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Original Research Article

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APP Expression in Primary Neuronal Cell Cultures from P6 Mice during in vitro Differentiation

Key Words
Alzheimer's disease
Amyloid protein precursor
Cerebellar neurons
Sialic acid

Abstract
Primary neuronal cell cultures from P6 mice were investigated in order to study amyloid protein precursor (APP) gene expression in differentiating neurons. Cerebellar granule cells which strongly express APP 695 allowed the identification of three distinct isoforms of neuronal APP 695. The high-molecular-weight form of APP 695 is sialylated. The expression pattern of neuronal APP 695 changes during in vitro differentiation. Sialylated forms become more abundant upon longer cultivation time. The secreted forms of sialylated, neuronal APP 695 are shown to comigrate with APP isolated from cerebrospinal fluid. We suggest that the different sialylation states of APP 695 may reflect the modulation of cell-cell and cell-substrate interactions during in vitro differentiation and regeneration.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by intracellular neurofibrillary tangles, extracellular plaques and cerebrovascular amyloid in the brains of affected individuals. The principal component of the deposits is the βA4 protein, which is derived from a larger amyloid protein precursor (APP) [1]. Several alternative splice forms of this membrane-spanning glycoprotein have been identified so far [2–7]. As shown by different authors, APP 695 is the predominantly expressed form in the developing central nervous system, whereas peripheral tissues mainly express the KPI-containing APPs [4, 6, 8–11]. Biochemical analyses have shown that APP is posttranslationaly modified by N- and O-glycosylation as well as tyrosine sulfation and serine phosphorylation [12, 13]. Proteolytic cleavage within the amyloidogenic region leads to secretion of a large soluble NH_2-terminal APP fragment into the surrounding environment [12, 14, 15]. Such carboxyl-terminal truncated molecules are found in cerebrospinal fluid (CSF) and blood [12, 16, 17]. APPs circulating in blood are mainly derived from APP 751 and 770. They may be secreted by platelets and leukocytes and were shown to be identical with protease nexin 2 [18–21]. In contrast, soluble APP found in CSF mainly lacks the KPI insert, reflecting the predominant expression of APP 695 in the central nervous system.

It has been suggested by several authors that APP 695 plays an important role in neuronal differentiation and regeneration [22–24].

Cerebellar cells derived from early postnatal brain share many of the features characteristic for their counter-

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parts in vivo such as cell migration, axonal sprouting, synapse formation and electric activity [25–28]. Thus they provide an ideal model for investigating APP biosynthesis during differentiation processes and formation of neuronal networks. Using this approach, we have studied the biogenesis of APP in developing neurons. Our findings show that APP is indeed strongly expressed by neuronal cells. Differentiation of these cells in vitro was associated with changes in posttranslational modification of amyloid protein precursor.

Materials and Methods

Cell Culture

The cell culture protocol employed was a modification of the methods described by Schnitzer et al. [29].

Cerebelli were obtained from postnatal Balb/c mice (P6). At this stage of development, granule cells constitute approximately 90% of all cerebellar neurons [30]. After removal of the meninges, cerebelli were incubated for 15 min in a solution of 10 mg/ml trypsin in Ca^{2+}- and Mg^{2+}-containing Hanks balanced salt solution ($HBSS^+$). Cerebelli were rinsed three times in Ca^{2+}- and Mg^{2+}-free HBSS, followed by dissociation of the cells. This was done in 0.5 mg/ml DNAse I in $HBSS^+$ by trituration through the narrowed bore of a fire-polished Pasteur pipette. Cells were distributed to polylysine-coated (100 µg/ml) culture dishes containing 2 ml of Earl's Basal Medium (BME; Gibco) buffered with sodium bicarbonate (10 m*M*) and supplemented with 10% horse serum (Gibco). Neurons were plated at a density of about 5×10^4 cells/cm^2. Cultures were maintained in a humified incubator at 37 °C in 5% CO_2.

Immunocytochemistry

Coverslips were removed from culture dishes, briefly rinsed with phosphate-buffered saline (PBS; pH 7.2) and fixed with freshly prepared 4% paraformaldehyde (in PBS; pH 7.2) at room temperature for 15 min. After permeabilization with 0.3% TX-100 in PBS for 5 min, nonspecific binding sites were blocked with 1% human serum albumin, 10% FCS in PBS for 30 min at room temperature (RT). Cells were then incubated with primary antibodies in PBS (0.3% TX-100, 1% human serum albumin) at 4 °C overnight, extensively washed with PBS and incubated with fluorescein- and rhodamine-conjugated secondary antibodies for 1 h at RT. Coverslips were washed for 45 min in PBS before being mounted upside down on a drop of aqueous mounting medium (Moviol including 2.5% DABCO). Cells were examined on a Zeiss axioplan microscope equipped with phase contrast and epifluorescence optics for rhodamine and fluorescein.

Antibodies were used at the following dilutions: polyclonal anti-Fd-APP [12], 1:400; monoclonal anti-GFAP (Serva), 1:50; monoclonal anti-neurofilament (Dakopatts), 1:50; fluorescein-conjugated goat anti-rabbit (Sigma) 1:100; rhodamine-conjugated goat anti-mouse (Jackson), 1:100.

Biosynthetic Labeling

After removal of the culture medium, cells were radioactively labeled with 120 µCi of [^{35}S]methionine (Amersham) in 1.5 ml of methionine-free Dulbecco's modified Eagle's medium (DMEM) for 4 h. The conditioned medium was then cleared by centrifugation and stored at –20 °C. Cells were washed once with phosphate-buffered saline. For lysis, cells were resuspended in 0.2 ml of lysis buffer [50 m*M* Tris, pH 7.5, 150 m*M* NaCl, 2 m*M* EDTA, 1% Nonidet P-40, 1% Triton X-100. 2 m*M* phenylmethanesulfonylfluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1.6 mg/ml iodoacetamide] and incubated for 30 min on ice. Cell lysates were centrifuged at 10,000 *g* for 5 min, and the supernatants were stored at –20 °C until further use. The extraction pellet was discarded.

Immunoprecipitation

For immunoprecipitation of [^{35}S]methionine-labeled APP, 100 µl of cell lysate or 750 µl of conditioned medium were used. The cell lysate was diluted 1:1 with ice-cooled washing buffer B (20 m*M* Tris, pH 7.5, 500 m*M* NaCl, 0.5% Nonidet P-40, 2 m*M* PMSF). Lysate was preincubated for 2 h at room temperature with 10 µl of preimmune serum and 3 mg of protein A-Sepharose. Insoluble complexes were spinned down and discarded. The supernatants were incubated for 1 h at room temperature with 5 µl of undiluted anti-CT or anti-FdAPP. The polyclonal anti-CT antiserum was raised against a synthetic peptide, which corresponds to the COOH-terminal 43 residues of APP. Polyclonal anti-FdAPP antiserum was raised against purified *Escherichia coli* FdAPP fusion protein consisting of APP 695 and the Fd fragment of the murine IgM heavy chain [12].

After addition of 2 mg of protein A-Sepharose, the mixture was incubated for 30 min at RT. Nonbound proteins were removed from Sepharose beads by sequential washing with buffer A (20 m*M* Tris, pH 7.5, 150 m*M* NaCl, 0.4% Nonidet P-40, 0.4% Triton X-100, 2 m*M* PMSF), washing buffer B and finally with TSA solution (20 m*M* Tris, pH 8.0, 150 m*M* NaCl). The immunoprecipitates were fractionated by SDS-PAGE (8%). Gels were soaked in enhancer solution, dried and exposed to Kodak X-OMAT AR film at –70 °C.

Secreted APP was precipitated under nondetergent conditions and analyzed by subsequent immunoblotting. Immunoblotting was performed as described by Mönning et al. [34].

Enzymatic Digestion

Prior to enzymatic digestion with neuraminidase from *Clostridium perfringens* (Sigma), immunoprecipitated proteins were denaturated in 20 µl of 0.2% SDS, 2% MCE for 10 min at 100 °C followed by addition of 100 µl of sodium-acetate buffer (pH 6.0; 100 m*M*). Enzymatic digestion was performed with 10 mU of neuraminidase at 37 °C for 16 h. The proteins were dried and resuspended in Laemmli sample buffer. After boiling, labeled proteins were subjected to SDS-PAGE.

Results

Immunocytochemistry

For immunocytochemical localization of APP in primary neuronal cell culture, cerebellar cells from P6 mice were isolated and grown on poly-l-lysine-coated coverslips in the presence of 10% horse serum. Medium was not changed until cells were used for staining procedure, usually carried out after six days of cultivation. Staining

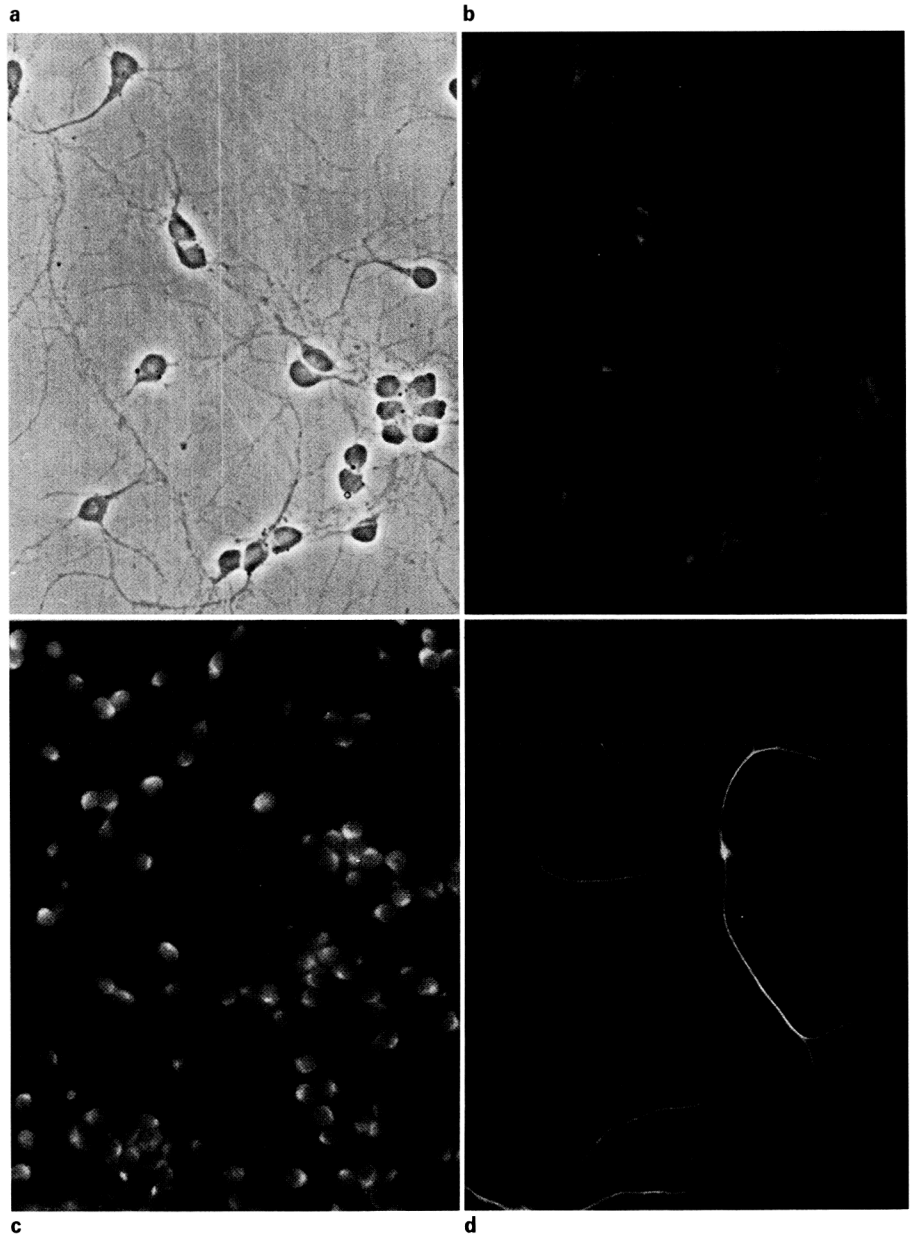


Fig. 1. Indirect immunofluorescence of primary cerebellar cell cultures. Cells were obtained from P6 mice and cultivated for 6 days. **a** Phase contrast. **b** Immunostaining with polyclonal anti-FdAPP serum. APP immunoreactivity is detected in granular cells. **c/d** Double staining with anti-Fd APP serum (**c**) and (**d**) monoclonal anti-GFAP (**d**). Neurons are the main source of APP-immunoreactivity (**c**). Astroglia, identified by GFAP-staining (**d**) show a weak APP staining.

with polyclonal anti-Fd-APP showed an intense labeling of granular cells which represented about 90% of cells in culture (fig. 1a, b). Within these neurons, strong APP reactivity could be detected close to the nucleus at a somewhat excentric position. Double staining with anti-GFAP (glial fibrillary acidic protein) revealed only weak labeling of astroglia (fig. 1c, d). Fibroblasts and oligodendroglia, as identified by their morphology, also showed low APP reactivity (results not shown). Thus, under our in vitro conditions granular cells were the main source of APP.

Metabolic Characterization

In order to analyze APP expression within these cells proteins were metabolically labeled with [^{35}S]-methionine and immunoprecipitated with anti-CT, an antiserum raised against the synthetic cytoplasmic part of APP. As shown in figure 2a, three distinct bands with a molecular weight of approximately 102, 112 and 120 kD could be detected in detergent extracts from cell pellets. They correspond to translation products of APP 695 mRNA as was confirmed by RNA analysis (results not shown). These findings are in accordance with results

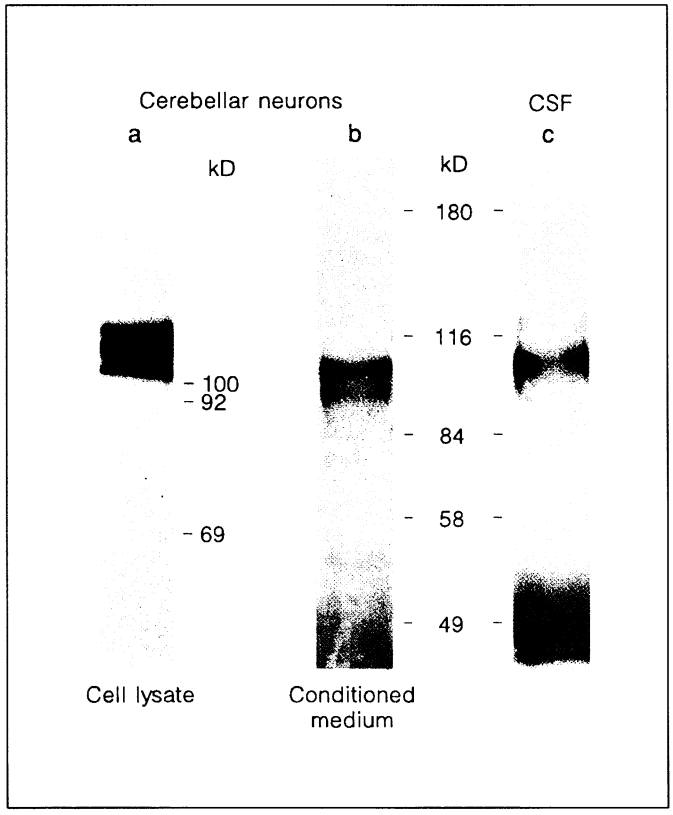


Fig. 2. Analysis of APP biosynthesis of cerebellar neurons. **a** Immunoprecipitation of APP from [^{35}S]methionine-labeled cell lysate of cerebellar neurons. Biosynthetic labeling was performed after 6 days of cultivation. The immunoprecipitates were analysed by autoradiography. Precipitation was done with the polyclonal anti-CT antiserum. **b** Identification of secretory APP from cerebellar neurons cultivated for 6 days. Conditioned medium of neurons was subjected to immunoprecipitation with anti-FdAPP serum. The immunoprecipitates were analyzed by immunoblotting. Detection of APP was performed with the monclonal antibody 22C11. **c** Western Blot analysis of APP from cerebrospinal fluid. Detection of APP was done as described in **b**.

from other groups who were able to show, that APP 695 is the predominantly expressed form in neurons [3, 4, 6, 8, 9, 31].

For detection of secretory APP released by neuronal cells, conditioned medium was investigated. Since polyclonal anti-Fd APP did not precipitate soluble isoforms of mouse APP under high detergent concentrations necessary for radioactive immunoprecipitations followed by autoradiography, secretory APP was identified by Western blot analysis with monoclonal 22C11. Two immunoreactive bands with an apparent molecular mass of 95 and 105 kD were identified (fig. 2b). The upper band of secretory APP was shown to comigrate with APP derived from cerebrospinal fluid (fig. 2c).

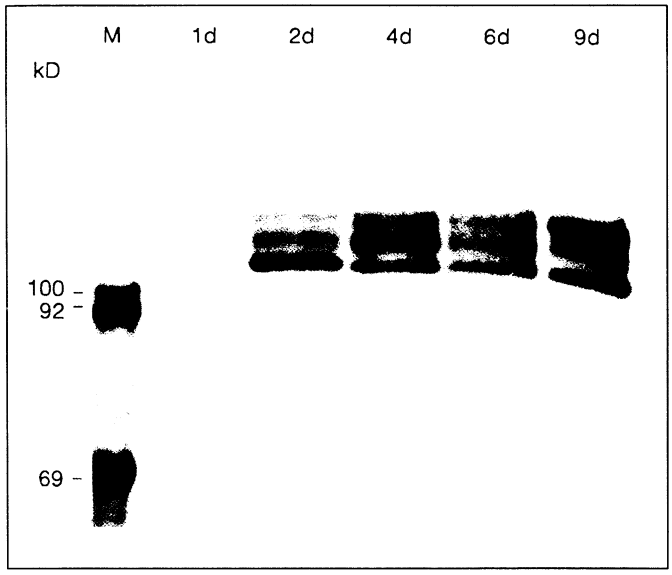
In order to assess APP biosynthesis during differentiation, cells were cultivated for different time periods (1, 2, 4, 6 and 9 days). At the end of each cultivation period, cells were metabolically labeled, immunoprecipitated with anti-CT and analyzed by SDS-PAGE. The autoradiography of the cell lysates is shown in figure 3a. The densitometric scanning analysis of the autoradiography is graphically demonstrated in figure 3b. At all time points investigated, cells in vitro expressed all three forms of APP 695. However, within prolonged cultivation time the ratio between these different forms changed. Whereas the lower band was dominating up to 48 h of cultivation, the higher molecular weight APP isoforms (112 kD and 120 kD) became more prominent upon longer cultivation time. At day 9 in culture, cells expressed large amounts of the high-molecular weight APP isoforms. The amount of secretory APP remained unchanged over the whole time investigated (results not shown).

Posttranslational Modifications

As could be shown by different authors, APP is posttranslationaly modified by N- and O-glycosylation as well as tyrosine sulfation and serine phosphorylation [12, 13]. APP has been proposed to be a cell adhesion molecule involved in cell-cell and cell-substrate interactions [31–34]. We have investigated whether neuronal APP is sialylated since sialic acid residues are known to play a key role in adhesion processes mediated by molecules like N-CAM (neuronal cell adhesion molecule) [35–37].

Immunoprecipitated APP was enzymatically digested with neuraminidase from *Clostridium perfringens* prior to separating proteins on SDS-PAGE. As shown in figure 4, the high molecular weight APP isoform (120 kD) disappeared after treatment with neuraminidase. Concomitant with the decrease of cell-associated 120-kD APP isoform there was an increase in the amount of cell-associated 112-kD APP. These experiments may indicate that the 112-kD APP isoform is the nonsialylated precursor of the high-molecular-weight APP isoform (120 kD). Sialylation of the 112-kD form gives rise to the apparent molecular weight shift of approximately 8 kD resulting in high-molecular-weight APP 695.

Cell associated, sialylated APP 695 was less prominent after 24 h of cultivation (8% of total APP) but contributes to about 45% of total APP upon 9 days of in vitro cultivation. This indicates that sialylation of neuronal APP 695 is regulated in a time-dependent way during in vitro differentiation.



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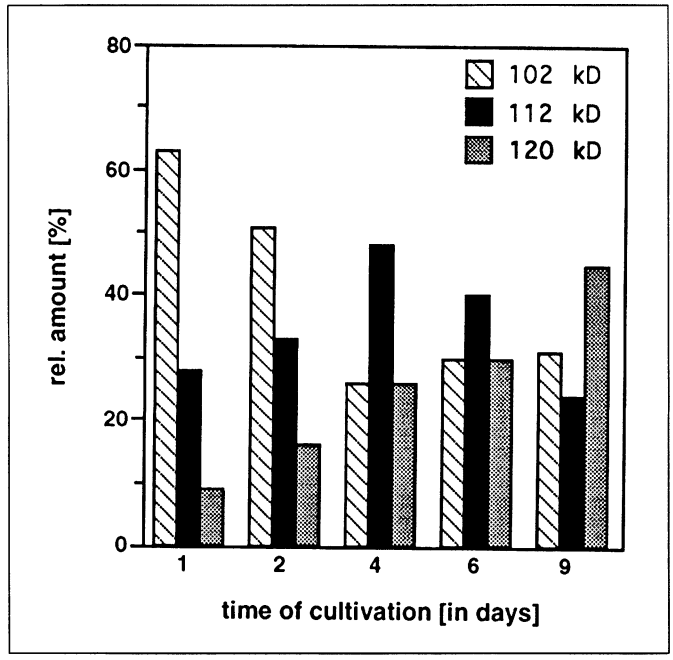

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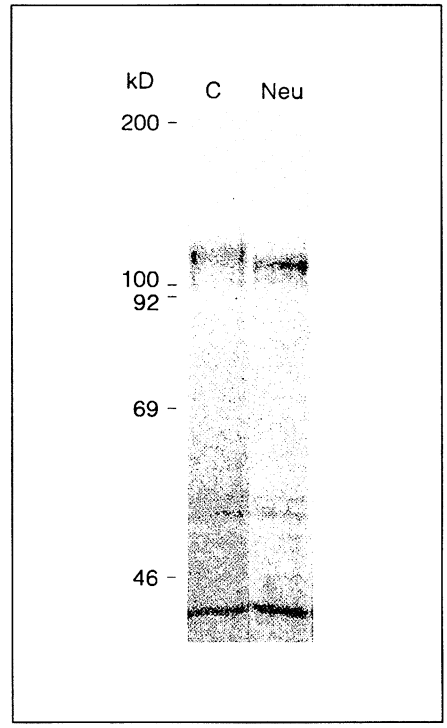

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Fig. 3. Expression of APP in cerebellar neurons during in vitro differentiation. **a** Cells were cultivated for different time periods. Biosynthetic labeling with [^{35}S]methionine was done at times indicated at the top of each lane. APP was isolated from cell pellets by immunoprecipitation with anti-CT antiserum. The immunoprecipitates were analyzed as in figure 2a. **b** Densitometric quantitation of the APP immunoprecipitation analysis (**a**) of cerebellar neurons cultivated for different time periods.

Fig. 4. Neuraminidase-treatment of cell-associated APP695 APP was immunoprecipitated from radiolabeled detergent extracts of cerebellar neurons with polyclonal anti-CT serum prior to enzymatic digestion with neuraminidase from *Clostridium perfringens* (Neu). Precipitates were analyzed by SDS-PAGE (8%) followed by autoradiography (C, non-digested APP).

Discussion

Primary neuronal cell culture from P6 mice was investigated to gain insight into APP expression during neuronal differentiation processes. Cerebellar neurons showed an intense labeling with anti-Fd APP, whereas astrocytes revealed only a weak APP immunoreactivity. This is in agreement with results from other groups, who have shown that neurons are the main source of cerebral APP production [10, 16, 31, 38, 39]. Large amounts of neuronal APP are associated with the Golgi apparatus as revealed by light- and electron-microscopic immunocytochemical techniques [39, 40]. Our data support these results since neuronal APP immunoreactivity was intracellularly located proximal to the nucleus. Rapid turnover of transmembrane APP by cleavage within the βA4 sequence might explain the weak cell surface staining. This is evidenced by the fact that large amounts of secretory APP could be detected already on day 1 in vitro.

We have shown that APP 695 from cerebellar neurons is posttranslationally modified by sialylation. Sialic acid residues are known to have great influence on adhesion properties of cell surface molecules. The best studied example is N-CAM (neuronal cell adhesion molecule) which exists in a highly sialylated form as well as several isoforms containing less sialic acid [41, 42]. The sialylation of N-CAM is developmentally regulated [43]. Polysialylation of N-CAM is known to promote axonal branching and decrease cell adhesivity, whereas removal of polysialic acid increases axonal fasciculation [35–37].

APP was shown to be involved in cell-cell and cell-substrate interactions [31–34]. We therefore suggest that sialylation of APP 695 might be a mechanism by which neuronal cell adhesion properties are modulated. Three distinct forms of neuronal APP 695 can be identified by Western blot analysis. The 120-kD APP isoform was shown to be sialylated. During in vitro differentiation, changes in the expression pattern of neuronal APP 695 were observed. Sialylated APP was rarely detectable after cultivation for 24 h, but was increased up to 45% of total APP upon 9 days of cultivation. These changes could provide the basis for regulation of cell-cell and cell-substrate interactions during in vitro differentiation of cerebellar neurons. The sialylation state seems to depend upon the state of differentiation of the neurons investigated. The presence of polysialylated cell associated APP may be a requirement for axonal branching and cell migration whereas a low sialylation state may be necessary for axonal fasciculation and cell adhesion.

We postulate that changes in the sialylation state of APP 695 play an important role in cellular interactions associated with neuronal cell migration and axonal guiding during early postnatal brain development. APP could thus belong to the group of cell surface molecules which are involved in the establishment of the neuronal network.

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