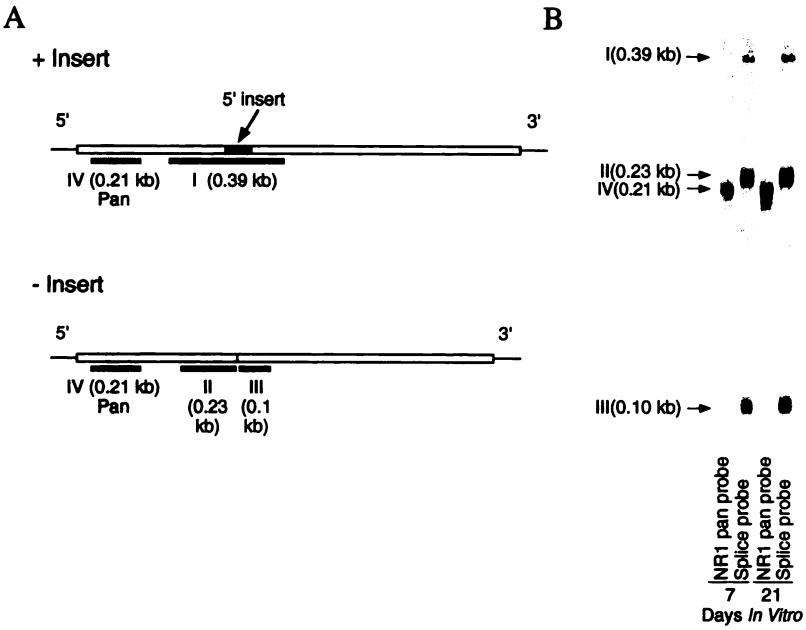

Fig. 5. Detection of NR1 splice variants with and without the 5′ insert. A, Schematic diagram of NR1 mRNA, showing the position of the 5′ insert and the location of RNA segments protected by the radiolabeled antisense probe. In variants that contain the insert, one segment of 388 bases is protected (*I*). In variants lacking the insert, two segments of 226 and 99 bases (*II* and *III*) are protected. The position of the pan probe (*IV*), which detects all NR1 variants, is also shown. B, Autoradiogram of a solution hybridization/RNase protection assay of NR1 mRNA using the pan probe and the probe for the 5′ insert. RNA was extracted from cells cultured for 7 or 21 days *in vitro*. Protected bands corresponding to NR1 variants with (*band I*) and without (*bands II* and *III*) the 5′ insert and to total NR1 mRNA (*band IV*) are shown.

expression of NR2B mRNA was not affected by growth in medium containing 1% or 0% FBS between days 7 and 21 (Fig. 8).

Discussion

Time-dependent changes in levels of mRNA encoding subunits of the NMDA receptor occur in cultured cortical neurons. Expression of NR1 mRNA increased progressively between days 1 and 21 *in vitro*. Expression of NR2A mRNA increased later than did expression of NR2B mRNA, and NR2B mRNA was expressed at higher levels than was NR2A mRNA. These changes are temporally similar to changes in the expression of NR1, NR2A, and NR2B subunits seen in mouse (16) and rat[1] cerebral cortex during development *in vivo*, where delayed postnatal expression of the NR2A (ϵ1) subunit has been observed. However, the level of NR2A mRNA in 21-day cultures (equivalent to postnatal day 17) was ≈4-fold lower than that of NR2B mRNA. In contrast, levels of NR2A and NR2B mRNA are similar at postnatal day 14 *in vivo*.[2] Thus, the mechanisms that control the differential temporal expression of NR2 subunits *in vivo* are partially operative in neurons maintained *in vitro*.

In cultured neurons the proportion of NMDA receptors having a low affinity for ifenprodil increases over time, as is seen during development *in vivo* (8). Because receptors having a low affinity for ifenprodil are expressed later than receptors having a high affinity for this antagonist, the delayed appearance of the NR2A subunit may underlie the delayed expression of receptors having a low affinity for ifenprodil. In adult rat forebrain, receptors having high and low affinities for ifenprodil are present in a ratio of 1:1 (8). In cortical neurons maintained for 21 days *in vitro*, where the level of NR2A mRNA is ≈4-fold lower than that of NR2B mRNA, receptors having a low affinity for ifenprodil represent only 25% of total NMDA receptors. Furthermore, recombinant NR1/NR2B receptors expressed in *Xenopus* oocytes have a high affinity for ifenprodil, whereas NR1/NR2A receptors have a low affinity for this antagonist (15). Taken together, these observations strongly suggest that the inclusion of NR2A subunits in a subpopulation of native NMDA receptors is responsible for the appearance of receptors with a low sensitivity to ifenprodil. It remains possible that other factors, for example changes in the levels of NR1 variants having different carboxyl-terminal splice patterns or in the levels of the NR2D subunit, which is widely expressed in neonatal brain (16), also contribute to the pharmacological heterogeneity seen with ifenprodil.

The expression of NMDA receptor subtypes and subunit mRNAs was selectively altered by maintaining neurons in media with different concentrations of serum. Maintaining neurons in medium with reduced serum prevented the time-dependent increase in the expression of NR1 and NR2A mRNAs, while having no effect on the expression of NR2B mRNA. Serum deprivation also prevented the increase in the number of receptors having a low affinity for ifenprodil. This

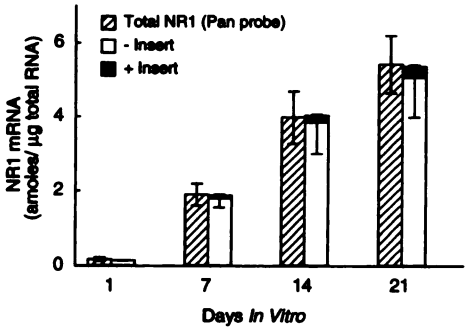

Fig. 6. Levels of NR1 mRNA with and without the 5′ insert. The levels of NR1 mRNA corresponding to variants with and without the 5′ insert were determined using the splice probe (Fig. 5, *bands I* and *II*) and total NR1 mRNA was determined using the pan probe (Fig. 5, *band IV*), in samples extracted from neurons cultured for 1–21 days *in vitro*. Values are means ± standard errors from four or five batches of cultured cells.

[1] J. Zhong, D. B. Pritchett, K. Williams, and P. B. Molinoff, unpublished observations.

[2] J. Zhong, D. B. Pritchett, K. Williams, and P. B. Molinoff, unpublished observations.

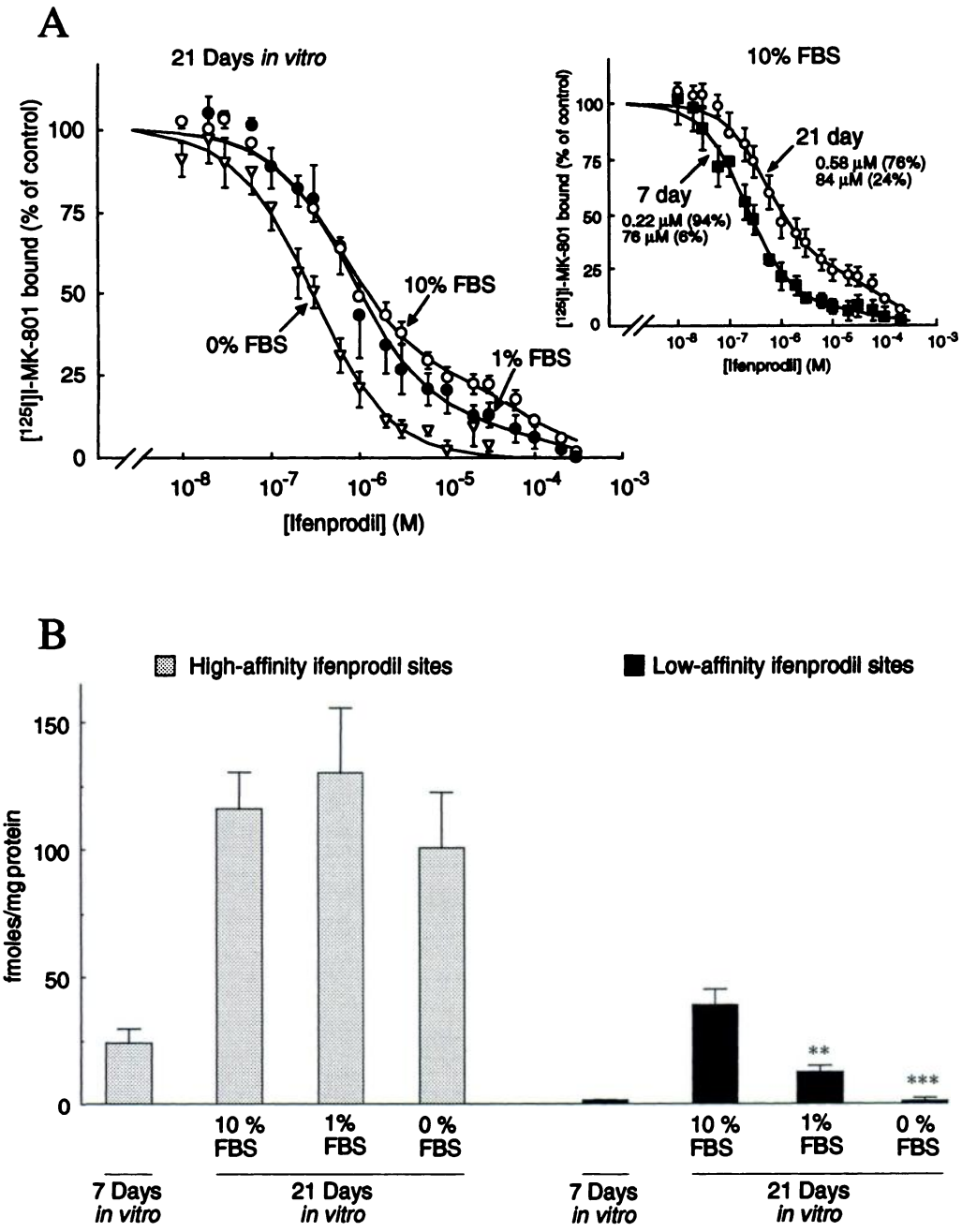

Fig. 7. NMDA receptors expressed on cultured neurons. The effects of ifenprodil on the binding of ^{125}I-MK-801 to NMDA receptors on membranes prepared from cultured neurons maintained for 7–21 days *in vitro* were determined. A, Inhibitory effects of ifenprodil on neurons maintained for days 1–7 in medium containing 10% FBS and then for days 7–21 in medium containing 0%, 1%, or 10% FBS. *Inset*, neurons were maintained for 7 or 21 days *in vitro* in medium with 10% FBS. B, The number of NMDA receptors (fmol/mg of protein) having high or low affinity for ifenprodil was calculated from the total specific binding of ^{125}I-MK-801 and the proportion of high and low affinity sites. **, $p < 0.01$; ***, $p < 0.001$, compared with the number of sites seen with 10% FBS (one-way analysis of variance with *post hoc* Tukey test). Data in A and B are means ± standard errors from four to seven separate batches of cultured cells.

TABLE 1

High and low affinity ifenprodil sites in cultured cortical neurons

Neurons were maintained in medium containing 10%, 1%, or 0% serum during days 7–21 *in vitro*. Values are means ± standard errors (four to seven experiments).

Serum in growth medium	High affinity ifenprodil sites		Low affinity ifenprodil sites	
	Proportion	IC_{50}	Proportion	IC_{50}
	%	µM	%	µM
10%	75 ± 2	0.8 ± 0.2	25 ± 2	99 ± 5
1%	92 ± 2[a]	1.1 ± 0.5	8 ± 2[a]	233 ± 33
0%[b]	99 ± 1[a]	0.3 ± 0.1	1 ± 1[a]	

[a] $p < 0.001$, compared with 10% FBS (one-way analysis of variance with *post hoc* Tukey test).

[b] In four of five batches of cells grown in 0% FBS inhibition by ifenprodil was monophasic, and in one batch there was a small (5%) low affinity component of inhibition.

is again consistent with the hypothesis that native NMDA receptors containing NR2A have a low affinity for ifenprodil. The number of receptors having a high affinity for ifenprodil and the levels of NR2B mRNA were not altered by serum deprivation. This suggests that the reduction of NR2A mRNA and of low affinity ifenprodil sites seen in reduced serum is not due to a general nonspecific effect on transcription or protein synthesis. Serum deprivation may alter the transcription of NR1 and NR2A genes or the stability of the resulting mRNAs. Alternatively, serum deprivation may alter cell-specific expression of particular NMDA receptor subunits or may selectively influence the survival of subpopulations of neurons expressing particular NMDA receptor subunits. If NMDA receptor subunit gene expression in neurons is "preprogrammed," then growth in serum-containing medium may be permissive or necessary for a predetermined sequence of transcriptional events.

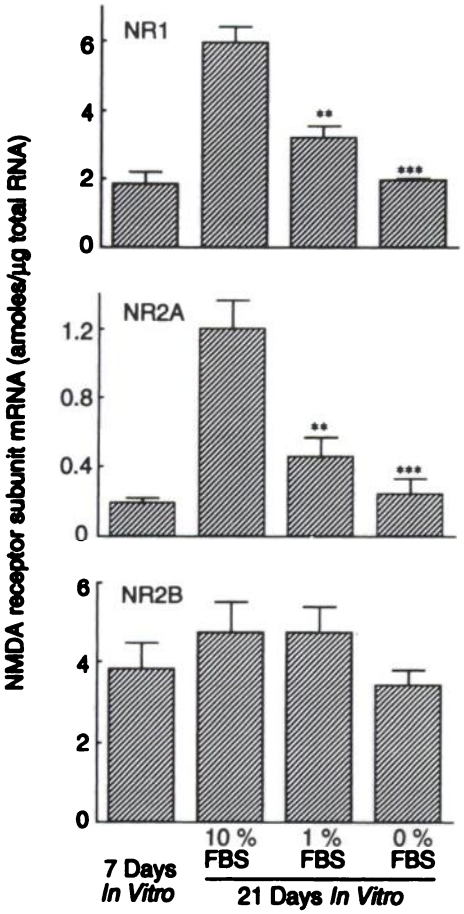

Fig. 8. Expression of subunit mRNA in cultured neurons. Cells were maintained for days 1–7 in medium containing 10% FBS and then for days 7–21 in medium without FBS or medium containing 1% or 10% FBS. Cells were harvested on day 7 or 21. **, $p < 0.01$; ***, $p < 0.001$, compared with 10% FBS (one-way analysis of variance with *post hoc* Tukey test). Values are means ± standard errors from four or five batches of cultured cells.

An increase in the density of receptors was observed whether cells were grown in the presence or absence of serum. The increase was less in serum-deprived cells than under control conditions. The increase observed in the absence of serum occurred despite the fact that there was no change in the expression of NR1, NR2A, or NR2B mRNAs. This may be due to more efficient translation of mRNAs, assembly of receptors, or incorporation of receptors into cell membranes in mature cultures. Alternatively, the discrepancy could reflect the presence of NR2D or an as yet unidentified subunit in some populations of NMDA receptors.

The NR1 gene is expressed as eight alternatively spliced mRNAs containing different exons. The inclusion of an exon in the 5′ region results in a 21-amino acid insert (12–14). The presence of this insert has been shown to alter the sensitivity of NMDA receptors to polyamines and to Zn^{2+} (14, 20, 21). In cultured neurons, NR1 variants without the 5′ insert represent the predominant forms of NR1, compared with those with the insert. No change in the relative expression of variants with and without the 5′ insert was seen as a function of time in culture. This suggests that the splicing pattern with regard to the exon encoding the 5′ insert is not altered over time in culture. Furthermore, variants without the 5′ insert represent the major species of NR1 mRNA in adult rat cerebral cortex (12, 13, 22),[3] suggesting that the splicing pattern of the NR1 gene in cultured neurons may be similar to that seen *in vivo*. The observation that in rat cerebral cortex the splicing pattern of NR1 remains constant while dramatic changes in the expression of NR2 subunits occur suggests that developmental changes in the expression of NR2 subunits are the determining factors that control properties of NMDA receptors *in vivo*.

Neurons from fetal rodent brain maintained in primary culture have been widely used as systems to study excitotoxicity mediated by NMDA receptors (3–5, 23). A number of paradigms have been developed in different laboratories for the growth and maintenance of cultured neurons. In some paradigms, neocortical or hippocampal neurons are maintained for up to several weeks in serum-free medium before being used for studies of excitotoxicity (e.g., Refs. 4 and 23). There is also variation in the length of time neurons are cultured before being used for toxicity studies. The results of the present work show that differences in the duration of culture and in the composition of culture medium can influence the expression of subunits and subtypes of the NMDA receptor. These results have implications for studies of excitotoxicity using cultured neurons, in particular for studies in which the effects of subtype- or subunit-selective antagonists of the NMDA receptor are investigated.

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Send reprint requests to: Keith Williams, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104–6084.