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Sialic acid metabolism is involved in the regulation of gene expression during neuronal differentiation of PC12 cells

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Abstract Sialic acid precursors are mediators of the sialic acid pathway. In this manuscript we present evidence that the application of sialic acid a precursor modulates gene expression and cell differentiation. The concept that sugars are involved in cellular transcription was first proposed by Jacob and Monod nearly 40 years ago studying the regulation of the lac-operon in prokaryotes. Surprisingly, these findings have never been transferred to eukaryotic systems. For our studies we have chosen PC12 cells. PC12-cells differentiate after application of NGF into a neuron-like phenotype. It is shown that treatment of PC12 cells with two different sialic acid precursors *N*-acetyl- or *N*-propanoylmannosamine, without application of NGF also induces neurite outgrowth. Moreover, the PC12 cells show the same morphology as the NGF-treated cells. Surprisingly, after application of both sialic acid precursors the phosphorylation and translocation of erk1/2 into the nucleus are activated, thus influencing the expression of genes involved in the differentiation of cells, such as the transcription factor c-Jun or TOAD-64/Ulip/CRMP (Turned ON After Division, 64 kd/ unc-33-like phosphoprotein/Collapsin Response Mediator Protein). These are the first experimental data showing that the sialic acid metabolism is closely associated with signal transduction and regulation of neuronal differentiation.

Keywords Proliferation · Gene expression · Differentiation

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Introduction

Sialic acids (Sia) are the most abundant terminal monosaccharides on glycoconjugates of eukaryotic cell surfaces [1]. Sia are involved in a variety of cellular functions, such as cell–cell interaction, virus infection or the stability of glycoproteins [2–4]. Sia are synthesized in the cytosol from UDP-*N*-acetylglucosamine (Fig. 1). The physiological precursor of Sia is *N*-acetylmannosamine (ManNAc) [5]. The *N*-acyl side chain of ManNAc can be extended to *N*-propanoylmannosamine (ManNProp). Application of ManNProp to cells results in the formation and incorporation of *N*-propanoyl Sia in glycoconjugates and replaces up to 85% of the natural Sia [6–8]. This procedure (biochemical engineering of the *N*-acyl side chain of Sia) has been explored and is a well-established method to modify sialylation of glycoconjugates [9–12]. *N*-Acyl-modified sialic acids have striking biological consequences for glia cells of the mammalian central nervous system. For example, oligodendrocytes showed increased signs of a non-mature cell stage when ManNProp was applied. The incorporation of *N*-propanoyl Sia, followed by the application of GABA (γ-amino butter acid), leads to calcium oscillations in oligodendrocytes [13]. Collins *et al.* [11] demonstrated the conversion of neuronal sialic acids from *N*-acetyl- to *N*-glycolyl Sia resulting in an inhibition of the binding of myelin-associated glycoprotein (MAG) to neuronal cells, a requirement for remyelinization.

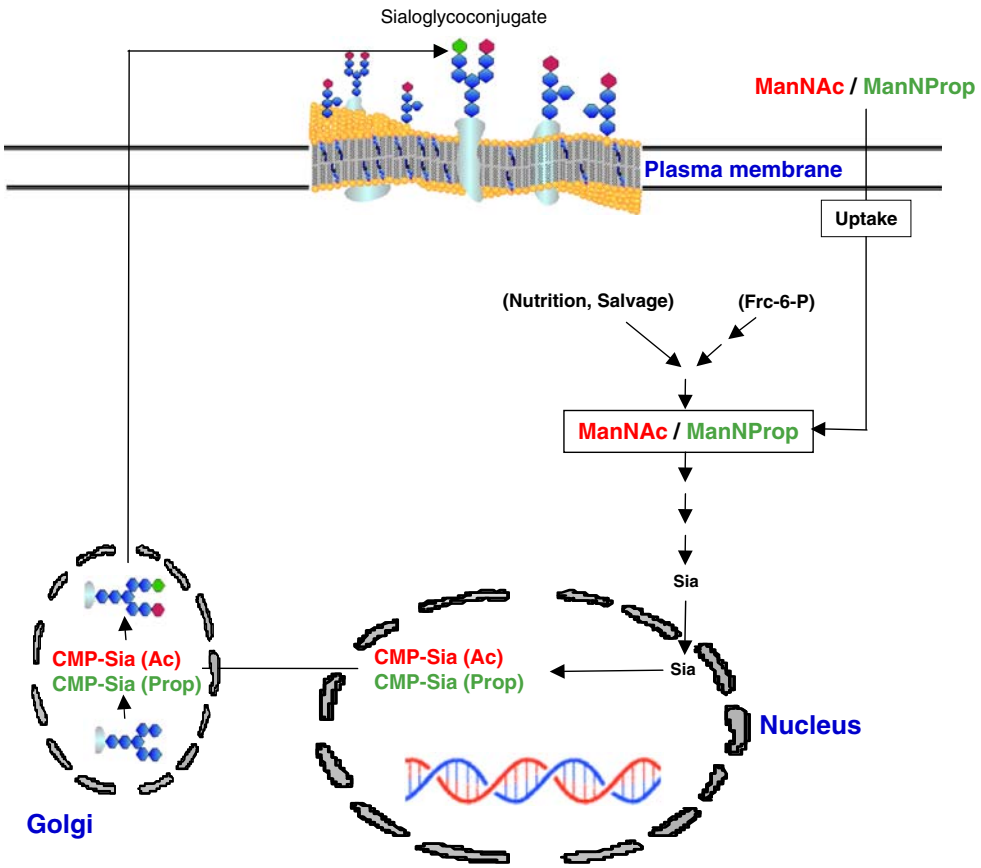

Fig. 1 Schematic representation of the Sia biosynthesis. Application of the Sia-precursors ManNAc (shown in *red*) or ManNProp (shown in *green*) leads to increased incorporation of Sia in glycoconjugates. Note that one crucial step of the Sia synthesis (activation with CMP) takes place in the nucleus

PC12 cells, which were derived from rat pheochromocytoma [14], are commonly used to investigate neurite outgrowth. On exposure to NGF, PC12 cells differentiate into a neuron-like phenotype. Neurite outgrowth of PC12 cells is associated with growth arrest, transcriptional and translational changes [15], which are initiated by the binding of NGF to its high-affinity receptor TrkA [16] and activation of *ras* and the MAPK cascade [17]. In addition, it has been shown that NGF stimulates both the expression and the phosphorylation of c-Jun in PC12-cells [18]. The over expression of c-Jun in PC12-cells resulted in the formation of c-Jun/c-Jun homodimers and induction of neurite outgrowth [19].

In the presence of either ManNAc or ManNProp together with NGF, PC12-cells extended up to 60% longer neurites. Similar results were obtained with primary neurones [20]. In brain slices we could show that the re-establishment of the perforant pathway was stimulated when the slices were cultured in the presence of ManNProp or ManNAc [20].

In this study we analyzed the neurite promoting activity of Sia-precursors in more detail. We were able to stimulate neurite outgrowth of PC12-cells without any application of NGF. Both Sia-precursors, ManNAc and ManNProp, stimulated the phosphorylation of erk1/2, one of the final targets of NGF. In addition, application of ManNAc or ManNProp stimulated the expression of c-Jun and ULIP. This demonstrates that the sialic acid metabolism is involved in intracellular signal transduction and it is intimately connected with the regulation of cellular processes during neurite outgrowth.

Results

Recently we showed that ManNAc and ManNProp stimulate neurite outgrowth in the presence of suboptimal concentrations of NGF [20]. We now tested the possibility whether ManNAc or ManNProp also stimulate neurite outgrowth in the absence of NGF. We therefore cultivated PC12-cells on collagen IV in the presence of 5 mM ManNAc or ManNProp for 120 h and added every 24 h new *N*-acyl-mannosamines to the cultures. We used 5 mM ManNAc or ManNprop since these concentrations, which are unphysiologically high, have been effectively used in all our previuos studies [8, 20]. Figure 2 shows the result of a typical experiment. PBS-treated cells grown in the absence of NGF or Sia-precursors did not extent any neurites. In the presence of NGF we found nicely differentiated PC12-cells. Even in the absence of NGF, but in the presence of ManNAc or ManNProp, we found neurite outgrowth

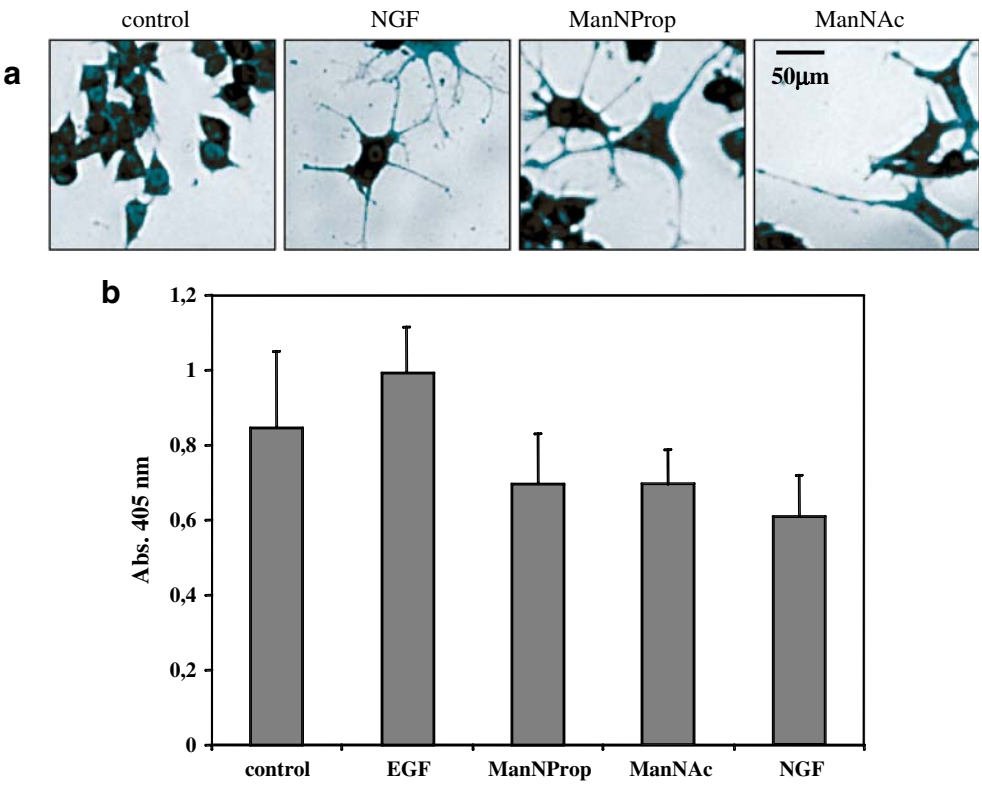

Fig. 2 **a** Neurite outgrowth in the presence of Sia-precursors. PC12-cells were grown in the absence (control) or presence of 100 ng/ml NGF, 5 mM ManNProp or ManNAc, respectively, on collagen. Representative micrographs of each PC12-cultures are shown. **b** BrdU-Proliferation Assay. PC12-cells cultured in the absence (control) or presence of EGF (100 ng/ml), NGF (100 ng/ml), ManNProp (5 mM) or ManNAc (5 mM) were analyzed. Induction of DNA synthesis was measured by the incorporation of pyrimidine analogue 5-bromo-2′deoxyuridine (BrdU), instead of deoxythymidine, into the DNA of proliferating cells. The cells were incubated with BrdU for 4 h and the incorporation of BrdU into the DNA was detected by immunoassay. At least three experiments were performed in triplicates

comparable to the NGF-induced neurite outgrowth. The morphology of the PC12-cells cultured in the presence of ManNAc or ManNProp was the same as in the cultures that were differentiated with NGF (Fig. 2a).

Differentiation of PC12 cells is accompanied with growth arrest. Therefore, we analyzed the proliferation of PC12-cells grown in the absence or presence of the Sia-precursors ManNAc or ManNProp (Fig. 2b). As expected, separate application of both Sia-precursors resulted in a reduction of proliferation by 20% compared to PBS-treated cells (Fig. 2b). As expected, application of NGF to PC12-cells resulted in a similar reduction of proliferation (30%). As a positive control we used EGF, which stimulated proliferation. ManNAc- or ManNProp-treated cells showed a 30% reduction of proliferation compared to EGF-treated cells. We then analyzed the expression and phosphorylation of erk1/2 in the absence or presence of ManNAc or ManNProp (Fig. 3). As a positive control we used NGF

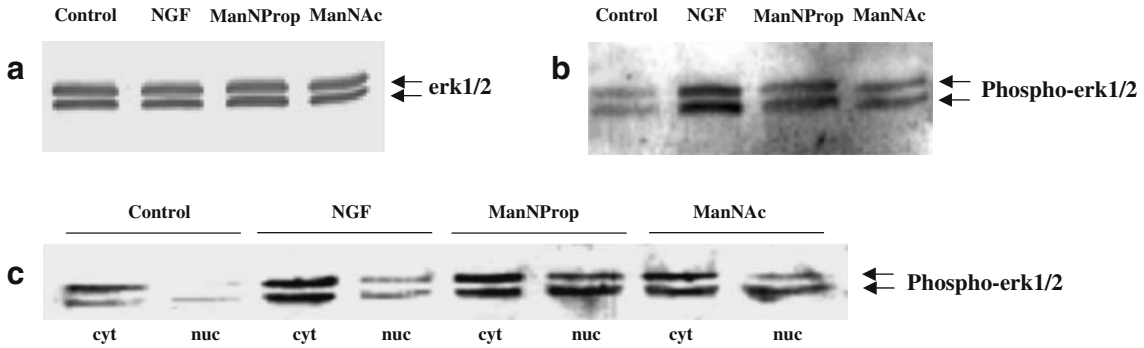

Fig. 3 Analysis of erk1/2 after application of Sia-precursors. PC12-cells were grown in the absence (control) or presence of 100 ng/ml NGF, 5 mM ManNProp or ManNAc, respectively. After 48 h of culture, cells were harvested and analyzed by Western blotting. **a** Expression of erk1/2. Total cell extracts were separated and analyzed using an erk1/2-specific antibody. Note that no change in the amount of total erk1/2 could be detected. **b** Phosphorylation of erk1/2. Total cell extracts were separated and analyzed using a phospho-erk1/2-specific antibody. Note that phosphorylation of erk1/2 was strongly stimulated in the presence of NGF and moderately in the presence of the Sia-precursors. **c** Distribution of erk1/2 after application of Sia-precursors. Cytosolic and nuclear extracts were prepared and separated and analyzed using an erk1/2-specific antibody. Note that translocation of erk1/2 into the nucleus occurred after application of NGF, ManNAc and ManNProp

for activation of the MAPK pathway. Neither NGF nor ManNAc or ManNProp stimulated expression of erk1/2 (Fig. 3a). The application of ManNAc or ManNProp increased the phosphorylation of erk1/2 compared to PBS-treated cells (Fig. 3b). Next we analyzed the cellular distribution of phospho-erk1/2 in PC12-cells cultured in the absence or presence of ManNAc or ManNProp. In unstimulated PC12-cells most phospho-erk1/2 is present in the cytosol. After stimulation with NGF, ManNAc or ManNProp, we found significantly more phospho-erk1/2 in the nucleus compared to PBS-treated control cultures (Fig. 3c). The distribution of total erk1/2 (not phosphorylated) was unchanged (data not shown).

It has been shown by several groups that c-Jun expression is up-regulated upon differentiation of PC12-cells and that overexpression of c-Jun in PC12-cells also stimulate neurite outgrowth [18, 21]. We therefore quantified the expression of c-Jun in PC12-cells after NGF, ManNAc- or ManNProp-induced neurite outgrowth. Figure 4a shows a Western blot analysis of c-Jun. As expected, we found in non-differentiated PC12-cells only weakly c-Jun expression. However, after ManNAc- or ManNProp-induced neurite outgrowth c-Jun expression increased between 2-fold (ManNAc and ManNProp) and 2.5-fold (NGF, positive control). Phosphorylation of c-Jun is a crucial step in the c-Jun-mediated differentiation [22]. We next analyzed the phosphorylation of c-Jun in PC12-cells after NGF, ManNAc- or ManNProp-induced neurite outgrowth. Only in the NGF-treated cultures we found an increase of c-Jun phosphorylation, whereas the phosphorylation of c-Jun remained unchanged in the case of ManNAc- or ManNProp-induced neurite outgrowth (Fig. 4b). To validate the c-Jun upregulation by Sia-precursors in PC12-cells treated with either PBS or ManNProp, we quantified the c-Jun-expression using an ELISA assay (Fig. 4c). PC12-cultures, which were treated with Man NProp, the expression of c-Jun protein was 40% higher compared to PBS treatment (Fig. 4c). Not only the protein, but also the expression of c-Jun mRNA is up-regulated in the presence of ManProp. This was shown by Northern blot analysis of c-Jun of PC12 cells, which were grown in the absence or presence of ManNProp (Fig. 4d). The up-regulation of c-Jun is specific for the Sia-precursors ManNAc or ManNProp. Other sugars like glucose, galactose or glucosamine did not influence the expression of c-Jun (Fig. 4e).

We next addressed the question, whether also the expression of other genes are influenced after giving Sia-precursors. Previously we analyzed by peptide mass fingerprinting the expression of several proteins in PC12-cells before and after application of ManNProp [20]. One of the proteins could be identified as a member CRMP/Ulip family [20]. Therefore, we analyzed the expression of ULIP-

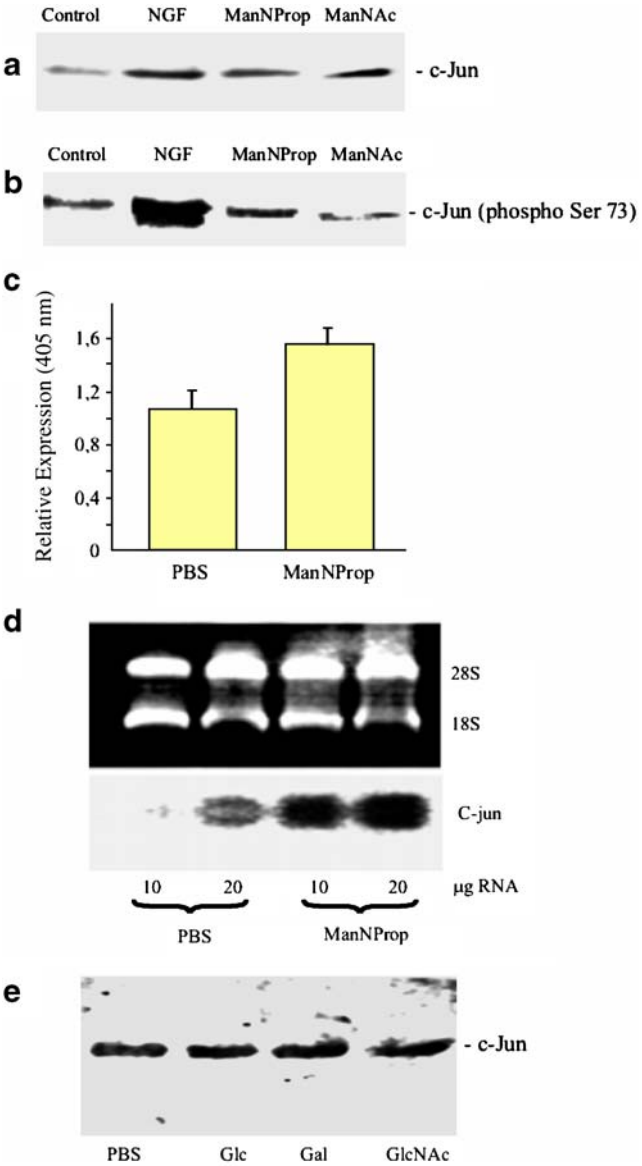

Fig. 4 Analysis of c-Jun after application of Sia-precursors. PC12-cells were grown in the absence (control) or presence of 100 ng/ml NGF, 5 mM ManNProp or ManNAc, respectively. After 24 h of culture, cells were harvested and analyzed by Western blotting. **a** Expression of c-Jun. Total cell extracts were separated and analyzed using a c-Jun-specific antibody. Note that NGF, ManNAc and ManNProp stimulate the expression of the c-Jun protein. **b** Phosphorylation of c-Jun. Total cell extracts were separated and analyzed using a phospho-c-Jun-specific antibody. Note that phosphorylation of c-Jun was only stimulated in the presence of NGF. **c** Quantification of the c-Jun-expression in the presence of ManNProp by ELISA. PC12-cells were grown in the absence or presence of 5 mM ManNProp on collagen. After 24 h of culture, cells were harvested and lysates were prepared. Expression of c-Jun was analyzed by sandwich-ELISA. Bars represent mean values±SD of five independent experiments. **d** Quantification of the c-Jun-expression in the presence of ManNProp by Northern blot. PC12-cells were grown in the absence or presence of 5 mM ManNProp on collagen. After 24 h of culture cells were harvested and RNA was isolated. Expression of c-Jun was analyzed by Northern blotting. One representative out of two independent experiments is shown. **e** Expression of c-Jun in the presence of control sugars. An experiment as shown in **a** was performed in the presence of glucose (*Glc*), galactose (*Gal*) or *N*-acetylglucosamine (*GlcNAc*). Note that none of these sugars modify the expression of c-Jun

6 by RT-PCR. The application of both Sia-precursors, ManNAc and ManNProp, resulted in an increased expression of Ulip-6 m-RNA (Fig. 5). NGF-treated-cells showed also an increase in ULIP-6 gene expression (Fig. 5). The expression of β-actin mRNA as an internal standard was unaffected (Fig. 5).

Discussion

By the use of the two different precursors of Sia, ManNAc and ManNProp, we could show that the Sia metabolism is involved in cell regulation and differentiation. This was underlined by the following experiments suggesting that Sia-precursors or its metabolites act as signaling molecules: First, application of both, ManNAc or ManNProp, stimulated neurite outgrowth of PC12-cells. Second, application of ManNAc or ManNProp resulted in an increased phosphorylation of erk1/2 and furthermore in an accumulation of erk1/2 within the nucleus. Third, the expression of c-Jun was enhanced after application of ManNAc or ManNProp. whereas the phosphorylation of c-Jun was unaffected.

PC12-cells can be differentiated into a neuronal-like phenotype by application of NGF. The signal transduction cascade of NGF in PC12-cells has been intensively studied over the past decade. After binding to its receptors, trkA or p75NTR, NGF stimulates the MAP-kinase and the JNK pathway, leading finally to the activation of erk1/2 and c-Jun. The synergistic activation of both pathways by NGF is necessary to reach maximal neurite outgrowth of PC12-cells [22]. Here we present evidence that application of Sia-precursors (ManNAc or ManNProp) stimulates the erk1/2 pathway (summarized in Fig. 6). This was demonstrated by the phosphorylation of erk1/2 and its subsequent translocation into the nucleus. However, we could not show any phosphorylation of c-Jun. These observations are in agreement with earlier results suggesting erk1/2 as regulator of the expression, but not of the phosphorylation of c-Jun [23]. Taken together, application of Sia-precursors (ManNAc or ManNProp) activates erk1/2 thereby leading to differentiation of PC12-cells. It seems that activation of the MAPK pathway by the Sia precursors is sufficient to induce neurite outgrowth of PC12 cells. We could also show that the Sia precursors led to an inhibition of proliferation, a necessary condition for differentiation.

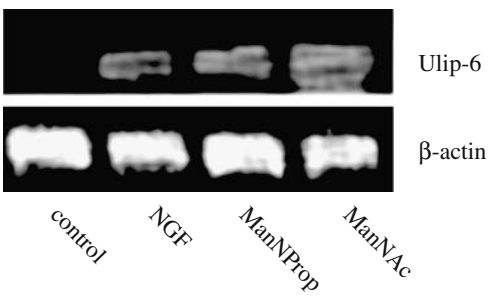

Fig. 5 Upregulation of ULIP-6 after application of Sia-precursors. PC12-cells were grown in the absence (control) or presence of 100 ng/ml NGF, 5 mM ManNProp or ManNAc, respectively. After 24 h of culture cells were harvested and RNA was isolated. Expression of ULIP-6 was quantified by semi-quantitative RT-PCR. One representative out of three independent experiments is shown

Nevertheless, application of Sia-precursors led to an increased expression of specific genes, such as Ulip-6. Ulip genes are probably involved in multiple cellular and molecular events involved in apoptosis/proliferation, cell migration, and differentiation. In the adult brain, the expression of CRMPs/Ulips is dramatically down regulated. However, they remain expressed in structures that retain their capacity for differentiation and plasticity and also in a subpopulation of oligodendrocytes [21, 24]. Here we used Ulip-6 as model gene, which is known to have similar expression patterns as Ulip 1–4 [25] and are up regulated in the differentiation state.

Taken together, sialic acid metabolism is closely related into the modulation of transcription during neurite outgrowth. These results support our earlier observation that application of Sia precursors leads to an altered expression of regulatory important molecules [20].

The enhanced cellular activity is more expressed by using the novel sialic acid precursor ManNProp than by the physiological precursor ManNAc. The reason why the neurite outgrowth promoting activity of ManNProp is higher compared to ManNAc could lie in the uptake of both Sia-precursors. ManNProp is, due to its prolonged *N*-acyl side chain, more lipophilic. Therefore one could argue that the cellular uptake of ManNProp is better compared to ManNAc and in agreement with this, the cellular response to ManNProp is stronger. It should be noted that the activation of the MAP-kinase pathway by ManNAc or ManNProp is specific for the CMP-Sia-precursors. No other sugar, including glucosamine, galactose or glucose has any effect on the differentiation of PC12-cells.

In our study we present novel regulatory aspects of sialic acid metabolism in a cellular environment. In conclusion, the metabolisation of Sia-precursors is related to the modulation of gene expression leading to the differentiation of PC12 cells.

Material and methods

Cell culture, antibodies Rat PC12-cells were cultivated in Falcon plastic flasks or dishes in RPMI 1640 supplemented with 10% horse serum.

Antibodies to erk1/2 were obtained from Santa Cruz; to phospho-erk1/2 und phospho-c-Jun from Cell signaling; to

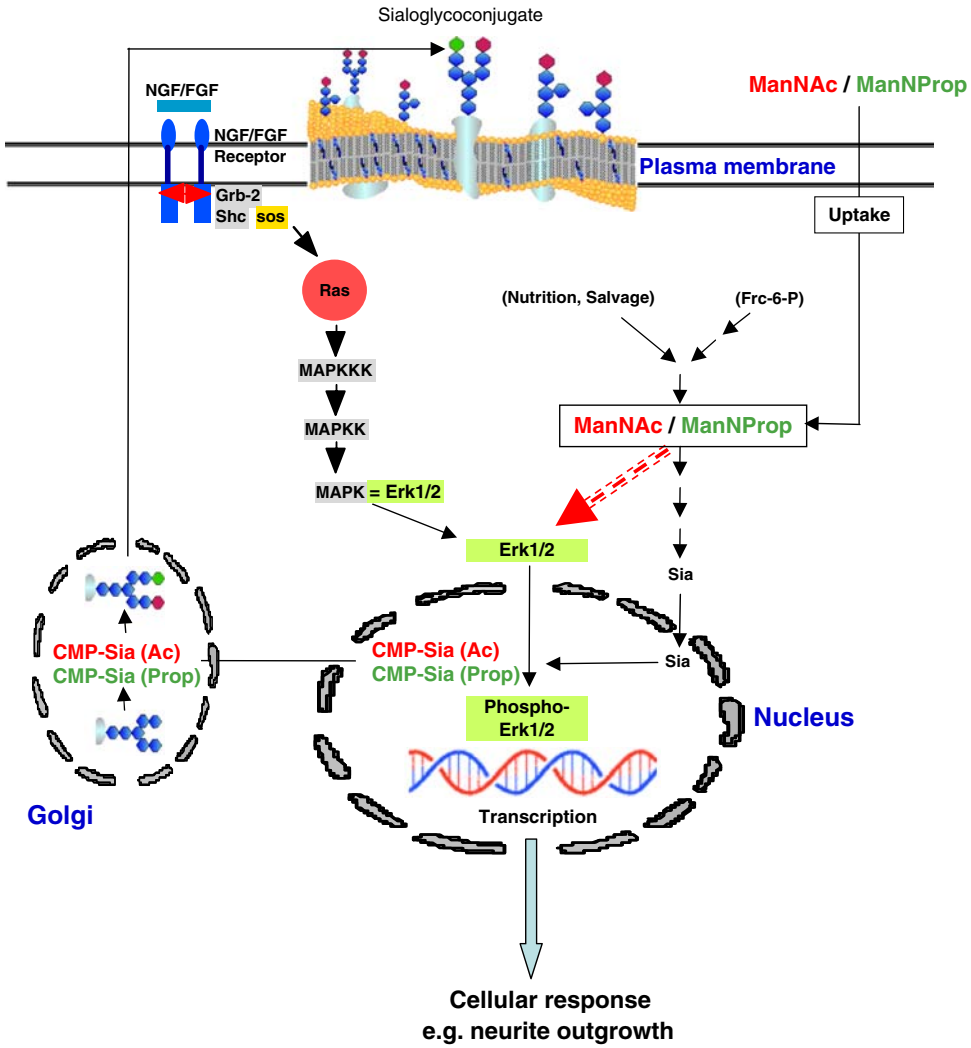

Fig. 6 Model of the involvement of Sia-precursors in the MAP kinase pathway of PC12-cells. PC12-cells differentiate upon stimulation with NGF into a neuronal-like phenotype. Sia-precursors (ManNAc in *red* and ManNProp in *green*) are taken up and metabolized to the respective Sia. Furthermore they are not only metabolized but also modulate the Map-kinase pathway, thereby activating transcription of specific genes and finally support differentiation of PC12-cells into a neuronal-like phenotype

c-Jun from abcam and Cell signaling. All secondary antibodies were obtained from Sigma.

Sialic acid precursors The physiological precursor of sialic acid, *N*-acetylmannosamine (ManNAc), was purchased from ICN Biomedicals Inc.

Synthesis of *N*-acyl-D-mannosamine derivatives

D-Mannosamine hydrochloride (NZP, New Zealand) was suspended to 10 mM in 30 ml methanol. At 0°C NaOMe/methanol (to 11 mM) and the corresponding carbonic acid anhydride (to 12 mM) were added. After stirring for 2 h at 0°C the solution was dried in a vacuum evaporator. The raw product was purified by column chromatography on silica gel 60 (Merck, Germany) with an elution solvent of acetic acid ethyl ester/methanol/HO (5:2:1 to 10:2:1, depending on the polarity of the anhydride). Characterization was carried out by H^1 NMR spectroscopy. All *N*-acyl-D-mannosamines were stored at 4°C.

Preparation of cytosolic and nuclear extracts Nuclear and cytosolic extracts of PC12-cells were prepared as described by [26]. Cell pellets were solubilized in hypotonic buffer (20 mM Hepes, 10 mM KCl, 1 mM MgCl2, 0,5 mM DTT, 0,1% triton, 20% glycerol and a protease inhibitor cocktail (P 8340, Sigma) at pH 7.0) and incubated for 30 min on ice. After centrifugation (9,000×*g*, at 4°C) the supernatants were transferred in new reaction tubes. The nuclear pellets were solubilized in hypertonic buffer (hypotonic buffer containing 450 mM NaCl) and the solubilisates were incubated for 30 min at 4°C. The probes were centrifuged at 9,000×*g* and 4°C for 5 min and supernatants were collected. Protein concentration was determined according to Bradford.

Preparation of cell extracts Cells were harvested and centrifuged at 900×*g* for 3 min. Cell extracts were isolated from pellets with cell lyses buffer (cell signaling) and incubated for 30 min on ice. Solubilisates were centrifuged at 10,000×*g* for 15 min and supernatants were collected and further analyzed by Western blot analysis.

Semi-quantitative RT-PCR Total RNA was isolated from cultured PC12 cells using Qiagen extraction kit following the manufacturer's manual. RNA (5 μg) was reverse transcribed using Superscript (Invitrogen) after priming with an Oligo dt primer. The reverse transcribed products were diluted directly in PCR buffer and amplified with the 5′primer for (ATGCTTGCCAATTCAGCCAGTGTG) and the 3′primer (CAGCAGACCTTGGGCTCAGCTAGAG) for ULIP and with the 5′primer (ACACGGCATTGTAAC CAACTGG) and 3′primer (CTCATTGCCGATAGTGAT GACC) for β-actin. Amplification was allowed for 28 or 30 cycles respectively. Those cycles were previously tested to be in the linear range of saturation.

Northern blotting Total RNA was isolated from culture cells using the RNeasy kit (Qiagen, Hilden, Germany). For denaturing gel electrophoresis, we used 1.2% formaldehyde-containing agarose gels running in 1× MOPS-buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA, pH 8.0). Transfer to Hybond N membrane (Amersham-Buchler, Braunschweig, Germany) was done by capillary blotting overnight in 10× SSC-buffer (3 M NaCl, 0.3 M Na-citrate, pH 7.0), and the RNA was visualized with ethidium bromide under UV-light. Fixed RNA was prehybridized in Express HybTM-solution (Clontech, Mountain View, USA) for 1 h at 42°C and hybridized with a 32P-labeled probe. Probes for hybridization were generated by PCR reaction under standard conditions. 5′primer (GACTGCAAAGATGGA AACGACCTTCTAC) 3′primer (GAAGTTGCTGAGG TTGGCGTAGAC) for c-Jun. PCR product were analyzed with agarose gel electrophoresis before hybridization. A random priming kit (Amersham Biosciences, USA) was used for labeling, and hybridization was again performed using ExpressHybTM-solution (Clontech) at 42°C. After hybridization, filters were washed several times in 0.2% SSC, 0.1% SDS and subjected to phosphoimaging (Molecular Devices).

Immunoblotting Samples were separated on SDS-polyacrylamide gels (BioRad) and transferred to nitro-cellulose filters. The blots were blocked with 5% fat-free dry milk powder in TBST, incubated with the respective primary antibodies, washed with TBST and incubated with the appropriate secondary antibodies. After washing, proteins were detected by the use of alkaline phosphatase-conjugated antibodies.

Neurite outgrowth PC12-cells (4×10^4 /well) were plated on collagen IV-coated (20 μg/ml) dishes and incubated with 100 ng/ml nerve growth factor, Alexis), 5 mM ManNAc (Sigma) or 5 mM ManNProp [6]. Every 24 h the medium and supplements were renewed. After 120 h, cells were fixed with 4% PFA and stained with 0.1% crystal violet.

Proliferation assay Cells were seeded in 96-well plates at a cell density of 2×10^4 cells per well in standard medium and incubated for 24 h. The assay was performed using the Cell Proliferation (BrdU) ELISA kit from Roche Diagnostics according to the instruction manual. Each experiment was performed in triplicates.

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