Resveratrol Improves Hippocampal Atrophy in Chronic Fatigue Mice by Enhancing Neurogenesis and Inhibiting Apoptosis of Granular Cells

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Neuroimaging evidence showed structural and/or functional abnormalities existing in the central nervous system, especially the hippocampus, in chronic fatigue syndrome (CFS) patients. However, its pathophysiologic mechanisms are unclear in part due to the lack of an applicable animal model. We established a chronic fatigue murine model by six repeated injections of Brucella abortus antigen to mice, which was manifested as reduced daily running activity and hippocampal atrophy. Thereafter, resveratrol, a polyphenolic activator of sirtuin 1, was used for treatment in this model. Daily running activity was increased by more than 20%, and the hippocampus was enlarged after 4-week resveratrol therapy. Furthermore, resveratrol inhibited neuronal apoptosis and expression of hippocampal acetylated p53 in the fatigue mice. Resveratrol also improved neurogenesis and expression of brain-derived neurotrophic factor mRNA in the hippocampus. We concluded that repeated injection of B. abortus antigen could induce hypoactivity and hippocampal atrophy in mice. Resveratrol may be effective for improving fatigue symptoms and enlarging the atrophic hippocampus by repressing apoptosis and promoting neurogenesis.

Key words chronic fatigue syndrome; resveratrol; sirtuin 1; p53; brain-derived neurotrophic factor

Chronic fatigue is reported in more than 20% of patients examined in primary care, and this common problem affects the quality of life and work productivity.1 A large part of the chronic fatigue population meets the diagnostic criteria for chronic fatigue syndrome (CFS), which is defined by unexplained physical or mental fatigue of at least 6-month duration and a combination of nonspecific symptoms.2 Several CFS symptoms including impaired concentration, attention, and memory as well as headache suggest that the central nervous system (CNS) may be involved in CFS pathophysiology.3 Moreover, neuroimaging evidence indicates that structural and/or functional abnormalities exist in the CNS of CFS patients. For example, marked reductions in the gray matter volume are observed;4,5 Cook et al.5 found an association between subjective feelings of mental fatigue and brain responses during a fatiguing cognitive task; CFS patients always have reduced concentrations of N-acetylaspartate in the hippocampus, a putative marker of neuronal metabolism6; and a 23% reduction in 5-HT 1A receptor number and affinity was found in the hippocampus of CFS patients.7 However, neural mechanisms, especially the hippocampal substrates in CFS, are unclear in part due to the lack of a suitable animal model.

In our previous study, a chronic fatigue mouse model was induced by six repeated injections of fixed killed Brucella abortus antigen solution via the tail vein every 2 weeks.8,9 We found that brain atrophy and reduction of the hippocampal Bel-2 and brain-derived neurotrophic factor (BDNF) mRNA expression levels were accompanied by lower spontaneous activity.8 However, the hippocampal weight was not measured in those previous studies.

Resveratrol (RSV), an active component of grapevines and peanuts, is recognized as a polyphenolic activator of sirtuin 1 (Sirt1), a protein deacetylase.10 It can enhance running activity of high fat diet-fed mice two-fold by activating Sirt1.11 Moreover, Sirt1 can protect neurons from DNA damage-induced apoptosis by downregulating p53 acetylation, an apoptotic mediator.12 RSV treatment of neural progenitor cells (NPCs) is capable of increasing differentiation and directing neurogenesis through a mechanism requiring Sirt1.13 Thus RSV may be beneficial for physical activity of our fatigue mice and even play a role in improving structural and/or functional defects of the hippocampus.

We aimed to clarify the hippocampal structural and/or functional defects in this fatigue model. Another purpose of our study was to determine whether RSV intervention could improve the fatigue and reverse the abnormalities by anti-apoptosis and promoting neurogenesis.

MATERIALS AND METHODS

Animals, Living Conditions, and Spontaneous Running Activity Eight-week old BALB/c mice (female, 20–24 g, CLEA Japan, Tokyo, Japan) were housed singly in previously described cages8,9 including running wheels and counters. The cages were maintained under a light–dark photoperiod (lights on from 09:00 to 17:00), and daily spontaneous running activity defined by the number of wheel turns was measured at 09:00 when environmental lighting was turned on. Our research protocol was approved by the Animal Experimental Committee of Kanazawa Medical University.

Induction of Chronic Fatigue with B. abortus Antigen Chronic fatigue was induced by six repeated injections of B. abortus antigen solution (0.2 ml/mouse) via the tail vein every 2 weeks, as previously described.8,9

Experimental Design Thirty mice were randomly as-
signed to two groups after 2 weeks of housing, since their activity stabilized after 2–3 weeks of housing\(^{14}\): one was the model group, which was established with the above method \((n=22)\), and another was the normal group \((n=8)\). During the 12-week induction period, 7 mice died immediately the day after antigen injection. After fatigue induction, daily activity was monitored for 3 weeks to evaluate its stability, and model mice were randomly subdivided into the model \((n=7)\) and RSV groups \((n=8)\). The RSV group orally received RSV 0.1 ml \((Sigma, St. Louis, MO, U.S.A.)\) at a daily dose of 40 mg/kg through a soft feeding needle. The dosage and treatment course (4 weeks) were determined based on a previous report.\(^{15}\) RSV was suspended in 10 g/l of carboxymethyl cellulose \((Wako Pure Chemical Industries, Ltd., Osaka, Japan)\). The same volume of carboxymethyl cellulose was applied as a vehicle and administered to mice in the other two groups.

### Body Weight, Food Consumption, and Body Temperature

Body weight and food consumption were measured weekly from the start to the end of the experiment. Body temperature was also recorded every day from the third injection to the fourth injection of \(B. abortus\) antigen using a digital thermometer \((BDT-100, Bio-research Center, Osaka, Japan)\).

#### 5-Bromodeoxyuridine (Brd U) Labeling

To demonstrate whether RSV altered hippocampal neurogenesis, a number of proliferative cells incorporated with BrdU was evaluated. Before they were killed, all mice were injected intraperitoneally with a thymidine analogue BrdU marker \((Tokyo Chemical Industry Co., Ltd., Japan)\) at a dose of 60 mg/kg/d for 7 consecutive days. The incorporation was determined by immunohistochemistry with a BrdU in situ detection kit \((BD Biosciences, San Jose, CA, U.S.A.)\).

#### Hippocampal Tissue Sampling

The body weight of each mouse was measured at death. The whole brain was quickly isolated after decapitation and divided into two hemispheres along the cerebral longitudinal fissure. The right hemisphere was frozen in liquid nitrogen and then cryostat sections were prepared. The left hemisphere was weighed and then dissected to isolate the hippocampus on an ice-cold plate. The left hippocampus was immediately weighed, stored in RNAlater \((Ambion, Inc., Austin, TX, U.S.A.)\) at 4 °C overnight, and then stored at −20 °C until use. The ratio of hippocampal weight to body weight was calculated.

#### Preparation of Cryostat Sections

Cryostat sagittal sections were made from frozen blocks of the brain embedded in cryomold Tissue-Tek O.C.T. compound \((Sakura Finetech Inc., Tokyo, Japan)\). The frozen blocks were sliced sequentially using a cryostat microtome to prepare serial frozen-thin sections. These sections were mounted on 3-amino-propyltriethoxy-silane-coated microslide glass. The anatomic brain regions and brain areas were identified using a mouse brain atlas.\(^{16}\) The corresponding sections were maintained at −80 °C until use.

#### Apoptotic Morphology

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling \((TUNEL)\) \((Takara in situ Apoptosis detection kit, Takara-bio Inc., Otsu, Japan)\) assay was performed, and apoptotic nuclei in the hippocampus were identified using criteria previously proposed.\(^{17}\)

#### Cell Counting

The right hemisphere was sagittally sectioned at 16-μm thick. From the lateral to sagittal plane-midline, 15 slices located from 1.56 to 1.80 mm were processed for BrdU-labeling; 15 slices located from 1.80 to 2.04 mm were used for the TUNEL assay. All positive cells in BrdU-labeling and the TUNEL assay in the dentate gyrus \((DG)\) were counted under a light microscope with a 40× objective lens with a counting frame by a pathologist who was blinded to each group. The average count of cells calculated from all sections on BrdU labeling was considered the newborn cell count for each. The ratio of positive cell count in the TUNEL assay to total cell count in the DG was also calculated.

### Western Blot Analysis

To determine deacetylation of p53 by Sirt1 in the hippocampus, expressions of the hippocampal Sirt1, p53, and acetylated p53 were assayed using Western blotting. The other half of the left hippocampus was transferred from RNAlater into ice-cold buffer to lyse the tissue and briefly sonicated. Twenty minutes after placing in ice, the homogenates were centrifuged at 15000 \(g\) for 20 min at 4 °C, and the supernatants were collected. The protein concentration was measured using the Bradford assay, with bovine serum albumin as the standard. Equivalent amounts of protein for each sample were resolved in 7.5% \((\text{for Sirt1 and p53})\) or 10—20% \((\text{for acetylated p53})\) sodium dodecyl sulfate–polyacrylamide gels, blotted electrophoretically to immobilize, and blocked for 30 min in 5% skim milk \((\text{in phosphate buffered saline (PBS)-TWEEN-20})\). Blots were incubated with anti-Sirt1 antibody \((07-131;1:1000)\); Upstate Biotechnology, Inc., Waltham, MA, U.S.A.), anti-p53 antibody \((sc-6243-G;1:200)\); Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or anti-acetylated p53 \((Lys373)\) antibody \((06-916;1:1000)\); Upstate Biotechnology) overnight at 4 °C. \(\beta\)-Actin was used as a control. The secondary antibody used was stabilized goat anti-rabbit horse-radish peroxidase \((HRP)-\text{conjugated antibody (No. 1858415; Pierce, Rockford, IL, U.S.A.)}\). Bands were visualized with an enhanced chemiluminescence Western blotting analysis system and luminescent image analyzer. In some experiments, membranes were washed and re probed with anti-\(\beta\)-actin antibody as a control for equal loading. Densitometry of images was performed using ImageJ version 1.41 software \((\text{National Institutes of Health, Bethesda, MD, U.S.A.})\). Values for Sirt1, p53, and acetylated p53 immunoreactivity were normalized to \(\beta\)-actin immunoreactivity. The procedure was repeated at least three times for each protein in each sample.

### Statistical Analysis

Data are expressed as mean±standard deviation. A two-tailed \(t\)-test for independent samples was used for comparison of the normal and model groups before treatment. Differences were determined by one-way ANOVA followed by Bonferroni’s test for multiple comparisons. A \(p\) value less than 0.05 was considered statistically significant.
RESULTS

**RSV Enhanced Daily Activity of Fatigue Mice** At baseline, daily running activity was 16041 ± 2408 wheel turns in the normal group and 17593 ± 2176 in the model group. During the 12-week model preparation, the activity of model mice decreased gradually to 8293 ± 1356 wheel turns, which was significantly lower than that of the normal controls (p < 0.01). A distinctly lower level of activity was noted in the 3-week stability evaluation of model mice (p < 0.01). RSV therapy for 4 weeks was associated with a marked increase in activity, from 10148 ± 1891 to 12449 ± 1338 wheel turns, which was significantly higher than that of model mice, from 10285 ± 2052 to 9446 ± 1067 wheel turns (p < 0.05) (Fig. 1).

After each injection, both body weight and food consumption decreased significantly in the first week, while in the second week, they recovered. Body temperature was raised by 2—2.5 °C on the first day after injection of *B. abortus* and gradually decreased to the normal level within the first week. RSV treatment did not change body weight, food consumption, and body temperature significantly compared with the vehicle.

**RSV Improved the Hippocampal Weight of Fatigue Mice** As shown in Table 1, the left hippocampal weight was markedly decreased after repeated injections of *B. abortus*. In model mice, the ratio of the left hippocampal weight to body weight was significantly lower than that in normal mice. RSV treatment for 4 weeks enlarged the hippocampus of fatigue mice and increased the ratio of hippocampal weight/body weight.

**RSV Improved Neurogenesis and Inhibited Apoptosis in the Hippocampus, Especially in the DG** As shown in Fig. 2, after BrdU labeling, positive cells were mainly found in the subgranular zone of the DG. The number of newborn cells was significantly decreased in model mice, while RSV treatment increased it markedly in comparison to vehicle. A significant increase in apoptotic cells in the TUNEL assay was seen in model mice, and 4-week RSV therapy improved

![Figure 1](image1.png)

**Figure 1. Decreased Daily Running Activity in Chronic Fatigue Mice and Upregulation by Resveratrol Treatment**

Model mice exhibited significantly decreased daily running activity compared to that in normal control after six repeated injections. Hypoactivity was stable in the 3-week evaluation of the model group. Running activity was markedly increased by 4-week resveratrol therapy, even though it was still less than the normal level. Black arrows denote the injection of *B. abortus* antigen. Asterisk means that significance exists not only in the resveratrol group versus normal group but also in the model group versus normal group. w, weeks.

<table>
<thead>
<tr>
<th>n</th>
<th>Body weight (g)</th>
<th>Left brain weight (mg)</th>
<th>Left hippocampal weight (mg)</th>
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<tr>
<td>Normal group</td>
<td>8</td>
<td>21.6 ± 1.5</td>
<td>240.7 ± 7.6</td>
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<td></td>
<td></td>
<td>(1.12 ± 0.06%)</td>
<td>(0.11 ± 0.02%)**</td>
</tr>
<tr>
<td>Model group</td>
<td>7</td>
<td>23.0 ± 1.8</td>
<td>237.1 ± 10.2</td>
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<tr>
<td></td>
<td></td>
<td>(1.03 ± 0.06%)</td>
<td>(0.07 ± 0.01%)</td>
</tr>
<tr>
<td>RSV group</td>
<td>8</td>
<td>22.1 ± 0.8</td>
<td>250.9 ± 11.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.13 ± 0.04%)*</td>
<td>(0.12 ± 0.01%)**</td>
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Data are expressed as mean ± standard deviation. Parentheses show the ratio of each organ weight to body weight (g/g). * p < 0.05, ** p < 0.01 compared with the model group.

**Figure 2. Apoptotic and Neurogenetic Morphology on BrdU Labeling and in the TUNEL Assay**

DG progenitors on BrdU labeling were decreased in the subgranular zone in model mice and treatment with resveratrol increased the count of BrdU-positive cells in the DG, especially in the subgranular zone. Many apoptotic granular cells in the TUNEL assay were found in the DG, and resveratrol treatment inhibited apoptosis of granular cells. Scale bar = 100 μm. Blank, filled, and striated bars denote the normal, model, and resveratrol group, respectively. * p < 0.05, ** p < 0.01.
the apoptosis.

RSV Increased BDNF mRNA Expression in the Hippocampus Reduced BDNF mRNA expression was found in the hippocampus of fatigue mice. RSV treatment significantly increased the expression level of BDNF mRNA (Fig. 3).

RSV Downregulated the Level of Acetylated P53 in the Hippocampus We found that repeated injections of *B. abortus* did not induce a marked reduction in Sirt1 protein and its mRNA expression in the hippocampus, and administration of RSV upregulated its mRNA expression significantly, but not its protein (Figs. 3, 4). As shown in Fig. 4, acetylated p53 protein was increased significantly in the hippocampus of fatigue mice, and there was a notable decrease in acetylated p53 in RSV-treated mice. However, the level of total p53 protein did not change remarkably after the injection of RSV and RSV administration. Hence the level of acetylated p53 is more tightly linked with p53-dependent apoptosis compared with that of p53. Our model was mainly induced by the postinfective immune reaction and injection-based stress, both of which can lead to DNA damage and p53-dependent apoptosis. Hence the level of acetylated p53 is more tightly linked with p53-dependent apoptosis compared with that of p53. Our model was mainly induced by the postinfective immune reaction and injection-based stress, both of which can lead to DNA damage and p53-dependent apoptosis.

Fig. 3. Effects of Resveratrol on Relative Expression Levels of Sirt1, Mash1, and BDNF mRNA in the Hippocampus

A significant decrease in expression level of BDNF mRNA, but not of Sirt1 and Mash1 mRNA, was detected in the hippocampus of model mice. In addition, relative expression levels of Sirt1 and BDNF mRNA were significantly higher in resveratrol-treated mice than in model mice. Neither injections of *B. abortus* nor resveratrol treatment changed expression levels of Mash1 mRNA in the hippocampus significantly.

Fig. 4. Effects of Resveratrol on the Expression of Sirt1, p53, and Acetylated p53 Protein in the Hippocampus in Western Blotting

Resveratrol treatment increased Sirt1 protein expression in the hippocampus, although not significantly. The hippocampal p53 level was not changed after resveratrol treatment. On the other hand, the acetylated p53 level was increased significantly after 4-week resveratrol therapy. RSV, resveratrol.

**DISCUSSION**

Postinfective CFS is a disease model for investigating one pathophysiologic pathway. The most common symptoms in 21 patients with chronic brucellosis were fatigue, depression, arthralgia, and muscular pain. Clinical examination was largely negative, but lymphadenopathy was found. The fatigue condition induced by *B. abortus* antigen administration could be applicable to the CFS model. Moreover, acute physical or psychological stress may trigger CFS onset. Thus injection *via* the tail vein was selected as a special stress. Ottenweller et al. developed a CFS murine model using *B. abortus* injection. We confirmed that injections of *B. abortus* gradually decreased daily running activity and that the hypoaactivity persisted for at least 7 weeks after the last injection, which was evident by the measurement of hippocampal weight.

We found hippocampal atrophy in model mice, which was evident from the hippocampal weight. The infection-induced hippocampal atrophy, which is mainly ascribed to the neurotoxic effects of immune challenge including inflammatory cytokine response, was reported. Physical activity that is considered beneficial for mental health induces increased endogenous neurogenesis. There were an increased number of apoptotic granular and pyramidal cells in the TUNEL assay and a decreased number of newborn cells on BrdU labeling in the hippocampus in our study.

p53 plays a key role in the apoptosis of postmitotic neurons. In response to DNA damage, p53 undergoes phosphorylation, which increases its ability to recruit CBP/p300 acetyltransferase. p53 is acetylated by CBP/p300, which enhances sequence-specific DNA binding of p53 to activate transcription of its targets with proapoptotic properties. Hence the level of acetylated p53 is more tightly linked with p53-dependent apoptosis compared with that of p53. Our model was mainly induced by the postinfective immune reaction and injection-based stress, both of which can lead to DNA damage and p53-dependent apoptosis. In our fatigue mice, acetylated p53 expression was significantly enhanced in the hippocampus, and the local apoptosis was markedly increased as shown by the images in the TUNEL assay.

NPCs, especially in the DG, that are capable of continuous proliferation and neuronal differentiation are sources of hippocampal structural plasticity. They lie along the border between the hilus and granular cell layer, a two cell body-wide zone, the so-called subgranular zone, migrate into the granular cell layer, and differentiate into the glial and granular cells, resulting in the generation of several thousands of newborn cells each day. BDNF plays an important role in the proliferation, differentiation, and survival of NPCs and even in hippocampal plasticity. Its synthesis is stimulated in an activity-dependent manner. In some treatments of de-
pression, it is also used to mediate the biological response. Therefore we investigated whether the RSV-induced increase in newborn neuronal cells in fatigue mice was related to a change in BDNF mRNA expression in the hippocampus. Chronic stress is linked to downregulation of BDNF protein expression in the hippocampus, and chronic as well as acute stress can reduce hippocampal BDNF mRNA expression.

Neonatal infection and an immune challenge in adult rats is associated with impaired induction of BDNF in the hippocampus (CA1, CA3, and DG), especially following fear conditioning. Our established model is a combination of chronic/acute stress and postinfec tive immune challenge. Consistently with the above findings, the expression level of BDNF mRNA in the hippocampus in the fatigue model was decreased by repeated B. abortus injections. In the hippocampus, a number of positive cells on BrdU labeling were markedly reduced in the DG of model mice compared with that in normal mice, as previously described. In those in vivo studies, elevation of BDNF expression was associated with an increased number of positive cells on BrdU labeling in the hippocampus. Hence we conclude that repeated injections of B. abortus downregulate BDNF expression in the hippocampus, resulting in reduction of hippocampal proliferation/differentiation and subsequent atrophy.

RSV can enhance the running activity of high fat diet-fed animals two-fold by activating Sirt1. Sirt1 is a nicotinamide adenine dinucleotide-dependent histone deacetylase that downregulates acetylation levels of many regulatory proteins involved in cell survival, energy homeostasis, DNA repair, and life span extension. It is widely expressed in the CNS to promote its development. Sirt1 can protect cells from stress damage through deacetylating its targets. Sirt1 can deacetylate p53 at the Lys residues (Lys373 and/or Lys382) and protect various cells from DNA damage-induced apoptosis. Recently, Hasegawa and Yoshikawa have demonstrated that Sirt1 directly regulates p53 deacetylation in neurons under various developmental and neuropathologic conditions. However, we confirmed that the injections of B. abortus did not induce a significant reduction in Sirt1 protein and its mRNA expression in the hippocampus. In our fatigue model, it is necessary to determine in the future whether Sirt1 or other molecules deacetylating p53 contribute to the hippocampal abnormalities.

RSV has Sirt1-dependent and -independent effects on neurogenesis. Mash1 has recently been reported to be involved in Sirt1-dependent neurogenesis and play a role in the differentiation of NPCs. However, in our study, expression levels of Mash1 mRNA were not significantly changed either by injections of B. abortus or by RSV administration. It is necessary to determine whether Mash1 is involved in hippocampal pathology based on its protein expression. In another report, RSV induced neurogenesis by enhancing AMP-activated protein kinase (AMPK) in neurons and in Sirt1-deficient mice brain. AMPK activation stimulates neuronal differentiation as well as mitochondrial biogenesis in neurons. More cellular energy including ATP can be produced and neuronal activity is enhanced accordingly. More ATP produced by mitochondria can activate BDNF and synthesis of BDNF stimulated in an activity-dependent manner will increase. We observed more newborn cells and higher BDNF mRNA levels in the hippocampus in RSV-treated fatigue mice than in vehicle-treated mice. It is important to confirm whether these findings are associated with the AMPK activation in the future.

It is difficult to conclude definitely that RSV-induced improvement of hippocampal atrophy and pathologic change affected the spontaneous activity of fatigue mice. RSV increased the level of BDNF mRNA expression in the hippocampus in this study. On the other hand, infusion of BDNF into the DG or the CA3 pyramid cell layer does not influence the distance traveled or time spent in the center of an open field, showing that there is no general effect on locomotor activity. Previous studies showed that BDNF is beneficial for improvement of depression and fine motor and cognitive skills. During cognitive behavior therapy, fatigue-related cognition is challenged to diminish somatic attributions, improve the sense of control over symptoms, and facilitate behavioral change. Interestingly, a neuroimaging study in CFS patients indicated that cognitive behavior therapy is associated with a reversal of gray matter atrophy. All of these findings indicate that improved cognition is beneficial for enhancement of physical activity and recovery of the brain structure in CFS patients. In our CFS model, RSV reversed hippocampal atrophy and increased the level of cognition-related neurotrophin, BDNF, which may be one possible mechanism for increased physical activity.

In summary, we observed hippocampal atrophy in a CFS murine model induced by the injection of B. abortus and demonstrated that this atrophy was mainly due to augmented apoptosis and inhibited neurogenesis. RSV was effective in improving daily running activity. It could improve the atrophic hippocampus by repressing apoptosis and promoting neurogenesis, which may be one of the possible mechanisms in the recovery of daily running activity. Basic and pharmacologic studies are needed to elucidate other potential mechanisms.

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REFERENCES

8. Chen R., Moriya J., Yamakawa J., Takahashi T., Li Q., Morimoto S.,...