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In FUS[1−359]-tg mice O,S-dibenzoyl thiamine reduces muscle atrophy, decreases glycogen synthase kinase 3 beta, and normalizes the metabolome

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ABSTRACT

Mutations in the gene encoding the RNA/DNA-binding protein Fused in Sarcoma (FUS) have been detected in familial amyotrophic lateral sclerosis (ALS) patients. FUS has been found to be a critical component of the oxidative damage repair complex that might explain its role in neurodegeneration. Here, we examined what impact antioxidant treatment with thiamine (vitamine B1), or its more bioavailable derivative O,S-dibenzoylthiamine (DBT), would have on the hallmarks of pathology in the FUS[1−359]-transgenic mouse model of ALS. From 8-weeks old, in the pre-symptomatic phase of disease, animals received either thiamine, DBT (200 mg/kg/day), or vehicle for 6 weeks. We examined physiological, behavioral, molecular and histological outcomes, as well as the serum metabolome using nuclear magnetic resonance (NMR). The DBT-treated mice displayed improvements in physiological outcomes, motor function and muscle atrophy compared to vehicle, and the treatment normalized levels of brain glycogen synthase kinase-3β (GSK-3β), GSK-3β mRNA and IL-1β mRNA in the spinal cord. Analysis of the metabolome revealed an increase in the levels of choline and lactate in the vehicle-treated FUS mutants alone, which is also elevated in the cerebrospinal fluid of ALS patients, and reduced glucose and lipoprotein concentrations in the FUS[1−359]-tg mice, which were not the case in the DBT-treated mutants. The administration of thiamine had little impact on the outcome measures, but it did normalize circulating HDL levels. Thus, our study shows that DBT therapy in FUS mutants is more effective than thiamine and highlights how metabolomics may be used to evaluate therapy in this model.

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disorder characterized by the rapid degeneration of motor neurons. While most cases are sporadic, approximately 10% of cases have been found to be familial. Currently, the mutations in over 20 genes have been identified to be causal in ALS; among them a number of mutations in the gene encoding the DNA/RNA-binding protein, Fused in Sarcoma (FUS) have been recognised. The identification of other causal genes related to ALS pathology suggest that aberrant RNA processing may underlie common mechanisms of neurodegeneration in ALS [10,33,39].

Abnormal RNA processing has been shown to be closely associated with compromised oxidative stress mechanisms. Since the identification of the SOD1 mutations in ALS in 1993, oxidative stress has also been considered to be a possible contributor to or even a primary cause of pathogenesis ALS pathology. In sporadic ALS patients, an increase GSSG/GSH ratio and increased levels of 4-hydroxynonenal in the cerebrospinal fluid, a marker of lipid peroxidation, have been reported [59]. These data suggest that oxidative stress and lipid peroxidation are associated with and may promote motor neuron degeneration in ALS. Oxidative stress and DNA damage are also increased in iPSC-derived C9ORF72 motor neurons in an age-dependent manner and pharmacological or genetic reduction of oxidative stress has been shown to partially rescue DNA damage in neurons in flies or human neurons with C9ORF72 mutations [34]. Oxidative stress was shown to be increased in iPSC-derived C9ORF72 motor neurons in an age-dependent manner and pharmacological reduction of oxidative stress has been shown to partially rescue neuronal damage in flies or human neurons with C9ORF72 mutations [34]. For FUS, it is known that FUS has been found to be a critical component of the oxidative damage repair complex and suggests that a loss of function might lead to susceptibility to oxidative stress where the mutation results in a loss of translocation of FUS to the nucleus. However, studies with conditional FUS knockout mutant mice also suggest that FUS-dependent motor degeneration cannot be attributed to a loss of FUS function, but to a gain of toxic properties conferred by ALS [56].

Some have argued that the early FUS-knockout mice have limited validity in mimicking ALS-like pathology [56]. However, the recently described FUS [1−359]-transgenic (FUS [1−359]-tg) mouse line lacking RNA binding capacity [57] recapitulates many of the key hallmarks of this disease including signs of elevated oxidative stress [53]. Specifically, FUS [1−359]-tg mice revealed elevated malondialdehyde (MDA) concentrations in the spinal cord [53], one of the final products of polyunsaturated fatty acids peroxidation in cells, which is increased by free radicals generation. These mutants also display FUS-positive inclusions, microglial activation, abrupt disease onset and fast progression [11,36,44], motor deficits including asymmetrical limb paralysis, muscle wasting and denervation, loss of spinal motor neurons and selective involvement of brainstem nuclei ([11,57]; de Munter et., 2020a), as well as elevated levels of pro-inflammatory cytokines IL-1β, IL-6 and glycogen synthase kinase-3β (GSK-3β) in the brain and spinal cord (de Munter et., 2020a,b). Importantly, the key pathology hallmarks of FUS [1−359]-tg mice were found to be responsive to the ALS treatment riluzole, as well as to a stem cell therapy [12,13,53].

Neuroinflammation is considered to be a key feature of ALS pathogenesis and is associated with elevated oxidative stress. For example, in the blood and cerebrospinal fluid of ALS patients increases in the levels of expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF have been reported [25,35,42,58]. Thus, the use of anti-inflammatory and antioxidant drugs may be considered as promising treatment strategy counteracting the ALS progression [53]. Thiamine and its derivatives as powerful antioxidant compounds might have this dual anti-oxidant and anti-inflammatory action [3,20,53]. Indeed, the administration of thiamine-based compounds have been reported to be beneficial in patients with neurodegenerative disorders and other neurological or psychiatric conditions, as well as in pre-clinical studies in animals [4,18,38,47,48,67].

For example, a 12-month-long treatment with benfotiamine, a bioavailable thiamine precursor, at the dose of 600 mg/day improved primary clinical outcomes in Alzheimer's disease patients, and reduced the levels of advanced glycation end products in the blood of these patients [18]. The administration of this drug for 3 weeks at the dose of 300 mg/day decreased the neuropathy score in patients with diabetic polyneuropathy [23,62], and a 2-month-long treatment with sulbutiamine, another lipophilic thiamine-derived compound, at the dose of 400 mg/day, improved fatigue impact scale in patients with multiple sclerosis [55]. Administration of this thiamine compound at the dose of 600 mg/day reduced social inhibition in patients with major depressive disorder [34] and thiamine, at 300 mg/day, improved Hamilton depression score in depressed patients [17].

In the preclinical studies, the anti-inflammatory and antioxidant properties of thiamine compounds have also been reported in which treatment has been shown to prevent stress-induced suppression of hippocampal neurogenesis, characteristic feature of neurodegeneration, and to decrease brain GSK-3β expression in a model of predator exposure [67]. The thiamine compounds have also been shown to prevent stress-induced increases in the expression of the proinflammatory markers cyclooxygenase-2 (COX-2), IL-1β, and tumor necrosis factor (TNF) [48], as well as in the concentration of oxidative stress markers total glutathione and protein carbonyl, in the brains of mice [20]. It is also of note that an 8-week-long treatment with benfotiamine, at the dose of 100 mg/kg/day, reduced ß-amyloid deposition and improved cognitive performance of APP/PS1 double-transgenic mice, which is often used model of Alzheimer's disease [46].

The thiamine derivative, O,S-dibenzoylthiamine (DBT) was recently demonstrated to have more marked anti-oxidative and anti-inflammatory properties [53,54]. Specifically, the use of 50 μM of DBT in LPS-stimulated microglial cell line showed greater anti-inflammatory effect than that of thiamine, benfotiamine, or sulbutiamine by reducing nitric oxide or TNF production [54]. Moreover, administration of DBT, but not thiamine, counteracted the development of ALS-like symptoms in FUS-transgenic mice, and relieved depressive-like behavior in mice subjected to chronic ultrasound stress [53].

Here, we have sought to explore the cellular and molecular mechanisms of beneficial effects of DBT treatment in the FUS [1−359]-tg mice including nuclear magnetic resonance spectroscopy-based metabolome analysis of the blood [5,41]. Metabolomics has been employed to identify biomarkers of disease progression and provide insight into the molecular mechanisms that underpin the treatment response in a number of neurological conditions such as Alzheimer disease [70], Parkinson's [45] and Huntington's diseases [40] and multiple sclerosis [6,28,71]. For ALS, in CSF predictive metabolomics models could be generated, using NMR, were found using those patients with at least one year's interval between recruitment and the second sample in which lactate and citrate were elevated in the ALS patients [21]. The serum metabolome has also been investigated in ALS [30], but no study has explored the effect of a therapy on outcome in either humans or animal models. The metabolome reveals the downstream metabolic processes that result from expression of the genome and transcriptome under influence of environmental factors, such as therapeutic interventions, in healthy or pathological conditions and, here, we were interested to understand the impact of the therapies on the metabolome.

2. Material and methods

2.1. Animals

FUS[1−359]-tg (FUS-tg) male mice and their wild-type littermates (WT) were provided by the FDA-certified IPAC RAS facilities (http://www.ipac.ac.ru/index.html). Mice were single housed under standard conditions (22 ± 1 °C, 55% humidity, reversed 12-h light/dark cycle

with lights on at 19:00, food and water ad libitum). Experimental procedures were set up in accordance with a Directive 2010/63/EU and ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-animal-research-reporting-vivo-experiments) and approved by the local veterinarian Committee for Bioethics of IPAC RAS (N19–16.06.2017) and MSMU (22/10/17–MSMU-35). All efforts were undertaken to ensure compliance with above-mentioned regulations concerning human endpoint in animal research.

2.2. Generation of FUS-tg mice

Generation of FUS-tg mice was performed as described elsewhere [57]. Briefly, a fragment of human FUS [1−359] cDNA including 9 bp of 5′-UTR was cloned into Thy-1 promoter plasmid 323-pTSC21k. A gel-purified fragment obtained by digestion of the resulting plasmid DNA with *Not*I was used for microinjection of mouse oocytes. Transgenic animals were identified by PCR analysis of DNA from ear biopsies by the presence of 255-bp product (primers 5′-TCTTTGTGCAAGGCCTGGGT-3′ and 5′-AGAAGCAAGACCTCTGCAGAG-3′).

2.3. Study design

Male FUS-tg mice and their wild type (WT) littermates at the age of 8 weeks received (1) regular tap water, (2) thiamine (200 mg/kg/day) or (3) DBT (200 mg/kg/day) for 6 weeks via drinking water as described elsewhere [20,38,67]. On weeks 5 (pre-symptomatic stage) and week 6 (humane end point stage), water and diet intake, body weight and motor functions in the pole test, wire test and rotarod were assessed as described elsewhere [12,66], dosing with pharmaca was continued. Signs of paralysis were assessed daily. Paralysis was defined by walking ability and in the test for limb flexion [7]. On week 6, mice were sacrificed, either perfused with NaCl or 4%-paraformaldehyde; muscle gastrocnemius, spinal cord and brain were harvested for histology, RT-PCR and ELISA assay, respectively. Group sizes are indicated in figure legends.

2.4. Drug administration

Experimental solutions replaced normal drinking water. Thiamine and DBT (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in tap water and changed every 4–5 days as described elsewhere [20,53]. Concentrations of treatments were adjusted to the daily liquid intake and desirable dose of treatment of 200 mg/kg/day. Drinking behavior was monitored by weekly evaluation of the 24-h liquid intake performed. No group differences in these measures were found suggesting normal drinking behavior in dosed mice, in line with previous observations [20,48,49,53].

2.5. Pole test

Mice were placed on a top of the vertical bar (diameter 1.1 cm, height 60 cm) and allowed to climb down to a horizontal surface. The latency of descending the bar and the number and percent of mice with sliding events were scored as described elsewhere [12].

2.6. Rotarod

Mice were placed on constantly rotting rod (speed 15 rpm) of rotarod (Columbus Instruments, Columbus, OH, USA) for 600 s. The latency to fall was registered as described elsewhere [12,66].

2.7. Blood and tissue collection

Mice were terminally anesthetized by isoflurane and blood plasma was collected. Thereafter, mice were perfused with NaCl, and thoracal part of spinal cord, hippocampus and prefrontal cortex were dissected and frozen immediately of dry ice as described elsewhere [12,20]. Blood collection was performed transcardially, blood was stored in heparinized vials prior to centrifugation (1500 rcf, 15 min, 4 °C); 100 μl of plasma was removed and immediately stored at − 20 °C until use. Lumbar part of the spinal cord and gastrocnemius muscles were collected as described elsewhere (de Munter et al., 2020) and stored at 4% paraformaldehyde.

2.8. ELISA assay

To study the concentrations of total GSK-3β in the prefrontal cortex and hippocampus, mouse enzyme-linked immunosorbent assay (ELISA) was performed using Invitrogen™ Total GSK-3β ELISA Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The microwell absorbance was measured at 450 nm with Synergy 4 Hybrid Multi-Mode Microplate Reader (BioTek, Thermo Fisher Scientific, MA, USA).

2.9. Quantitative real-time PCR

Total mRNA was isolated from each sample with TRI Reagent (Invitrogen, Carlsbad, CA, USA). During first-strand cDNA synthesis 1 μg total RNA was converted into cDNA using random primers and Superscript III transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, PA, USA) and the CFX96 Deep Well Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). qRT-PCR was performed in a 10 μl reaction volume containing a SYBR Green master mix (5 μl), RNase-free water (3 μl), specific forward and reverse primers used at the concentration 20 pmol/μl (1 μl), cDNA (1 μl). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as a reference gene, since in previous experiments it was observed relatively low variability in its brain expression [20,38]. The initial denaturation step for qRT-PCR was at 95 °C for 4 min followed by 40 cycles of denaturation at 95 °C for 20 s, annealing was at 54 °C for 90 s. Sequences of all primers used (Evrogen, Moscow, Russia) are listed in Table 1. All samples were run in triplicate. Data were normalized to GAPDH mRNA expression and calculated as relative-fold changes compared to control vehicle-treated mice, as described elsewhere [20,47].

2.10. Histological assay of muscle atrophy

Muscle samples were washed in water for 3 h and then dehydrated in a 70% alcohol solution for 8 h that was followed by washing in a 96%-alcohol solution for 2 h. The samples were then further dehydrated in 100%-alcohol solutions for 12 h and additionally washed for 2 h, that was followed by the dehydration in a 50%-chloroform solution in alcohol for 30 min; solutions were changed every 15 min. Next, samples were placed in a 50% chloroform Histomix® solution (BioVitrum Ltd., St. Petersburg, Russia) for 4 h at 60 °C, solutions were changed every 2 h. Next, muscle samples in Histomix were embedded in plastic blocks in the tissue embedding station Tissue-Tek TEC 5 (Sakura Finetek, Tokyo, Japan). Eight Section (5 μm each) from each sample were cut using a microtome Accu-Cut SRM (Sakura Finetek, Tokyo, Japan). Finally, muscle slices were stained with hematoxylin-eosin in Tissue-Tek Prisma Plus Automated Slide Stainer (Sakura Finetek, Tokyo, Japan) and

Table 1
Sequences of primers for qRT-PCR.

Gene	Forward primer 5–3′	Reverse primer 5–3′
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
IL-1β	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG
GSK-3β	TCCATTCCTTTGGAATCTGC	CAATTCAGCCAACACACAGC
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF	AGCCGATGGGTTGTACCTTG	GTGGGGTGAGGAGCACGTAGTC

embedded in BioMount covering media (Bio-Optica, Milan, Italy).

Scoring for atrophy was performed by two independent pathologists, blinded to sample identity using a light microscope (Axiovision 4.3, Zeiss, Berlin, Germany) at 40x magnification; ranking histograms were generated ranging samples were from 0 to 3 from "normal" to "severe atrophy" as described elsewhere [12]. Specifically, for each slide, score 0 was assigned to normal appearing muscle fibers, score 1 – to small number of atrophic fibers with few rounded hypertrophied fibers among normal, score 2 – to small groups of angulated atrophic fibers, and score 3 – to widespread whorled and necrotic muscle fibers among many angulated atrophic fibers (see examples for each score on Fig. 3A). Mean score for each group was then taken as 'atrophy score', in compliance with generally accepted criteria [32].

2.11. Metabolome assay

Plasma samples were defrosted at room temperature. 150 μl of the plasma supernatant was then diluted with 450 μl of 75 mM sodium phosphate buffer prepared in D_2O (pH 7.4). Samples were then centrifuged at 16,000 x g for 3 min to remove any precipitate before transferring to a 5-mm NMR tube.

All NMR spectra were acquired using a 700-MHz Bruker AVII spectrometer operating at 16.4 T equipped with a ^1H ($^{13}C/^{15}N$) TCI cryoprobe (Department of Chemistry, Oxford). Sample temperature was stable at 310 K. ^1H NMR spectra were acquired using a 1D NOESY presaturation scheme for attenuation of the water resonance with a 2 s presaturation. Broad signals arising from plasma proteins, such as albumin, were suppressed using a spin-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence with a 40 ms total effective filter time, 32 data collections, an acquisition time of 1.5 s, a relaxation delay of 2 s, and a fixed receiver gain. CPMG spectra provide a measurement of small molecular weight metabolites and mobile side chains of lipoproteins in the plasma sample and were used for all further analysis. ^1H total correlation spectroscopy (TOCSY) spectra were acquired on at least one sample in each classification to aid in metabolite identification along with reference to literature values and the Human Metabolome database [68]. For quality control, pooled plasma samples were spread throughout the run to monitor technical variation. Resulting free induction decays (FIDs) were zero-filled by a factor of 2 and multiplied by an exponential function corresponding to 0.30 Hz line broadening prior to Fourier transformation. All spectra were phased, baseline corrected (using a 3rd degree polynomial), and chemical shifts referenced to the lactate-CH_3 doublet resonance at $\delta = 1.33$ ppm in Topspin 4.1 (Bruker, Germany). Spectra were visually examined for errors in baseline correction, referencing, spectral distortion, or contamination. Technical details on spectral processing and analysis have been previously published [72]. In brief, spectra were divided in to 0.02 ppm 'bins' and constant-sum-normalization used to compute the integral of each 'binned' region.

2.12. Statistical analysis

Multivariate analysis of metabolomics data was performed using in-house R scripts (R foundation for statistical computing, Vienna, Austria) [51] and the ropls package [63]. Unsupervised principal component analysis (PCA) was applied to the binned CPMG data to reduce the complexity of the data with the aim of identifying distinct metabolic clusters within the sample set. The metabolites loading on the discriminatory component are then extracted and univariate analysis applied.

All univariate analysis was performed in GraphPad Prism 8.0 software (San Diego, CA, USA) was used; two-way ANOVA followed by post hoc Tukey's test or Kruskal-Wallis test. Normality of distribution was checked by Kolmogorov-Smirnov test. χ^2 test was used to treat qualitative measures. The level of significance was $p \leq 0.05$. The results are presented as bars with standard error of means (SEM).

3. Results

3.1. The onset of paralysis and basic physiological parameters are improved in the dibenzoyl thiamine-dosed mutants

χ^2 test revealed significant differences in the percentage of animals exhibiting signs of paralysis, between groups ($p = 0.0498$). A greater percentage of untreated animals displayed ALS-like paralysis symptoms than controls ($p = 0.0171$). While thiamine treatment had no significant effect on the percentage of animals with paralysis, FUS-tg mice treated with DBT had this measure significantly decreased at the symptomatic stage compared to non-treated mutant group ($p = 0.0334$, Fig. 1A).

A significant genotype effect was shown in body weight at the symptomatic stage and at the humane end point stage ($F=12.57$, $p = 0.0007$ and $F=85.64$, $p < 0.0001$, respectively, two-way ANOVA). At the symptomatic stage, post hoc Tukey's test showed that body weight of both non-treated and thiamine-treated mutants was significantly lower than in non-treated controls ($p = 0.0423$ and $p = 0.0202$, respectively; Fig. 1B). At the humane end point stage, significant decrease of body weight was found in non-treated FUS-tg mice compared to non-treated controls ($p < 0.0001$), as well as in mutants treated with thiamine or DBT in comparison with wild types that received same treatments ($p < 0.0001$ and $p = 0.0054$, respectively; Fig. 1C). Thus, body weight of mutants decreased at the end of the experiment, but DBT delayed this decrease.

At the symptomatic stage, two-way ANOVA demonstrated significant genotype effect, treatment effect and genotype x treatment interaction in food intake ($F=11.00$, $p = 0.0014$; $F=5.058$, $p = 0.0085$ and $F=5.735$, $p = 0.0047$, respectively). Food intake was significantly lower in non-treated mutants than in non-treated wild types ($p = 0.0010$, post hoc Tukey's test). Although no significant effect on food intake was observed with thiamine treatment, food intake in FUS-tg mice treated with DBT was significantly higher than in untreated FUS-tg mice ($p < 0.0001$; Fig. 1D) and indistinguishable from untreated controls. Significant genotype and treatment effects were revealed in food intake at humane end point stage ($F=104.3$, $p < 0.0001$ and $F=3.88$, $p = 0.0247$, respectively, two-way ANOVA). Post hoc analysis showed that parameter was lower in non-treated mutant group than in non-treated controls ($p < 0.0001$), and in mutants treated with thiamine or DBT than in wild types that received same treatments (both $p < 0.0001$; Fig. 1E).

While no significant genotype, treatment or interaction effects were found in water intake at the symptomatic stage ($p > 0.05$, two-way ANOVA; Fig. 1F), genotype, treatment, and interactions effects were significant at humane end point stage ($F=32.35$, $p < 0.0001$; $F=5.027$, $p = 0.0088$ and $F=3.63$, $p = 0.0312$, respectively). Non-treated mutants and mutants that received thiamine displayed decreased water intake compared to respective wild type groups ($p < 0.0001$ and $p = 0.0363$, respectively, post hoc Tukey's test), but mutants that received DBT did not differ significantly from controls in this measure ($p = 0.87$, Fig. 1G). Again, decrease of food and water intake, characteristic features of mice with ALS-like symptoms, was prevented or delayed by chronic administration of DBT.

3.2. Deficient motor functions of FUS-tg mice are partly ameliorated by the dibenzoyl thiamine administration

In the pole test, genotype effect was shown in latency to descent at the symptomatic stage and at the humane end point stage ($F=37.54$, $p < 0.0001$ and $F=60.34$, $p < 0.0001$, respectively, two-way ANOVA). At both stages, a significant decrease in this score was found in non-treated FUS-tg mice compared to non-treated controls ($p = 0.0018$ and $p < 0.0001$), as well as in mutants treated with thiamine ($p = 0.0218$ and $p < 0.0001$). FUS-tg mice treated with DBT exhibited significantly lower latency to descent than DBT-treated CD-1 controls only at the humane end point stage ($p = 0.0056$; Fig. 2A,B). χ^2 test

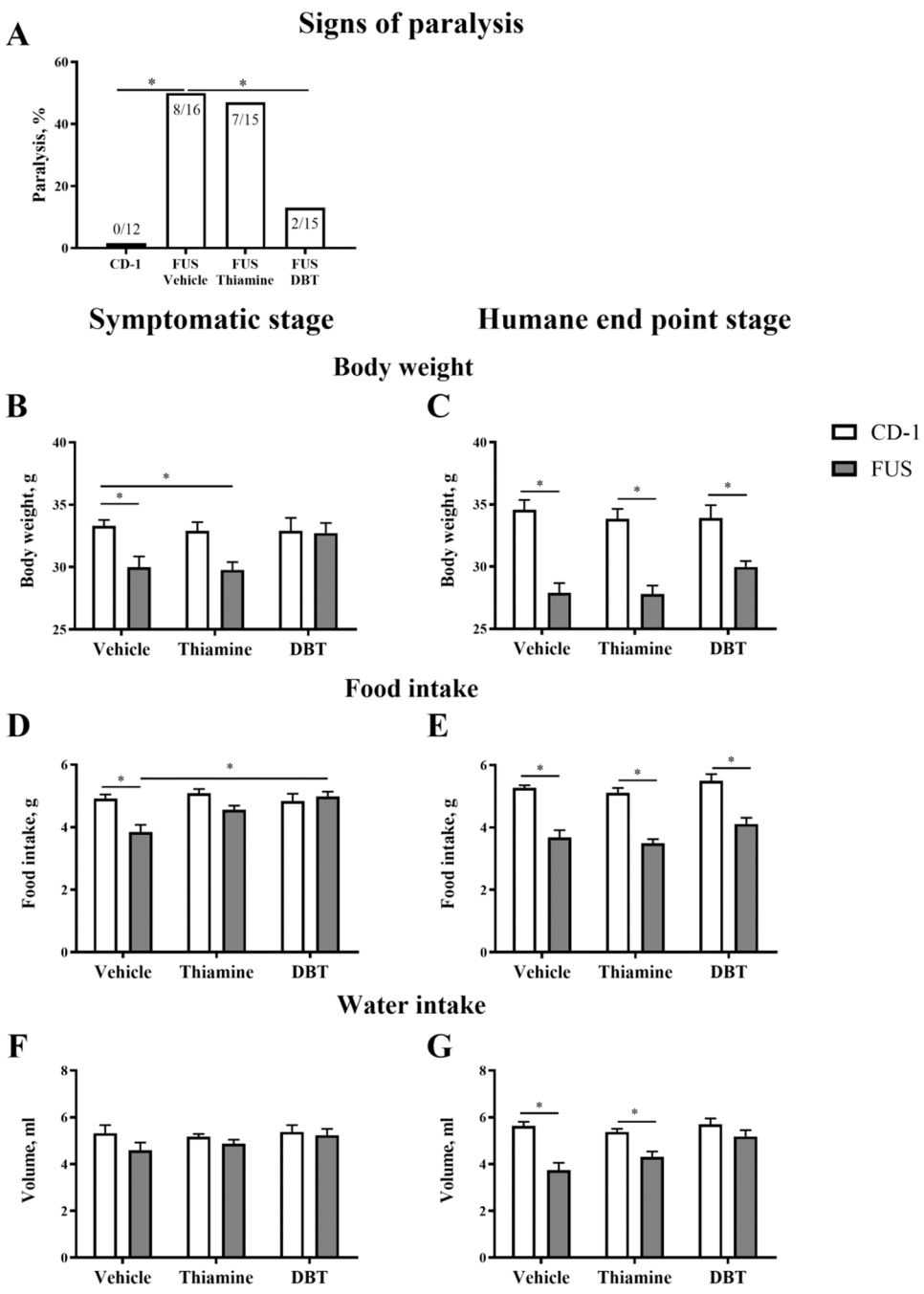

Fig. 1. The onset of paralysis and basic physiological parameters are improved in the DBT-dosed mutants. (A) FUS-tg mice treated with DBT had significantly decreased percent of mice with ALS-like symptoms compared to non-treated mutant group. (B) At the symptomatic stage, non-treated and thiamine-treated FUS-tg mice showed lower body weight than non-treated controls. (C) At the humane end point stage, non-treated, thiamine- and DBT-treated mutants had decreased body weight compared to respective controls. (D) At the symptomatic stage, non-treated FUS-tg mice had lower food intake than non-treated controls and mutants treated with thiamine or DBT. (E) At the humane end point stage, non-treated, thiamine- and DBT-treated mutants showed decreased food intake than wild types that received same treatments. (F) No differences were found between groups in water intake at the symptomatic stage. (G) At the humane end point stage, non-treated and thiamine-treated FUS-tg mice had lower water intake that non-treated and thiamine-treated wild types. $p < 0.05$, χ^2 test or two-way ANOVA and post hoc Tukey's test, 7–9 mice per groups of controls, 12–19 animals per groups of mutants. Bars are Mean ± SEM. DBT = dibenzoyl thiamine.

revealed significant differences in the percentage of mice with sliding events between groups at the humane end point stage, but not at the symptomatic stage ($p = 0.009$ and $p = 0.46$, respectively; Fig. 2C,D). At the humane end point stage, the percentage of animals with sliding events was significantly increased in non-treated mutants relative to untreated CD-1 controls ($p = 0.0155$; Fig. 2D).

Two-way ANOVA showed significant genotype effect in the latency to fall from the rotarod at the symptomatic stage ($F=4.65$, $p = 0.043$), as well as a strong trend for the treatment effect ($F=2.57$, $p = 0.083$), but no significant group differences were found by post hoc Tukey's test ($p > 0.05$; Fig. 2E). At the humane end point stage, a significant genotype effect was revealed ($F=50.71$, $p < 0.0001$, two-way ANOVA). Non-treated mutants and mutants that received thiamine demonstrated shorter latency to fall than respective wild type groups ($p < 0.0001$ and $p = 0.0002$, respectively, post hoc Tukey's test). However, FUS-tg mice treated with DBT did not display significant differences in this score from controls ($p > 0.05$, Fig. 2F). Thus, DBT effectively delayed the development of ALS-like symptoms and partly ameliorated motor functions of FUS-tg mice.

3.3. The study of treatment effects on the muscle atrophy

Kruskal-Wallis test revealed significant group differences in the score of muscle atrophy ($F=20.18$, $p = 0.0002$; Fig. 3A), showing increased signs of muscle degeneration in mutant mice. Non-treated and thiamine-treated mutants had increased atrophy scores compared to non-treated wild types ($p = 0.0001$ and $p = 0.0269$, respectively), while muscle atrophy in DBT-treated FUS-tg did not differ significantly from controls ($p > 0.05$), suggesting possible positive effects of the compound on this score.

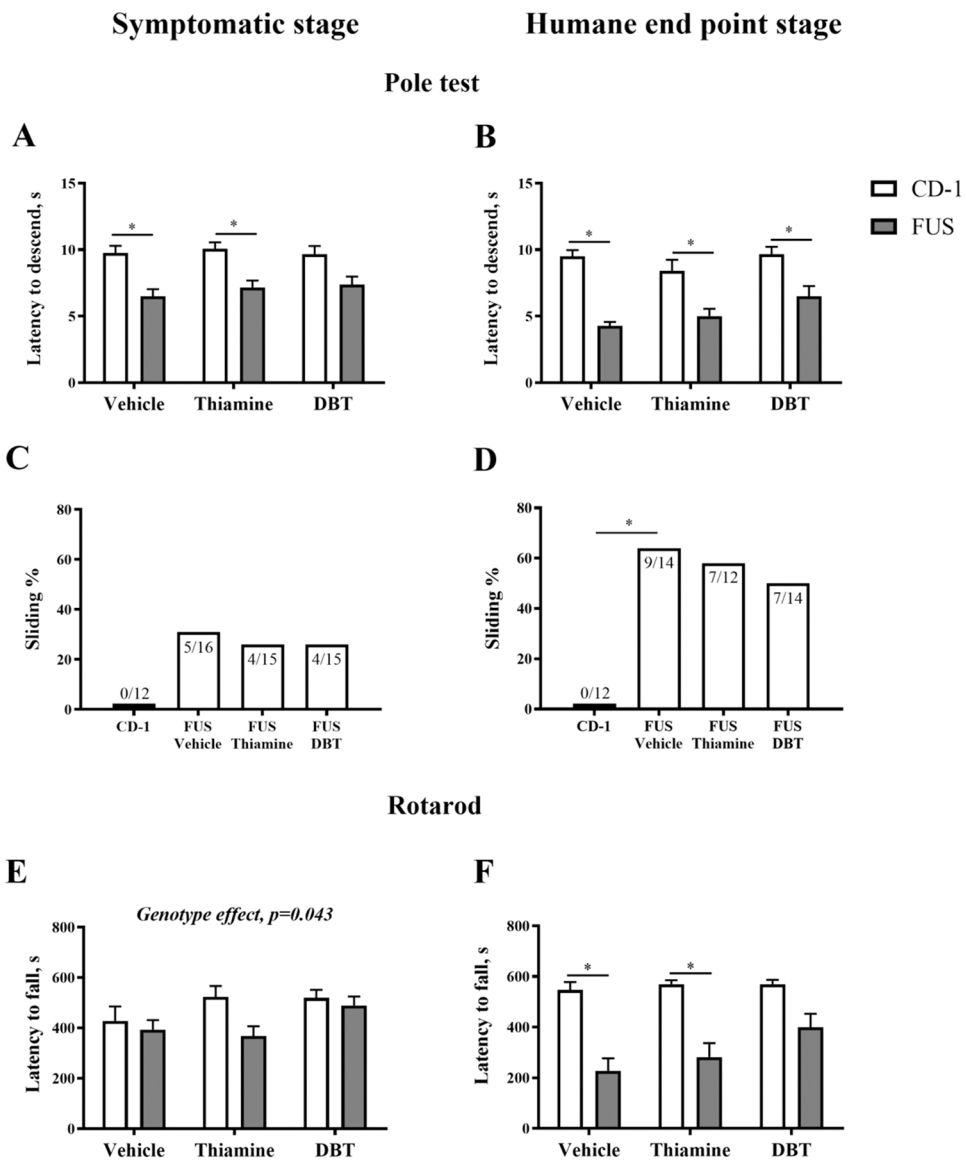

Fig. 2. Deficient motor functions of FUS-tg mice are partly ameliorated by the dibenzoyl thiamine administration. (A) At the symptomatic stage, non-treated and thiamine-treated mutants had decreased latency to descend in the pole test than wild types that received same treatments. (B) At the humane end point stage, non-treated, thiamine- and DBT-treated mutants had decreased latency to descend than respective wild types. (C) At the symptomatic stage, no significant differences were found in the events of sliding. (D) At the humane end point stage, non-treated mutants showed significantly increased percent of events of sliding than wild type mice. (E) At the symptomatic stage, significant genotype effect was found in the latency to fall from the rotarod. (F) At the humane end point stage, non-treated and thiamine-treated FUS-tg mice had lower latency to fall from the rotarod that non-treated and thiamine-treated wild types. $p < 0.05$, χ^2 test or two-way ANOVA and post hoc Tukey's test, 7–9 mice per groups of controls, 12–19 animals per groups of mutants. Bars are Mean ± SEM. DBT = dibenzoyl thiamine.

3.4. The administration of dibenzoyl thiamine reduces the expression of neuroinflammatory markers in the CNS of FUS-tg mice

Two-way ANOVA revealed significant genotype effect in the IL-1β expression in the thoracic spine ($F=20.59$, $p < 0.0001$, two-way ANOVA), while post hoc test showed significant increase of this measure in non-treated FUS-tg mice compared to non-treated wild types ($p = 0.0059$, post hoc Tukey's test), as well as in thiamine-treated mutants compared to thiamine-treated controls ($p = 0.044$; Fig. 4A). Significant genotype and treatment effects were revealed in GSK-3β relative expression ($F=10.81$, $p = 0.0026$ and $F=7.86$, $p = 0.0018$, respectively). Non-treated mutants showed significant increase of this parameter in comparison with controls ($p = 0.0134$, post hoc Tukey's test), and in the spinal cord of FUS-tg mice treated with DBT GSK-3β expression was lower than both in non-treated mutants and in thiamine-treated mutants ($p = 0.0019$ and $p = 0.0284$, respectively; Fig. 4B). Significant genotype effect without significant group differences was demonstrated both in TNF and IL-6 expression ($F=4.39$, $p = 0.0448$ and $F=17.05$, $p = 0.0003$, respectively; Fig. 4C,D).

Next, significant genotype and treatment effects were revealed by two-way ANOVA in GSK-3β content in the prefrontal cortex measured by ELISA assay ($F=4.37$, $p = 0.0474$ and $F=4.46$, $p = 0.025$; two-way ANOVA, respectively). This measure was higher in non-treated FUS-tg mice than in non-treated wild types ($p = 0.0236$, post hoc Tukey's test), while mutant mice treated with DBT displayed decrease in this parameter compared to non-treated mutants ($p = 0.0492$; Fig. 4E). At the same time, no statistically significant group differences were found in GSK-3β concentration in the hippocampus ($p > 0.05$; Fig. 4F). Overall, we demonstrated significant increases in pro-inflammatory cytokine expression in the spinal cord and brain GSK-3β, as well as anti-inflammatory action of DBT.

3.5. Metabolome changes in FUS-transgenic mice ameliorated, in part, by dibenzoyl thiamine treatment

Both significant genotype and treatment effects were revealed by two-way ANOVA in high density lipoprotein (HDL) dominated methylene $(-CH_2-)_n$ NMR resonance ($F=4.57$, $p = 0.0399$ and $F=7.35$, $p = 0.0022$, respectively). Plasma HDL levels were significantly decreased in non-treated mutants in comparison with non-treated controls and thiamine-treated FUS-tg mice ($p = 0.0145$ and $p = 0.0385$, respectively; Fig. 5A). No significant difference in HDL concentration was observed between CD-1 DBT-treated and FUS-tg DBT-treated animals.

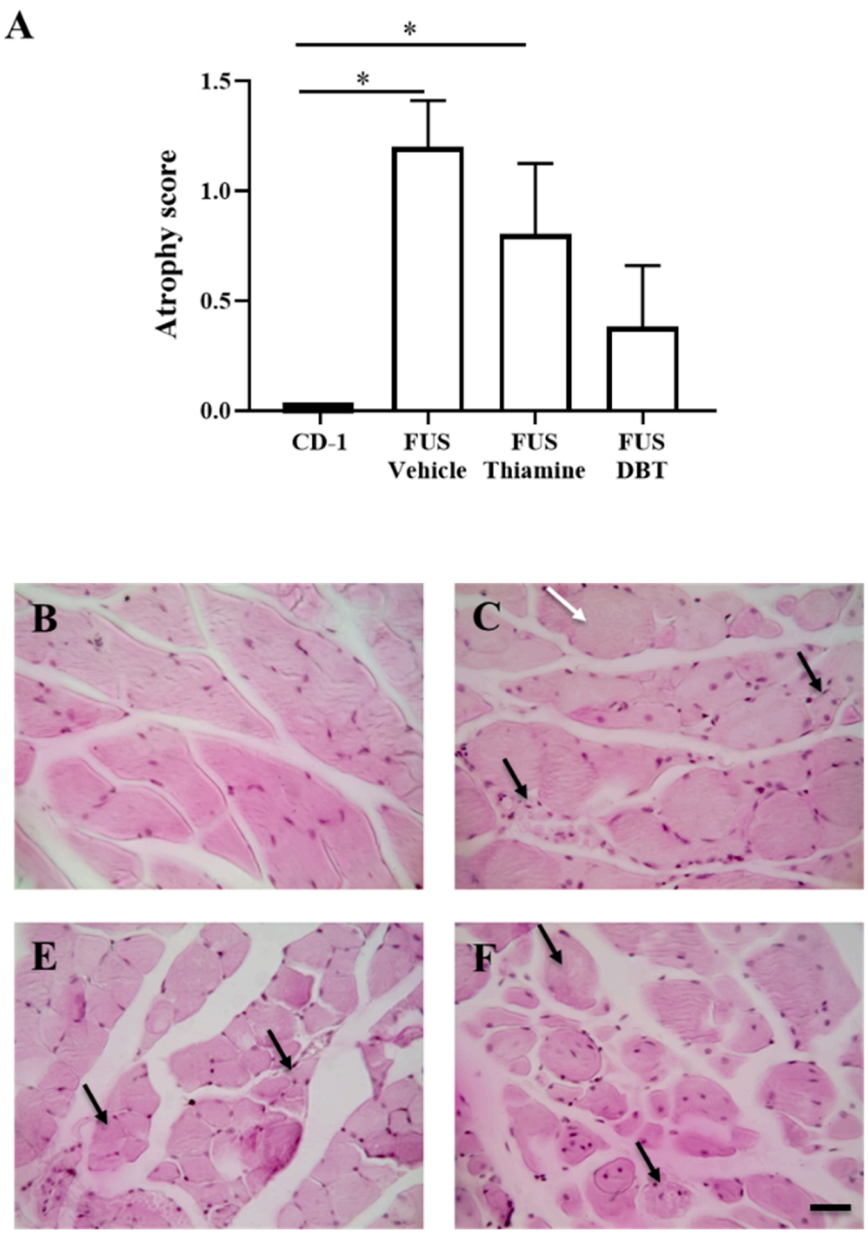

Fig. 3. Assessment of atrophy score in FUS-tg groups of mice. (A) Muscle atrophy score was significantly increased in non-treated and thiamine-treated mutants compared to controls. (B) Normal appearing muscle fibers (score 0); (C) A small number of atrophic fibers (black arrows) and a few more rounded hypertrophied fibers (white arrow) among normal (mild, score 1); (D) Small groups of angulated atrophic fibers (black arrows) (moderate, score 2); (E) Widespread whorled and necrotic muscle fibres (black arrows) among many angulated atrophic fibers (severe, score 3). Bar = 50 μm. $p < 0.05$, Kruskal-Wallis test, 6–8 animals per group. Bars are Mean ± SEM. DBT = dibenzoyl thiamine.

Next, a significant genotype x treatment interaction was shown for plasma lactate and free choline levels (F=4.85, $p = 0.014$ and F=4.54, $p = 0.0178$, respectively), which were both higher in non-treated mutants compared to controls ($p = 0.0224$; Fig. 5B, and $p = 0.0034$; Fig. 5C, respectively).

ANOVA showed significant treatment effect in the overlapping lysine/arginine resonance (two-way ANOVA, F=13.01, $p < 0.0001$) with a significant increase in DBT-treated controls than in thiamine-treated wild types ($p = 0.001$, post hoc Tukey's test; Fig. 5D). Significant treatment effect was also demonstrated in phenylalanine (F=14.48, $p < 0.0001$). The plasma phenylalanine concentration was significantly lower in thiamine-treated wild types in comparison with both non-treated and DBT-treated controls ($p = 0.0446$ and $p = 0.0003$, respectively; Fig. 5E).

Two-way ANOVA demonstrated significant treatment effect in mobile lipoprotein /=CH-CH2-CH2- (F=4.65, $p = 0.0164$; Suppl. Fig. 1A). While PCA revealed separation between untreated WT and FUS plasma metabolomes driven by mobile lipoprotein βCH2, lactate/-CH_2 mobile lipoprotein, NAC1/=CH-CH2-CH2-, unsaturated lipid, valine/proline, very low density lipoprotein (VLDL) and Chyl/VLDL NMR resonances, no significance was observed by two-way ANOVA likely due to lack of power ($p > 0.05$; Suppl. Fig. 1 B-H).

4. Discussion

Here, we found that the FUS mutants treated with DBT exhibited fewer signs of paralysis compared to the other groups. Unaltered motor behavior in the Pole test, body weight and diet intake were also rescued in the DBT-treated mutant mice. At the humane end point, DBT-treated mutants exhibited no change in water intake or rotarod behavior, in contrast to the non-treated and thiamine-treated FUS-tg animals. These physiological and behavioral data reveal that the DBT treatment delays progression of the ALS-like syndrome. In keeping with these findings, the mutant DBT-treated group exhibited a lower muscle atrophy score, and the expression of IL-1β, GSK-3β and TNF in the spinal cord was significantly elevated in the untreated or thiamine-treated mutants, but unaltered in the DBT-treated FUS-tg animals. There was also no change in the concentration of GSK-3β protein in the prefrontal cortex of the

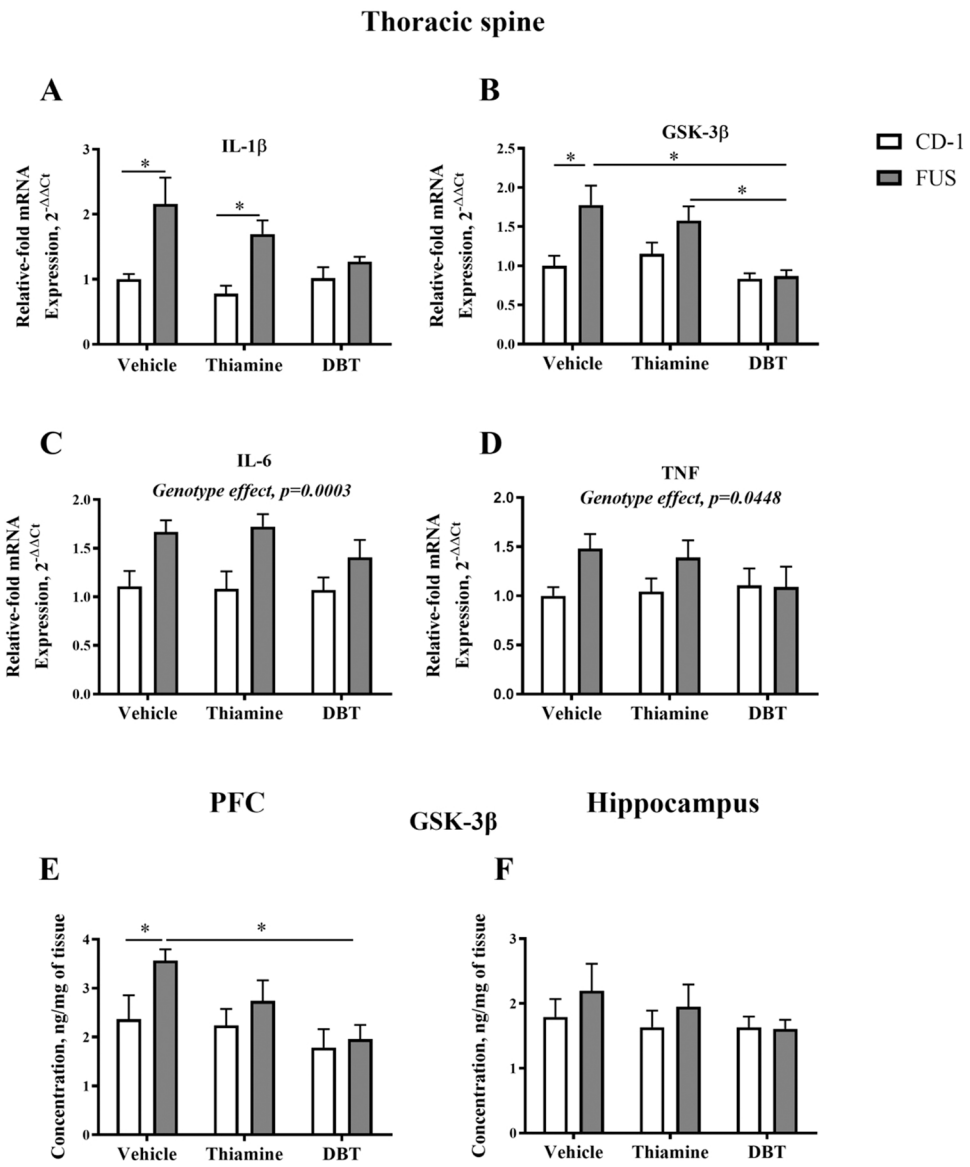

Fig. 4. The administration of DBT reduces molecular changes in the CNS of FUS-tg mice. (A) Non-treated and thiamine-treated FUS-tg mice displayed increased expression of IL-1β compared to respective controls. (B) GSK-3β expression in the spinal cord was elevated in comparison controls. It was significantly lower in DBT-treated mutants than in non-treated and thiamine-treated FUS-tg mice. Significant genotype effect was found in the (C) IL-6 and (D) TNF expression. (E) GSK-3β concentration in the prefrontal cortex was higher in non-treated mutants than in non-treated wild types and FUS-tg mice treated with DBT, while (F) this measure did not differ significantly between groups in the hippocampus. $p < 0.05$, two-way ANOVA and post hoc Tukey's test, 6–7 animals per group. Bars are Mean ± SEM. DBT = dibenzoyl thiamine.

DBT-treated animals compared to the changes observed in the untreated mutants versus the CD1 controls. Thus, animals administered with long-term DBT therapy exhibited no significant change in markers of neuroinflammation and cellular stress, which are known to be associated with ALS pathogenesis [12,13,61]. Proinflammatory cytokines and chemokines, in particular, have been shown to be increased in serum samples of ALS patients as well as in other ALS mouse models [26,65]. IL-1β, in particular, has been shown to play an important role in ALS. For example, caspase–1 or IL-1β deficiencies, as well as blocking the IL-1-receptor have been shown to prolong the survival in animal models [65]. Here, IL-1β was significantly elevated in the spinal cord of the vehicle- and thiamine-treated FUS mutants, but not in the DBT-treated animals suggesting that the effect of DBT is upstream of IL-1β induction and, thus, might be expected to have a greater impact on outcome than downstream anti-IL-1β therapies, which are expensive, high molecular weight, and raise brain bioavailability concerns.

Neuroinflammation is suggested to be associated with motor dysfunction in ALS. Analysis of cerebrospinal fluid and postmortem spinal cord samples from ALS cases has revealed an increase in microglial activation and lymphocyte recruitment [50], and overproduction of inflammatory cytokines has been demonstrated to occur in association with neuronal loss [29]. In particular, elevated levels of inflammatory mediators were observed in motor regions of the CNS [2]. Our previous results have shown that the ameliorative effects of therapeutic interventions on the motor scores in the FUS[1−359]-tg mice are accompanied by normalization of IL-1β and Iba-1 expression in the spinal cord [12] and in the brain [13]. Previously we established that there is an elevated malondialdehyde content in the spinal cord of FUS [1−359]-tg mice [53], which indicates that there is as increase in oxidative stress that may be functionally related to neuroinflammation ([60]; He et al., 2021).

The present study has also revealed significant changes in the blood metabolome in the FUS-tg mice, which was, largely, normalized by DBT administration. Compared to other neurodegenerative diseases, there are relatively few studies that have explored the ALS metabolome and the impact of therapy on the ALS metabolome [31]. Here, HDL, free choline and lactate concentrations were altered in the plasma of the FUS-tg mice; changes in the levels of these metabolites have been previously reported to be associated with ALS and the presence of other neurodegenerative conditions in humans. In ALS, a recent study has shown that, controlling for age and sex, higher levels of HDL in blood in presymptomatic individuals are associated with a reduced risk for ALS [64]. Decreased HDL content has also been described in patients with Alzheimer's disease and Huntington's disease [1]. Notably,

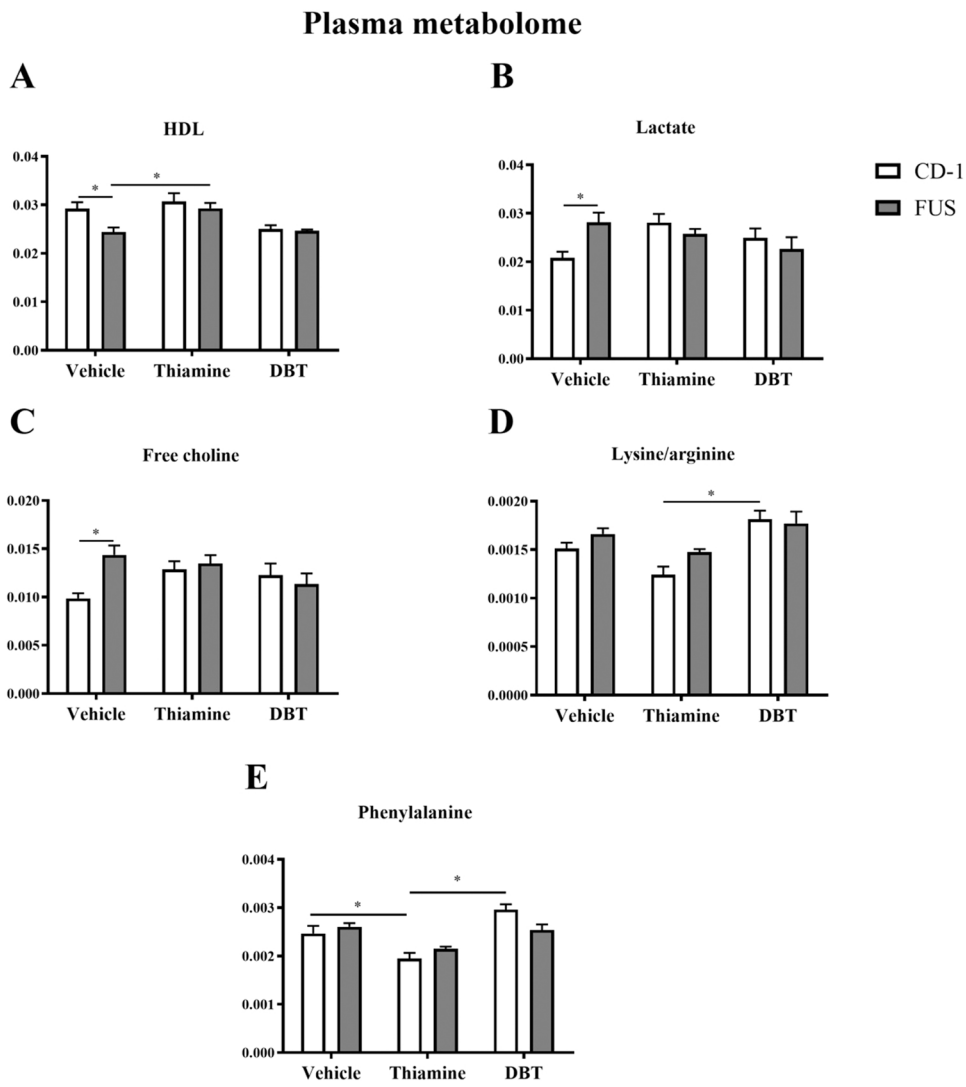

Fig. 5. Metabolome changes in FUS-tg mice and effects of the treatment with DBT. (A) Non-treated mutants had decreased HDL in comparison with non-treated controls and thiamine-treated FUS-tg mice. (B) Lactate was higher in non-treated mutants compared to controls, and (C) free choline was elevated in non-treated FUS-tg mice compared to non-treated wild types. (D) Lysine/arginine ratio was significantly higher in DBT-treated controls than in thiamine-treated wild types. (E) Phenylalanine was significantly lower in thiamine-treated wild types in comparison with both non-treated and DBT-treated controls. $p < 0.05$, two-way ANOVA and post hoc Tukey's test, 5–6 animals per group. Bars are Mean ± SEM. DBT = dibenzoyl thiamine.

dyslipidemia is considered an important risk factor for dementia [52], and protective effect of HDL on brain pathology and dementia have been suggested [1,73], as lower levels of HDL correlated with a higher risk of neuronal degeneration and oxidative stress [22]. In the cerebrospinal fluid, changes in HDL levels were found to alter intracellular homeostasis of cholesterol that in turn can regulate the metabolism of amyloid-precursor-protein which promotes amyloid beta fibrils formation, the main component of amyloid plaques [15]. Given that a soluble proteolytic fragment of the amyloid-β precursor protein is also implicated in the pathophysiology of neuromuscular junction in the ALS syndrome [43], normalization of HDL could lead to beneficial effects via this mechanism in ALS patients. Here, thiamine compounds are likely to have increased HDL levels by normalizing oxidative stress, which is suggested by our previous data that showed a counteracting effect of chronic DBT treatment on the increase of malondialdehyde in the spinal cord of FUS-tg mice [53].

Jia et al. found that lactate was increased in the serum of serum samples obtained from ALS patients compared to healthy controls [24,27]. The increase in lactate concentration that we observed in the model here and that in individuals with ALS seems somewhat counterintuitive given that lactate is administered to treat traumatic brain injury as a neuroprotective agent [8]. However, we may speculate that the increase in lactate in blood may reflect a response to an unmet energy demand by neurons or a failure to utilize lactate in the ALS brain owing to a dysfunctional relationship between astrocytes and neurons in ALS. The CNS lactate transporter, monocarboxylate transporter is argued to have lowered functionality in ALS patients and in a mouse model of ALS [69]. This dysfunction was shown to result in axon damage and neuron loss [37]. Reduced glucose content and general suppression of glucose metabolism has been reported in patients with Alzheimer's, Parkinson's and Huntington's disease resulting in the elevated lactate levels [19], and patients with Alzheimer's disease have been shown to exhibit increased lactate levels in the cerebrospinal fluid [16,37].

Choline is elevated in the blood of ALS patients compared to the levels in the blood of a spouses, but not compared to healthy controls [9]. However, the changes reported were not marked. An explanation for the presence of increased free choline in blood in the mutant mice is not clear, but it may reflect accumulation owning to a failure of the brain or muscle choline utilization. Choline deficiency is known to cause muscle wasting, and the degeneration of muscle observed in the model in might result in higher circulation levels as demand falls. Increased choline levels have also been reported in patients with Alzheimer's disease [14]. A better understanding of the causes of these metabolite changes, and whether the changes are upstream or downstream of the pathology, is likely lead to new avenues for therapeutic intervention. In this study, for example, FUS animals administered DBT did not show any change in lactate or choline, and thus measurement of these metabolites may be useful as a biomarker in other studies.

To conclude, current study explored ameliorative effects of long-term administration of DBT and thiamine on key molecular and

histological hallmarks of the ALS-like syndrome in FUS[1−359]-tg mice. Given that any effective treatment of this fatal disease is currently lacking, it is hoped that DBT administration, in particular, might warrant further investigation in the clinical as a promising therapy for ALS. In addition, the present study has identified important metabolic changes in the mutant that were normalized by the DBT therapy. The changes we observed largely overlap with those reported for ALS and other neurodegenerative disorders, including Alzheimer's disease. The metabolites that exhibited the most significant changes in the mutant animals may, therefore, prove to be useful biomarkers in future clinical and pre-clinical studies.

CRediT authorship contribution statement

DA, TS, AD and LB conceived the study. FP, AD, TS, DA and TS designed the experiments. FP, AG, EV, KC, TI and AN carried out the experiments, data analysis and performed the literature study. FP, AG, MS, DA performed the graph preparation and statistical analyses. DA, LB, TS supervised the project. DA, TS, AD got the funding. DA, AG, and TS wrote the initial draft of the manuscript and all other authors listed here revised it.

Conflict of interest statement

Here, we declare that none of the authors involved in the work have any competing interest. We confirm that founding bodies had no role in the data collection, manuscript preparation and decision to publish the results.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113986.

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