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Regulated Appearance of NMDA Receptor Subunits and Channel Functions during *In Vitro* Neuronal Differentiation

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ABSTRACT: The schedule of NMDA receptor subunit expression and the appearance of functional NMDA-gated ion channels were investigated during the retinoic acid (RA) induced neuronal differentiation of NE-4C, a p53-deficient mouse neuroectodermal progenitor cell line. NR2A, NR2B, and NR2D subunit transcripts were present in both nondifferentiated and neuronally differentiated cultures, while NR2C subunits were expressed only transiently, during the early period of neural differentiation. Several splice variants of NR1 were detected in noninduced progenitors and in RA-induced cells, except the N1 exon containing transcripts that appeared after the fourth day of induction, when neuronal processes were already formed. NR1 and NR2A subunit proteins were detected both in nondifferentiated progenitor cells and in neurons, while the mature form of NR2B subunit protein appeared only at the time of neuronal process elongation. Despite the early presence of NR1 and NR2A subunits, NMDA-evoked responses could be detected in NE-4C neurons only after the sixth day of induction, coinciding in time with the expression of the mature NR2B subunit. The formation of functional NMDA receptors also coincided with the appearance of synapsin I and synaptophysin. The lag period between the production of the subunits and the onset of channel function suggests that subunits capable of channel formation cannot form functional NMDA receptors until a certain stage of neuronal commitment. Thus, the *in vitro* neurogenesis by NE-4C cells provides a suitable tool to investigate some inherent regulatory processes involved in the initial maturation of NMDA receptor complexes. © 2002 Wiley Periodicals, Inc. J Neurobiol 51: 54–65, 2002

Keywords: cell line; progenitor; NR1 splice variants; NR2 subunits; neuronal development

INTRODUCTION

NMDA (*N*-methyl-D-aspartate) gated ion channels—a subtype of ionotropic glutamate receptors—play im-

portant roles in excitatory neurotransmission, neuronal survival, excitotoxicity, synapse formation as well as in the processes of learning and memory (Balazs et al., 1988; Scheetz and Constantine-Paton, 1994; Vallano, 1998). The NMDA receptor comprises subunits from two gene families, designated NR1 and NR2 (or GluRζ and GluRε, respectively) (Mori and Mishina, 1995). Some data has also been published on the involvement of a third subclass of subunits, NR3 (Ciabarra et al., 1995; Sucher et al., 1995). In NR1

gene products one N-terminal (N1 or exon 5) and two C-terminal exons (C1 or exon 21 and C2 or exon 22) can be transcribed independently, leading to the formation of eight splice variants (Sugihara et al., 1992; Hollmann et al., 1993). The NR2 gene family codes four distinct types of subunits called NR2A-D (or GluRϵ1−4) (Monyer et al., 1992; Nakanishi, 1992). Functional NMDA receptors are heteromeric complexes, containing at least one NR1 and one or more types of NR2 subunits (Mori and Mishina, 1995). The subunit composition influences the functional properties as well as the intracellular and cell surface localization of the receptor complexes (Ehlers et al., 1998; McIlhinney et al., 1998; Okabe et al., 1999; Yamakura and Shimoji, 1999; Perez-Otano et al., 2001).

In the course of neural tissue-genesis, the pattern of NR1 and NR2 subunit expression reflects both regional determination and temporal shifts in development (Watanabe et al., 1992; Laurie and Seeburg, 1994; Monyer et al., 1994). NR2B and NR2D mRNAs are expressed predominantly in the embryonic brain. NR2A mRNAs appear in the entire brain after birth, while NR2C mRNAs are expressed postnatally, primarily in the cerebellum. In postnatal rodent brain, NR2B transcripts are restricted principally to the forebrain, and the level of NR2D mRNAs is greatly decreased throughout the whole brain. Consequently, neurons can display functionally different NMDA receptor complexes during development and maturation (Williams et al., 1993; Feldmeyer and Cull-Candy, 1996; Vallano, 1998).

Although progenitor cells of the early embryonic nervous system are not available for molecular analyses, one-cell-derived neuroectodermal progenitor cell lines can provide appropriate *in vitro* models to understand the importance of the subunit composition of NMDA receptors and to investigate the time schedule of the subunit expression during some early phases of neuronal commitment and differentiation.

NE-4C cell line, derived from forebrain vesicles of 9-day-old p53-deficient mouse embryos, provides a well-established model to study *in vitro* neuron formation (Schlett and Madarasz, 1997; Schlett et al., 1997). Neuronal differentiation of NE-4C cells can be induced by treatment with *all-trans* retinoic acid. The first immature neurons appear on the third to fourth day of RA treatment, and dense networks of neuronal processes are formed by the end of the first week of induction. Morphological maturation, changes in the expression of intermediate filaments, the production of neuron-specific markers and the development of bioelectrical properties have been thoroughly investigated and led to the description of a reproducible time table of *in vitro* neurogenesis (Schlett and Madarasz, 1997; Schlett et al., 1997; Jelitai et al., 2000).

In this study, the expression of various NMDA receptor subunits and the appearance of functional NMDA gated ion channels were investigated and correlated with defined stages of RA-induced neuronal differentiation of NE-4C cells. Some parts of the work have been presented as conference abstracts (Jelitai et al., 2000; Schlett et al., 2000).

METHODS

Maintenance of the NE-4C Cell Line

NE-4C cells were grown in Minimum Essential Medium (MEM; Sigma) supplemented with 5% fetal calf serum (FCS, Gibco), 4 mM glutamine (Sigma-Aldrich Co, Hungary) and 40 μg/mL gentamycin (Sigma-Aldrich Co, Hungary), at 37°C with 5% CO_2. Subconfluent cultures were regularly transferred by trypsinization [0.05% trypsin (Sigma-Aldrich Co, Hungary) in phosphate buffer saline (PBS)] and replated at 10^4 cells/cm^2 cell density into poly-L-lysine (PLL) (Sigma-Aldrich Co, Hungary)-coated dishes.

Treatment with *all-trans* Retinoic Acid (RA)

10^{-2} M stock solution of *all-trans* retinoic acid (Sigma-Aldrich Co, Hungary) dissolved in DMSO was stored under liquid N_2. 10^{-4} M working solution of RA (dissolved in MEM) was stored in dark at −80°C, and was diluted directly to the final 10^{-6} M concentration into the medium of the cells. Cells were seeded at 2.5×10^4 cells/cm^2 density onto poly-L-lysine-coated surfaces and were induced to differentiate by a 48-h treatment with RA. Cultures were maintaned without RA from the third day of induction.

Semiquantitative RT-PCR Analysis

Total RNA from nondifferentiated and RA treated NE-4C cells was isolated using Trizol-LS (Gibco BRL), while total RNA from the cerebella and the forebrains of 30-day-old C57/Bl6 mice was isolated by PeqGold RNAPure (PEQLAB Biotech.; Erlangen, Germany) according to the manufacturer's recommendations. After digestion with RNase-free DNase I (Boehringer, Germany) at 37°C for 15 min, 3 μg RNA was reverse-transcribed using the First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) both with random hexamer and oligodT primers, according to the manufacturer's instructions. Negative controls consisted of the same reaction mixture except that reverse transcriptase was omitted. PCR reactions were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research), using HotStarTaq polymerase (Qiagen; Hilden, Germany; 1 U/50 μL PCR reaction mixture). The primer sets used for the PCR analyses are listed in Table 1. The

Table 1

Set	Subunit	Primer Sequences	Position (bp)	Expected Size (bp)
1	NR1 N1 exon	Forward 5'gacaagagcatccacctgagcttcc3'	481–505	360 bp (N1+)
		Reverse 5'agcgtcgtcctcgcttgcagaaagg3'	777–753	297bp (N1−)
2	NR1 C1 exon	Forward 5'tgtgtccctgtccatactcaag3'	2406–2427	307 bp
		Reverse 5'gtcgggctctgctctaccactc3'	2712–2693	
3	NR1	Forward 5'atgcccctgccaccctcacttttg3'	2501–2524	381bp (C1+, C2+)
	C1, C2 exons	Reverse 5'gcagctggccctcctccctctca3'	2881–2859	270bp (C1−, C2+)
4	NR2A	Forward 5'ggagaagggtactccagcgctgaac3'	81–105	280 bp
		Reverse 5'agtctgtgaggagataaaatccagc3'	360–335	
5	NR2B	Forward 5'gcaagcttctgtcatgctcaacatc3'	456–480	220 bp
		Reverse 5'gctctgcagcttcttcagctgattc3'	675–651	
6	NR2C	Forward 5'actccttggtgcttgggcagg3	811–831	421 bp
		Reverse 5'cagcacctgcagctgctgctc3'	1231–1211	
7	NR2D	Forward 5'cttggctcctccacagagcaacagc3'	481–505	298 bp
		Reverse 5'cctcttctgccgcccggaaaacagg3'	778–754	
8	GAPDH	Forward 5'tgatgacatcaagaaggtggtgaag3'	805–829	240 bp
		Reverse 5'tccttgggaggccatgtaggccat3'	1044–1022	

Primer sets used for the amplification of NMDA receptor subunit mRNAs. The annealing position for each of the NR1 primer sets (1–3) is relative to the sequence of Yamazaki et al., (1992). Set 1 detects NR1 mRNAs both with and without the N1 (exon5) splice casette (367 bp and 297 bp, respectively). Set 2 detects NR1 mRNAs with the C1 (exon 21) splice casette. Set 3 gives a positive PCR reaction only if the C2 (exon22) splice casette is expressed—the larger and smaller fragments refer to the presence or absence of the C1 (exon 21) splice casette, respectively. The EMBL/GenBank accession numbers of the sequences used to design the primers are as follows: NM_008169, NM_008170, NM_008171, NM_010350, NM_008172, and M32599 (primer set 1–3, 4, 5, 6, 7, and 8, respectively). Sets 2 and 3 are taken from Liesi et al. (1999).

reaction mixture contained 2.5 mM MgCl$_2$, 0.4 mM dNTPs (PEQLAB Biotech.; Erlangen, Germany) and 0.4 μM of each primer for primer sets 1 and 8, and 1.5 mM MgCl$_2$, 0.2 mM dNTPs and 0.2 μM of each primer for primer sets 2–7 (see Table 1). PCR temperature protocols were consisted of an initial activation of the HotStarTaq polymerase at 95°C for 15 min, followed either by 40 cycles with 94°C 1 min, 72°C 2 min (primer sets 1, 3, and 7), 40 cycles with 94°C 1 min, 62°C 45 s, 72°C 2 min (primer set 2), 40 cycles with 94°C 45 s, 67°C 1 min, 72°C 1 min (primer set 4 and 5), 40 cycles with 94°C 1 min, 63°C 1 min, 72°C 1 min (primer set 6) or 30 cycles with 94°C 1 min, 55°C 1 min, 72°C 1 min (primer set 8). A final elongation period of 10 min at 72°C was included in all PCR protocols. The reactions were set in a way to maintain linear transcript amplification during the PCR reactions. Forebrain cDNA samples were used as positive controls except in the case of NR2C subunit detection, where cerebellar cDNA was included. PCR products were analyzed on 2% agarose gel stained with ethidium-bromide.

Each experiment was repeated a minimum of three times with cDNAs from independent RT reactions using either random hexamer or oligo dT primers. To be sure that the observed mRNA expression patterns are typical during the differentiation of NE-4C, a second set of cDNA samples from independent NE-4C cultures was also reverse transcribed with oligo dT primers, and the PCR reactions were repeated. In all cases, the results were concordant. For better visualization, representative negative images are presented on Figure 1.

Protein Sample Preparation

NE-4C cells were harvested with Ca^{2+}, Mg^{2+}-free PBS containing 0.1% EDTA, and collected by centrifuging at 800 × g. Unless indicated, all steps were carried out on ice. Cell-containing pellets were stored at −80°C until use. For subcellular fractionation, pellets were lysed using a hand-operated pestle (Sigma, Germany) in 10 mM HEPES, 250 mM sucrose containing buffer (pH = 7.4) supplemented with 0.5 mM phenylmethyl-sulfonyl fluoride, 10 μg/mL leupeptine, 2 μg/mL aprotinin, and 10 μM bestatin. Nuclei were removed by centrifugation at 2000 × g for 5 min, and supernatants were centrifuged at 16,000 × g for 30 min. The resulting pellet was enriched in mitochondria and larger membrane particles (crude membrane fraction), while the supernatant contained microsomal fraction and soluble components of the cytoplasm (cytoplasmic fraction). Pellets were homogenized in 150 mM NaCl, 10 mM HEPES containing buffer (pH = 7.4) supplemented with protease inhibitors (as above). Protein extracts from forebrains and cerebella of 2-month-old C57/Bl6 mice were prepared by homogenization in 8–10 volumes of ice-cold buffer containing 0.32 M sucrose, 1 mM NaHCO$_3$, 1 mM MgCl$_2$, 0.5 mM CaCl$_2$, and protease inhibitors (as above), using a Potter homogenizer with 12 strokes at 800 rpm. Brain homogenates were centrifuged at 1400 × g for 10 min, and supernatants were used for Western blot analyses. Protein content of the samples was determined according to Bradford (Bradford, 1976).
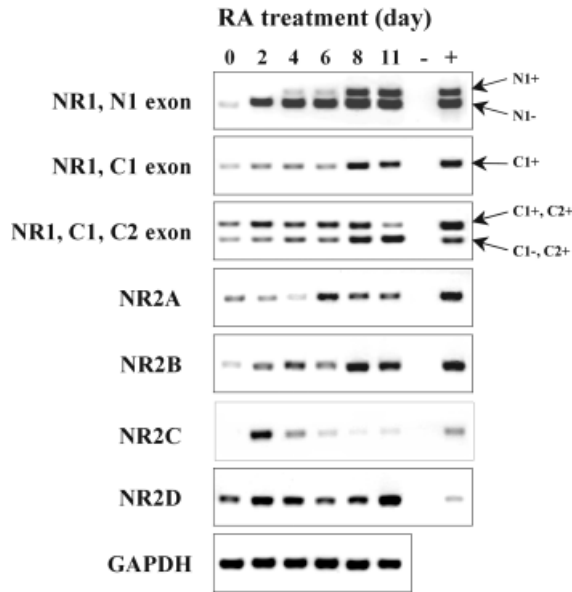
RA treatment (day)

0 2 4 6 8 11 - +

NR1, N1 exon

NR1, C1 exon

NR1, C1, C2 exon

NR2A

NR2B

NR2C

NR2D

GAPDH

Figure 1 Expression of various NMDA receptor subunit mRNAs in NE-4C cells at different stages of RA-induced differentiation. Samples were taken from non-differentiated (0 day) or from RA-treated cultures on the second, fourth, sixth, eighth, or 11th days, respectively. The primer sets used for amplification are listed in Table 1. The signs adjoining the arrows indicate the existence ($+$) or absence ($-$) of the different exons (N1, C1, or C2, respectively). GAPDH-transcripts (glycerol-aldehyde-phosphate dehydrogenase) were amplified to show the comparable amounts of cDNA in the samples. cDNA isolated from 30-day-old mouse forebrain was used as positive control ($+$) except in the case of NR2C subunit detection, when cerebellar cDNA was used. Negative control ($-$) included a mixture of NE-4C samples prepared without reverse transcriptase.

Western Blot Analysis

Equal amounts of protein samples (10 μg protein in the case of NR1 and 60 μg protein in the case of NR2 subunits) dissolved in Laemmli buffer (Laemmli, 1970) were loaded onto 7.5% polyacrylamide gels, separated by SDS-PAGE and transferred to nitrocellulose membrane (Immobilon NC-pure, Millipore, Hungary; or Hybond ECL extra, Amersham, Germany). Nonspecific antibody binding was blocked by incubating with 0.05% Tween-20 and 3% nonfat milk powder in TBS (Tris-buffered saline) for 1 h. Membranes were incubated overnight at $+4°C$ with the primary antibodies listed in Table 2. After three washes with TBS containing 0.05% Tween-20 (TBST), blots were incubated with anti-rabbit, anti-mouse, or anti-goat IgGs conjugated with either alkaline phosphatase (for colorimetric detection; Jackson ImmunoResearch Laboratories, USA) or horse radish peroxidase (for chemiluminescence detection; Pierce, Rockford, USA) in a dilution of 1:10,000 for 1 h. After washing three times with TBST, antibody binding was visualized either by 5-bromo-4-chloro-3-indolyl phosphate

(Sigma; 0.165 mg/mL) and nitro blue tetrazolium (Sigma; 0.33 mg/mL) dissolved in a buffer containing 0.1 M Trizma-Base, 5 mM MgCl$_2$, and 0.1 M NaCl, pH = 9.5, or using ECL chemiluminescence detection system (Amersham, Germany).

Immunocytochemical Staining

Cells grown on PLL-coated coverslips in 24-well plates were fixed with 4% paraformaldehyde (Taab, UK; w/v in PBS) for 20 min, at room temperature (RT). After rinsing, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. For staining with antibodies against NR1, NR2A, or NR2B generously provided by Prof. Watanabe (Hokkaido University, Japan), 2–3 min of 1 mg/mL pepsin pretreatment (dissolved in 0.2 N HCl) at 37°C was undertaken. Nonspecific antibody binding was blocked by incubating with 2% BSA (bovine serum albumin) in PBS for 1 h at RT. Primary antibodies (see Table 2) were applied overnight at $+4°C$. Fluorescent detection was carried out by incubation either with biotinilated anti-mouse or anti-rabbit IgGs (1:500, 1 h; Vector Laboratories) followed by Fluorescein-conjugated Avidin D (1:1000, 1 h; Vector Laboratories) or with FITC labeled anti-mouse or anti-rabbit antibodies (1:500, 1 h; Sigma). Preparations were mounted with Mowiol 4.88 (Polysciences, Germany) and were investigated under a Leitz Laborlux K microscope equipped with fluorescent optics. For viewing under a light microscope, biotin-labeled secondary antibodies were applied for 1 h followed by the addition of ABC complex (Vector Laboratories) and were visualized by the 3-3′-diamino-benzidine (DAB, 0.55 mg/mL; Sigma) reaction in the presence of 0.3% H$_2$O$_2$.

Measurement of Intracellular Free Calcium ($[Ca^{2+}]_I$) Level in Single Cells

NE-4C cells grown on PLL-coated glass coverslips in 35-mm dishes were washed with Mg^{2+}-free physiological solution (NaCl 140 mM, KCl 4.7 mM, CaCl$_2$ 2.6 mM, HEPES 10 mM, D-glucose 10 mM, glycine 10 μM, pH = 7.2) and loaded with 5 μM Fura-2/AM (Molecular Probes, Eugene, OR) for 45 min in a CO$_2$-incubator at 37°C in the above buffer solution. At the end of the incubation, cultures were washed and incubated for a further 30 min to ensure full hydrolysis of the Fura-ester. $[Ca^{2+}]_I$ was determined at 37°C by a PTI (South Brunswick, NJ) microspectrofluorimeter fitted to an inverted Nikon TDM Diaphot epifluorescent microscope equipped with Fluor objectives (Nikon, Japan) and a thermo-regulated sample chamber. Fluorescence of single cells emitted in response to excitation at 340 and 380 nm was measured at 510 nm by a photomultiplier (D104 Microscope Photometer, PTI, South Brunswick, NJ). Drugs (100 μM NMDA, 10 μM nifedipine, 500 μM DL-AP5, 1 μM ω-conotoxin, all from Tocris, UK) were added for 20 s via a pressure ejection system. Data were evaluated by a softwer (Felix) provided by PTI.

Table 2   List of Primary Antibodies Used Either for Western Blotting or Immunocytochemistry

Antibody	Specificity (Amino Acids)	Species	Producer	Western Blotting	Immunocytochemistry
NR1	4–51	rabbit	Watanabe, 1998 (GluRζ1N)	0,5 μg/mL	0,5 μg/mL
NR1 C2 exon	891–920	rabbit	Chemicon (AB1516)	1:200	—
NR1	660–811	mouse, clone 54.1	PharMingen (60021A)	—	1:1000
NR2A	last 200	rabbit	Upstate Biotech. (06-313)	1:3000	1:1000
NR2A	1126–1408	rabbit	Watanabe, 1998 (GluRε1C)	—	0,5 μg/mL
NR2B	1325–1461	mouse	Laurie, 1997 (BM 1B3.3B6)	1:10000	—
NR2B	1–48	rabbit	Watanabe, 1998 (GluRε2N)	—	0,25 μg/mL
NR2C	C terminal	rabbit	Chemicon (AB1592P)	1:1000	—
NR2D	C terminal	goat	Santa Cruz (sc-1471)	1:1000	—
Synaptophysin		mouse, clone SVP-38	Sigma (S5768)	1:5000	1:2000
Synapsin I	C terminal	mouse	Chemicon (MAB355)	—	1:2000

RESULTS

The Expression of NR1 and NR2 NMDA Receptor Subunit mRNAs during *In Vitro* Induced Neuronal Differentiation

Neuronal differentiation of NE-4C embryonic neuroectodermal progenitor cells was induced by *all-trans* retinoic acid (RA). Semiquantitative assays were used to monitor the changes in the level of NMDA receptor subunit mRNAs expressed in different stages of retinoic acid-induced neuronal differentiation. Total RNA samples obtained from NE-4C cells at different stages of neuronal differentiation were analyzed by RT-PCR (Fig. 1).

NR1 transcripts were detected with three different sets of primers, each recognizing distinct splice variants (Table 1). Assays with primers detecting all variants of NR1 mRNAs (set 1 in Table 1) showed that the total amount of NR1 transcripts was highly elevated by retinoic acid treatment. Nondifferentiated NE-4C cells expressed mRNA containing C1 (exon 21) and/or C2 (exon 22) but variants containing the N-terminal N1 exon (exon 5) were not detected. The proportion of the different splice variants changed with the advancement of neuronal differentiation: the amount of mRNAs containing C2 but lacking C1 exon

increased in comparison to those containing both C1 and C2 exons. The N1 exon cassette appeared only 4 days after the addition of RA, and the quantity detected was highly elevated after the first week of induction. NR2A, NR2B, and NR2D transcripts were present in both nondifferentiated and differentiated cells. The level of NR2D transcripts was consistently high during the whole induction period, while the level of NR2A mRNAs increased after the sixth day of induction. NR2B mRNA level was very low in nondifferentiated cells but elevated steadily with the advancement of differentiation. NR2C subunit mRNAs were detectable only transiently: a 2-day treatment with retinoic acid-induced NR2C expression strongly but the quantity of detectable transcripts became negligible by the eighth day of induction.

NR1 Subunit Protein Was Detected Both in Nondifferentiated and Neuronally Differentiated NE-4C Cells

To detect the presence of NR1 subunit proteins, cytoplasmic and crude-membrane fractions of NE-4C cells were prepared at different stages of neural differentiation and were analyzed on Western blots using two different antibodies [Fig. 2(A)]. The apparent
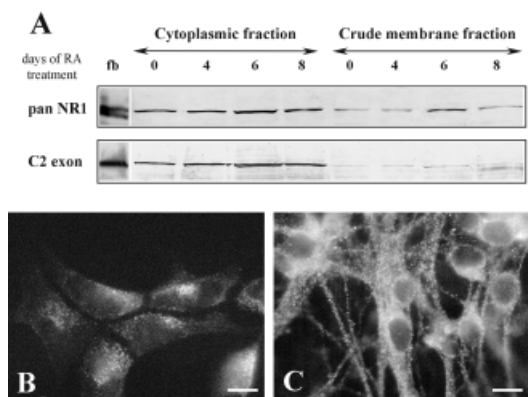
Figure 2 Expression of NR1 subunit proteins in NE-4C cells at different stages of RA-induced differentiation. (A) Western blot detection of NR1 subunit protein in subcellular fractions of NE-4C cells obtained from noninduced (0 day) or RA-treated cultures on the second, fourth, sixth, or eighth day of cultivation. Protein (10 μg) was loaded from both NE-4C and control forebrain samples (fb). The bands were visualized by alkalic phosphatase reaction. The upper panel shows the results obtained by the antibody recognising all forms of NR1 (pan NR1; Watanabe et al., 1998). A single protein band at about 116 kDa was detected both in the cytoplasmic/microsomal and in the crude-membrane fractions of NE-4C cells at all developmental stages. In adult forebrain homogenates used as controls (fb), double bands were visible. The lower panel presents data obtained by the antibody recognizing the C2 cassette of the NR1 rat protein (C2 exon; Chemicon Ltd). A single protein band at about 116 kDa was demonstrated primarily in the cytoplasmic/microsomal fractions. (B,C) Immunocytochemical detection of NR1 subunit in nondifferentiated (B) and in differentiated cells on the seventh day of induction (C). Cells were stained with either the N-terminal specific antibody (B; Watanabe et al., 1998) or with Clone 54.1 monoclonal antibody (C; Pharmingen Ltd) and were visualized by immunofluorescent microscopy. The bars indicate 10 μm.

molecular weight of the NR1 protein produced by NE-4C cells corresponded to that found in adult forebrain samples that were used as positive controls. One of the antibodies produced against the N-terminal 4–51 amino acids of mouse NR1 subunit protein (Watanabe et al., 1998) recognized all NR1 splice variants. The antibody stained double bands in the forebrain homogenates that may correspond to the relatively high level of different NR1 splice variants in adult forebrain [Fig. 2(A)]. The other antibody reacting with the C-terminal 891–920 amino acids (Chemicon Int., Ltd) was specific to the C2 exon cassette containing protein form (Table 2), and gave a specific band at an apparent molecular weight of 116 kDa.

NR1 subunits were readily detected by both anti-

bodies in 10 μg protein samples. In the cytoplasmic fraction, the amount of NR1 protein did not change in the course of retinoic acid induced differentiation [Fig. 2(A)]. In the crude membrane fraction, on the other hand, less NR1 protein was detected and the two antibodies revealed different distribution patterns. While NR1 subunit protein was found in samples prepared from both noninduced and differentiated NE-4C cells, the C2 cassette containing NR1 protein was hardly detectable in the crude membrane fraction of noninduced cultures, and its amount was limited even on the eighth day of induction.

To visualize the distribution of NR1 subunit protein, NE-4C cells were immunostained either with the N-terminal specific antibody (Watanabe et al., 1998) or with a monoclonal antibody directed against amino acids 660–811 between the III–IV transmembrane domain (PharMingen Ltd; Table 2). Both antibodies recognized all variants of NR1 subunits and gave similar, dot-like immunostaining [Fig. 2(B)–(C)]. In noninduced progenitor cells [Fig. 2(B)], immunoreactivity was localized in the perinuclear region, in a rather nonhomogeneous pattern. In retinoic acid-treated cultures, neuron-like cells displayed strong, evenly distributed immunoreactivity both in the perykarion and along the neurites [Fig. 2(C)].

NR2A Subunit Protein Was Present Both in Noncommitted Progenitors and in RA-Induced Neurons

To detect NR2A subunit protein by Western blotting, a C-terminal specific antibody raised against the last 200 amino acids of the C-terminus of mouse NR2A (Upstate Biotech.) was used (Table 2). In mouse forebrain samples, a single band with an apparent molecular weight of 170 kDa was strongly stained [Fig. 3(A)]. The same specific band was detected in all NE-4C samples but at a very low level. Specific signals were detected only if 60 μg or more protein was loaded indicating that the relative amount of NR2A protein in NE-4C cells was low.

In noninduced cells and in cells on the second day of RA induction, the majority of the detectable NR2A protein was found in the crude membrane fraction. After the fourth day, the amount of NR2A protein was markedly elevated in the cytoplasmic fraction. Similar signal intensity in the crude membrane fraction was found only on the ninth day of induction. An NR2A-immunreactive protein band with lower molecular weight was present in all samples throughout the induction period. The presence of the immunoreactive band at about 160 kDa suggested that some immature
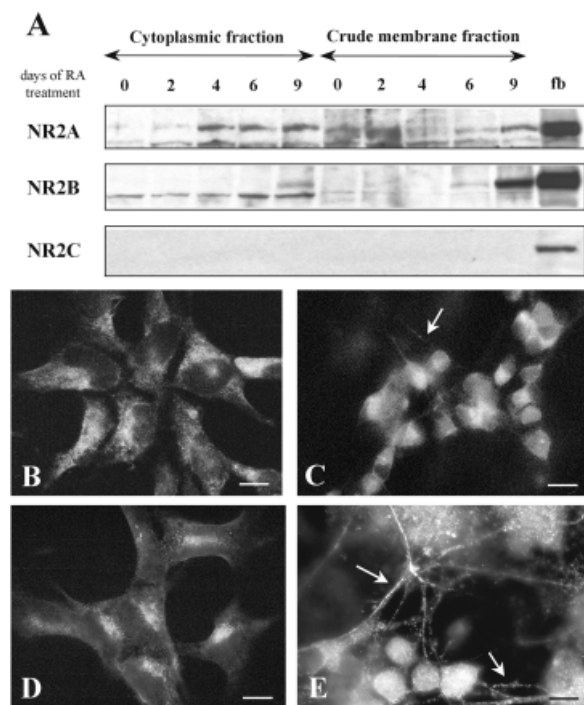
Figure 3 NR2A, NR2B, and NR2C subunits in NE-4C cells at different stages of neuronal differentiation. (A) Western blot detection of NR2A (upper panel), NR2B (middle panel), and NR2C (lower panel) subunits in subcellular fractions of NE-4C cells obtained from non-induced (0) or RA-treated cultures on the second, fourth, sixth, or ninth days of cultivation. Lysates of mouse brain: forebrain (fb) (in the case of NR2A and NR2B detection) or cerebellum (in the case of NR2C detection) were used as controls. Protein (60 μg) was loaded in each lane and immunoreaction was visualized by ECL method. The specific bands in the forebrain samples were detected at 170 kDa (NR2A) or at 180 kDa (NR2B). In the cerebellar samples, NR2C protein was detected approximately at 140 kDa. (B–E) Immunocytochemical staining of NR2A (B,C) or NR2B (D,E) subunit in nondifferentiated (B,D) and in differentiated cells on the seventh (C) or ninth (E) day of induction. Anti-NR2A (B) and anti-NR2B (D–E) antibodies were gifts from Prof. Watanabe (Watanabe et al., 1998) or were from Upstate Biotech (C). Staining was visualized by immunofluorescent microscopy. Arrows point to the neurites stained by the antibodies (C,E). The bars indicate 10 μm.

or partly degraded forms of NR2A protein were also present.

Immunocytochemical staining was carried out either by the antibody used for Western blotting, or by an antibody directed against the 1126–1408 amino acids of NR2A (Watanabe et al., 1998) (Table 2). The two antibodies gave similar staining patterns. Fine grain-like staining was localized evenly over the cytoplasm of noninduced progenitor cells [Fig. 3(B)], while a more diffuse staining was detected in RA-induced NE-4C neurons, mainly in the perikarya [Fig. 3(C)].

NR2B Subunit Protein Showed a Distinct Expression Pattern

NR2B protein was detected by the antibody directed against the 1325–1461 amino acid residues (Laurie et al., 1997) in Western blot analyses (Table 2). Immunoreactive protein bands with an apparent molecular weight of about 180 kDa were detected in the control forebrain samples and also in samples of differentiated NE-4C cells [Fig. 3(A)]. Similarly to NR2A detection, at least 60 μg protein of NE-4C samples was required to get reliable signals.

NR2B immunoreactive bands with a molecular weight (180 kDa) identical to that seen in the forebrain sample were mainly localized in the crude membrane fraction. These specific bands were not detected earlier than the sixth day of RA induction. On the ninth day, the majority of NR2B protein was found in the crude membrane fraction and the relative amount was similar to that found in the forebrain sample.

In the cytoplasmic fractions obtained from noninduced or from differentiated cells, an immunoreactive band with a lower (about 160 kDa) apparent molecular weight was found. The amount of the 160-kDa immunopositive protein increased during the whole period of differentiation. These bands, however, were not observed in the crude membrane fraction.

Using an antibody directed against the N-terminal 1–48 amino acids of NR2B (Watanabe et al., 1998) (Table 2), immunreactivity was demonstrated in both nondifferentiated and differentiated NE-4C cells [Fig. 3(D)–(E)]. The antibody gave nonhomogeneous, dot-like staining in noninduced progenitors [Fig. 3(D)]. After the appearance of neurons with long processes—from the second week of induction—strong immunoreactivity was observed in the perikarya and also in the neurites [Fig. 3(E)].

Although NE-4C cells expressed NR2C mRNA only transiently, the presence of NR2C protein was checked by Western blotting. In cerebellar homogenates used as positive control, a single band with 140-kDa apparent molecular weight gave a strong signal. In NE-4C samples taken from any period of neuronal differentiation, the subunit could not be detected even if 60 μg protein was loaded [Fig. 3(A)].

The commercially available antibody (Santa Cruz Biotech.) against NR2D failed to detect any specific protein bands in any samples prepared from NE-4C cells or 3-day-old forebrain tissue.
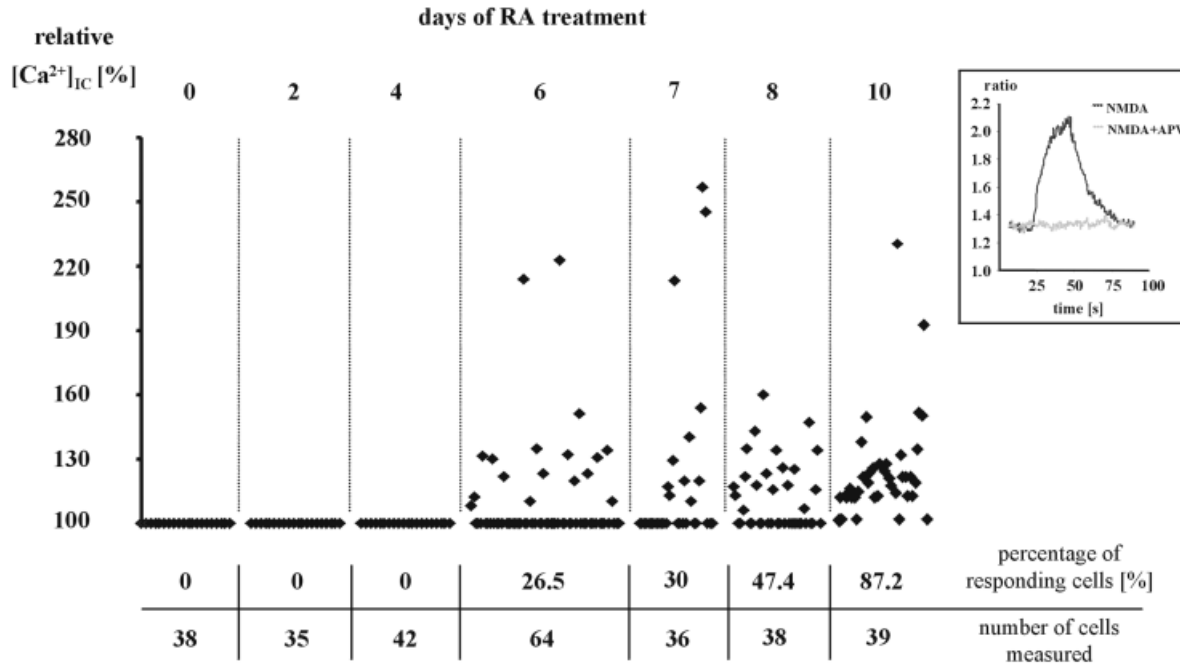
Figure 4 The relative $[Ca^{2+}]_I$ rises in noninduced and RA-treated cells, in response to the addition of 100 μM NMDA. Each dot represents the maximum of the Ca-signal recorded from a separate cell and is presented as the percentage of the resting $[Ca^{2+}]_I$ in the same cell (100%), detected just before the addition of NMDA. Insert: a representative NMDA evoked response from a single NE-4C cell on the sixth day of induction. DL-AP5 (500 μM) completely abolished (gray line) the NMDA evoked $[Ca^{2+}]_I$ rise (black line).

NMDA-Gated Channel Activity Was Delayed in Comparison to the Detection of the Subunit Proteins

The appearance of functional NMDA channels during neuronal differentiation was investigated by microspectrofluorimetry. The changes in the intracellular Ca^{2+} level ($[Ca^{2+}]_I$) were measured in single cells. In noninduced, homogeneous cultures, cells were chosen randomly for the analysis. After the appearance of process-bearing cells in retinoic acid treated cultures, Ca^{2+} responses were measured only in cells showing neuron-like morphology.

In noninduced cells or during the first 4 days of induction, we failed to detect any changes in the $[Ca^{2+}]_I$ in response to the addition of 100 μM NMDA (Fig. 4), even under Mg^{2+}-free and depolarizing conditions (30 mM KCl; data not shown).

The first NMDA-evoked responses appeared on the sixth day of induction (Fig. 4), when a large number of neurons was already present in the cultures and dense networks of neuronal started to form. NMDA receptors in NE-4C cells displayed Mg^{2+} sensitivity (data not shown). DL-AP5 (DL-2-Amino-5-phosphonovaleric acid), a competitive antagonist of

NMDA, could completely block NMDA evoked Ca^{2+} responses (see insert in Fig. 4). With the advancement of neuronal differentiation, the number of NMDA responsive cells kept growing (Fig. 4). By the 10th day, almost all recorded cells gave responses to NMDA, albeit the amplitude of Ca^{2+} answers varied considerably.

Addition of specific blockers to voltage-dependent calcium channels (VDCCs), nifedipine or ω-cono-toxin MVIIC, resulted in a partial reduction of NMDA-induced Ca^{2+} responses (data not shown) indicating that NMDA-elicited changes stimulated further Ca influx through VDCCs. To demonstrate the neuronal maturation of NE-4C cells, the presence of synaptophysin and synapsin I proteins was investigated. Synaptophysin was not detectable during the first 4 days of RA treatment by Western blotting [Fig. 5(A)]. Specific signals at an apparent molecular weight of 38 kDa were detected in the crude membrane fractions from the sixth day on, coinciding in time with the formation of network of neuronal processes.

Synaptophysin was first detected around the seventh day of induction by immunocytochemical staining. From this time on, diffuse immunoreactivity was
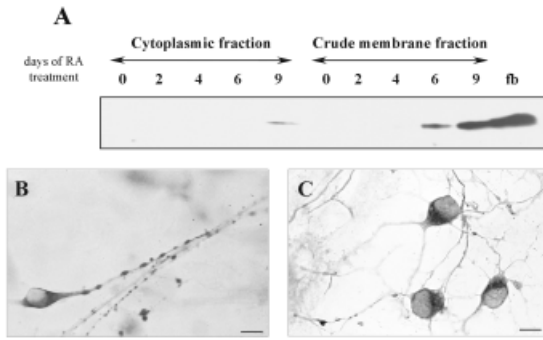
Figure 5 Expression of synaptic marker proteins in NE-4C cells at different stages of neuronal differentiation. (A) Western blot analysis of synaptophysin protein in the cytoplasmic/microsomal and in the crude membrane fractions obtained from nondifferentiated (0) or RA-treated NE-4C cells on the second, fourth, sixth, or ninth day of treatment. Forebrain sample was used as positive control. Protein (70 μg) was loaded from each sample, and detection was carried out by ECL technique. Strong, specific signal at the apparent molecular weight of 38 kDa was detected in samples obtained from the forebrain and from the crude membrane fractions of NE-4C cells from the sixth day of induction on. A weaker band appeared also in the cytoplasmic samples of NE-4C cells but not sooner than the ninth day of RA treatment. (B,C) Immunocytochemical detection of synaptophysin (B) and synapsin I (C) protein in NE-4C neurons on the 10th (C) or 14th (B) day of induction. The bars represent 10 μm.

found in the perikarya as well as in the neuronal processes. Varicose processes were stained from the end of the second week [Fig. 5(B)]. Synapsin I, another characteristic presynaptic marker, was present in a similar pattern in the perikarya and in the processes by the second week of induction [Fig. 5(C)].

DISCUSSION

In the present study, the expression of NMDA receptor subunits and the formation of functional NMDA-gated ion channels were investigated during retinoic acid induced *in vitro* differentiation of NE-4C neuroectodermal progenitor cells. NR1 as well as different NR2 subunits were demonstrated both at mRNA and protein level. Despite the early presence of the subunits, functional NMDA-gated channels appeared only later, in parallel with the formation of dense networks of neuronal processes. Among the NR2 subunits, the appearance of NR2B protein showed a remarkable coincidence in time with the onset of receptor activity. The timing suggested that NMDA receptor activity required the presence of NR2B sub-

unit and took place only after a defined stage of neuronal maturation.

In the course of *in vitro*-induced neuronal differentiation, heterogeneous types of cells developed from the originally homogeneous NE-4C progenitors. Also, a number of nondifferentiated progenitors persisted during the entire period of induction (Schlett et al., 1997; Schlett and Madarasz, 1997). The subunit expression by noncommitted NE-4C cells therefore contributed to the subunit pattern displayed by the cultures in any stage of neuronal induction. Above this background, the amount of N1 and C1 exon containing NR1 mRNAs as well as the level of 180-kDa NR2B protein were specifically elevated in the course of neuronal differentiation. These changes coincided with the formation of NMDA-gated ion channels and the neuronal maturation of NE-4C cells.

The N1 exon-containing NR1 splice variant was detected when the first N-tubulin-positive cells appeared and became abundant by the time when dense networks of neuronal processes were formed (Schlett et al., 1997; Schlett and Madarasz, 1997). Similar maturation-dependent shifts from the N1 exon lacking towards the N1 exon containing transcripts were also described *in vivo* and *in vitro* (Asahi et al., 1998; Prybylowski et al., 2000). The mass appearance of NMDA responsive NE-4C neurons also coincided in time with a prominent increase in the relative amount of C1 exon containing mRNAs, while the total amount of C2-containing splice variants did not show a similar trend. These findings are in accordance with other observations (Ehlers et al., 1998; Okabe et al., 1999; Scott et al., 2001) and argue for the potential regulatory role of C1 exon in the assembly of functional NMDA-gated channels.

NMDA receptor function was not observed before the appearance of the 180-kDa NR2B protein. NR1 and NR2A subunits, on the other hand, were present throughout the whole investigated period in the crude membrane fraction, suggesting that at least a portion of these subunits was located in the surface membrane. The contradiction between the presence of potential channel forming subunits and the lack of functional receptors argues for control mechanisms hindering the operation of NMDA receptors before a certain stage of neuronal differentiation.

Interestingly, we failed to detect a sharp increase in the amount of the NR1 protein despite the clear-cut increase in the amount of NR1 mRNAs in the course of RA-induced development. Moreover, we failed to detect NR2C protein in NE-4C cultures, even if NR2C transcripts were observed during the initial stages of neuronal differentiation. Our findings are in accordance with data demonstrating that the level of

receptor subunit mRNAs does not strictly correlate with the level of the corresponding subunit proteins (Sucher et al., 1993; Resink et al., 1995).

Our data argue for the presence of some mechanisms, which seem to regulate the maturation and cell surface expression of the subunits on a differentiation-dependent way. C2 exon containing NR1 subunits were localized mainly in the cytoplasmic fraction. This observation is in accordance with the hypothesis that NR1 subunits containing C2 cassette are not targeted to the cell surface (Chazot and Stephenson, 1997). Although the apparent molecular weight of NR1 protein in NE-4C samples corresponded to that found in the adult forebrain, some NR2A and NR2B immunoreactive bands with a lower (approximately 160 kDa) molecular weight were also detected. The fact that the intensity of the lower molecular weight bands increased in the cytoplasmic fraction while it decreased in the crude membrane fraction argues against the possibility of nonspecific antibody binding. Although the partial degradation of the subunits cannot be ruled out, the lower immunoreactive bands can also represent immature forms of the subunit proteins. Unfortunately, we do not have any direct evidence concerning the glycosylation or phosphorylation of these subunits, which are known to influence the molecular weight of the NR2 subunits (Kennedy, 1997; Laurie et al., 1997). NMDA receptors are believed to assemble in the endoplasmic reticulum, where NR2 subunits seem to be the limiting factors in the coassembly and cell surface targeting of the receptor complexes (McIlhinney et al., 1996, 1998). Other observations also support that interactions between various parts of the NR1 and NR2 subunits are required for the efficient assembly and trafficking of the receptor complex (Forrest at al., 1994; Mori et al., 1998; Sprengel et al., 1998; Steigerwald et al., 2000; Scott et al., 2001). As in NE-4C cells the majority of the NR1 protein was found in the cytoplasmic fraction during all investigated periods, it can be hypothesized that the assembly and/or the successful transport of NR1 protein to the cell surface was hindered either by the presence of the lower molecular weight forms of NR2 subunits or by the lack of the mature form of NR2B protein. Further investigations are needed to determine why no functional NMDA receptors were detected in NE-4C cells, even if mature forms of NR2A and NR1 were present. One explanation can be that the mature form of NR2B subunit is the limiting factor for receptor functioning in NE-4C cells.

NMDA responsiveness was not observed before the detection of synapsin I and synaptophysin. The onset of receptor functioning required a certain stage of neuronal maturation even if some NR1 and NR2 subunits had already been present. The observed diffuse immunostaining of the presynaptic proteins, however, indicates that the clustering of these proteins, and consequently, the formation of presynaptic machineries, did not start at a stage when NMDA responsiveness had been a general feature of NE-4C neurons. Our observations prove that NMDA receptors were functional in NE-4C neurons well before the establishment of synaptic connections.

The schedule of NMDA receptor subunit expression by NE-4C cells did not strictly correlate with *in vivo* patterns described for various regions of the developing rodent brain (Watanabe et al., 1992; Laurie and Seeburg, 1994; Monyer et al., 1994). NE-4C progenitors, derived from forebrain vesicles of 9-day-old embryos, represent an early neuroectodermal phenotype. Therefore, the *in vitro* neurogenesis by NE-4C cells provides a model system to study mainly the early steps of pan-neuronal commitment by neuroectodermal progenitors but not the more region- and function-specific neuronal differentiation. Studies carried out on another $p53^{-/-}$ embryonic neuroectodermal progenitor line, NE-7C2 (Varju et al., 2001), demonstrated that while the expression pattern of the NMDA receptor subunits differed, functional NMDA channels appeared at a similar stage of *in vitro* neuron formation. Studies on neuronally differentiated embryonic stem cells (Strübing et al., 1995; Finley et al., 1996; Okabe et al., 1996) or P19 EC cells (Morley et al., 1995; Finley et al., 1996) also indicated that NMDA-gated ion channels appeared on differentiating neurons, but only after a defined stage of commitment. Consequently, neural progenitor cell lines are appropriate objects for studies on the relation between neuronal cell fate decision and NMDA receptor functioning, even if the data obtained on defined cell lines necessarily reflect some clone-specific characteristics.

Several cell lines screened for NMDA receptor expression displayed only a few NMDA receptor subunits (Turetsky et al., 1993; Younkin et al., 1993; Leclerc et al., 1995; Morley et al., 1995; Asahi et al., 1998). NE-4C cells, on the other hand, expressed all forms of NR2 mRNAs and several NR1 mRNA splice variants on a highly reproducible schedule, and many subunits were present also at the protein level. In induced NE-4C cells, these processes are controlled by inherent regulatory machinery. The *in vitro* induced neuron formation by NE-4C cells can be used to study the early steps of subunit production, transport, or assembly into functional NMDA receptors in developmental stages preceding synapse formation.

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