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 reduced fasting blood glucose levels. Moreover, physical activity showed 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 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.

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 prevent 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). 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. In vitro studies have 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). In the CNS coumarins seem to improve cell survival, promoting neuroprotection and promoting favorable conditions for neuronal expansion in mice and rats (Gao et al. 2014; Skalicka-Woźniak et al. 2016; Qin et al. 2017).

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, such as 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 (coumarin treatment and physical activity, respectively) in promoting metabolic fitness and brain plasticity in normal mice. We show that, either isolated or combined, coumarin and physical activity produce behavioral improvements and neuroplasticity responses, particularly in neurotrophic factors 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 7,8-Dihydroxy-4-methylcoumarin (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 DHMC.

Experimental animals

Adult male C57BL/6 mice 8 weeks old, obtained from Federal University of Minas Gerais (UFMG), were kept in cages of one at as 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

For the administration of coumarin and voluntary physical activity wheel, the mice were divided into four groups (n =8/group): vehicle, coumarin, physical activity + vehicle, physical activity + coumarin (30 mg / weight / day). During the habituation period (2 weeks), a Z distribution of the individuals in the groups was performed according to the running volume for 72 hours before the beginning of the experiment. "The animals were distributed in the four groups, following Z distribution of the physical activity volume acquired 72 hours before the beginning of the experiment (from higher volume to lower volume of physical activity)". For the other experiments, the animals were randomly divided into smaller groups, meeting some technical limitations. Being distributed: elevated plus maze experiment, five (n=5) animals from each group were used. In the molecular tests, the eight initial animals were divided into two groups of four animals (n=4), distributed between immunofluorescence assays and detection of neurotrophic markers by polymerase chain reaction (PCR). Coumarin was administered by gavage once a day, for 29 days (dissolved in saline solution containing 0.3% sodium carboxymethylcellulose, CMC-Na). Control groups received an equal volume of 0.3% CMC-Na saline (vehicle) (Fig. 1A). BrdU (5-bromo-2'-deoxyuridine; Sigma) was used 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 [23]. All animals received BrdU (0.1 M, phosphate buffered saline [PBS], pH = 7.2; 50 mg / weight, 2x / day ip, 12/12 hours) and were sacrificed 20 days later to test cell proliferation and early neuronal differentiation,

To promote physical activity through voluntary exercises, two groups (physical activity and physical activity + coumarin) had free access to the racing wheel for 29 days. The casters were installed inside the cage and have a speed sensor connected to an interface and program to record this information (Columbus Instruments®). At the end of the experiment, the total volume of voluntary physical activity in the period and the average speed were quantified and stratified in the light and dark periods to assess behavioral paradigms.

Behavioral analysis

For analysis of anxious behavior and locomotion, we use the Elevated Plus Maze (EPM). EPM is made of polished wood panels with a matte dark acrylic surface and consists of two open arms (50×10×0 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 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). The data were considered in relation to the total number of entries. Illumination was reduced by means of high lights with equal light intensity

between a periphery and a central part, as well as between open and closed arms. At the end of each behavioral session, the devices were cleaned with a 70% alcohol solution and an interval of 5 minutes between the tests.

Immunofluorescence staining

On the 30th day of the experiment, mice were anesthetized with a mixture of ketamine (45 mg/wt,ip) and xylazine (5 mg/wt,ip) and perfused through the left cardiac ventricle with 0.9% saline solution, followed by 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4). After perfusion, 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) of 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 detection 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 room temperature (RT) for 1 h and pre-incubation with 10% blocking solution (0.1M 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) primary antibodies. The sections were then incubated with secondary antibodies anti-rat (donkey, 1:500; A-21208, Invitrogen) e 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 experimental 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 neurogenic niches (estimated as 120 sections).

The results were expressed as the total number of labeled cells by multiplying the total number of labeled cells obtained from the sum of every one-in-six section by the serial sectioning factor (obtained total cell counting x 6). 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

RNA samples were prepared using TRIzol (Invitrogen) according to the manufacturer'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 Transcription Synthesis kit (Applied Biosystems) according to the manufacturer's recommendations. Real-time PCR reactions were run using the TaqMan system (Applied Biosystems). Primers used were DCX (Mm00438400_m1); NEUROD1 (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 H2O, and 6.25 μL 2X TaqMan Universal

MasterMix. 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 hours of food deprivation with access to water ad libitum. The deprivation period started after 4 hours of inversion of the light / dark cycle. After the controlled period of deprivation, 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 (GraphPad Software Inc.,CA, USA). The statistical analyses were carried out using 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 test. Data are presented as means ± standard error of the mean (SEM). A p value < 0.05 was considered to be statistically significant.

Results

Coumarin treatment reduces fasting blood glucose levels

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. Statistical analysis using two-way ANOVA, being an experimental group (vehicle x DHMC) X time interaction (weight gain), showed that there was no significant difference in body weight between the groups (Fig.1b) $[F_{(3,140)}=1,641; p=0.1827]$, 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.1c) $[F_{(1, 28)}=4.515; p=0.0426]$. To investigate the effect of DMHC in isolation, we used a paired t test (vehicle x DMHC) considering all animals, exercised or not from each group (Fig.1d). The animals supplemented with DHMC showed a significant reduction in fasting glycemia when compared to their vehicle controls $[t = 2,158, df = 30; p = 0.0391]$.

Effect of coumarin treatment and physical activity in anxiolytic behavior

To determine the effects of voluntary physical activity and DHMC on behavioral responses related to anxiety, the rats underwent two interventions. 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 initially submitted to two-way ANOVA (vehicle x 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.2a) [$F_{(1,286)}$ =9.711; p = 0.0020] and confirmed by the analysis of the total volume by the paired t-test, considering the sum of the 29 experimental days (Fig.2c) [t=2.861, df=10; p=0.0169]. Interestingly, no differences were identified during the dark period (Fig.2b) [$F_{(28,338)}$ = 4.610; p<0.1781] and (Fig.2d) [t=0.08443, df=13; p=0.9340]. In a second approach, the mice's locomotion index and anxiolytic response were assessed in the EPM task. As expected, the physical activity group showed an increased locomotion index, considering the total number of entries in all arms of the labyrinth (Fig.2e) [$F_{(1,16)}$ = 22.15; p=0.0002]. In addition, physical activity reduced the animals' anxious behavior, characterized by a longer stay in the open arms of the labyrinth (Fig.2f) [$F_{(1,16)}$ = 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.2g) [$F_{(1,16)}$ = 0.02631; p=0.8732].

Coumarin treatment increases hypothalamic VEGF gene expression

To evaluate the neurotrophic pathways that could be activated by treatment with coumarin or physical activity, the hypothalamic tissue was prepared for analysis of gene expression (Fig.3). We used bidirectional ANOVA considering the treatment (vehicle x DHMC) X neurotrophic markers (fold change). We observed a higher average gene expression of DCX, CNTF, BDNF and VEGF (Fig.3a, c, e and f) in groups treated with coumarin, 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], although our number of replicates only allows for a statistical difference when comparing sedentary vehicles and groups treated with coumarin for VEGF expression by means of a paired t test considering animals exercised or not together (Fig.3g) [t = 3.094, df = 6; p = 0.0213]. The NEUROD1 mRNA levels (Fig. 3B) [$F_{(1, 12)}$ = 0.001866; p = 0.9663] and CNFTR (Fig.3d) [$F_{(1, 12)}$ = 1,205; p = 0.2940] remained unchanged. Interestingly, we found a positive correlation when comparing VEGF expression and fasting glucose levels (Fig.3h) [F = 6,802; R = 0.3270 p = 0.0207].

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 central nervous system 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 a molecular and cellular level.

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 results 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 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). In concert, 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). However, we recognize that the anxiolytic effect of DHMC was only found in one of its measures (voluntary activity wheel) and other models of behavioral experiments 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 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 2005a).

The mice hypothalamus presents some neurogenic activity during adulthood, which has been reported to play a role in long-term metabolic control (Kokoeva 2005b; 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 enhanced 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.

Although 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 Francia 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 to govern appetite and energy metabolism (Xu et al. 2003; Kokoeva 2005b; 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 tanycytic 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 impaiment 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).

Conclusions

In conclusion, our data reinforce the positive outcomes of physical activity in promoting neuroplasticity improvements that could mitigate the development of neuropsychiatric conditions, such as anxiety, and bring up DHMC as a potentially useful pharmacological approach that could be assessed in further studies targeting neurodegenerative conditions associated with metabolic disorders.

Declarations

Author contributions

PKFL, DFE, MSAM, NOB and CAP performed experiments and analyzed the data; DFE and PKFL wrote the manuscript; RFM designed the study; all authors revised and approved the final version of the manuscript.

Conflicts of interest

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

Availability of data and material

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

Ethics 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 nº 078/17.

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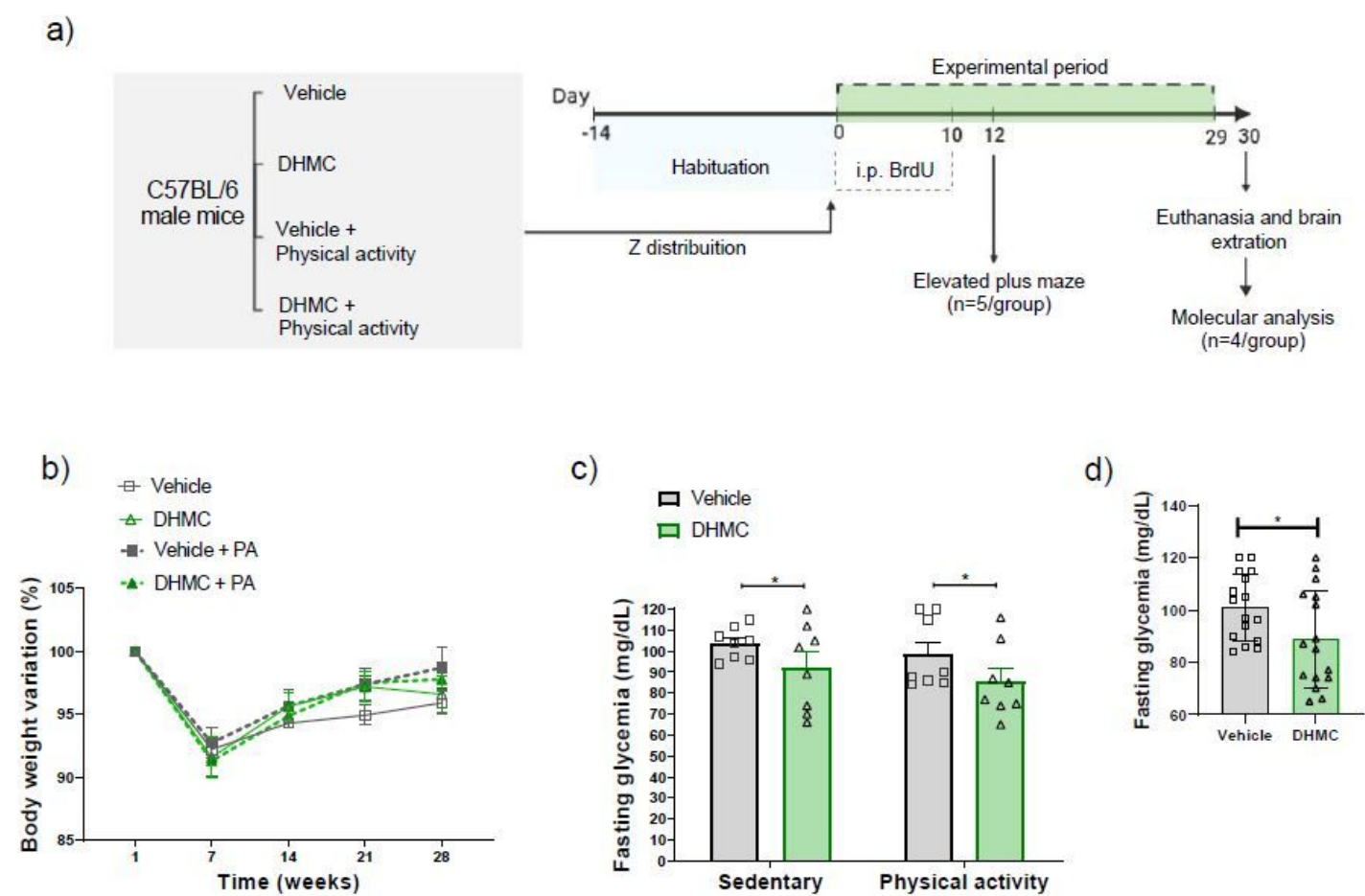
Figures

Figure 1

DHMC reduced fasting blood glucose. Panel A shows the experimental design. C57BL/6J mice received a 29-day repeated treatment of 7,8-Dihydroxy-4-methylcoumarin (DHMC) or vehicle i.g. In the first 10-day mice received BrdU (5-Bromo-2'-deoxyuridine) 50mg/wt, 2x/day i.p.,12/12 hours. 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 exposed to elevated plus maze paradigm (EPM) in order to assess anxiety-related behaviors (n=5 per group). The animals were sacrificed 20 days after the last BrdU injection by transcardial perfusion and their brains were processed for molecular analyses. (B) Anova two-way weekly bodyweight gain (vehicle x DHMC) X time interaction (weight gain) (C) Anova two-way fasting blood glucose levels (vehicle x DHMC) X fasting glycemia (mg/dL) (D) Paired t test (vehicle x DMHC). Statistical significance

defined when *p<0.05. Data are presented as means +SEM. Kolmogorov-Smirnov normality and variance equality F-test. DHMC= 7,8-dihydroxy-4-methylcoumarin; BrdU= 5-bromo-2'-deoxyuridine
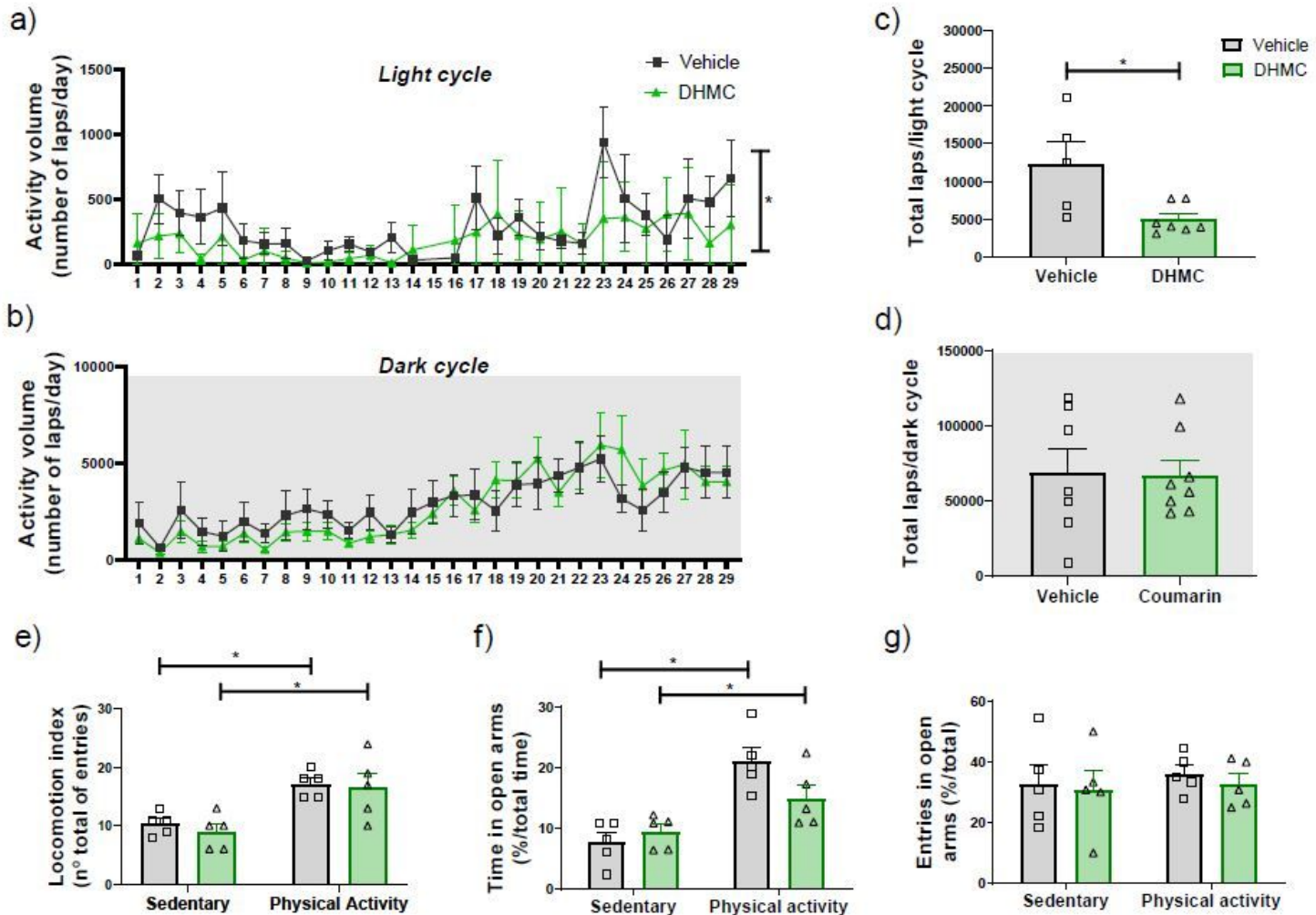

Figure 2

Effect of DHMC treatment and physical activity on anxiolytic behavior. (A) Two-way ANOVA (vehicle x 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 (B) Paired t test (vehicle x DMHC) for total volume in the dark cycle. (C) Two-way ANOVA (vehicle x DHMC) X revolutions per minute per day (RPM/day) over the 29 days of the experiment for total volume of voluntary physical activity during the light or (D) Paired t test (vehicle x DMHC) for total volume in the dark cycle (E) Two-way ANOVA (vehicle x DHMC) X locomotion index (total number of entries) for locomotion index obtained by the total number of entries in the open and closed arms (F) Two-way ANOVA (vehicle x DHMC) X time in open arms (%/total time) for total time in the open arms and (G) Two-way ANOVA (vehicle x DHMC) X entries in open arms (%/total) for number of entries in the open arms; both represented as a percentage of total entries in both arms. Data are presented as means + SEM. N=8 per group for analyzes related to the voluntary physical activity wheel (A-D) and N=5 per group for analyzes related to the elevated plus maze test (E-G).Statistical significance defined when *p<0.05. Data are presented as means +SEM. Kolmogorov-Smirnov normality and variance equality F-test. DHMC= 7,8-dihydroxy-4-methylcoumarin
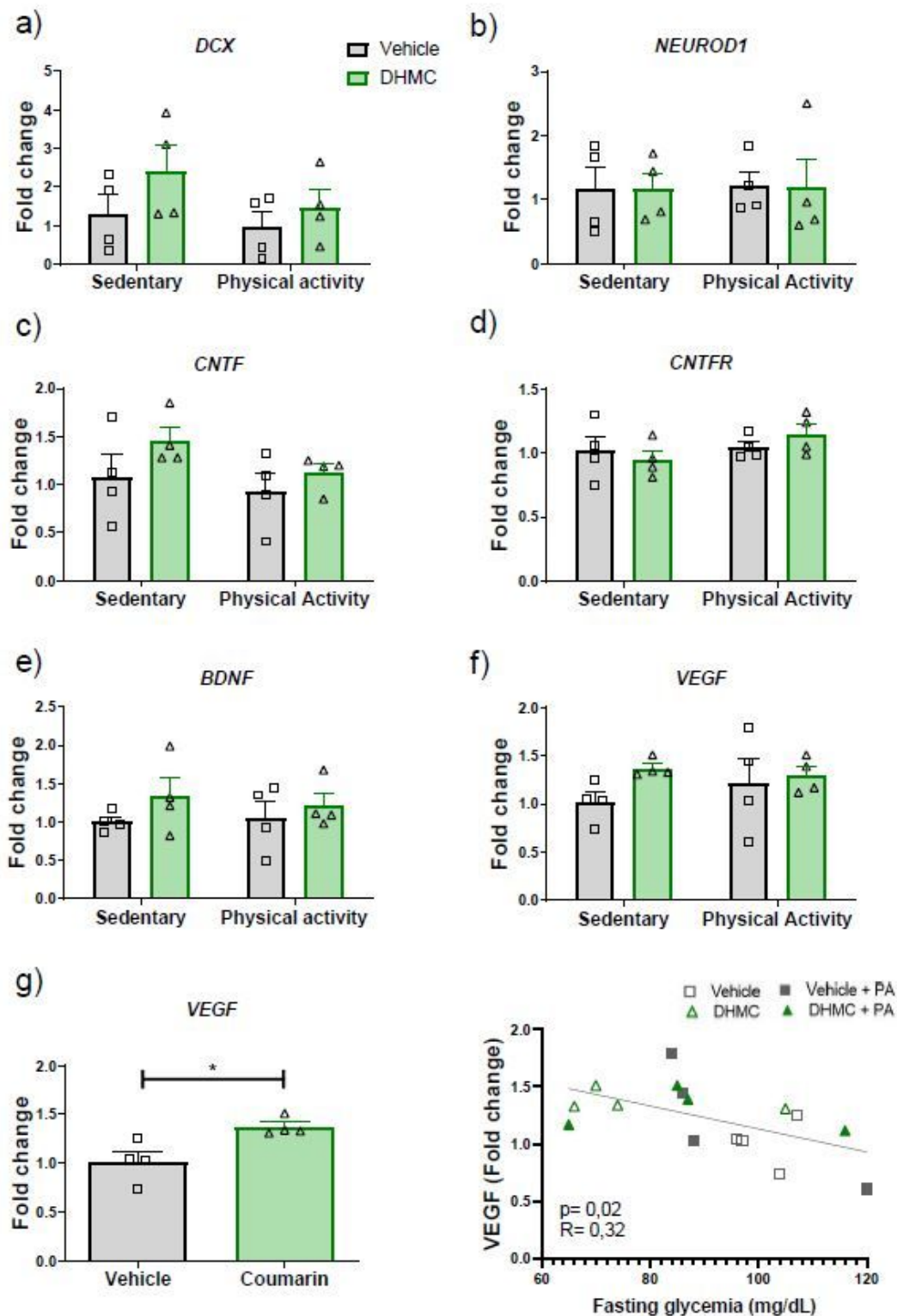
Figure 3

DHMC treatment increases hypothalamic VEGF gene expression. Two-way ANOVA (vehicle x DHMC) X neurotrophic marker (fold change) for evaluation of hypothalamic mRNA levels of DCX, NEUROD1, CNTF, CNTFR, BDNF and VEGF (A-F). Paired t-test (vehicle x DHMC) considering animals exercised or not grouped, disregarding the physical activity variable. Representation of VEGF mRNA levels presented in F in animals treated with vehicle and DHMC (G). Pearson's correlation coefficient analysis between VEGF

mRNA expression and fasting glucose levels (H). N=4 per group. Statistical significance defined when *p<0.05. Data are presented as means +SEM. Kolmogorov-Smirnov normality and variance equality F-test. DHMC= 7,8-dihydroxy-4-methylcoumarin

Supplementary Files

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- FigSI1.pdf