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Methylene blue improves sensorimotor phenotype and decreases anxiety in parallel with activating brain mitochondria biogenesis in mid-age mice

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ABSTRACT

Age-related brain dysfunctions are associated with mitochondria malfunctions and increased risk of developing neurodegenerative diseases (ND). Recently, a mitochondria-targeting drug methylene blue has been drawing considerable interest as a potential treatment for ND. We found that aged mice manifested a decrease in physical endurance, spontaneous locomotor activity, and exploration concomitant with an increase in anxiety-related behavior, as compared to adult mice. Treating mice for 60 days with MB slowed down these changes. There were no significant changes in the animals' body weight, oxygen consumption rates, or respiratory quotient index, in adult or aged MB-treated mice. However, MB treatment significantly increased the generation of reactive oxygen species in brain mitochondria. The expression of several genes relevant to mitochondria biogenesis, bioenergetics, and antioxidant defense (NRF1, MTCOX1, TFAM, and SOD2) was greatly suppressed in aged mice; it was restored by MB treatment. It seems plausible that the effects of MB could be mediated by its ability to increase H2O2 production in brain mitochondria, thereby activating Nrf2/ARE signaling pathway and mitochondria biogenesis. Our data and earlier findings support the idea that MB can be an attractive prototype drug for developing safe and efficient gerontoprotective compounds.

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1. Introduction

Physiological aging affects the functioning of central nervous system (CNS) in multiple ways, greatly increasing a risk of developing various neurodegenerative diseases (ND) (Navarro et al., 2002; Boveris and Navarro, 2008). There is a consensus that neural mitochondria dysfunction, although not necessarily being a cause of ND, significantly contributes to the development of ND. There are two most experimentally documented mitochondria dysfunctions that are associated with aging: one is a decline in mitochondria's ability to produce energy (Dencher et al., 2007), and another is an increase in mitochondria's generation of reactive oxygen species (ROS) in aged tissues (Sohal and Sohal, 1991). In mitochondria, the energy is produced by means of electron transport from metabolic substrates to oxygen. This is an inherently dangerous process, as it is accompanied by the production of reactive oxygen species (ROS) (Andreyev et al., 2005; Murphy, 2009), which are damaging to cell components and to mitochondria themselves. Therefore, significant efforts are currently invested in developing therapies to counteract mitochondria-derived ROS production. One of the most recent compounds of significant interest is methylene blue (MB), a well-known drug with the ability to affect mitochondria functions. The MB can cross blood brain barrier, which is important in relation to ND (Starkov, 2008; Oz et al., 2009; Medina et al., 2011; Sontag et al., 2012; Stack et al., 2014). Previous publications have suggested that low doses of MB are neuroprotective (Rojas et al., 2009, 2012) and can improve spatial memory retention in a toxic model of brain damage in rats (Callaway et al., 2002, 2004), cause extinction mem-

Abbreviations: MB, methylene blue; ND, neurodegenerative diseases; CNS, central nervous system; ETC, electron transport chain; ROS, reactive oxygen species; Nrf2/ARE, NF-E2-related factor 2/antioxidant response element; NRF-1, nuclear respiratory factor 1; TFAM, transcription factor A, mitochondrial; SOD1/2, superoxide dismutase1/2; COX1, cytochrome c oxidase 1; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog.

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ory improvement (Gonzalez-Lima and Bruchey, 2004), and improve spatial discrimination memory (Wrubel et al., 2007).

In vitro, MB was shown to interact with mitochondrial electron transferring chain (ETC) (Visarius et al., 1997; Rojas et al., 2012). In isolated mitochondria, flavin-dependent enzymes utilize NADH to reduce MB to leucomethylene blue (MBH_2), whereas cytochrome c reoxidizes MBH_2 to MB. Thereby MB functions as an alternative electron carrier, which accepts electrons from NADH (Atamna et al., 2008; Wen et al., 2011) or $FADH_2$ (Tretter et al., 2014) and transfers them to cytochrome c. This causes an increase in the rate of mitochondrial respiration, especially in the presence of complex I/III inhibitors (Wen et al., 2011; Tretter et al., 2014) and increases the activity of cytochrome c oxidase (Atamna et al., 2008; Atamna and Kumar, 2010). Some data also suggest an antioxidant effect of MB in HT-22 cell mitochondria (Poteet et al., 2012). However, a recent study with isolated guinea pig brain mitochondria clearly demonstrated that MB can also elevate H_2O_2 production in mitochondria (Tretter et al., 2014). In relation to this, it is interesting that MB was shown to increase mitochondria biogenesis in a mouse model of tau pathology (a model of Alzheimer's disease) (Stack et al., 2014), likely by activating Nrf2/ARE signaling. This is of particular interest because mitochondrial biogenesis is impaired in aged postmitotic tissue (Miquel et al., 1980; Gonzalez-Freire et al., 2015). It was also shown that MB can act as a gerontoprotector in cell culture model by delaying the replicative senescence of human fibroblasts by more than 20 divisions (Atamna et al., 2008).

The mechanistic aspects of the effects of MB on cellular metabolism are well studied in cell culture models and isolated mitochondria; there are also a number of well-performed studies addressing its effect on the cognitive behavior of rodents. However, many aspects of the MB treatment, especially on the sensorymotor behavior of non-diseased animals, remain to be elucidated. In this study, we focused on the effects of MB in non-diseased mid-aged mice, such as muscular strength, motor coordination, anxiety-related behavior and exploration, changes in the rate of mitochondrial H_2O_2 production, and Nrf2/ARE-controlled gene expression in brain.

2. Material and methods

2.1. Animals and experiment design

Animal maintenance, treatment, and sacrifice were performed strictly in accordance with the rules set by Voronezh State University Ethical Committee on Biomedical Research (Section of Animal Care and Use). Males of C57Bl/6 mice were obtained from Stolbovaya (Moscow, Russia). The following animal groups were used for physiological experiments and quantitative PCR: control 7-month-old-mice ($n = 7$), MB-treated 7-month-old mice ($n = 6$), control 15-month-old mice ($n = 6$), and MB-treated 15-month-old mice ($n = 6$). For measuring the rates of mitochondrial H_2O_2 production for the estimation of mtDNA abundance in brain tissue, we used control 15-month-old mice ($n = 4$), and MB-treated 15-month-old mice ($n = 4$).

All animals were maintained under controlled conditions 12 h light/12 h dark and received water and a standard laboratory diet (Ssniff Spezialdiäten GmbH, Germany) *ad libitum*. MB treatment was performed as follows: first, 15 month old mice were given 5 mg/kg/day of MB (>97% purity, CAS No.: 61-73-4, obtained from MCD Company, Russia) in drinking water, for 14 days. At the second stage, MB-treated groups of 7 and 15 month-old mice were provided with water containing MB at concentration of 15 mg/kg/day for 60 consecutive days. The concentration of MB in the drinking water was 310 μM. In preliminary experiments, we estimated that mice were drinking on average 4.4 ml of water per day per animal (evaluated experimentally); that means that every mouse was receiving MB at 0.446 mg/day. Considering that average weight of our mice was 29 g/mouse, the animals received MB at 15 mg/kg/day. Mice were kept in groups of 4 per cage; the body weight variability was insignificant and did not change appreciably over 60 days of treatment (the data are presented in Table 2). The total amount of consumed food and water was measured daily and divided by the number of mice per cage. In a pilot experiment (data not reported), the mice were kept alone per cage and the amount of consumed food and water per day was evaluated. It was similar for all mice of the same age. Control mice groups received pure water. Measurements of the oxygen consumption rates of live animals were performed on day 5, 14 and 50. The open field test was performed on day 54 and 55, elevated plus-maze test on day 57 and 58. The string test was performed on day 58 and 59. After 60 days, the mice were sacrificed for biochemical, bioenergetics and qPCR analysis. A diagram explaining the time line of the experiments is presented in Fig. 1.

2.2. Respiration evaluation in live mice

Measurements of oxygen consumption and carbon dioxide emission were performed with a gas-phase oximeter ("Vernier", USA). The animals were placed in air-tight vessel (2 l volume) and their oxygen consumption and CO_2 emission were recorded for 8 min. The rates were expressed in μmol O_2(CO_2)/min/g body weight.

2.3. String test

A mouse was placed by its front paws on a string 50 cm long ~42 cm above a padded surface for 1 min. The test scoring was performed as described by (Cardozo-Pelaez et al., 1999). The mouse was then rated according to the following scheme: 0—falls; 1—hangs by two forepaws; 2—attempts to climb onto the string; 3—two forepaws + one or both hind paws; 4—four paws + tail around the string; 5—escape.

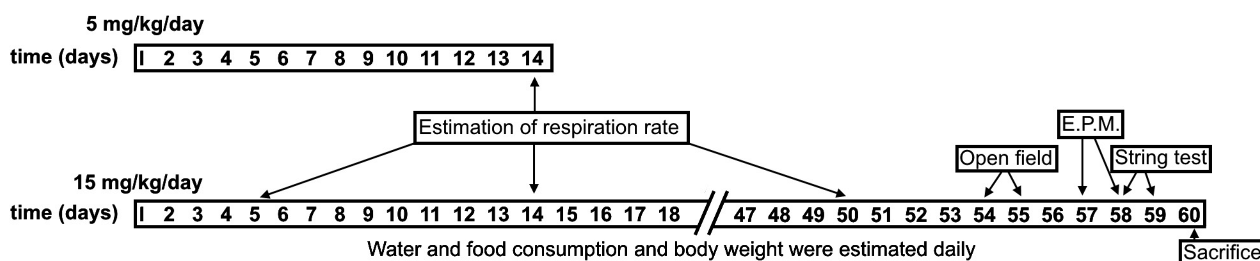

Fig. 1. Time-line of the treatments and behavioral testing of mice. The respiration rate (and CO_2 emission) of live animals was evaluated as described in Section 2. E.P.M. stands for elevated plus maze test. Brain tissue for mitochondria isolation and other *in vitro* assays was collected at day 60 immediately after sacrifice.

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