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Midbrain expression of Delta-like 1 homologue is regulated by GDNF and is associated with dopaminergic differentiation

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Abstract

Affymetrix GeneChip technology and quantitative real-time PCR (Q-PCR) were used to examine changes in gene expression in the adult murine substantia nigra pars compacta (SNc) following lentiviral glial cell line-derived neurotrophic factor (GDNF) delivery in adult striatum. We identified several genes that were upregulated after GDNF treatment. Among these, the gene encoding the transmembrane protein Delta-like 1 homologue (Dlk1) was upregulated with a greater than 4-fold increase in mRNA encoding this protein. Immunohistochemistry with a Dlk1-specific antibody confirmed the observed upregulation with increased positive staining of cell bodies in the SNc and fibers in the striatum. Analysis of the developmental regulation of Dlk1 in the murine ventral midbrain showed that the upregulation of Dlk1 mRNA correlated with the generation of tyrosine hydroxylase (TH)-positive neurons. Furthermore, Dlk1 expression was analyzed in MesC2.10 cells, which are derived from embryonic human mesencephalon and capable of undergoing differentiation into dopaminergic neurons. We detected upregulation of Dlk1 mRNA and protein under conditions where MesC2.10 cells differentiate into a dopaminergic phenotype (41.7±7.1% Dlk1+ cells). In contrast, control cultures subjected to default differentiation into non-dopaminergic neurons only expressed very few (3.7±1.3%) Dlk1-immunopositive cells. The expression of Dlk1 in MesC2.10 cells was specifically upregulated by the addition of GDNF. Thus, our data suggest that Dlk1 expression precedes the appearance of TH in mesencephalic cells and that levels of Dlk1 are regulated by GDNF.

Keywords: Delta-like 1 homologue; Fetal antigen 1; GDNF; Dopamine; Neural progenitor; Immortalization; Differentiation

Introduction

Several *in vitro* and *in vivo* studies have shown that glial cell line-derived neurotrophic factor (GDNF) exerts distinct neurotrophic and pharmacological effects on both intact and injured nigrostriatal dopamine (DA) neurons. GDNF may therefore be a useful agent to prevent the neurodegeneration underlying Parkinson's disease (PD), although the results from the clinical trials are controversial (Sherer et al., 2006). Preservation or regeneration of striatal DA axon terminals is crucial for the recovery of motor function (Bjorklund et al., 2000) and is observed following striatal GDNF administration to the DA axon terminals but not when administrated to the cell bodies in the SNc (Kirik et al., 2000a,b; Oo et al., 2003). Delivery of GDNF by lentiviral vectors to the striatum has shown that stable long-term expression of high levels of GDNF can be achieved in rodents and in primates that are sufficient for almost full protection of the DA neurons in the SNc against toxic damage (Bjorklund et al., 2000; Georgievska et al., 2002; Kordower et al., 2000). In addition to providing protection against lesions, GDNF administration leads to increased DA turnover and to increased release of DA and its metabolites (Kirik et al., 2000b; Martin et al., 1996) and GDNF induces sprouting near the site of

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administration i.e. globus pallidus in lesioned rats after prolonged exposure in the striatum (Georgievska et al., 2002). Despite extensive studies of the functional effects of GDNF on DA neurons *in vivo* very little is understood about the molecular mechanisms underlying the observed effects. The transmembrane protein Dlk1 is expressed in monoaminergic nuclei in the adult brain, including the SNc and the ventral tegmental area (VTA) (Jensen et al., 2001). The extracellular part of Dlk1 corresponds to the soluble protein fetal antigen-1, which was originally isolated from second trimester human amniotic fluid. The soluble form of Dlk1 has been shown to be involved in proliferation and differentiation of various non-neuronal cell types in autocrine/paracrine/juxtacrine fashions (Jensen et al., 1997; Laborda et al., 1993; Smas and Sul, 1993). The role of Dlk1 in the SNc and VTA is of particular interest in relation to PD, which involves progressive degeneration of DA neurons in these regions. In our study, we used Affymetrix GeneChip technology and Q-PCR to identify genes upregulated in the midbrain by intrastriatal injection of a viral vector expressing GDNF. The mRNA for the *Dlk1* gene was identified as one of the most upregulated. The developmental expression and regulation of Dlk1 were further characterized in cells derived from a human mesencephalic cell line (MesC2.10) (Lotharius et al., 2002) as they differentiated into DA neurons in the presence of GDNF. Our results show that Dlk1 is expressed in midbrain neurons just prior to their differentiation into DA neurons and that the expression is enhanced by GDNF.

Materials and methods

Animals and surgical procedures

All work involving experimental animals was conducted according to the guidelines set by the Danish Ethical Committee. Animals were housed in a 12 h light/darkness cycle with access to food and water. Female NMRI mice (weighing 25 g at the time of surgery) were used. VSV-G pseudotyped rLV vectors were produced as previously described (Rosenblad et al., 2003). The mice were anesthetized using Isofluoran and stereotaxically injected with 0.5 μl rLV-GFP or rLV-GDNF of viral stocks diluted to equals titers (2×10^8 TU/ml) along two tracts in the right striatum at the following coordinates (expressed in mm from bregma): (i) AP=+0.5, ML=−2.0, DV=−3.5, TB=0.0 and (ii) AP=+0.5, ML=−2.0, DV=−3.0, TB=0.0. To prepare tissue for Q-PCR, animals were deeply anesthetized with pentobarbital (70 mg/kg), decapitated and the brains quickly dissected out. Animals used for immunohistochemical analysis were also deeply anesthetized using sodium pentobarbital and perfused transcardially with saline at room temperature for 1 min followed by 50 ml ice-cold phosphate-buffered 4% paraformaldehyde (pH 7.2–7.4).

GDNF ELISA

After removing the brains as described above, tissue was prepared and GDNF ELISA was carried out as described previously (Georgievska et al., 2002).

Preparation of RNA

For the GeneChip analysis, RNA was prepared from tissue pieces dissected from the brain SNc region (see Fig. 1A). After dissection, the tissue pieces were instantly frozen in liquid nitrogen and stored at −80 °C. To generate material for the microarray analysis, tissue from 3 animals was pooled and total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. To concentrate RNA and to remove traces of chromosomal DNA, RNase on column DNAse digestion with RNase-Free DNase was used (Qiagen) and RNA subsequently eluted in ultra pure H_2O. The quality of RNA samples for GeneChip analysis was assessed using the Agilent Bioanalyzer 2100 and the RNA 6000LABChip. To extract RNA derived from the ventral mesencephalon of embryonic day (E) 11.5, E13.5, postnatal day (P) 1 and adult mice, tissue pieces dissected from the ventral mesencephalon were instantly frozen in liquid nitrogen and stored at −80 °C before using Trizol. For human cell cultures, total RNA was extracted from either proliferating, 2- and 4-day differentiated MesC2.10 cells plated in media conditions as indicated. The quality of the RNA samples for Q-PCR was estimated from OD260/280 ratio and Agilent Bioanalyzer 2100.

Microarray

RNA samples were amplified according to the MessageAmpTM aRNA protocol (Ambion). Briefly, total RNA was used for first strand cDNA synthesis with a poly-dT primer containing a T7 RNA-polymerase promoter sequence. After second strand synthesis, the resulting dsDNA was purified by phenol/chloroform extraction and used for *in vitro* transcription in the presence of biotin-labeled nucleotides to create labeled cRNA. The 2 μg total RNA used as starting material resulted in production of approximately 30 μg cRNA. After purification and fragmentation, 15 μg biotinylated cRNA was hybridized for 16 h at 45 °C to the Affymetrix Mouse Genome 430A Chip containing almost 22,600 probe sets representing transcripts from over 14,000 well-characterized mouse genes (www.affymetrix.com). The arrays were washed and subsequently stained with a streptavidin–phycoerytrin conjugate using the GeneChip Fluidics Station 400. Scanning was done with the Affymetrix GeneArray Scanner, all according to manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Data acquisition and primary analysis was performed as described previously (Jorgensen et al., 2006). Briefly, two rounds of hybridizations with independent RNA samples were performed. Each scanning generated a CEL file with measurements of intensities corresponding to the hybridization of targets with the respective probes. Primary analysis of the datasets was carried out using the software package GenePublisher (Knudsen et al., 2003). For each probe ID, the Excelfile contained signal values, fold change, *p*-value and gene annotation. To select genes for further analyses, a low-stringency filter was applied. First, the average signal values should be larger than 50 to ensure a detectable expression level. Second, the *p*-value should be smaller than 0.1 to exclude outliers in one of the experiments.