Involvement of the NGFI-A gene in the differentiation of neuroblastoma cells

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Abstract The transcription factor NGFI-A is an early response gene that has been implicated in the regulation of cell growth and differentiation and, more recently, in apoptosis. This gene is expressed in many tissues, and is very abundant in the brain. However, little is known about its functional role in the differentiation of this tissue. In the present work we investigated the role of NGFI-A in serum withdrawal-induced differentiation in N2A neuroblastoma cells. To do so, we studied the effect of NGFI-A antisense oligonucleotides and NGFI-A overexpression on this process. We show that neuroblastoma cells treated with an NGFI-A antisense oligonucleotide do not undergo normal morphological differentiation after serum withdrawal, whereas N2A cells overexpressing this gene extend long neurites, even in the presence of serum. We also show that NGFI-A overexpression is accompanied by an increase in the amount of phosphorylated microtubule-associated protein MAP1B, which has been associated with neurite outgrowth. Our results suggest that the NGFI-A gene plays an important role in neurite extension.

Key words: Differentiation; Neuronal; NGFI-A

1. Introduction

The development and function of the central nervous system require a balance between cellular proliferation, differentiation and cell death [1]. The molecular mechanisms that control neuronal cell differentiation are complex and as yet poorly understood. In mammalian cells, many of the extracellular stimuli that control proliferation, differentiation and cell death result in a rapid but transient induction of a group of genes termed 'immediate early response' genes. This group of genes includes transcription factors such as the protooncogenes c-jun and c-fos [2,3] as well as NGFI-A [4], also known as krox-24, zif-268, egr-1 and TIS-8 [2,5–7]. Transient induction of this group of genes is thus among the very first changes in gene activity observed in response to extracellular stimulation.

The NGFI-A transcript encodes a protein with an apparent molecular weight of 82 kDa containing a sequence-specific DNA-binding domain composed of three tandemly repeated zinc fingers [4]; these latter represent a common feature of many DNA-binding transcription factors. The NGFI-A protein specifically binds to the GCG/TGGGGCG sequence, resulting in the induction of target genes [8]. Like many other early genes, NGFI-A is induced in different cell types by a variety of signals, including growth factors, retinoic acid and seizure induction [4,6,9–11]. Several studies have associated NGFI-A gene expression with the regulation of cell proliferation and different aspects of development [2,12–16]. It has also been shown that NGFI-A plays a role in macrophage differentiation [17,18] and programmed cell death in melanoma cells [19,20], although the biological role and the regulatory mechanisms of NGFI-A in mammalian cells remain to be fully elucidated.

Several lines of evidence suggest that the NGFI-A gene may play an important role in the central nervous system. Although the NGFI-A gene is expressed ubiquitously in rat tissues, the adult brain displays the highest levels of expression and an elevated concentration of NGFI-A transcripts can also be detected in many areas of the developing brain [16]. Further, NGFI-A gene expression is highly responsive to neuronal stimulation and the levels of NGFI-A are excellent markers of neuronal activity [11]. Earlier, we have also reported an up-regulation of NGFI-A gene expression by thyroid hormone, whose receptors probably play a role in processes such as neuronal proliferation, the determination of cell lineages and the establishment of interneuronal connections [16,21–23]. To date, however, no research has addressed the involvement of NGFI-A in the differentiation in cells of neuronal origin.

In the present work we studied the effect of NGFI-A on the differentiation of Neuro2A cells. These cells can be induced to differentiate by serum withdrawal. Our results reveal that the morphological differentiation of N2A cells can be blocked by the presence of NGFI-A antisense oligonucleotides in the culture medium. This effect must be specific since no effect was observed with NGFI-A sense or c-myc antisense oligonucleotides. Moreover, stably transfected N2A cells overexpressing the NGFI-A protein extend very long neurites and the number of cells showing these extensions is much higher in these cell lines. Our results suggest that NGFI-A could play a role in neuronal differentiation in mammals.

2. Materials and methods

2.1. Cell culture and transfection

Mouse Neuro2A (N2A) cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA), 2 mM glutamine, and 0.01 mg/ml sodium pyruvate. Cells were seeded at a density of 20 000/cm^2 and grown for 24 h in complete medium. Following this, some of the plates were placed in serum-free medium. In the absence of serum, N2A cells first began to differentiate (12–24 h) and then began to die (24–32 h). Antisense or sense oligonucleotides were added to the culture medium at a concentration of 40 μM at the time of serum with-

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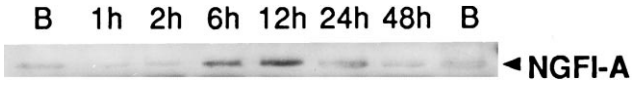

Fig. 1. NGFI-A protein concentrations in Neuro2A cells following serum withdrawal. Immunoblots were performed on lysates (70 μg/lane) prepared from N2A cells deprived of serum for different times. A representative blot probed with an NGFI-A-specific antibody is shown.

drawal. To select stably transfected cells, 0.5×10^6 cells were seeded onto a 6 cm diameter tissue culture plate, incubated overnight, and transfected with 2 μg of pLNCX NGFI-A using the calcium phosphate precipitation technique, as previously described [24]. Twelve hours after transfection, the medium was replaced with regular growth medium and the cells were incubated for 24 h, after which they were subcultured at 1:10 dilution with the addition of geneticin (1 mg/ml). The growth medium was renewed every 3 days and fresh geneticin was added. Individual colonies were transferred into 24-well plates, expanded, and screened for NGFI-A expression.

2.2. Oligonucleotides

NGFI-A antisense or sense oligodeoxyribonucleotides were synthesized with the following sequences: Antisense: 5'-GCG GGG TGC AGG GGC ACA CT-3'; sense: 5'-AGT GTG CCC CTG CAC CCC GC-3'. This was accomplished with an Applied Biosystems 391 DNA synthesizer and the oligodeoxyribonucleotides were purified on acrylamide gels. The antisense oligonucleotide is complementary to a sequence located 120–101 bases upstream from the AUG initiation codon. A 15-mer antisense oligodeoxyribonucleotide with the sequence 5-GGG GTA GTT GTC CAT-3' complementary to a sequence starting at the AUG initiation codon was also used, with the same results. C-myc antisense and sense oligonucleotides were as follows: Antisense: 5'-AAC GTT GAG GGG CAT-3'; sense: 5'-ATG CCC CTC AAC GTT-3'.

2.3. Western immunoblot analysis

N2A cells were lysed in 500 μl of PBS containing 1% NP40; 0.5% sodium deoxycholate; 0.1% SDS, and protease inhibitors, spun at $12\,000\times g$ for 20 min. The supernatants were removed and stored at −70°C until analysis. Equal amounts of protein (70 μg) were electrophoresed in 10% SDS-polyacrylamide gels, and blotted onto nitrocellulose membranes. Transferred membranes were blocked with 5% non-fat milk in TBST buffer (20 mM Tris-HCl pH 7.6; 130 mM NaCl; 0.1% Tween-20) for 1 h at room temperature. They were then incubated with NGFI-A-specific antibody (Santa Cruz Biotech. Inc) used at 1:1000 dilution, or antibody 125 at a 1:25 dilution, in TBST with 4% non-fat milk for 2 h. After several washes, the membranes were incubated with the second antibody coupled to horseradish peroxidase. After washing, immunoreactive bands were visualized using the Amersham ECL detection kit according to the manufacturer's instructions. For loading controls, all blots were stained with Ponceau Red and incubated with an actin antibody.

3. Results

We first determined NGFI-A gene expression during N2A differentiation induced by serum deprivation. As shown in Fig. 1, NGFI-A protein levels were very low in non-stimulated cells and the expression of the gene increased 4- and 5-fold after 6 and 12 h, respectively, of serum deprivation. This increase was transient, and the levels observed at 48 h after serum withdrawal were seen to be similar to basal values.

We next examined the function of the NGFI-A gene on N2A cell differentiation. To do so, we used antisense NGFI-A oligodeoxynucleotides (AS NGFIA) in the culture medium in order to interfere with the normal expression of NGFI-A. As controls, cells were incubated with the same NGFI-A sequence in the sense orientation (S NGFI-A). An equal num-

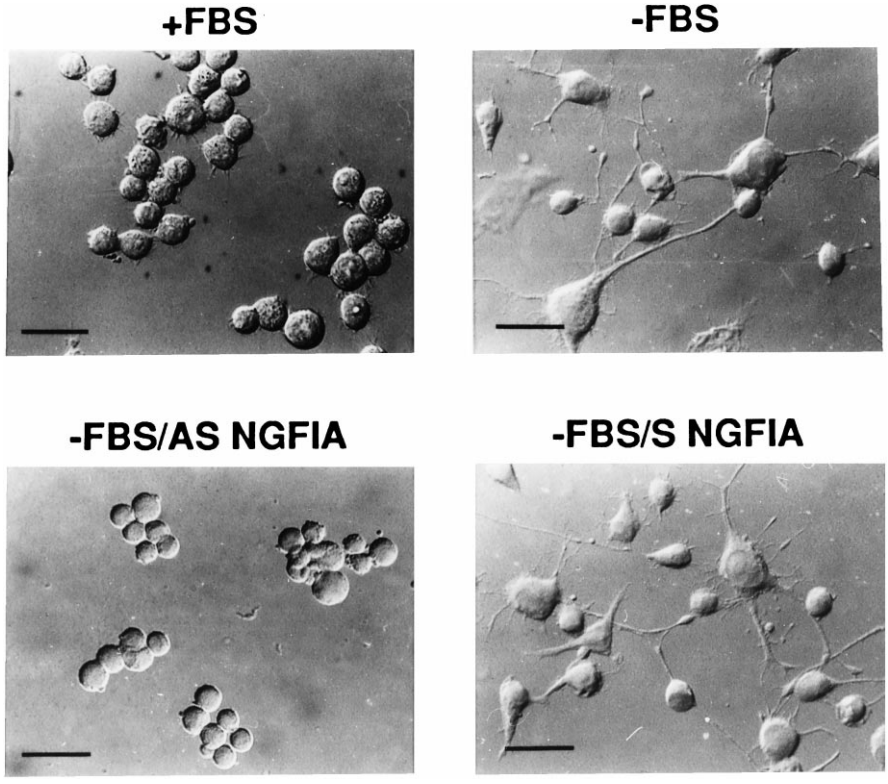

Fig. 2. Effect of NGFI-A antisense oligodeoxynucleotides on the phenotype of differentiating Neuro2A cells. Cells were seeded and grown in medium containing 10% fetal bovine serum over 24 h. Some of the cultures were then induced to differentiate by serum withdrawal and oligonucleotides were added to the culture medium at a final concentration of 40 μM. The images are Nomarski photomicrographs taken 12 h after cell growth in normal or serum-free medium (×200). Scale bar = 20 μm.

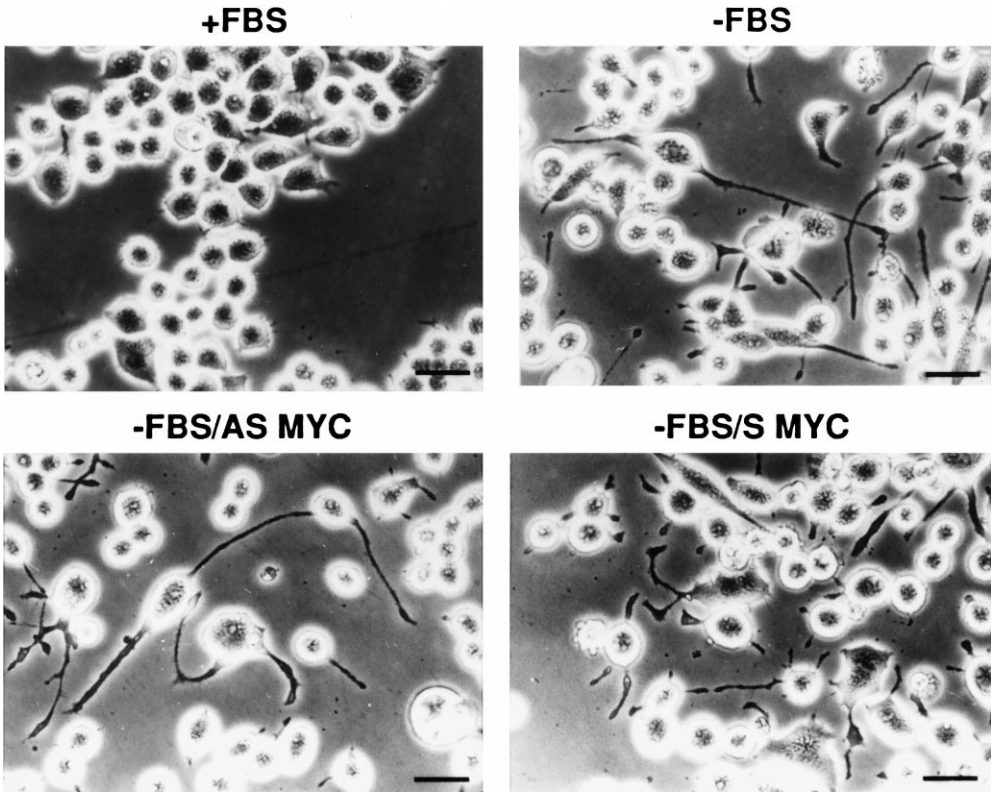

Fig. 3. Effect of c-myc antisense oligodeoxynucleotides on the phenotype of differentiating Neuro2A cells. Cells were seeded and grown in medium containing 10% fetal bovine serum over 24 h. Some of the cultures were then induced to differentiate by serum withdrawal and oligonucleotides were added to the culture medium at a final concentration of 40 μM. The images are phase contrast photomicrographs taken 12 h after cell growth in normal or serum-free medium (×200). Scale bar = 20 μm.

ber of cells was plated, induced to differentiate by serum withdrawal, and photographed 12 h after induction. As shown in Fig. 2, cells grown in the presence of 10% FBS did not extend neurites, or only very short ones. However many of the cells grown in the absence of serum had extended long neurites that were often branched and longer than the cell body. However, as can be seen from Fig. 2 cells incubated without FBS but in the presence of NGFI-A antisense oligomers displayed a morphology similar to that of control cells incubated in the presence of serum. Thus, the presence of NGFI-A antisense oligomers in the culture medium of cells induced to differentiate resulted in a dramatic inhibition of neurite outgrowth. By contrast, normal neuritogenesis was observed in the presence of sense oligonucleotides. Fig. 2 shows the morphology of N2A cells extending neurites in a similar fashion to cells grown in serum-free medium. The same results were observed when the cells were analyzed 24 h after serum withdrawal (data not shown).

We also analyzed the effect of c-myc antisense oligodeoxynucleotides on N2A differentiation. A concentration of 40 μM c-myc antisense oligonucleotides (AS MYC), which reduced the proliferation of N2A cells by 60%, had no effect on neurite extension (Fig. 3). This clearly suggests that the observed effect of NGFI-A antisense oligonucleotides is specific.

To further study the effect of NGFI-A on N2A cell differentiation, we determined the effect of NGFI-A overexpression in these cells. To this end, Neuro2A cells were stably transfected with an expression construct for this transcription factor (pLNCX-NGFI-A). Several clones were examined by Western blot analysis. Representative data of some of the clones are shown in Fig. 4A. The morphologies of wild-type and transfectant N2A cells were observed 24 h after they had been plated in the presence of 10% fetal bovine serum. As is apparent from the micrographs in Fig. 4B, a pronounced difference was seen in the phenotype of the parental N2A cells and the three clones overexpressing the NGFI-A protein. Control cells showed a round morphology, typical of an undifferentiated state, whereas all the clones extended more elongated neurites than in many cases were branched. Additionally, when the cells were grown in serum-free medium all the clones began to extend neurites much earlier than the parental N2A cells (data not shown). All the NGFI-A transfectants examined (three independent lines) showed the same phenotype, suggesting that the observed effect was the result of the overexpression of NGFI-A and not due to disruption of another gene by the integration event. The aspect of the clones that did not overexpress NGFI-A was similar to that of wild-type N2A cells (data not shown).

It has been reported that neurite outgrowth depends on the increased assembly of microtubules, which constitute the cytoskeletal framework of developing neurites. Enhanced phosphorylation of the microtubule-associated protein MAP1B has been shown to occur during neurite outgrowth and it has also been demonstrated that this protein is required for the proper assembly of microtubules [25–27]. To assess the effect of NGFI-A overexpression on the MAP1B protein, we used a monoclonal antibody (Ab 125) that reacts with a phosphorylated epitope on MAP1B, which is believed to be necessary for the function of this protein [28]. Western blot analysis revealed a significant increase in the amount of phos-

phorylated MAP1B protein in cells overexpressing NGFI-A (Fig. 5).

4. Discussion

In the present work we used a neuroblastoma cell line (Neuro2A), which is able to extend neurites under serum-free conditions, in an attempt to demonstrate that the NGFI-A transcription factor significantly stimulates neurite outgrowth. The rationale of this was to uncover the involvement of this gene in the differentiation of neuronal cells.

To our knowledge, this is the first time that the involvement of the NGFI-A gene in a differentiation process, which resembles a step in neuronal differentiation, has been reported. As mentioned in Section 1, NGFI-A has been implicated in the differentiation of other cell types, such as that of macrophages [18]. In human myeloblastic leukemia HL60 cells, NGFI-A is transcriptionally silent but is activated when these cells are induced to differentiate along either the macrophage or granulocyte lineage. Conversely, NGFI-A is constitutively active in U-937 and MI cells, which are predetermined for macrophage differentiation. Additionally, in other cell lines the expression of this gene is induced by stimuli known to promote differentiation processes, such as retinoic acid [29]. Following retinoic acid treatment NGFI-A expression rapidly increases in P19 cells, and NGFI-A protein levels remain at high constitutive levels in differentiated P19 cells, indicating a distinct role for this transcription factor in the induction and maintenance of the differentiated state. In rat brain the levels of NGFI-A transcripts are induced by neuronal activation [30–32] and thyroid hormone [16,23], which is known to be required for adequate development of the dendritic arbor of different neuronal types [33]. Together with the foregoing references, the results offered here are very suggestive of an in vivo role for NGFI-A in the late stages of neuronal differentiation, when neuronal processes begin to develop and con-

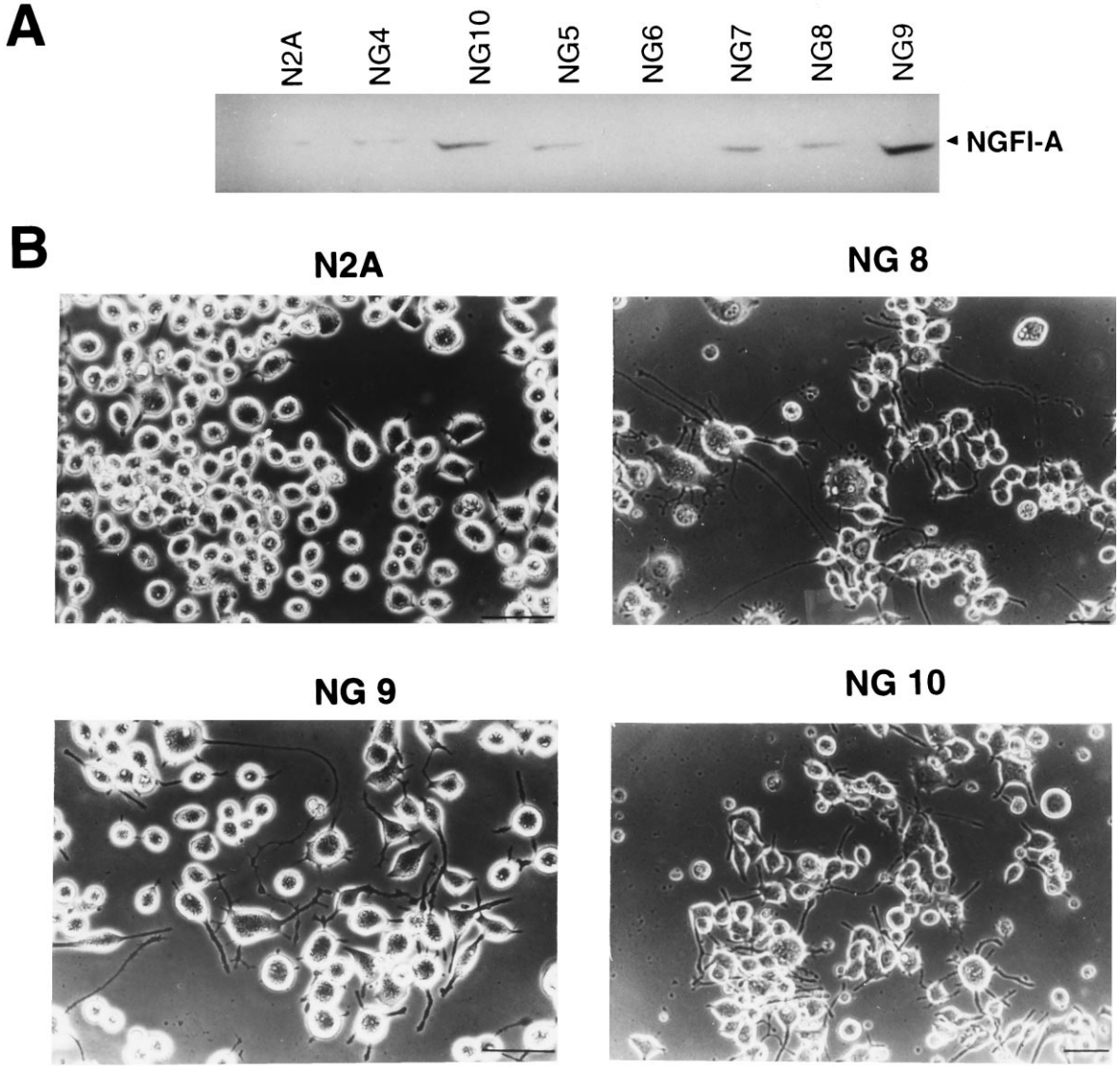

Fig. 4. Effect of NGFI-A overexpression on the phenotype of Neuro2A cells. A: Representative Western blot showing NGFI-A levels in different clones of transfected cells and N2A progenitor cells. B: Three different clones and parental cells were subcultured for 24 h in medium containing 10% fetal bovine serum. Then, phase contrast pictures at ×100 (NG8 and NG10) or ×160 (N2A and NG9) magnification were taken. Scale bar = 30 μm.

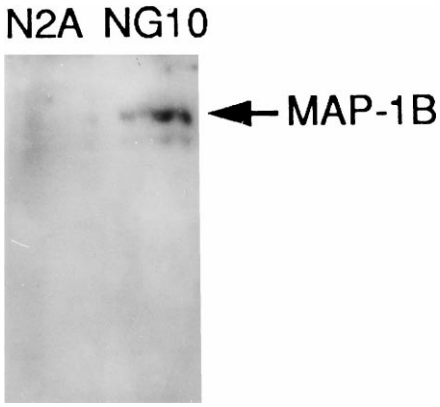

Fig. 5. Immunoreactivity for antibody 125 recognizing phosphorylated MAP1B in N2A progenitor cells and clone NG10. Immunoblot analysis showing the reaction of antibody 125 with aliquots containing the same amounts of protein of extracts from N2A cells and clone 10. Note the reaction of antibody 125 with polypeptides derived from MAP1B proteolysis (reported previously [44]).

nections among cells begin to be established. Also in support of this idea is the presence of NGFI-A-binding sites in the promoters of numerous neuronal genes, such as synapsin I, synapsin II, synaptobrevin II and neurofilament light [34–37]. However, the function of NGFI-A in vivo is either more subtle or is masked by the action of other gene family members that bind to very similar DNA sequences, since knockout mice for this gene do not show major changes either in morphology or neuronal activity [38–40].

Unlike the effect of NGFI-A antisense oligonucleotides on neurite formation, no effect was observed with c-myc antisense oligonucleotides, further underlining the specificity of the effect of NGFI-A. These results are in agreement with the lack of effect of c-myc antisense oligonucleotides on neurite outgrowth in N1E-115 neuroblastoma cells [41]. We decided to study the c-myc protooncogene for two reasons. First, c-myc expression also seems to be associated with the induction of proliferation and the inhibition of terminal differentiation [42]; second, and more importantly, the promoter of this gene contains a putative NGFI-A-binding site and hence the effects of NGFI-A on the differentiation of these cells could possibly be mediated by the repression of c-myc. Despite this, our results suggest that the mechanisms by which the NGFI-A gene induces N2A differentiation would not involve regulation of the c-myc protooncogene.

We further tested whether overexpression of the NGFI-A gene might affect neurite outgrowth via the control of the microtubule-associated protein MAP1B. This protein has been implicated in the assembly of microtubules, leading to neurite outgrowth, and its activity seems to be controlled by phosphorylation [25,27]. Ulloa et al. have shown that depletion of casein kinase II by antisense oligonucleotides is accompanied by a site-specific dephosphorylation of the MAP1B protein, leading to a decreased association of MAP1B with microtubules and a lack of differentiation in N2A cells [28]. Our results showing that overexpression of NGFI-A elicits an increased amount of phosphorylated MAP1B suggest that the promotion of neurite outgrowth by NGFI-A could be mediated by MAP1B activation.

The studies reported here, together with previous works on the regulation of NGFI-A gene expression and the possible role of this gene in neuronal tissues [16,31,43], suggest that NGFI-A levels may participate significantly in neuronal cell differentiation and plasticity during the development of the central nervous system. The precise mechanism(s) of the differentiation-enhancing action of this gene are currently unknown. In this context, it should be stressed that NGFI-A belongs to a family of transcription factors thought to couple extracellular stimuli to long term responses by altering gene expression. Thus, the differentiating effects of NGFI-A may well involve changes in the expression of genes expressed further along the cascade of events leading to a neuronal phenotype. Future studies aimed at identifying of these targets in neuronal cells in differentiation pathways should help to further our understanding of the complexity of these cellular processes.

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