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Original Article

Nrf1 and Nrf2 Knockdown Effect in Anxiety-related Behavior and Mitochondrial Function

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Abstract

Introduction: Nuclear factor, erythroid-derived 2, -like2 (Nrf2) and Nuclear erythroid 2-related factor 1 (Nrf1) stand as two important regulators of antioxidant defense system.

Materials and Methods: Small interfering RNA (siRNA) targeting Nrf1 and Nrf2 (Nrf1&2) was injected in dorsal third ventricle of adult male albino Wistar rats. Anxiety-related behaviors and protein level of mitochondrial biogenesis, apoptotic marker factors and also electron transport chain (ETC), Citrate synthase (CS) and Malate dehydrogenase (MDH) enzymes activity in three brain regions: hippocampus, prefrontal cortex, and amygdala were evaluated.

Results: Nrf1&2-silenced rats induced anxiety-like behaviors compared to the control group. The level of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) protein increased in those three regions. Although Nrf1&2-silencing decreased MDH activity in hippocampus and prefrontal cortex, the activity of CS was increased in all three mentioned areas. However, Nrf1&2 silencing had no effect in complex I and II-III activity, but complex IV activity was increased, particularly in amygdala. Furthermore, Bax/Bcl2 ratio and cleavage of caspase-3 was increased in all mentioned areas of the brain in Nrf1&2-silenced group.

Conclusion: In conclusion, the presented data evaluated the complexity of mitochondrial functions and Nrf1 and Nrf2 in rat's brain and points to mitochondrial crucial role in oxidative stress, energy metabolism, and behavior.

Keywords: Anxiety, Nrf1 , Nrf2, siRNA, Apoptosis

1. Introduction

According to Bouayed et al. (2009) anxiety is a class of human psychiatric disorder, which involves about one-eighth of the whole world population. Stress related hormones could cause structural and functional changes in hippocampus [1]. Different parts of brain are involved in anxiety including an excitatory projection to basal forebrain, hypothalamus and brainstem structures [2]. In addition, prefrontal cortex, which is involved in responding to threatening motives, is severely activated through stress [3].

Stressful environment changes activities of cells including antioxidant defense system. [4]. Mitochondria are the most important energy producing sites; these organelles are main intracellular sources of reactive oxygen species (ROS) [5]. Anxiety can

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elevate the expression levels of electron transport chain (ETC) complexes, devastate antioxidant defense system and disrupt mitochondrial transport processes [6]. Consequently, extreme and sustained stress can lead to mitochondrial dysfunction and activate mitochondrial-mediated apoptotic pathway. Some proteins participate in apoptotic pathways, such as Bax and B cell lymphoma 2 (Bcl2) which are pro- and anti-apoptotic molecules, respectively; moreover, Bcl2 modulates specific anxiety features are restricted to particular brain regions, such as hippocampus and prefrontal cortex. Anxiety-like behaviors increase the consequence of deletion of Bcl2 gene which may not be correlated to Bcl2 anti-apoptotic effects [6].

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcription coactivator that plays an essential role by stimulating mitochondrial respiration and turnover. Likewise, PGC-1α interacts with a broad range of transcription factors, including Nuclear respiratory factor-1 (NRF-1), (NRF-2), and mitochondrial transcription factor A (TFAM) [7,8]. Under oxidative stress conditions, PGC-1α increases some ROS-detoxifying proteins, including glutathione peroxidase, catalase, and superoxide dismutase (SOD) [9].

Citrate synthase (CS) is an enzyme, which exists in nearly all living cells, with a role as a peacemaking in the first step of the citric acid cycle [10]. Another enzyme, which stands as a catalyzer in the citric acid cycle, is named Malate dehydrogenase (MDH): its role is to catalyze the interconversion of malate to oxaloacetate.

Nuclear factor, erythroid-derived 2,-like 1(Nrf1) and nuclear factor, erythroid-derived 2,-like 2 (Nrf2) are members of the NF-E2 basic leucine zipper family of proteins which interact with the antioxidant response element (ARE) [11]. In addition, Nrf1 and Nrf2 have some physiological activities, such as development and inflammation; they participate in transcription of proteasome subunits and induction of auto-phagosome formation [12].

It has been shown that knockdown of Nrf2 by short interfering RNA molecule (siRNA) significantly increases anxiety-like behavior in rats. [13]. It is worth to note that this evidence was associated with mitochondrial dysfunction and neuronal apoptosis in hippocampus, amygdala and prefrontal cortex. This study aimed to silence Nrf1 and Nrf2 by intra-dorsal third ventricle (D3V) siRNA injection in order to investigate the effect of this silencing on anxiety and on the apoptotic markers in the three brain regions including hippocampus, prefrontal cortex and amygdala. Furthermore, CS and MDH and ETC activities were detected.

2. Materials and Methods

2.1 Reagents

Anti-Nrf1, Nrf2 and PGC-1α antibodies were made by ABCAM (Cambridge, UK). NRF-1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrochemiluminescence (ECL) kit was purchased from Amersham Bioscience (Piscataway, USA). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Billerica, MA, USA). All other reagents were made by Sigma Aldrich (St. Louis, USA).

2.2 Animals and Experimental Design

Male albino rats of Wistar strain were bought from Pasteur Institute (Tehran, Iran), with weight of about 200-250 g. Three groups were allocated to this experiment: control group 1 who received intra-D3V injection of 5 µl scrambled siRNA (note that scrambled siRNA has no homology to any known mammalian gene); control group 2 who received 5 µl RNase-free water in their D3V; and third group which received intra-D3V injection of 5 µl Nrf1-siRNA and 5 µl Nrf2-siRNA (5 nmol siRNA/200 µl RNase-free water). Each experimental group mentioned above were divided into two groups: one was decapitated, 4 h after

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siRNA injection, and second one was decapitated 8 h after injection.

2.3 Stereotaxic Surgery and siRNA Administration in Rat Brain

Rats were anesthetized through an intraperitoneal Ketamine Hydrochloride (50 mg/kg) and intraperitoneal injection of xylezine (4 mg/kg) and placed in stereotaxic apparatus. Next, guide cannula was placed in their D3V (anteroposterior: –0.5 mm relevant to bregma, mediolateral: 0 mm, and dorsoventral: –3 mm from the skull surface) [14 To knock down Nrf2 in the rat's brain, Silencer Select predesigned siRNA specific to Nrf2: 5′-GCUGAACUCCUUAGACUCAtt-3′ (ID: s136127) was obtained from Ambion (Austin, TX, USA) and scrambled siRNA as a control was obtained from QIAGEN (Germany): 5′-UUCUCC GAACGUGUCACGUdTdT-3. To knockdown Nrf1 in the rats' brain, Silencer Select pre-designed siRNA specific to Nrf1: 50-CAACCUGCCUGUAGAAGAAtt-30 (ID: s165931) was obtained from Ambion (Austin, TX, USA) and scrambled siRNA as control was obtained from Qiagen (Germany): 50 UUCUCCGAACGUGUCACGUdTdT-30.

2.4 Behavioral Testing by Elevated Plus Maze (EPM)

As described before, EPM test has been founded on creating a conflict between the rats' drive and its innate fear of opened and exposed areas [15]. It has been documented that the apparatus of EPM test has two open arms (50-cm long, 10-cm wide, 0.5-cm high borders) and two closed arms (50-cm long, 10-cm wide, 40-cm high walls), about 50 cm above the ground [16]. The maze was in a separated room (the maze was placed in rodent behavioral assessment room). The experimenter did not make any noise and movement. At first, rats were placed into the central area (10 × 10 cm), facing the open arm and observed for 5 min. Then, rats were returned to their home cages. Percentage of time spent in open arms [%OAT: (time in open arm/time in "open and closed" arm) × 100] of all the alliances during the 5-min exposure were considered as an index of anxiety. In addition, the whole number of enclosed arm entries were considered as a measurement of locomotor activity. Four hours after injection, all groups were examined for anxiety related behavior by EPM.

2.5 Western Blotting

The protein extraction buffer was used to extract the protein tissues from hippocampus, prefrontal cortex, and amygdala and then were centrifuged at 4000 × g at 4 ˚C (Note that, the protein extraction buffer contains protease inhibitor cocktail) [17]. According to Bradford's method, the protein content of the supernatant was purified using bovine serum albumin as a reference standard [18]. In the next step, 60 µg of total protein of each sample was transferred to sodium dodecyl sulfate-polyacrylamide (SDS) PAGE separation. After an extensive washing, using enhanced chemiluminescence method, results were visualized by immune-reactivity. The bands were analyzed by film scanning. Densitometry analysis was also performed and the results were analyzed by ImageJ software.

2.6 Analysis of ETC Complexes Activities through Spectrophotometer, Complex I Activity Assay

Mitochondria was isolated according to the method described by Clarke and Nicklas [19]. Also, NADH ubiquinone reductase activity was measured by NADH oxidation assay according to Birch-Machin's method [20]. Through this method, decylubiquinone was used as the electron acceptor for determination of complex I activity. The assay medium (KH_2 PO4 35 mM, $MgCl_2$ 5 mM, KCN 2 mM, pH 7.2) was supplemented with decylubiquinone 65 mM and NADH 10 mM. The reaction was initiated by the addition of sample and the

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oxidation rate of NADH was measured at 340 nm.

2.7 Complex II-III Activity Assessment

The process of complex II-III was performed in medium buffer containing Tris-HCL (50 mM), succinate (2mM), and dodecylmaltoside (1 %). The reaction was started by the addition of cytochrome c (100 mM). According to Veereshwaraya 's method, the changes in complexes activity were monitored at 550-nm wavelength [21].

2.8 Complex IV Activity Determination

Complex IV activity investigation was done through Rustin et al.'s method [22]. The medium buffer was composed of potassium phosphate 40 mM at pH 7 and isolated mitochondria. Furthermore, the reaction was started by the addition of reduced cytochrome c. The oxidation of cytochrome c was measured spectrophotometrically at 550 nm (37 ˚C) according to Rustin et al.'s method [22].

2.9 Assessment of CS Activity

According to method described by Craig, CS activity was measured [23]. Protein extract was added to assay buffer containing Triton X-100, Tris-HCL, acetyl-CoA, 5-5´-dithiobis (2-nitrobenzoic acid) and oxaloacetate. At last, alterations were recorded in the absorbance at 412 nm wavelength.

2.10 Measurement of MDH Activity

Protein extract was added to the mixture, composed of oxaloacetate, Tris-HCL and NADH. Absorbance was recorded at 340 nm spectrophotometrically [23].

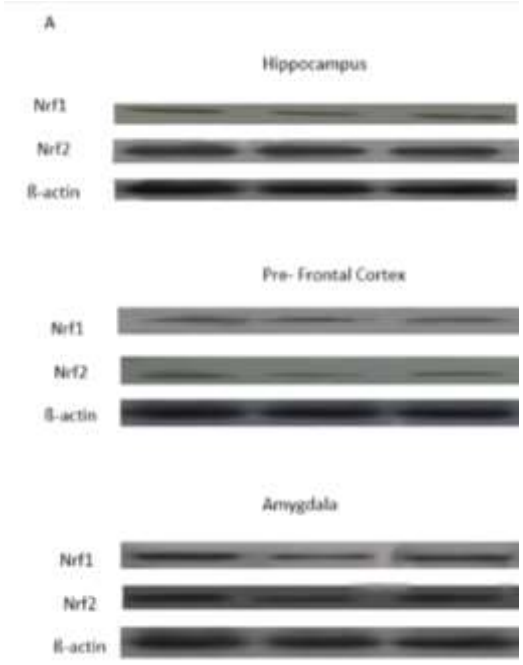
2.11 Ethical code

This research was supported financially by Iran National Science Foundation (INSF, Grant Number was 91003013).

2.12 Data Analysis

Each experiment was repeated at least three times. Mean ± SEM (standard error of mean) was used in order to express the data, which was processed by Graph Pad Prism® 5.0. One-way analysis of variance (ANOVA) and post hoc analysis Turkey's were used to analyze Enzymatic and Western blot data. P value less than 0.05 ($P < 0.05$) was considered as statistically significant.

3. Results



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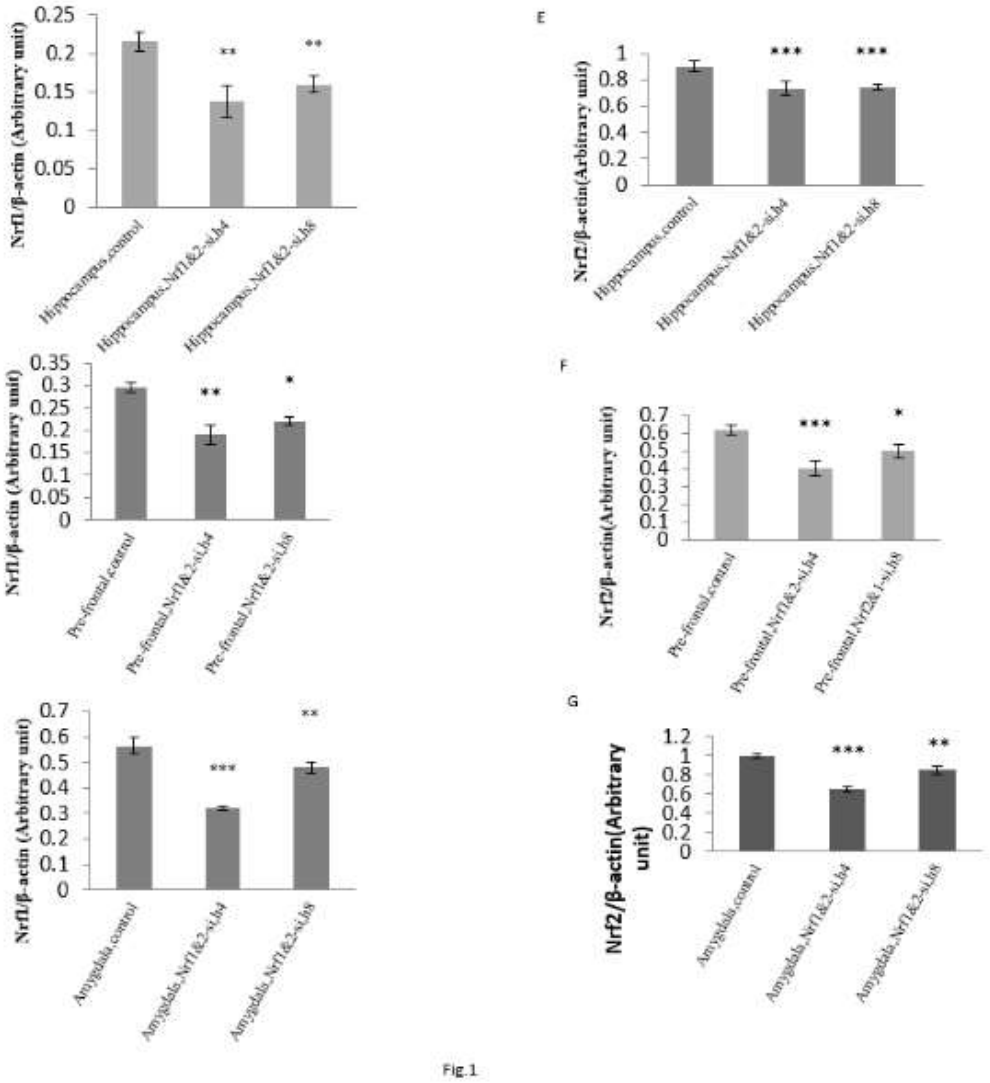

Fig.1

Figure1b–d. siRNA-mediated down regulation of Nrf1 and Nrf2 in 3 areas of rat's brain. Evaluation of protein level (Western blotting) after direct in *vivo* injection of scrambled siRNA (control) and Nrf1&2 siRNA in hippocampus(**b**), prefrontal cortex(**c**), and amygdala(**d**). We separated Sixty µg proteins on SDS-PAGE, Western blotted, probed with anti Nrf1 and Nrf2 antibody, and reprobed with anti-β-actin antibody (one representative Western blot was shown). The densities of corresponding bands were calculated and the ratio to β-actin bands was reported. Fig.1e–g siRNA-mediated a decrease in Nrf2 in all three brain regions. We assessed the protein level (Western blotting) after *in vivo* injection of scrambled siRNA (control) and Nrf1&2 siRNA in three mentioned areas of rats' brain. *P < 0.05; **P < 0.01; ***P < 0.001 compared to the control group

Nrf1 and Nrf2 mRNA Knockdown Significantly Reduced their Protein Level in the Brain

The level of Nrf1 and Nrf2 was assessed by western blotting, 4 and 8 hours after Nrf1&2-siRNA injection. According to Fig. 1b–g, protein level of Nrf1 and Nrf2 was reduced in consequence of siRNA injection in three brain regions (hippocampus, prefrontal cortex and amygdala). The most reduction in Nrf1 and Nrf2 protein level was in Amygdala, 4 h after siRNA injection compared to the control group.

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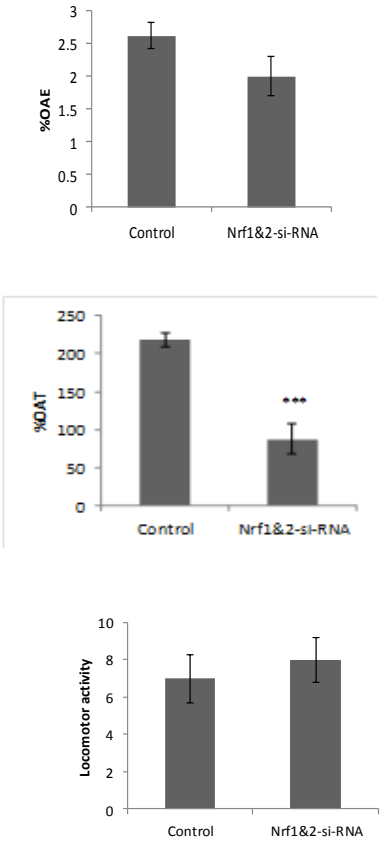
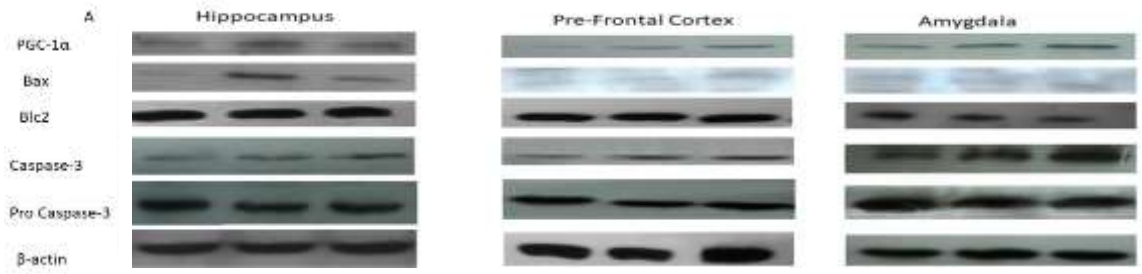


Figure 2. Effect of Nrf1&2-siRNA administration on rat anxiety-like behavior in the EPM. The animals received scrambled siRNA or Nrf1&2 into D3V. Mean ± SEM of a percentage of open arm time, b percentage of open arm entries, c locomotor activity

Nrf1&2 Silencing Induced Anxiety-Related Behavior in Rats

In order to understand more about the molecular basis of anxiety-related behavior, targeted genes were knocked down by siRNA. Our data showed that in the group that received Nrf1&2-siRNA, the %OAT decreased. %OAE and locomotor activity did not change significantly between experimental groups (fig. 2).



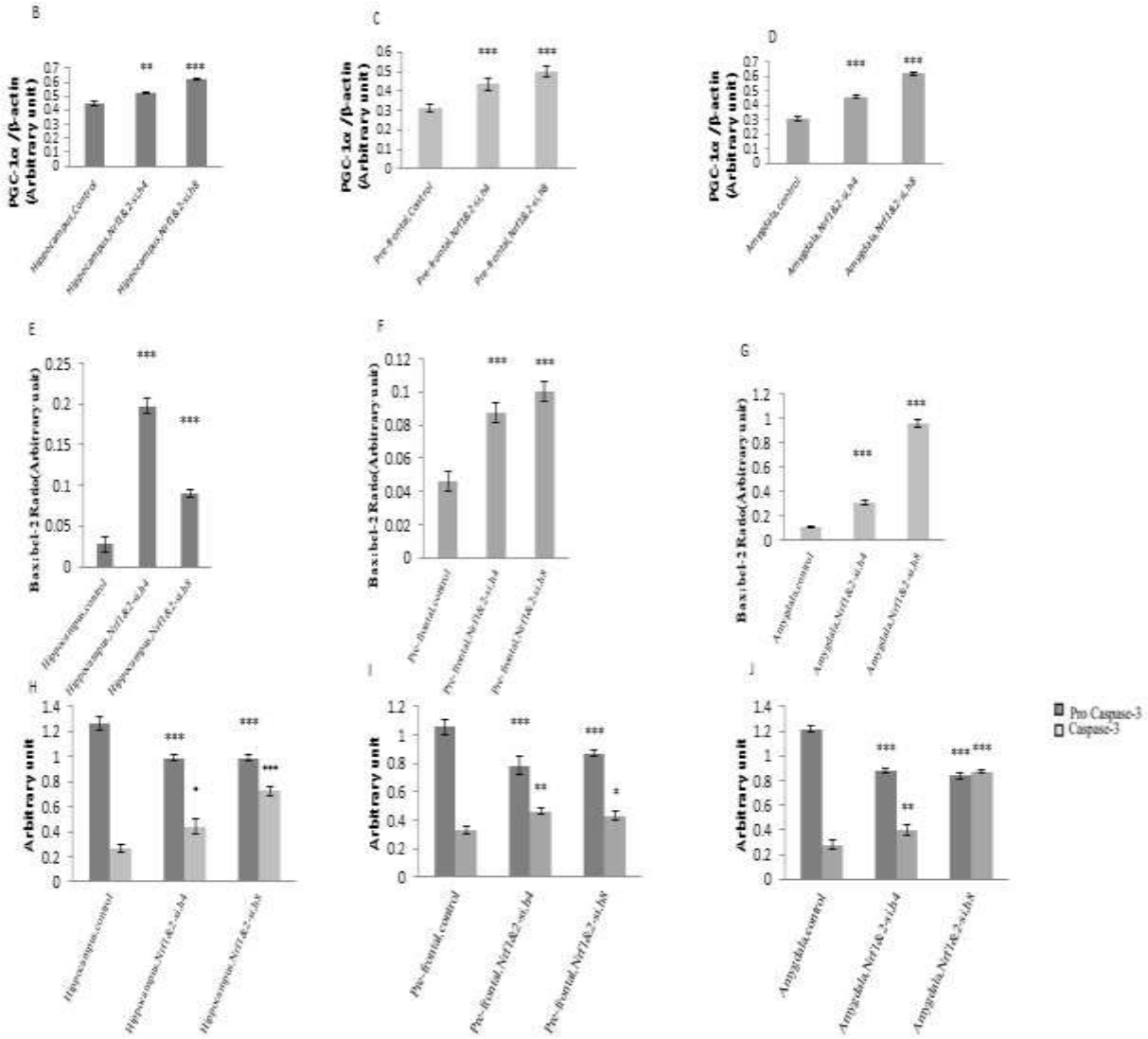


Figure 3. Bax/Bcl2 ratio, Caspase-3 cleavage and PGC-1α level in hippocampus, prefrontal cortex and amygdala after siRNA injection are shown by Western blotting. Method was according to explanation in Fig. 1 legend. Shown mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared to the control group

Nrf1&2-siRNA Injection Changed Apoptotic Factors and PGC-1α Level

Apoptosis cascade is mediated by some intracellular proteins including Bax, Bcl2 and caspase-3[24]. As shown in Fig. 3, the level of proapoptotic factor Bax and antiapoptotic factor Bcl2 were both increased through Nrf1&2 silencing. Also, the data exhibited an increase in Bax/Bcl2 ratio in 3 mentioned brain regions in all Nrf1&2-siRNA injected rats, 4 and 8 h after siRNA administration, compared to the control group (Fig. 3). Furthermore, as shown in Fig. 3, cleavage of caspase-3 notably increased 4 h after siRNA injection in hippocampus area specifically.

The biogenesis of mitochondria was concluded from organizes from activity of nuclear and mitochondrial genomes [25]. The level of some important factors with a role in mitochondrial biogenesis after Nrf1&2-siRNA injection was evaluated. As shown in Fig. 3, Nrf1&2-siRNA injection increased PGC-1α level compared to the control group. The most raise in PGC-1α level was in amygdala (2-fold compared to

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the control group) 8 h after Nrf1&2 siRNA injection. Furthermore, the level of PGC-1α increased 1.1-, 1.3-, and 1.5- fold in hippocampus, prefrontal cortex and amygdala, respectively, 4 h after siRNA injection compared to the control group. Eight h after siRNAs injection, the level of PGC-1α increased about 1.3-, 1.6-, and 2-fold in hippocampus, prefrontal cortex and amygdala, respectively, compared to the control group.

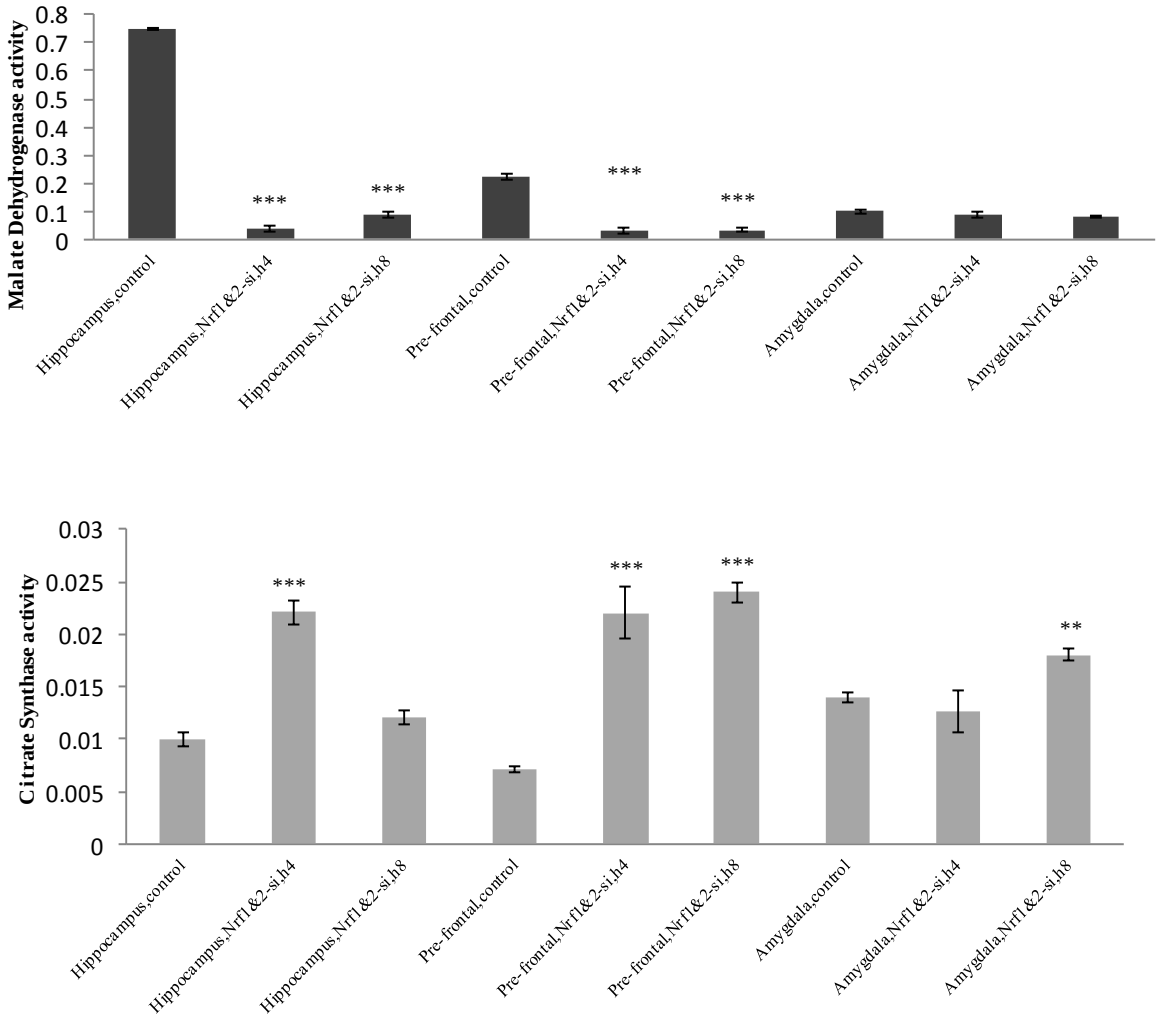

Figure 4. Effects of Nrf1&2 silencing on CS and MDH enzymes activities in hippocampus, amygdala and prefrontal cortex of rat brain. ***P > 0.01 compared to the control group.

Nrf1&2-siRNA Injection CS and MDH Activities

The level of CS activity was increased about 2.2- and 3.1-fold in prefrontal cortex and amygdala, respectively (but decreased in amygdala 4 h after siRNA injection). Moreover, the level of CS activity increased about 1.2-fold in both hippocampus and prefrontal cortex, 8 h after siRNA injection. The most increase in level of CS activity was detected in amygdala (about 3.2-fold) 8 h after siRNA injection. As shown in Fig. 4, the level of MDH activity decreased highly in all 3 brain regions. The most reduction was in hippocampus and prefrontal cortex 4 h and 8 h after siRNA injection.

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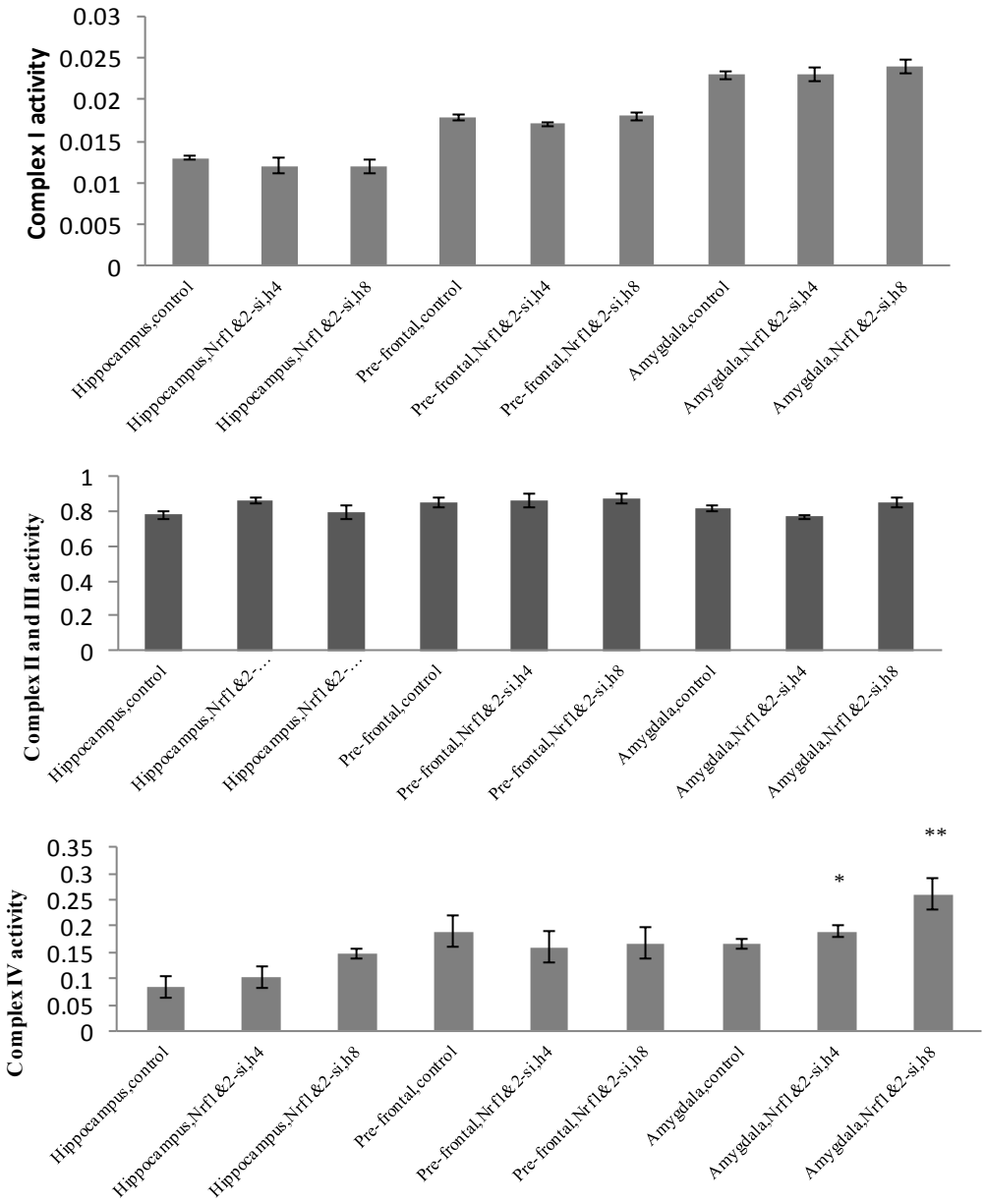

Figure 5. Effects of Nrf1&2 silencing on ETC enzymes activities in hippocampus, amygdala and prefrontal cortex of rat brain. *P < 0.05 and **P < 0.01; compared to the control group.

Nrf1&2 Silencing Modulated Mitochondrial Respiratory Complexes' Activity

It is well known that mitochondrial ETC is the most important energy producer process in the cell, which produces energy through multi-subunit enzyme complexes [26]. As shown in Fig. 5, complex I activity did not change considerably in the three brain regions. In addition, complexes II-III activity showed no significant change in those three brain regions. However, complex IV activity slightly increased in hippocampus and amygdala, 1.8- and 1.6-fold, respectively 8 h after siRNA injection compared to control group.

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4. Discussion

This study's results have indicated anxiety in male Wistar anxiety-like behavior and mitochondrial function are related to each other. Cui et al., Rasbach, and Schnellmann have referred to PGC-1α as the most important regulator of mitochondrial biogenesis and ETC complexes that has a wide variety of biological responses, such as cell death and cellular adaptation under conditions of stress and mitochondrial dysfunction [27]. Several genes that mediate the defense mechanism against oxidative stress such as SOD, are downstream targets of PGC-1α [28]. The data revealed a significant increase in PGC-1α following Nrf1&2 knockdown. Adamovich et al. discussed that PGC-1α protects NAD (P)H dehydrogenase [quinone] 1 (NQO1), which is one of the Nrf2 dependent downstreams [29].

The most highly glucose consumer cells are neurons as of maintaining their intrinsic functions [13]. The core part of the aerobic respiration in cells is TCA, which includes a series of enzyme-catalyzed chemical reactions, starting from CS, and ending to MDH enzymes [30]. The present study also revealed that the activity of MDH was decreased in prefrontal cortex and hippocampus, while CS enzyme activity was increased in those regions following Nrf1&2 knockdown. On the other hand, Bélanger et al. and Wright et al. have reported that Nrf2 participates in anabolic pathways of glucose and glutamine metabolisms [31,32]. Subsequently, Nrf2 silencing leads to intensifying intracellular catabolism pathways that can cause an increase in level of acetyl CoA. Hayes John D et al. stated that Acetyl CoA is one of the main substrates for CS activity [33]. Wright et al. indicated that PGC-1α may regulate the activity of CS enzyme [32]. The study results also illustrated that MDH activity was reduced in hippocampus and prefrontal cortex following Nrf1&2 knockdown. Besides, Wright et al. and Hatazawa et al. have reported that PGC-1α overexpression leads to upregulation of TCA cycle enzymes activity and expression [34,35]. Wright et al. and Hatazawa et al. discussed the Nrf2 regulatory effect on MDH expression [32,36]. According to Roselli CE et al. and Franklin RB et al., together with the evidence from the decrease in protein level of PGC-1α, a decrease in activity of MDH was expected which was due to downregulation of PGC-1α following Nrf1&2 knockdown [35,37].

The present study's data indicated that silencing of Nrf1 and Nrf2 by means of specific siRNA developed a significant increase in ETC complex IV activity in hippocampus, prefrontal cortex and amygdala. Kuloglu et al. and Bouayed et al. demonstrated oxidative stress as an inducer of anxiety-like behavior [38,39]. However, though Rezin et al. indicated that anxious mice revealed inhibition of some ETC components [40], the present findings negate these. Since Hayes and Ashford introduced Nrf2 as a director of glucose and glutamine metabolism along anabolic pathways, its basal expression has specific effect on ETC chain and the cellular glycolytic [33].

Khalifeh et al. have reported that Nrf2 has a significant role in regulating mitochondrial associated pathways of developing anxiety [13]. Recently, it has been revealed that one of the leading causes of anxiety is the elevation of hippocampal apoptosis with a decrease in its volume [41,42]. Despite this apoptotic basis, Khalifeh et al. suggests a significant link between prefrontal Bax, Bcl2 level, respiratory complex activity, and Nrf2 non-oxidative function in controlling anxiety [13]. According to Hovatta et al. and Manoli et al., it has been indicated that relatively low hippocampal apoptosis accompanies anxiety-like behaviors as previously observed in PTDS models of anxiety [43,44]. Nevertheless, the mitochondrial pathway of apoptosis has been contented as the leading mechanism in apoptotic neurons. The deletion of Bcl2

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gene resulted in an increase in anxiety-like behavior in mice [6]. The present study revealed that Nrf1&2 silencing upregulated caspase-3 cleavage which is an irreversible step in apoptosis induction and also a significant increase in Bax/Bcl2 ratio. Here, a divergence was observed between the peak of caspase-3 and Bax/Bcl2 in hippocampus, prefrontal cortex and amygdala. In fact, the data demonstrated higher activation of Bax/Bcl2 in all 3 studied regions, in the absence of Nrf1 and Nrf2, while the peak of caspase-3 was observed in all those regions. The survival effect of Bcl2 is not limited to its antiapoptotic effect: it improved mitochondrial activity and oxidative phosphorylation [45]. Accordingly, the inhibition of Bcl2 has distinct mechanisms in the development of anxiety, which is due to Bcl2 non-antiapoptotic activities [13]. Recently, Bcl2 has been discussed as a transcriptional downstream of Nrf2 [46]. Furthermore, this idea has been originated from Nrf2-associated upregulation of Bcl2 through the interaction of Nrf2 inhibitor, Keap1 [47]. Khalifeh et al have reported that Nrf2-mediated activation of Bcl2 and concurrent Bax inhibition caused induction of protective mechanisms which was accomplished by decrease in anxiety-like behavior, which may be distinct from the anti-apoptotic role of Bcl2 [13]. The tight association has been observed between anxiety-like behavior and antioxidant defense mechanism including glutathione reductase and glyoxalase. Accordingly, glyoxalase has been referred to as a biological marker for anxiety [43]. In addition, Xue et al. have revealed that glutathione reductase and glyoxalase 1 regulate Nrf2 [48].

5. Conclusion

According to khalifeh et al. development of anxiety pathophysiological is beyond Bcl2 or apoptosis point of view and interaction of some compensatory mechanisms in Nrf1 knockdown [13]. Here, under the stress condition the level of PGC-1α as the most important regulator of mitochondrial biogenesis increased; also, series of enzyme-catalyzed chemical reactions such as MDH and CS activity have been altered. Furthermore, the level of Acetyl CoA has been elevated following Nrf1&2 silencing. This study results' emphasized on the specific role of Nrf2 in modulation of anxiety and mitochondrial biogenesis, through the increase in Bax and caspase-3 cleavage and decrease of Bcl2. Correspondingly, according to the correlation between Nrf2 and mitochondrial biogenesis [3,49], Khalifeh et al. have speculated that in compensation of Nrf2 silencing, this pathway may be involved in hyperactivity of ETC complexes especially complex IV activity [13]. Moreover, khalifeh et al. have demonstrated an anxiety-related behavior in Nrf2 knockdown [13]. However, this behavior has not been reported in Nrf1 knockdown [50].

In this current study, specific siRNA was used to knockdown Nrf1&2 and induced anxiety-like behavior in rats. These results may suggest an important role for Nrf2 in regulation of mitochondrial biogenesis, anxiety, and compensation of Nrf1 knockdown.

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Conflict of interest

The authors declare no conflict of interest.

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