

Synthesis of apolipoprotein E (ApoE) mRNA by human neuronal-type SK N SH-SY 5Y cells and its regulation by nerve growth factor and ApoE

Cathia Soulié^a, Valérie Mitchell^a, Lætitia Dupont-Wallois^a,
Marie-Christine Chartier-Harlin^b, Jean-Claude Beauvillain^a,
André Delacourte^a, Marie-Laure Caillet-Boudin^{a,*}

^aINSERM U 422, Place de Verdun, F-59045 Lille Cedex, France

^bINSERM U508, Rue C. Guérin, 59019 Lille Cedex, France

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Abstract

By in situ hybridization, we show the ability of human neuroblastoma SY 5Y cells to synthesize apolipoprotein E (apoE) mRNA. This synthesis varied during cell NGF-differentiation: the mRNA level decreased during the first 4 days of NGF treatment (NGF 4 days) and then increased during the 3 following days (NGF 7 days). Furthermore, a treatment of 4-day NGF differentiated cells with exogenous apoE during 3 additional days induced a clear decrease in apoE mRNA synthesis when compared with control cells. This effect was more or less pronounced according to the apoE tested variants: apoE4 was more efficient to decrease the apoE mRNA synthesis as compared with the control cells than apoE3 which was itself more efficient than apoE2. These results suggest that apoE mRNA synthesis in human neuronal-type cells could be regulated by different mechanisms such as those induced by NGF- and apoE-treatments. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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In humans, apolipoprotein E (apoE) exists as three major structural isoforms: apoE2, apoE3, apoE4 [16]. For a few years, apoE has been thought to play an important role in the central nervous system (CNS). Indeed, according to the expressed isoforms, apoE might be involved; (i) in maintaining the integrity of the aging CNS [10]; (ii) in repair, growth and maintenance of myelin and axonal membranes during development and after an injury [8,14]; (iii) in neurite outgrowth [3,12]; (iv) in neurotoxicity [9], and (v) in pathological processes such as Alzheimer's disease [15].

By immunohistochemistry, cerebral apoE protein was mainly located in glial cells, particularly in astrocytes [14]. Nevertheless, apoE protein has also been detected in some neurons of Alzheimer brain [7], control brain [11], patients with pontosubicular necrosis [1]. In situ hybridization studies on brain slices have showed that apoE mRNA synthesis mainly occurred in astrocytic cells [4,14,19]. It

was never detected, by this method, in neurons on brain slices. Then, neuronal apoE protein has been postulated to be taken up by neurons. However, two types of experiments allow to think that a synthesis of apoE might be possible in neurons. First, we have shown that human neuroblastoma cells, such as SK N SH-SY 5Y (SY 5Y) and Kelly cells, expressed apoE mRNA and apoE protein detected, respectively, by RT-PCR and Western blotting experiments [6]. Secondly, apoE protein and mRNA are both detected in some neurons of transgenic mice expressing human apoE [17,18]. These experiments could be in favor of a neuronal apoE synthesis in some particular conditions. As a clear understanding of the sites of apoE mRNA synthesis and its regulation is probably important to determine the role of apoE in the CNS, we have investigated the expression of apoE mRNA in neuronal-type SY 5Y cells, by in situ hybridization and we have looked for factors apt to regulate the cellular apoE synthesis. Particularly, we have tested the effects of cellular differentiation and exogenous apoE protein presence.

The present study was performed on human SY 5Y

* Corresponding author. Tel.: +33-3-20-62-2073; fax: +33-3-20-62-2079.

E-mail address: caillet@lille.inserm.fr (M.L. Caillet-Boudin)

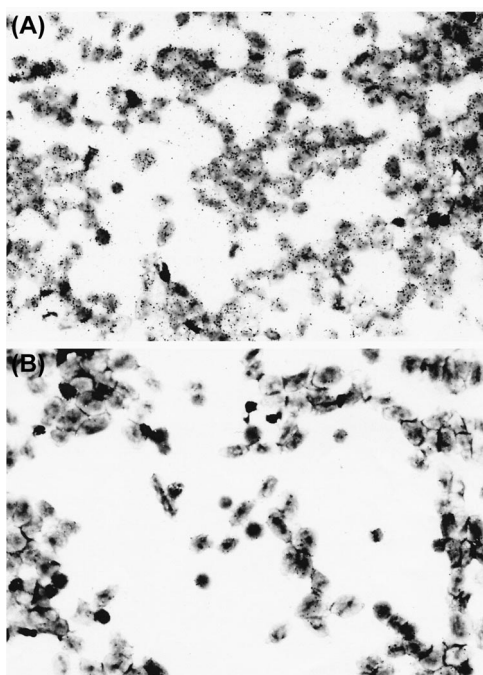


Fig. 1. Detection of apoE mRNA (A) by in situ hybridization experiments. Silver grains were located on most cells. Nuclei were stained with Azur blue. The background was shown in (B).

neuroblastoma cells which are able to differentiate in neuronal cells by nerve growth factor (NGF) treatment [13]. The mRNA apoE expression was investigated by in situ hybridization using a mixture of three 40-mer antisense oligonucleotide probes (5'-CCAGGAATGTGACCAGCAACG-CAGCCACAAACCTTCAT-3'; 5'-TTCAACTCCTTCA-TGGTCTCGTCCATCAGCGCCCTCAGTT-3'; 5'-CATG-TCTTCCACCAGGGGCTCGAACCAGCTCTTGAGGC-GG-3'), corresponding, respectively, to the nucleotides 1871–1910, 3780–3813, and to 4375–4414 on apoE gene sequence. The probes (50 ng) were labeled at the 3' end with [³⁵S]dATP (Amersham, France) using terminal transferase (Amersham, France) and purified from non-incorporated nucleotides. 0.5.10⁶ cpm of each probe per 30 μl of the hybridization mixture were used for incubation on the SY

Table 1
Quantification of apoE mRNA synthesis during cellular differentiation^a

	Mean ± SEM
ND	75.35 ± 1.96*
NGF 4 days	35.22 ± 0.94*
NGF 7 days	53.00 ± 1.89*

^a The cells were undifferentiated (ND) or differentiated by NGF treatment for 4 days (NGF 4 days) or for 7 days (NGF 7 days). Each value represents the mean ± SEM of silver grain densities which is representative of the mRNA level. *Significantly different, $P < 0.01$, ND versus NGF 4 days, NGF 4 days versus NGF 7 days, ND versus NGF 7 days (determined by one-way ANOVA analysis and post-hoc Bonferroni's t -test).

5Y cells grown on glass slides. Before the hybridization step, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 5 min, rinsed in PB, incubated in glycine 0.1 M, Tris 0.2 M, pH 7.4 for 10 min and then 15 min with proteinase K at 0.05 μg/ml in Tris/EDTA buffer before being fixed again for 15 min with 4% paraformaldehyde. Then, the slides were incubated for 55 min in prehybridization buffer consisting of 4 × standard saline citrate buffer (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7). The hybridization buffer consisted in 50% deionized formamide, 1 × Denhardt's solution (2% each of polyvinyl pyrrolidone, bovine serum albumin and Ficoll), 4 × SSC, 1 × sarkosyl, 0.1 M phosphate buffer and 10 mM dithiothreitol (DTT). After hybridization for 16 h at 42°C, the slides were rinsed in 1 × SSC with 10 mM DTT, then in 1 × SSC for twice 55 min at 20 and 45°C, and allowed to dry. Slides were dipped in LM1 (Amersham, France) and exposed for 4 weeks. The slides (four slides by experimental condition) were developed in D19 (Kodak), for 4 min and fixed in 30% sodium thiosulfate. Finally, the slides were stained with Azur blue 2/1000, mounted and coverslipped. Quantification of cell apoE mRNAs was performed under epifluorescence by counting silver grains in at least 80–100 cells by slide. We determined the grain density overlaying each cell by reflected light under darkfield epiillumination. Video images were obtained with a camera attached to the microscope. The computerized program was Densirag of Biocom (Les Ulis, France). The results were expressed as means of grain density per cell ± SEM and the statistical analysis was assessed by the one-way ANOVA analysis followed by post-hoc Bonferroni's t -test. The reproducibility of the results was controlled in two distinct experiments for each experimental condition. To determine the effects of cell differentiation on apoE mRNA synthesis, cells were differentiated by adding NGF (10 ng/ml) in a serum free culture medium (DMEM + F12 medium containing insulin, transferrin, selenite, progesterone) as described in Ref. [5]. Cells

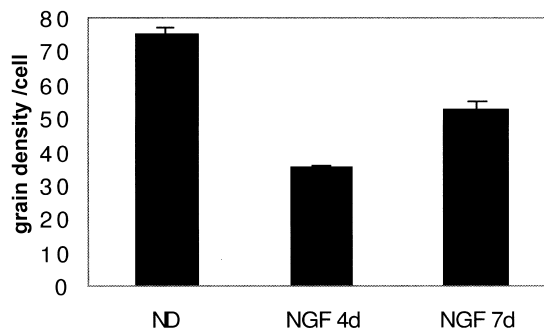


Fig. 2. Quantification of apoE mRNA expression during cellular differentiation. The cells were undifferentiated (ND) or differentiated by NGF treatment for 4 days (NGF 4 d) or for 7 days (NGF 7 d). The results are presented as silver grain densities. The values correspond to Table 1. These data show significant variations of apoE mRNA level during the differentiation time. Data are mean ± SEM (bars).

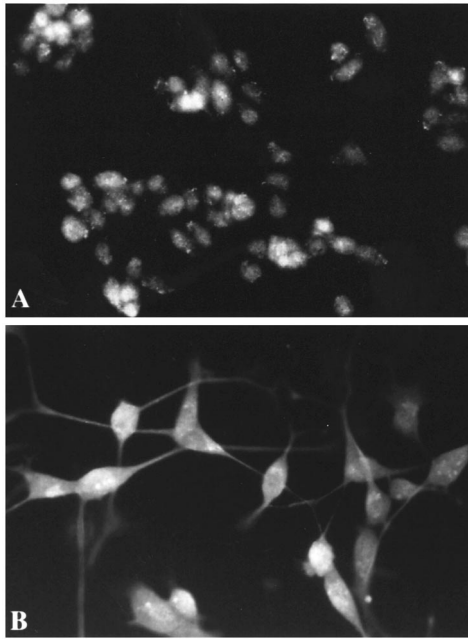


Fig. 3. Detection of apoE protein by immunofluorescence in both undifferentiated (A) and 4-day NGF differentiated cells (B). In (B), the neuritic extensions of differentiated cells were also immunolabeled.

were examined after 0, 4 or 7 days of NGF treatment. To determine the effects of exogenous apoE protein on cellular apoE mRNA synthesis, SY 5Y cells were previously treated by NGF for 4 days before adding 250 nM of one of the different isoforms of apoE (purchased from PanVera, Madison, Biogene Science) in the cell medium for 3 additional days. ApoE protein expression was investigated by immunofluorescence in both undifferentiated and NGF-differentiated cells. After fixation with 4% paraformaldehyde, cells were first incubated in PBS containing 5% donkey serum

Table 2
Quantification of apoE mRNA during apoE treatment^a

	Mean \pm SEM
NGF 4 days	46.87 \pm 1.43*
NGF 7 days	76.77 \pm 1.27*
apoE2	35.00 \pm 1.38*
apoE3	24.3 \pm 0.66*
apoE4	10.1 \pm 0.97*

^a The human recombinant apoE (apoE2, apoE3 or apoE4) (250 nM) (Panvera corporation) was added in the culture medium of 4-day NGF differentiated cells and the cells were maintained for 3 additional days. NGF-treated cells maintained in culture during the same time in absence of exogenous apoE were used as control cells (NGF 7 days). The apoE mRNA level before the treatment was determined and corresponded to the 4-day NGF treated cells (NGF 4 days). Data are mean \pm SEM values of silver grain densities. *Significantly different. *P*-value was calculated for all the possible arrangements by post-hoc Bonferroni's *t*-test after performing a one-way ANOVA analysis.

during 30 min to decrease the background. Then, cells were incubated for 90 min with rabbit polyclonal purified antibodies directed against apoE protein (1/2500) (generous gift from Drs. J.C. Fruchart, J. Najib, H. Parra), washed three times with PB buffer and incubated for 90 min with FITC-conjugated donkey anti-rabbit IgG (1/300) (Interchim, France). Cells were examined under an axiophot (Zeiss, Göttinger, Germany) epifluorescence microscope.

By in situ hybridization performed on untreated SY 5Y cells, we detected the expression of apoE mRNA (Fig. 1A). All the cells and not only a subpopulation synthesized apoE mRNA (Fig. 1A). The specificity of the probes was established by use of an excess of unlabelled probes (20 times more) over the radioactive probes, which resulted in the abolition of the specific signal (Fig. 1B).

During the differentiation process (Table 1, Fig. 2), a significant decrease of apoE mRNA was observed after 4 days of differentiation (35.22 \pm 0.94 grain density/cell versus 75.35 \pm 1.96, *P* < 0.01) and then this expression increased until 7 days of differentiation (53.00 \pm 1.89 grain density). These results were reproducible in two different experiments. Furthermore, we observed by immunofluorescence that apoE proteins were actually expressed in both undifferentiated or differentiated cells (Fig. 3). All cells are immunolabeled, even after 4-day NGF treatment (Fig. 3B)

Exogenous apoE also seemed to modify the cellular apoE mRNA synthesis level. NGF-differentiated cells for 4 and 7 days were used as control to determine the basic level of apoE synthesis in apoE-untreated cells, respectively, at the beginning and the end of the experiment. As seen in Fig. 4 and Table 2, exogenous apoE induced a significant decrease of cellular apoE mRNA expression when compared with the level of untreated cells (NGF 4 days or NGF 7 days), but apoE4 was more efficient to decrease the apoE mRNA expression as compared with the control cells (10.1 \pm 0.97 versus 46.87 \pm 1.43 (NGF 4 days) and versus 76.77 \pm 1.27 (NGF 7 days)) than apoE3 (24.3 \pm 0.66) which was itself more efficient than apoE2 (35.00 \pm 1.38).

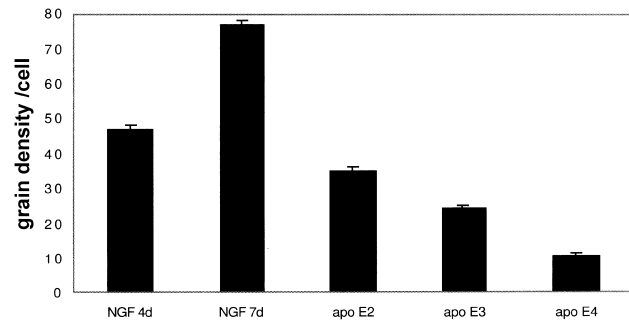


Fig. 4. Quantification of apoE mRNA during apoE treatment, the values correspond to Table 2. Data are mean \pm SEM (bars). We observe a clear decrease of cellular apoE mRNA synthesis in presence of exogenous apoE when compared with the 7-day NGF treated cells. This effect was more pronounced with apoE4 isoform than apoE3, and with apoE3 than apoE2.

Thus, this result is consistent with the hypothesis that, during a possible neuronal expression of apoE, a regulation by exogenous apoE could take place and that this negative control would be dependent of the apoE variant. As apoE is thinking to play a role during neuronal differentiation or regeneration processes [8,12], this result could explain why allele $\epsilon 4$ acts as risk factor [15] whereas $\epsilon 2$ would be protective [2] as well as why apoE3 increases neurite extension more than apoE4, whereas apoE4 increases more branching than apoE3 [12].

In conclusion, the present study demonstrates; (i) a synthesis of apoE mRNA by neuronal-type SY 5Y cells, and (ii) a regulation of this cellular apoE mRNA synthesis. The neuronal features of these cells are largely admitted in the literature [13]. At least, two factors, NGF and ApoE, seemed to be efficient to modulate the cellular apoE mRNA synthesis. Furthermore, this study shows that these cells could be used as a biological tool to understand the role and regulation of apoE, in neuronal growth, differentiation and survival.

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