7,8-dihydroxyflavone exhibits therapeutic efficacy in a mouse model of Rett syndrome

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7,8-dihydroxyflavone exhibits therapeutic efficacy in a mouse model of Rett syndrome. J Appl Physiol 112: 704–710, 2012. First published December 22, 2011; doi:10.1152/japplphysiol.01361.2011. — Rett syndrome (RTT), caused by mutations in the methyl-CpG binding protein 2 gene (MECP2), is a debilitating autism spectrum developmental disorder predominantly affecting females. MeCP2 mutant mice have reduced levels of brain-derived neurotrophic factor (BDNF) in the brain; conditional deletion and overexpression of BDNF in the brain accelerates and slows, respectively, disease progression in MeCP2 mutant mice. Thus we tested the hypothesis that 7,8-dihydroxyflavone (7,8-DHF), a small molecule reported to activate the high affinity BDNF receptor (TrkB) in the CNS, would attenuate disease progression in MeCP2 mutant mice. Following weaning, 7,8-DHF was administered in drinking water throughout life. Treated mutant mice lived significantly longer compared with untreated mutant littermates (80 ± 4 and 66 ± 2 days, respectively). 7,8-DHF delayed body weight loss, increased neuronal nuclei size and enhanced voluntary locomotor (running wheel) distance in MeCP2 mutant mice. In addition, administration of 7,8-DHF partially improved breathing pattern irregularities and returned tidal volumes to near wild-type levels. Thus although the specific mechanisms are not completely known, 7,8-DHF appears to reduce disease symptoms in MeCP2 mutant mice and may have potential as a therapeutic treatment for RTT patients.

MeCP2 mutant mice; irregular respiratory pattern; Trkb; tidal volume

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MATERIALS AND METHODS

Animals, 7,8-DHF administration, and body mass measurement. All procedures were performed in accordance with the University of Wisconsin’s Animal Care and Use Committee. The MeCP2 mutant mice (MT) and their wild-type littermates (WT) were maintained in the C57BL/6 background (MeCP2tm1.1/Jae) and genotyped as previously described (9). On the basis of the preliminary dose-response studies, 7,8-DHF was first dissolved in DMSO to generate a stock solution at the concentration of 100 mg/ml. Stock solution (80 μl) was added to 100 ml of Hydroparc water containing 1% of sucrose (pH = 7.4), and given to mice as drinking water. The 7,8-DHF containing drinking water was replaced every 3–4 days. Mice were monitored daily, and body mass was recorded every 3–4 days. The water bottle was weighed every 3–4 days to monitor water intake. Four groups of
mice were studied: mutants with either 0 or 80 mg/l 7,8-DHF in their drinking water (MT0, MT80) and wild-type mice with either 0 or 80 mg/l 7,8-DHF in their drinking water (WT0, WT80). Survival curves were generated, and a log rank test (16) was performed to assess the statistical significance in the difference between the curves. A two-way ANOVA was performed to compare body mass between groups (WT0, WT80, MT0, MT80) at each week (week 0/Weaning, 1, 2, 3, and 4 wk of treatment). Although every attempt was made to randomize investigations in mutant and wild-type mice, parts of the studies were not blinded because the Mecp2 mutant mice can be easily distinguished from the wild type mice because of their obvious phenotypes. However, the investigators conducting the running wheel assay and measuring the neuronal nuclei size did not have knowledge of the genotypes of the mice or the brain sections. When plethysmography recordings were performed, the investigators were not blinded so that mice could be randomly assigned to different plethysmography chambers.

Neuronal nuclei size measurement in the CA1 region of the hippocampus. For CA1 neuronal nuclei size measurement, brains were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 4% paraformaldehyde overnight at 4°C, washed in PBS, processed by a TISSUE-TEK VIP machine (MilesCo Scientific, Princeton, MN), and embedded in paraffin. Serial coronal sections (5 μm) were taken through the whole brain. Sections of the comparable rostral-caudal position were stained with cresyl violet. Digital pictures were taken for the CA1 region of the hippocampus. The nuclei area (μm²) of projection neurons was determined by tracing the outline of the nuclei, using ImageJ (NIH, Bethesda, MD). At least 20 neurons per image were analyzed. At least two independent images were scored per mouse. Thus 177 neurons from 4 mice (at least 40 neurons per mouse) in the WT0 group, 126 neurons from 3 mice in the WT80 group, 305 neurons from 7 mice (at least 40 neurons per mouse) in the MT0 group, and 298 neurons from 7 mice (at least 40 neurons per mouse) in the MT80 group were included in the double-blind analysis. Nuclear sizes between groups were compared using a one-way ANOVA.

Voluntary running wheel activity. At 8 wk of age (~4 wk following treatment start), total spontaneous wheel running distance over a 24-h period was recorded in a wheel running cage connected with a Mini-EDL IV (Mini Mitter, Bend, OR) in each experimental group (WT0: n = 4; MT0: n = 10; WT80: n = 3; MT80: n = 10). Wheel circumference was 72 cm/revolution. Distances were compared between groups with a one-way ANOVA.

Plethysmography recording and data analysis. At 6 wk of age, ventilatory responses were assessed in unanesthetized, unrestrained Mecp2 mutant mice and their wild-type littermates administered either 0 mg/l (WT0, MTO) or 80 mg/l 7,8-DHF (WT80, MTO) in their drinking water using a whole body flow through plethysmograph (BUXCO Electronics, Troy, NY). Thus, four groups of mice were studied: WT0 (n = 9), WT80 (n = 6), MT0 (n = 12), and MT80 (n = 8). Whole body plethysmography allows for quantitative measurement of ventilation in unanesthetized, free-behaving animals while simultaneously controlling inspired gas concentrations. A pressure calibration signal, the plethysmograph temperature, mouse body temperature, ambient and chamber pressures, and mouse body mass were then used by the computer software to continuously calculate breath-by-breath ventilatory variables using the equations described by Drorbaugh and Fenn (12) as modified by Jacky (17). Data were rejected if there was evidence of pressure fluctuations caused by gross body movements or snifing behavior. Mice were weighed, body temperatures recorded, and then placed into the plethysmograph chamber while breathing room air (21% oxygen, balance nitrogen). Mice were allowed to acclimate to the chamber. Once the mice were quiet but awake, ventilatory measurements were recorded continuously for 30 min during baseline conditions (21% oxygen, 0% carbon dioxide, balance nitrogen), followed by consecutive 10 min periods of hypoxia (11% oxygen, balance nitrogen) and hypercapnia. Statistical analyses were performed on data collected during the most quiet 3-min baseline period and the last 3 min of each exposure period. At the conclusion of the study, mice were removed from the chambers and body temperatures recorded.

Ventilatory variables in 6-wk-old mice were averaged over 3 min of baseline, hypoxia, and hypercapnia. Measurements included breathing frequency, tidal volume (Vt), and minute ventilation (the product of breathing frequency and tidal volume, VE). Breathing volumes (Vt and VE) were normalized per 100 g of body mass. The total respiratory cycle duration (TTOT) was measured for 100 continuous breaths during baseline breathing and averaged in each mouse to reflect differences in stability of the breathing patterns based on the distribution of TTOT and the respective 95% confidence intervals (CI). The coefficient of variation (CV) was calculated by dividing the standard deviation of TTOT by TTOT. After TTOT, CI, and CV were determined for each mouse, mean TTOT, CI, and CV were calculated for each group. Statistical comparisons of baseline ventilatory variables were made between groups (WT0, MT0, WT80, MT80) at each condition (baseline, hypoxia, and hypercapnia) with a two-way ANOVA. Baseline variability analyses (TTOT, CI, CV) were compared between groups (WT0, MT0, WT80, MT80) with a one-way ANOVA.

Statistical analyses. All statistical analyses were performed using SigmaStat 3.0 (SPSS Chicago, IL). Differences were considered statistically significant at P < 0.05 and Student-Neuman-Keuls post hoc tests were performed when applicable. Except for survival curves, all data are presented as means ± SE.

RESULTS

Survival and body mass. 7,8-DHF (80 mg/l) was administered to Mecp2 mutant mice and their wild-type littermates in drinking water from weaning (~4 wk of age) and continued until the end of the study. The average water intake per mouse per day was 2–3 ml. Compared with Mecp2 mutant mice receiving no 7,8-DHF (MT0, n = 35), Mecp2 mutant mice receiving 7,8-DHF in drinking water (MT80, n = 32) lived significantly longer (average survival times: MT0 = 66 ± 2 days, MT80 = 80 ± 4 days, Fig. 1A, P = 0.003). The beneficial effect of 7,8-DHF on the life span of the Mecp2 mutant mice was similar to genetic overexpression of BDNF in the brains of Mecp2 mutant mice (8). Because both wild-type littermates receiving no 7,8-DHF (WT0) and 7,8-DHF (WT80) in drinking water had a normal life span, their survival curves were combined in Fig. 1A (n = 55).

We also compared the body mass of the four mouse groups from ~4 wk of age until 8 wk of age (4 wk of treatment). When all data are combined over time, all four experimental groups were significantly different from each other (all P < 0.007; Fig. 1B) with a significant interaction between group and time (Fig. 1B; P = 0.033). Before treatment (week 0) and 1–2 wk after treatment with 7,8-DHF, no significant difference was detected between the MT0 and MT80 groups (Fig. 1B; P = 0.351, P = 0.215, and P = 0.069, respectively). However, at 3 and 4 wk following the start of treatment, the MT80 weighed significantly more than the MT0 group (Fig. 1B; P = 0.002 and P < 0.001, respectively), whereas the WT0 and WT80 groups never differed from each other (all P > 0.05). Mutant mice (MT0 and MT80) weighed significantly less than wild-type mice (WT0 and WT80) at each time point studied (Fig. 1B; all P < 0.05). In a separate two-way ANOVA comparing only the WT0 and WT80 groups, there was no significant interaction term (P = 0.966), nor were there differences at individual times post-treatment (P = 0.363, 0.071, 0.065, 0.160, and
0.197, for time points 0, 1, 2, 3, and 4, respectively), suggesting that the treated wild-type mice were simply slightly larger than the untreated wild-type mice for unexplained reasons. These data suggest that 7,8-DHF only partially rescued the growth deficit in Mecp2 mutant mice because mutants always had smaller body mass than the wild types. However, MT80 mice continued to gain weight at a similar rate as the WT0 and WT80 groups (linear slopes: MT80, n = 32; WT0 and WT80, n = 35). The dashed black line represents wild-type mice (WT0 and WT80, n = 35). MT80 mice lived significantly longer than the MT0 group (66 ± 2 vs. 80 ± 4 days, log rank test; P = 0.003); all WT mice had normal survival. B: growth curve of the treated and nontreated mutant and wild-type groups comparing the average body mass and the weeks following 7,8-DHF treatment. When data are combined over time, all 4 experimental groups were significantly different from each other (†, all P < 0.007). For 2 wk following the start of treatment, there were no significant differences between MT0 and WT0 groups (P = 0.215 and P = 0.069, respectively). However, MT80 mice had significantly larger body mass at 3 and 4 wk following the start of treatment compared with their untreated mutant littermates (P = 0.002 and P < 0.001, respectively). The MT0 and MT80 groups had significantly lower body mass than the WT0 and WT80 groups at every time point (all P < 0.05), resulting in a significant interaction between group and time (P = 0.033). *P < 0.05 vs. MT0 group, †P < 0.05 vs. both MT0 and MT80 group for each point in growth curve.

Voluntary wheel-running activity. Because impaired motor function has been observed in RTT patients (6) and Mecp2 mutant mice (8), we examined the effect of 7,8-DHF treatment on voluntary locomotor distance in Mecp2 mutant mice. In a voluntary running wheel assay that measures spontaneous wheel distance run per night, we found that the MT80 group ran significantly longer distances than the MT0 group at 8 wk of age (~4 wk after the start of 7,8-DHF treatment; 151 ± 20 vs. 72 ± 21 meters/24 h; P = 0.008; Fig. 2A). The improvement in voluntary wheel running in Mecp2 mutant mice attributable to 7,8-DHF treatment was also reminiscent of the rescue observed following BDNF overexpression in an earlier study (8). However, this rescue is only partial, because the running wheel distances in both the MT80 and MT0 groups remained significantly lower than the WT0 mice (242 ± 11 m/24 h; P = 0.019 and P < 0.001, respectively; Fig. 2A). WT80 mice ran quantitatively more than all other groups (532 ± 71 m/24 h; all P < 0.001; Fig. 2A). However, the proportional increase in voluntary wheel running associated with 7,8-DHF administration was similar in WT and MT groups (1.2- and 1.1-fold increase from nontreated mice, respectively).
Hippocampal neuronal nuclei size. RTT patients and MeCP2 mutant mice exhibit smaller brain (6, 9), hippocampal neuron soma (8, 9), and neuronal nuclei (2) sizes. We examined the size of neuronal nuclei in the hippocampal CA1 region from 8- to 10-wk-old mice. Nuclear sizes in mice from the WT0 and WT80 groups were not statistically different (121 ± 1 and 118 ± 2 μm²; P = 0.15). The MT80 group (116 ± 1 μm²) had significantly larger neuronal nuclei than the MT0 mice (109 ± 1 μm²; P < 0.001; Fig. 2B), and the MT80 group did not differ from the WT80 group (P = 0.361), thus confirming results from the aforementioned studies. WT0 mice (120 ± 1 μm²) had significantly larger neuronal nuclei than the MT0 group (P < 0.001) as well as the MT80 group (P = 0.0150; Fig. 2B). Together, these data suggest significant, but not complete recovery of neuronal nuclei size by 7,8-DHF treatment.

Ventilatory capacity and breathing pattern. Respiratory rhythm generation and pattern formation within the brain stem are severely affected in RTT patients (6, 33) and MeCP2 mutant mice (19). We performed whole body plethysmography to assess breathing in unanesthetized mice. We made measurements in 6-wk-old unanesthetized and unrestrained mice to obtain quantitative ventilatory measurements under conditions of 1) baseline (21% oxygen, balance nitrogen), 2) hypoxia (11% oxygen, balance nitrogen), and 3) hypercapnia (21% oxygen, 7% carbon dioxide, balance nitrogen). Although there were no significant differences among the MT0, MT80, WT0, and WT80 groups in breathing frequency under baseline conditions (P = 0.235; Fig. 3), frequency was significantly elevated in MT0 and MT80 groups vs. WT0 (P < 0.001 and P = 0.004, respectively) and WT80 groups during hypoxia (P < 0.001 and P = 0.001, respectively; Fig. 3). In contrast, breathing frequency was significantly decreased in MT0 and MT80 mice vs. WT0 (P = 0.005 and P = 0.004, respectively) and WT80 during hypercapnia (P = 0.020 and P = 0.011, respectively; Fig. 3). Thus there was a significant interaction between mouse group (MT0, MT80, WT0, WT80) and condition (baseline, hypoxia, hypercapnia) on breathing frequency (interaction: P < 0.001). There were no significant interactions between group and condition in tidal volume (Vt) or minute ventilation (Ve) when all conditions were combined.

Fig. 3. Effects of 7,8-DHF treatment on baseline, hypoxic, and hypercapnic breathing in 6-wk-old mice. Top: schematic diagram of the conditions used in the plethysmography recording experiments. Bottom: bar graphs of breathing parameters recorded in each group of mice under the 3 conditions illustrated on top. Open bars: WT0 (n = 9); open shaded bars: WT80 (n = 6); hatched open bars: MT0 (n = 12); hatched grey bars: MT80 (n = 8). There were no significant differences among experimental groups in breathing frequency under baseline conditions (P = 0.235). However, frequency was significantly elevated in MT0 and MT80 groups during hypoxia (vs. WT0: P < 0.001 and P = 0.004, respectively; vs. WT80: P < 0.001 and P = 0.001, respectively). In contrast, breathing frequency was significantly decreased in MT0 and MT80 groups during hypercapnia (vs. WT0: P = 0.005 and P = 0.004, respectively; vs. WT80: P = 0.020 and P = 0.011, respectively). Thus, there was a significant interaction between mouse group (MT0, MT80, WT0, WT80) and condition (baseline, hypoxia, hypercapnia) on breathing frequency (interaction: P < 0.001). Combining measures across conditions (baseline, hypoxia, hypercapnia) revealed significant increases in Vt and Ve in the MT0 group vs. the WT0 and MT80 groups (Vt: P = 0.002 and P = 0.013, respectively; Ve: both P = 0.007; Fig. 3), suggesting that 7,8-DHF treatment (MT80 group) attenuated Vt and Ve responses in the MeCP2 mutant mice, and returned values nearly to wild-type levels when all conditions were considered. Highly variable breathing patterns with prominent apneas are characteristic of MeCP2 mutant mice (29). Therefore, we analyzed the variability of breathing pattern in 6-wk-old mice by quantifying the total duration of each breath (TTOT), the

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95% CI of T$_{TOT}$, and the CV of T$_{TOT}$ for each mouse and then for each group. Representative plethysmography traces from a single 6-wk-old mouse from each experimental group are shown in Fig. 4A; three representative 6-wk-old mice from each group are incorporated into the scatterplots for T$_{TOT}$ in Fig. 4B. Under baseline conditions, the MT0 group had significantly longer T$_{TOT}$ than all other groups (WT0: $P < 0.001$, WT80: $P = 0.014$ and MT80: $P = 0.038$; Figs. 4, A and B, left, and C). Similarly, the MT0 group also showed greater variability in breathing pattern as the CI and CV were significantly greater than for all other groups (all $P < 0.001$; Figs. 4, A and B, left, and C). These deficits were partially rescued by 7,8-DHF because T$_{TOT}$ in the MT80 group was not significantly different from the WT0 or WT80 groups ($P = 0.080$ and $P = 0.349$, respectively; Figs. 4, A–C). However, the CV of MT80 remained significantly higher than the WT0 and WT80 groups (both $P < 0.001$, Figs. 4, A–C), as did the CI ($P < 0.001$ and $P = 0.002$, respectively; Figs. 4, A–C), suggesting that although 7,8-DHF reduced breathing variability in mutant mice (lower CV and CI in the MT80 group vs. the MT0 group), it did not return breathing stability completely to normal.

DISCUSSION

Here we demonstrate that 7,8-DHF administered in drinking water significantly decreases the severity of pathologies in Mecp2 mutant mice, including improvements in life span, body mass, voluntary running wheel distance, hippocampal neuronal nuclei size, and breathing abnormalities. Interestingly, although there are abundant literature reports that increased voluntary or induced physical activity increase BDNF levels in the hippocampus (for review, see Ref. 28), we are unaware of converse reports whereby TrkB receptor activation increases voluntary wheel running in rodents. However, our laboratory has observed that genetic overexpression of BDNF in mice increases running wheel activity to a similar extent (Chang et al., unpublished data) as does 7,8-DHF administration.

Although restoring BDNF in mutant Mecp2 mice appears to slow disease progression (8, 21, 22, 32), BDNF is unlikely to become a systemic treatment for RTT because of its inability to cross the blood-brain barrier (23). 7,8-DHF has been implicated as a TrkB agonist (18) and has similar effects as BDNF in certain forms of learning and memory (3, 11). Intraperitoneal or oral 7,8-DHF administration is reported to activate...
TrkB in the mouse brain, indicating either that this compound itself or its metabolites may cross the blood-brain barrier and access CNS TrkB receptors (18). In the current study, we tested the therapeutic efficacy of 7,8-DHF in Mecp2 mutant mice to reduce pathologies such as respiratory and somatic motor impairment, breathing irregularities, and growth/viability deficiencies. Although additional pharmacological actions of 7,8-DHF are possible, its effects on these hallmark pathological features of RTT disease are similar to the effect of genetic overexpression of BDNF in Mecp2 mutant mice. In the future, it will be of interest to study the effects of 7,8-DHF on female Mecp2 heterozygous mutant mice as most RTT patients are female and to directly test the hypothesis that its actions are via TrkB receptor activation. Regardless, the present study demonstrates therapeutic efficacy in males, where disease is more severe (usually lethal at a young age).

Consistent with previous reports (5, 30), our study found greater ventilatory responses to hypoxia in Mecp2 mutant mice. We further report that Mecp2 mutant mice have significantly larger tidal volumes than wild-type mice when the baseline, hypoxic, and hypercapnic conditions were combined. Previous reports (22, 30) detected a similar trend, although those reports did not show statistical significance. Without measurements of metabolic rate or arterial blood gases, it is not possible to determine if the increased tidal volume (and minute ventilation) reflect a true hyperventilation, or if the increase is appropriate to match a greater metabolic demand. Unlike a previous study (22), we did not observe elevated breathing frequencies at rest in Mecp2 mutant mice. Because genetic background affects ventilatory control in mice (7, 35), it is worth noting that Ogier et al. (22) used the Mecp2tm1.1/Jae mice in a mixed background of 129Sv/Jae, C57BL/6, and BALB/c, whereas we used Mecp2tm1.1/Jae mice in a pure C57BL/6 background. In a different RTT mouse model (Mecp2tm1.1/Bird), Voiturou et al. (30) did not detect elevated breathing frequency at baseline. Developmental age also affects breathing pattern and frequency between P30 and P60 (30), potentially explaining (at least in part) different baseline frequencies in these studies. Finally, our P42 Mecp2 mutant mice had highly variable breathing patterns. If breathing frequency data were taken during a period of low breathing frequency with prolonged apneas and TTOT, the overall frequency will be slower vs. baseline breathing during an episode of high breathing frequency. Although every attempt to avoid sampling bias was made and breaths were averaged over 3 min, sampling variation could have affected the mean values of breathing frequency reported here. Interestingly, breathing frequencies were higher in MT mice during hypoxia but were lower in MT mice during hypercapnia when compared with wild-type groups. Because chemosensory pathways from the peripheral carotid body chemoreceptors are primarily responsible for breathing changes in response to hypoxemia, whereas central chemoreceptors mainly respond to changes in carbon dioxide, these chemosensory pathways may be differentially regulated in Mecp2 mutant mice.

7,8-DHF administration tended to return the augmented tidal volumes (but not frequency) and irregular respiratory rhythms seen in mutant mice toward wild-type breathing patterns. Because 7,8-DHF was systemically delivered in the drinking water, the site of action is unknown. However, likely locations include integrative centers in the brain stem that regulate breathing pattern. Nonetheless, we cannot rule out other sites of action (e.g., direct actions on chemoreceptors, bulbospinal pathways to phrenic motor neurons, neuromuscular junctions on respiratory muscles) as tidal volumes were abnormally increased in mutant mice, but returned toward wild-type levels with 7,8-DHF administration.

The actions of BDNF (presumably via TrkB receptor activation) on the brain stem respiratory control network are complex. BDNF decreases respiratory frequency and enhances glutamate-evoked currents of pre-Bötzinger neurons in postnatal day 1–4 mice (26). BDNF also reduces the amplitude of evoked inward currents in nucleus tractus solitarius (NTS) neurons of postnatal day 35–48 Mecp2 mutant mice (21). Although previous reports suggest that 7,8-DHF activates TrkB receptors in the CNS (similar to BDNF), our study does not address the pharmacological mechanisms of 7,8-DHF actions; further investigations are needed.

In summary, 7,8-DHF significantly extends life span, improves growth (body mass), increases the size of neuronal nuclei, increases voluntary wheel running, and partially corrects breathing instability. The effects of 7,8-DHF on these hallmark pathological features of RTT disease are similar to genetic overexpression of BDNF in Mecp2 mutant mice. Our results support 7,8-DHF as a candidate drug that may eventually be tested in RTT patients once mechanistic studies are confirmed. Because drinking water appears to be an acceptable route for delivering 7,8-DHF in mice, oral administration of this compound in humans may be possible. Moreover, because noninvasive assessment of breathing stability can be readily performed in RTT patients, improvement in ventilatory abnormalities may provide valid endpoint measures to test the therapeutic efficacy of 7,8-DHF.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

7,8-DHF EFFICACY IN A MOUSE MODEL OF RETT SYNDROME


