Amino Acids (2000) 18: 299–304

Amino
Acids

Short Communication

Excitatory amino acid stimulation of the survival of rat cerebellar granule cells in culture is associated with an increase in SMN, the spinal muscular atrophy disease gene product

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Accepted September 26, 1999

Summary. Excitatory amino acids which promote the survival of cerebellar granule cells in culture, also promote the expression of the survival of motor neuron (SMN) protein. Immunolocalization studies using SMN monoclonal antibody showed that SMN is decreased in cultures grown in low K^+ or chemically defined medium with respect to cultures grown in high K^+ medium and that an increase of SMN can be induced by treatment of low K^+ cultures with glutamate or N-methyl-D-aspartate.

Keywords: Amino acids – Cerebellar granule cell – Excitatory amino acid – Trophic factor – Survival of motor neuron protein – Spinal muscular atrophy

Abbreviations: SMN, survival of motor neuron protein; EAA, excitatory amino acids; NMDA, N-methyl-D-aspartate; SMA, spinal muscular atrophy; CGCs, primary cerebellar granule cell cultures; FCS, fetal calf serum; CDM, serum-free chemically defined medium; DIV, days *in vitro*; CNS, central nervous system.

Introduction

Cerebellar granule cells develop specific survival requirements in culture (Balazs et al., 1988a) as nerve cells generally do *in vivo* (Oppenheim, 1991). These requirements can be met *in vitro* either by K^+-induced depolarization of the cells (Gallo et al., 1987) or by stimulation of excitatory amino acid (EAA) receptors, the N-methyl-D-aspartate (NMDA)-preferring subtype being especially effective (Balazs et al., 1988b).

The survival of motor neuron gene (SMN) has been identified as the disease-causing gene in spinal muscular atrophy (SMA), a common autosomal

recessive neuromuscular disorder (1/6000 live births) characterized by degeneration of motor neurons of the spinal cord and atrophy of proximal limb muscles (Lefebvre et al., 1995). SMN is expressed in all mammalian tissues, particularly high levels are expressed in certain areas of CNS including spinal cord and cerebellum (Francis et al., 1998). In individuals affected by the most severe form of SMA, the SMN protein is barely detectable in motor neurons (Lefebvre et al., 1997; Mirabella et al., 1999). SMN is found both in the nucleus and in the cytoplasm. In the nucleus the protein is concentrated in structures called "gems", similar in size and number to coiled bodies and often associated with them (*gem*ini of coiled bodies) (Liu and Dreyfuss, 1996).

In the present study we explore the relationship between the expression of SMN and specific aspects of neuronal cell survival, using cerebellar cultures as model. In particular, we evaluate whether trophic effects induced by stimulation of EAA receptors on cerebellar granule cells may be related with an increase in expression of the SMN protein.

Material and methods

Primary cerebellar granule cell cultures (CGCs) were prepared according to the procedure described by Levi et al. (1984). Dissociated cells were resuspended and plated in Basal Eagle's Medium containing 10% fetal calf serum (FCS), 2 mM glutamine and K^+ concentrations adjusted either to 10 mM or 25 mM (K10 and K25, respectively) or in serum-free chemically defined medium (CDM) (Gallo et al., 1986). Cells were plated at a density of 250.000 cells/cm^2 on poly-L-lysine-coated 12 mm round coverslips placed in 35 mm dishes. After 24 hr NMDA or glutamate (140 μM final concentration) was added to some K10 cultures (K10-NMDA or K10-Glu, respectively). At the same time all the cell cultures were treated with cytosine arabinoside (10 μM final concentration) to prevent the growth of non-neuronal cells. Qualitative evaluation of neuronal cell survival was done by daily inspection of the cultures using phase-contrast microscopy. The experiments were carried out on 4-day-old (days *in vitro*, DIV) CGCs. For the immunofluorescence staining, CGCs grown on coverslip were washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 5 mM HEPES, pH 7.4), fixed with a mixture of methanol/H_2O (80:20 v/v) and exposed to 2B1 antiSMN antibody (overnight, 4°C, dilution 1:1000) kindly provided by Dr. Gideon Dreyfuss, Department of Biochemistry and Biophysics, University of Pennsylvania, PA (Liu and Dreyfuss, 1996). The coverslips were then treated with fluorescein-conjugated goat antimouse IgG (30 min, 37°C, dilution 1:50). At the end, cells were examined for fluorescence pattern and intensity. Nuclear SMN was evidenced as "gems" (one or two/nucleus). We evaluated nuclear SMN by counting the number of cells positive for gems over 400 total cells (i.e., as percent of positive cells). Values are means ± SEM. All the experiments were carried out in duplicate or triplicate on at least two cell batches. Statistical analysis was performed by one-way ANOVA. A p value ≤ 0.05 was considered as significantly different.

Results

The normal *in vitro* developmental pattern of isolated cerebellar perikarya consists of rapid attachment to the polylysine coated surface followed by profuse elaboration of cellular processes of various diameter (1–2 DIV). Generally within 3–5 days the formation of cell clusters occurs in conjunction

with the fasciculation of the neuritic processes to form an extensive fibrous network interconnecting the cell clusters (6–8 DIV) (Burgoyne et al., 1988). Serum (fetal calf serum, FCS) is an essential factor for neuronal cell survival and maturation *in vitro*. In fact, the initial development of cerebellar granule cells in cultures containing 10% FCS is similar irrespective of the K^+ concentration in the medium (Gallo et al., 1987). However, usually after 5–6 DIV, there is an abrupt wave of degeneration of cells grown in the presence of low K^+ (here K^+ 10 mM, K10) so that cell number beyond that time is only about one-third of that obtained in high K^+ medium (here K^+ 25 mM, K25). Cell rescue can be effected by treatment of low K^+ cultures with the EAA receptor agonist NMDA or glutamate itself (Balazs et al., 1988b) (here both added at 140 μM final concentration 24 hr after plating, K10 + NMDA or K10 + Glut, respectively). A very different result is obtained when the cells are cultured in a serum free-chemically defined medium (here CDM). In this case the survival is not compromised but the great majority of the cells remain dispersed on the surface of the culture dish and do not aggregate to form clumps, even after 10 days in culture (Gallo et al., 1986). From these findings it emerges that in the first days of culturing major differences are not noticed regardless of whether the cells are cultured under "trophic" (K25/K10 + NMDA/K10 + Glut) or "non trophic" (K10/CDM) conditions. For this reason we carried out experiments to evaluate the expression of SMN at 4 DIV. At this stage, the vast majority (70%) of cerebellar granule cells cultured in K25 medium expressed SMN protein, as appreciated by immunofluorescence (Fig. 1). SMN was found both in the nucleus concentrated in one or two dot-like structures, "gems" (arrow), and in the cytoplasm evidenced as diffuse but intense staining (arrowhead). The change of growth conditions determined a dramatic effect on the expression of nuclear gems (as percent of positive cells) whereas it did not influence the cytosolic pattern. As shown in Fig. 2, nuclear SMN was greatly reduced in cells cultured in a low K^+ medium (K10, 13% cell positivity) or maintained in CDM (CDM, 8% cell positivity) with respect to the control (K25, 70% cell positivity). Addition of NMDA or glutamate to K10 cultures restored the expression of nuclear gems (40% cell positivity for both K10 + NMDA and K10 + Glut conditions). Thus, neurotrophic effects of EAA receptor stimulation on developing cerebellar granule cells are associated with an increased expression of the SMN protein.

Discussion

Cerebellar granule cell cultures provide a useful model to study the role of excitatory amino acids during development and the putative involvement of these substances in pathological processes of CNS. Using cerebellar culture as a model, we have demonstrated that treatments with EAA that restore the survival of cerebellar granule cells grown under suboptimal conditions result in specific increase in SMN, the spinal muscular atrophy disease gene product. The finding that both glutamate and NMDA gave almost the same effect on

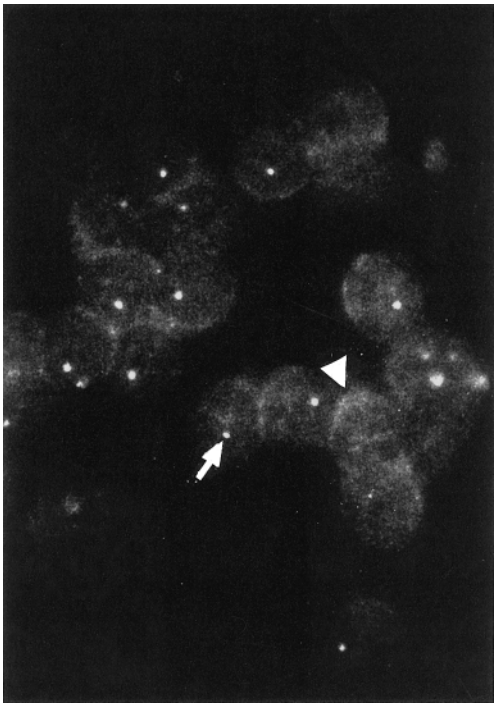

Fig. 1. Fluorescent localization of SMN in cerebellar granule cells cultured for 4 days in K25 medium. SMN protein is found in discrete nuclear structures (arrow) and diffuse throughout the cytoplasm (arrowhead). K25 medium = 10% FCS-25 mM KCl. For further details see text. Magnification 400×

the expression of SMN suggests that the NMDA glutamate receptor subtype is preferentially involved (cf. Balazs et al., 1988b). It has been shown that influences of EAA treatment are mediated through Ca^{2+} (Pearson et al., 1992) and also proposed that this situation mimics the *in vivo* effects of the first innervation of cerebellar granule cells by the glutamatergic mossy fibers (Gallo et al., 1987). Little is known of other components of the survival signalling pathways triggered by EAA.

Recent studies from developing human spinal cord suggested that in late foetal and early postnatal life the repertoire of glutamate receptors expressed by motor neurons may lead to NMDA-receptor mediated excitotoxicity and that such an occurrence may be relevant to the pathogenesis of SMA (Kalb and Fox, 1997). One the other hand, SMN protein has been demonstrated to be specifically involved in the regeneration of some components of the splicing machinery (Pellizzoni et al., 1998). Our data on cerebellar granule cells link the expression of SMN to activation of EAA receptors and ultimately to neuronal cell survival. These findings suggest that trophic influences of EAA may be mediated through the function of SMN (e.g. by controlling specific mRNA synthesis of proteins needed for cell survival) and that alteration of such pathway(s) in SMA patients may be relevant to the pathogenesis of the disease.

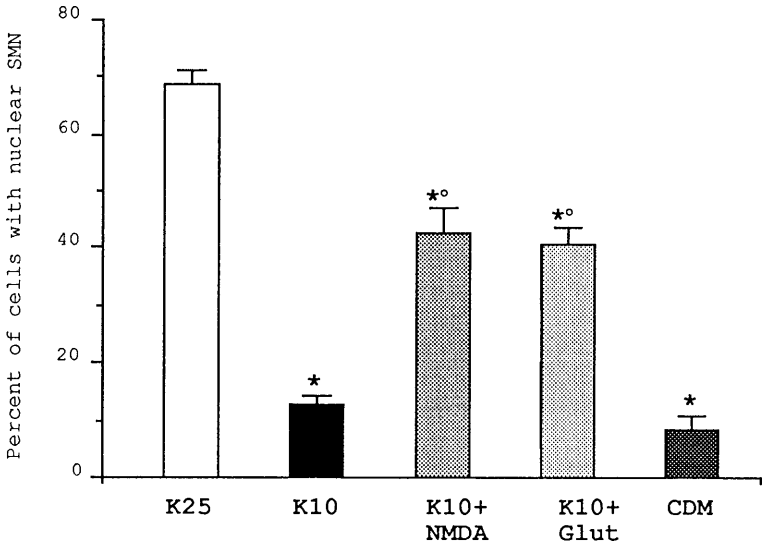

Fig. 2. Effect of NMDA and glutamate on the percent of cerebellar granule cells expressing nuclear SMN. Cells were cultured for 4 days in different media, stained with antiSMN antibody and evaluated for SMN immunofluorescence. *K25* 10% FCS-25 mM KCl; *K10* 10% FCS-10 mM KCl; *CDM* serum-free chemically defined medium. NMDA or glutamate (*Glut*) were added to the medium 24 h after plating (140 μM). Values are means ± SEM of the estimates related to the number of cells expressing nuclear gems. *$p < 0.05$ *vs* K25; °$p < 0.05$ *vs* K10

Acknowledgements

This work was supported by a MURST 60% given to M.L.E. and Telethon-grant 828 given to C.B.

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Received March 31, 1999