

Beneficial Effects of Multitarget Iron Chelator on Central Nervous System and Gastrocnemius Muscle in SOD1^{G93A} Transgenic ALS Mice

Sagit Golko-Perez¹ · Tamar Amit¹ · Moussa B. H. Youdim¹ · Orly Weinreb¹

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Abstract Accumulation of evidence has demonstrated high levels of iron in the central nervous system of both sporadic and familial amyotrophic lateral sclerosis (ALS) patients and in ALS mouse models. In accordance, iron chelation therapy was found to exert beneficial effects on ALS mice. Our group has designed and synthesized series of multifunctional non-toxic, brain permeable iron-chelating compounds for neurodegenerative diseases. Recent study has shown that co-administration of one of these drugs, VAR10303 with high calorie/energy-supplemented diet (VAR-ced), initiated after the appearance of disease symptoms improved motor performance, extended survival, and attenuated iron accumulation and motoneuron loss in SOD1^{G93A} mice. Since VAR was found to exert diverse pharmacological properties associated with mitochondrial biogenesis in the gastrocnemius (GNS) muscle, we further assessed in the current study the impact of VAR-ced on additional neurorescue-associated molecular targets in the GNS and frontal cortex in SOD1^{G93A} mice. The results show that VAR-ced treatment upregulated the expression of various HIF-1 α -target glycolytic genes and elevated the levels of Bcl-2, neurotrophic factors, and AKT/GSK3 β signaling in the GNS and frontal cortex of SOD1^{G93A} mice, suggesting that these protective regulatory parameters regulated by VAR-ced treatment may be associated with the beneficial effects of the drug observed on ALS mice.

Keywords Amyotrophic lateral sclerosis · SOD1^{G93A} mice · Iron chelation · Neurorescue

Introduction

Several studies have demonstrated high levels of iron in the central nervous system (CNS) of both sporadic and familial amyotrophic lateral sclerosis (ALS) patients (Oba et al. 1993; Ince et al. 1994; Imon et al. 1995; Kasarskis et al. 1995; Ignjatovic et al. 2012) and mice overexpressing mutated human SOD1 gene (Lee et al. 2015; Jeong et al. 2009). Iron chelation was found to exert beneficial effects on ALS mouse models (Kupersmidt et al. 2009; Wang et al. 2011).

Our group has designed and synthesized multifunctional non-toxic, brain permeable iron-chelating compounds for neurodegenerative diseases, M30 series (Zheng et al. 2005a, b). Among these drugs, VAR10303 (VAR) possesses the neuroprotective *N*-propargyl moiety of rasagiline and the antioxidant/radical-scavenging iron-chelating moiety of VK28 (Bar-Am et al. 2015). Recent studies have provided positive outcomes for VAR, regarding ALS (Golko-Perez et al. 2014). Thus, co-administration of VAR with high calorie/energy-supplemented diet (VAR-ced), initiated after the appearance of disease symptoms (at day 88), was demonstrated to provide several beneficial effects on SOD1^{G93A} transgenic ALS mice (Golko-Perez et al. 2014). These included an improvement in motor performance, extension of survival, and attenuation of spinal iron accumulation and motoneuron loss. Moreover, VAR-ced treatment attenuated neuromuscular junction (NMJ) denervation and exerted a significant preservation of myofibril regular morphology, associated with a reduction in the expression levels of genes related to denervation and atrophy in the gastrocnemius (GNS) muscle in SOD1^{G93A} mice. These effects were accompanied by

✉ Orly Weinreb
worly@tx.technion.ac.il

¹ Eve Topf Center, Faculty of Medicine, Technion-Israel Institute of Technology, P.O.B. 9697, 31096 Haifa, Israel

upregulation of mitochondrial DNA/biogenesis and related genes and proteins and elevation of complexes I and II activities in the GNS muscle (Golko-Perez et al. 2014).

Considering the diverse pharmacological properties of VAR, here, we have analyzed the regulatory effects of VAR-ced on hypoxia inducible factor-1 α (HIF-1 α) target genes encoded to glycolytic enzymes, Bcl-2 family proteins, neurotrophic factor levels, and phospho-protein kinase B (AKT)/glycogen synthase kinase-3 beta (GSK3 β) signaling pathway in the GNS muscle and frontal cortex (FC) of SOD1^{G93A} mice.

Materials and Methods

Animal Treatment

All procedures were carried out in accordance with the National Institutes of Health Guide for care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of the Technion, Haifa, Israel (Ethics Num. IL-066-06-12; 16.07.2012-16.07.2016). SOD1^{G93A} transgenic familial ALS female mice (high copy number; B6SJL Tg SOD1^{G93A} Gur/J) (Gurney 1997) and littermate (WT; non-Tg) mice were obtained from The Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed at an ambient temperature of 22 °C with a 12-h light/dark cycle and humidity-controlled environment. Food and water were available ad libitum.

SOD1^{G93A} mice (22 mice/group) were administered by oral gavage with VAR (0.5 and 2.5 mg/kg, two times a week) +ced (the diet consisted of regular chow supplemented with 21 % (*wt/wt*) fat and 0.15 % (*wt/wt*) cholesterol (Research Diets, INC), and 1.25 % creatine given in sterile drinking water and replaced by a freshly prepared solution every 2 days) or VAR+ced (VAR-ced). Control mice were treated by oral gavage with vehicle (no drug, no diet supplementation). The doses of VAR used in the experiments were selected based on our previous studies with M30 and VAR in SOD^{G93A} mice (Golko-Perez et al. 2014; Golko-Perez et al. 2015). VAR-ced or vehicle was administered to SOD1^{G93A} from 88 days of age, when motor impairment was observed by behavioral tests (rotarod performance, screen grasping, tail suspension, balance beam, and gait footprint) and when mice started to lose weight, compared to WT Mice. At 120 days of age, mice were sacrificed by decapitation, and GNS muscle and FC tissues were dissected, frozen immediately in liquid nitrogen, and stored at –80 °C for further biochemical analyses.

Quantitative Real-Time Reverse Transcriptase-PCR

Isolation of total RNA was performed using PurfectPure RNA Tissue Kit, as recommended by the manufacturer

(5'PRIME Inc., USA). After extraction of total RNA from tissues, the RNA concentrations were determined by NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). Reverse transcription (RT) was performed using PrimeScriptTM RT reagent kit (Takara Bio Inc., Korea). The resulting cDNA was amplified with specific primers of genes examined in the present study, by using 7500 Real-Time PCR system (Applied Biosystems, USA). SYBR Premix Ex Taq (Tli RNase H Plus) (Takara Bio Inc. Korea) was performed according to the manufacturer's protocol. Real-time PCR was performed with specific primers, for the genes in search, including the following: brain neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), aldolase-A (ALDA), phosphoglycerate kinase-1 (PGK1), enolase 1 (ENO1) and pyruvate kinase muscle-2 (PKM2), HIF-1 α , Bcl-2, and Bax (QIAGEN, CA, USA). Two reference genes were used: γ -tubulin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (QIAGEN, CA, USA).

Western Blotting Analysis

For Western blotting analysis, the GNS muscle and FC tissues were homogenized in Tris-sucrose buffer pH = 7.4, as previously described (Kupersmidt et al. 2009). Protein concentrations of the samples were determined using the Bradford method. Equal amounts of proteins per sample were loaded and subjected to sodium dodecyl sulfate (SDS)-PAGE (4–12 % Bis-Tris gels) and blotted on Protran nitrocellulose membrane (Schleicher & Schuell, Germany). Membranes were treated with blocking buffer and incubated with primary antibodies for overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies diluted in the same buffers for 1 h at 25 °C. The following antibodies were used: rabbit anti-HIF-1 α (Santa Cruz biotechnology, USA); rabbit monoclonal anti-BDNF (Epitomics Inc. Burlingame, CA, USA); rabbit anti-phospho-GSK3 β (pGSK3 β) (ser9); rabbit anti-GSK3 β ; rabbit anti-phospho-Akt (pAKT) (ser473); rabbit anti-Akt; and mouse monoclonal anti-GAPDH (Merck Millipore, Germany). Detection was completed by using Western blotting ECL reagent system (Amersham Pharmacia, UK). Band intensities were quantified, using the computerized imaging program Bio-1D (Vilber Lourmat Biotechnology, France).

Statistical Analysis

A one-way ANOVA was used to determine significant differences among means. When significance occurred ($p \leq 0.05$), a Tukey-Kramer post hoc test was used to determine the significance.

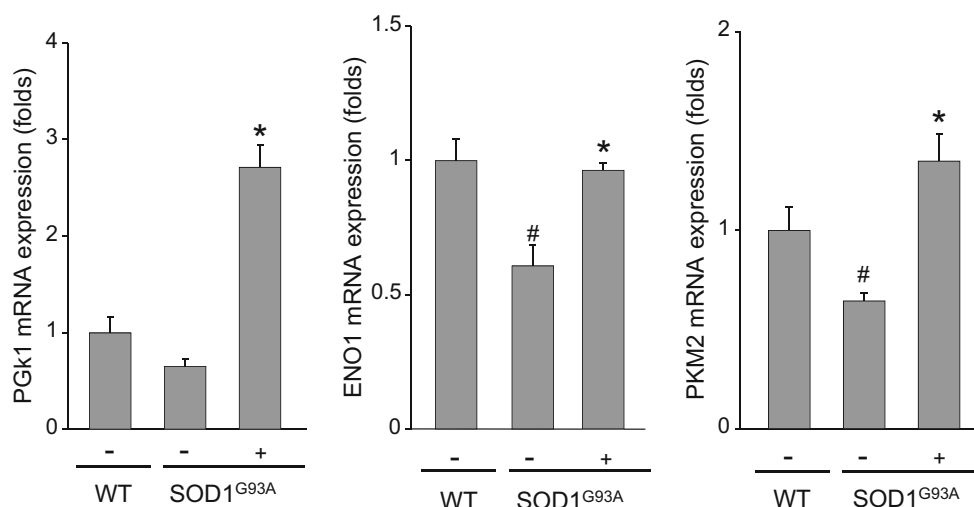


Fig. 1 Effect of VAR-ced treatment on mRNA expression levels of glycolytic enzymes in the GNS muscle of SOD1^{G93A} mice. SOD1^{G93A} mice were treated by oral gavage with vehicle (no drug, no diet supplementation) and VAR-ced (0.5 mg/kg) two times a week, starting at 88 days of age and continuing until death. GNS muscles were collected

at symptomatic age of 120 days. The amount of mRNA products was assessed by real-time RT-PCR and normalized to γ -tubulin and GAPDH, and values represent relative expression levels vs. WT mice. Results are expressed as folds \pm SEM; ($n = 4$ /each experimental group); # $p < 0.05$ vs. WT mice; * $p < 0.05$ vs. vehicle-treated SOD1^{G93A} mice

Results

Regulatory Effect VAR-ced on mRNA Expression of Glycolytic Enzymes in GNS Muscle of SOD1^{G93A} Mice

Initially, we have analyzed the regulatory effect of VAR-ced treatment on expression levels of HIF-1 α target genes encoding for enzymes involved in glycolysis (Palamiuc et al. 2015) in the GNS muscle of VAR-ced (0.5 mg/kg)-treated and vehicle-treated SOD1^{G93A} mice. As shown in Fig. 1, VAR-ced administration significantly upregulated messenger RNA (mRNA) expression levels of several HIF-1 α target genes encoding for the glycolytic

enzymes, PGK1, ENO1, and PKM2 in the GNS muscle in SOD1^{G93A} mice. In addition, VAR-ced treatment increased the expression levels of HIF-1 α in GNS muscle in SOD1^{G93A} mice (2.08 ± 0.34), compared with vehicle-treated SOD1^{G93A} mice (1.30 ± 0.20).

Effect of VAR-ced on Bcl-2 Family Members, BDNF, and pAkt/pGSK-3 β Signaling Pathway in GNS Muscle of SOD1^{G93A} Mice

Figure 2 demonstrates that VAR-ced (0.5 mg/kg) administration significantly prevented the decrease in the ratio of mitochondrial anti-apoptotic Bcl-2 and pro-apoptotic Bax

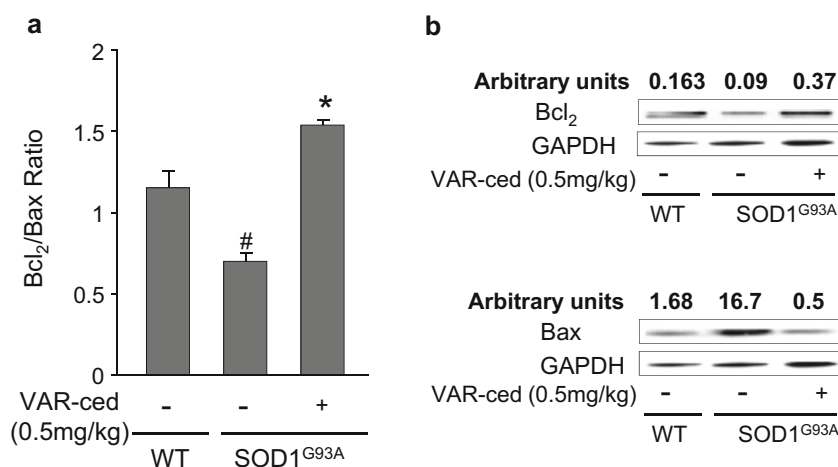


Fig. 2 Effect of VAR-ced treatment on Bcl-2 and Bax in the GNS muscle of SOD1^{G93A} mice. SOD1^{G93A} mice were treated as described in Fig. 1, and GNS muscles were collected at symptomatic age of 120 days ($n = 4$ /each experimental group). **a** The ratio of the mRNA expression levels of Bcl-2/Bax; the amount of mRNA products were assessed by real-time

RT-PCR and normalized to γ -tubulin and GAPDH. # $p < 0.05$ vs. WT-vehicle mice and * $p < 0.05$ vs. vehicle-treated SOD1^{G93A} mice. **b** A representative Western blotting analysis of Bcl2 and Bax, and results are expressed as arbitrary units

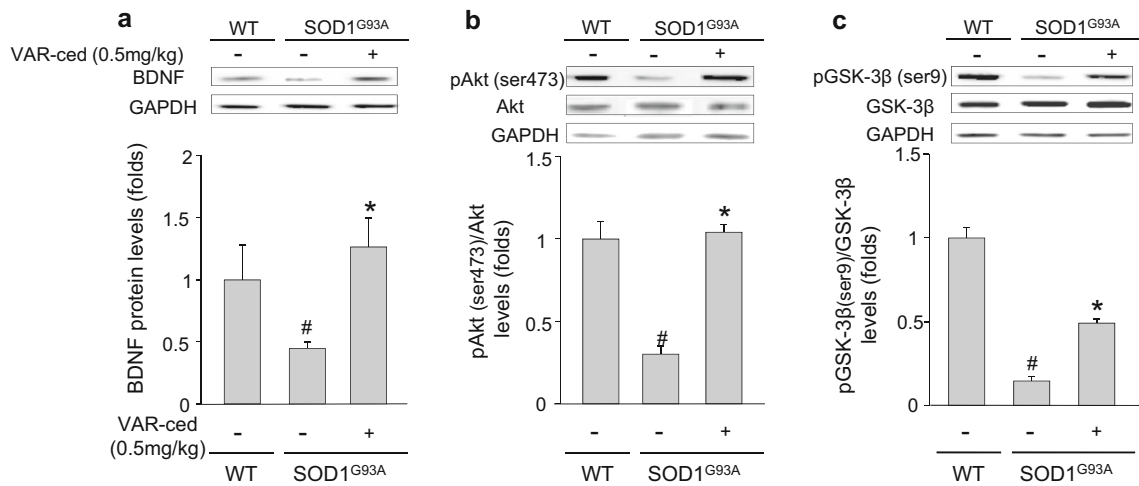


Fig. 3 Effect of VAR-ced treatment on BDNF, pAkt, and pGSK-3β in the GNS muscle of SOD1^{G93A} mice. SOD1^{G93A} mice were treated as described in Fig. 1, and GNS muscles were collected at symptomatic age of 120 days. Western blotting analysis of BDNF (a), pAkt (ser473) (b), and pGSK-3β (ser9) (c). The graphs represent densitometry

quantification of the lanes, normalized to GAPDH. The values represent relative levels vs. WT mice, and results are expressed as folds ± SEM; (n = 4/each experimental group); #p < 0.05 vs. WT mice; *p < 0.05 vs. vehicle-treated SOD1^{G93A} mice

mRNAs, in the GNS muscle of vehicle-treated SOD1^{G93A} mice, as compared to WT mice. In accordance, VAR-ced treatment increased protein levels of Bcl-2 and reduced those of Bax in the GNS muscle, compared to vehicle-treated SOD1^{G93A} mice (Fig. 2b).

As shown in Fig. 3, the levels of BDNF, pAkt, and pGSK-3β were significantly decreased in the GNS muscle of vehicle-treated SOD1^{G93A} mice, as compared to WT mice. VAR-ced treatment significantly elevated the expression levels of BDNF (Fig. 3a) and the levels of pAkt (Fig. 3b) and pGSK-3β (Fig. 3c) in the GNS muscle of drug-treated compared to vehicle-treated SOD1^{G93A} mice.

Regulatory Effects of VAR-ced on mRNA Expression Levels of Glycolytic Enzymes, BDNF, GDNF, and pAkt in the FC in SOD1^{G93A} Mice

Since ALS is characterized by impairments in the cerebral cortex (Nefussy and Drory 2010), we have further examined the regulatory effect of VAR-ced (0.5 mg/kg) treatment on various biochemical alterations in the FC of SOD1^{G93A} mice.

Similar to its effect on the GNS, VAR-ced treatment prevented the downregulation in the mRNA expression levels of several genes encoded to the glycolytic enzymes, PGK1, ENO1, and PKM2 in the FC of SOD1^{G93A} mice, compared to vehicle-treated SOD1^{G93A} mice (Fig. 4). VAR-ced treatment

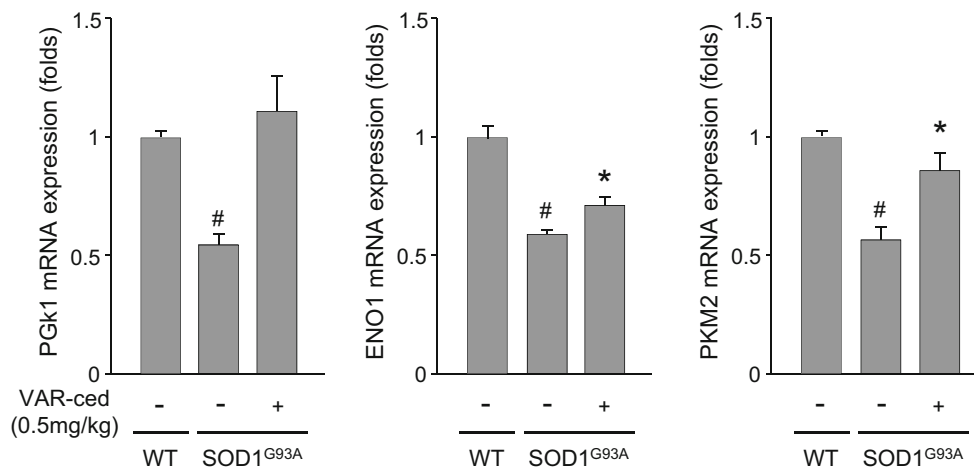


Fig. 4 Effect of VAR-ced treatment on mRNA expression levels of glycolytic enzymes in the FC of SOD1^{G93A} mice. SOD1^{G93A} mice were treated as described in Fig. 1, and FC tissues were collected at symptomatic age of 120 days. The amount of mRNA products was

assessed by real-time RT-PCR and normalized to γ-tubulin and GAPDH, and values represent relative expression levels vs. WT mice. Results are expressed as folds ± SEM; (n = 4/each experimental group); #p < 0.05 vs. WT mice; *p < 0.05 vs. vehicle-treated SOD1^{G93A} mice

also increased the levels of HIF-1 α in the FC of drug-treated (1.90 ± 0.50), compared to vehicle-treated SOD1^{G93A} mice (0.72 ± 0.13).

In addition, VAR-ced administration upregulated mRNA expression levels of the neurotrophic factors, BDNF, and GDNF (Fig. 5a) and levels of pAkt (Fig. 5b) in the FC, compared to vehicle-treated SOD1^{G93A} mice.

Discussion

Recently, we have demonstrated that VAR-ced treatment provided several beneficial effects on ALS mouse model (Golko-Perez et al. 2014). Using a neurorescue protocol (drug treatment was initiated after the appearance of disease symptoms), it was

shown that VAR-ced increased survival, attenuated spinal cord motoneuron loss, and improved motor performance in SOD1^{G93A} mice. Considering the diverse pharmacological properties of the multitarget compound VAR, we further assessed in the current study the impact of VAR-ced on additional neurorescue-associated molecular targets in the GNS muscle and FC in SOD1^{G93A} mice.

VAR was shown to afford iron chelation and iron-induced lipid peroxidation inhibitory potency (Bar-Am et al., 2015). Recently, we have shown that VAR-ced treatment lowered the elevated iron levels in the spinal cord and significantly reduced L-ferritin expression levels in the GNS muscle of SOD1^{G93A} mice (Golko-Perez et al. 2014). In the current study, we have demonstrated that VAR-ced treatment increased the expression levels of various glycolytic genes targeted by the iron-sensitive transcription factor, HIF-1 α , including PGK1, ENO1, and PKM2 in the GNS muscle and FC of SOD1^{G93A} mice. In line with this, previous studies have demonstrated that iron-chelating compounds significantly increased the expression of HIF-1 α -responsive genes (see review (Weinreb et al. 2010)). Thus, it was found that the multitarget iron chelator M30 enhanced the expression of HIF-1 α target genes involved in glycolysis in the FC of APP/PS1 Alzheimer's disease mice (Mechlovich et al. 2014), indicating that activating brain HIF-1 α signal transduction pathway may be associated with the neuroprotective effects of M30 (Weinreb et al. 2015). In addition, it has been shown in vitro that M30 differentially induced several neuroprotective-adaptive HIF-1 α -dependent target genes in cortical neurons (Avramovich-Tirosh et al. 2010) and motor-neuron-like NSC-34 cells (Kupersmidt et al. 2009), and in vivo, in various brain regions and in the spinal cord in mice (Kupersmidt et al. 2011).

Regarding the importance of Bcl-2 family proteins in the regulation of programmed cell death in ALS (Kostic et al. 1997), we have examined in the present study the anti-apoptotic effects of VAR-ced treatment on SOD1^{G93A} mice. We demonstrated that the VAR-ced administration upregulated mRNA and protein levels of the pro-survival Bcl-2 and reduced the pro-apoptotic Bax in the GNS muscle and FC in SOD1^{G93A} mice. In addition, VAR-ced treatment enhanced the expression levels of BDNF and GDNF and modulated the levels of various molecular systems (pAkt and pGSK-3 β) that are involved with their signaling pathways. Indeed, VAR, as a derivative of rasagiline, possesses the *N*-propargyl moiety that has been previously shown to exert neuroprotective properties (Bar-Am et al. 2005; Bar-Am et al. 2010). It was previously reported that the mechanism of neuroprotection by the propargyl moiety and propargyl-containing derivatives (e.g., rasagiline, ladostigil, and M30) may be associated with various protective-regulated effects, such as induction of anti-apoptotic Bcl-2 family proteins, upregulation of neurotrophic factors, and activation of pAkt and pGSK-3 β

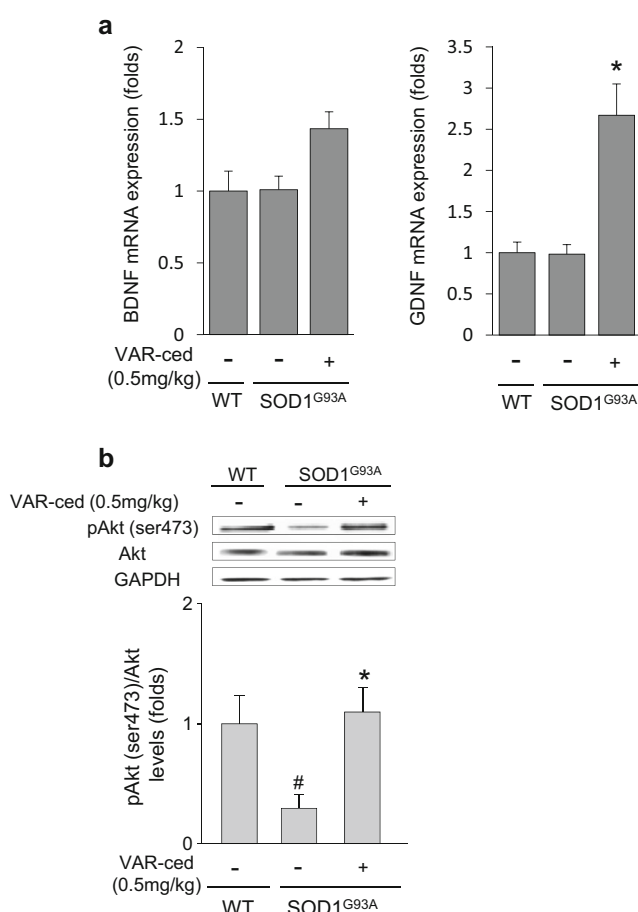


Fig. 5 Effect of VAR-ced treatment on BDNF, GDNF, and pAkt in FC of SOD1^{G93A} mice. SOD1^{G93A} mice were treated as described in Fig. 1, and FC tissues were collected at symptomatic age of 120 days. **a** mRNA expression of BDNF and GDNF; the amount of mRNA products was assessed by real-time RT-PCR and normalized to γ -tubulin and GAPDH, and values represent relative expression levels vs. WT mice. **b** Western blotting analysis of pAkt (ser473); the graph represents densitometry quantification of the lanes, normalized to GAPDH. The values represent relative levels vs. WT mice, and results are expressed as mean of folds \pm SEM; ($n = 4$ /each experimental group); # $p < 0.05$ vs. WT mice and * $p < 0.05$ vs. vehicle-treated SOD1^{G93A} mice

signaling pathways (Bar-Am et al. 2010). In this context, M30 was shown to upregulate Bcl-2 and conversely downregulated Bax expression levels in SH-SY5Y cells exposed to long-term serum deprivation (Avramovich-Tirosh et al. 2007). Moreover, it was found in ALS mice that M30 treatment could increase the expression of Bcl-2 and reduce the levels of Bax in the spinal cord (Wang et al. 2011).

In summary, we presented data demonstrating that VAR-ced treatment upregulated the expression levels of glycolytic-related genes, attenuated the apoptotic cascades (induction of Bcl-2 and reduction of Bax), and increased the expression levels of BDNF/GDNF and phosphorylation of Akt and GSK-3 β in the GNS muscle and frontal cortex of SOD1^{G93A} mice. It is indicated that these protective regulatory parameters regulated by VAR-ced treatment may be associated with the beneficial effects of the drug observed in ALS mice.

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Compliance with Ethical Standards All procedures were carried out in accordance with the National Institutes of Health Guide for care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of the Technion, Haifa, Israel (Ethics Num. IL-066-06-12; 16.07.2012-16.07.2016).

Conflict of Interest MBH Youdim is the scientific founder of Abital Pharma Pipelines and commercial interest in VAR10303 drug.

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