Behavioral, neuroplasticity and metabolic effects of 7,8-dihydroxy-4-methylcoumarin associated with physical activity in mice

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Abstract
The search for strategies to develop resilience against metabolic and neuropsychiatric disorders has motivated the clinical and experimental assessment of early life interventions such as lifestyle-based and use of unconventional pharmacological compounds. In this study, we assessed the effects of voluntary physical activity and 7,8-Dihydroxy-4-methylcoumarin (DHMC), independently or in combination, over mice physiological and behavioral parameters, adult hippocampal and hypothalamic neurogenesis, and neurotrophic factors expression in the hypothalamus. C57Bl/6J mice were submitted to a 29-day treatment with DHMC and allowed free access to a running wheel. We found that DHMC treatment alone reduced fasting blood glucose levels. Moreover, physical activity showed an anxiolytic effect in the elevated plus maze task and DHMC produced additional anxiolytic behavior, evidenced by reduced activity during the light cycle in the physical activity group. Although we did not find any differences in hypothalamic or hippocampal adult neurogenesis, DHMC increased gene expression levels of VEGF, which was correlated to the reduced fasting glucose levels. In conclusion, our data emphasize the potential of physical activity in reducing development of neuropsychiatric conditions, such as anxiety, and highlights DHMC as an attractive compound to be investigated in future studies addressing neuropsychiatric disorders associated with metabolic conditions.

Keywords Coumarin · DHMC · VEGF · Fasting glucose · Anxiolytic · Physical activity

Introduction
Disruption of synaptic plasticity processes, neuronal growth and remodeling, neurogenesis, and eventually, progressive neuronal loss, underlies the development of several neuropsychiatric and metabolic disorders (Myers and Olson 2012; Dugger and Dickson 2017; Estrada and Contreras 2019). As an attempt to counteract such outcomes, early life interventions, mainly lifestyle-based and new phytochemical compounds, have been investigated aiming at preventing neurodegeneration and promoting healthier aging (Jameel et al. 2016; Alkadhi 2018; Carrera et al. 2020).

Coumarins are polyphenols that constitute a large class of heterocyclic oxygen compounds, initially found as secondary plant metabolites. They have high bioavailability, low molecular weight and simple processes for synthesis (Wu et al. 2009). Coumarins have demonstrated experimental therapeutic potential in several diseases, including obesity,
diabetes, cardiovascular failure, renal failure, cancer, and neurological disorders (Kadakol et al. 2016; Ahmed et al. 2020). Notably it was reported that they increased insulin secretion in isolated cells from mouse pancreas (Ahmed et al. 2020) and prevented oxidative damage in myocardial infarction in rats (Rajadurai and Stanely Mainzen Prince 2006). Regarding the central nervous system (CNS) these molecules have shown experimental results in neuroprotection in human (Molina-Jiménez et al. 2005), mouse (Yao et al. 2015) and rat (Kang and Kim 2007) neuronal cells in vitro, adult neurogenesis, cognitive function improvement in mice (Gao et al. 2015) and antidepressant effect in rats (Yang et al. 2020). Furthermore, coumarins appear to improve cell survival, promoting favorable conditions for neuronal expansion in mice and rats (Gao et al. 2014; Skalicka-Woźniak et al. 2016; Qin et al. 2017). The 7,8-Dihydroxy-4-methylcoumarin (DHMC) is a coumarin synthetized by relatively simple, low-cost and good yielding processes (Potdar et al. 2001). It is considered a simple coumarin since structural substitution occurs only in the benzene ring using two hydroxyl radicals. Specifically, in in vitro studies have also pointed to a possible neuroprotective effect of this coumarin, mainly related to its antioxidant and anti-inflammatory properties (Tyagi et al. 2005; Togna et al. 2014; Jin et al. 2015).

Physical activity is widely known to reduce the risk of metabolic abnormalities, such as obesity (Shapiro et al. 2011; Swift et al. 2018), as well as to prevent or slow down the progression of neuropsychiatric conditions, such as dementia, depression and anxiety (Angevaren et al. 2008; Cunha et al. 2013; Kandola et al. 2018). Among mechanisms that have been shown to mediate physical activity effects in the CNS are the increased expression of neurotrophic factors and synaptic plasticity markers, decrease in neuroinflammatory processes (Cotman et al. 2007; Choi et al. 2018) and enhancement of adult neurogenesis (van Praag et al. 1999; Kronenberg et al. 2006; Niwa et al. 2016). The proliferation and differentiation of adult-born neurons can be stimulated by physiological and external stimuli, related to physical activity and antidepressants (van Praag et al. 1999; Santarelli et al. 2003; Niwa et al. 2016). Frequently these stimuli-induced neuroplasticity responses are mediated by trophic factors, such as ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) (Fabel et al. 2003; Kokoeva et al. 2007; Choi et al. 2018).

There is a growing interest in the search for strategies to efficiently improve resilience against the development of metabolic and neuropsychiatric diseases. In the present study we evaluated the impact of a pharmacological and a behavioral intervention (DHMC treatment and physical activity, respectively) in promoting metabolic fitness and brain plasticity in normal mice. We show that both DMHC and physical activity produce behavioral improvements and that DHMC triggers trophic factor expression in the hypothalamus. These results expand the window of opportunities for the development of preventive and therapeutic approaches in metabolic and neuropsychiatric conditions.

**Methods**

**Chemicals**

The coumarin DHMC was synthesized at the Chemistry Department of the Federal University of Lavras (UFLA) as previously described (Potdar et al. 2001). The synthesis process resulted in 97% purity and approximately 75% yield of DHMC.

**Experimental animals**

Adult male C57BL/6 mice 8 weeks old, obtained from Federal University of Minas Gerais (UFMG), were housed individually at a temperature of 22 ± 2 °C, under a 12 h light/dark cycle and with free access to food and water. The procedures were approved and carried out in accordance with the Ethics Committee of the Federal University of Lavras/Brazil and Animal Experiments Control Council (CONCEA), according to protocol No. 078/17. All procedures were focused on reducing the number of animals used and minimizing their suffering.

**Experimental protocol**

To avoid novelty stress, the mice were moved to the housing facility, where treatments and physical activity were later performed, 2 weeks before any experimental intervention. In the last 72 h of this 2-week period all animals were presented with running wheels and the physical activity volume acquired. Then, each of the four experimental groups were settled based on a Z distribution of the running volume.

The mice were assembled into four groups (n = 8/group): vehicle, DHMC, physical activity + vehicle, physical activity + DHMC (30 mg/weight/day). DHMC was administered orally by gavage once a day, for 29 days (dissolved in a saline solution containing 0.3% sodium carboxymethylcellulose, CMC-Na). Control groups received an equal volume of 0.3% CMC-Na saline (vehicle) (Fig. 1a).

Additionally, BrdU (5-bromo-2′-deoxyuridine; Sigma) was administered in the first 10 days to assess cell proliferation and neurogenesis. BrdU is an analogue of thymidine that is incorporated into the DNA double helix during the S phase of the cell cycle and therefore marks cells in active proliferation (Cooper-Kuhn et al. 2002). The animals included in the neurogenesis analysis received BrdU (0.1 M,
DHMC treatment decreased the total volume of physical activity in the light period. Panel A shows the experimental design: C57BL/6 male mice received a 29-day repeated treatment of 7,8-Dihydroxy-4-methylcoumarin (DHMC) or vehicle by oral gavage. In the first 10 days, mice received BrdU (5-Bromo-2′-deoxyuridine) 50 mg/wt or vehicle, 2x/day i.p.,12/12 h. Part of the animals were exposed to the voluntary physical activity wheel during the whole experiment (physical activity groups) while the others did not have access to the wheel (sedentary groups) (n=8 per group). On the 12th day the mice were tested in the EPM paradigm in order to assess anxiety-related behaviors (n=5 per group). The animals were euthanized 20 days after the last BrdU or vehicle injection by transcardial perfusion and their brains were processed for immunohistochemistry or molecular analyses, respectively. B, D Two-way ANOVA (vehicle × DHMC) X revolutions per minute per day (RPM/day) over the 29 days of the experiment for daily volume of voluntary physical activity during the light or dark cycle. C, E Unpaired t test (vehicle × DHMC) for total volume in the light and dark cycle. Data are presented as means + SEM. N=8 per group. Statistical significance defined when \(^*p<0.05\). Data are presented as means + SEM. Normality and variance equality were assessed with the Kolmogorov–Smirnov F-test. BrdU = 5-bromo-2′-deoxyuridine; DHMC = 7,8-dihydroxy-4-methylcoumarin

**Behavioral analysis**

For analysis of anxious behavior and locomotion, we used the Elevated Plus Maze (EPM). The EPM apparatus is made of polished wood panels with a matte dark acrylic surface and consists of two open arms (50 × 10 × 40 cm) and two closed arms (50 × 10 × 40 cm) that extended from a common central platform (5 × 5 cm) elevated 50.0 cm from the floor. Mice, N = 5 per group were used in this test, were individually placed on the central platform facing an open arm and allowed to freely explore the maze for 5 min. Behavior in the EPM trials was scored using video recordings as follows: entries into
open arms (all four limbs crossing into an open arm),
time in open arms (duration of time spent in open arms),
entries into closed arms (all four limbs crossing into a
closed arm), time in closed arms (duration of time spent
in closed arms).

Each measure (time in open arms, for instance) was com-
pared to the total number of inputs (sum of time in open and
closed arms) and presented as a percentage. The room light-
ing was adjusted to assure homogeneous incidence across
the apparatus, avoiding shadows or unlit corners. At the end
of each behavioral session, the devices were cleaned with a
70% alcohol solution.

Immunofluorescence staining

On the 30th day of the experiment, a subset of the animals
(N = 4 per group) was anesthetized with a mixture of keta-
mine (45 mg/weight, ip) and xylazine (5 mg/weight, ip).
For cell proliferation analyses through immunofluorescence
staining, the mice (n = 4) were perfused through the left car-
diac ventricle with 0.9% saline solution, followed by 4% par-
aformaldehyde (PFA) in 0.1 M PBS (pH 7, 4). After perfu-
sion, the brains were removed, post-fixed in the same fixative
solution for 24 h at room temperature (RT) and immersed
in a 30% sucrose solution in PBS at 4 °C. Serial coronal
sections (20 μm) were obtained with a cryostat (LEICA
Microsystems, CM1860). To analyze cell proliferation and
neurogenesis in the hippocampus and hypothalamus, a series
of one-in-six free floating sections were processed for detec-
tion of the BrdU immunoreactivity.

The neural progenitor phenotype was assessed by double
labeling BrdU and doublecortin (DCX, an early neuronal
differentiation marker). Briefly, after DNA denaturation in
2 N HCl at RT for 1 h and pre-incubation with 10% blocking
solution (0.1 M PBS with 10% normal goat or donkey serum
and 0.2% Triton X-100), sections were incubated overnight
at 4 °C in anti-BrdU (rat, 1:200; AB6326; ABCAM) and
anti-DCX (rabbit, 1:100[PK1]; 4604S; Cell Signaling) pri-
mary antibodies. The sections were then incubated with sec-
ondary antibodies anti-rat (donkey, 1:500; A-21208, Invit-
rogen) and anti-rabbit (donkey, 1:500, A10040; Invitrogen)
for 2 h at RT.

The morphological analyses were performed on coded
slides, with the executing researcher blinded to the experi-
mental group. The total numbers of BrdU-immunopositive
cells were estimated by manually counting all positive
cells in coronal sections from 1.06 to 3.52 mm posterior to
Bregma, to include the hypothalamic and hippocampal neu-
rogenic niches (estimated as 120 sections). The total number
of labeled cells was multiplied by 6 (serial-sectioning fac-
tor). Double-labeling was confirmed by three-dimensional
reconstruction of z-series of confocal microscopy covering
the entire nucleus (or cell) of interest (confocal microscope
Upright LSM780-NLO).

RNA extraction and quantitative real-time PCR

Following euthanasia by deep anesthesia, the mice hypo-
thalami (n = 4) were dissected and prepared for gene
expression analysis. Briefly, RNA samples were prepared
using TRIzol (Invitrogen) according to the manufactur-
er’s recommendations. Spectrophotometry was employed
for RNA quantification. For each sample, 2 μg of RNA
was employed for the synthesis of complementary DNA
(cDNA) using the High Capacity cDNA Reverse Transcrip-
tion Synthesis kit (Applied Biosystems) according to
the manufacturer’s recommendations. Real-time PCR reac-
tions were run using the TaqMan system (Applied Biosys-
tems). Primers used were DCX (Mm00438400_m1); NEU-
ROD1 (Mm01946604_s1); CNTF (Mm00446373_m1),
CNTFR (Mm00516693_m1), BDNF (Mm01334043_m1)
and VEGF (Mm00437306_m1). Analyses were run using
4 μL (10 ng/μL) cDNA, 0.625 μL primer/probe solution,
1.625 μL H2 O, and 6.25 μL 2X TaqMan Universal Mas-
terMix. GAPDH (Mm99999915_g1) was employed as a
reference gene. Gene expression was obtained using the
SDS System 7500 software (Applied Biosystems).

Blood glucose analysis

For the fasting glucose test the animals were subjected to
8 h of food deprivation with access to water ad libitum.
The deprivation period started after 4 h of inversion of the
light/dark cycle. After the controlled period of depriva-
tion, all animals were subjected to the collection of a drop
of blood from the caudal vein and the measurement was
performed immediately using the Accu-Chek Performa
 glucometer (ROCHE).

Statistics analysis

Data were analyzed using GraphPad Prism 8.0.1 (Graph-
Pad Software Inc., CA, USA). Statistical analysis was
performed initially by applying the Kolmogorov–Smirnov
and F-test, to certify normality and equality of data vari-
ance, respectively. Then, data were analyzed by unpaired
two-tailed Student’s t-test, two-way analysis of variance
(ANOVA) or ANOVA with repeated measures when
appropriate. Post hoc comparisons were performed using
the Bonferroni’s test. Data are presented as means ± stan-
dard error of the mean (SEM). A p value < 0.05 was con-
sidered significant.
Results

DHMC treatment decreased the total volume of physical activity in the light period

Briefly, the amount of daily physical activity was obtained, which corresponds to the total number of complete rotations and volume performed in the light and dark periods of the day. The physical activity group was monitored through daily records, obtained from the voluntary physical activity wheel, and the sedentary group was not exposed to the equipment. The data were analyzed by two-way ANOVA (vehicle × DHMC) X revolutions per minute per day (RPM/day) over the 29 days of the experiment. We found that DHMC administration decreased the total volume of physical activity in the light period (Fig. 1b) [F (1, 286) = 9.711; p = 0.0020], which was confirmed by the analysis of the total volume by the unpaired t-test, considering the sum of the 29 experimental days (Fig. 1c) [t = 2.861, df = 10; p = 0.0169]. Interestingly, no differences were found during the dark period (Fig. 1d) [F (28,338) = 4.610; p < 0.1781] and (Fig. 1e) [t = 0.0844, df = 13; p = 0.9340].

DHMC treatment reduces fasting blood glucose levels without any effects on body weight

Body weight was measured weekly, while fasting blood glucose was measured on the last day of the experimental protocol. Blood samples were obtained by caudal puncture. The two-way ANOVA analysis, considering treatment (vehicle × DHMC) X time interaction (weight gain), showed that there was no significant difference in body weight gain between groups (Fig. 2a) [F (3, 140) = 1.641; p = 0.1827], but a significant main effect of time [F (4, 140) = 33.93; p = 0.0001] reflecting a change in weight, regardless of treatment (vehicle or DHMC). However, fasting blood glucose levels were reduced in animals treated with DHMC, for both sedentary and voluntary physical activity groups (Fig. 2b) [F (1, 28) = 4.515; p = 0.0426], on the other hand, voluntary physical activity alone did not change blood glucose levels [F (1, 28) = 1.072; p = 0.3094]. While the average glycemia for control sedentary mice was 103.8 mg/dL, in DHMC treated mice it was reduced to 92.3 mg/dL, both values remaining around the expected range for healthy, chow fed adult mice (80–100 mg/dL) (Andrikopouloes et al. 2008; Togashi et al. 2016). To further investigate the effect of DHMC, disregarding physical activity, we used an unpaired t test (vehicle × DHMC) considering all animals, exercised or not, from vehicle and DHMC treated groups (Fig. 2c) and confirmed that the DHMC treated mice showed a significant reduction in fasting glycemia when compared to their vehicle controls [t = 2.158, df = 30; p = 0.0391].

Effect of DHMC treatment and physical activity in anxiolytic behavior

To determine the effects of voluntary physical activity and DHMC on behavioral responses related to anxiety, locomotion index and anxiolytic response were assessed in the EPM task. The DHMC treatment had no effect on EMP parameters such as total number of entries/locomotion (Fig. 3a) [F (1.16) = 0.4274; p = 0.5226]; total number of entries in open arms (Fig. 3b) [F (1, 17) = 4.610; p < 0.1781] and (Fig. 3c) [t = 0.0844, df = 13; p = 0.9340]. DHMC treatment (vehicle × DHMC) however the mice were not divided in sedentary and physical activity; Unpaired t test (vehicle × DHMC). Statistical significance considered when *p < 0.05. Data are presented as means ± SEM. Kolmogorov–Smirnov normality and variance equality F-test. DHMC = 7,8-dihydroxy-4-methylcoumarin;
(16) = 1.475; p = 0.2422], and percentage of entries between the open and closed arms (Fig. 3c) [F (1, 16) = 0.2754; p = 0.6070]. As expected, the physical activity group showed an increased locomotion index, considering the total number of entries in all arms of the labyrinth (Fig. 3a) [F (1, 16) = 22.15; p = 0.0002] and reduced the animals’ anxious behavior, characterized by a longer stay in the open arms of the labyrinth (Fig. 3b) [F (1, 16) = 22.76; p = 0.0002], although there was no difference in the percentage of entries between the open and closed arms in relation to the total number of both arms (Fig. 3c) [F (1, 16) = 0.02631; p = 0.8732].

**DHMC treatment increases hypothalamic VEGF gene expression**

To evaluate the neurotrophic pathways that could be activated by treatment with DHMC or physical activity, the hypothalamic tissue was prepared for analysis of gene expression (Fig. 4). We used bidirectional ANOVA considering the treatment (vehicle × DHMC) X neurotrophic markers (fold change). Although our number of replicates only allows for a statistical difference when we perform a separate analysis of vehicle versus DHMC treatment in sedentary mice (Fig. 4g) [t = 3.094, df = 6; p = 0.0213], overall, we observed a higher average gene expression for DCX, CNTF, BDNF and VEGF (Fig. 4a-f) in groups treated with DHMC, respectively [F (1, 12) = 0.3550; p = 0.5624], [F (1, 12) = 0.2763; p = 0.6087], [F (1, 12) = 0.2212; p = 0.6466] and [F (1, 12) = 0.8718; p = 0.3689]. The NEUROD1 (Fig. 4b) [F (1, 12) = 0.001866; p = 0.9663] and CNFTR mRNA levels (Fig. 4d) [F (1, 12) = 1.205; p = 0.2940] remained unchanged. Interestingly, we found a positive correlation between VEGF expression and fasting glucose levels (Fig. 4h) [F = 6,802; R = 0.3270 p = 0.0207].

**DHMC treatment or physical activity have no effect on adult neurogenesis**

To address the putative involvement of adult neurogenesis on coumarin or physical activity effects, we determined the rates of hippocampal and hypothalamic cell proliferation in 8-week-old C57BL/6J mice treated with DHMC or vehicle kept in control housing or allowed free access to a running wheel. A subset of animals from all experimental groups were injected with BrdU and euthanized 20 days later. In addition to quantification of BrdU positive cells, their phenotype was characterized by colocalization with the early neuronal marker DCX. In the present study, and due to our limitation on the final sample number we did not find any difference between the experimental groups in adult hippocampal (Fig. 5b and d) [F (1, 8) = 0.2113; p = 0.6580 and [F (1, 6) = 0.8583; p = 0.3900] or hypothalamic cell proliferation and overall neurogenesis (Fig. 5c and e) [F (1, 7) = 0.1492; p = 0.7108] and [F (1, 6) = 0.9323; p = 0.3716].
Discussion

The overall increase in life expectancy has been accompanied by growing incidence of aging-related neuropsychiatric conditions such as Alzheimer and Parkinson’s diseases, depression, and anxiety (Armstrong and Okun 2020; Curran et al. 2020). Throughout life the complex CNS environment seems to depend on a balance of factors to maintain optimal neuronal function (Myers and Olson 2012). Among the various strategies on debate to prevent, delay or reverse neurodegeneration development, systemic approaches, such as physical activity, and new pharmacological molecules outstand from classic interventions, given their lower potential for adverse effects (Chen and Shan 2019; Bhatti et al. 2020). In this study, we assessed the effects of voluntary physical activity and DHMC, independently or in combination, over physiological and behavioral parameters, as well as an investigation of their neuroplasticity effects in the hypothalamus and hippocampus at molecular and cellular levels.
First, we asked if physical activity would produce anxiolytic-like behavior and the potential incremental effect of DHMC treatment. Physical activity comprehends body movements produced by skeletal muscles that result in energy expenditure and it is positively correlated with physical fitness (Caspersen et al. 1985). Mounting data have shown that physical activity/exercise interventions promote improvements in chronic diseases such as metabolic, cardiovascular, and neurodegenerative diseases (Angevaren et al. 2008; Shapiro et al. 2011; Spartano et al. 2017). Additionally, it is remarkable the effectiveness of physical activity in improving anxiety symptoms in people with a current diagnosis of anxiety and/or stress-related disorders (Kandola et al. 2018). At the preclinical level, the voluntary running wheel is a useful method of increasing physical activity in rodents (Cunha et al. 2013; Alkadhi 2018). As expected, we observed an anxiolytic effect of physical activity evidenced by an increased time spent in the open arms in the EPM, corroborating previous studies (Hötting and Röder 2013; Caliskan et al. 2019). The circadian clock and

**Fig. 5** DHMC treatment or physical activity have no effect on adult neurogenesis. Panel A shows representative images of BrdU-positive cells and BrdU/doublecortin (DCX) double-labeling in the hippocampus and hypothalamus of mice 20 days after the last BrdU injection. No significant differences were found either for physical activity nor DHMC treated groups in cell proliferation (B) or overall neurogenesis (D) in the hippocampus or in the hypothalamus. Scale bars = 100 μm (A). Data are presented as means ± SEM. N = 2–3 per group. *p < 0.05; Two-way ANOVA, followed by Bonferroni post hoc test.
inadequate rest time can influence several processes involved in neurodegeneration and morphological and functional changes in the neuronal environment (Musiek and Holtzman 2016). Interestingly, the DHMC treatment produced an additional anxiolytic effect in the physical activity group, observed as a decreased volume of voluntary physical activity during the light cycle (rest period for rodents). Although this is the first study showing a potential anxiolytic effect of DHMC, it was previously reported that DHMC alleviated chronic unpredictable mild stress-induced depression-like behaviors and alterations in spine density in rats (Yang et al. 2020). Moreover, other coumarins with neuroprotective properties alleviate anxiety-like behaviors in mouse models (Randjelovic et al. 2020; Adu-Nti et al. 2021). However, we recognize that the anxiolytic effect of DHMC was only found in one of its measures (voluntary activity wheel) and other behavioral analysis could be considered in the future.

Next, we investigated if our interventions were able to induce neuroplasticity alterations. In animal models, physical activity mitigates age related impairment in adult neurogenesis and cognition in the hippocampus, preserving functions such as learning, memory and emotional behavior (Praag et al. 2005; Hill et al. 2015). Although we confirmed the anxiolytic effect of physical activity and a further action of DHMC, in our experimental conditions, they were not accompanied by an increment in adult hippocampal or hypothalamic neurogenesis. Adult neurogenesis is the process that leads to the formation of functional new neurons, in certain regions of the adult brain, such as the subgranular zone of the dentate gyrus (DG), subventricular zone of the lateral ventricles and, at a lower rate, at the ventricular zone of the hypothalamus (Ming and Song 2011). Adult-born neurons integrate into the established circuitry and modulate structure-related functions, for instance cognition and emotional behavior in the DG (Sahay and Hen 2008; Deng et al. 2010), and body energy homeostasis in the hypothalamus (Kokoeva 2005).

The mice hypothalamus presents some neurogenic activity during adulthood, which has been reported to play a role in long-term metabolic control (Kokoeva 2005; Lee et al. 2012; Li et al. 2012). Hypothalamic neurons are critical sensors of nutrient availability implicated in energy balance and glucose metabolism control (Schneeberger et al. 2014). While we could not see differences in adult hypothalamic neurogenesis, we found that DHMC produced a reduction in fasting glucose, a positive metabolic feature when considering the risk for development of metabolic conditions (Wilson 2017). Similar improvements in glucose metabolism have been described for other methyl coumarins that particularly enhance insulin secretion (Ahmed et al. 2020). Of note, metabolic disorders are also correlated to a higher risk of developing neuropsychiatric conditions (Bădescu et al. 2016; Lyra e Silva et al. 2019), which makes DHMC an interesting candidate to be assessed as a new pharmacological approach in the association of metabolic and neurodegenerative disorders. Of note, an important limitation of our study is that, despite the sex differences in neurodegenerative and metabolic-related disorders, we used only male mice. Hence, the data should be amplified in female mice for a more translational interpretation. Additionally, although here we focused on a preventive strategy, another future perspective would be to challenge the animals with neurodegenerative insults (such as aging, chronic stress, high-fat diet exposure), which could reveal a stronger effect of the association of DHMC and physical activity, especially in neuroplasticity assessments.

Albeit limbic structures are the major sites comprehending the neural circuitry regulating behavioral outcomes in mood disorders (Price and Drevets 2010), dysfunction in the hypothalamus–pituitary–adrenal (HPA) axis is commonly known to be associated with stress-related development of behavioral conditions, such as anxiety and depression (Kessing et al. 2011; Russell and Lightman 2019). In this scenario, important findings mediating cellular neurodegeneration are the impairment of neuroplasticity and neurotrophic factors supply (Duman et al. 1997; Alleva and Francis 2009). Among the neurotrophins that are important regulators of neural survival, development, function, and plasticity are BDNF, CNTF, VEGF and others (Duman 2009; Fargali et al. 2012). In addition to the widely studied hippocampal related neurotrophic activity, several studies have uncovered a central role for hypothalamic neurotrophins, specially BDNF, upon multiple circuits that control appetite and energy metabolism (Xu et al. 2003; Kokoeva 2005; An et al. 2020). Therefore, it is known that behavioral disturbances causing derangement in the HPA axis are, as well, correlated to metabolic outcomes (Ivić et al. 2016; Jelenik et al. 2018). It was previously shown that chronically stressed rats presented reduced hypothalamic and pituitary VEGF and BDNF mRNA levels (Nowacka et al. 2015). Conversely, in our study, we found that DHMC treatment increased hypothalamic VEGF gene expression, which was also positively correlated with reduced fasting glucose levels. In this regard, Langlet and colleagues demonstrated an important role of tanyctyic VEGF in blood-hypothalamus barrier plasticity and hypothalamic metabolic response to fasting (Langlet et al. 2013) corroborating the correlation found in our study. Moreover, it has been shown that VEGF regulates GLUT-1 expression and glucose uptake in blood brain barrier cells of obese mice, and this VEGF mediated homeostatic regulation limits cognitive impairment in obesity models (Jais et al. 2016). Supporting this line of experimental evidence, in humans, high fat diet consumption modulated tissue expression of GLUT1 and serum VEGF levels, which was correlated to performance in cognitive tests (Schüler et al. 2018). Although the association of
DHMC treatment and physical activity did not produce any additional effects in behavioral and neuroplasticity outcomes in most of the parameters measured, individually, both strategies still showed interesting results.

Conclusion

In conclusion, our data reinforce the positive outcomes of physical activity in mitigating anxiolytic behaviors, that could prevent the development of anxiety-related neuropsychiatric conditions and bring up DHMC as a potentially useful pharmacological approach that could be assessed in further studies targeting neurodegenerative conditions associated with metabolic disorders.

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Author contributions  PKFL and DFE performed experiments and analyzed the data; MSAM and NOB assisted voluntary physical activity experiments. SST guided the DMHC synthesis process. LAV provisionalyzed the data; MSAM and NOB assisted voluntary physical activity; PKFL and DFE wrote the manuscript; RFM designed the study; all authors revised and approved the final version of the manuscript.

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Data availability  The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest  The authors have no conflict of interest to declare that are relevant to the content of this article.

Ethical approval  All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Ethics Committee of the Federal University of Lavras/Brazil and Animal Experiments Control Council (CONCEA), according to protocol n° 078/17.

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