Binding and Repressive Activities of Apolipoprotein E3 and E4 Isoforms on the Human ApoD Promoter

Louis-Charles Levros Jr. · Marilyne Labrie · Cyndia Charfi · Eric Rassart

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Abstract Apolipoprotein D (ApoD) gene expression is increased in several neurological disorders such as Alzheimer’s disease (AD) and multiple sclerosis. We previously showed that transgenic mice that overexpress human ApoD show a better resistance against paraquat or OC43 coronavirus-induced neurodegeneration. Here, we identified several nuclear factors from the cortex of control and OC43-infected mice which bind a fragment of the proximal ApoD promoter in vitro. Of interest, we detected apolipoprotein E (ApoE). Human ApoE consists of three isoforms (E2, E3, and E4) with the E4 and E2 alleles representing a greater and a lower risk for developing AD, respectively. Our results show that ApoE is located in the nucleus and on the ApoD promoter in human hepatic and glioblastoma cells lines. Furthermore, overexpression of ApoE3 and ApoE4 isoforms but not ApoE2 significantly inhibited the ApoD promoter activity in U87 cells (E3/E3 genotype) cultured under normal or different stress conditions while ApoE knock-down by siRNA had a converse effect. Consistent with these results, we also demonstrated by ChIP assay that E3 and E4 isoforms, but not E2, bind the ApoD promoter. Moreover, using the Allen Brain Atlas in situ hybridization database, we observed an inverse correlation between ApoD and ApoE mRNA expression during development and in several regions of the mouse brain, notably in the cortex, hippocampus, plexus choroid, and cerebellum. This negative correlation was also observed for cortex layers IV–VI based on a new Transcriptomic Atlas of the Mouse Neocortical Layers. These findings reveal a new function for ApoE by regulating ApoD gene expression.

Keywords Apolipoprotein D · Apolipoprotein E · OC43 · Mass spectrometry · ChIP · Glioblastoma astrocytic cells

Introduction

Human apolipoprotein D (ApoD) is a 29-kDa secreted glycoprotein and a member of the lipocalin superfamily [1, 2]. ApoD mRNA is expressed at high levels in several tissues particularly in the central nervous system (CNS) where it is produced in glia but also in scattered neurons (for review, see [3]). However, in contrast to other apolipoproteins, such as apolipoprotein E (ApoE), it is poorly expressed in liver and intestine. Moreover, its gene expression is increased in several neuropathologies such as Alzheimer’s disease (AD), meningoencephalitis, stroke [4], Parkinson’s [5], Niemann–Pick’s type C [6], and multiple sclerosis [7]. Furthermore, ApoD expression and proliferation are inversely correlated in response to cellular stress. This regulation of ApoD gene expression is reflected by the numerous cis elements located within its promoter [3, 8]. Several nuclear factors that bind the human ApoD promoter under growth arrest were identified. Notably, PARP-1, APEX-1, and ERK1/2 were found to regulate ApoD gene expression [9]. Despite several putative roles attributed to ApoD, such as repair and reinnervation as well as lipid turnover after nervous tissue injury [10, 11], the precise role of ApoD in the CNS remains elusive but accumulating evidences suggest a neuroprotective role as a repair protein.
ApoE is a 34-kDa secreted protein that was first identified in 1973 in association with very-low-density lipoprotein [12]. After the liver, the brain is the second site of expression for ApoE [13]. Three major ApoE isoforms exist in human population: ApoE2, ApoE3, and ApoE4, thus giving rise to six different genotypes. This genetic heterogeneity of ApoE is due to amino acid composition at positions 112 and 158 (E2, cys112/cys158; E4, arg112/arg158; and E3, cys112/arg158) [14, 15]. More importantly, ApoE is the major genetic marker associated with AD, the E4 and E2 alleles representing a greater and a lower risk for developing the disease, respectively [16, 17]. Since both ApoD and ApoE share common biological functions and are also implicated in neurodegenerative situations, it was of interest to investigate their respective regulation.

We previously showed that overexpression of human ApoD in transgenic mice treated with paraquat or infected with OC43 coronavirus (HCoVOC43) which both provoke brain inflammation and neurodegeneration confers a neuroprotection [18, 19]. Paraquat treatment is known to generate ROS and thus protein and lipid peroxidation [20]. HCoVOC43 are enveloped positive-stranded RNA viruses responsible for respiratory, enteric, and neurological diseases [21, 22]. Indeed, HCoVOC43 has neurotropic capabilities [23–25] and was also found in human brains [24]. Most importantly, several studies detected the OC43 virus in subjects with multiple sclerosis [26, 27] suggesting the involvement of a coronavirus in neurological disorders along with environmental and genetic factors [28].

Using this mouse model of neurodegenerative disease, we identified several nuclear factors from the cortex of control and OC43-infected-mice which bind in vitro a DNA fragment of the human ApoD promoter. We demonstrate that human ApoE isoforms E3 and E4 but not E2 bind the ApoD promoter in vivo and downregulate its gene expression. We also observed an inverse correlation between ApoD and ApoE expression in the mouse cortex using several databases. Overall, these results demonstrate a new function for ApoE by regulating the ApoD gene expression.

Materials and Methods

Cell Culture and Reagents

Human glioblastoma cells (U87 and U373M) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10 % inactivated fetal bovine serum, penicillin G (100 units/ml) and streptomycin (100 μg/ml). All cells were maintained at 37 °C in a 5 % CO2-humidified atmosphere and were fed every 2 days with fresh medium.

Virus and Mice Infection

The American Type Culture Collection HCoV-OC43 strain (ATCC VR-759) was grown on the HRT-18 cell line, and virus stocks (10–6.5 tissue culture infectious dose 50 (TCID50/ml) were kept at −80 °C as previously described [29]. Mice were infected at 22 days postnatal with intracerebral (IC) inoculation of 10 μl containing ten TCID50 of HCoV-OC43. Control mice received an IC inoculation of 10 μl of cell culture medium. Mice (C57BL/6) were killed at 7 days postinfection, and cortex was collected.

Nuclear Extract

Briefly, 200 mg of cortex of control (sham) or OC43-infected mice were homogenized in 500 μl of NE1 buffer (250 mM sucrose, 15 mM Tris–HCl pH 7.9, 140 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 20 % glycerol, 0.15 mM spermine, 0.5 mM spermidine, 1 mM DTT, 0.4 mM PMSF, 25 mM KCl, and 2 mM MgCl2). After homogenization, 25 μl of Nonidet P-40 (Sigma Chemical) at 10 % were added followed by a second step of homogenization. Lysates were cleared by 8 min of centrifugation at 1,000×g. The nucleus pellet was wash with 1 mL of NE1 buffer and was resuspended in 100 μL of NE2 buffer (buffer NE1 with 350 mM KCl) followed by homogenization and centrifugation for 5 min at 12,000×g. Organels were eliminated with centrifugation at 180,000×g for 90 min. The lysate were dialyzed for 1 h with buffer D (50 mM KCl, 4 mM MgCl2, 20 mM K3PO4 pH 7.4, and 1 mM de β-mercaptoethanol) using 0.025 μm filter (Millipore) and kept at −80 °C until use. All steps of the extraction protocol were done at 4 °C. The protein concentration was determined at 595 nm using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Affinity Purification of Nuclear Factors and Mass Spectrometry

The region −817 to +64 of the human ApoD promoter was amplified by PCR in six overlapping regions (1–6) using biotinylated primers (see Supplementary Fig. S1a, b). Fragments of approximatively 200bp were obtained for each region. Streptavidin beads (200 μg; Dynabeads M-280 Streptavidin, Dynal Biotech, Oslo, Norway) were resuspended and incubated with 6 μg of biotinylated DNA (regions 1–6) for 15 min in W&B buffer (1×; 10 mM Tris–HCl, pH 7.5, 1 M NaCl, and 1 mM EDTA) as recommended by the manufacturer. The washing steps were done in the same buffer, and the beads linked with the biotinylated DNA were incubated for 15 min at room temperature with 100 μg of cortex nuclear extracts containing 20 μl of annealing buffer 10× (50 mM Tris–HCl, pH 7.5, 1 M NaCl, 100 mM MgCl2, 0.2 mM EDTA, and 1 mM DTT), 1 mg of BSA, and 20 μg of
Poly(dI/dC) in a final volume of 200 µl. The beads were washed with a low salt washing buffer (20 mM Tris–HCl, pH 8.0, 0.05 % NP-40, 1 mM EDTA, and 75 mM KCl) and eluted with 60 µl of SDS at 0.1 %. The proteins were loaded and migrated on a SDS-PAGE (12 %) followed by Coomassie blue staining. Each lane was cut in forthen distinct bands eluted, trypsinized, and analyzed by mass spectrometry (LC-MS/MS velos) at the Genome Québec Innovation Centre Proteomics Platform (Montreal, QC, Canada) for protein identification. Proteins were identified by the presence of at least 1 distinct peptide in the sample with 99.9 % probability. Of note, no differences in band intensity were observed between each regions or conditions used for purification due to the sensibility of Coomassie blue staining. However, silver staining allowed to see clear differences between control or OC43 conditions for region 1 (Fig. 1c).

siRNA Transfections

For the siRNA experiments, two predesigned siRNA were used for human ApoE gene (si-ApoE1 and si-ApoE2; Qiagen), and negative control siRNA (Allstar negative control, Qiagen) were included in each experiment.

Luciferase Assays

The cDNA of human ApoE isoforms (E2, E3, and E4) were cloned in pCMV6 expression vector (Origene, Rockville, MD). Each plasmid was transiently co-transfected in U87 cells in the presence of Polyclect (Qiagen) as recommended by the manufacturer, with a vector containing the human ApoD promoter upstream of the luciferase reporter gene (−728/+4-ApoD-Luc). Twenty-four hours later, cells were

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**Fig. 1** Sequence of the proximal ApoD promoter and biotinylated primers used for amplification of smaller overlapping regions. a Schematic representation of the 5′ flanking region of the ApoD promoter (−828/+64) showing several known responsive elements [8, 41] and the positions of the primers (indicated with arrows of different colors). Six overlapping regions (1–6) of the ApoD promoter were amplified by PCR. b Nucleotide sequence of the primers with their respective positions on the ApoD promoter. c Silver-stained SDS-PAGE showing differences in band intensity between control and OC43 nuclear extracts. Proteins were eluted from region 1.
rinsed twice with phosphate-buffered saline and maintained in a medium supplemented with 10 % serum containing 10 μg/ml lypopolysaccharide (LPS), low-serum medium containing 0.2 % serum, or normal medium containing 10 % serum. Transfected cells were lysed at different time post-transfection and luciferase activity was measured with the Dynex MLX microplate luminometer (MLX Dynex Technologies, Chantilly, VA). Each sample was co-transfected with a β-gal-expressing vector for normalization (pcDNA-lacZ). The β-galactosidase activity was measured using the Galacto-Light™ kit (Applied Biosystems, Bedford, MS) according to manufacturer's suggestions. Luciferase activity is presented in relative light units and represents the calculated mean±SD of eight transfected samples normalized by the measured β-galactosidase activity.

Immunofluorescence and Confocal Microscopy

For immunofluorescence staining, U87 and U373M cells were grown on six-well plates (immunofluorescence) and Labtek chamber slides (confocal) and fixed with methanol for 30 min at −20 °C. After three washes with PBS, fixed cells were incubated in blocking (2 % goat serum) for 1 h. Cells were sequentially incubated with primary antibody (goat polyclonal antibody anti-ApoE, 1:100; obtained from Ross Milne, Ottawa Heart Institute) and then with fluorophore-labeled secondary antibody (alexa 488). For nucleus staining, cells were incubated with propidium iodide (1:300) (Sigma-Aldrich) for 10 min at room temperature.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed on human glioblastoma U87 and hepatic HepG2 cells according to the manufacturer protocol (Zm Tech Scientifique). Briefly, cells were cross-linked with 1 % formaldehyde and quenched with 0.125 M glycine. After sonication, chromatin was immunoprecipitated with anti-ApoE polyclonal or anti-DDK monoclonal antibodies (Origene). After several washes and DNA purification, semi-quantitative PCR was performed using specific primers for the ApoD promoter (−397 to +64; Fig. 1b).

Analysis of Gene Expression in Allen Brain Atlas, GENSAT, and the Transcriptomic Atlas of the Mouse Neocortical Layers

The Allen Brain Atlas (ABA; www.brain-map.org) [30] and GENSAT (www.gensat.org) [31] projects are public databases and useful resources to study the expression of genes in the mouse brain as well as during development. The ABA database mapped the expression of the whole mouse transcriptome (over 20,000 genes mRNA expression) by in situ hybridization (ISH) technique. The expression levels of ISH images are also quantified by color intensity as described in the website. The ISH images for ApoD and ApoE were selected from this database from 56 days old mice in order to compare their expression pattern in the adult mouse brain. Comparisons were also carried throughout development (from P4 to 24 months for ApoD and 33 months for ApoE) (see Figs. S1 and S2).

In the Transcriptomic Atlas of the Mouse Neocortical Layers (http://genserv.anat.ox.ac.uk/layers), cortical layers were separated in sections (A–F) from somatosensory (S1), dorsal cortex (DC), and lateral cortex (LC) and in which the transcriptome was sequenced [32]. The relative expression for each genes is represented in fragments per kilobase of exon model per million reads mapped (FPKM) values (Table 2). Sections A to F correspond to layers I–III, layer IV, upper layer V, lower layer V, layer VI, and layer VIb, respectively [32]. Correlation between expression of each gene was calculated using the Pearson’s correlation test.

Statistical Analysis

Statistical significance of the experiments was evaluated using an unpaired Student's t test. Results were considered statistically significant at P<0.05.

Results

Purification and Identification of Nuclear Factors Binding the Proximal Human ApoD Promoter In Vitro

We previously showed that ApoD gene expression is upregulated in the brain of human OC43 virus-infected mice [19]. In order to better understand the regulation of ApoD gene expression, we purified the nuclear factors that bind the ApoD promoter using nuclear extracts from the cortex of control and OC43-infected mice. Six overlapping regions of the ApoD promoter (−816/+64) were amplified by PCR using biotinylated primers and then linked to streptavidin beads (see “Materials and Methods”; Fig. 1a, b). After incubation with nuclear extracts, the specific proteins were eluted from the DNA-streptavidin complex, visualized by SDS-PAGE followed by Coomassie blue staining. Protein bands were then eluted and analyzed by mass spectrometry. Because of low sensibility of Coomassie blue, silver staining was used for samples obtained from region 1 and allowed to see clear differences between control and OC43 conditions (Fig. 1c).

Several transcription factors were identified, such as Stat1, Daxx, transcriptional activator Pur-α and Pur-β, Ilf-2, and Ilf-3 (also known as nuclear factors 45 and 90,
respectively), and several members of the HDAC, heat shock proteins, DDX, MAPK, importin, dynein, and kinesin families, just to name a few (unpublished data). Proteins implicated in neurodegenerative diseases, including AD, Parkinson’s disease, and Huntington’s disease and known not only for their tendency to aggregate but also to bind DNA nonspecifically (for review, see [33]), were also purified. In particular, we detected microtubule-associated protein tau, amyloid-β (Aβ) A4, alpha- and beta-synuclein, superoxide dismutase (Cu–Zn and Mn), Huntingtin, Huntingtin-interacting protein K, Huntingtin-interacting protein-related-1, and ataxin-10 (unpublished data). More interestingly, we identified proteins which are not recognized in the literature to bind DNA. Of particular interest, we found ApoE, clusterin (CLU; also known as ApoJ), phosphatidylinositol-binding clathrin assembly protein (PICALM), bridging integrator 1, high-density lipoprotein-binding protein (HDL-BP), and alpha-2-macroglobulin receptor-associated protein also known as low-density lipoprotein receptor-related protein-associated protein 1 (LRPAP1) (Table 1). Due to the novelty of ApoE binding to DNA and its association with neurodegenerative diseases, we further investigated the implication of this protein on ApoD gene regulation. ApoE was detected in both conditions in regions 1, 2, and 3 and only in control nuclear extracts in regions 4, 5, and 6 (Table 1).

**ApoE Localizes in the Nucleus of U87, U373M, and HepG2 Cell Lines**

Several studies already reported the nuclear localization of ApoE in different cell types such as human foreskin fibroblast (AG01518), Chinese-hamster ovary cells, ovarian cancer cells, and from various rat tissue cells such as brain and liver [34–38]. We performed immunofluorescence and confocal microscopy to investigate the possible nuclear localization of ApoE in glioblastoma astrocytic cells (U87 and U373M; Fig. 2) cultured in normal conditions. ApoE was found predominantly in the cytoplasm and to a lesser extent in the nucleus in a punctate pattern. The same pattern of nuclear localization was also seen in human hepatic cells HepG2 (data not shown).

**ApoE3 and E4 Bind the ApoD Promoter In Vivo**

Since ApoE was found in the nucleus, we investigated its binding to DNA. The binding specificity of ApoE on the ApoD promoter was verified by ChIP assay. We found that the ApoD promoter was immunoprecipitated by both ApoE and TFIID (positive control) antibodies in U87, U373M, and HepG2 cells (Fig. 3a). When overexpressing the different isoforms of ApoE (E2, E3, and E4) tagged with DDK in U87 cells, we demonstrated that only E3 and E4 but not E2 could be detected on the ApoD promoter compared with the control (empty pCMV6 vector; Fig. 3b). These results are consistent with the fact that the U87 and HepG2 cells genotype is E3/E3 [39]. However, to our knowledge, the ApoE genotype of U373M cells is unknown.

**ApoE3 and E4 Repress the ApoD Promoter Activity**

Since both mouse and human ApoE proteins were detected on the human ApoD promoter by mass spectrometry analysis and ChIP assay, we next attempted to determine if ApoE could regulate ApoD expression in U87 cells under different stress conditions. Indeed, we previously demonstrated that both mouse and human ApoD gene expression is upregulated in several stress conditions such as growth arrest and inflammation in different cell lines, more precisely in human astrocytic cell lines.

### Table 1

| Protein ID | Acct. No. | Region 1 | Region 2 | Region 3 | Region 4 | Region 5 | Region 6 |
|------------|-----------|----------|----------|----------|----------|----------|----------|
| ApoE       | P08226    | 4        | 3        | 2        | 3        | 3        | 2        |
| Clu/apoJ   | Q06890    | 4        | 2        | 6        | 0        | 5        | 3        |
| HDL-BP     | Q3V1M8    | 7        | 15       | 8        | 15       | 7        | 12       |
| LRPAP1     | Q3V1M8    | 0        | 2        | 1        | 0        | 2        | 0        |
| BIN1       | O08539    | 33       | 26       | 20       | 25       | 28       | 27       |
| PICALM     | Q3TD51    | 4        | 6        | 3        | 5        | 5        | 3        |

Nuclear extracts from the cortex of control (Ctrl) or OC43-infected mice were incubated with six overlapping regions (1–6) of the ApoD promoter (approximately 200 bp for each region) bound to streptavidin beads. The bound proteins were eluted and analyzed by SDS-PAGE and silver staining. Each well was cut in 14 distinct bands, eluted, and analyzed by mass spectrometry analysis (LC-MSMS velos) after tryptic digestion. For each region, the number of peptides which allowed protein identification are indicated. Unless otherwise indicated, all proteins were identified with peptides with a minimum of 99.9 % probability.

**LRPAP1 low-density lipoprotein receptor-related protein-associated protein 1, MW molecular weight**

*A A probability of 99.0 %"
cells (U373) and NIH/3T3 murine fibroblasts [40, 41]. Therefore, U87 cells were co-transfected with the different ApoE isoforms cDNA in pCMV6 vector and a construct containing the ApoD promoter upstream of the luciferase reporter gene (−728/+4-ApoD-pGL3). The luciferase activity was analyzed in normal (10 % serum), serum-starved (0.2 % serum), and inflammatory conditions (LPS; 10 μg/ml). At 24-h post-transfection (Fig. 4a), both ApoE3 and E4 repressed the ApoD promoter activity in normal and serum-starved conditions compared with the control (empty vector) while E2 isoform had no effect. In inflammatory conditions, the ApoD promoter was repressed in the control, but this repression was less prominent in the presence of E2. Moreover, E4 isoform repressed the ApoD promoter activity compared with the control, with E3 having no effect.

Furthermore, we used specific siRNA against ApoE gene to verify if the repressive activity of E3 isoform in U87 cells could be reversed since this cell line harbors a E3/E3 genotype. Indeed, blocking the expression of ApoE in U87 cells for up to 48 h post-transfection of specific siRNA (siApoE) (Fig. 4b), significantly transactivated the ApoD promoter in all conditions tested (Fig. 4c). These results show that ApoE3 and E4 negatively regulate ApoD gene expression.

**Discussion**

The ApoD gene is upregulated in several neuropathological disorders, such as AD, meningoencephalitis [4], multiple...
sclerosis [7], and Parkinson’s disease [5]. ApoD concentrations are increased by 350 and 60 % in the cerebrospinal fluid (CSF) and hippocampus, respectively, of patients with AD compared with normal subjects [4]. ApoD gene expression is also upregulated in the CNS of mice infected with viruses, which cause encephalitis, such as herpes simplex type-I virus [42], Sindbis virus [43], Japanese encephalitis virus [44], rabies virus [45], and human coronavirus OC43 [19]. In OC43-infected mice, ApoD gene expression was found upregulated by five- and sevenfold in the cortex and hippocampus, respectively. Several studies have speculated a neuronal protective role for ApoD [5, 11, 46–51] and overexpression of ApoD in neurons of transgenic mice confers neuroprotection [18, 19] and/or an improved repair potential.

The study presented herein reports the identification of several nuclear factors, from the cortex of control and OC43-infected mice, that bind the human ApoD promoter. Among the nuclear factors identified, we were particularly interested in the ApoE DNA-binding properties mostly because these two genes share common biological features. More precisely, both ApoD and ApoE proteins vary in concentration in several neurodegenerative diseases, bind to HDL [52], are involved in regenerative and reinnervation in the peripheral nervous system [10, 53]. Their expression is regulated after enthorinal cortex lesioning of the rat brain [11] and in several stress conditions, such as growth arrest caused by serum deprivation [35, 41, 54] and inflammation [40]. Moreover, under stress conditions, ApoD [40] and ApoE [34, 35, 40] were shown to enter the nucleus. Finally,
both proteins colocalize with Aβ within senile plaques and neurofibrillary tangles of AD brain tissue [51, 55, 56].

Using streptavidin beads and biotinylated PCR-amplified DNA complex, we showed that ApoE binds to all six regions of the ApoD promoter from nuclear extracts obtained from the cortex of either control or OC43-infected mice (Table 1). These results show that ApoE may be constitutively bound on the ApoD promoter in both normal and pathological conditions and demonstrate for the first time a DNA-binding activity for ApoE. Since ApoE does not contain any DNA-binding domain or NLS, we speculate that it binds the ApoD promoter by interaction with another nuclear protein. Despite this fact, ApoE nuclear localization has already been demonstrated in several cell types and tissues, including brain and liver [35–38]. Here, we confirm that ApoE is localized to the nucleus of human hepatic and glioblastoma cells (Fig. 2) and we clearly demonstrate by ChIP assays that E3 and E4 isoforms, but not E2, could bind the ApoD promoter in vivo (Fig. 3).

The mechanism by which ApoE may escape the secretory or endocytic pathways and be directed to the nucleus is still obscure. It is quite possible that ApoE enters the cytoplasm through interaction with cellular receptors such as low density lipoprotein receptor (LDLr) or LDLr-related family members (LRP) and once inside the cytoplasm could migrate to the nucleus via importins or nucleolin [57, 58]. We did not identify any known ApoE binding receptors among those factors binding the ApoD promoter but several functional related ApoE proteins were identified such as LRPAP1, ApoJ, and HDL-BP (Table 1). Interestingly, ApoJ is also implicated in several aspects of AD. Effectively, its gene expression is induced in AD [59] and plays pleiotropic functions such as removal of Aβ peptides and fibrils, brain cholesterol and lipid homeostasis in association with ApoE (for review, see [60]). Moreover, ApoJ is localized in the nucleus in complex with Ku70, a DNA binding subunit of DNA-dependent protein kinase (DNA-PK) [61]. Also, several genome-wide association studies have identified and associated PICALM, BIN1, and CLU genetic polymorphisms with AD (for review, see [62]), which were all found on the ApoD promoter. PICALM and BIN1 are both associated with clathrin endocytic pathways [63, 64].

Despite the fact that several studies have clearly demonstrated the presence of ApoE in the nucleus, its underlying role in this organelle remains unclear. It was speculated that ApoE could play a role in transcriptional activity [34, 37, 65]. In this present work, we clearly demonstrated that ApoE is implicated directly at the promoter level in gene transcription regulation. Indeed, overexpression of E3 and E4 isoforms significantly inhibited the ApoD promoter in normal and serum-starved conditions 24 h post-transfection while E2 had no effect (Fig. 4a). Unexpectedly, in inflammatory conditions, the ApoD promoter appeared repressed in contradiction with previous findings [40]. This apparent

| Gene | Gene ID | FPKM |
|------|---------|------|
| Somatosensory cortex | A | 78.5686 |
| | B1 | 20.2918 |
| | B2 | 33.3066 |
| | C | 46.2856 |
| | D | 58.6014 |
| | E | 101.871 |
| | F | 293.871 |
| Dorsal cortex | DCA | 169.173 |
| | DCB | 43.2737 |
| | DCC | 67.7391 |
| | DCD | 110.61 |
| | DCE | 164.592 |
| | DCF | 273.522 |
| ApoD | ENSMUSG000000002985 | 1,484.84 |
| | A | 897.763 |
| | B | 664.742 |
| | C | 563.017 |
| | D | 507.324 |
| | E | 1,501.04 |
| | F | 585.15 |
| Lateral cortex | LCA | 150.411 |
| | LCB | 96.6178 |
| | LCC | 107.019 |
| | LCD | 113.292 |
| | LCE | 174.006 |
| | LCF | 181.347 |
| ApoE | ENSMUSG000000002985 | 4,637.85 |
| | A | 3,191.89 |
| | B | 2,196.49 |
| | C | 1,668.19 |
| | D | 1,826.03 |
| | E | 1,501.04 |
| | F | 750.515 |

The FPKMs values were obtained from Ref. [32]. FPKMs values for ApoD and ApoE in the six-section layers (from superficial to deep layers (A–F)) of somatosensory cortex (S1), dorsal cortex (DC), and lateral cortex (LC). For somatosensory cortex (S1), section B was done in duplicate.
discrepancy is very likely due to the incubation time used (24 h in this study versus 3–6 days). Moreover, ApoE gene expression is upregulated by LPS in astrocytes and monocytes but repressed in macrophages [66]. Therefore, inhibition of ApoD promoter activity in presence of LPS may be due to endogenous induction of ApoE gene in U87 cells. Also specific siRNA directed against ApoE gene relieved the ApoD promoter from repression (Fig. 4c). In this experiment, only the ApoE3 isoform could be targeted with a specific siRNA due to the E3/E3 genotype of U87 cells [39]. Since a neuroprotective role for ApoD has been proposed, the fact that the E4 and E2 alleles represent a greater and a lower risk for developing AD, respectively is in agreement with our results of the effect of these isoforms on the ApoD promoter activity. In contrast, the repressive activity of E3 isoform on the ApoD promoter does not correlates well with ApoD neuroprotective role since the E3 allele is considered more beneficial than E4 in AD. Considering the clearly established neuroprotective role of ApoD, it is tempting to speculate that the neuroprotective action of ApoE2 is due to the fact that it does not inhibit ApoD expression. It would be interesting to further investigate this mechanism of regulation of the ApoD promoter by ApoE isoforms using glioblastoma cell lines with different ApoE genotype (E2/E2 and E4/E4) and also using knock-in mice for each human ApoE isoforms. Such experiments will clearly clarify the overall picture regarding the role of each ApoE isoforms on ApoD gene regulation.

In human brain, ApoD gene regulation is positively correlated with age at the opposite for ApoE [56, 67–70]. Interestingly, it was demonstrated that the increase of ApoD and decrease of ApoE concentrations in the CSF and brain respectively of AD patients are correlated in a dose-dependent manner with inheritance of the E4 allele [4, 39, 71, 72]. This correlation is also observed in the cortex, cerebellum and hippocampus of ApoE-KO mice where ApoD protein expression is increased [4, 73]. Also, the expression of ApoD and ApoE genes is differentially modulated in rats hippocampus 4 days after entorhinal cortex lesioning where ApoD is upregulated ipsilaterally and ApoE downregulated bilaterally [4]. Perdomo and Dong [74] also reported an increase of ApoD plasma levels in a mouse model of atherosclerosis using 3 months old ApoE-KO mice.

Based on the ABA database, we observed qualitatively that ApoD and ApoE mRNA expression are inversely correlated in the mouse cortex during development (Fig. S1) and also in several brain regions of 56 days old mice (Fig. S2). Effectively, ApoE mRNA appears strongly expressed in the cortex, hippocampus and plexus choroid (Fig. S2b–d) in contrast to ApoD gene expression. Furthermore, this inverse correlation was also observed in a transcriptomic atlas of the mouse brain neocortex for layers IV–VI corresponding to section B–F (Table 3). However, a positive correlation was shown in cortex layers I–IV corresponding to section A–B which is apparently attributable to higher ApoD expression in sample A relative to sample B (Table 3). ISH data from the ABA database in developing brain (Fig. S1) and previous work suggest that ApoD mRNA, like ApoE, is expressed in the pia mater of the subarachnoid space in monkey and rabbit brain [75, 76], some of which could have been swept into the dissection of section B. These cells and the perivascular fibroblasts of the subarachnoidal space derive from different lineages. It is thus possible that, in those particular cell lineages such a negative regulatory relationship is overpowered by other transcription factors. The significant negative correlation seen elsewhere (samples B–F corresponding to cortex layers IV–VI) is certainly consistent with our experimental findings of a negative regulatory relationship in glia. However, the cell-type specificities of ApoE and ApoD could be an alternative explanation. Indeed, the expression profile of ApoE is quite similar to the expression profile of many astrocyte-specific genes while the expression profile of ApoD is similar to the expression profile of many oligodendrocyte-specific genes [77].

In conclusion, several proteins were identified that bind the ApoD promoter in vitro from a mouse model of acute encephalitis induced upon infection by the human OC43 coronavirus. Our results demonstrate a novel function for ApoE through binding and regulating the ApoD promoter. We also observe an inverse correlation between the ApoE and ApoD gene expression in most regions of the mouse brain as well as during development. This inverse correlation certainly requires further investigations. Moreover, ApoE could, as a potential transcription factor, be involved in several neuropathological disorders.

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