Overexpression of Neuronal Pentraxin 1 Is Involved in Neuronal Death Evoked by Low K⁺ in Cerebellar Granule Cells*

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Mature cerebellar granule cells in culture die by a process that requires new RNA and protein synthesis when deprived of depolarizing concentrations of potassium. We investigated gene expression during the early phase of the cell death program evoked by potassium deprivation. Using a differential gene display technique, we isolated a cDNA that was increased by potassium deprivation. This cDNA was homologous to the 3' mRNA end of neuronal pentraxin 1 (NP1), a gene encoding a secreted glycoprotein whose expression is restricted to the nervous system. Reverse-Northern and Northern blot analyses confirmed that treatment with low potassium induces overexpression of NP1 mRNA, with a subsequent increase in NP1 protein levels. Time-course studies indicated that overexpression of NP1 protein reaches a maximum after 4 h of exposure to potassium deprivation and 4 h before significant cell death. Incubation of cerebellar granule cells with an antisense oligodeoxynucleotide directed against NP1 mRNA reduced low potassium-evoked NP1 protein levels by 60% and attenuated neuronal death by 50%, whereas incubation with the corresponding sense oligodeoxynucleotide was ineffective. Furthermore, acute treatment with lithium significantly inhibited both overexpression of NP1 and cell death evoked by low potassium. These results indicate that NP1 is part of the gene expression program of apoptotic cell death activated by nondopolarizing culture conditions in cerebellar granule cells.

In the central nervous system, during normal embryonic development, the number of neurons is adjusted by activation of a built-in gene expression program that kills unnecessary cells in a process described as programmed cell death (1). This process determines the size and shape of the vertebrate nervous system (2), and the cell death that results from it exhibits the morphological features of apoptosis (3). Apoptotic death is characterized by cell shrinkage, nuclear condensation, DNA fragmentation at internucleosomal sites, and degeneration of cell membrane-bound particles that are phagocytized by macrophages without an inflammatory response. Recent evidence indicates that gene expression-dependent apoptosis is also involved in the pathological death of mature neurons observed in various neurodegenerative disorders, such as Alzheimer's disease, or following brain ischemia or traumatic injury of the central nervous system (4–8). Nonetheless, the mechanisms involved in gene expression-dependent apoptotic death of mature neurons are not well characterized.

One of the most documented in vitro models to investigate gene expression-dependent apoptosis in mature neurons is death induced by nondopolarizing culture conditions (9–11). Cerebellar granule cells require depolarizing concentrations of potassium (25–30 mM) to be maintained in culture (12). When these cells are mature, reduction of the extracellular concentration of potassium produces a cell death that is morphologically apoptotic. This cell death evoked by low potassium is associated with DNA fragmentation and requires both new RNA and protein synthesis (9), because addition of protein or RNA synthesis inhibitors within the first 4 h of exposure to potassium deprivation prevents cell death and results in a complete recovery of the damaged DNA (9, 13–15). Likewise, replacement of high concentrations of potassium within 4 h after treatment with low potassium results in no cell loss. However, the activation of the cell death program becomes irreversible in ~50% of cerebellar granule cells after 6 h of exposure to low potassium (14).

In neurons, apoptotic death may be mediated by posttranslational mechanisms as well as by "de novo" expression of death genes. However, it has been proposed that the mechanisms involved in low potassium-mediated apoptosis of granule neurons are similar to those operating in neuronal death during development or following blockade of neuronal activity, which require new mRNA and protein synthesis (9). Consequently, strategies directed to identify genes whose expression is increased before neuronal cells reach the commitment to die may help to identify new targets for neuroprotection. Based on this assumption, we have investigated gene expression during the early phase of the cell death program.

Overexpression of several genes has been associated with cerebellar granule cell death induced by low potassium. Thus, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) overexpression has been shown to be involved in the initiation of the apoptotic process evoked by low potassium (16, 17). Other genes whose overexpression has been found to mediate apoptosis by potassium deprivation include those for the ICE-re-
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labeled protease CPP32 (18), c-Jun (10), Erk-1 (19), and the transcription factor of cyclin-dependent kinases E2F-1 (20). However, the identification of other genes of the neuronal death program is necessary to understand the relationship among the different biochemical mechanisms of the apoptotic signal pathway activated by reduction of synaptic activity.

Using a differential gene display technique (21), we have investigated gene expression evoked by low potassium in cerebellar granule cells. We now report that treatment with low potassium induces the overexpression of neuronal pentraxin 1 (NP1), a gene that was originally identified and isolated as a rat protein that mediates the calcium-dependent uptake of the snake venom toxin, taipoxin (22). NP1 encodes a glycoprotein of apparent molecular mass of ~50 kDa that is predicted to be secreted and whose expression is restricted to the nervous system (22). Our studies demonstrate that antisense oligodeoxyribonucleotides against NP1 inhibit low potassium-induced cell death. Thus, the present results provide evidence of a new function for NP1 and indicate that NP1 is part of the gene program of apoptotic death in cerebellar granule cells kept under nondepolarizing culture conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Primary cultures of cerebellar granule neurons were prepared from 7-day postnatal Harlan Sprague-Dawley rat pups (Harlan) as described previously (23, 24). Procedures involving animals and their care were approved by the ethics committee of the University of Barcelona and conducted in conformity with institutional guidelines that are in compliance with national (Generalitat de Catalunya decree 214/1997, DOGC 2450) and international (Guide for the Care and Use of Laboratory Animals, National Institutes of Health publication 85-23, 1985) laws and policies. Cells were dissociated in the presence of trypsin (1985) and DNase I, and plated in dishes coated with poly-L-lysine (100 μg/ml). The primary cultures were kept at 37 °C in a humidified incubator with 5% CO2, 95% air and remained undisturbed until experiments were performed. The replication of non-neuronal cells was prevented by addition of 10 μM cytosine-D-arabinofuranoside to the culture medium 24 h after plating. Cells were used for experiments after 8 days in culture (8DIV).

**Induction of Neuronal Death by Low Potassium—**Previous studies have shown that cerebellar granule cells maintained in medium supplemented with 25 mM K+ undergo apoptotic death when switched to 5 mM K+ (9). In addition, exposure of cerebellar granule cells to fresh serum-containing medium triggers excitotoxicity (25). This neurotoxicity does not occur if conditioned medium is used (25). Thus, after 8 days in culture, the medium in which cerebellar granule cells had grown, referred to as conditioned medium (Scconditioned medium), was replaced with one of the following media: fresh unconditioned serum-free medium supplemented with 25 mM potassium (S K+) or fresh unconditioned serum-free medium containing 5 mM potassium (S K−). Immediately after replacement, cells were incubated at 37 °C for different times up to 24 h.

Treatments with lithium and antisense oligodeoxyribonucleotides (ODNs) were performed at 8DIV immediately after the replacement of the media described above. LiCl was added to the cultures at a concentration of 5 mM K+ (9). In pilot experiments, we found that the optimal concentration of ODNs was 10 μM.

**Oligodeoxyribonucleotide Synthesis—**A 21-base-long phosphorothioate antisense ODN against the NP1 mRNA and its corresponding sense ODN were obtained from Roche Molecular Biochemicals. The sequences were 5′-GCCGTGGCCGCAGCCGCAGCG-3′ for the antisense ODN (NP1AS) and 5′-CTGGCGCGCGCCGCAGCGC-3′ for the corresponding sense ODN (NP1S). The phosphorothioated nucleotides are underlined. The NP1 antisense ODN sequence corresponds to nucleotides 4–24, which immediately follow the first initiation codon of the coding sequence of the NP1 cDNA.

**Determination of Cell Death—**Cell death was assessed using propidium iodide (PI) staining. PI is excluded by the plasma membrane of viable cells. Injury to the cytoplasmic membrane allows the entry of PI, which, by interacting with nuclear DNA, yields a bright red fluorescence. In time-course experiments, PI fluorescence was measured in 24-well plates using a CytoFluor 2350 scanner (Millipore, Barcelona, Spain) with 530 nm (25-nm band pass) excitation and 645 nm (40-nm band pass) emission filters. The percentage of nonviable cells was measured using a modification of the method described by Rudolph et al. (26). Base-line fluorescence (I0) was measured immediately after treatment with the corresponding concentration of PI (1 μM). Subsequent fluorescence readings were obtained at different times after the beginning of treatment. At the end of the experiment, cells were permeabilized with 375 μM digitonin for 10 min at 37 °C to obtain the maximum fluorescence corresponding to 100% of cell death (I0). Percentage of cell death was calculated as follows: % cell death = 100 × (I0/If)max, where If is fluorescence at any given time. Cells were kept in the incubator between measurements. In some experiments, percentage of dead cells was measured counting PI stained over a total number of cells using simultaneous fluorescence and phase contrast observation in an epifluorescence microscope. In these experiments, cells were incubated with 10 μM PI for 30 min and fixed in 3.7% paraformaldehyde for 20 min at room temperature before addition of a final glycerol protective layer.

**Gene Expression Analysis—**Gene expression was assessed with the differential display technique as described by Liang and Pardee (21) using the RNA image kit from GenHunter. Total RNA from cerebellar granule cells exposed to S K+ and S K− for 2 and 4 h was isolated using the Ultraasy mini kit (Qiagen) and treated with DNase I (GenHunter, Nashville, TN). First-strand cDNA synthesis and 32P-labeling differential display PCR were performed using the RNA image kit (GenHunter). The PCR reactions were performed in duplicate for each treatment. The amplified cDNAs were resolved by electrophoresis using a 6% denaturing polyacrylamide gel. After immobilizing the gel on Whatman no. 3MM paper and drying it for 30 min under vacuum at 80 °C, the gel was exposed to X-Omat AR film (Eastman Kodak Co.) overnight. The autoradiogram and the dried gel were oriented with needle punches to be able to locate in the gel the bands identified in the film. After developing the film, the patterns of amplified cDNA bands were compared among treatments. We chose cDNA bands exhibiting a higher intensity in S K− compared with S K+, which indicates that low potassium treatment induces the overexpression of a gene represented in the band. The cDNA bands of interest were excised from the gel, reamplified by PCR with the same set of primers and PCR conditions used in the mRNA display but with a higher concentration of dNTPs, and ligated into the pCR-TRAP cloning vector (GenHunter). Ligated plasmids were transformed into DH-competent cells and plated on LB plates containing 20 μg/ml tetracycline. The pCR-TRAP vector includes a tetracycline-dependent positive selection of plasmids with DH-competent cells. Only recombinant plasmids confer antibiotic resistance.

**Reverse Northern Dot Blot and Northern Blot—**To verify overexpression of the cDNA fragments identified by differential display, we used two different procedures: reverse Northern dot blot and Northern blot. Bands excised from the gel may contain different cDNA species. To identify the cDNA that is overexpressed in the band, we used the reverse Northern dot blot technique (27). This procedure allows the isolation of efficiently expressed mrRNAs, from bands excised from a differential display gel. For reverse Northern dot blot experiments, tetracycline-resistant colonies were randomly picked from each plate and lysed by boiling in 50 μl of lysis buffer (0.1% Tween 20 in TE buffer, pH 8.0). The cloned cDNA fragments were amplified using primers flanking the cloning site of the vector. Each PCR product was dot-blotted onto duplicate nylon membranes using a microfiltration system. The membranes were UV-cross-linked and probed with total [32P]cDNA. The [32P]cDNA probes were prepared by reverse transcription of 20 μg of total RNA obtained from cerebellar granule cells control (S K+) and treated with low potassium (S K−) for 4 h, in the presence of [α-32P]dCTP (3000 Ci/mmol, PerkinElmer Life Sciences). Equal counts (5–10 × 106 cpm) of the cDNA probes from each treatment, (S K+) and (S K−), were heat-denatured and used to probe the duplicate blots.

Once cDNA overexpression was confirmed by reverse Northern dot blot, we performed Northern blots to verify whether the selected cDNAs represent overexpression of a single mRNA. Northern blot experiments were also performed to study time course of NP1 expression. Total RNA was isolated using TRIzol reagent (Life Technologies, Inc.). Denatured RNA was separated on a 1% agarose gel (20 μg of total RNA). Cerebellar granule cells control (S K+ and S K−) and treated with low potassium (S K−) were electrophoresed in 1.3% agarose and 0.66× formaldehyde gels, transferred to nylon membranes (Hybond-XL, Amersham Pharmacia Biotech), and the RNA was fixed to the membranes by baking for 2 h at 80 °C. Hybridization with 32P-labeled probes and washing conditions were performed as described by the membrane manufacturer. Filters were
exposed to BioMax films (Amersham Pharmacia Biotech) with intensifying screens for 12–48 h at ~80 °C.

**Preparation of NP1 and β-Actin Probes**—The NP1 probe used for Northern blot analysis corresponds to nucleotides 5127–5339 of the NP1 cDNA. The NP1 probe was obtained by PCR amplification of a positive clone identified by reverse Northern dot blot. The PCR product was electrophoresed in agarose and purified using the QIAEX II gel extraction kit (Qiagen) and α-32P-labeled using Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech). The β-actin probe was obtained by digestion of a pUC19 vector containing a 1.9-kilobase pair human β-actin insert between BamHI sites. Densitometric values of NP1 and β-actin mRNA bands were obtained using Kodak DS1 computer software and were normalized with the densitometric values of the corresponding β-actin mRNA band.

**Data Base Searches and Nucleotide Alignment**—DNA sequencing was performed with Thermo-Sequenase (Amersham Pharmacia Biotech) using an ABI Prism 377 fluorescent sequencing instrument at the Serveis Científics Tecnics (University of Barcelona, Barcelona, Spain). Data base searches and sequence comparisons were performed using BLAST and BLASTX search servers at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD).

**SDS-PAGE and Western Blot Analyses**—After the corresponding treatments, cerebellar granule cells were solubilized in lysis buffer (5 mM Tris-HCl, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 2 mm aprotinin, 1 μg/ml apristatin, 100 μg/ml phenylmethylsulfonyl fluoride) and briefly sonicated. The homogenate was centrifuged at 15,000 × g for 20 min at 4 °C. Total protein concentration was determined using the BCA protein assay kit (Pierce). The polypeptides were separated on 10% SDS-polyacrylamide gel electrophoresis and then electroblotted onto PVDF membranes (Millipore, Bedford, MA) according to the manufacturer’s protocol. Blots were preincubated with 5% nonfat dry milk in Tris-buffered saline before immunostaining. For specific immunodetection of NP1 protein, mouse anti-rat NP1 monoclonal antibody (Transduction Laboratories, Los Angeles, CA) and rabbit anti-rat polyclonal NP1 antibody (provided by Drs. Carsten Hof and Paul Worley, Department of Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD), were used at their appropriate concentrations in a solution containing 0.5% nonfat dry milk and 1% bovine serum albumin in Tris-buffered saline containing 1% Tween 20. Peroxidase-conjugated goat anti-mouse IgG (Transduction Laboratories, Los Angeles, CA) or peroxidase-conjugated mouse anti-rabbit IgG (Sigma) were used as secondary antibodies. In addition to the measurement of the amount of protein before loading, we used a rabbit anti-actin antibody (Sigma) to control for the amount of protein loaded. Immunoreactive polypeptides were visualized using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech). Quantification of the intensity of the bands on the films was performed with Kodak DS1 computer software. Densitometry values of the bands representing NP1 immunoreactivity were normalized with the values of the corresponding actin bands.

**Statistical Analysis**—Results are expressed as mean ± S.E. of at least three separate experiments. Statistical significance of differences was examined using independent t tests or using one-way analysis of variance when required. Post hoc multiple comparisons were performed using Student-Newman-Keuls tests.

**RESULTS**

**Exposure to Low Potassium Induces Cell Death in Mature Cerebellar Granule Cells**—Treatment of mature cerebellar granule cells with low potassium resulted in a time-dependent increase in neuronal death, measured with propidium iodide fluorescence (Fig. 1). The loss of neurons was ~60%, 24 h after switching from high to low extracellular concentration of potassium. In comparison, in the same time period, control cultures kept in conditioned medium containing serum and high potassium (S2K+) exhibited 15% cell death (Fig. 1). As additional controls, we used cultures in which conditioned medium was replaced with fresh medium supplemented with high (25 mM) potassium, but without serum (S-K+). In these conditions, cell death was 20% after 24 h of treatment. Serum removal did not have a significant effect on cell death until after 12 h of treatment. Moreover, after 24 h of treatment, cell death by serum removal was only 7% compared with undisturbed S2K+ controls. Treatment with low potassium (5 mM) did not produce any significant cell death until after 8 h of treatment compared with controls. However, 24 h of exposure to low potassium (S K+) induced 40% cell death, compared with exposure to serum-free medium containing high potassium (S2K+).

![FIG. 1. Time course of neuronal death by low potassium in mature cerebellar granule cells.](Image)

**Neuronal Pentraxin 1 Gene Expression Is Up-regulated by Low Potassium in Cerebellar Granule Cells**—To identify genes whose expression is induced before neuronal death, we systematically compared mRNA display patterns between cerebellar granule cells exposed to high potassium (S2K+), as controls, and cells treated with low potassium (S-K+). RNA from cultures treated with either S2K+ or S-K+ was isolated after 2 and 4 h of treatment and subjected to differential gene display analysis. α-32P-labeled PCR was performed with 24 different 5′-anchored primers combined with each one of three different 3′-anchored primers, for a total of 72 different primer combinations. PCR reactions were performed in duplicate for each treatment group and time point. The analysis of differential gene expression was performed by comparing the band pattern of all these primer combinations for each treatment. We chose only those bands that showed a consistent differential expression, in both duplicates and in the two time points analyzed, between the two treatments. We found 102 bands that in both duplicates exhibited consistent differential expression between high and low potassium treatments at both 2 and 4 h of treatment. Among all of these, 62 bands indicated mRNA overexpression. We chose only those bands that exhibited at least a 4-fold higher densitometric intensity in low over high potassium-treated cultures. We amplified and sequenced the cDNA species from 12 bands that attained the criterion difference between high and low potassium treatments at both time periods. Finally, we isolated a cDNA band that showed overexpression after both 2 and 4 h of low potassium treatment, named AP21G1 (Fig. 2). To identify the cDNA fragment overexpressed in this band, several clones obtained from the
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NP1 (22, 28). In cerebellar granule cells treated with either conditioned medium or serum-free medium with high potassium, NP1 protein levels are low but detectable (Fig. 4C). However, exposure to low potassium for 6 h induced a marked increase in the levels of NP1 (Fig. 4C). Subsequent studies using a rabbit polyclonal antibody against NP1 showed a unique band of immunoreactivity and further confirmed that 6 h of treatment with low potassium increases the levels of NP1 protein.

Time-course Analysis of NP1 mRNA Expression and Protein Levels Induced by Low Potassium—The coordinated program of biochemical events that leads to cell death is activated immediately after exposure to nondepolarizing culture conditions. However, cytoplasmic membrane damage and neuronal loss is detected only after 8 h of exposure to nondepolarizing culture conditions (Fig. 1). Thus, we studied the expression of NP1 during the period of the first 8 h of exposure to low potassium, before there is any significant membrane damage and granule cell loss.

First, expression of NP1 mRNA was examined by Northern blot analysis as a function of time of exposure to low potassium (Fig. 5A). Expression of NP1 gene in cerebellar cultures was up-regulated at 2 h after potassium deprivation and exhibited a peak of expression at 6 h of treatment (Fig. 5B). The levels of β-actin mRNA remained relatively constant throughout the time course examined.

The levels of NP1 protein were also studied by Western blot analysis as a function of time of exposure to low potassium (Fig. 5C). A statistically significant increase in the levels of NP1 was first observed after 4 h of potassium deprivation. The amount of NP1 protein peaked after 4 h of exposure to low potassium and the peak levels were sustained for up to 8 h of treatment.

Fig. 2. Differential display analysis of gene expression between high and low extracellular potassium treatment in cerebellar granule cells. Total RNA was extracted from 8DIV cerebellar granule cultures after 2 and 4 h of high (S K+) or low potassium (S K-) treatments and subjected to differential display analysis. The autoradiogram shows the band pattern obtained from [α-32P]dATP-labeled differential display reactions performed in duplicate using 5'-AAGCTTTTTTTTTTTG-3' as an anchored primer (H-T,1,G) and 5'-AAGCTTTTCTCTGG-3' as a random arbitrary primer (H-AP21). The arrow indicates a band (AP21G1) corresponding to a cDNA fragment that is overexpressed both after 2 h (lanes 3 and 4) and 4 h (lanes 7 and 8) of potassium deprivation.

A

B

Fig. 3. Reverse Northern dot blot of clones from AP21G1 band. A, the cDNA from the band AP21G1 represented in Fig. 2 (lane 8) was excised, reamplified, and ligated into the pCR-TRAP cloning vector. The PCR products from five randomly picked colonies (AP21G1-1, -2, -3, -4, and -5) were blotted onto duplicate filters. One of the filters (S K+) was hybridized with 32P-labeled cDNA from cerebellar granule cell cultures control. The other filter was hybridized with 32P-labeled cDNA from cerebellar granule cells treated with low potassium for 4 h (S K-). B, nucleotide sequence of AP21G1–1 clone cDNA fragment. The cDNA inserts of the AP21G1–1, -2, -3, and -5 clones showing differential expression were sequenced. The nucleotide sequence of the four cDNA inserts was identical. The sequence of the primers used in differential gene display analysis are underlined (H = HindIII site at the 5’ end of the primers).

re amplified cDNA from this band were subjected to reverse Northern dot blot (Fig. 3A). Reverse Northern analysis showed that 4 out of 5 clones (AP21G1–1, -2, -3, and -5) obtained from the AP21G1 band exhibited higher signal when hybridized with cDNA from low potassium treated cultures than when hybridized with cDNA from control cultures (Fig. 3A). This result indicates that these clones correspond to the gene whose overexpression was induced by treatment with low potassium and, therefore, the gene that confers the high intensity signal to the AP21G1 band (Fig. 2). PCR amplification of the inserts of the positive clones revealed a fragment of ~200 base pairs in the four cDNAs. The sequence of the cDNA inserts of the four positive clones was identical, confirming that reverse Northern actually identified a single cDNA that was overexpressed in the AP21G1 band. The sequence of this cDNA overexpressed by low potassium treatment is 213 base pairs long and it is represented in Fig. 3B. A search of the GenBank® data base at the National Center for Biotechnology Information using the BLAST program revealed that the AP21G1 fragment is 100% homologous with the 3’-untranslated region of rat NP1 mRNA (GenBank® accession no. U18772) (22).

To further confirm that treatment of cerebellar granule cells with low potassium induces overexpression of the NP1 gene, we performed Northern blot analysis (Fig. 4A). The cDNA fragment homologous to NP1 mRNA was 32P-labeled and used as hybridization probe. A single NP1 mRNA transcript was detected at ~5.5 kilobases, which is consistent with the expected size of the NP1 mRNA (5341 bases) (22). Comparison with untreated cultures (S K+) showed that overexpression of the NP1 gene was induced only in cultures treated with low potassium (S K-). The level of NP1 expression in cultures subjected to serum deprivation (S K+) was as low as in untreated cells. Densitometric analysis of Northern blot autoradiograms indicated that the ratio of NP1 over β-actin mRNA was ~10-fold higher in cultures exposed to low potassium for 4 h compared to both S K+ and S K- controls (Fig. 4B).

To examine whether overexpression of NP1 mRNA is associated with an increase in NP1 protein levels, we performed Western blot analyses using a mouse monoclonal antibody against NP1. A unique band of immunoreactivity of apparent molecular mass of ~50 kDa was detected in extracts of cerebellar granule cell cultures, consistent with the expected size of NP1 (22, 28). In cerebellar granule cells treated with either conditioned medium or serum-free medium with high potassium, NP1 protein levels are low but detectable (Fig. 4C). However, exposure to low potassium for 6 h induced a marked increase in the levels of NP1 (Fig. 4C). Subsequent studies using a rabbit polyclonal antibody against NP1 showed a unique band of immunoreactivity and further confirmed that 6 h of treatment with low potassium increases the levels of NP1 protein.

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Low Potassium Evoked Cell Death in Cerebellar Granule Cells—To examine the role of NP1 in the neurotoxicity induced by exposure to low potassium, we used the antisense knock-down strategy. Treatment with an antisense ODN (NP1AS) against NP1 mRNA significantly inhibited cell death induced by 24 h of treatment with low potassium by ~60% when compared with cultures treated with vehicle (Fig. 6A). In contrast, treatment with the corresponding sense ODN (NP1S) was ineffective. At the same time, treatment with antisense ODN against NP1, but not sense, significantly reduced the levels of NP1 protein induced by potassium deprivation by ~60%. Thus, the neuroprotective effect of the antisense ODN against NP1 is preceded by inhibition of the overexpression of NP1 evoked by low potassium (Fig. 6, B and C).

Lithium Protects Mature Cerebellar Granule Cells from Cell Death and Inhibits Overexpression of NP1 Protein Induced by Low Potassium—Acute treatment with lithium (5 mM) significantly inhibited cell death induced by low potassium in mature cerebellar granule cells. Lithium reduced cell death after 24 h of treatment with low potassium by ~60%, when compared with the respective control (Fig. 7A). To determine whether the neuroprotective effects of lithium are associated with modulation of NP1 expression, we performed Western blot experiments in cerebellar granule cells treated with potassium deprivation in the presence of lithium (Fig. 7B). Densitometric analysis of the ratio between NP1 over actin immunoreactivity showed that lithium significantly reduced by ~64% the levels of NP1 protein evoked by low potassium treatment (Fig. 7C).

DISCUSSION

Consistent with results of previous studies (9, 13, 14), reduction of the extracellular concentration of potassium induced a pronounced cell death after 24 h of treatment (Fig. 1). Time-course analysis of this process indicated that viability of cerebellar granule cells was unaffected during the first 6 h of treatment. Significant cell death was first detected only after 8 h of exposure to low potassium, and this effect increased with time (Fig. 1). These results are consistent with previous temporal analyses of the biochemical and molecular events that occur during the process of cell death evoked by low potassium in cerebellar granule neurons (9–11, 14, 29). Results from these previous studies indicate that potassium deprivation induces a coordinated program of events before cerebellar granule cells commit to die and apoptotic death becomes irreversible. Inhibitors of protein and RNA synthesis reverse DNA fragmentation and block cerebellar granule cell death when added within the first 3–4 h of treatment (14, 15). This indicates that "de novo" expression of death genes, rather than inhibition of expression of survival genes, mediates cell death by low potassium.

In the present study, we have investigated gene expression during the early phase of the death program induced by potassium deprivation, to identify genes specifically involved in neuronal death triggered by the lowering of synaptic activity. Thus, after isolation and identification of cDNAs differentially expressed by low potassium, we focused on genes whose expression is restricted to the nervous system. First, the analysis of differential gene expression revealed 102 bands that showed a consistent difference between high and low potassium treatments. Among these bands, 62 exhibited mRNA overexpression and only 12 bands attained the criteria of an at least 4-fold higher expression in nondepolarizing than in depolarizing culture conditions. After reamplification and subcloning, the cDNA species obtained from these 12 bands were sequenced and compared with the GenBank data base. Blast searches revealed that among the cDNAs with high homology to known genes, only one of them (Fig. 3B), corresponding to rat NP1 was

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Fig. 4. Low potassium increases NP1 protein and mRNA levels in mature cerebellar granule cell cultures. A, Northern blot analysis showing overexpression of NP1 mRNA after 4 h of potassium deprivation. Conditioned medium (S−K+) or fresh unconditioned serum-free medium supplemented with 50 mM potassium (S+K+) or fresh unconditioned serum-free medium containing 5 mM potassium (S−K+). Total RNA (20 μg/lane) was isolated for each treatment and run in a 1.3% agarose gel and transferred to nylon filters. The filters were hybridized with the 32P-labeled AP21G1–1 cDNA fragment homologous to NP1 identified by differential display. The blot was also rehybridized to β-actin probe as control. B, quantitative analysis of NP1 mRNA expression normalized with β-actin mRNA. The autoradiographic signal intensities were determined by densitometric analysis. The ratio of NP1 over β-actin intensity was expressed as percentage of control values. Values are the mean ± S.E. of three independent experiments. *, significantly different from control values (S−K+), p < 0.05, Student’s t test. C, Western blot showing the immunoreactivity of two different antibodies against NP1. Proteins were extracted from undisturbed mature cerebellar granule cells (S−K+), or after 6 h of high (S+K+) or low potassium (S−K+). Protein extracts were run in a 10% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with two different primary antibodies: a mouse anti-NP1 monoclonal antibody and a rabbit anti-NP1 polyclonal antibody. Chemiluminescent detection shows a unique band of ~50 kDa. Actin was used as control for protein loading.

(Fig. 5D). The levels of actin protein did not significantly change between treatments during the period investigated.
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expressed exclusively in the nervous system. Thus, in subsequent experiments, we investigated the effect of treatment with low potassium on NP1 expression.

Our results show that treatment of cerebellar granule cells with low potassium induces a >15-fold increase of NP1 mRNA compared with cells kept in high potassium either with or without serum (Fig. 5B). The increase in NP1 mRNA transcripts is followed by an increase in NP1 protein levels between 3- and 6-fold over control levels (Figs. 5D and 6C). This difference between the amounts of NP1 message and protein is consistent with previous findings showing that in cortical or cerebellar homogenates NP1 protein is rare (22). The discrepancy between the high amount of message and the relatively low levels of NP1 protein has been interpreted as indicating a rapid turnover, as might be expected for a secreted protein (22).

The increased expression of NP1 protein peaks after 4 h of exposure to low potassium (Fig. 5, C and D). This up-regulation of NP1 precedes cytoplasmic membrane damage by at least 4 h, and the peak of NP1 protein corresponds approximately to the period when cerebellar granule cells commit to die (14). Induction of other genes has been reported to occur before commitment to die. For example, expression of c-Jun mRNA is induced rapidly and peaks at 2 h after survival signal withdrawal (15). Likewise, expression of GAPDH reaches a maximum after 2 h of exposure to low potassium (17). At present, the relationship between these genes and NP1 is unknown, but comparison of their temporal pattern of expression after treatment with low potassium suggests that NP1 is downstream of both c-Jun and GAPDH expression.

To investigate whether overexpression of NP1 is directly involved in low potassium-evoked cell death, we used the antisense knockdown strategy. We found that antisense oligodeoxyribonucleotides against NP1 mRNA markedly inhibited the increase in NP1 protein and significantly reduced cerebellar granule cell death evoked by low potassium (Fig. 6). In contrast, NP1 sense oligonucleotides did not significantly modify neither cell death nor NP1 protein levels induced by low potassium (Fig. 6). These results strongly suggest that the neuroprotective effects of these antisense oligonucleotides are due to knockdown of NP1 gene expression and provide evidence to indicate that NP1 is part of the cell death program induced by nondepolarizing culture conditions in cerebellar granule cells.

Further evidence showing the involvement of NP1 in low potassium-evoked cerebellar granule cell death was obtained after neuroprotective treatment with lithium. Treatments that inhibit low potassium-evoked apoptosis such as insulin-like growth factor or brain-derived neurotrophic factor suppress expression of cell death genes by activating different intracellular signaling pathways (30–32). Likewise, lithium promotes survival of mature cerebellar neurons (33) by a dual mechanism comprising increased transcription of survival genes such as bac2 and inhibition of expression of pro-apoptotic genes such as bax and p53 (34–36). In the experiments reported here, we found that acute treatment with lithium significantly inhibits the expression of NP1 evoked by low potassium and this effect is followed by a significant reduction of cerebellar granule cell death (Fig. 7). Thus, inhibition of NP1 expression by lithium is associated with the neuroprotective effects of this drug.

The pentraxin family of proteins may be divided into two structural classes based on size (see Ref. 37 for review). Recent evidence indicates that long pentraxins such as NP1, which share a high homology at the C-terminal half, possess diverse functions. Thus, NP1 was originally identified as a calcium-dependent binding protein for the snake venom toxin taipoxin and was found to be expressed exclusively in neurons (22). Based on NP1’s homology to short pentraxins such as C-react-
tive protein and serum amyloid P protein, the function of NP1 has been proposed to mediate the uptake of synaptic material during synapse remodeling (22, 28, 38). The results reported here showing that NP1 is involved in cerebellar granule cell death induced in nondepolarizing culture conditions provide evidence of a new function for NP1. On the other hand, neuronal activity-related pentraxin (Narp), also called neuronal pentraxin 2, another member of the long pentraxin family, has been proposed to mediate the synaptic clustering of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate glutamate receptors at a subset of excitatory synapses, supporting thereby a synaptogenic signaling function (39, 40). Interestingly, Narp is rapidly induced and dynamically regulated by depolarizing conditions (39, 40). However, in contrast with the marked increase that treatment with low potassium evokes on NP1 expression, such treatment did not have the same effect on the expression of Narp (results not shown). This raises the possibility that Narp and NP1 could function as part of genetic switch to sensor changes in synaptic activity; Narp associated with neurite outgrowth after depolarization and NP1 associated with neuronal death under nondepolarizing conditions. Experiments using NP1 and Narp gene transfection into host neurons will allow to investigate this hypothesis and to deter-

FIG. 6. NP1 antisense ODNs inhibit cerebellar granule cell death evoked by low potassium. Mature (8DIV) cerebellar cells were incubated in serum-free medium supplemented with 25 mM potassium (S′K′), or serum-free medium containing 5 mM potassium (S′K′). Antisense ODNs (NP1AS, 10 μM, AS) or corresponding sense ODNs (NP1S, 10 μM, S) were added immediately after switching from high to low extracellular concentration of potassium. A, neuroprotective effect of NP1 antisense ODNs on neuronal death by low potassium. The number of dead cells was assessed by PI staining after 24 h of exposure to the different treatments. Values are the mean ± S.E. of three independent experiments. +, significantly different from S′K′; * , significantly different from S′K′; p < 0.05, using independent t test analysis. B, Western blot showing the effect of antisense ODNs on the increase of NP1 protein levels evoked by low potassium. Protein extracts were run in a 10% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with mouse anti-NP1 antibody. Actin was used as control for protein loading. C, quantitative analysis of the effects of antisense and sense ODNs on the increase of NP1 protein levels evoked by low potassium. NP1 protein was normalized with actin. The autoradiographic signal intensities were determined by densitometric analysis. The ratio of NP1 over actin intensity was expressed as percentage of control values. +, significantly different from S′K′; * , significantly different from S′K′; p < 0.05, using independent t test analysis.

FIG. 7. Effects of lithium on cell death and NP1 protein levels evoked by low potassium in mature cerebellar granule neurons. Mature (8DIV) cerebellar cells were incubated in high (S′K′), or low potassium (S′K′). Lithium (Li, 5 mM) was added in both treatments as indicated. A, lithium inhibits cell death evoked by low potassium. Cell death was assessed with PI fluorescence after 24 h of treatment. Cell death was expressed as a percentage of maximum cell death obtained with digitonin. Values are mean ± S.E. of three independent experiments. * , significantly different from S′K′; +, significantly different from S′K′; p < 0.05, Student’s t test. B, Western blot showing the effects of lithium on the increase of NP1 levels evoked by low potassium. Protein extracts were run in a 10% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with mouse anti-NP1 antibody. Actin was used as control for protein loading. C, quantitative analysis of the effects of lithium on the increase of NP1 protein levels evoked by low potassium. NP1 protein was normalized with actin. The autoradiographic signal intensities were determined by densitometric analysis. The ratio of NP1 over actin intensity was expressed as percentage of control values. +, significantly different from S′K′; * , significantly different from S′K′; p < 0.05, using independent t test analysis.
mine whether NP1 overexpression is sufficient to elicit its apoptotic effect or if it requires the concerted action of other cellular proteins.

In summary, the present results show that treatment with low potassium induces overexpression of NP1 before cell death. In addition, inhibition of NP1 overexpression by either antisense oligodeoxyribonucleotides or lithium prevents cerebellar granule cell death evoked by low potassium. These findings provide evidence of a new function for NP1 and indicate that NP1 is part of the gene program that leads to apoptotic cell death in cerebellar granule cells kept under nondepolarizing culture conditions.

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