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In the Rat Brain Acetyl-L-carnitine Treatment Modulates the Expression of Genes Involved in Neuronal Ceroid Lipofuscinosis

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Abstract Acetyl-L-carnitine (ALC) is a naturally occurring substance that, when administered at supraphysiological concentration, is neuroprotective. It is a molecule of considerable interest for its clinical application in various neural disorders, including Alzheimer's disease and painful neuropathies. Suppression subtractive hybridization methodology was used for the generation of subtracted cDNA libraries and the subsequent identification of differentially expressed transcripts in the rat brain after ALC treatment. The method generates an equalized representation of differentially expressed genes irrespective of their relative abundance and it is based on the construction of forward and reverse cDNA libraries that allow the identification of the genes which are regulated by ALC. We report that ALC treatment: (1) upregulates *lysosomal H^+/ATPase* gene expression and (2) downregulates *myelin basic protein* gene expression. The expression of these genes is altered in some forms of neuronal ceroid lipofuscinosis (NCL) pathologies. In this case, ALC might rebalance the disorders underlying NCL disease represented by a disturbance in pH homeostasis affecting the acidification of vesicles transported to lysosomal compartment for degradation. This study provides evidence that ALC controls genes involved in these serious neurological pathologies and provides insights into the ways in which ALC might exert its therapeutic benefits.

Keywords Neuronal ceroid lipofuscinosis · Suppression subtractive hybridization · Lysosomal storage disorders · Acetyl-L-carnitine · Rat

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Introduction

Neuronal ceroid lipofuscinoses (NCLs) are a group of autosomal-recessive hereditary lysosomal storage diseases. They are caused by at least eight mutant genes (CLN1–CLN8) [1]. These disorders are characterized by massive accumulation of autofluorescent lysosomal storage bodies in most cells of the CNS. This accumulation is associated by severe degeneration of the CNS. The most significant component of the storage material is a protein that appears to be similar to a hydrophobic inner mitochondrial membrane protein: the subunit *c* of mitochondrial adenosine triphosphate (ATP) synthase [2–5]. This anomalous storage of mitochondrial ATP synthase subunit *c* is not a result of enhanced expression of nuclear genes encoding the protein nor does the stored protein exhibit changed encoded sequences. A slower degradation of the mitochondrial ATP synthase seems to occur in NCL fibroblasts in comparison with normal cells [6].

A link between acetyl-L-carnitine (ALC) and lipofuscinosis has been singled out by two important reports: (1) the storage body accumulation in both juvenile and late-infantile human ceroid lipofuscinosis seems to be correlated to altered

carnitine biosynthesis [7]; (2) an inherited disease in the motor neuron degeneration (*mnd)* mouse strain has been proposed as a model for certain types of human ceroid lipofuscinosis [7, 8]. In these *mnd* mice, it was observed that dietary carnitine supplements could slow the disease progression and reduce the accumulation of autofluorescence storage bodies in neurons prolonging their lifespan [7].

To enlighten these correlations between ALC and the lipofuscinosis disease, we have performed experiments in order to determine whether, in the rat brain, ALC treatment might modulate expression of genes involved in NCL.

ALC is the acetyl ester of the trimethylated amino acid L-carnitine that plays an essential role in energy production as "shuttles" of long-chain fatty acids between the cytosol and the mitochondria for subsequent β-oxidation [9, 10]. ALC is involved in the control of ATP levels, mitochondrial acyl-CoA to CoA ratio, peroxisomal oxidation of fatty acids, and mitochondrial enzyme activities [11, 12]. ALC has neuromodulatory, neurotrophic, cytoprotective, antioxidant, antiapoptotic, and antiaging effects [12–17]. In addition, ALC exhibits cardioprotective activity [18]. Cardiomyopathy is frequently observed in storage disorders, including the NCL [19].

The present study has been carried out to detect differentially expressed genes in young rats treated with ALC, by using the subtractive suppression hybridization (SSH) method [20, 21]. The main advantage of SSH technology is the enrichment of transcripts expressed at much lower levels, potentially obscured by more abundant genes, and therefore higher sensitivity is attained with respect to other methods of differential screening [22–24].

In a previous work, we identified genes involved in mitochondrial and lysosomal function; in particular, we showed that ALC treatment switches on the *heat shock protein 72* (Hsp72) gene [20]. The Hsp72 belongs to the vitagene system: a cluster of genes controlling cellular homeostasis during stressful situations. We observed that ALC treatment downregulates the expression of the *2AA9* clone, corresponding to the *ATP synthase lipid-binding protein, subunit c* gene.

In this paper, we provide further evidence that ALC might control the molecular mechanism which is responsible for mitochondrial and lysosomal impairment. In particular, we observed that ALC modulates expression of genes involved in NCL, suggesting a pathway for a potential therapy.

Materials and Methods

Animals

Fifty-day-old male Wistar rats from the same litter breed, housed at a temperature of 22°C, were intraperitoneally injected daily for 21 days either with ALC (100-mg/kg body weight Sigma-Tau Laboratories, Pomezia, Italy, treated group) or saline (control group), as described previously [20]. The investigation has been performed in conformity with the principles expressed in the Helsinki Declaration.

Subtracted cDNA Library Construction

Total RNA was isolated from rat brains of four control and four ALC-treated animals as previously described [20]. Poly $(A)^+$ RNAs were purified from the pools of total RNAs of both control group and treated rat brains using the PolyATtract mRNA Isolation System (Promega Corp., Madison, WI, USA). ALC-modulated cDNA were generated by SSH using the polymerase chain reaction (PCR)-select cDNA subtraction kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Poly$(A)^+$ mRNA samples were used to synthesize *tester* and *driver* cDNA pools [20]. For forward subtraction, *tester* cDNA was synthesized from poly$(A)^+$ mRNA isolated from treated rat brains and *driver* cDNA was produced from poly$(A)^+$ mRNA isolated from control rat brains; on the contrary, reverse subtraction was performed using control cDNA as *tester* and treated cDNA as *driver*. The quality of the subtraction was evaluated by PCR using primers for *glyceraldehyde-3-phosphate dehydrogenase* (*G3PDH*) housekeeping gene supplied from the kit.

Cloning, Differential Screening, and Sequence Analysis

Amplified cDNA sequences from the forward and reverse subtraction were directly inserted into a T/A cloning vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). A differential screening was performed for all collected clones using the cDNA Array approach (Clontech PCR-Select Differential Screening kit, BD Biosciences Clontech, Palo Alto, CA, USA). The positive clones were sequenced by the dideoxy chain termination method with an automated DNA sequencer and sequences were analyzed with BLAST and FASTA algorithms at the National Center of Biotechnology Information and European Bioinformatics Institute servers.

Semiquantitative RT-PCR

Reverse transcriptions (RT) were carried out with total RNA (4 μg) isolated from treated and control rat brains using the SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen Carlsbad, CA, USA) and with the $Oligo(dT)_{12–18}$ Primer (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions as reported in Traina et al. [21]. One microliter of first-strand cDNAs were used for each PCR amplifications. PCRs were performed using gene-

specific primers for: *3BE3* clone (3BE3f, 5′-agcatcatttaact cagtgtgcaggttcg-3′; 3BE3r, 5′-ggttctgcccatttaaatatgactcat gaa-3′); *4AH7* clone (4AH7f, 5′-agaacagtaggtgcttctgt ccagcc-3′; 4AH7r, 5′-ttgttaatccgttctaattccgagg-3′) and specific primers for rat G3PDH cDNA sequence for *3BE3* relative RT-PCR ($G3PDHf_3$, 5′-ggagaaggctggggctcacctg-3′; G3PDHr, 5′-tccaccaccctgttgctgta-3′) and for *4AH7* relative RT-PCR ($G3PDHf_4$, 5′-accacagtccatgccatcac-3′; G3PDHr). Primers were designed to yield a 663- or 452-bp fragment for G3PDH (for *3BE3* and *4AH7* relative RT-PCR, respectively) and variable length fragments for *3BE3* clone (430 bp) and for *4AH7* clone (235 bp). PCRs were performed in 50 μl of 1× reaction buffer containing 250 μM of each dNTP, 1.7 mM $MgCl_2$, 0.4 μM of each primer and 2.5 U EuroTaq (EuroClone, Milano, Italy). Amplifications were carried out according to the following temperature profile: 94°C for 4 min, then 24 cycles each consisting of 30 s at 94°C, 30 s at 59°C, 30 s at 72°C, and with a final extension of 7 min at 72°C.

The relative amounts of each PCR product were readily quantified by direct scanning with a densitometer of ethidium-bromide-stained 2% TAE–agarose gels with a UVP Image Store 5000 (Ultra Violet Product Ltd., Cambridge, England) equipped with the UVP GelBase-GelBlot TM Windows Software. To normalize for equal amounts of total RNA and efficiency of cDNA synthesis from various tissue samples, the band intensities were normalized with the average intensity of the G3PDH product across the samples investigated. The ratio between the value of the analyzed gene product level and the G3PDH product level of each sample was calculated from four or five independent experiments performed for each gene. Statistical analyses were performed by Mann–Whitney tests because the experimental data were not normally distributed (according to *Kolmogorov–Smirnov test*), and the level of significance was given by $p<0.05$. Data are presented as means±SEM.

Western Blot Analysis

Tissue samples were homogenized in an Ultra-Turrax homogenizer in the presence of a lysis buffer containing: 1 mM ethylenediaminetetraacetic acid, 5 mM NaF, 6 M urea, 1 mM Na-orthovanadate, 2.5 mM Na-pyrophosphate, 0.5% Triton X-100, Protease Inhibitor Cocktail *Sigma* and all diluted in phosphate-buffered saline (PBS; Dulbecco A, Oxoid) and incubated at room temperature for 1 h. The proteins were extracted from the supernatant obtained by centrifugation at 5,000×*g* for 30 min at 4°C and quantitated by Bio-Rad Protein Assay. After the addition of sodium dodecyl sulfate (SDS) and β-mercaptoethanol, the samples were boiled, and 60 μg of protein–lane was separated by 15% SDS-polyacrylamide gel electrophoresis with the Bio-Rad Mini-PROTEAN 3 system. After the transfer of proteins onto nitrocellulose membranes (Amersham Biosciencies, Milano, Italy), gels and blots were routinely stained with Bio-Safe Coomassie and Ponceau S red, respectively.

Blots were blocked for 2 h with PBS and 0.05% Tween 20 which contained 3% skimmed milk powder. The membranes were then incubated overnight at 4°C using either anti-MBP (1:1,000) or anti-G3PDH (1:500) antibodies (Santa Cruz Biotechnology Inc., CA, USA) or anti-H^+/ATPase V1 subunit (1:3,000) antibody (Abnova Corporation, Taiwan), diluted in the presence of PBS and 0.05% Tween 20. After incubation with the appropriate secondary antibodies (1:1,000), blots were developed by the SuperSignal West Pico system from Pierce Biotechnology (Rockford, IL, USA), according to the manufacturer's instructions.

The membrane used for H^+/ATPase and MBP detection was then stripped and reused for the detection of G3PDH. The images were scanned using an image analysis system (UVP Image Store 5000). The optical density of bands was measured whit Quantity One® Software (Bio-Rad, Milano, Italy).

Results

Upregulation of H^+/ATPase by ALC

The treatment with ALC upregulates the expression of *3BE3* clone, corresponding to the *lysosomal H^+/ATPase*, *V1 subunit D* (as determined by the identification in GenBank, European Molecular Biology Laboratory for closest similarity (accession no BC063177; similarity 99.391%; *E* value: 1.1e−49)). The modulation of gene expression is confirmed by semiquantitative RT-PCR analysis, as shown by comparative analysis with the G3PDH gene (Fig. 1a). The figure illustrates a representative result with primers for *H^+/ATPase* and *G3PDH* housekeeping genes. In Fig. 1b, the relative expression levels of *3BE3* transcripts were estimated by measuring the staining of the PCR products resolved by gel electrophoresis.

To detect lysosomal H^+/ATPase protein, Western blot method has been performed (Fig. 3a). The blots show a clear increase of the level protein after ALC treatment. The relative expression level of H^+/ATPase was estimated by measuring the staining of blots (Fig. 3b).

Downregulation of Myelin Basic Protein by ALC

The treatment with ALC downregulates the expression of *4AH7* clone, corresponding to the *myelin basic protein* (*MBP*), as determined by the identification in GenBank for

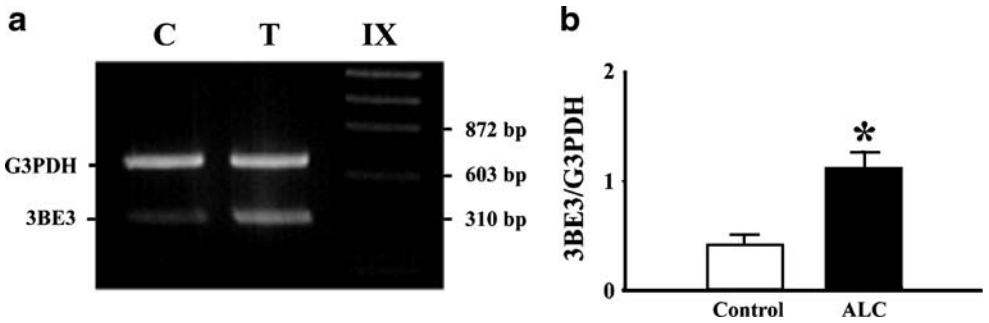

Fig. 1 **a** Relative RT-PCR analysis of *3BE3* clone. *Lanes C* and *T*, control and treated animals, respectively; *lane IX*, marker IX. **b** Relative expression levels of *3BE3* transcript. The relative expression levels of *3BE3* transcript were estimated by measuring the ethidium bromide staining of the PCR products resolved by gel electrophoresis in **a** as the ratio of each analyzed transcript with respect to the G3PDH product level. The housekeeping gene, G3PDH, showed no significant difference between controls and ALC-treated samples. Gene-specific signals were thus normalized with corresponding G3PDH signals for each sample. Probability value was obtained by Mann–Whitney test (p=0.01, n=4). Values shown are means±SEM (control, 0.397±0.104; ALC, 1.101±0.159)

closest similarity (accession no BC094522.1; similarity 99.8%; *E* value 8.4e−33). The modulation of this gene expression is confirmed by relative RT-PCR analysis, as shown by comparative analysis with the *G3PDH* housekeeping gene (Fig. 2a). The figure illustrates a representative result with primers for MBP and G3PDH genes. The relative expression levels of *4AH7* transcripts were reported in Fig. 2b. Protein content of the tissue was determined by Western blot analysis (Fig. 3). Following electrophoresis, proteins were transferred to a membrane and immunoblotted as reported in Fig. 3a. The relative expression level of MBP was estimated by measuring the staining of blots (Fig. 3b).

Discussion

By using the SSH method to search for differentially expressed genes in the brain of ALC-treated young rats, we observed the effects of ALC on important molecular elements involved, to different degrees, in NCL. ALC treatment: (1) upregulates the expression of *3BE3* clone, corresponding to the *lysosomal H^+/ATPase*, *V1 subunit D* gene and (2) downregulates the expression of *4AH7* clone, corresponding to the *myelin basic protein* (*MBP*) gene. In addition, in a previous work [20], we have observed that ALC treatment downregulates the expression of the *2AA9* clone corresponding to the *ATP synthase lipid-binding protein*, *subunit c* gene.

Upregulation of *Lysosomal H^+/ATPase*, *V1 Subunit D*

V-ATPases are multisubunit membrane proteins that use ATP binding and hydrolysis to transport protons across membranes against a concentration gradient. Generally, all eukaryotes require V-ATPases to maintain an acidic pH in membrane-bound compartments of endocytic and secretory networks to facilitate protein trafficking and processing. In fact, a loss of H^+/ATPases determines a strong accumulation of subunit c of the mitochondrial ATP synthase and

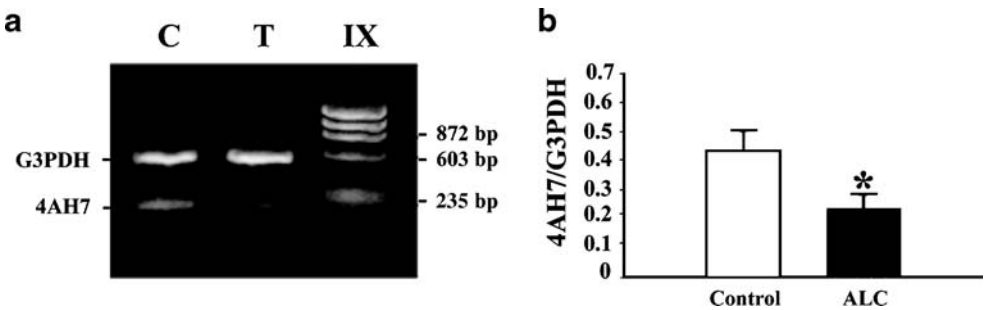

Fig. 2 **a** Relative RT-PCR analysis of *4AH7* clone. *Lanes C* and *T*, control and treated animals, respectively; *lane IX*, marker IX. **b** Relative expression levels of *4AH7* transcript**.** The relative expression levels of *4AH7* transcript were estimated by measuring the ethidium bromide staining of the PCR products resolved by gel electrophoresis in **a** as ratio of each analyzed transcript with respect to the G3PDH product level. The housekeeping gene, G3PDH, showed no significant difference between controls and ALC-treated samples. Gene-specific signals were thus normalized with corresponding G3PDH signals for each sample. Probability value was obtained by a Mann–Whitney test (p=0.039, n=5). Values shown are means±SEM (control, 0.434± 0.0664; ALC, 0.23±0. 0492)

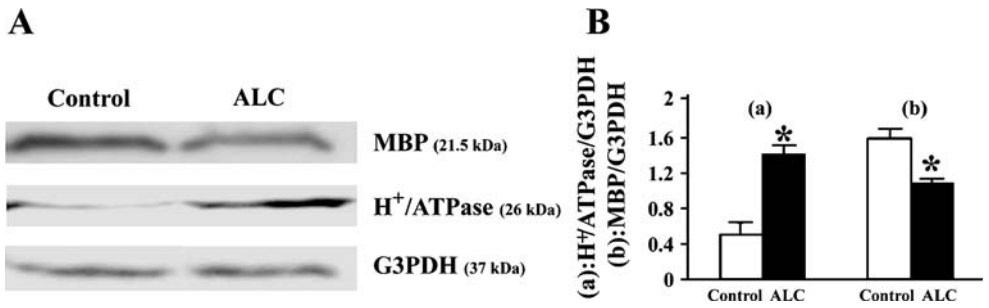

Fig. 3 Western immunoblot analysis of tissues after probing with the indicated antibodies. **a** The blots corresponding to *G3PDH* protein showed no significant difference between control and ALC-treated samples. In contrast, the blots of *H^+/ATPase* show a clear increase of the level protein after ALC treatment. The blots of *MBP* show a decrease after ALC treatment. **b** The relative expression levels of proteins were estimated by measuring the blot signals of proteins H^+/ATPase (*a*) and MBP (*b*) with respect to the blots of G3PDH, used as internal standard. Probability values was obtained by Mann–Whitney test (p=0.0286, n=4). Values shown are means±SEM (H^+/ATPase: control, 0.5073±0.1128; ALC, 1.425±0.09861; MBP: control, 1.613±0.1026; ALC, 1.088±0.05749)

increased amounts of lysosomal enzymes [25]. Mutations in subunit D lead to uncoupling to proton transport and ATP hydrolysis, suggesting that this subunit works as the central rotor in the V-ATPase [26]. Lysosomal pH has been shown to be alkalinized in mucolipidosis, which is a severe lysosomal storage disorder. The low pH of lysosomes seems necessary to maintain the activity of acid hydrolases in the lumen of the lysosome and to provide a driving force for coupling the transport of ions and molecules [26, 27]. Holopainen and colleagues [28] have measured intracellular and lysosomal pH in fibroblast cell lines of patients with six different types of NCLs. The greatest alkalinization was found in lysosomes of the most severe form of NCL. The elevated lysosomal pH might disturb the catalytic activity of the lysosome by inactivating hydrolases and seems to be one important factor that increases accumulation of lipofuscin storage material in the tissues of NCL patients [28]. There are studies suggesting that the regulation of lysosomal pH may be the underlying cause of Batten disease [6, 29]. A deficit in proton pump leads to severe neurodegeneration [25].

The potentiation of the lysosomal protonic pump by ALC treatment might be a compensatory mechanism of the abnormal higher lysosomal pH, and the increased H^+/ATPase might rebalance the pH homeostasis within the cell and normalize the pool of enzymes. In the CLN3 form, the lysosomal pH has been shown to be elevated. This finding is not in agreement to the results reported in yeast. In the btn1-Δ yeast strain, an acidification of vacuolar pH has been measured during the early phase of growth. The yeast vacuolar acidification is transient and is fully compensated during growth.

In a preceding paper, it has been observed that ALC exerts a downregulation of the *ATP synthase* gene [20].

This protein is one of the chains of the nonenzymatic membrane component (F0) of mitochondrial ATPase with similarity to the ATPase C chain family. In particular, this protein is the major protein accumulated in the storage bodies of animals or humans affected by NCL [5].

The fact that mitochondrial ATP synthase lipid-binding protein, subunit *c*, initially located in the mitochondria, is accumulated in lysosomes of NCL cells strongly suggests that the intracellular trafficking of specific molecules to lysosome, where the degradative process occurs, is severely altered. Therefore, an impairment of lysosomal pH homeostasis might be the basic mechanism of the lysosomal storage disorder [30]. Physiological and biochemical studies carried out mainly on cultured cells have shown that the known gene products are novel members of the intracellular trafficking pathway [28].

In a canine model for the juvenile form of the human disease, a major constituent of the storage bodies is the subunit *c* protein of mitochondrial ATP synthase that contains an ε-*N*-trimethyllysine (TML) residue [31]. TML is a precursor in carnitine biosynthesis. The changes in plasma carnitine and TML levels in the carriers are consistent with the possibility that the disease involves a defect in the carnitine biosynthetic pathway. Both TML and carnitine levels were significantly depressed in the affected individuals [7, 32]. In addition, dietary supplementation with carnitine delayed the progression of cognitive decline in NCL dogs [33]. Prolonged treatment with L-carnitine in isolated fibroblast cells from NCL patients fully restored the mitochondrial enzyme activities [34].

Downregulation of Myelin Basic Protein

Lamers and colleagues [35, 36] have observed an increase in cerebrospinal fluid and in blood of neuron-specific enolase, protein S-100B, and MBP in patients with neurometabolic diseases. In particular, enhanced MBP occurred frequently in mitochondrial encephalomyopathy, infantile Tay Sachs, *ceroid lipofuscinosis*, adrenoleukodystrophy, and nonketotic hyperglycemia. In addition, MBP in cerebrospinal fluid is an indicator of disease activity in multiple sclerosis. Therefore, our finding of a downregulation of MBP by ALC treatment is a further positive

action of this substance on the control of mitochondrial disorders and strengthens the validity of ALC as putative therapeutical agents which, acting on gene expression, might produce sustained beneficial effects.

Concluding Remarks

The results of this paper and those observed in a previous one [20] are the first studies in which, by applying a molecular biological approach, it has been possible to identify a direct effect of ALC on gene expression related to neuronal activity. The results are of relevant importance for possible therapeutic intervention, making a significant contribution to the neuroscience literature on the increasingly recognized importance of ALC role on neuroprotection and suggesting a pathway for the treatment of NCL, among the most serious child disorders. Given the complexity of the forms of NCL disease, we cannot generalize our results. But it should be very interesting to carry on similar studies either on pathological animal models or on clinical cases.

We demonstrated that ALC produces a rebalance of the pH disorders. In this contest, the mechanism of action of ALC seems to be much more extent, controlling at genetic level the pH. This finding is not conflicting with the studies carried out by Wei et al. [37], in which it has been shown that endoplasmic reticulum (ER) and oxidative stresses (ER stress) are common manifestation in cells from both neurodegenerative and nonneurodegenerative lysosomal storage disorders (LSD). These ER stress might cause apoptosis. Wei et al. [37] claim that lysosomal dysfunction, through the alteration of pH, produces ER and oxidative stress, supporting the concept that all the abnormality origins by the alteration of the pH of the organelle. According to these findings, neuroprotective therapeutic strategies, involving the rise of natural or synthetic substances that reduce the risk of neurodegeneration, are emerging [38]. These substances are chemical and pharmacological chaperones that stabilize the conformation of proteins, increase the protein folding capacity of the ER, and facilitate the trafficking of mutant proteins, including one of the main intracellular redox systems involved in neuroprotection, the vitagene system as a potential target for novel cytoprotective intervention. Vitagenes encode for cytoprotective heat shock protein (Hsp) 70 and heme oxygenase 1 as well as thioredoxin reductase and sirtuins proteins. In addition, there is neuroprotective roles of dietary antioxidants including curcumin, ALC, and carnosine. These chemical chaperones might protect LSD cells (by reducing the ER stress). ALC acts both at genetic level, switching on the Hsp72 [20], and as chemical chaperone, modulating the redox status and the cellular stress response.

Finally, even if it is well known that in eukaryotic cells the mRNA stability is very constant, we cannot exclude that the effect of ALC might be posttranscriptional. The control of mRNA stability is known to be modulated by a wide variety of physiological stimuli, including cytokines, growth factors, hormones, and hypoxia. A clinical relevance of posttranscriptional gene regulation by mRNA stability is furthermore highlighted by various pathologies whose occurrence tightly correlates with mutations in the *cis*-regulatory regions responsible for mRNA decay or by a dysregulation of *trans*-acting proteins specifically binding to these regulatory elements.

Strategies aiming at a specific inhibition of members of these signaling modules, in addition to interfering with the transcriptional activation of many genes also via mRNA stability, may affect the steady-state mRNA (and protein) levels of only a limited number of target genes [39].

Some of the human exosomal homologs are supposed to specifically bind to adenylate- and uridylate-rich elements (ARE) thus regulating the degradation of mRNA. The stability of V-ATPase mRNA is regulated through ARE in 3′ untranslated regions. ARE can contribute to the maintenance of even long-lived mRNAs under conditions of cell stress. Because these transporters are so crucial to cell survival and so closely linked to cellular energetics, their levels are likely to be specifically preserved under a number of adverse conditions that could otherwise alter gene expression profiles throughout the cell. Also, MBP stability is regulated through ARE regions. In light of this, it is not possible to exclude that ALC modulates mRNA stability. Further studies will be aimed to clarify if a similar situation is concerning ALC action. These considerations do not alter our finding that ALC treatment might exert therapeutic benefit in NCL pathologies.

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