

# Differential Effects of Resveratrol on the Expression of Brain-Derived Neurotrophic Factor Transcripts and Protein in the Hippocampus of Rat Brain

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## What's Known

- Resveratrol induces the expression of the exon IX transcript of the *BDNF* gene in hippocampal tissues from male rats.

## What's New

- Resveratrol causes an increase in the expression levels of the brain-derived neurotrophic factor exons III, IV and IX but not the exon I in the hippocampi of rats.

## Abstract

**Background:** The induction of brain-derived neurotrophic factor (BDNF) expression in the hippocampus has shown to play a role in the beneficial effects of resveratrol (RSV) on the learning and memory. The BDNF gene has a complicated structure with eight 5' noncoding exons (I-IXa), each of which can splice to a common coding exon (IX) to form a functional transcript. Estrogens increase levels of BDNF transcripts in the hippocampus of rats. The aim of this study was to evaluate the effects of the phytoestrogen, RSV, on the splicing pattern of BDNF transcripts and on the pro-BDNF protein in the hippocampi of mother rats and their embryos.

**Methods:** RSV (60 or 120 mg/kg BW/day) was administered orally to pregnant rats from days 1 to 20 of gestation. Hippocampi of adults and embryos were dissected 24 h after the last administration of RSV. Extracts from hippocampi were subject to quantitative (q) RT-PCR and Western blotting to assess splicing pattern of the BDNF transcripts and levels of pro-BDNF protein, respectively.

**Results:** RSV (120 mg/kg BW/day) caused a statistically significant increase in the expression levels of BDNF exons III, IV and IX, but not the exon I in the hippocampi of adult rats ( $P \leq 0.05$ ). Levels of pro-BDNF protein remained unchanged in the hippocampal tissues from both adult and embryonic rats treated by RSV (60 or 120 mg/kg BW/day).

**Conclusion:** Our results showed that RSV differentially activates promoters of the BDNF gene in the hippocampus of pregnant rats, but fails to affect the pro-BDNF level neither in adult nor in the embryonic hippocampal tissues.

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**Keywords** • Resveratrol • Brain-derived neurotrophic factor • Hippocampus • Rat

## Introduction

Neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's are globally prevalent disorders that result in region specific neuronal loss and consequent movement and behavioral disorders. Dysfunction of learning and memory is one of the most prominent symptoms of Alzheimer's and

Parkinson's diseases.<sup>1</sup> One population that is prone to neurodegenerative abnormalities is the fetus. This feeble group may be exposed to insults like drugs, infection and trauma that may cause neurodegeneration and hence impair the development of CNS.<sup>2</sup> Induction of neurogenesis could be a new approach to overcome these symptoms.<sup>3</sup>

Brain derived neurotrophic factor (BDNF) is a growth factor that belongs to the nerve growth factor (NGF) family of peptides. This peptide has established effects on neuronal proliferation, regulation of neuronal function and synaptic plasticity. BDNF has shown important roles in learning and memory by enhancing the efficacy of synapses in the hippocampus.<sup>4</sup> The BDNF gene in the rat contains eight 5' noncoding exons (I-IXa), each with a separate promoter and one 3' coding exon (IX) that comprises the entire open reading frame for BDNF protein. The expression pattern of BDNF gene in rodents is complex in that splicing of each of the eight noncoding I-IXa exons with the coding exon IX creates 10 transcript variants that all are translated to one same mature protein.<sup>5</sup> Since the discovery of these noncoding exons, many studies have been conducted to elucidate their function. Although roles like effects on transcription regulation and stability,<sup>6</sup> translation efficiency,<sup>7</sup> and differential regulation of BDNF expression in the somatic or dendritic compartments<sup>8</sup> were attributed to the untranslated regions of the BDNF gene, but their exact character is yet vague. A few studies have also reported the differential expression of noncoding exons I to IXa in response to some therapeutic/toxic factors.<sup>9</sup>

Resveratrol (RSV) is a phytoestrogen with antioxidant activity and neuroprotective features<sup>10</sup> that can pass blood brain barrier<sup>11</sup> and placenta.<sup>12</sup> Parts of the neuroprotective effects of resveratrol have been attributed to its antioxidant properties. However, the compound has also shown to exert neuroprotection via other mechanisms. Thus, neuroprotective effects of resveratrol have been shown to be linked to increased neuronal survival through induction of SIRT1 activity and subsequent decreases in neuroinflammation and apoptosis.<sup>10</sup>

A growing body of data shows defective BDNF signaling in the neurodegenerative disorders.<sup>13</sup> Therefore, synaptic repair by BDNF is a new approach in the treatment of neurodegenerative disorders.<sup>14</sup> Endogenous production of BDNF may be a useful and noninvasive way to induce neurogenesis and promote synaptic repair and neuronal function improvement. We have already shown that oral resveratrol induces the expression of the

exon IX transcript of the BDNF gene in rat hippocampal tissue.<sup>15</sup> To better characterize this effect of RSV, we sought to determine its differential effects on the expression of exons I, III, and IV and the common exon IX of the BDNF gene in the hippocampi of adult rats. Further, we attempted to define any difference between the effects of RSV on the hippocampal expression of BDNF protein in adult and neonatal rats.

## Materials and Methods

### Animals

Thirty six female Sprague-Dawley rats weighing 200-250 g were provided by the laboratory animal center of Shiraz University of Medical Sciences. Every manipulation of rats before decapitation was done in the laboratory animal center of Shiraz University of Medical Sciences in February 2013. Rats were mated and after detection of vaginal plaque, i.e, the first day of pregnancy, were divided into 3 groups of 12 as follows, (i) the control group that were gavaged with ethanol 10% as the vehicle, (ii) RSV 60 mg/kg BW/day, and (iii) RSV 120 mg/kg BW/day groups that were treated orally with resveratrol (98% purity from Biotivia, USA). Rats had free access to water and food in a 12-h cycle of light and darkness. Oral administrations were started on day 1 of pregnancy and continued until day 20 at 9 am daily. 24 h after the last gavage, all rats were sacrificed by CO<sub>2</sub> inhalation and hippocampal tissues were dissected from both mothers and embryos. Hippocampi from 4 adult rats and hippocampi from embryos of 4 mothers, in each group were pooled together to make one specimen. In the case of adult rats, the right and the left hippocampi were collected separately and allocated to the analysis of mRNA and/or protein, respectively. In the case of embryos, half (2-4) of the embryonic hippocampi of each mother were collected for mRNA analyses and the same number of tissues were pooled for protein assay. All procedures were approved by the Animal Ethics Committee of Shiraz University of Medical Sciences.

### RNA Extraction and cDNA Synthesis

Right-hippocampal specimens from adult rats and whole hippocampi from half of the embryos were collected in Biozol reagent (BioFlux, Japan) immediately after dissection and kept in -80 °C until RNA extraction. RNA extraction was done by the Biozol kit based on manufacturer's protocol and the samples were analyzed by spectrophotometry to define their concentration and purity. The integrity of RNAs

was checked by denaturing gel electrophoresis.<sup>16</sup> After treatment with DNase I (Fermentas, EU) to remove any DNA contamination, cDNA synthesis was carried out using 5 ug of RNA and 1 ul of oligo dT with Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Fermentas, EU). All procedures were done based on the manufacturer's protocol.

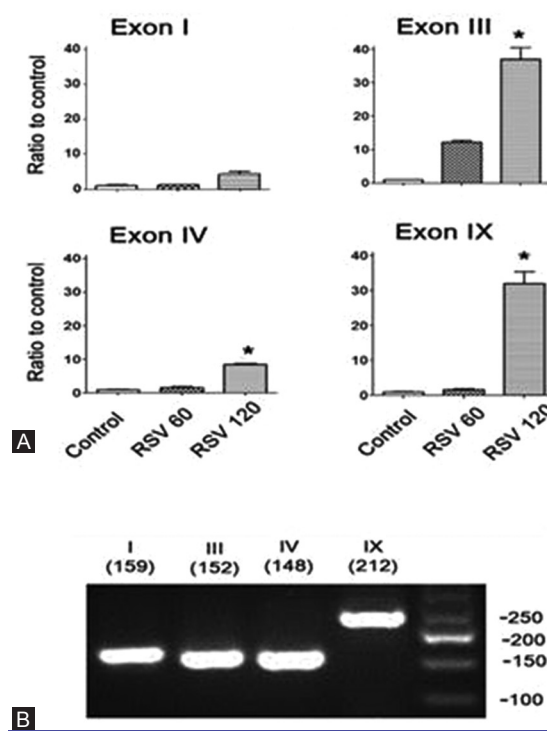
**Quantitative Real-Time RT-PCR**

We performed qRT-PCR as described previously.<sup>17</sup> Primer sequences are shown in Table 1. The interested cDNAs were amplified under the following conditions: an initial denaturation at 95 °C for 30s, followed by 40 cycles of 95 °C for 5s, annealing and extinction at 60 °C for 30s. Hypoxanthine guanine phosphoribosyltransferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping gene and data were normalized relative to the expression level of the HPRT gene. All PCR reactions were run in duplicate. The ratio of expression level of BDNF exons was calculated by  $\Delta\Delta CT$  method.<sup>18</sup> The quality and correct size of the PCR products were checked by electrophoresis on 1.6% agarose gels (Figure 1B).

**Protein Extraction and Western Blotting**

Left-hippocampal specimens from adult rats and whole hippocampi from half of embryos were frozen in liquid nitrogen immediately after dissection and kept in -80 °C until protein extraction. Protein extraction was done using extraction buffer containing NP40 lysate buffer and protease inhibitor cocktail (Sigma-Aldrich) as proposed by the manufacturer. Western blot analysis was performed as previously described.<sup>19</sup> Briefly, each homogenized sample was sonicated 3 times for 5s, and then centrifuged for 10 min at 10,000 ×g at 4 °C and the supernatant was collected. Protein concentration was measured by Bradford method and 40 ug of each sample was fractionated on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane at 100 V for 90 min (Mini Trans-Blot Cell Bio-Rad, USA). Membranes were blocked in 5% skimmed

milk in 0.2% TBST for 1 h at room temperature and then membranes were incubated with either a 1:250 titer of the polyclonal rabbit antiserum against BDNF (sc-546, Santa Cruz, USA) or a 1:500 dilution of the anti β-actin antibody (ab1801, Abcam, USA) in 1% skimmed milk-TBST at 4 °C overnight. This procedure was followed by washing the membrane 3 times for 20 min by 0.2% TBST and treatment with the secondary antibody; 1:7500 goat anti-rabbit IgG: HRP antibody (Aviva System Biology, USA) for 60 min at room temperature. After 3 times of washing with 0.2% TBST for 20 min, the



**Figure 1:** Expression of mRNAs containing transcripts of exons I, III, IV and IX of the BDNF gene in the adult rat hippocampus. A) qRT-PCR analysis of the expression of exons I, III, IV and IX in animals treated by either ethanol 10% (as a vehicle) or RSV for twenty days from day 1 to day 20 of gestation. Data are presented as Mean±SEM. Asterisks show statistical significance at P<0.05. B) Electrophoresis of the qRT-PCR products of exons I, III, IV and IX of the BDNF gene in representative cases. The presence of PCR products with predicted lengths of about 159 bp, 152 bp, 148 bp and 212 bp, respectively, for exons I, III, IV and IX of the BDNF gene was confirmed.

**Table 1:** Sequences of primers used for qRT-PCR amplification of transcripts of interest

Gene name	Primer forward (5' to 3')	Primer reverse (5' to 3')
BDNF EXON I	TGTTGGGGAGACGAGATTTT	CGTGGACGTTTGCTTCTTTTC
BDNF EXON III	CTGAGACTGCGCTCCACTC	GTGGACGTTTGCTTCTTTCA
BDNF EXON IV	GAGCAGCTGCCTTGATGTTT	GTGGACGTTTGCTTCTTTCA
BDNF EXON IX	GTGACAGTATTAGCGAGTGGG	GGGTAGTTCGGCATTGC
HPRT	CCCAGCGTCGTGATTAGTGA	TGGCCTCCCATCTCCTTCAT
GAPDH	CGTGATCGAGGGCTGTTGG	CTGCTTCAGTTGGCCTTTTCG

membrane was developed by homemade ECL (250 mM 3-aminophthalhydrazide (luminol), 90 mM p-coumaric acid, 30% H<sub>2</sub>O<sub>2</sub> and 100 mM Tris, pH 8.6, and 13×18 AGFA X-ray films). Intensities of bands were quantified by AlphaEaseFC software. The level of pro-BDNF protein of each sample was normalized to its cognate  $\beta$ -actin and fold change of BDNF in the treatment groups were calculated relative to the control group.

#### Statistical Analyses

Results were expressed as Mean $\pm$ SEM and nonparametric analysis of variance (Kruskal-Wallis test/Mann-Whitney U test with Bonferroni correction) was performed to diagnose any differences between groups. Whenever significant differences were detected between groups by Kruskal-Wallis, Bonferroni multiple comparison test was used as post hoc. All analyses were done using SPSS (PASW statistic 18) and  $P < 0.05$  was considered to indicate significance.

## Results

#### *mRNA Expression of BDNF Exons I, III, IV and the Common Exon IX in the Hippocampi of Adult Rats*

Real-time RT-PCR was performed on the adult rat hippocampus samples to assess the expression levels of mRNAs containing transcripts encoded by any of the exons I, III, IV and IX of the BDNF gene. As shown in the Figure 1, RSV (120 mg/kg BW/day) increased the levels of BDNF exons I, III, IV and the common exon IX as compared to the control group. This increase was statistically significant with respect to exons III ( $P=0.03$ ), IV ( $P=0.03$ ) and IX ( $P=0.04$ ), but not to the exon I ( $P=0.06$ ) (Kruskal-Wallis test/Mann-Whitney U test with Bonferroni correction). RSV at the dose of 60 mg/kg BW/day tended to increase the levels of transcripts of the exons tested in this study, but the effect failed to achieve significance (Kruskal-Wallis test/Mann-Whitney U test with Bonferroni correction,  $P \leq 0.05$ ) under our experimental conditions.

#### *mRNA Expression of BDNF Exons I, III, IV and the Common Exon IX in the Hippocampi of Embryonic Rats*

We tried to evaluate the effects of RSV on the expression levels of the above exons in the hippocampi of embryos in the RSV treated rats. However, we were not able to do so because mRNA expression of all of the three housekeeping genes we used showed strong

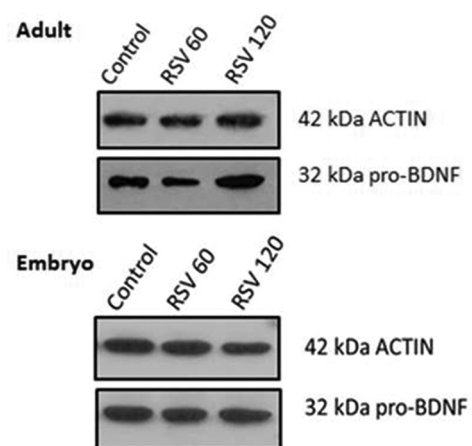
changes in the RSV (60 mg/kg BW/day) treated group when compared with the control group. The housekeeping genes we used were, HPRT and GAPDH.

#### *Analyses of Pro-BDNF Protein in the Hippocampus of Adult Rats and Their Embryos in Response to Resveratrol*

We did Western blotting to analyze levels of pro-BDNF protein in the hippocampi of adult and embryonic rats in response to two different doses of RSV. As depicted in Figure 2, RSV (120 mg/kg BW/day) increased the levels of pro-BDNF protein in the hippocampi from adult rats, an effect that was not statistically significant (data not shown). Levels of pro-BDNF protein in the hippocampal tissues of embryos from rats treated by two doses of 60 and 120 mg/kg BW/day of RSV also remained unchanged as compared to control specimens (Figure 2).

## Discussion

We have used transcript-specific primers of the BDNF gene to show that hippocampal levels of BDNF transcripts containing different 5' exons are differentially affected by oral resveratrol. Recently, increasing body of data is pointing to the idea that changes in the splicing pattern of BDNF exons may be a new molecular mechanism of diseases.<sup>20</sup> These data include those concerning the association of specific BDNF exon expression with neurological disorders,<sup>21,22</sup> and those on the pharmacological manipulations that affect the exon specific expression of BDNF in the rodent tissues.<sup>7,22</sup>



**Figure 2:** Expression pattern of pro-BDNF protein in hippocampi from adult rats and their embryos. Anti BDNF antibody used in immunoblotting assays against extracts from hippocampal tissues, recognized a protein band of ~32 kDa for pro-BDNF as expected. The antibody against  $\beta$ -actin revealed the expected 42 kDa band of the actin protein.

RSV has been earlier reported to induce the level of the mRNA encoded by the exon IX of the BDNF gene in the hippocampus of naive adult male<sup>15</sup> and depression model of rats.<sup>23</sup> Here, we have extended these studies to the effects of the phytoestrogen, resveratrol, on the expression pattern of BDNF transcripts containing exons I, III, IV and IX in the hippocampi of female rats. Notably, sex hormones like estrogens have shown to strongly affect the expression and activity of BDNF through both transcriptional and translational mechanisms.<sup>24</sup> Our data are not sufficient to clarify the extent to which RSV has acted through estrogen receptors to produce the effects observed in this study.

Resveratrol has shown a spectrum of beneficial effects against neurodegenerative diseases.<sup>10</sup> It protects neurons through multiple mechanisms like free radical scavenging, anti-inflammatory effects and modulation of programmed cell death and longevity via apoptosis inhibiting factor and SIRT1, respectively.<sup>10</sup> Additionally, RSV can work as an estrogen receptor agonist<sup>25</sup> to show its neuroprotective features. Our findings show that RSV increases the expression levels of BDNF transcripts containing exons III, IV, and IX in a dose dependent manner, but fails to affect the expression of BDNF transcript containing exon I. This effect of RSV may either be attributed to its phytoestrogenic structure, as has been mentioned earlier that estrogen upregulates BDNF gene expression and activity,<sup>24</sup> or back to its effect on gene transcription via upregulation of TCF-Egr-1 transcription factors.<sup>26</sup> Exons I and IV show respectively 95% and 91% homology in rodents and humans.<sup>27</sup> These exons have shown to be the most inducible BDNF transcripts in response to KCl-mediated membrane depolarization in embryonic cortical neuron cultures and to kainite treatment.<sup>28</sup> While exons III and IX showed to be most highly induced by RSV under our experimental conditions. Differential expression of the BDNF exons has also been shown in other experimental systems.<sup>6,29</sup>

Despite the inducing effects of resveratrol on levels of the mRNAs encoded by the coding BDNF exon IX as well as the non-coding exons III and IV of the BDNF gene, pro-BDNF protein showed a small (not significant) increase in this study. The inconsistency between levels of BDNF protein and its encoding mRNA has also been observed in rats treated by estrogens.<sup>30</sup> These animals showed an increase in their hippocampal levels of BDNF mRNA that was accompanied by a significant decrease in the BDNF protein.<sup>30</sup> Further, Perovic et al.

observed accumulation of pro-BDNF in the hippocampus of aged rat with no increase in the mRNA level of BDNF.<sup>31</sup> While exon I is reported to change remarkably in response to some treatments,<sup>5,32</sup> it failed to show any significant changes under treatments applied in this study. On the other hand, it has been reported that an exon I specific translation start site more efficiently contributes to the synthesis of BDNF protein than the common translation start site located downstream of the splicing site of exons III, IV and IX.<sup>33</sup> Our results show a slight non-significant increasing effect of RSV on the expression of the BDNF transcript containing exon I. This data is reminiscent of the changes we observed in the level of pro-BDNF protein. However, it is noteworthy that genes of regulation as those of BDNF usually show poor mRNA-protein expression correlation, a topic that are discussed elsewhere in the literature.<sup>34</sup>

Our study is the first on the effects of a phytochemical on the expression pattern of BDNF exons in the rat tissues. Results of the present work are in line with the published data on the increasing effects of RSV on the expression of hippocampal levels of the exon IX containing BDNF transcripts in the naive rats<sup>15</sup> and/or in the rats exposed to chronic unpredictable mild stress.<sup>35</sup> Comparable results were also obtained when intraperitoneal RSV was tested in mice models of depression.<sup>23</sup>

When testing effects of RSV on the expression pattern of exons of the BDNF gene in the rat embryonic hippocampi, we were encountered with dramatic changes in the expression of housekeeping genes. Although, we tried three different housekeeping genes reported to be the most stable ones,<sup>36</sup> but all of them showed instability in our RSV administered groups. The reason is speculated to be that levels of many housekeeping genes change during progression through the cell cycle. Hence, we are not able to present our data on the expression of exons of BDNF in the embryonic rat hippocampus. More housekeeping genes need to be tested to find the one that shows stability under the experimental conditions of this study. At the protein level, we found no significant changes in the expression of pro-BDNF protein between control and RSV treated embryos. There is no report in the literature on the effects of RSV on the embryonic expression of BDNF.

However, RSV has shown to improve the brain expression of BDNF in the postnatal day 40 rats whose mothers have been subject to restraint stress during gestational period.<sup>37</sup> Promoters of the BDNF gene have also shown to be a

subject of epigenetic modifications.<sup>38</sup> Therefore, possible effects of RSV on the histone code and DNA methylation need further investigation.

Although, results of most of the studies are in line with the neuroprotective effects of RSV, but some adverse effects of the compound have also been presented recently. Thus, Park et al. reported the antiproliferative effects of RSV on the neuronal progenitor cells as well as the inhibitory effects of the phytochemical on hippocampal neurogenesis. These findings that are in contrast with the established neuroprotective effects of RSV may back to the dose of treatments, as RSV in low doses did not produce any inhibitory effect in the same work.<sup>39</sup> These data reflect the importance of fine-tuning of RSV doses when used as a therapeutic agent. This study can be extended further to evaluate the effects of RSV on the expression of other non-coding exons of the BDNF gene as well as to assess the levels of the BDNF protein in both adult and embryonic rats.

### Conclusion

Taken together, our data imply that promoters related to BDNF exons III, IV, and IX may serve as points of regulation for transcription of the BDNF gene in the rat hippocampus. In addition, our findings indicate that RSV mimics effects of estrogen on the upregulation of BDNF expression.

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**Conflict of Interest:** None declared.

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