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APP RNA splicing is not affected by differentiation of neurons and glia in culture

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Amyloid precursor protein (APP) gene expression was investigated in primary cultures of neurons, astrocytes, microglial cells and oligodendrocytes. Neurons from various rat brain regions, as well as oligodendrocytes, contained RNA encoding APP_{695}, while astrocytes and microglial cells expressed high levels of RNAs for APP_{770} and APP_{751}. It was studied whether the cell type-specific regulation of APP gene expression could be modified by induction of cellular differentiation in vitro. While neuronal differentiation of PC12 cells has been shown to correspond with an altered pattern of APP splicing, in the primary cultures neither the time in culture nor a treatment of the cells with appropriate differentiation factors affected this pattern.

APP gene expression; Neuron; Astrocyte; Microglial cell; Oligodendrocyte

1. INTRODUCTION

Alzheimer's disease (AD) is correlated with massive deposition of fibrillar aggregates of the βA4 protein in the brain [1]. This protein is derived by proteolysis from a large transmembrane precursor protein, the βA4 precursor protein (APP). Three major forms of APP transcripts have been identified, which code for precursor proteins with 695, 751 and 770 amino acids. The two larger isoforms contain a sequence homologous to protease inhibitors of the Kunitz type. Gene analysis has revealed that the different APP transcripts arise from a single gene by alternative splicing [2–4].

These observations encouraged studies to investigate whether the levels and ratios of APP transcripts in AD brains differ from control brains. Alterations in the abundance of specific APP transcripts are discussed as a mechanism leading to the generation of the mature βA4.

APP is secreted from many cell types by the cleavage at a site which is located within the βA4 sequence [5,6]. This proteolysis can therefore not be responsible for the production of the mature protein and an alternative pathway for APP processing is likely to exist. In situ hybridization and PCR studies demonstrating the overexpression of APP_{751} in white matter of AD patients [7,8] implied that the increased expression of APP containing the Kunitz type protease inhibitor domain could favour the generation of βA4 by blocking the normal proteolytic pathway. The cellular origin of the amyloid deposits is still unclear; based on their distribution they are most likely produced by neurons [9–11]. Since histopathology of AD is accompanied by reactive gliosis, however, the participation of activated astrocytes and microglia in APP synthesis and generation of βA4 must be taken into account [12,13].

We decided, therefore, to use individual cell types of the CNS to investigate expression and splicing of APP in vitro. For the relative quantification of APP spliceforms we used PCR amplification of APP cDNA [7] from primary cultures of neurons (from cortex, hippocampus, septum, substantia nigra and rostral raphe), cortical astrocytes, microglia and oligodendrocytes. To mimic the in vivo situation of reactive gliosis, astrocytes were activated with bFGF or cAMP [14], and microglial cells with LPS, a highly potent activator of macrophages [15]. Neurons were studied with respect to their age in culture and to the influence of appropriate survival and differentiation factors.

2. MATERIALS AND METHODS

2.1. *Materials*

Media and other sterile solutions were either from Gibco or Flow Laboratories, and Nu-serum was from Collaborative Research. Bovine recombinant basic fibroblast growth factor and mouse GFAP-antibody were from Boehringer. O1, a mouse monoclonal antibody, was a gift from M. Schachner, Zürich. Other immunochemicals were from Vector Laboratories.

2.2. *Preparation of neuronal cell cultures*

Primary cultures of neurons from septum, hippocampus and cortex were prepared from rat brain at embryonic day 17 [16]. Cultures of dopaminergic or serotonergic neurons were isolated from the ventral mesencephalon or the rostral rhombencephalon, respectively, at E14

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[17,18]. After dissection, the appropriate tissue pieces were dissociated by gentle tituration in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline. The cell suspension was centrifuged for 10 min at 200 × g. The cells were plated at a density of 3×10^6 viable cells per 16-mm well in 24-well plates precoated with 30 μg/ml of poly-D-lysine. The culture medium contained 70% Eagle's minimum essential medium, 25% HBSS, 5% Nu-Serum, 6 mg/ml glucose, 70 U/ml penicillin, and 0.07 mg/ml streptomycin. Cultures were grown at 35°C in a 95% air/5% CO_2 humidified atmosphere.

2.3. *Preparation of astrocyte cultures*

Cultures were prepared from newborn Sprague–Dawley rats according to the method of Hertz et al. [19]. Briefly, after removal of the meninges, the cerebral hemispheres were cut into 1 mm cubes and vortexed at maximum speed. The tissue was then dissociated by tituration with a Pasteur pipette. After passing the cell suspensions through Nitex meshes (80–10 μm), cells were seeded in poly-D-lysine-coated culture dishes. These primary cultures were grown to confluency in DMEM containing 10% FCS and then passaged by trypsinisation. Cultures were characterized by GFAP immunocytochemistry.

2.4. *Preparation of oligodendrocytes*

Oligodendrocytes were prepared from mixed glial cultures isolated from neonatal rat cortex according to [20]. After 8 days in vitro cultures were washed once with Ca^{2+}/Mg^{2+}-free HBSS. Subsequently, cultures were shaken vigorously for 20 min in HBSS to remove the bipolar oligodendrocyte precursor cells. The supernatants were replated at 2×10^4 cells/cm² in poly-D-lysine-coated culture dishes. To obtain differentiated oligodendrocytes, the cells were cultivated in DMEM containing 15 μg/ml insulin, 1 μg/ml transferrin and 30 nM selenite for several days. Mature oligodendrocytes were identified as O1-positive cells and represented about 60% of the total cell population.

As an alternative method to obtain differentiated oligodendrocytes we applied the purification from a crude suspension of glial cells on a percoll density gradient [21]. A band formed, with a density between δ 1.033 and δ 1.050, which delivered a population of almost pure $O1^+$ oligodendrocytes (>90%).

2.5. *Preparation of microglial cells*

Cultures of microglial cells were prepared from newborn ICR mice as described in detail [22]. For isolation of microglial cells, confluent glial cell cultures between day 12 and 14 were vigorously shaken on a rotary shaker for 1 h. The floating cells were collected and cultured in 6-well tissue culture plates at a density of 1.5×10^6 cells/well in DMEM containing 5% FCS. After 2 h, the non-adherent cells were washed out two times with Hank's balanced salt solution. The adherent microglial cells were nearly 100% pure when examined by non-specific esterase staining and immunofluorescence with anti-C3b receptor antibodies (Mac-1, Sera Laboratory, Crawleydown, Sussex, GB) and anti-F_c (IgG1/2b) receptor antibodies (NEN, Boston, MA).

2.6. *RNA extraction and PCR (polymerase chain reaction)*

Total cellular RNA was extracted from cell cultures by the acid phenol method [23].

Reverse transcription was performed using hexanucleotides as primers and M-MLV reverse transcriptase (BRL). The 20 μl reaction contained enzyme buffer (BRL), 1 μg of RNA, 100 pmol of the hexanucleotides, 10 U RNAsin (Promega), 200 U M-MLV RT, and dNTPs at a final concentration of 500 μM. The reaction was terminated after 1 h at 37°C. For PCR 1 μl of cDNA was used and the samples were brought to a final concentration of 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 6.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.01% gelatin, 6.8 μM EDTA, 10% DMSO and 100 μM dNTPs. Additionally, the reaction mixture (50 μl) contained 20 pmol of the forward and reverse primer and 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus). To monitor the reaction, the forward primer was 5' end-labeled with ^{32}P using polynucleotide kinase (Boehringer).

PCR was carried out in a programmable heating block (PREM III, LEP Scientific) using cycles consisting of denaturation at 94°C for 1 min, followed by annealing at 59°C and primer extension at 72°C for 2 min. The samples were separated on 4% agarose gels. The gels were dried and exposed to X-ray films. The amount of DNA in the bands was quantified by cutting the bands from the dried gels and subsequent Cerenkov counting.

Oligonucleotides were synthesized on an automated solid-phase synthesizer (Applied Biosystems 380B). The 20-mer oligonucleotides used for PCR were passed through Quick spin columns (Boehringer), precipitated with ethanol and dissolved in water.

The sequence for the forward primer was TACCACTGAGTCTGTGGAGG, corresponding to bases 834–853 of the APP_{695} sequence [9]. The sequence of the reverse primer was GGGGGTCTCCAGGTACTTGT, complementary to bases 921–902 of the APP_{695} sequence.

3. RESULTS

We used RT-PCR to examine the pattern of APP mRNA splicing in primary cultures of neurons, astrocytes, microglia and oligodendrocytes. The primers used for PCR flanked the alternative splice site [7]. They gave rise to PCR products of 87, 255 and 312 bp, corresponding to the APP_{695}, APP_{751} and APP_{770} mRNAs, respectively. The validity of this PCR method for the relative quantification of the APP slice forms has been demonstrated previously [7]. To ensure that it worked in our hand, we reproduced the well-characterized shift towards the expression of APP_{695} occurring in PC12 cells [24] upon induction with NGF (data not shown).

We then analysed primary cultures of neurons from different brain regions. Purity of the neuronal cultures was ensured by PCR analysis of the mRNAs with primers for GFAP, delivering no positive signals (data not shown). GFAP is an intermediate filament protein specific for astrocytes. Examining neurons from hippocam-

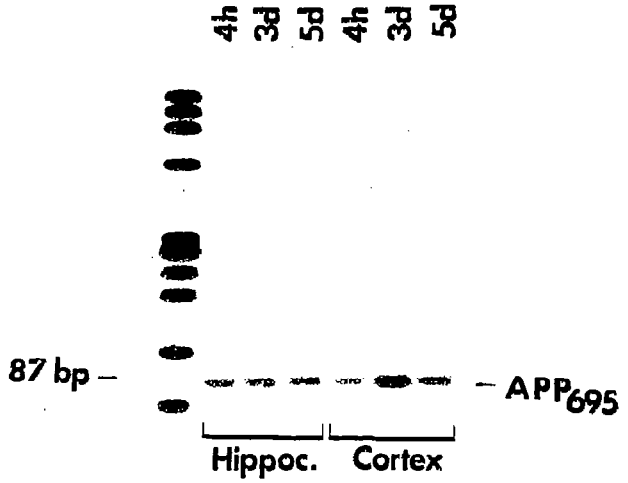

Fig. 1. PCR analysis of APP mRNAs in neurons from hippocampus and cortex. Cells were cultivated for 4 h, 3 or 5 days, respectively. Total RNA was extracted from cultured cells, reverse transcribed and amplified by 30 cycles of PCR with ^{32}P-labelled APP-specific primers spanning the sites of differential splicing. The indicated fragments were generated by PCR. The slot on the left contained ØX174DNA digested with *Hae*III as a marker.

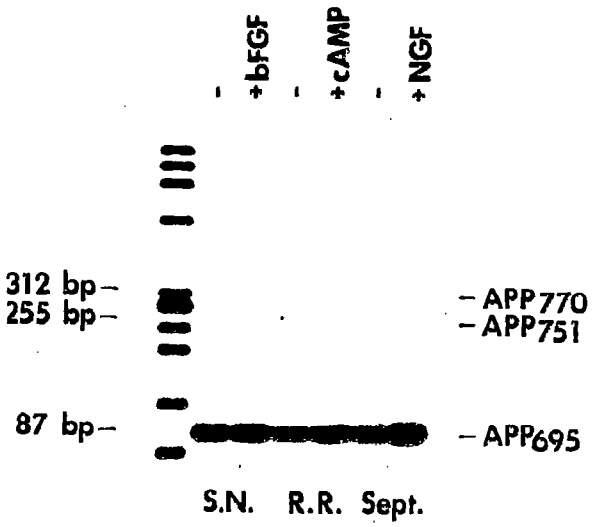

Fig. 2. PCR analysis of APP mRNAs in neurons from substantia nigra (S.N.), rostral raphe (R.R.) and septum. Neurons were cultivated for 4 days in the absence or presence of bFGF (50 ng/ml), cAMP (1 mM), or NGF (10 ng/ml), respectively. Total RNA was extracted, reverse transcribed and amplified with APP-specific primers.

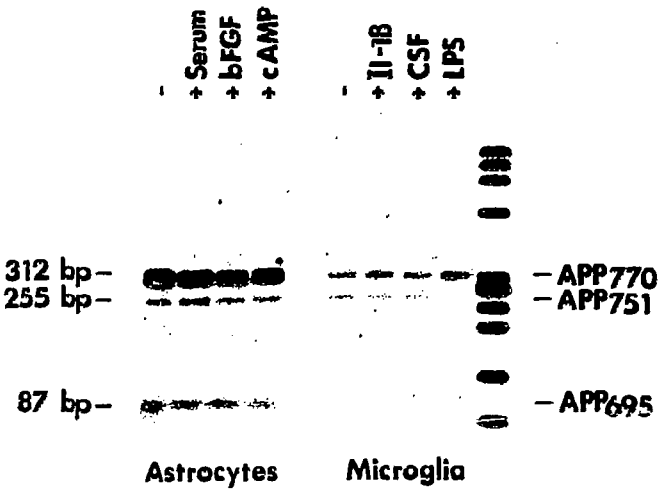

Fig. 3. PCR analysis of APP mRNAs in astrocytes and microglia. Astrocytes were grown in the absence of serum or in the presence of either 10% fetal calf serum, bFGF (50 ng/ml) or cAMP (1 mM). Microglial cells were stimulated with Il-1β (10 ng/ml), M-CSF (100 U/ml) or lipopolysaccharide (LPS; 10 μg/ml) for 24 h.

pus and cortex, we showed that APP_{695} was exclusively expressed in these cultures. The two larger isoforms, APP_{751} and APP_{770}, could not be detected (Fig. 1). This expression pattern did not change with time in culture (4 h, 3 or 5 days).

Primary cultures of neurons from substantia nigra, rostral raphe and septum were also examined. PCR analysis of these neurons again revealed the expression of only the APP_{695} isoform (Fig. 2). Treatment of the different types of neurons with appropriate survival or differentiation factors (50 ng/ml of bFGF, 1 mM of db-cAMP, or 10 ng/ml of NGF, respectively) did not alter the splicing pattern of APP. The absolute amounts of the transcript for APP_{695} were not determined since the PCRs were not performed in the presence of an internal standard.

In contrast to neurons, astrocytes in culture expressed not only APP_{695}, but also the two isoforms with the Kunitz domain, APP_{751} and APP_{770}. APP_{770} was the most abundant form in astrocytes (Fig. 3). Upon stimulation of the cells with bFGF (50 ng/ml) or cAMP (1 mM) the expression pattern of APP remained unchanged, while this treatment altered the morphology and antigen expression of astrocytes in a way that resembled a reactive gliosis [14].

Furthermore, we analysed the APP splice pattern of microglia in culture. APP_{751} and APP_{770} were the major APP forms in microglial cells. Expression of APP_{695} was only very sparse (Fig. 3). APP expression was not altered upon stimulation of the cells with IL-1β (10 ng/ml), macrophage colony-stimulating factor (100 U/ml) or LPS (10 μg/ml) for 24 h.

As oligodendrocytes are another major cell type in the brain we examined if oligodendrocytes in culture express APP, using bipolar progenitor cells and differentiated oligodendrocytes (60% pure). Moreover, differentiated oligodendrocytes were enriched by density gradient chromatography (90% pure population). Fig. 4 shows that all three samples mainly expressed APP_{695}. The two isoforms with the Kunitz domain were found only in the 60% pure oligodendrocyte population. They were probably derived from astrocytes and microglial cells present in these cultures. The APP_{695} seen here could not be of neuronal origin since only very few neurons, if any, were present in these cultures.

4. DISCUSSION

APP gene expression was investigated by RT-PCR in different types of primary neurons, astrocytes, microglial cells and oligodendrocytes.

Pure neuron cultures derived from fetal rat brain exclusively expressed APP_{695}. The expression of this isoform was independent of the state of differentiation of the cells and of the time in culture. We did not investigate neuronal cultures that were cultivated for more than 8 days since contaminating astrocytes proliferated with time and reduced the purity of the cultures.

As described for other non-neuronal cells [2,25,26] APP_{770} and APP_{751} were the major APP isoforms in astrocytes and microglia. In addition, we detected significant levels of APP_{695} in astrocytes, and small amounts in microglia. While it has been reported that astrocyte cultures did not express APP forms containing the Kunitz domain [27], our study clearly demonstrated the expression of APP_{770} and APP_{751} in astrocytes. LeBlanc et al. [28] obtained similar results using Northern blots and RNase protection assays.

To mimic the in vivo situation of reactive gliosis,

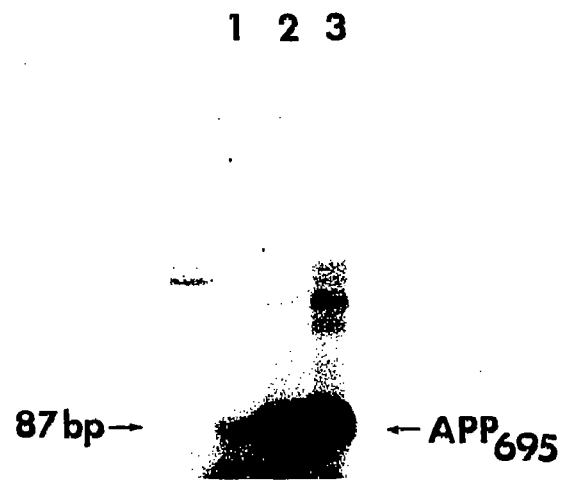

Fig. 4. PCR analysis of APP mRNAs in oligodendrocytes (obtained by percoll density chromatography), bipolar precursor cells (O-2A) and oligodendrocytes (obtained by the shaking method, 60% pure).

astrocytes were treated with bFGF or cAMP. This treatment imitates several of the effects described in reactive gliosis [14]. The activation did not affect the splice pattern of APP. The same could be demonstrated for activated microglia in culture. These results indicated that glial activation did not involve an alteration of the splice pattern of APP mRNA. It is of particular interest that oligodendrocytes expressed the neuron-specific isoform, APP_{695}, rather than the APP isoforms typical for non-neuronal cells. Even the bipolar progenitor cells (O-2A cells), that generate both oligodendrocytes and type 2-astrocytes in culture [29], expressed only APP_{695}.

Our results confirmed the existence of a cell-type specific post-transcriptional regulation of APP gene expression at the level of splicing. Although the pattern of APP splicing is modified during the induction of a neuronal phenotype in various tumor cell lines [24,30,31], a specific splice pattern apparently does not depend on the state of differentiation or activation of the respective cell type. Therefore, it is reasonable to argue that a switch in APP splicing may not be responsible for the increased occurrence of APP_{751} observed in AD cases. The observed shift to the APP isoforms containing the Kunitz domain may instead be explained by an elevated number of astrocytes and microglia due to reactive gliosis and concomitant neuron loss.

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