CMLS, Cell. Mol. Life Sci. 54 (1998) 721–725
1420-682X/98/070721-05 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Developmental expression of NMDA receptor subunits and the emergence of glutamate neurotoxicity in primary cultures of murine cerebral cortical neurons

I. Mizuta[a,*,], M. Katayama[a,***], M. Watanabe[b], M. Mishina[c] and K. Ishii[a]**

[a]Department of Cell Biology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606 (Japan)
[b]Department of Anatomy, Hokkaido University School of Medicine, Sapporo 060 (Japan)
[c]Department of Molecular Neurobiology and Pharmacology, School of Medicine, University of Tokyo, Tokyo 113 (Japan)

Received 7 April 1998; accepted 30 April 1998

Abstract. Using primary cultures of murine cerebral cortices, we investigated the developmental expression of *N*-methyl-D-aspartate (NMDA) receptor subunits in relation to the appearance of NMDA receptor-mediated glutamate neurotoxicity. The cultures were not affected by glutamate exposure on culture days 7–9, but became sensitive to glutamate neurotoxicity on day 11. The expression of NMDA receptor subunit messenger RNAs (mRNAs) was investigated by means of reverse transcription polymerase chain reaction (RT-PCR). The ε3-NR2C and ε4-NR2D transcripts could not be detected in the culture. The ε2-NR2B and ζ1-NR1 subunit mRNAs, on the other hand, could be detected clearly and continuously from the culture initiation, and the ε1-NR2A subunit mRNA became clearly detectable on culture day 4. The expression of these three subunits' proteins in the glutamate-insensitive stage (culture day 8) and the sensitive stage (day 11) were studied by means of Western blotting. The ε2-NR2B and ζ1-NR1 subunit proteins were clearly expressed on culture days 8 and 11, but the ε1-NR2A subunit protein could hardly be detected on either day 8 or day 11. These results suggest that the glutamate neurotoxicity in the primary culture was mediated mainly by ε2/ζ1 NMDA receptors. The time lag between the protein expression of the ε2-NR2B and ζ1-NR1 subunits and the emergence of glutamate neurotoxicity may be necessary for the maturation of functional NMDA receptor systems, including heteromeric receptor formation, increase in receptor density and maturation of the postreceptor signal transduction system.

Key words. NMDA receptor subunit; developmental expression; glutamate neurotoxicity; primary culture; cerebral cortical neuron.

* Corresponding author.
** Present address: Laboratory for Cerebrovascular Disorders, National Cardiovascular Center, Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565 (Japan), Fax +81 6 872 7485.
*** Present address: Department of Neurophysiology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 (Japan).

Glutamate, a major excitatory neurotransmitter in the brain, mediates important physiological roles, including learning and memory, as well as pathological neurotoxic effects [1–3]. Glutamate receptors are classified into ionotropic receptors, including NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-

isoxazole propionate) and KA (kinate) subtypes, and metabotropic receptors [4, 5]. Glutamate neurotoxicity is mainly mediated by NMDA ionotropic receptors, which are widely distributed in the brain [6, 7]. Five NMDA receptor subunits have been cloned and named $\zeta 1$, $\varepsilon 1$, $\varepsilon 2$, $\varepsilon 3$ and $\varepsilon 4$ in mouse, and NR1, NR2A, NR2B, NR2C and NR2D in rat [8–11].

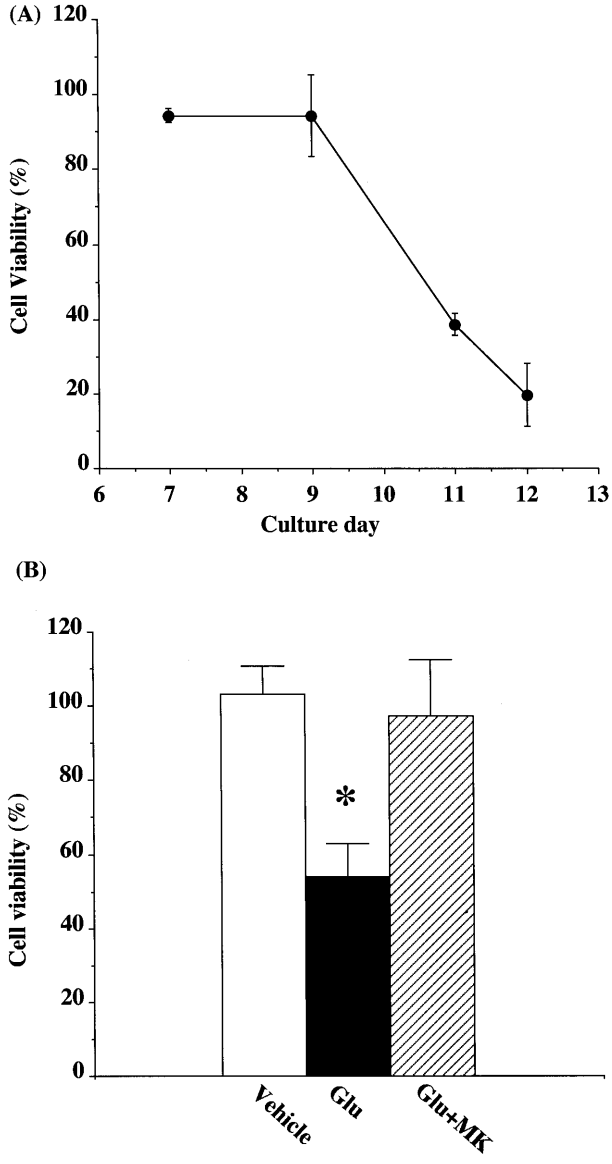

Figure 1. NMDA receptor-mediated glutamate neurotoxicity in primary cultured cortical neurons. (*A*) Cell viabilities after glutamate exposure relative to vehicle treatment were plotted against the culture days. Data are shown as mean ± SD ($n = 2$–4). (*B*) Primary culture protected against glutamate neurotoxicity by MK801 on day 11. Cell viabilities after vehicle (white column), glutamate (black column) and glutamate + MK801 (striped column) exposure relative to before treatment are presented as mean + SD ($n = 4$). Cell viability after glutamate exposure decreased significantly in comparison with those after the other two treatments (Student's *t* test, $^*P < 0.001$).

Previous studies have shown that the functional NMDA receptor is composed of $\zeta 1$-NR1 and one (or more) of the four ε-NR2 subunits [12–15]. Electrophysiological and pharmacological properties of NMDA receptors differ according to each subunit's composition, mainly in terms of ε-NR2 subunits [12–15]. However, molecular diversities of NMDA receptors in relation to glutamate neurotoxicity have been less thoroughly studied. Although some recent reports have referred to this issue by using cultures of cerebellar granule cells [16, 17], little has been reported in the case of cerebral cortical neurons. The NMDA receptor subunits, especially the ε-NR2 subunits, are expressed in a temporal- and spatial-specific manner [18–21]. Therefore, the same or a similar analysis as for cerebellar neurons is needed for cortical neurons. One approach to studying the contributions of each of the ε-NR2 subunits to NMDA receptor-mediated glutamate neurotoxicity is developmental investigation. In the case of cultures of cerebral cortical neurons, several developmental studies of either NMDA receptor expressions or glutamate neurotoxicity have been reported [22, 23], but no report has dealt with these two parameters simultaneously. Because NMDA receptor expression patterns are influenced by various culture conditions [23, 24], it is necessary to examine NMDA receptor expression and the emergence of neurotoxicity in the same culture system. In the present study, we investigated the expression of NMDA receptor subunit messenger RNAs (mRNAs) and proteins, as well as glutamate neurotoxicity, in the development of cortical neurons in the same primary culture system.

Materials and methods

Cerebral cortices were removed from ddY mice which were killed by cervical dislocation under ether anaesthesia. Primary cultures were prepared from the cerebral cortices of embryonic day 16 (E 16) sibling mice. The culture medium was Dulbecco's modified Eagle medium containing 5.4 mM KCl, supplemented with 20 mM Hepes (Dojindo Laboratories, Kumamoto, Japan), 5% fetal calf serum and 10 μM 2-mercaptoethanol [25]. To inhibit the growth of glial cells, 10 μM arabinosyl cytosine was added to the medium on culture day 4.

To assay the glutamate neurotoxicity, the cells were inoculated at a density of 3×10^5 cells per 30-mm dish (Corning) coated with poly-D-lysine (Sigma), and cultured for 7, 9, 11 and 12 days. As described previously [26, 27], the cells were exposed to Locke's solution with or without 100 μM of glutamate for 15 min, followed

by incubation in serum-free medium for 24 h, after which the viable cells were counted under a phase-contrast microscope. In some cases, NMDA antagonist MK801 (20 μM) was added to the glutamate solution.

mRNAs were extracted from primary cultures and cerebral cortices by using a Quick Prep Micro mRNA purification kit (Pharmacia). The first-strand complementary DNAs (cDNAs) primed with random hexamers or specific antisense primers were obtained by using avian myeloblastosis virus (AMV) reverse transcriptase under the conditions recommended for the commercial kit (Reverse Transcription System, Promega). Amplification of each subunit transcript was performed in 20 μl of a polymerase chain reaction (PCR) cocktail containing cDNA derived from 50 ng of mRNA, 4 pmols of each primer and 0.5 U of Ampli Taq Gold DNA polymerase (Perkin-Elmer). After incubation at 95 °C for 9 min, 25 cycles of amplification (30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C) were performed. Sense and antisense PCR primers were as follows: ε1: 5′-CCCAC-CTACTCAGGCCACTT-3′ and 5′-CCGACTGTCC-CTGGAGCAAT-3′ (nt 3664–3683 and 4251–4232 in databank accession D10217); ε2: 5′-CTCTACGGTG-GCAGGGCAAA-3′ and 5′-GGGGTTGGACTG-GTTCCCTA-3′ (nt 3678–3697 and 4356–4337 in D10651); ζ1: 5′-CTGGTGCTGGATAGGCCTGA-3′ and 5′-GCTGCATCTGCTTCCTACGG-3′ (nt 2056–2075 and 2641–2622 in D10028).

For Western blotting, cells were lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.4), 1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulphonyl fluoride. Protein samples (45 μg per lane) were fractionated by 7% SDS-polyacrylamide gel electrophoresis (PAGE), and then electroblotted to nitrocellulose membranes (S&S). The membranes were blocked with 10% skim milk in phosphate-buffered saline (PBS) and then incubated with 1 μg/ml of affinity-purified antibodies against NMDA receptor subunits which are known as ζ1N, ε1C and ε2N antibodies [28]. Immunoreactive bands were visualized with an ECL Western blot detection kit (Amersham).

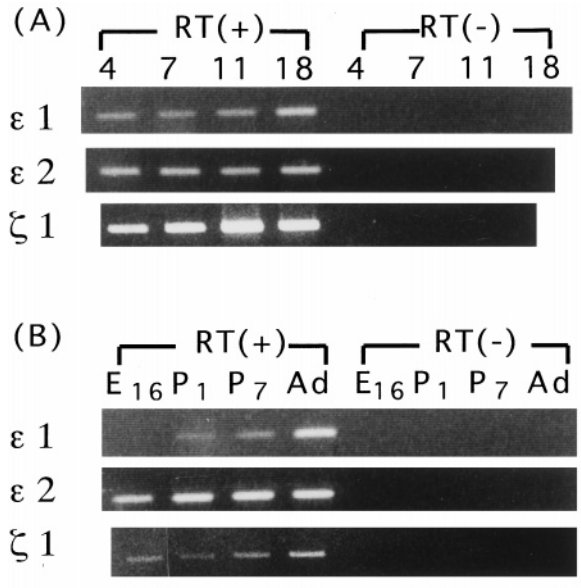

Figure 2. RT-PCR analysis of NMDA receptor subunit mRNA expression. PCR reaction was performed with [RT(+)] or without [RT(−)] reverse transcription reaction. Amplified products were electrophoresed in 1.2% agarose gel containing ethidium bromide. (*A*) Primary cultures of cerebral cortical neurons on days 4, 7, 11 and 18. (*B*) Cerebral cortices on embryonic day 16 (E 16), postnatal day 1 (P 1), postnatal day 7 (P 7) and adulthood (Ad, 13 weeks).

Results

Glutamate neurotoxicity was assayed in primary cultured cortical neurons on days 7, 9, 11 and 12 (fig. 1A). Exposure to glutamate did not affect cell viability on either day 7 or 9. However, the cell viabilities on days 11 and 12 decreased to 40 and 20%, respectively. This effect was completely blocked by the addition of MK801, an antagonist of the NMDA receptor (fig. 1B). This leads to the conclusion that glutamate neurotoxicity was not seen in immature neurons; it appeared on culture day 11 and was mainly mediated by NMDA-type glutamate receptors.

The developmental expression of NMDA receptor subunit mRNAs in the primary culture of the cerebral cortex was investigated by using the reverse transcription-polymerase chain reaction (RT-PCR) technique. The transcripts of ε1-NR2A, ε2-NR2B and ζ1-NR1 subunits were clearly detected (fig. 2), but the transcripts of ε3-NR2C and ε4-NR2D could not be detected (data not shown). No amplified products were obtained without reverse transcription reaction, indicating that the PCR products were not derived from contaminated genomic DNAs. At the beginning of the culture (E 16), both ε2-NR2B and ζ1-NR1 subunit transcripts were detected, but the ε1-NR2A transcript was not. However, all three subunit transcripts were expressed clearly and continuously from culture day 4. In cortices of developing brains, the ε1-NR2A transcript became detectable at P 1 and increased until adulthood, whereas ε2-NR2B and ζ1-NR1 subunit transcripts were expressed from E 16 (fig. 2), which agrees with the previous report [21].

We further investigated the protein expression of the ε1-NR2A, ε2-NR2B and ζ1-NR1 subunits in the glutamate-insensitive stage (culture day 8) and sensitive stage (day 11) by Western blot analysis (fig. 3). The ε1-NR2A protein was clearly detected in the adult cortex, but not in the primary culture on either day 8 or 11. We could

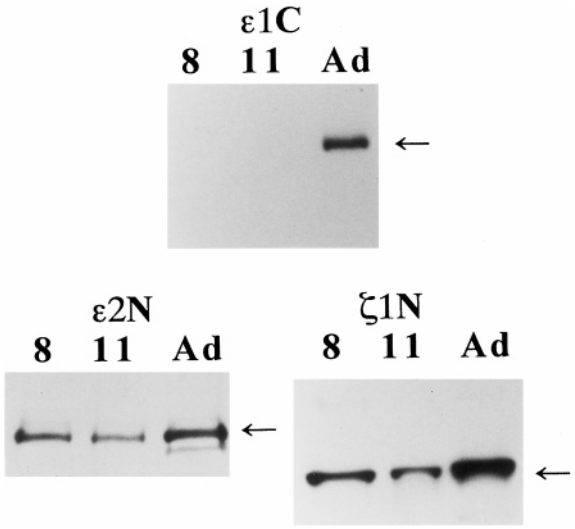

Figure 3. Western blot analysis for expression of $\varepsilon 1$, $\varepsilon 2$ and $\zeta 1$ subunits. The protein samples were extracted from the primary culture on day 8, day 11 and from the adult (Ad) cortex. Sizes of the bands labelled by the $\varepsilon 1$C, $\varepsilon 2$N and $\zeta 1$N antibody, respectively, were 175, 180 and 120 kDa (arrows).

detect only very thin $\varepsilon 1$-immunoreactive bands on days 8 and 11 even after a long exposure (not shown), while the $\varepsilon 2$-NR2B and $\zeta 1$-NR1 subunit proteins could be detected clearly in the primary culture both on days 8 and 11.

Discussion

The primary culture system has major advantages for the study of the molecular diversity of NMDA receptors in relation to NMDA receptor-mediated glutamate neurotoxicity. Both developmental studies and inhibition/induction experiments can be performed using primary cultured neurons. In the case of primary cultured cerebellar granule cells, it is known that an exposure to high concentrations of KCl or NMDA renders the cells sensitive to NMDA neurotoxicity after prolonged K^+ depolarization [29, 30]. Bessho et al. reported that these treatments selectively induced NR2A subunit mRNA, and that the cells failed to acquire sensitivity to NMDA toxicity after depression of NR2A by antisense oligonucleotide treatment [16]. Furthermore, Didier et al. recently reported that antisense oligonucleotide treatment of either $\varepsilon 1$-NR2A, $\varepsilon 2$-NR2B or $\varepsilon 3$-NR2C reduced protein expression, followed by attenuation of NMDA neurotoxicity to a similar extent [17]. Studies of cerebral cortical neurons reported that depression or destruction of the $\zeta 1$-NR1 subunit resulted in, respectively, reduced or no sensitivity to neurotoxicity [31, 32], but little is known about the relation between ε-NR2 subunits and glutamate neurotoxicity.

The NMDA receptors in adult cerebral cortex are mainly composed of $\varepsilon 1$-NR2A, $\varepsilon 2$-NR2B and $\zeta 1$-NR1 subunits. During the development of the cerebral cortex, the expression of $\varepsilon 1$-NR2A mRNA follows that of the $\varepsilon 2$-NR2B and $\zeta 1$-NR1 subunits [21, 31, 32]. Delayed expression of the $\varepsilon 1$-NR2A mRNA was also observed in our primary culture (fig. 2). We hypothesized that the additional expression of the $\varepsilon 1$-NR2A subunit might be necessary for the primary culture to become sensitive to glutamate neurotoxicity. To ascertain this possibility, the protein expression of these three subunits in the glutamate-insensitive stage (day 8) and the sensitive stage (day 11) was investigated (fig. 3). The $\varepsilon 2$-NR2B and $\zeta 1$-NR1 subunit proteins were detected clearly on days 8 and 11, but the $\varepsilon 1$-NR2A subunit protein could be hardly detected on day 8 or day 11, indicating that the $\varepsilon 1$-NR2A subunit mRNA was only slightly translated even after the emergence of glutamate neurotoxicity. Sucher et al. previously reported that the NR1 subunit mRNA was not translated in PC12 cells [33]. Similarly, posttranscription regulation might suppress the translation of the $\varepsilon 1$-NR2A subunit transcript in primary culture. These results suggest that the glutamate neurotoxicity of the primary culture was mainly mediated by the $\varepsilon 2/\zeta 1$ subunits. The time lag between the protein expression of the $\varepsilon 2$-NR2B and $\zeta 1$-NR1 subunits and the emergence of glutamate neurotoxicity may be necessary for formation of the heteromeric $\varepsilon 2/\zeta 1$ receptors and/or increase in the receptor density. However, the possibility cannot be excluded that the emergence of glutamate toxicity was not merely due to the formation of functional NMDA receptors but also to a reduced intracellular Ca^{2+} buffering capacity [34] and/or related changes in the metabolism of cortical neurons.

Acknowledgements. This work was partly supported by research grants from the Ministry of Education, Science and Culture of Japan.

1 McDonald J. W. and Johnston M. V. (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res. Brain Res. Rev. **15:** 41–70
2 Monaghan D. T., Bridges R. J. and Cotman C. W. (1989) The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. Annu. Rev. Pharmacol. Toxicol. **29:** 365–402
3 Nakanishi S. (1992) Molecular diversity of glutamate receptors and implications for brain function. Science **258:** 597–603
4 Collingridge G. L. and Lester R. A. J. (1989) Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol. Rev. **41:** 143–210
5 Honore T. (1989) Excitatory amino acid receptor subtypes and specific antagonists. Med. Res. Rev. **9:** 1–23

6 Choi D. W., Koh J. Y. and Peters S. (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. J. Neurosci. **8:** 185–196

7 Olney J. W. (1990) Excitotoxic amino acids and neuropsychiatric disorders. Annu. Rev. Pharmacol. Toxicol. **30:** 47–71

8 Hollmann M. and Heinemann S. (1994) Cloned glutamate receptors. Annu. Rev. Neurosci. **17:** 31–108

9 Seeburg P. H. (1993) The molecular biology of mammalian glutamate receptor channels. Trends Neurosci. **16:** 359–365

10 Mishina M., Mori H., Araki K., Kushiya E., Meguro H., Kutsuwada T. et al. (1993) Molecular and functional diversity of the NMDA receptor channel. Ann. NY Acad. Sci. **707:** 136–152

11 Nakanishi S. and Masu M. (1994) Molecular diversity and functions of glutamate receptors. Annu. Rev. Biophys. Biomol. Struct. **23:** 319–348

12 Ishii T., Moriyoshi K., Sugihara H., Sakurada K., Kadotani H., Yokoi M. et al. (1993) Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. J. Biol. Chem. **268:** 2836–2843

13 Kutsuwada T., Kashiwabuchi N., Mori H., Sakimura K., Kushiya E., Araki K. et al. (1992) Molecular diversity of the NMDA receptor channel. Nature **358:** 36–41

14 Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H. et al. (1992) Heteromeric NMDA receptors: molecular and functional distribution of subtypes. Science **256:** 1217–1221

15 Meguro H., Mori H., Araki K., Kushiya E., Kutsuwada T., Yamazaki M. et al. (1992) Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature **357:** 70–74

16 Bessho Y., Nawa H. and Nakanishi S. (1994) Selective upregulation of an NMDA receptor subunit mRNA in cultured cerebellar granule cells by K^+-induced depolarization and NMDA treatment. Neuron **12:** 87–95

17 Didier M., Xu M., Berman S. A., Saido T. C. and Bursztajn S. (1997) Involvement of three glutamate receptor ε subunits in the formation of *N*-methyl-D-aspartate receptors mediating excitotoxicity in primary cultures of mouse cerebellar granule cells. Neuroscience **78:** 1129–1146

18 Wenzel A., Fritschy J. M., Mohler H. and Benke D. (1997) NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B and NR2C subunit proteins. J. Neurochem. **68:** 469–478

19 Moriyoshi K., Masu M., Ishii T., Shigemoto R., Mizuno N. and Nakanishi S. (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature **354:** 31–37

20 Monyer H., Burnashev N., Laurie D. J., Sackmann B. and Seeburg P. H. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron **12:** 529–540

21 Watanabe M., Inoue Y., Sakimura K. and Mishina M. (1992) Developmental changes in distribution of NMDA-receptor channel subunit mRNAs. Neuroreport **3:** 1138–1140

22 Choi D. W., Maulucci-Gedde M. and Kriegstein A. R. (1987) Glutamate neurotoxicity in cortical cell culture. J. Neurosci. **7:** 357–368

23 Zhong J., Russel S. L., Pritchett D. B., Molinoff P. B. and Williams K. (1994) Expression of mRNAs encoding subunits of the *N*-methyl-D-aspartate receptor in cultured cortical neurons. Mol. Pharmacol. **45:** 846–853

24 Vallano M. L., Lambolez B., Audinat E. and Rossier J. (1996) Neuronal activity differentially regulates NMDA receptor subunit expression in cerebellar granule cells. J. Neurosci. **16:** 631–639

25 Katayama M. and Ishii K. (1994) 2-Mercaptoethanol-independent survival of fetal mouse brain neurons cultured in a medium of human serum. Brain Res. **656:** 409–412

26 Savio T. and Levi G. (1993) Neurotoxicity of HIV coat protein gp120, NMDA receptors and protein kinase C: a study with rat cerebellar granule cell cultures. J. Neurosci. Res. **34:** 265–272

27 Katayama M., Mizuta I., Sakoyama Y., Kohyama-Koganeya A., Akagawa K., Uyemura K. et al. (1997) Differential expression of neuroD in primary cultures of cerebral cortical neurons. Exp. Cell Res. **236:** 412–417

28 Watanabe M., Fukaya M., Sakimura K., Manabe T., Mishina M. and Inoue Y. (1998) Selective scarcity of NMDA receptor channel subunits in the stratum lucidum (mossy fibre-recipient layer) of the mouse hippocampal CA3 subfield. Eur. J. Neurosci. **10:** 478–487

29 Gallo V., Kingsbury A., Balázs R. and Jørgensen O. S. (1987) The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. J. Neurosci. **7:** 2203–2213

30 Balázs R., Jørgensen O. S. and Hack N. (1988) *N*-methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. Neuroscience **27:** 437–451

31 Wahlestedt C., Golanov E., Yamamoto S., Yee F., Ericson H., Yoo H. et al. (1993) Antisense oligonucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infarctions. Nature **363:** 260–263

32 Tokita Y., Bessho Y., Masu M., Nakamura K., Nakao K., Katsuki M. et al. (1996) Characterization of excitatory amino acid neurotoxicity in *N*-methyl-D-aspartate receptor-deficient mouse cortical neuronal cells. Eur. J. Neurosci. **8:** 69–78

33 Sucher N. J., Brose N., Deitcher D. L., Awobuluyi M., Gasic G. P., Bading H. et al. (1993) Expression of endogenous NMDAR1 transcripts without receptor protein suggests post-transcriptional control in PC12 cells. J. Biol. Chem. **268:** 22299–22304

34 Hartley D. M., Kurth M. C., Bjerkness L., Weiss J. H. and Choi D. W. (1993) Glutamate receptor-induced $^{45}Ca^{2+}$ accumulation in cortical cell culture correlates with subsequent neuronal degeneration. J. Neurosci. **13:** 1993–2000