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Modulation of Myelin Basic Protein Gene Expression by Acetyl-L-Carnitine

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Abstract Acetyl-L-carnitine (ALC), the acetyl ester of L-carnitine, is a naturally occurring molecule which plays an essential role in intermediary and mitochondrial metabolism. It has also neurotrophic and antioxidant actions, demonstrating efficacy and high tolerability in the treatment of neuropathies of various etiologies. ALC is a molecule of considerable interest for its clinical application in various neural disorders, although little is known regarding its effects on gene expression. Suppression subtractive hybridization methodology was used for the generation of subtracted complementary DNA libraries and the subsequent identification of differentially expressed transcripts in the rat brain after chronic ALC treatments. We provided evidence for a downregulation of the expression of all of the isoforms of myelin basic protein gene following prolonged ALC treatment, indicating a possible role in the modulation of myelin basic protein turnover, stabilizing and maintaining myelin integrity.

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Introduction

Acetyl-L-carnitine (ALC) is the acetyl ester of the trimethylated amino β-hydroxybutyrate, L-carnitine (LC). It plays an essential role in energy production as shuttle of long-chain fatty acids between the cytosol and the mitochondria for β-oxidation. Similarly to LC, ALC is involved in the control of mitochondrial acyl-CoA/CoA ratio and peroxisomal oxidation of fatty acids [1]. The dietary supplementation with ALC reduces oxidative stress and inhibits apoptotic cascade induced by hypoxia [2]. ALC exerts cytoprotective, antioxidant, and antiapoptotic activities, and many studies have focused on its neurotrophic effects in the nervous system. In addition, ALC improves different aspects of the neuronal metabolism and has wide neuromodulatory effects [1]; it enhances the cholinergic transmission and provides a possible tool for Alzheimer's disease treatment [3]. ALC has also antiaging effects improving cognitive function and cardioprotective activity [4, 5] and restoring the age-associated decline in learning and memory [6]. ALC treatment produces sustained effects, suggesting that it might modulate protein synthesis through changes of gene expression. We therefore worked out an extensive study in the brain of rats subjected to a chronic ALC treatment in order to evaluate the possibility that ALC might affect the gene expression. We performed a comprehensive analysis of all the genes that might be up- or downregulated after long-lasting ALC treatment by using the suppressive subtractive hybridization (SSH) methodology [7]. In this paper, we report that ALC downregulates all the isoforms

of myelin basic protein (MBP) gene expression, suggesting a possible role in the control of myelin stability.

Materials and Methods

Dissection of the Brain

Fifty-day-old male Wistar rats weighting approximately 200–250 g from the same litter bred, housed at a temperature of 22°C, were intraperitoneally injected daily for 21 days either with ALC (Sigma-Tau Laboratories, Pomezia, Italy, at doses of 40 and 100 mg/kg body weight (bw)) or LC (100 mg/kg bw; treated groups) or saline (control group), as previously described [7]. The animals were anesthetized with ether and then killed by decapitation. Brains were quickly removed, frozen, and stored at −80°C until use. Procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with national (D.L. no. 116, Gazzetta Ufficiale (G.U.), suppl. 40, 18 Febbraio, 1992, Circolare no. 8, G.U., 14 Luglio 1994) and international (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996) laws and policies.

Subtracted cDNA Library Construction

Total RNA was isolated from rat brains of four control and four ALC-treated (100 mg/kg bw) animals following [7]. Briefly, 2 μg of poly $(A)^+$ RNAs was purified from the pools of total RNAs of brain (except cerebellum and spinal cord) of both control and treated rats using the PolyATtract mRNA Isolation System (Promega Corp., Madison, WI). ALC-modulated cDNA was generated by SSH using the PCR-Select cDNA Subtraction kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol (Fig. 1).

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) as reported in [7].

Multiplex Semiquantitative RT-PCR

Reverse transcriptions were carried out with total RNA (2 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. PCRs were performed using MPB specific primers: MBP 5′ (Pro3, CCGAAGGCCTGGATGTGATG; Start, GCATCACAGAAGAGACCCTCACAGC) and MBP 3′ (Term, TCAGCGTCTCGCCATGGGAG; End7, ATCCAGAGCGGCTGTCTCTCTTCCTCC) and rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH)-specific primers for housekeeping gene (G3PDHf, 5′-CCGTGGGGCAGCCCAGAACAT-3′; G3PDHr, 5′-GGGCCCACTAAAGGGCATCC-3′). Primers were designed to yield a 112-bp fragment for G3PDH. PCRs were performed in 25 μl containing 5 μl of first-strand cDNAs, 1× Reaction buffer 250 μM of dNTPs, 1.7 mM $MgCl_2$, 0.625 μM of each primer, and 1.25 U EuroTaq (EuroClone, Milano, Italy). Amplifications were carried out according to the following temperature profile: 95°C for 4 min, then 30 cycles each consisting of 30 s at 95°C, 30 s at 60°C, 60 s at 72°C, and with a final extension of 7 min at 72°C, according to [8].

The relative amounts of each PCR product were quantified by direct scanning of ethidium bromide-stained 12% TBE polyacrylamide gel electrophoresis with a UVP Image Store 5000 (Ultra-Violet Products Ltd, Cambridge, England) equipped with the UVP GelBase–GelBlot TM Windows Software. To equalize the amounts of the total RNA and the efficiency of cDNA synthesis from various tissue samples, the band intensities were normalized with the average intensity of the co-amplified housekeeping G3PDH fragment across the samples investigated. The relative expression levels have been calculated as ratio of each analyzed transcripts with respect to the G3PDH product level from three independent experiments performed for each transcript. The statistical analysis was performed with the Mann–Whitney *U* test. All data are expressed as mean values±SD.

Western Blot Analysis

The proteins extracted from rat brains of control and ALC-treated (100 mg/kg bw) animals were analyzed by SDS–PAGE, transferred onto filter membrane, and stained with Ponceau S red according to [3, 9]. 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), 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 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

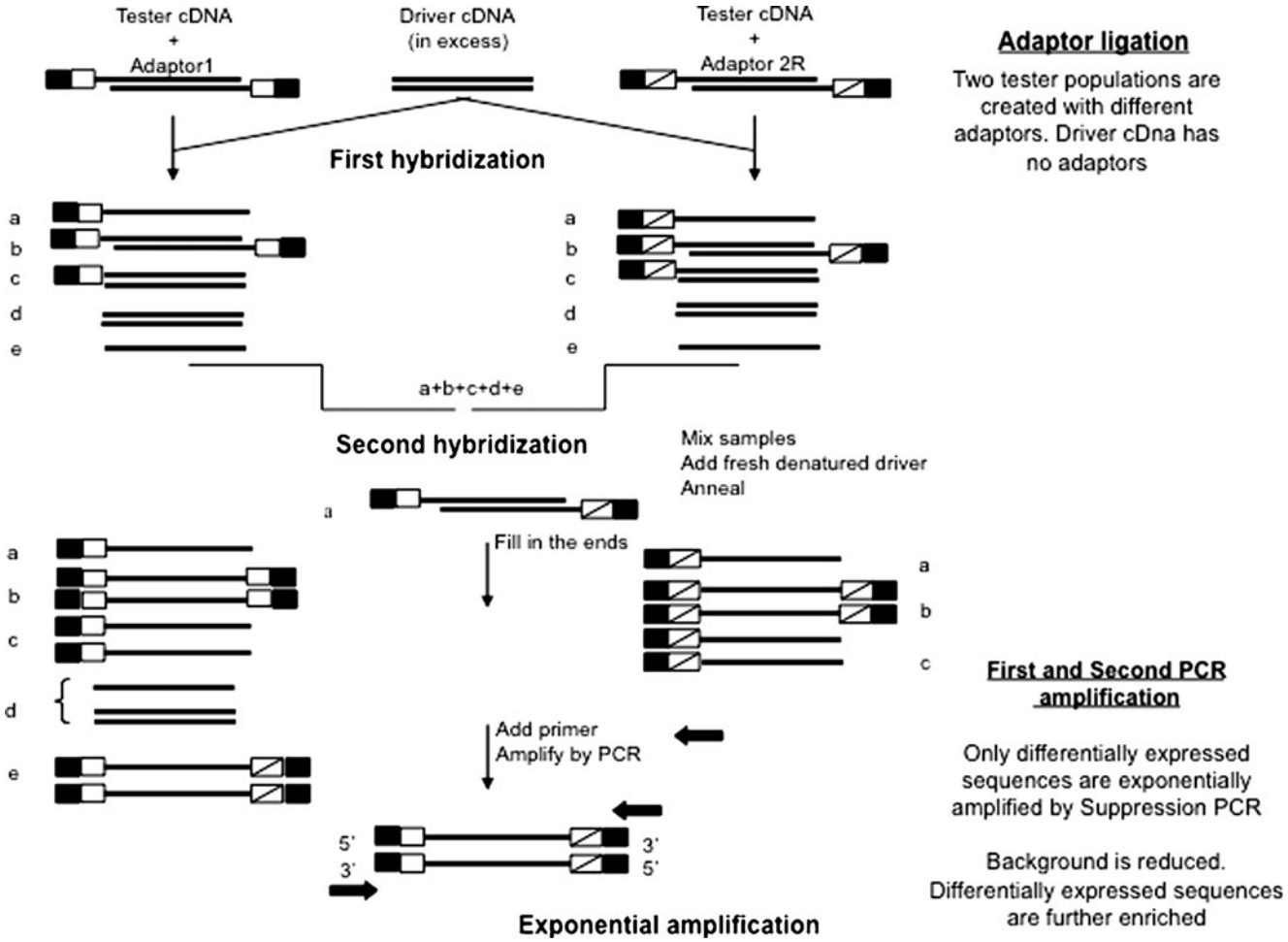

Fig. 1 A schematic summary of the suppression subtractive hybridization (SSH) method. It is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. Both mRNA populations are converted into cDNA: it refers to the cDNA that contains specific (differentially expressed) transcript as *tester* and to the reference cDNA as *driver*. Tester and driver cDNAs are hybridized, and the hybrid sequences are removed. As a result, the remaining unhybridized cDNA represents genes that are expressed in the tester but not in the driver mRNA. At that time, two hybridizations are performed. In the first one, an excess of driver is added to each sample of tester. The samples are heat denatured and annealed, generating the *a*, *b*, *c* molecules in each sample. During the second hybridization, the primary hybridization samples are mixed together without denaturing. Only the remaining equalized and subtracted single-strand tester cDNA can reassociate and arise new type (**e**) of hybrids. Soon after that, the entire population of molecules is subjected to PCR to amplify the desired differentially expressed sequences. Next, a secondary PCR amplification is performed using nested primers to further reduce any background PCR products and enrich for differentially expressed sequences (following BD Biosciences Clontech, Palo Alto, CA, USA)

was measured with Quantity One® Software (Bio-Rad, Milano, Italy).

Results

In order to identify the differentially expressed genes in the rat brain in response to ALC treatment, gene expression was compared at the mRNA level using the SSH method [7]. After the construction of forward and reverse cDNA libraries, the screening of the positive clones was carried out by dot blot screening. Following comparison with the sequences in the GenBank nucleotide database, we identified various clones as true positive, corresponding to differentially expressed genes after ALC treatment [3, 7, 9]. In this study, we reported a downregulation in the expression of the genes coding for all the MBP isoforms. This finding has been confirmed by relative reverse transcription polymerase chain reaction (RT-PCR) analysis, as shown by comparative analysis with G3PDH gene (Fig. 2a). The relative expression levels are shown in Fig. 2b. In Fig. 2a, b, five isoforms can be observed: isoform 1 (585 bp), isoform 2 (510 bp), isoform 3 (474 bp), isoform 4 (462 bp), and isoform 5 (384 bp). To detect protein expression in the rat brain, Western blot analysis was performed (Fig. 3a). The expressed levels of the proteins were calculated (Fig. 3b). We can single out four

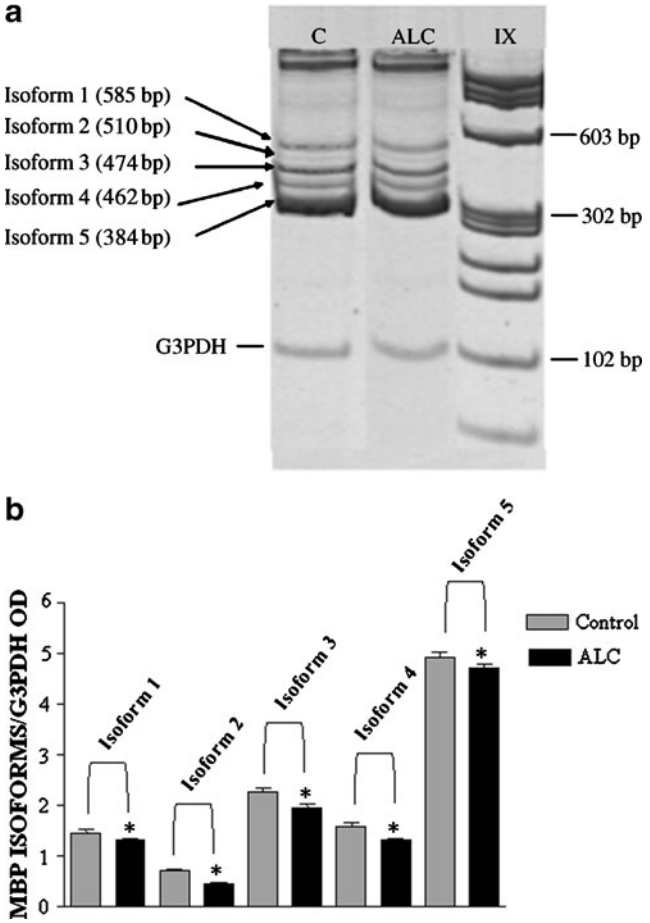

Fig. 2 Relative RT-PCR analysis of MBPs (**a**) and relative expression levels (**b**) are shown. The relative expression levels have been calculated as ratio of each analyzed transcript with respect to the G3PDH housekeeping product level. The expression of the MBP transcript was higher in control samples than in ALC-treated (100 mg/kg bw) samples. Values shown are means±SD (*isoform 1*, control 1.460±0.044; ALC 1.320±0.072; *isoform 2*, control 0.710±0.047; ALC 0.450±0.059; *isoform 3*, control 2.270±0.037; ALC 1.950±0.054; *isoform 4*, control 1.590±0.027; ALC 1.310±0.041; *isoform 5*, control 4.930±0.039; ALC 4.710±0.056). Unpaired *t* test, $p<0.005$, $n=4$). *Asterisks* indicate statistical significance. *Lane IX* marker IX

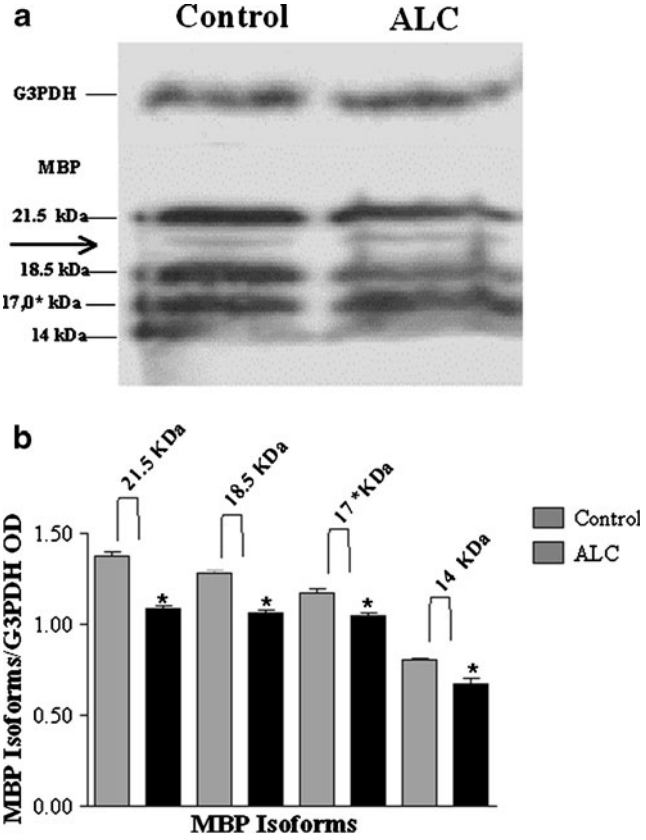

Fig. 3 Analysis levels of synthesis of MBP isoforms performed with Western blot. **a** Western blot analysis. G3PDH showed no significant difference between control and ALC-treated samples. In contrast, the blots of MBP show a clear decrease of the protein level after ALC treatment. It is possible to single out a single, nonspecific 19.7-kDa band (*arrowhead*). The isoform 17* could represent the average of the two isoforms 3–4 shown in Fig. 2a (not detectable in our electrophoretic conditions). This is in accordance with Kimura et al. [10] who refer a splitting of isoform 17* in 17.2 and 17.3 kDa. **b** Expressed levels of the proteins are calculated divided by optical density of the blots of G3PDH protein used as internal control. Values shown are means±SD (21.5 kDa, control 1.381±0.039; ALC 1.176±0.026; 18.5 kDa, control 1.335±0.032; ALC 1.087±0.024; 17 kDa, control 1.212±0.048; ALC 1.076±0.031; 14 kDa, control 0.876±0.018; ALC 0.603±0.049). Statistic analysis was performed with the unpaired *t* test ($p<0.05$, $n=4$). *Asterisks* indicate statistical significance

isoforms: 21.5 kDa, 18.5 kDa, 17* kDa, and 14 kDa. In Fig. 3a, it is possible to single out a single, nonspecific 19.7 kDa band (arrowhead). The isoform 17* could represent the average of the two isoforms 3–4 shown in Fig. 2a (not detectable in our electrophoretic conditions). This is in accordance with Kimura et al. [10] who refer a splittingc of isoform 17* in 17.2 and 17.3 kDa. The blots of MBP exhibited a clear decrease of the protein level following ALC treatment, whereas in G3PDH housekeeping product level, no significant difference between control and ALC-treated samples has been observed.

Rats treated with ALC at the dose of 40 mg/kg bw did not show qualitatively significant differences in the expression of MBP gene compared with controls. Similarly, rats treated with LC at the dose of 100 mg/kg bw did not exhibit any effects, and it is not possible to appreciate any change

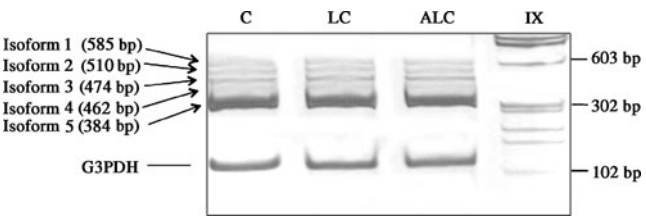

Fig. 4 Relative RT-PCR analysis of MBP. No significant difference between control, LC (100 mg/kg bw), and ALC-treated (40 mg/kg bw) samples has been observed. *Lane C* control animals, *LC* and *ALC* treated animals, *lane IX* marker IX

in the different isoforms of MBP with respect to controls (Fig. 4).

Discussion

The obtained results show that ALC chronic treatment brings about a decrease of MBP gene expression in the rat brain. MBP is a key component of myelin, representing 30% of the protein [11]. In the central nervous system, myelin is a dynamic structure arising from oligodendrocyte membrane processes. The gene of oligodendrocyte lineage (Golli) gives rise to a variety of developmentally regulated splice isoforms of MBP. In particular, the MBP family includes a number of developmentally regulated members arising from different transcription start sites, differential splicing, and posttranslational modifications [12]. In rodents, four isoforms of this protein resulting from alternative splicing of the gene Golli have been identified [12]. The MBP isoforms differ in their molecular weight: the most abundant in the CNS of rodents are the L isoform (14 kDa) and S isoform (18 kDa), which represent the major part of MBP present in myelin. Studies in cultured rat neurons showed that the isoforms of MBP containing exon II, the 21.5 kDa and 17 kDa, were distributed diffusely in the cytoplasm and accumulate in the nucleus only during the process of maturation of oligodendrocytes which play a key regulatory role in initiating the myelination. Even in humans, the isoforms containing exon II are mainly expressed during the myelinogenesis. These isoforms are again expressed during remyelinisation process, as has been demonstrated in experimental model of multiple sclerosis (MS). In contrast, L and S isoforms, lacking exon II, play an essential role in the compact myelin sheath. These isoforms are expressed only at the outer cell membrane of oligodendrocytes. The *classic* isoforms of MBP include the 18.5 kDa form, which prevails in adult human myelin and facilitates compaction of the mature myelin sheath in the central nervous system, thereby maintaining its structural integrity. In addition to membrane association, the 18.5 kDa and all other classic isoforms are able to interact with a number of proteins, including Ca^{2+}–calmodulin, actin, tubulin, and SH3 domain-containing proteins, and thus, they may represent signaling linkers during myelin development and remodeling. All proteins in this family are intrinsically disordered, creating a large effective surface to facilitate multiple protein associations, and are posttranslationally modified to various degrees by methylation, phosphorylation, and deamination [12].

The complex gene structure is conserved among species, suggesting that the MBP transcription unit is an integral part of the Golli transcription unit, and that this arrangement is important for the function and/or regulation of these genes. Mutation of the MBP gene is associated with the *shiverer* and *myelin-deficient* phenotypes in mouse [13, 14].

Since the normal processes of nerve impulse conduction depend on the insulating properties of the myelin sheath that surrounds the axon, an alteration in the composition of myelin may lead to the appearance of severe motor and sensory functions. Studies have shown that these proteins are able to awake a strong immune response because when injected into healthy rats, they determine the appearance of allergic encephalomyelitis, characterized by local inflammatory foci and destructive processes of the myelin sheath in the CNS. This disease has been used as an experimental model for studying MS, a chronic disease characterized by myelin loss and excessive levels of MBP in cerebrospinal fluid of patients. It was shown that in human cultures of neurons, an excess of MBP refers to cells of inflammatory response which directly attack the myelin sheath, resulting in the neural degeneration [15]. MBP in cerebrospinal fluid results as an indicator of disease activity in MS since in blood of patients with neurometabolic diseases, an increase of MBP has been observed [16]. In this context, it is very interesting to observe a modulation of MBP isoforms by ALC.

In our previous studies, we observed that a chronic ALC treatment modulates different gene expressions in the rat brain, and that the majority of detected clones are involved in neuroprotection and neuromodulation [3, 9].

In the present work, since the ALC treatment was executed at the beginning on 50-day-old rats, we observed that the down-modulation of MBP gene expression by ALC occurs at the end of the myelination process, that is completed in rats at about 40 days old [12]. This result suggests that ALC might exert a control role in the early stages of MS. In fact, ALC could lead to a reduction of the synthesis of MBP in excess and, therefore, of inflammatory or degenerative outbreaks. ALC may therefore have the function of stabilizing the process that leads to the integrity of myelin. Finally, the finding of a downregulation of MBP gene expression by ALC treatment is a further positive action of this substance on the control of mitochondrial disorders.

In conclusion, the 21-day treatment with ALC might be important in pathological conditions in which changes in MBP levels occur. In this context, we refer the experiment by Ikeda et al. [17] in which the authors described that in compression-induced cord injury, a reduction of MBP expression was greatly reduced by brain-derived neurotrophic factor administration, and this neurotrophin promoted the recovery of MBP expression nearly to a control level after 2 weeks. The same effects could be induced by ALC treatment.

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