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Short communication

Acute and chronic changes in K^+-induced depolarization alter NMDA and nNOS gene expression in cultured cerebellar granule cells

F. Tascedda [a], R. Molteni [a], G. Racagni [a], M.A. Riva [a,b,*]

[a] Center of Neuropharmacology, Institute of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy
[b] Di.Bi.T. San Raffaele Scientific Institute, Via Olgettina 58, Milan, Italy

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Abstract

The influence of low or high (10 or 25 mM) K^+-induced membrane depolarization on the mRNA levels for NMDA receptor subunits was investigated by RNase protection assay in cultured rat cerebellar granule cells. Cells, maintained for 7 days in K^+_{25}, a condition that promotes their survival and maturation, express the highest levels of NR-1 and NR-2A mRNA, whereas NR-2B is maximally expressed in cells grown in K^+_{10}. Acute changes in medium K^+ concentration had a significant effect on the mRNA levels for NMDA receptor subunits. A concomitant reduction of NR-2A mRNA and induction of NR-2B was observed following a 24-h shift of the culture medium from K^+_{25} to K^+_{10}. Under these circumstances NR-2C, not detected in basal conditions, became expressed. Neuronal nitric oxide synthase, an enzyme linked to NMDA receptor activation, was also influenced by growth conditions. Its expression, higher under low excitation (K^+_{10}), is induced in the shift from K^+_{25} to K^+_{10} and is markedly decreased in the opposite situation. These data indicate that several factors may influence the expression of NMDA receptor subunits and consequently may modulate the function of this receptor complex and its adaptation to acute and chronic changes in neuronal activity.

Keywords: Glutamate; Nitric oxide synthase; Apoptosis; NMDA; mRNA; Granule cell

The *N*-methyl-D-aspartate (NMDA) receptor is a glutamate-gated cation-specific ion channel which is important in neuronal plasticity. It is composed by two families of subunits, termed NMDA-R1 (NR-1) [16], which occurs in eight splice isoforms [22], and NMDA-R2 (NR-2; A, B, C, D) [11,13,14]. NR-1 serves as the key subunit that possesses the characteristic features of the NMDA receptor, whereas NR-2 subunits potentiate receptor activity in heteromeric configurations and are regarded as modulatory [20]. Following prolonged activation, this receptor may cause neuronal degeneration and cell death [5]. Primary cultures of neuronal cells from different brain structures have been employed to investigate the mechanisms involved in excitotoxic cell damage. Cerebellar granule cells are very useful for this purpose as they contain a relatively homogeneous population of neurons and are known to express different glutamate receptors, including the NMDA subtype [21]. In order to gain insight in the mechanisms that control the expression of NMDA receptor subunits during cerebellar granule cell maturation, we investigated their mRNA content in different growth conditions known to affect the survival and maturation of this neuronal phenotype in culture [2,9]. In parallel, and under the same experimental conditions, we determined the mRNA levels for neuronal nitric oxide synthase (nNOS), an enzyme linked to NMDA receptor activation and responsible of NO formation [8,10].

Dissociated cultures, enriched in granule cells, were obtained from cerebella of 8-day-old Sprague–Dawley rats (Charles River) according to standard protocols [9]. Cerebellar granule neurons were plated at a density of 2.5×10^6 cells/ml (basal Eagle's medium; Irvine) supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml gentamicin) and grown under different KCl concentration (10 or 25 mM). Cytosine arabinoside (10 μM) was added 20 h after seeding to inhibit glial proliferation. Acute changes of KCl concentration were performed after 7 days in culture. The shift from K^+_{10} to K^+_{25} was obtained by adding the appropriate concentration of KCl from a stock solution. The shift from K^+_{25} to

* Corresponding author. Fax: +39 (2) 2940-3673 or 2040-4961; E-mail: riva@isfunix.farma.unimi.it

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K^+_{10} was done by substituting (after filtration) the medium (K^+_{25}) with conditioned medium obtained from sister cultures grown for 7 days in K^+_{10}. No fresh medium or serum was added to the cultures. Total RNA was isolated by phenol–chloroform extraction according to Chomczynski and Sacchi [6]. Quantification was carrier out by absorption at 260 nm and the mRNA content for NMDA receptor subunits and nNOS was determined by RNase protection assay on total RNA (10 μg) as previously described [19]. All cRNA probes were generated by a T7 RNA polymerase and ^{32}P-CTP was used as radiolabelled nucleotide. The cRNA probes and the relative protected fragment (p.f.) were the following: NR-1 = 438, p.f. = 414; NR-2A = 187, p.f. = 171; NR-2B = 310, p.f. = 264; NR-2C = 244, p.f. = 213. For the determination of nNOS expression a plasmid containing its cDNA [4] was linearized with *Nco*I and used as a template for T7 polymerase. The in vitro transcription yielded a 811-base cRNA probe which included 760 b of nNOS sequence and 51 b of the polylinker region.

The levels of mRNA were calculated by measuring the peak densitometric area of the autoradiography analysed with a LKB laser densitometer. The statistical analysis was performed by ANOVA and Dunnett *t*-test.

We cultured cerebellar granule cells for 7 days in K^+_{10} or K^+_{25}. As depicted in Table 1, the mRNA levels for NR-1 and NR-2A are higher under 'classical' depolarizing conditions whereas cells grown in K^+_{10} yielded a higher expression of NR-2B. In the latter experimental condition, the levels of nNOS were 2.5-fold higher than in K^+_{25}.

Fig. 1, representing a typical RNase protection assay with the concomitant determination of NMDA receptor subunits (1, 2A, 2B and 2C) and nNOS, shows that significant effects on the mRNA levels for NMDA receptor subunits were evident after acute changes in growth conditions. In this experiment the concentration of KCl was shifted for 24 h (between div 7 and 8) from K^+_{25} to K^+_{10} or from K^+_{10} to K^+_{25}. The results, summarized in Fig. 2, indicate that there is a 40% reduction in the mRNA levels for NR-2A with a concomitant 2.5-fold elevation in the expression of NR-2B subunit when cells grown for 7 days in K^+_{25} were shifted for 24 h to K^+_{10}. The expression of NR-1 was only slightly increased as a result of the medium shift (+33%). Most importantly the shift from

Table 1
Effect of KCl concentration on NMDA-R and nNOS mRNA levels in cultured rat cerebellar granule cells. The results, expressed as % of the mRNA content of cells cultured in K^+_{25}, represent the mean ± S.E.M. of at least three independent determinations, as measured at 7 div

K^+ (mM)	NR-1	NR-2A	NR-2B	nNOS
10	60 ± 4 *	61 ± 6 *	172 ± 26 *	245 ± 27 * *
25	100 ± 4	100 ± 8	100 ± 12	100 ± 9

* $P < 0.05$ and * * $P < 0.01$ vs. cerebellar granule cells grown in K^+_{25} (ANOVA with Student *t*-test).

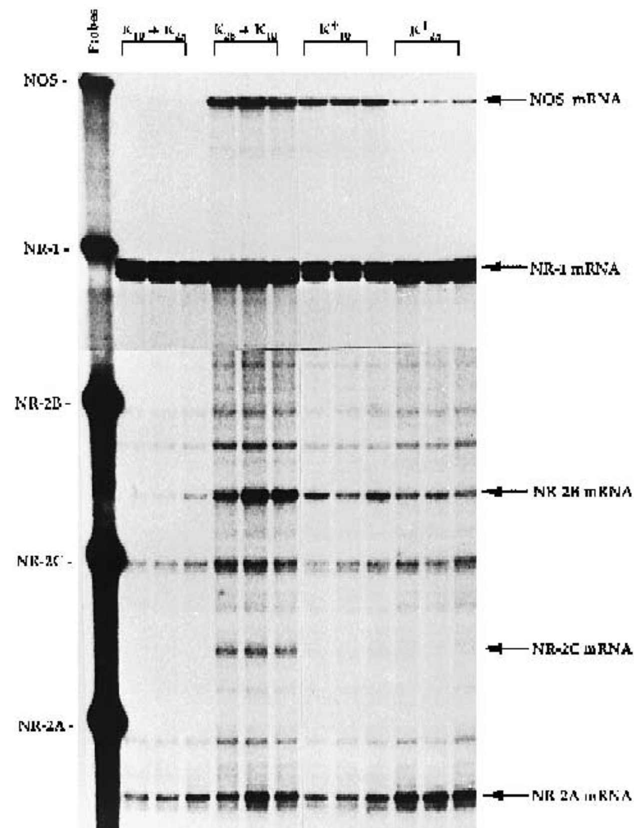

Fig. 1. Determination of the mRNA levels for NMDA receptor subunits and neuronal nitric oxide synthase (nNOS) by RNase protection assay. The autoradiograph shows the effect of acute changes in growth conditions on the expression pattern in cultured rat cerebellar granule cells. Arrows indicate the protected fragments for each receptor subunits or nNOS. The lane marked as Probes indicates an aliquot (10000 c.p.m.) of the hybridization solution containing the antisense cRNA probes. The X-ray film was exposed for 16 h (nNOS and NR-1) or 30 h (NR-2A, B, C) at −70°C with intensifying screens.

K^+_{25} to K^+_{10} induced the expression of NR-2C. As the mRNA for NR-2C could not detected under control conditions (see Fig. 1), its induction could not be accurately quantitated and, for this reason, the data is not included in Fig. 2. If cells, grown in K^+_{10}, were cultured for 24 h in K^+_{25} a 60% reduction was observed in NR-2B mRNA, no change was detected for NR-1 and a slight reduction was evident in NR-2A expression. Beyond changes in the NMDA receptor subunit, marked differences of nNOS mRNA content were detected as a consequence of the acute changes in growth conditions. The shift from K^+_{25} to K^+_{10} produced a significant induction of nNOS mRNA levels (3.6-fold) whereas a marked decrease (−90%) was observed after the shift from K^+_{10} to K^+_{25}.

In agreement with previous reports [3,18], the levels for NR-1 and NR-2A mRNAs are high in cells grown under depolarizing conditions, suggesting a link between growth conditions, which favor survival and maturation of the cells in vitro [2,9], and the expression of these receptor subunits. However, two major differences exist between

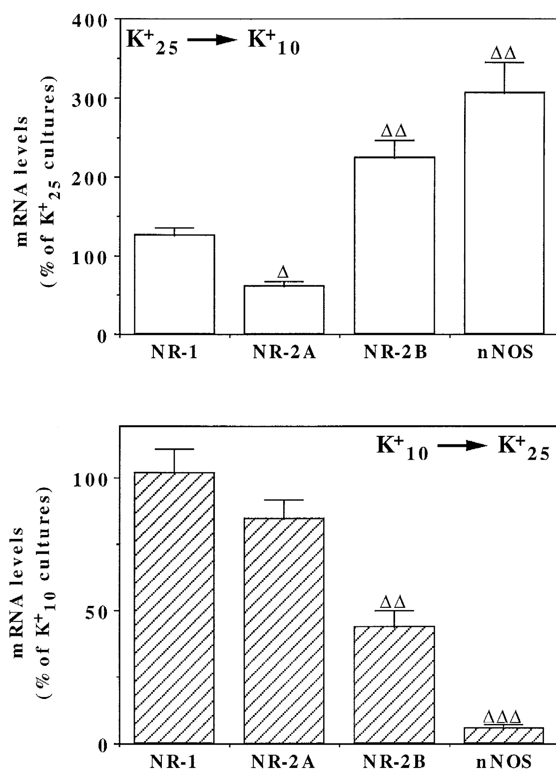

Fig. 2. Expression of NMDA receptor subunits and nNOS in cultured rat cerebellar granule cells following a 24-h change in KCl concentration. Cell were cultured for 7 days in K^+_{10} or K^+_{25} before the shift in KCl concentration, as indicated in the text. The data, expressed as % of the original culture (before the shift), represent the mean ± S.E.M. of four to seven independent determinations. $^{\Delta}$ $P < 0.05$, $^{\Delta\Delta}$ $P < 0.01$ and $^{\Delta\Delta\Delta}$ $P < 0.001$ vs. respective control cultures without changes in KCl concentration (ANOVA with Dunnett *t*-test).

our data and the report from Resink et al. [18]. These authors did not find any modification in NR-2B mRNA content in cells grown under K^+_{10} vs. K^+_{25} whereas we observed a higher expression of this subunit in K^+_{10}. Moreover, they report a marked elevation of NR-2C mRNA levels between div. 5 and 9, an effect which is more evident in granule cells grown in K^+_{10}. In our cultures the mRNA for NR-2C was virtually absent in basal conditions both in K^+_{10} and in K^+_{25}. The reasons for such discrepancies are presently unknown and could be the results of several factors (cell density, type of serum), able to influence the stage of maturation reached by the cells. In line with our results is the report from Audinat et al. [1] showing that granule cells in cultured cerebellar slices express NR-2A and NR-2B, but not NR-2C, with a predominance of NR-2B following elimination of spontaneous electrical activity by tetrodotoxin.

A comparison between the in vivo and in vitro situation strengthens the notion that NR-2B is predominant at an immature stage of development. The high expression of this subunit observed in cells grown in K^+_{10} or during the first days in culture (data not shown), correlates with the transient appearance of its mRNA in vivo during the second week of postnatal life [15]. NR-2A and NR-2C show a similar developmental profile in the cerebellum, with a progressive increase of their mRNA levels from the end of the second week of life to adulthood [19]. However, their regulation is markedly different in cultured cerebellar granule cells where the developmental maturation of NR-2A is not paralleled by a similar elevation of NR-2C gene expression.

A 24-h transition from K^+_{25} to K^+_{10} increases NR-2B mRNA levels by 2-fold whereas addition of depolarizing concentration of K^+ to cells grown for 7 days in a K^+_{10} reduces the mRNA levels of NR-2B by 50% indicating that a lower state of excitation might favor NR-2B expression. Conversely, sustained membrane depolarization and consequent Ca^{2+} influx might be responsible for the maintenance of NR-2A as its mRNA levels are decreased as a result of the shift from high to low KCl. Bessho et al. [3] have indeed demonstrated that the increase in Ca^{2+} through the activation of voltage-dependent Ca^{2+} channels is responsible for NR-2A upregulation, which in turn contribute to the induction of functional NMDA receptor channels.

Alterations in the state of cellular activation can lead to changes of subunit expression that may alter NMDA receptor function. The specific properties of the NMDA receptor appears to be the result of a heteromeric receptor complex formed by the NR-2 subunit which is co-expressed with the NR-1 subunit [20]. The changes in their expression pattern may influence response of the cells to glutamate. This aspect is relevant for the use of cerebellar granule cells as a model to investigate the mechanisms involved in glutamate-dependent excitotoxicity. Indeed the vulnerability of these cells to excitatory amino acids may depend upon growth conditions employed [17].

Another important consequence of changes in neuronal excitation is the modulation of nNOS, a enzyme system located downstream to NMDA receptor activation. Neuronal NOS is responsible for the formation of the cellular messenger NO [8,10] and it is highly expressed in the cerebellum [4]. We show for the first time that the mRNA levels for this enzyme can be regulated in cerebellar granule neurons according to their maturation state. Its expression at 7 div is high in cells grown under K^+_{10} suggesting its preferential expression in neurons under a low state of activation. As intracellular Ca^{2+} concentrations can reflect the state of activity of the cell, it may in turn modulate the expression of enzymes, such as nNOS, which are important in coupling external signals to intracellular responses [8]. As nitric oxide can inhibit NMDA receptor [12], the lower functionality of NMDA receptor in K^+_{10} may be the consequence of a higher expression of nNOS in this condition.

The shift from K^+_{25} to K^+_{10} in cultured cerebellar granule neurons induces apoptosis, a degenerative process that involves both transcriptional and translational mechanisms [7]. It become therefore important to characterize the tem-

poral sequence of the events taking place at the nuclear level during apoptosis. If NO is one of the agents capable of triggering cell death, it is possible that the induction of nNOS observed following the shift from K^+_{25} to K^+_{10} can be related to the undergoing apoptotic events. The appearance of NR-2C under the same experimental conditions suggests that changes in the properties of the NMDA receptor channel may also be involved in apoptotic cell death.

In conclusion, we think that monitoring the expression pattern of NMDA receptor subunits may be a useful approach to investigate the plasticity inherent to changes in neuronal activity and, in a more general way, may provide a molecular correlate for short- and long-term adaptation of the CNS to different stimuli.

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