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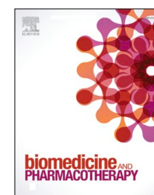

Decreased long-chain acylcarnitine content increases mitochondrial coupling efficiency and prevents ischemia-induced brain damage in rats

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ABSTRACT

Long-chain acylcarnitines (LCACs) are intermediates of fatty acid oxidation and are known to exert detrimental effects on mitochondria. This study aimed to test whether lowering LCAC levels with the anti-ischemia compound 4-[ethyl(dimethyl)ammonio]butanoate (methyl-GBB) protects brain mitochondrial function and improves neurological outcomes after transient middle cerebral artery occlusion (MCAO). The effects of 14 days of pretreatment with methyl-GBB (5 mg/kg, p.o.) on brain acylcarnitine (short-, long- and medium-chain) concentrations and brain mitochondrial function were evaluated in Wistar rats. Additionally, the mitochondrial respiration and reactive oxygen species (ROS) production rates were determined using *ex vivo* high-resolution fluorespirometry under normal conditions, in models of ischemia-reperfusion injury (reverse electron transfer and anoxia-reoxygenation) and 24 h after MCAO. MCAO model rats underwent vibrissae-evoked forelimb-placing and limb-placing tests to assess neurological function. The infarct volume was measured on day 7 after MCAO using 2,3,5-triphenyltetrazolium chloride (TTC) staining. Treatment with methyl-GBB significantly reduced the LCAC content in brain tissue, which decreased the ROS production rate without affecting the respiration rate, indicating an increase in mitochondrial coupling. Furthermore, methyl-GBB treatment protected brain mitochondria against anoxia–reoxygenation injury. In addition, treatment with methyl-GBB significantly reduced the infarct size and improved neurological outcomes after MCAO. Increased mitochondrial coupling efficiency may be the basis for the neuroprotective effects of methyl-GBB. This study provides evidence that maintaining brain energy metabolism by lowering the levels of LCACs protects against ischemia-induced brain damage in experimental stroke models.

1. Introduction

Stroke results in brain tissue damage that leads to physical, emotional, and cognitive symptoms with a slow recovery process [1,2]. Ischemic stroke is caused by a reduced blood supply to the affected brain region, which deprives it completely or partially of oxygen and nutrients. Ischemic stroke accounts for 80 % of all strokes [3], and 23 % of ischemic stroke patients will have a recurrent stroke, which is more severe and less responsive to treatment than the initial event [4]. New therapies are needed both to prevent the occurrence of stroke and to treat the resulting symptoms.

One of the emerging directions in stroke pharmacotherapy involves targeting energy metabolism pathways in the brain, especially mitochondria-related pathways [5]. Under pathological conditions, including stroke and ischemia-eperfusion-induced injury, mitochondria act as a source of reactive oxygen species (ROS) [6]. Under conditions of limited oxygen supply, detrimental accumulation of energy metabolism substrates disturbs mitochondrial energy pathways and drives oxidative stress reactions [7]. Long-chain acylcarnitines (LCACs) are harmful fatty acid intermediates that inhibit oxidative phosphorylation (OXPHOS) and stimulate ROS production [8–10]. In the brain, LCAC concentrations are relatively high [11], reaching levels that are comparable to those in muscles [12]. However, the physiological and pathological roles of LCACs in the central nervous system remain substantially less studied than their roles in muscles. Recent studies have shown that the brain content of LCACs in aged male mice is significantly elevated after stroke

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[13,14]. Thus, mitochondria-targeted therapies that decrease LCAC accumulation could have the potential to modulate and counteract stroke-induced ROS production.

We previously showed that the anti-ischemia compound meldonium (mildronate), a regulator of energy metabolism pathways [15], accelerated the recovery of sensorimotor and proprioceptive functions in rats, but it failed to decrease the infarcted tissue area in an experimental model of transient middle cerebral artery occlusion (MCAO) [16]. Meldonium is a relatively weak inhibitor of the L-carnitine synthesizing enzyme γ-butyrobetaine (GBB) hydroxylase and of organic cation/carnitine transporter 2, and it is used clinically for both cardiometabolic and neurological conditions [17]. The effect of meldonium against anoxia–reoxygenation injury to brain mitochondria has been attributed to lowered L-carnitine concentrations and preconditioning-like effects on mitochondria [18]. We have developed a pharmacological modulator, 4-[ethyl(dimethyl)ammonio]butanoate (methyl-GBB, Fig. 1), which decreases acylcarnitine concentrations much more effectively than meldonium and exerts a cardioprotective effect after ischemia—reperfusion-induced damage to the heart at a dose of 5 mg/kg [8,19,20]. The purpose of this study was to test the potential protective effect of lowering LCAC levels on mitochondrial function in a model of anoxia-reoxygenation-induced brain mitochondrial damage. Furthermore, the effects of methyl-GBB treatment on motor, sensory, and tactile functions as well as the infarct size were tested in an experimental rat model of stroke.

2. Results

2.1. Treatment with methyl-GBB decreases acylcarnitine and L-carnitine content in brain tissue

Fourteen days of oral (p.o.) treatment with methyl-GBB at a dose of 5 mg/kg significantly decreased L-carnitine, short-chain acylcarnitine (SCAC), medium-chain acylcarnitine (MCAC) and LCAC levels in brain tissues by 69 %, 68 %, 66 % and 38 %, respectively ($p < 0.05$ vs. the control group, Fig. 2A, B, C, D). The methyl-GBB concentration in brain tissues was 14.8 ± 0.7 nmol/g 24 h after the last p.o. dose. Thus, methyl-GBB crosses the blood-brain barrier and significantly decreases acylcarnitine content in brain tissue.

2.2. Lowering acylcarnitine content reduces ROS production without affecting OXPHOS capacity

To determine whether methyl-GBB treatment affects mitochondrial function, mitochondrial respiration, and ROS production were measured in rat brain homogenates after 14 days of methyl-GBB treatment at a dose of 5 mg/kg. As shown in Fig. 3, methyl-GBB treatment did not induce any changes in the mitochondrial respiration rate (Fig. 3A), while H_2O_2 production (Fig. 3B) in NADH (N) pathway-, NADH/succinate (NS) pathway- and succinate (S) pathway-linked OXPHOS was significantly decreased by 40 % in the methyl-GBB-treated group ($p < 0.05$ vs. the control group). These results show that treatment with methyl-GBB reduces ROS production without affecting OXPHOS capacity, indicating that it increases mitochondrial electron transport chain coupling.

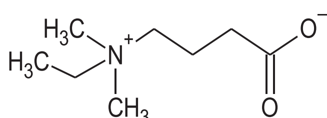

Fig. 1. Structure of methyl-GBB.

2.3. Lowering acylcarnitine content results in decreased ROS production during reverse electron transfer (RET) and after anoxia–reoxygenation

To determine whether the methyl-GBB-induced decrease in ROS production under normoxic conditions can maintain mitochondrial function following ischemia-reperfusion damage to the brain, ROS production and the mitochondrial respiration rate were assessed during RET (reperfusion injury) and after anoxia and reoxygenation. During RET, methyl-GBB treatment induced a significant 12 % decrease in ROS production ($p < 0.05$ vs. the control group, Fig. 4A) without affecting the respiration rate (Fig. 4B) or H_2O_2/O ratio (Fig. 4C). Moreover, after anoxia–reoxygenation, the H_2O_2 production rate ($p < 0.05$ vs. the control group, Fig. 4D) and H_2O_2/O ratio ($p < 0.05$ vs. the control group, Fig. 4F) were significantly reduced by 58 % and 65 % in the presence of N-pathway substrates and by 29 % and 25 % in the presence of S-pathway substrates, respectively, in the methyl-GBB-treated group. After anoxia and reoxygenation, the respiration rate in the presence of N-pathway substrates tended to be higher in the methyl-GBB group (Fig. 4E). Taken together, these results indicate that treatment with methyl-GBB protects brain mitochondria against ischemia-reperfusion damage by reducing ROS production.

2.4. Lowering acylcarnitine content protects against stroke-induced tissue damage

To further investigate whether the decrease in LCAC content and preservation of mitochondrial function could protect against stroke, the effects of methyl-GBB pretreatment were studied in a rat model of transient MCAO. Brain damage was localized to the cortex and the striatum on day 7 after transient MCAO for 120 min (Fig. 5B). The infarct region accounted for 36 % of the ipsilateral (damaged) hemisphere in the control group ($p < 0.05$ vs. the sham group, Fig. 5A), while in the methyl-GBB treatment group, the infarct region accounted for only 20 % of the ipsilateral hemisphere (Fig. 5A). There was a significant difference in the infarct size between the treatment and control MCAO groups ($p < 0.05$ vs. the control group, Fig. 5A), indicating that decreasing LCAC content protects brain tissue from ischemic damage.

2.5. Lowering long-chain acylcarnitine levels results in improved neurological function after MCAO

To assess whether decreased LCAC content is beneficial for neurological outcomes after MCAO, behavior, and body weight were measured. In the limb-placing test, animals in the control MCAO group exhibited statistically significant impairment of paw responses to tactile and proprioceptive stimuli on days 1, 3, and 7 ($p < 0.05$ vs. the sham group, Fig. 6A). As shown in Fig. 6A, methyl-GBB pretreatment at a dose of 5 mg/kg for 14 days before MCAO significantly diminished MCAO-induced tactile and proprioceptive impairment by 38 % ($p < 0.05$ vs. the control group).

In the vibrissae-evoked forelimb-placing test, a significant decrease in sensory and tactile function in control group animals was observed on days 1, 3, and 7 after MCAO ($p < 0.05$ vs. the sham group, Fig. 6B). As shown in Fig. 6B, methyl-GBB treatment 14 days before MCAO significantly improved sensorimotor function on day 1 after MCAO ($p < 0.05$). Additionally, the motor response scores of methyl-GBB-treated rats were more than 7 times higher than those of saline-treated rats (46 % vs. 6 %, respectively) on day 1 after stroke.

The body weight of the rats in the control MCAO group was significantly decreased compared with that of the rats in the sham-operated group ($p < 0.05$ vs. the sham group, Fig. 6C). There were no significant differences in body weight changes between the control and methyl-GBB-treated groups.

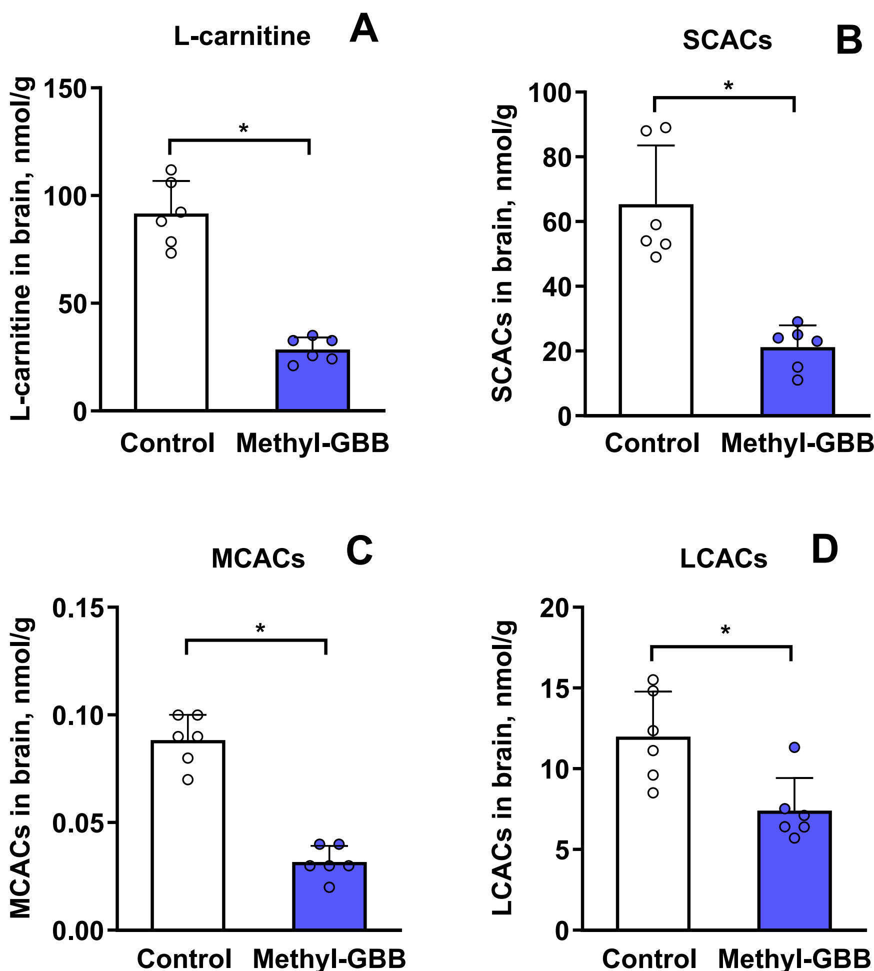

Fig. 2. Methyl-GBB treatment decreased the content of L-carnitine and acylcarnitines in brain tissues. (A) L-carnitine, (B) short-chain acylcarnitine (C2–C5, SCAC), (C) medium-chain acylcarnitine (C6–C12, MCAC), and (D) long-chain acylcarnitine (C13–C20, LCAC) content in brain tissue after 14 days of p.o. methyl-GBB treatment at a dose of 5 mg/kg. The results are presented as the mean ± SD of 6 animals. The differences between the methyl-GBB-treated group and the control group were analyzed using Student's t-test; differences were considered significant at $p < 0.05$.

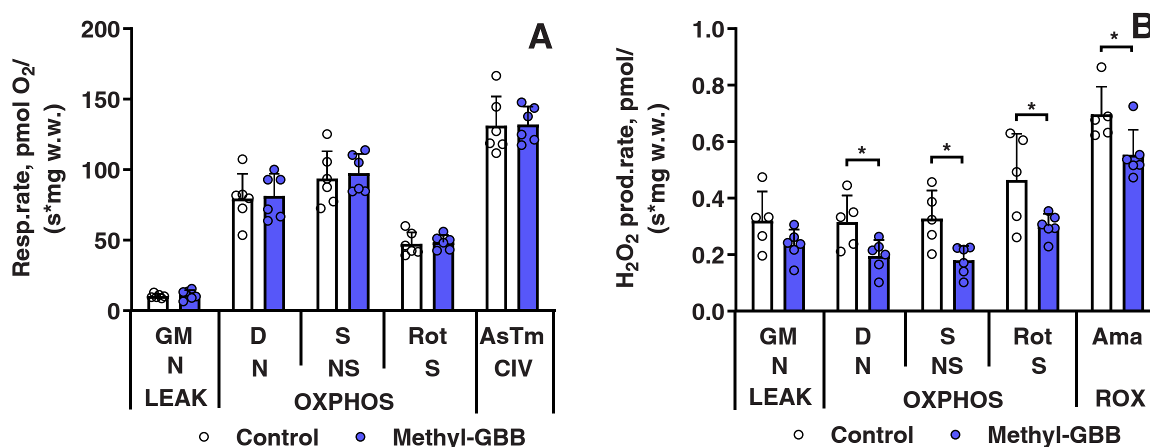

Fig. 3. The effects of methyl-GBB treatment on mitochondrial function in brain homogenates. Methyl-GBB treatment did not affect (A) the mitochondrial respiration rate (B) but significantly decreased the H_2O_2 production rate. The results are presented as the mean ± SD of 5–6 animals. The differences between the methyl-GBB-treated group and the control group were analyzed using Student's t-test; differences were considered significant at $p < 0.05$. Abbreviations: GM, glutamate and malate; D, ADP; S, succinate; Rot, rotenone; AsTm, ascorbate and TMPD; Ama, antimycin A; N, NADH pathway; S, succinate pathway; CIV, Complex IV; LEAK, substrate metabolism–dependent state; OXPHOS, (respiratory state dependent on) oxidative phosphorylation; ROX, residual oxygen consumption.

Fig. 4. The effects of methyl-GBB treatment on mitochondrial function in models of ischemia-reperfusion damage. Methyl-GBB treatment significantly decreased (A) the H_2O_2 production rate without affecting (B) respiration or (C) the H_2O_2/O ratio during RET. After anoxia and reoxygenation, the (D) H_2O_2 production rate and (F) H_2O_2/O ratio were significantly decreased in the methyl-GBB-treated group in the presence of both N-pathway and S-pathway substrates, (E) while the mitochondrial respiration rate was slightly increased in the presence of N-pathway substrates. The results are presented as the mean ± SD of 5 animals. The differences between the methyl-GBB-treated group and the control group were analyzed using Student's t-test; differences were considered significant at $p < 0.05$. Abbreviations: GM, glutamate, and malate; SRot, succinate, and rotenone; N, NADH pathway; S, succinate pathway.

Fig. 5. (A) The effect of methyl-GBB on the infarct size after MCAO. (B) Representative TTC-stained sections of the brain. Methyl-GBB was administered p.o. at a dose of 5 mg/kg for 14 days before MCAO (n = 10). The control MCAO (n = 8) and sham-operated (n = 14) rats received water. The infarct size was evaluated on day 7 after MCAO and was measured as the difference in the volume of undamaged tissue between the damaged and undamaged hemispheres. The values are presented as the mean ± SD. The data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test; differences were considered significant at $p < 0.05$ ($^*p < 0.05$ vs. the control group, $^{\#}p < 0.05$ between the control MCAO and sham-operated groups).

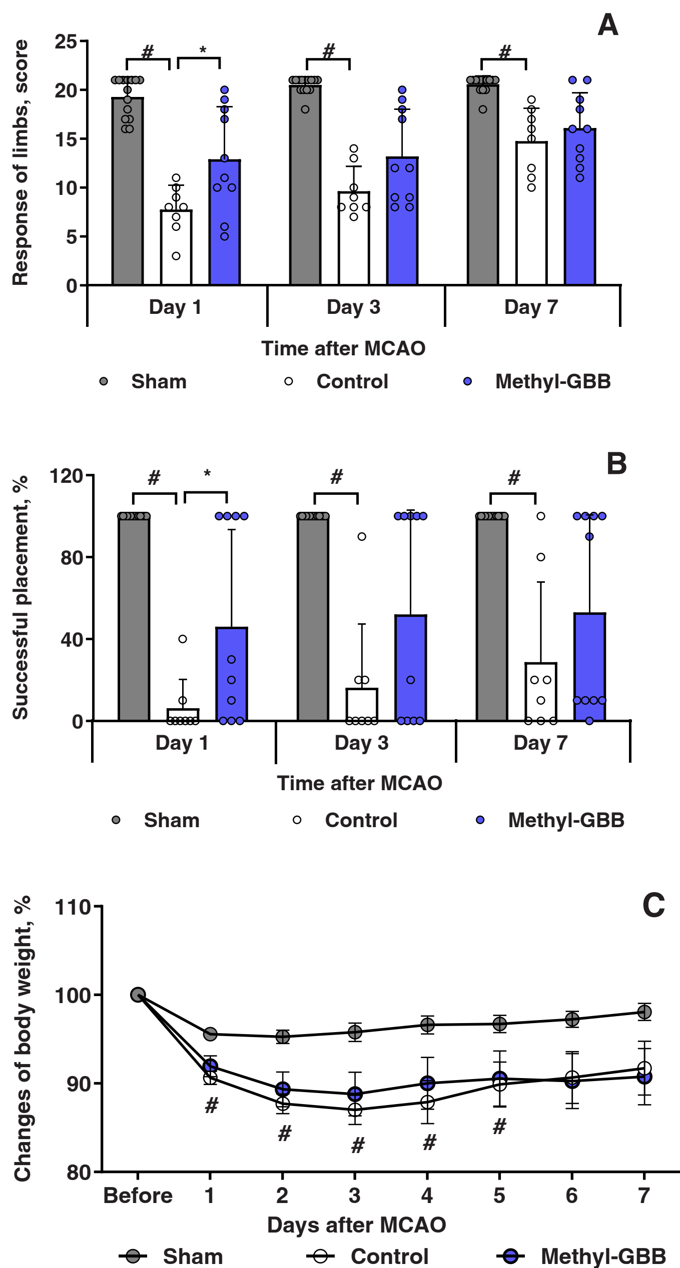

Fig. 6. Effects of methyl-GBB treatment on (A) limb-placing and (B) vibrissae-evoked forelimb-placing in rats after MCAO. Methyl-GBB was administered p.o. at a dose of 5 mg/kg 14 days before MCAO (n = 10). The control (n = 8) and sham-operated (n = 14) rats received water. The tests were carried out on days 1, 3 and 7 after MCAO. (C) Body weight changes in the experimental groups following MCAO. The values are presented as the mean ± SD. The data were analyzed using two-way repeated-measures ANOVA followed by Tukey's multiple comparisons test; differences were considered significant at $p < 0.05$ (*$p < 0.05$ vs the control group, $^{\#}p < 0.05$ between the control MCAO and sham-operated groups).

2.6. Treatment with methyl-GBB decreases acylcarnitine content in brain and mitochondrial ROS production 24 h after MCAO

After 14 days of methyl-GBB treatment, brain levels of L-carnitine, SCACs, MCACs, and LCACs decreased significantly by 79 %, 83 %, 68 %, and 57 %, respectively, compared to the untreated group at 24 h post-MCAO ($p < 0.05$). We found that the brain content of C:18 was notably elevated in the affected hemisphere, in comparison to the control group (non-affected hemisphere, Fig. 7A, #$p < 0.05$ vs. control

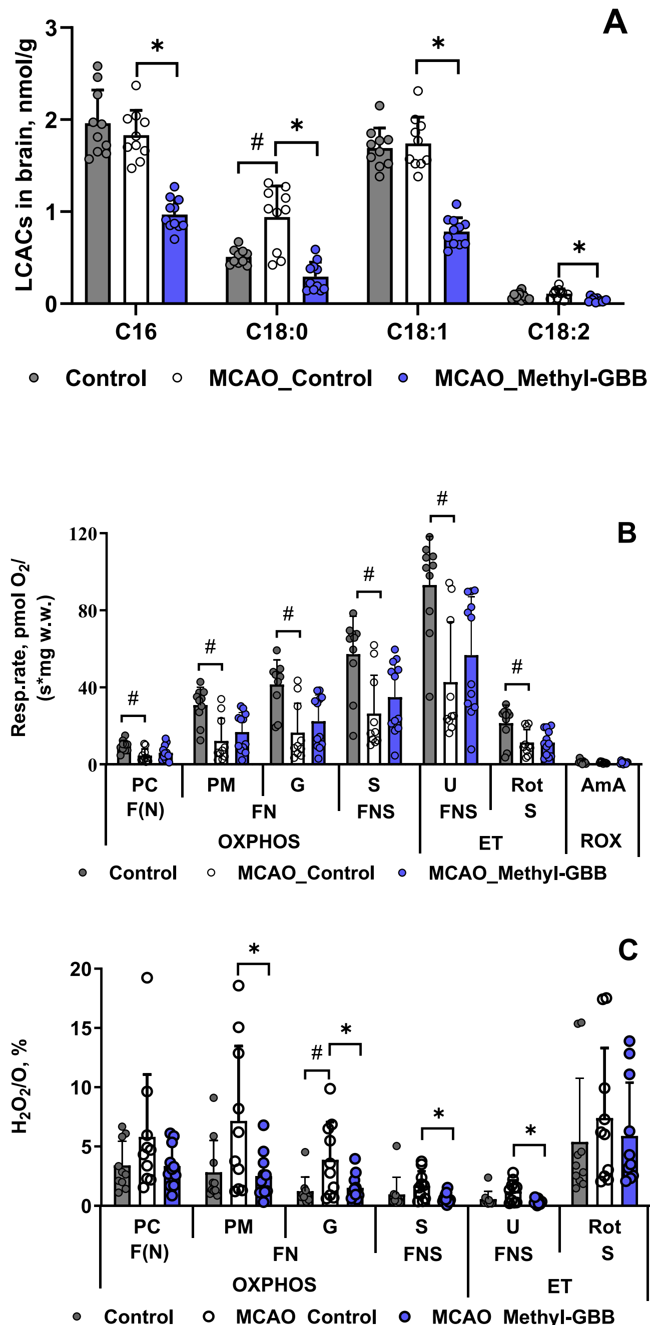

Fig. 7. Effects of methyl-GBB treatment on LCAC content in brain tissue and mitochondrial function in rats 24 h after MCAO. Methyl-GBB treatment reduced LCAC content in the brain (A) and did not affect the mitochondrial respiration rate (B) but significantly decreased the H_2O_2/O ratio (C). Methyl-GBB was administered p.o. at a dose of 5 mg/kg 14 days before MCAO (n = 12). The control rats (n = 10) received water. Control (grey bar) is the intact hemisphere in the control group, MCAO-control (white bar) is the affected hemisphere in the control group, and MCAO methyl-GBB (blue bar) is the affected hemisphere in the treatment group. The values are presented as the mean ± SD. The differences between groups were analyzed using Student's t-test; differences were considered significant at $p < 0.05$ (*$p < 0.05$ between the MCAO-control and MCAO-Methyl-GBB groups, $^{\#}p < 0.05$ between the intact control and MCAO-control groups). Abbreviations: PC, palmitoylcarnitine; PM, pyruvate and malate; G, glutamate; S, succinate; U, uncoupler (carbonyl cyanide m-chlorophenyl hydrazone); Rot, rotenone; AmA, antimycin A; F, fatty acid oxidation pathway; N, NADH pathway; S, succinate pathway; OXPHOS, (respiratory state dependent on) oxidative phosphorylation; ET, electron transfer; ROX, residual oxygen consumption.

group). The brain content of C:18 was significantly decreased by 69 % in the methyl-GBB treated group compared to the MCAO-control group (Fig. 7A, *$p < 0.05$).

To further assess whether lowering of acylcarnitine content by methyl-GBB would affect the mitochondrial respiration rate and ROS production, brain homogenates were subjected to high-resolution fluorespirometry analysis 24 h after MCAO. As expected, in the MCAO-control group we observed a significantly decreased ($p < 0.05$ vs. the control group) respiration rate in the fatty acid oxidation (FAO) (F)-pathway, as well as in the N- and NS-pathways in the OXPHOS state (Fig. 7B). In the MCAO-control group, we also observed a 50 % lower overall mitochondrial electron transfer (ET) system capacity, as indicated by the respiration rate after titration of the uncoupler (U) (Fig. 7B). Moreover, in the MCAO-control group H_2O_2/O ratio was higher than in the control group with FN- and FNS- pathway substrates, indicating that more oxygen is wasted for ROS production (Fig. 7C). Methyl-GBB treatment did not affect the respiration rate of brain mitochondria after MCAO (Fig. 7B), however, the H_2O_2/O ratio was normalized and close to that observed in the control group (Fig. 7C). These results indicate that treatment with methyl-GBB and reduction of LCACs results in lowered ROS production in the brain 24 h after stroke.

3. Discussion

For the first time, we showed that treatment with methyl-GBB, an inhibitor of GBB hydroxylase and organic cation/carnitine transporter 2 [19], reduces acylcarnitine content in the brain, resulting in increased mitochondrial electron transfer system coupling and reduced ROS production in a model of anoxia-reoxygenation-induced brain mitochondrial damage. Thus, lowering the concentration of LCACs attenuates brain damage and behavioral dysfunction caused by MCAO.

The detrimental effects of increased levels of LCAC in peripheral tissues, particularly the heart, are well supported by evidence [9,21,22]. The accumulation of LCACs after infarction has a strong effect on OXPHOS that results in increased ROS production in mitochondria [8, 10]. Reduction of LCAC levels by genetic means in an N6-trimethyllysine dioxygenase enzyme knockout mouse model or by treatment with methyl-GBB in rats prevents mitochondrial damage during myocardial infarction [19,23]. However, the physiological and pathological roles of LCACs in the central nervous system remain substantially less studied.

LCAC levels in the brain are higher than those in other tissues. In our study, the measured concentration of LCACs in the rat brain was 12 nmol/g, which is comparable to or exceeds the levels in the muscle, liver, and kidneys [12]. In line with the notion that the brain might be less protected against lipotoxicity than other tissues [24], elevated levels of LCAC might be even more detrimental to brain cells than to cardiomyocytes. In the brain, LCAC levels are more stable and less affected by physiological and pharmacological stimuli. In tissues with high fatty acid metabolism and turnover, such as the heart, muscle, and liver, fatty acid and acylcarnitine content during the fed-fasted cycle can vary up to 5-fold [12]; however, in the cortex of the brain, the difference in LCAC content between the fasted and fed states is only approximately 20 % [11]. Additionally, the decrease in acylcarnitine content in response to long-term pharmacological treatment with methyl-GBB and meldonium is much less pronounced in the brain than in other tissues. In a previous study, methyl-GBB administration at 5 mg/kg for 14 days decreased plasma and heart tissue LCAC concentrations by 4- to 15-fold [19], while in this study, the same treatment regimen reduced brain LCAC concentrations by only approximately 40 %. Nevertheless, it was sufficient to prevent ischemia-induced brain damage. Recent studies have revealed a 2-fold increase in LCAC content in the peri-infarct region of the mouse brain [13,14]; thus, LCACs might be effective targets for the amelioration of neurodegeneration after stroke. This is in line with the results of the present study, which demonstrates that the content of the LCAC C:18 was increased in the lesioned hemisphere compared to the intact hemisphere 24 h after MCAO, thus providing evidence that lowering LCAC levels by methyl-GBB treatment is an effective approach to prevent stroke-induced brain damage.

Neurons possess poor antioxidative defense mechanisms and are thus particularly susceptible to ROS, including those generated by mitochondrial processes affected by lipotoxic metabolites [24]. Mitochondria play a central role in cell energy homeostasis, regulate calcium turnover, and are the main source of ROS in cells [25]. During ischemia, deprivation of oxygen and substrates for energy metabolism leads to disrupted ATP synthesis and overproduction of ROS by mitochondria, leading to neuronal death following ischemic stroke [26]. In part, ROS production is induced by metabolic intermediates such as LCACs if they accumulate in ischemic mitochondria. In addition, an increase in coupling efficiency and a decrease in ROS production in heart muscles are associated with cardioprotection in patients with heart failure [27]. Therefore, the prevention of metabolic intermediate accumulation is suggested as a therapeutic approach to prevent excessive ROS production specifically during ischemia-reperfusion [7]. We showed that methyl-GBB, by decreasing LCAC content, ameliorated ischemia—reperfusion-induced mitochondrial dysfunction in the brain by reducing ROS production. This is in line with previous studies showing that in cardiac tissue, treatment with methyl-GBB prevents the ischemia—reperfusion-induced increase in mitochondrial ROS production under conditions of limited OXPHOS capacity [28]. The prevention of oxidative stress in ischemic hearts by methyl-GBB treatment was associated with the prevention of LCAC accumulation during ischemia [28]. It is known that the accumulation of LCACs in ischemic cardiac mitochondria induces membrane hyperpolarization and promotes ROS production due to inhibited OXPHOS-dependent mitochondrial respiration in the presence of NS- and S-pathway substrates [8,10]. Additionally, other changes induced by elevated concentrations of LCACs, including inhibition of sodium-potassium exchange [29,30], pyruvate and lactate oxidation [31], and insulin signaling [32–34], as well as facilitation of Ca^{2+} release from the sarcoplasmic reticulum [29,35,36], might contribute to the detrimental effects of LCAC in stroke-affected tissues. The results of this study provide evidence for the involvement of LCAC in detrimental stroke-induced processes in the brain and identify mitochondrial energy pathways as drug targets for stroke treatment.

Mitoprotective compounds that ameliorate oxidative stress have been tested in experimental models of stroke with some success. In a transient MCAO mouse model, a mitochondrion-targeted peptide, elamipretide, was found to reduce both the infarct size (by 50 % compared to the vehicle-treated group) and neurological deficits. The same study confirmed that elamipretide exerted an anti-free-radical effect in vitro, protecting cell survival and reducing ROS production in an H_2O_2-induced cell injury model [37]. Additionally, a mitochondrion-targeting ROS scavenger, mitoquinone (MitoQ), was shown to inhibit neuronal death related to oxidative stress in rats [38]. Numerous studies have shown that the natural polyphenolic antioxidant resveratrol exerts neuroprotection in ischemic stroke through different mechanisms, including attenuating oxidative stress and increasing antioxidant capacity and mitochondrial respiration efficiency [39]. In line with other studies that used elamipretide and mitoquinone, the anti-stroke efficacy of methyl-GBB observed in this study confirms that protecting mitochondria is a promising strategy to prevent stroke.

It is expected that neuroprotective agents can exert multimodal effects against ischemic stroke and promote cellular and metabolic health [40]. In addition, our study provides evidence that a reduction in energy metabolism intermediate accumulation can prevent stroke-induced mitochondrial damage. This can be attributed to various other metabolic intermediates that accumulate during brain ischemia [7]. For example, preventing succinate accumulation by malonate can decrease brain injury in ischemic stroke [41]. Importantly, in this study, the methyl-GBB-mediated decrease in LCAC levels at least partially prevented ROS formation during succinate-induced RET, indicating that an increase in LCAC content might facilitate succinate accumulation during brain ischemia. The mechanism of action of methyl-GBB in the brain is

different from that of other mitochondrion-targeted treatments. This diversity of mechanisms could be useful, allowing the combination of multiple treatments to more effectively prevent stroke-induced injury.

Methyl-GBB significantly decreases L-carnitine and LCAC content in brain tissue, thereby increasing the efficiency of mitochondrial coupling by preventing an increase in mitochondrial ROS production in the brain during ischemia-reperfusion. Moreover, the present study reveals that pretreatment with methyl-GBB protects brain tissue from infarction and neurological deficits induced by MCAO. Therefore, lowering LCAC levels could be considered a promising strategy to protect the brain against stroke-induced ischemic damage and neurological dysfunction.

4. Materials and methods

4.1. Chemicals

Methyl-GBB was obtained from JSC Grindeks (Riga, Latvia). Isoflurane was purchased from Abbott (Maidenhead, Great Britain). Atropine solution was obtained from Nycomed (Elverum, Norway). Tramadol solution was purchased from KRKA (Novo Mesto, Slovenia). Penicillin G was obtained from Sandoz (Kundl, Austria). Ethanol was purchased from LAKO Ltd. (Riga, Latvia). Physiological saline (0.9 %) was purchased from Fresenius Kabi (Warszawa, Poland). TTC was purchased from Alfa Aesar (Karlsruhe, Germany). Oxygen and nitrous oxide gases were purchased from AGA (Riga, Latvia).

4.2. Animals and treatment

For mitochondrial function analysis and bioanalytical measurements, 22 male Wistar rats weighing 440–540 g were obtained from the Laboratory Animal Centre, University of Tartu (Tartu, Estonia). For the MCAO experiments, 58 (32 in experiment I and 26 in experiment II) male Wistar rats weighing 380–450 g were obtained from Harlan Laboratories BV (Venray, Netherlands). The animals were housed under standard conditions (21–23 °C, 65 % ± 10 % relative humidity, 12 h:12 h light-dark cycle) with unlimited access to standard food (R70 diet, Lactamin AB, Stockholm, Sweden) and water. The experimental procedures were performed in accordance with the guidelines of EU Directive 2010/63/EU and local laws and policies. All procedures were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service in Riga, Latvia.

For mitochondrial function analysis ($n = 5$/ per group) and bioanalytical measurements ($n = 6$/ per group), the rats were divided into 2 experimental groups: a control group and a group that received methyl-GBB at a dose of 5 mg/kg p.o. for 14 days. The control rats received water.

In the MCAO experiment I, the rats were divided into 3 experimental groups: a sham-operated group ($n = 14$), a control MCAO group ($n = 11$), and an MCAO group that received oral methyl-GBB at a dose of 5 mg/kg ($n = 11$) for 14 days before MCAO. Rats that did not survive after surgery (3 rats in the control MCAO group and 1 rat in the treated MCAO group) were excluded. The control and sham-operated rats received water instead of treatment. Behavioral testing was performed at baseline and on days 1, 3, and 7 after the stroke during the light phase of the light–dark cycle. Body weight was measured before and up to 7 days after MCAO.

In the MCAO experiment II, the rats were divided into 2 experimental groups: a control MCAO group ($n = 13$) and an MCAO group that received oral methyl-GBB at a dose of 5 mg/kg ($n = 13$) for 14 days before MCAO. Rats that did not survive after surgery (3 rats in the control MCAO group and 1 rat in the methyl-GBB treated MCAO group) were excluded. The control MCAO rats received water instead of treatment. Mitochondrial function analysis and bioanalytical measurements were performed 24 h after MCAO induction. Body weight was measured before and 24 h after MCAO.

To eliminate systematic differences between the treatment groups, the animals were randomly assigned to the experimental groups. To avoid subjectivity in the rating process, all experimental procedures were performed in a blinded fashion.

4.3. Determination of the acylcarnitine profile and L-carnitine and methyl-GBB levels in brain tissue

The acylcarnitine profile and L-carnitine and methyl-GBB concentrations in brain homogenates were determined with ultrahigh-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) after 14 days of oral treatment with methyl-GBB as described previously [12,19].

4.4. Ex vivo mitochondrial measurements

For mitochondrial function analysis, animals received methyl-GBB at a dose of 5 mg/kg p.o. for 14 days. Afterward, the rats were sacrificed, and brain tissue was collected. Mitochondrial function in brain tissue homogenates prepared as described previously was assessed [42].

4.4.1. Mitochondrial respiration and H_2O_2 production measurements

Mitochondrial respiration and H_2O_2 production were measured simultaneously at 37 °C using Oxygraph-2k (O2k; Oroboros Instruments, Austria) with O2k-Fluo-Modules in MiR05Cr (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES; pH 7.1; 0.1 % BSA (essentially fatty acid-free) and 20 mM creatine)·H_2O_2 flux (ROS flux) was measured using the H_2O_2-sensitive probe Ampliflu™ Red (AmR) [43]. AmR (10 μM), 1 U/mL horseradish peroxidase (HRP), and 5 U/mL superoxide dismutase (SOD) were added to the chamber·H_2O_2 was detected based on the conversion of AmR into the fluorescent compound resorufin. Calibration was performed by adding H_2O_2 in 0.1 μM steps. The H_2O_2 flux was corrected for background (the AmR slope before the addition of the sample). The H_2O_2/O flux ratio [%] was calculated as H_2O_2 flux/(0.5 O_2 flux).

4.4.2. Substrate-Uncoupler-Inhibitor titration (SUIT) protocols for high-resolution respirometry

For initial fluorespirometry experiments in Control and methyl-GBB-treated rats, glutamate and malate (10 mM and 2 mM, respectively) were used to evaluate NADH(N)-pathway activity and complex I (CI)-linked LEAK (L) respiration. ADP was added at a concentration of 5 mM to measure OXPHOS-dependent respiration (OXPHOS state, P). Then, succinate (10 mM, complex II (CII) substrate, S pathway) was added to activate the convergent NS-pathway (CI&II-linked) respiration. Rotenone (Rot, 0.5 μM, inhibitor of complex I) and antimycin A (AmA, 2.5 μM, inhibitor of complex III) were added to measure CII-linked OXPHOS capacity and residual oxygen consumption (ROX), respectively. To measure complex IV (CIV)-linked respiration, the medium was reoxygenated, and ascorbate (2 mM) and N,N,N,N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mM) were added. Background oxygen consumption induced by TMPD autooxidation and ascorbate reactions was assessed after inhibition of CIV by sodium azide (100 mM) and reoxygenation. Wet-weight-specific oxygen fluxes were compared after correction for ROX.

For respirometry measurements in the MCAO II experiment a protocol focusing on the respiration in the OXPHOS state with additional information about the involvement of FAO in brain energy metabolism was chosen. Briefly, after the addition of the sample to the chambers, ADP was added to a concentration of 5 mM to initiate respiration in the OXPHOS state. Subsequently, malate (0.1 mM) was added, followed by palmitoylcarnitine (PC) (10 μM) to measure FAO-dependent mitochondrial respiration (F(N)-pathway). Further pyruvate (5 mM) and malate (0.5 mM) were added to reconstitute FAO&CI-linked respiration. Glutamate (10 mM) was added as an additional substrate for CI. Succinate (10 mM) was added to reconstitute convergent respiration through

FAO&CI&CII. Afterward, uncoupler (U) carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was added stepwise to determine the maximum electron transfer (ET) capacity. Finally, Rot and AmA were added to determine the CII ET capacity and ROX, respectively.

4.4.3. RET and anoxia–reoxygenation model

RET was induced by the addition of succinate (10 mM) without rotenone·H_2O_2 production was monitored for 10 min. In a different set of experiments, anoxia was induced by stimulating the maximal respiration rate of the sample by the addition of substrates, i.e., glutamate and malate (10 and 2 mM; N-pathway, complex I) or succinate (10 mM) with rotenone (0.5 μM) (S-pathway, complex II) and ADP (5 mM), and the sample was allowed to consume all O_2 in the respiratory chamber (which occurred within 10–20 min), causing it to enter a state of anoxia [18]. After 30 min of anoxia, O_2 was reintroduced to the chamber by opening the chamber to achieve reoxygenation. After 8 min of reoxygenation, the chamber was closed, and O_2 flux was monitored for an additional 2 min. At the end of the experiment, antimycin A (2.5 μM) was added to measure ROX.

4.5. Animal model of transient MCAO

MCAO was induced in Wistar rats by utilizing a filament (silicone-coated thread) to transiently (for 120 min) occlude the MCA. The filament tip was covered with 5 mm thick silicon rubber [44,45]. Anesthesia was induced with 5 % isoflurane in a mixture of 50 % nitrous oxide and 50 % oxygen and maintained with 2–3 % isoflurane using a face mask. The right common carotid artery (CCA) and the bifurcation area were exposed through a midline anterior cervical incision and carefully separated from the surrounding vagus nerve fibers. First, the external carotid artery (ECA) was sutured beyond the branch of the superior thyroidal artery, a loose suture was placed around the internal carotid artery (ICA), and the CCA and ICA were occluded with atraumatic clips. The ECA was dissected, and the distal stump was electrocoagulated. A 19 mm long filament was inserted into the lumen of the ECA and advanced into the ICA. The suture around the ICA was tightened, the clip on the ICA was removed, and the suture was gently advanced 19 mm from the bifurcation to occlude the origin of the MCA. One hundred twenty minutes later, the rats were anesthetized again, and the suture was removed to allow brain reperfusion. Sham-operated animals were subjected to the same surgical procedure except for the advancement of the suture into the MCA. The animals were then returned to their cages and given free access to food and water.

4.6. Behavioral experiments

Behavioral testing was performed by experienced testers blinded to the experimental groups. To evaluate sensorimotor, proprioceptive, and tactile function after ischemic stroke, the limb-placing and vibrissae-evoked forelimb-placing tests were performed at baseline and on days 1, 3, and 7 after stroke during the light phase of the light–dark cycle.

4.6.1. Limb-placing test

The test, which was used to assess forelimb and hind limb responses to tactile and proprioceptive stimulation, consisted of seven limb placement tasks [45]. Performance was scored as follows: 3 points, the rat completed the task normally; 2 points, the rat completed the task with a delay of 3–5 s; 1 point, the rat completed the task with a delay of more than 6 s and/or performed the task incompletely; and 0 points, the rat did not perform the task. Both sides of the body were tested. The maximum possible score, which was achieved by the sham-operated rats, was 21.

4.6.2. Vibrissae-evoked forelimb-placing test

As described previously [45], rats were suspended in the air, and the left or right vibrissae were brushed against the edge of a tabletop to trigger a placement response of the forelimb ipsilateral to the stimulated vibrissae. The vibrissae-evoked forelimb-placing response may be lost following certain types of lesions. The percentage of successful placements out of ten trials was recorded.

4.7. Ischemic infarct size evaluation

The infarct size was quantified on day 7 after MCAO according to a previously described method [46]. Briefly, the rats were decapitated, and their brains were removed and washed in ice-cold phosphate-buffered saline. The excised brain was cut into 2 mm thick coronal sections, stained with 1 % TTC, and then imaged and analyzed with ImagePro Plus 6.3 software. The total infarct size (%) was calculated as the difference in the volume of undamaged tissue between the damaged and undamaged hemispheres.

4.8. Statistical analysis

All results are expressed as the mean ± SD. The behavioral data were evaluated by two-way repeated-measures analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. One-way ANOVA followed by Tukey's multiple comparisons test was used to compare the infarct size. Student's t-test was used when only two groups were compared. *P* values less than 0.05 were considered to indicate significance. Statistical analysis was performed using the GraphPad Prism software package (GraphPad Software, Inc., La Jolla, California, USA).

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CRediT authorship contribution statement

Conceptualization, L.Z., E.L., and M.D.; methodology, validation and formal analysis, B.S., E.V., S.G., B.G., E.S. and L.Z.; behavioral analysis, L.Z., B.S.; histological analysis, E.L., L.Z.; data analysis and interpretation, B.S., E.V. and L.Z.; writing—original draft preparation, L.Z., E.L., M.O. and M.D.; writing—review and editing, E.V., B.S., E.L., M.O., L.Z. and M.D. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Institutional Review Board Statement

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