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Original Article

Altered mechanisms of protein synthesis in frontal cortex in Alzheimer disease and a mouse model

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Abstract: Expression of the nucleolar chaperones nucleolin (*NCL*) and nucleophosmin (*NPM1*), upstream binding transcription factor (*UBTF*), rRNA18S, rRNA28S, and several genes encoding ribosomal proteins (RPs) is decreased in frontal cortex area 8 at advanced stages of Alzheimer's disease (AD). This is accompanied by reduced protein levels of elongation factors eEF1A and eEF2. Changes are more marked in AD cases with rapid course (rpAD), as initiation factor eIF3η is significantly down-regulated and several RP genes up-regulated in rpAD when compared with typical AD. These changes contrast with those seen in APP/PS1 transgenic mice used as a model of AD-like β-amyloidopathy; *Ncl* mRNA, rRNA18S, rRNA28S and seven out of fifteen assessed RP genes are up-regulated in APP/PS1 mice aged 20 months; only eEF2 protein levels are reduced in transgenic mice. Our findings show marked altered expression of molecules linked to the protein synthesis machinery from the nucleolus to the ribosome in frontal cortex at terminal stages of AD which differs from that seen in APP/PS1 transgenic mice, thus further suggesting that molecular signals in mouse models do not apply to real human disease counterparts.

Keywords: Alzheimer's disease, protein synthesis, nucleolar chaperones, rRNAs, transcription, translation, APP/PS1 transgenic mouse

Introduction

Brain atrophy in Alzheimer's disease (AD) and other neurodegenerative diseases is commonly considered the result of a combination of factors including nerve cell atrophy, nerve cell loss and reduced myelin fibers in the white matter. Since nerve cell loss is produced as a consequence of neuron death whatever the mechanism involved, neuron atrophy implies increased catabolism, reduced protein and other biomolecule/substrate synthesis, or a mixed scenario. In certain settings, reduced protein synthesis may lead to neuronal atrophy but also to cell death. Despite the importance of altered protein synthesis in AD, most data come from individual observations which focus on the nucleolus and the ribosome. The surface of nuclear organizer region (NOR)/total nucleus surface is reduced [1, 2], the rRNA gene promoter is hypermethylated, suggesting epigenetic rRNA silencing [3], rRNA levels are reduced, there is increased RNA oxidation [4-10], the expression of certain transcription factors is reduced [11-13], and incorporation of S^{35} methionine into AD brain ribosomes is decreased [9, 14].

Here we report a detailed study of the dentate gyrus and hippocampus at different stages of AD showing altered mRNA and protein expression of nucleolar chaperones nucleolin (NCL), nucleophosmin (NPM1) and nucleoplasmin 3 (NPM3), and upstream binding transcription factor RNA polymerase I (UBTF); altered 28S-RNA; decreased expression of genes encoding ribosomal proteins; and altered protein expression of several initiation and elongation translation factors [15]. Since these alterations are

Table 1. Summary of human cases used in the present study

Case	Diagnosis	Gender	Age	PM delay	RIN	WB
1	MA	M	64	8 h 30	7.7	X
2	MA	M	56	5 h	7.1	X
3	MA	M	67	5 h	7	X
4	MA	M	62	3 h	7.2	X
5	MA	M	52	4 h 40 m	7.9	X
6	MA	M	30	4 h 10 m	8.4	X
7	MA	M	53	3 h	7.7	X
8	MA	F	49	7 h	8.2	X
9	MA	F	75	3 h	6.5	X
10	MA	F	46	9 h 35 m	7.2	X
11	MA	F	86	4 h 15 m	8.4	X
12	MA	F	79	3 h 35 m	8	X
13	AD	F	89	8 h 50 m	6.5	X
14	AD	M	81	7 h 30 m	6.1	X
15	AD	M	91	7 h	7.3	X
16	AD	F	86	9 h	7.3	X
17	AD	M	92	7 h 45 m	6.5	X
18	AD	F	84	7 h 45 m	7.1	X
19	AD	M	82	5 h	7.1	X
20	AD	M	75	8 h 15 m	6.5	X
21	rpAD	M	69	18 h	7.2	X
22	rpAD	F	85	6 h	6.4	X
23	rpAD	F	76	18 h	6	X
24	rpAD	F	79	5 h 30 m	6.9	X
25	rpAD	M	83	5 h 30 m	6.4	X
26	rpAD	M	83	8 h 20 m	6.6	X
27	rpAD	F	81	6 h	6.8	X
28	rpAD	M	78	3 h 30 m	7	X

MA: middle-aged individuals with no neurological symptoms and no brain lesions on neuropathological study. AD: Alzheimer's disease cases with typical prolonged advanced dementia, Braak stage V-VI of neurofibrillary tangle pathology, Thal phase of β-amyloid and CERAD score C; rpAD: cases with clinical and neuropathological characteristics similar to AD but with a rapid course equal to or less than 2 years. M: male; F: female; PM delay: post-mortem delay; RIN: RNA integrity number (cases assessed with RT-qPCR); WB: cases used for western blotting.

region-dependent (they differ in CA1 and dentate gyrus), we decided to analyze frontal cortex area 8 at terminal stages of AD using the same probes and methods. We also compared AD cases with the typical clinical course of cases with rapid clinical course (rpAD) [16] to learn whether differences in disease progression are associated with particular changes in the protein synthesis machinery. Finally, we compared changes in AD with those seen in the somatosensory cortex of APP/PS1 transgenic mice used as a model of AD-like β-amyloidopathy.

Material and methods

Subjects

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank and the Hospital Clinic-IDIBAPS Biobank following the guidelines of Spanish legislation on this matter and the approval of the local ethics committee. Processing of brain tissue has been detailed elsewhere [17]. The post-mortem interval between death and tissue processing was from 3 to 18 hours. One hemisphere was immediately cut in coronal sections, 1 cm thick, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags and stored at -80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphological studies. Neuropathological diagnosis in all cases was based on the routine study of 20 selected de-waxed paraffin sections comprising different regions of the cerebral cortex, diencephalon, thalamus, brain stem and cerebellum, which were stained with haematoxylin and eosin, and Klüver-Barrera, and for immunohistochemistry for microglia, glial fibrillary acidic protein, β-amyloid, phosphorylated tau (clone AT8), α-synuclein, TDP-43, ubiquitin and p62. Stages of neurofibrillary tangle pathology [18, 19], phases of AD-related β-amyloid plaques [20] and ABC score were assigned according to current consensus guidelines [21]. All AD cases were sporadic. Neuropathological data were assessed by two independent observers. AD cases were 5 men and 3 women, mean age 85 ± 5.7 years; rpAD cases were 4 men and 4 women, mean age 79.2 ± 5.1 years. AD and rpAD cases had similar degrees of AD-related pathology. We used as controls middle-aged cases (MA, n = 12: 7 men, 5 women; mean age 59.9 ± 15.5 years) who had not suffered from neurologic, psychiatric, or metabolic diseases (including metabolic syndrome), and did not have abnormalities in the neuropathological examination excepting Braak stage I-II of NFT pathology with no β-amyloid deposition. A summary of human cases is shown in **Table 1**.

APP/PS1 mice

APP/PS1 mice and wild-type (WT) littermates aged 3, 12 and 20 months (n = 7 for each phenotype and time-period; total number of ani-

Table 2A. Probes used for RT-qPCR analysis in human cases

Gene	Full name	Reference
Housekeeping genes		
GUS-B	β-glucuronidase	Hs00939627_m1
XPNPEP1	X-prolylaminopeptidase (aminopeptidase P) 1	Hs00958026_m1
AARS	Alanyl-tRNA synthetase	Hs00609836_m1
HPRT	Hypoxanthine phosphoribosyltransferase 1	Hs_02800695_m1
Nucleolar, rRNAs and genes encoding ribosomal protein		
NCL	Nucleolin	Hs01066668_m1
NPM1	Nucleophosmin (nucleolar phospho-protein B23, numatrin)	Hs02339479_m1
rRNA 28S	RNA, 28S ribosomal 5	Hs03654441_s1
rRNA 18S	Eukaryotic 18S rRNA	Hs99999901_s1
UBTF	Upstream binding transcription factor, RNA polymerase I	Hs01115792_g1
RPL5	Ribosomal protein L5	Hs_03044958_g1
RPL7	Ribosomal protein L7	Hs_02596927_g1
RPL21	Ribosomal protein L21	Hs_00823333_s1
RPL22	Ribosomal protein L22	Hs_01865331_s1
RPL23A	Ribosomal protein L23A	Hs_01921329_g1
RPL26	Ribosomal protein L26	Hs_00864008_m1
RPL27	Ribosomal protein L27	Hs_03044961_g1
RPL30	Ribosomal protein L30	Hs_00265497_m1
RPL31	Ribosomal protein L31	Hs_0101549_g1
RPS3A	Ribosomal protein S3A	Hs_00832893_sH
RPS5	Ribosomal protein S5	Hs_00734849_g1
RPS6	Ribosomal protein S6	Hs_04195024_g1
RPS10	Ribosomal protein S10	Hs_01652370_gH
RPS13	Ribosomal protein S13	Hs_01011487_g1
RPS16	Ribosomal protein S16	Hs_01598516_g1
RPS17	Ribosomal protein S17	Hs_00734303_g1

mals = 42) were used. Mice expressing human mutated forms APPswe and PS1dE9 [22] show the first β-amyloid deposits at the age of 3 months and altered memory and learning capacities by the age of six months [23]. Animals were maintained under standard animal housing conditions in a 12-hour dark-light cycle with free access to food and water. Transgenic mice were characterized by genotyping from genomic DNA isolated from tail clips using the polymerase chain reaction (PCR) conditions proposed. Animals were kept under the same conditions as stated above. All animal procedures were conducted according to ethical guidelines (European Communities Council Directive 86/609/EEC) and were approved by the local ethics committee.

RNA purification

Purification of RNA from right frontal cortex area 8 in human cases and somatosensory cortex in mice aged 3, 12 and 20 months (n = 7 per age and phenotype) was carried out using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer, and performing the optional DNase digest to avoid extraction and subsequent amplification of genomic DNA. The concentration of each sample was obtained from A260 measurements with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values for RNA integrity number (RIN) varied from 6.1 to 8.4 in human cases (**Table 1**). Post-mortem delay had no effect on RIN values in the present series (**Table 1**). RIN values in mice were greater than 8.

Retrotranscription reaction

Retrotranscription reaction of RNA samples selected based on their RIN values was carried

Table 2B. Probes used for RT-qPCR analysis in mice

Gene	Full name	Reference
Housekeeping genes		
Gus-B	β-glucuronidase	Mm01197698_m1
Xpnpep11	X-prolylaminopeptidase (aminopeptidase P) 1	Mm00460040_m1
Aars	Alanyl-tRNA synthetase	Mm00507627_m1
Hprt	Hypoxanthine phosphoribosyltransferase 1	Mm01545399_m1
Nucleolar, rRNAs and genes encoding ribosomal protein		
Ncl	Nucleolin	Mm01290591_m1
Npm3	Nucleophosmin/nucleoplasmin 3	Mm00784254_s1
rRNA 28S	RNA, 28S ribosomal 5	Mm03682676_s1
rRNA 18S	Eukaryotic 18S rRNA	Mm03928990_g1
Ubtf	Upstream binding transcription factor, RNA polymerase I	Mm00456972_m1
Rpl5	Ribosomal protein L5	Mm00847026_g1
Rpl7	Ribosomal protein L7	Mm02342562_gH
Rpl21	Ribosomal protein L21	Mm01216752_m1
Rpl22	Ribosomal protein L22	Mm04410429_m1
Rpl23a	Ribosomal protein L23A	Mm01614208_gH
Rpl26	Ribosomal protein L26	Mm02343715_g1
Rpl27	Ribosomal protein L27	Mm01245874_g1
Rpl30	Ribosomal protein L30	Mm01611464_g1
Rps3A	Ribosomal protein S3A	Mm00656272_m1
Rps5	Ribosomal protein S5	Mm00501433_m1
Rps6	Ribosomal protein S6	Mm01263489_m1
Rps10	Ribosomal protein S10	Mm02391992_g1
Rps13	Ribosomal protein S13	Mm01731324_g1
Rps16	Ribosomal protein S16	Mm01617542_g1
Rps17	Ribosomal protein S17	Mm01314921_g1

out with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, California, USA) following the guidelines provided by the manufacturer, and using Gene Amp® 9700 PCR System thermocycler (Applied Biosystems). A parallel reaction for one RNA sample was processed in the absence of reverse transcriptase to rule out DNA contamination.

Real time PCR

Real Time quantitative PCR (RT-qPCR) assays were conducted in duplicate on 1,000 ng of cDNA samples obtained from the retrotranscription reaction, diluted 1:20 in 384-well optical plates (Kisker Biotech, Steinfurt, GE) utilizing the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Parallel amplification reactions were carried out using 20× TaqMan Gene Expression Assays and 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Human and mouse TaqMan probes are shown in **Table 2A** and **2B**, respectively.

Parallel assays for each sample were carried out using probes for β-glucuronidase (*GUS-β*), X-prolyl aminopeptidase (aminopeptidase P) 1 (*XPNPEP1*), alanyl-transfer RNA synthase (*AARS*) and hypoxanthine-guanine phosphoribosyltranferase (*HPRT*) for normalization. Housekeeping genes were selected because they show no modifications in several neurodegenerative diseases in human post-mortem brain tissue [24, 25]. The reactions were performed using the following parameters: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. TaqMan PCR data were captured using the Sequence Detection Software (SDS version 2.2, Applied Biosystems). Subsequently, threshold cycle (CT) data for each sample were analyzed with the double delta CT (ΔΔCT) method. First, delta CT (ΔCT) values

Table 3. Primary antibodies used in the present study including reference, supplier, host and dilution

Antibody (anti-)	Reference	Supplier	Host	Dilution
β-actin	A5316	Sigma-Aldrich, St Louis, MO, USA	Ms	1/30000
eIF2α (eukaryotic initiation factor 2α)	5A5	Thermo Fisher Sci, Waltham, MA, USA	Ms	1/50
eIF3η (eukaryotic initiation factor 3η)	sc 2887	Santa Cruz, Dallas, TX, USA	Rb	1/200
eIF5 (eukaryotic initiation factor 5)	sc.282	Santa Cruz, Dallas, TX, USA	Rb	1/400
eEF1A (eukaryotic elongation factor 1A)	2551	Cell Signaling, Danvers, MA, USA	Rb	1/100
eEF2 (eukaryotic elongation factor 2)	2332	Cell Signaling, Danvers, MA, USA	Ms	1/1,000
GAPDH (glyceraldehyde 3-phosphate dehydrogenase)	ADI-CSA-335-E	Enzo Life Sciences, Incl., Farmingdale, NY, USA	Ms	1/5000
P-eIF2α (phospho-eukaryotic initiation factor 2α pSer51)	S.674.5	Thermo Fisher Sci, Waltham, MA, USA	Rb	1/50

were calculated as the normalized CT values for each target gene in relation to the mean of endogenous controls *GUS-β*, *XPNPEP1*, *AARS* and *HPRT* in humans, whereas in mice *hprt* was the housekeeping gene selected for normalization. Second, ΔΔCT values were obtained with the ΔCT of each sample minus the mean ΔCT of the population of control samples (calibrator samples). The fold-change was determined using the equation $2^{-\Delta\Delta CT}$.

Gel electrophoresis and western blotting from total homogenate

0.1 g of sample tissue (n = 28) from frontal cortex area 8 in human cases and 0.1 g of somatosensory cortex in mice aged 3, 12 and 20 months (n = 7 per age and phenotype) were lysed with a glass homogenizer in Mila lysis buffer (0.5 M Tris at pH 7.4 containing 0.5 methylenediaminetetraacetic acid at pH 8.0, 5 M NaCl, 0.5% Na doxicholic, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, bi-distilled water, protease and phosphatase inhibitor cocktails (Roche Molecular Systems, Pleasanton, CA, USA), and then centrifuged for 15 min at 13,000 g in human cases and for 10 min at 5,000 g in mice, at 4°C (Ultracentrifuge Beckman with 70Ti rotor, CA, USA). Protein concentration was measured with a Smartspect™plus spectrophotometer (Bio-Rad, CA, USA) using the Bradford method (Merck, Darmstadt, Germany). Samples containing 15μg of protein and the standard Precision Plus Protein™ Dual Color (Bio-Rad) were loaded onto 10-15% w/v acrylamide gels. Proteins were separated in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes using the Trans-Blot®Turbo™ transfer system (Bio-Rad) at 200 mA/membrane for 40 min for human samples and at 50 mA/membrane overnight for mouse samples. Non-specific bindings were blocked by incubation in 5 w/v% dry milk in Tris-buffered saline (TBS) containing 0.1% v/v Tween for 1 hour at room temperature. After washing, the membranes were incubated at 4°C overnight with one of the primary antibodies in TBS containing 3% w/v albumin and 0.1% v/v Tween. Primary antibodies are detailed in **Table 3**. Monoclonal antibody anti-β-actin diluted 1:30,000 (β-actin, A5316; Sigma-Aldrich, St. Louis, MO, USA) was blotted for the control of protein loading in human samples and monoclonal anti-GAPDH (glyceraldehydes 3-phosphate dehydrogenase, ADI-CSA-335-E; Enzo Life Sciences, Inc., Farmingdale, NY, USA) diluted 1:5,000 for mouse samples. Following incubation with one of the primary antibodies, membranes were incubated for 1 hour with the appropriate HRP-conjugated secondary antibody (1:2,000, Dako, Glostrup, Denmark), and the immune complexes were revealed with a chemiluminescence reagent (ECL, Amersham, GE Healthcare, Buckinghamshire, UK).

Statistical analysis

The normality of distribution of the mean fold-change values obtained by RT-qPCR for every region and stage between controls and pathological cases was analyzed with the Kolmogorov-Smirnov test. The non-parametric Mann-Whitney test was performed to compare each group when the samples did not follow a normal distribution whereas the unpaired *t* test was used for normal variables. Statistical analysis was performed with GraphPad Prism version 5.01 (La Jolla, CA, USA) and Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (Warrenton, VA, USA). Differences between groups were considered

Table 4. mRNA expression of genes coding for selected nucleolar and ribosomal proteins, rRNA18S and rRNA28S in frontal cortex area 8 in MA, AD and rpAD

	MA	AD	rpAD	MA vs AD	MA vs rpAD	AD vs rpAD
UBTF	1.069 ± 0.444	0.602 ± 0.124	0.574 ± 0.085	*,↓	**,↓	-
NPM1	1.078 ± 0.476	0.531 ± 0.077	0.593 ± 0.144	**,↓	*,↓	-
NCL	1.083 ± 0.472	0.646 ± 0.098	0.673 ± 0.123	*,↓	*,↓	-
rRNA18S	1.038 ± 0.270	0.702 ± 0.303	0.578 ± 0.126	*,↓	***,↓	-
rRNA28S	1.113 ± 0.617	0.423 ± 0.230	0.385 ± 0.085	**,↓	*,↓	-
RPL5	1.008 ± 0.130	1.010 ± 0.165	1.039 ± 0.155	-	-	-
RPL7	1.007 ± 0.120	1.029 ± 0.183	1.091 ± 0.051	-	-	-
RPL21	1.085 ± 0.492	1.606 ± 0.559	1.606 ± 0.545	*,↑	*,↑	-
RPL22	1.013 ± 0.165	0.835 ± 0.139	0.855 ± 0.083	*,↓	*,↓	-
RPL23A	1.044 ± 0.318	1.140 ± 0.179	1.482 ± 0.363	-	*,↑	*,↑
RPL26	1.020 ± 0.195	0.992 ± 0.295	0.947 ± 0.237	-	-	-
RPL27	1.009 ± 0.141	1.007 ± 0.129	0.746 ± 0.630	-	-	-
RPL30	1.032 ± 0.266	1.097 ± 0.547	0.921 ± 0.404	-	-	-
RPL31	1.010 ± 0.148	1.002 ± 0.115	1.170 ± 0.161	-	*,↑	*,↑
RPS3A	1.031 ± 0.271	1.119 ± 0.423	0.919 ± 0.310	-	-	-
RPS5	1.006 ± 0.110	1.071 ± 0.209	1.261 ± 0.155	-	***,↑	-
RPS6	1.009 ± 0.134	1.255 ± 0.375	1.430 ± 0.373	*,↑	**,↑	-
RPS10	1.017 ± 0.191	1.223 ± 0.131	1.324 ± 0.242	*,↑	**,↑	-
RPS13	1.002 ± 0.072	1.031 ± 0.069	1.255 ± 0.217	-	**,↑	*,↑
RPS16	1.019 ± 0.205	1.020 ± 0.243	0.845 ± 0.283	-	-	-
RPS17	1.004 ± 0.091	1.035 ± 0.133	1.252 ± 0.192	-	**,↑	*,↑
RPS20	1.008 ± 0.140	0.819 ± 0.132	0.674 ± 0.234	**,↓	**,↓	-

The non-parametric Mann-Whitney test was performed to compare each group when the samples did not follow a normal distribution whereas the unpaired t test was used for normal variables. Data are represented as the mean ± SD: *P<0.05, **P<0.01 and ***P<0.001. ↓, ↑ indicate gene up-regulation and down-regulation, respectively.

statistically significant at *p*-values: *P<0.05, **P<0.01 and ***P<0.001.

Densitometry of western blot bands was assessed with the TotalLab program (TotalLab Quant, Newcastle, UK) and subsequently analyzed with GraphPad Prism and Statgraphics Statistical Analysis, and Data Visualization Software version 5.1 (VA, USA), with one-way ANOVA with post-hoc Tukey's range test for multiple comparisons; differences were considered statistically significant with *p*-values: *P<0.05; **P<0.01; ***P<0.001.

Results

Human cases: AD and rpAD

mRNA expression levels of nucleolar proteins, 18S and 28S rRNA and ribosomal proteins in frontal cortex area 8 in MA, AD and rpAD: Genes encoding nucleolar proteins nucleophosmin 1 (NPM1) and nucleolin (NCL), and upstream binding transcription factor (UBTF) were significantly down-regulated in AD (*p* values ranged from <0.05 to <0.01) and in rpAD (*p* values from <0.05 to <0.01) compared with MA. rRNA18S and rRNA28S expression levels were reduced in AD and rpAD (*p* values from <0.001 to <0.001). No significant differences were observed when comparing AD with rpAD (**Table 4**).

Three genes encoding ribosomal proteins *RPL21*, *RPS6* and *RPS10* were up-regulated, and two encoding *RPL22* and *RPS20* down-regulated in AD when compared with MA (*p* values between <0.05 and <0.01). However, ten of seventeen genes encoding ribosomal proteins were deregulated in rpAD when compared with MA. The expression of most of these genes, including *RPL21*, *RPL23A*, *RPL31*, *RPS5*, *RPS6*, *RPS10*, *RPS13* and *RPS17*, was significantly increased, but *RPL22* and *RPS20* were down-regulated (*p* values between <0.05

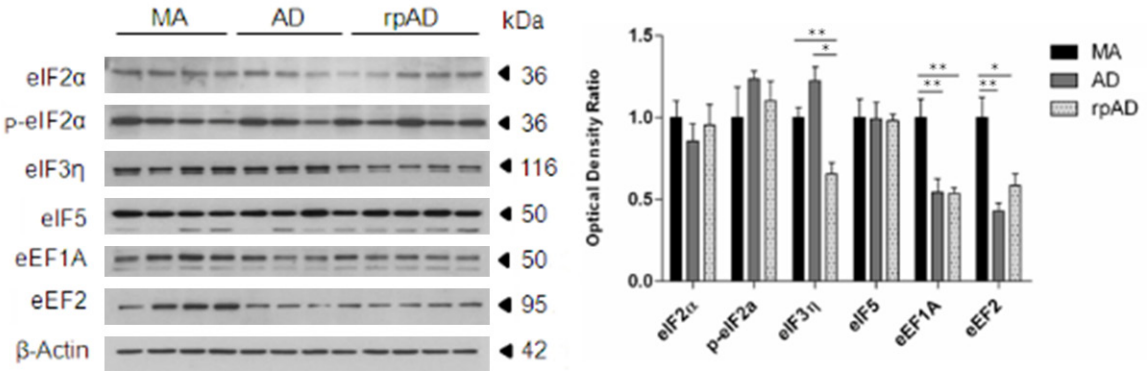

Figure 1. Protein expression levels of eukaryotic initiation factors eIF2α, phospho-eIF2α, eIF3η and eIF5, and eukaryotic elongation factors, eEF1A and eEF2 as revealed with gel electrophoresis and western blotting in frontal cortex area 8 in MA, AD and rpAD, and corrected with β-actin. Densitometry of western blot bands was assessed with the TotalLab program and subsequently analyzed with GraphPad Prism and Statgraphics Statistical Analysis, and Data Visualization Software version 5.1 with one-way ANOVA with post-hoc Tukey's range test for multiple comparisons: *P<0.05; **P<0.01; ***P<0.001.

Table 5. mRNA expression of genes coding for selected nucleolar and ribosomal proteins, rRNA18S and rRNA28S in the somatosensory cortex of WT and APP/PS1 mice aged 3, 12 and 20 months

	3 months		12 months		20 months	
	WT	APP/PS1	WT	APP/PS1	WT	APP/PS1
Ubtf	1.057 ± 0.111###	1.123 ± 0.211	1.628 ± 0.041	1.463 ± 0.083	1.342 ± 0.089	3.796 ± 0.873
Npm3	1.188 ± 0.305	1.023 ± 0.087&	0.862 ± 0.070	0.852 ± 0.082	2.518 ± 0.396	3.796 ± 0.873
Ncl	1.010 ± 0.059	1.023 ± 0.087	0.082 ± 0.070&	1.003 ± 0.238	0.960 ± 0.086	1.370 ± 0.080**,↑
rRNA18S	1.018 ± 0.075###	1.085 ± 0.164&	2.730 ± 0.188&&&	2.237 ± 0.456	1.259 ± 0.170	2.504 ± 0.496*,↑
rRNA28S	1.009 ± 0.052###,&&&	1.076 ± 0.150	2.367 ± 0.191	1.847 ± 0326	1.338 ± 0.175	2.401 ± 0.344*,↑
Rpl5	1.004 ± 0.032###	1.005 ± 0.038###	3.015 ± 0.327&&&	2.393 ± 0.122&&&	0.727 ± 0.047	1.190 ± 0.147
Rpl7	1.032 ± 0.093	1.029 ± 0.094	1.785 ± 0.279	1.305 ± 0.108	1.251 ± 0.211	1.366 ± 0.211
Rpl21	1.003 ± 0.031	1.018 ± 0.067	0.903 ± 0.113	0.635 ± 0.058	0.834 ± 0.116	0.956 ± 0.094
Rpl22	1.004 ± 0.037	1.010 ± 0.049	1.043 ± 0.180	0.758 ± 0.51	0.823 ± 0.073	1.011 ± 0.100
Rpl23a	1.009 ± 0.052	1.015 ± 0.049&&&	1.081 ± 0.085	0.794 ± 0.036&&&	1.005 ± 0.059	1.649 ± 0.108**,↑
Rpl26	1.024 ± 0.022	1.009 ± 0.49	1.459 ± 0.204	1.116 ± 0.108&	1.025 ± 0.172	1.679 ± 0.205
Rpl27	1.003 ± 0.28	1.004 ± 0.030	1.465 ± 0.158	1.255 ± 0.133	0.960 ± 0.434	1.129 ± 0.120
Rpl30	1.023 ± 0.098#	1.010 ± 0.049	0.729 ± 0.068	0.878 ± 0.205	0.838 ± 0.082	1.589 ± 0.263
Rps3a	1.001 ± 0.013##	1.000 ± 0.017	1.683 ± 0.199&&	1.384 ± 0.194	0.896 ± 0.036	1.336 ± 0.155*,↑
Rps5	1.002 ± 0.028##	1.006 ± 0.043#,&	1.987 ± 0.252&	1.698 ± 0.231	1.050 ± 0.053	1.576 ± 0.173*,↑
Rps6	1.003 ± 0.043	1.191 ± 0.245	1.184 ± 0.080	1.643 ± 0.127	1.006 ± 0.052	1.831 ± 0.255*,↑
Rps10	1.007 ± 0.045	1.013 ± 0.065###	3.730 ± 0.522&&&	3.344 ± 0.368	1.122 ± 0.083	1.884 ± 0.263*,↑
Rps13	1.001 ± 0.015###	1.004 ± 0.038#	0.803 ± 0.092&&	0.610 ± 0.021	0.778 ± 0.033	1.150 ± 0.128*,↑
Rps16	1.004 ± 0.032#	1.006 ± 0.042&	1.286 ± 0.106&&	1.268 ± 0.120	0.827 ± 0.057	1.433 ± 0.176*,↑
Rps17	1.004 ± 0.031	1.008 ± 0.049	1.202 ± 0.096	1.057 ± 0.084	0.901 ± 0.102	1.225 ± 0.154

The non-parametric Mann-Whitney test was performed to compare each group when the samples did not follow a normal distribution whereas the unpaired t test was used for normal variables. Data are represented as the mean values ± SD. *P<0.05, **P<0.01 compared with WT animals; &P<0.05, &&P<0.01, &&&P<0.001 compared with 20 months; #P<0.05, ##P<0.01, ###P<0.001 compared with animals aged 12 months. Expression levels were calculated using Hprt for normalization. ↑ indicate gene up-regulation.

and <0.001). Four genes, including *RPL23A*, *RPL31*, *RPS13* and *RPS17,* were differentially up-regulated in AD when compared with rpAD (*P*<0.05) (**Table 4**).

Expression levels of initiation and elongation factors of protein synthesis in frontal cortex area 8 in MA, AD and rpAD: Four eukaryotic initiation factors eIF2α, phospho-eIF2α, eIF3η and eIF5, and two eukaryotic elongation factors, eEF1A and eEF2, were assessed with western blotting.

eEF1A and eEF2 were significantly decreased in AD when compared with MA (P<0.01); eIF3η, eEF1A and eEF2 were significantly decreased in rpAD when compared with MA (*p* values from <0.01 to <0.001); eIF3η was significantly down-

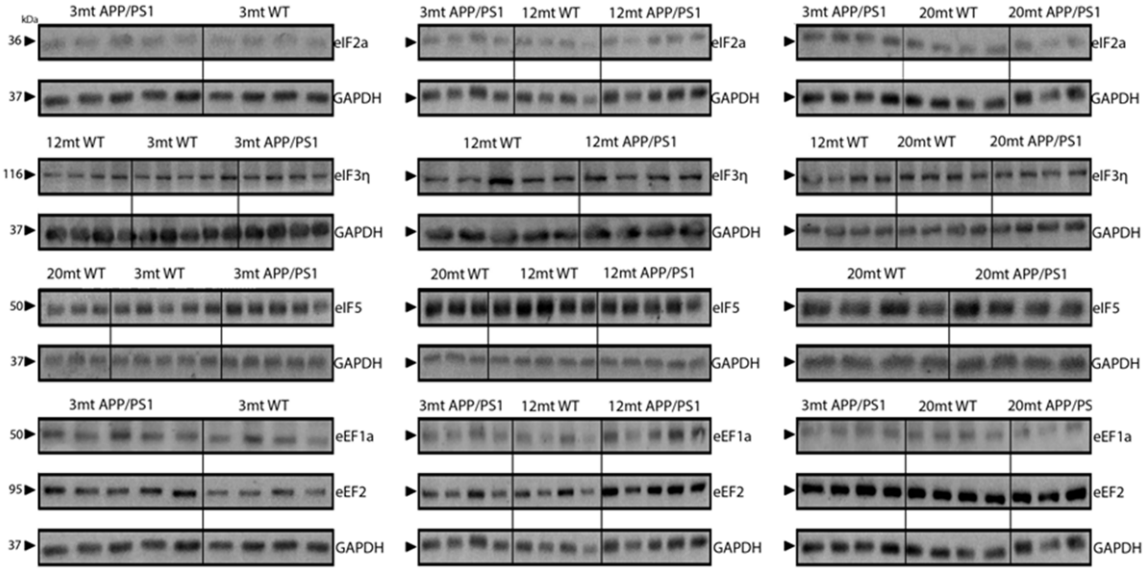

Figure 2. Western blots of the somatosensory cortex of WT and APP/PS1 transgenic mice at the ages of 3, 12 and 20 months showing the expression of eIF2α, eIF3η, eIF5, eEF1a and eEF2 proteins.

regulated in rpAD when compared with AD ($P<0.05$) (**Figure 1**).

mRNA expression levels of nucleolar proteins, rRNA18S and rRNA28S, and ribosomal proteins in somatosensory cortex of APP/PS1 mice at 3, 12 and 20 months: Expression levels of rRNAs and several genes coding for ribosomal proteins varied with age in WT and APP/PS1 mice, although no significant differences were seen between the two groups at the ages of 3 months and 12 months. Values were higher at the age of 12 months when compared with the age of 3 months in both phenotypes. Values decreased after the age of 12 months in both groups. However, a significant increase in the expression of Ncl, rRNA18S, rRNA28S, Rpl23a, Rps3a, Rps5, Rps6, Rps10, Rps13 and Rps16 mRNA was found in APP/PS1 transgenic mice when compared with WT littermates at the age of 20 months (p values from <0.05 to <0.01) (**Table 5**).

Protein expression of initiation and elongation transcription factors in somatosensory cortex in WT and APP/PS1 mice: eIF2α, eIF3η, eIF5, eEF1a and eEF2 protein levels were assessed with western blotting in WT and APP/PS1 at the ages of 3, 12 and 20 months. Western blots of all samples are shown in **Figure 2**. eIF2α was significantly increased in APP/PS1 animals at the age of 3 months when compared with age-matched littermates; eIF5 was significantly decreased (P<0.01), as was eEF2 (P<0.01) in APP/PS1 transgenic mice when compared to WT at the age of 12 months (**Figure 3**).

Discussion

Present observations show altered expression of genes and proteins involved in the protein synthesis machinery from the nucleolus to the ribosome in frontal cortex area 8 at advanced stages of AD and in the somatosensory cortex of APP/PS1 transgenic mice used as a model of AD-like β-amyloidopathy at advanced stages of the disease.

However, alterations differ in humans and mice. *NPM*1, *NCL* and *UBTF* mRNA expression is reduced in AD whereas *Ncl* mRNA is up-regulated in transgenic mice. rRNA18S and rRNA28S are reduced in AD but significantly increased in transgenic mice. Several genes encoding ribosomal proteins of the large and small subunits are up- or down-regulated in AD, but seven out of fifteen assessed genes encoding ribosomal proteins are up-regulated in the APP/P1 mouse. This is accompanied by a significant decrease in the protein levels of elongating factors eEF1A and eEF2 in AD but only of eEF2 in mice when compared with age-matched controls. Moreover, alterations are more marked in rpAD when compared with typical forms of AD, as several

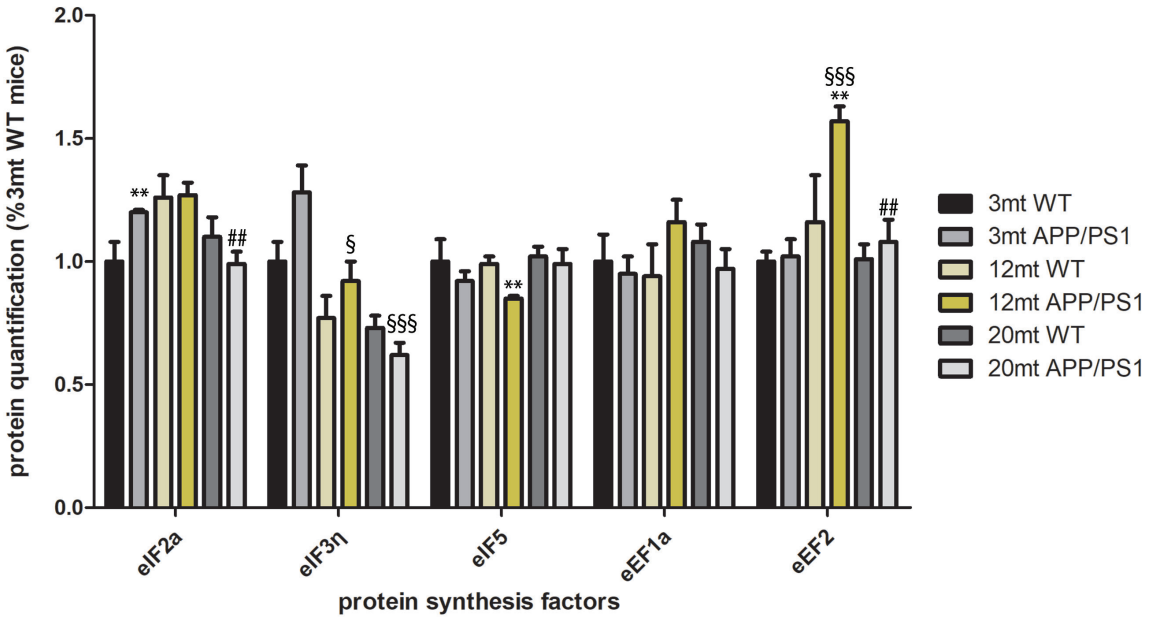

Figure 3. Protein expression levels of eukaryotic initiation factors eIF2α, eIF3η and eIF5, and eukaryotic elongation factors, eEF1A and eEF2 as revealed with gel electrophoresis and western blotting in somatosensory cortex of WT and APP/PS1 transgenic mice at the ages of 3, 12 and 20 months. Densitometry of western blot bands was assessed with the TotalLab program and subsequently analyzed with GraphPad Prism and Statgraphics Statistical Analysis, and Data Visualization Software version 5.1 with one-way ANOVA with post-hoc Tukey's range test for multiple comparisons: *P<0.05, **P<0.01 comparison with age-matched WT; §P<0.01, §§P<0.01, §§§P<0.001, comparison with mice aged 3 months; #P<0.05, ##P<0.01, comparison with mice aged 12 months.

ribosomal protein mRNAs are up-regulated, and initiation factor eIF3η protein expression is reduced, in rpAD when compared with typical AD.

Changes in AD cannot be attributed to the mere loss of nerve cells, as some genes are up-regulated in parallel to others which are down-regulated. It may be argued that differences in gene expression are related to differences in cell type, but that all these factors are expressed in neurons and glial cells.

Reduced expression of RNAs encoding nucleolar chaperones and *UBTF* in frontal cortex parallels decreases mRNA and protein expression in the CA1 region of the hippocampus at advanced stages of AD [15]. Reduced rRNA expression in frontal cortex is in agreement with previous observations [2-4, 9] but only with expression levels in the CA1 region of hippocampus at early and middle stages of the disease [15]. Increased expression of rRNA28S in CA1 at advanced stages of AD may be related to the accompanying astrogliosis [15]. Regarding transcription of genes encoding ribosomal proteins, marked differences are seen between the hippocampus and frontal cortex, as the majority of assessed genes are down-regulated in the CA1 region of the hippocampus and up-regulated in the frontal cortex at the same disease stage [15].

Finally, differences also exist in the expression of initiation and elongation factors between the hippocampus and frontal cortex at similarly advanced stages of the disease. eIF2 is markedly increased in the hippocampus but not in the frontal cortex, whereas eEF1A and eEF2 total levels do not differ in AD from those of age-matched controls in the hippocampus [15] but are markedly reduced in frontal cortex at the same stage of the disease. Regional differences in the neuronal responses may account for these differences in the populations of neurons and glial cells between the hippocampus and the frontal cortex at the same stage of the disease. Moreover, discrete differences might not be identified using total brain homogenates. As an example, no modifications in total levels of P-eIF2α were observed in the present study although P-eIF2α is expressed in only approximately 60% of neurofibrillary tangle (NFT)-containing neurons in the isocortex in

AD, thus indicating that not all neurons with NFTs over-express phosphorylated P-eIF2α [12].

In summary, our observations, when compared with previous similar studies of ours focused on the hippocampus (), show marked regional differences in the expression of players involved in the protein synthesis machinery at the same advanced stage of the disease. Alterations differ slightly between typical AD and those cases with rapid clinical course, while alterations differ between AD and APP/PS1 transgenic mice, implying there can be no direct application of the results in the mouse model to the human disease.

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Disclosure of conflict of interest

None.

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