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Pleiotrophin mediates the neurotrophic effect of cyclic AMP on dopaminergic neurons: Analysis of suppression-subtracted cDNA libraries and confirmation in vitro

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Abstract

To better understand the particular vulnerability of mesencephalic dopaminergic neurons to toxins or gene mutations causing parkinsonism, we have taken advantage of a primary cell culture system in which these neurons die selectively. Antimitotic agents, such as cytosine arabinoside or cAMP, prevent the death of the neurons by arresting astrocyte proliferation. To identify factors implicated in either the death of the dopaminergic neurons or in the neuroprotective effect of cAMP, we constructed cDNA libraries enriched by subtractive hybridization and suppressive PCR in transcripts that are preferentially expressed in either control or cAMP-treated cultures. Differentially expressed transcripts were identified by hybridization of the enriched cDNAs with a commercially available cDNA expression array. The proteoglycan receptors syndecan-3 and the receptor protein tyrosine phosphatase zeta/beta were found among the transcripts preferentially expressed under control conditions, and their ligand, the cytokine pleiotrophin, was highly represented in the cDNA libraries for both conditions. Since pleiotrophin is expressed during embryonic and perinatal neural development and following lesions in the adult brain, we investigated its role in our cell culture model. Pleiotrophin was not responsible for the death of dopaminergic neurons under control conditions, or for their survival in cAMP-treated cultures. It was, however, implicated in the initial and cAMP-dependent enhancement of the differentiation of the dopaminergic neurons in our cultures. In addition, our experiments have provided evidence for a cAMP-dependent regulatory pathway leading to protease activation, and the identification of pleiotrophin as a target of this pathway.

Keywords: Parkinson's disease; Mesencephalon; Differentiation; Cell death; Transcriptome; Macroarray; HB-GAM; HARP; Syndecan-3; RPTP-ζ/β

Introduction

In humans, degeneration of nigrostriatal dopaminergic (DA) neurons in the mesencephalon underlies the major motor dysfunctions characteristic of parkinsonism. These neurons are vulnerable to a number of toxic agents, such as the complex I inhibitors 1-methyl-phenylpyridinium (MPP^+), a metabolite of the prodrug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or rotenone (Heikkila et al., 1985), as well as to mutations in several genes, most notably alpha-synuclein (Polymeropoulos et al., 1997), parkin (Kitada et al., 1998), DJ-1 (Bonifati et al., 2003), and PINK1 (Valente et al., 2004). The mechanisms by which these ubiquitously distributed toxins or mutations cause the death of this population of neurons is not well understood, however, and the cause of DA neurodegeneration in idiopathic Parkinson's disease is completely unknown.

To identify factors that might shed light on the particular vulnerability of DA neurons, we have analyzed an in vitro

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model, primary cultures of mesencephalon, in which selective degeneration of these neurons occurs (Michel and Agid, 1996; Michel et al., 1995, 1997, 1999; Mourlevat et al., 2003). In this model, the DA neurons degenerate progressively and selectively over the first 10 days in culture, due to the presence of immature proliferating astrocytes (Mourlevat et al., 2003). Treatment of the cultures with cell-permeable dibutyryl (db) cAMP or agents that increase intracellular cAMP levels prevents the death of the DA neurons, as does the antimitotic drug cytosine arabinoside, by arresting the proliferation of the astrocytes (Michel et al., 1997; Mourlevat et al., 2003). Cyclic AMP also increases the state of differentiation of the neurons (Michel and Agid, 1996).

We have used this model to construct cDNA libraries enriched in transcripts preferentially expressed in our mesencephalic cell cultures: (1) under control conditions in which the astrocytes proliferate and induce the death of the DA neurons (control cDNA library); (2) and under neurotrophic conditions in which astrocyte proliferation and neurotoxicity were suppressed by treatment with dbcAMP (cAMP cDNA library). The technique used was subtractive hybridization with suppressive PCR (Diatchenko et al., 1996) to reduce the presence in the respective libraries of transcripts expressed in both conditions, and to enhance the representation of uniquely or preferentially expressed sequences. Differentially expressed transcripts were identified by hybridization of probes transcribed from each of the cDNA libraries to a commercially available macroarray of identified rat genes reflecting the major cell functions. The combination of suppression subtractive hybridization (SSH) and macroarray screening has been recommended for its efficacy in identifying differentially expressed genes (Beck et al., 2001).

The results of this differential transcriptome analysis led us to examine the role of the cytokine pleiotrophin (PTN) in the cAMP-dependent survival and differentiation of the DA neurons in our mesencephalic cultures.

Materials and methods

Cell cultures and treatments

Animals were treated in accordance with the Declaration of Helsinki and the *Guide for the Care and Use of Laboratory Animals* (U.S. National Institute of Health). Cultures of post-mitotic neurons were prepared from the ventral mesencephalon of Wistar rat embryos dissected at embryonic day 15.5 as previously described (Michel and Agid, 1996). After dissection, pieces of mesencephalic tissue were mechanically dissociated and plated in 24-well culture plates precoated with polyethylenimine (PEI, 1 mg/ml; Sigma-RBI-Aldrich; Saint Quentin Fallavier, France), at a density of 80,000–120,000 cells per 16-mm well. The cells were then maintained in N5 culture medium (Kawamoto and Barrett, 1986) supplemented with 5 mM glucose, 5% horse serum and 0.5% fetal calf serum (FCS), except for the first 3 days in vitro when the concentration of FCS was 2.5%. Cultures were fed every other day by replacing 350 of the 500 μl of medium in the culture wells. Cell-permeable dbcAMP (Sigma-RBI-Aldrich) was added to cultures when the medium was changed to a final concentration of 1 mM.

For some experiments, the cultures were grown on PTN instead of PEI, as previously described (Bernard-Pierrot et al., 2001) with minor modifications. ELISA microtiter plates [96-well, Nunc, Nalge (Europe) Ltd., Hereford, UK] that favor protein adhesion to the culture wells were incubated with PTN (200 ng in 200 μl of PBS) for 6 h at room temperature. Uncoated wells were used for controls. Both PTN-coated and uncoated wells were then incubated with BSA (200 ng in 200 μl PBS) for 1 h at 37°C (Raulo et al., 1992). The mesencephalic cultures were plated at a density of 25,000 cells per well, and cultivated as above.

Construction of SSH cDNA libraries

Suppression subtractive hybridization (Diatchenko et al., 1996) was used to enrich control and cAMP cDNA libraries in differentially-expressed transcripts, using the PCR-select cDNA subtraction kit (Clontech, Franklin Lakes, NJ, USA) as prescribed. Briefly, polyadenylated mRNA was isolated with the RNeasy kit and Oligotex polyT-coupled beads (Qiagen, Courtaboeuf, France) from both control and dbcAMP-treated cultures after 6 days in vitro (DIV6). After reverse transcription, the 5′ ends of two aliquots of the cDNAs to be subtracted were ligated to two different adaptors, then hybridized with an excess of cDNA from the other condition, leaving uniquely expressed sequences as adaptor-ligated single-stranded cDNAs that, when hybridized together, form double-stranded cDNAs with a different primer at each end. These differentially expressed cDNA were then amplified by nested PCR using oligonucleotide primers corresponding to the adaptor sequences.

cDNA macroarray screening

To identify some of the transcripts preferentially expressed in the SSH cDNAs prepared from the control and dbcAMP-treated cultures, four ^{32}P-labeled probes corresponding to the unsubtracted and suppression-subtracted cDNAs of the two conditions were prepared by random-priming (RadPrime DNA Labeling System, Invitrogen, Cergy-Pontoise, France). Each probe was hybridized to an Atlas 1.2 rat gene expression array (Clontech), as specified by the manufacturer. The membranes were then exposed to X-ray film (X-OMAT, Kodak, Paris, France) at −80°C with amplifying screens, for 3 h, 1, 3 and 8 days, and the identity of labeled spots was determined according to the manufacturer's grid.