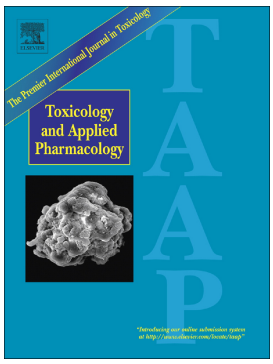

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Central administration of Tert-butylhydroquinone attenuates hypertension via regulating Nrf2 signaling in the hypothalamic paraventricular nucleus of hypertensive rats

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ABSTRACT

Reactive oxygen species (ROS) in the paraventricular nucleus (PVN) play a pivotal role in the pathogenesis of hypertension. Nuclear factor E2-related factor-2 (Nrf2) is an important transcription factor that modulates cell antioxidant defense response against oxidative stress. The present study aimed to explore the efficacy of PVN administration of tert-butylhydroquinone (tBHQ), a selective Nrf2 activator, in hypertensive rats. 16-week-old spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats were used in this study. These rats were chronic bilateral PVN infusion of tBHQ (0.8μg/day), or oxygen free radical scavenger tempol (20μg/h), or vehicle for 2 weeks. SHR rats had higher mean arterial pressure (MAP), plasma norepinephrine (NE) levels, and sympathetic nerve activity (RSNA) and lower PVN levels of Nrf2, hemeoxygenase-1 (HO-1), superoxide dismutase-1 (SOD1) and catalase (CAT) as compared with those in the WKY group. Bilateral PVN infusion of tBHQ or tempol significantly reduced MAP, RSNA, plasma NE levels in SHR rats. In addition, tBHQ treatment enhanced the nuclear accumulation of Nrf2 and increased the expression of HO-1, CAT and SOD1 in SHR rats. Furthermore, tBHQ attenuated PVN levels of ROS, the expression of proinflammatory cytokines and restored the imbalance of neurotransmitters in PVN. Knockdown of Nrf2 in the PVN by adeno-associated virus mediated small interfering RNA abrogated the protective effects of tBHQ on hypertension. These findings suggest that PVN administration of tBHQ can attenuate hypertension by activation of the Nrf2-mediated signaling pathway.

Keywords: Paraventricular nucleus, Hypertension, Nrf2, tert-butylhydroquinone, Oxidative stress

1. Introduction

Sympathoexcitation is one of the key factors that contribute to the pathogenesis of hypertension. The hypothalamic paraventricular nucleus (PVN) has been regarded as one of the most important region that can influence the sympathetic outflow in the central nervous system (Patel, 2000; Gabor and Leenen, 2012). Previous studies have shown that reactive oxygen species (ROS) and proinflammatory cytokines (PICs) in the PVN contributes to sympathoexcitation in different kinds of hypertension (Oliveira-Sales *et al.*, 2009; Su *et al.*, 2014; Dange *et al.*, 2015; Xue *et al.*, 2016). Our recent studies suggest that inhibition of reactive oxygen species in PVN can attenuate PICs and renin–angiotensin system (RAS), restore the imbalance between inhibitory and excitatory neurotransmitters, and thereby reduce sympathetic activity and blood pressure (Su *et al.*, 2016).

The nuclear factor E2 related factor-2 (Nrf2) is an important transcription factor that can modulate cell antioxidant defense response against oxidative stress by regulating the synthesis of a range of antioxidants and detoxification enzymes (Barancik *et al.*, 2016). Under normal conditions, Nrf2 binds to its inhibitor Kelch-like ECH-associated inhibitor 1 (Keap1). This Keap1/Nrf2 complex is targeted for proteasomal degradation. When stimulated by stress, the conformation of Keap1/Nrf2 complex changes, resulting in the increase in nuclear translocation of the activated Nrf2. Then, Nrf2 binds to a group of antioxidant response element (ARE)-dependent cytoprotective genes, including hemeoxy-genase-1 (HO-1), NAD(P)H: quinone oxidoreduc-tase-1 (NQO1) and glutathione S-transferasea-1 (GST-a1). Recently, activation of the Nrf2-ARE pathway has been documented to be beneficial in many cardiovascular diseases (Barancik *et al.*, 2016). A previous study has shown that reduction in nuclear translocation of Nrf2 in the rostral ventrolateral medulla (RVLM) neurons contributes to hypertension induced by LPS-mediated systemic inflammation (Wu *et al.*, 2016). Our group has found that oral administration of oleuropein can reduce oxidative stress and improve mitochondrial function in PVN by activation of the Nrf2-mediated signaling pathway (Sun *et al.*, 2016a). Despite the positive roles of Nrf2 in

preventing oxidative stress, there is little direct evidence that activation of the Nrf2-mediated signaling pathway in PVN has protective effects against hypertension. The underlying mechanisms of its potential protective effects on hypertension remain to be fully elucidated.

Tert-butylhydroquinone (tBHQ), a synthetic phenolic antioxidant, is widely used as a selective Nrf2 activator. Growing evidence has confirmed its antioxidant activity in different kinds of diseases (Li *et al.*, 2014b; Wang *et al.*, 2015; Wu *et al.*, 2015; Duan *et al.*, 2016; Ye *et al.*, 2016). Notably, a recent study indicates that oral administration of tBHQ can lower blood pressure in hypertensive mice (Xu *et al.*, 2016). However, the precise mechanisms by which it protects against hypertension remains unclear. In the present study, we investigated the effects of PVN infusion of tBHQ on hypertension and Nrf2 signaling pathway. We also determined whether ROS, PICs and neurotransmitters in the PVN were involved in the effects of tBHQ.

2. Materials and Methods

2.1. Animals

Male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) weighing 250 g-270 g were purchased from Charles River Laboratory Animal, Ltd. (Beijing, China). The rats were housed individually in a room with controlled temperature (20–23 °C) and light: dark cycle (12 h: 12 h). All animals were allowed access to standard chow and tap water ad libitum. All of the animal procedures were approved by the Animal Care and Use Committees of Xi'an Jiaotong University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drug and preparation

tBHQ was purchased from Sigma-Aldrich and first dissolved in 100% dimethyl sulfoxide (DMSO) as a stock. Then artificial cerebrospinal fluid (aCSF) was added to prepare for the working solution with a final concentration of 1.8 mM in aCSF with 1% DMSO. Oxygen free radical scavenger tempol (Sigma) was dissolved in aCSF. The osmotic minipumps (ALZET,

model 1004; infusion rate of 0.11μL/h) were then loaded with fresh tBHQ solution or tempol according to the manufacturer's protocols. The vehicle solution was a matching concentration of DMSO (1%) in aCSF. In general, the rats were PVN infusion of tBHQ (0.8μg/day), or tempol (20μg/h), or vehicle for two weeks. The doses used in this study were based on previous effective studies (Shih *et al.*, 2005; Su *et al.*, 2014) and our preliminary experiment results.

2.3. Experimental design

Experimental protocol 1: After anesthesia, cannulae were implanted bilaterally into the PVN (1.8 mm caudal to the bregma, 0.4 mm lateral to central line, and 7.8 mm below the skull surface) using brain stereotaxic apparatus, as described previously (Li et al., 2015b). Seven days after surgical recovery, the osmotic minipumps were then implanted subcutaneously and connected to the PVN cannulae for drug administration of tBHQ or tempol for 2 weeks. At the end of the second week, rats were anesthetized for the sympathetic nerve activity (RSNA) measurement and then euthanized to collect blood and tissue samples for further analysis. All the precise injection sites were verified by histological examination. The success rate of bilateral PVN microinjecton was about 70%. Animals with verifiable bilateral PVN injection sites were used for the final analysis.

Experimental protocol 2: Adeno-associated virus mediated small interfering RNA against Nrf2 (AAV-NRF2-siRNA) or control vectors (AAV-SCM-siRNA) with enhanced green fluorescent protein (eGFP) were purchased from Hanbio Biotechnology Co., Ltd. (Shanghai, China). The siRNA sequence for Nrf2 is 5′-GTCTTCAGCATGTTACGTGATGAGGATGG-3′ (Wruck *et al.*, 2007). The intra-PVN infusion of AAV was conducted as previously described (Chen *et al.*, 2014). Briefly, rats were anesthetized by intraperitoneal injection of a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture; the cannulae were implanted bilaterally into the PVN. Then they were connected to two 10μl Hamilton micro-syringes which were mounted on an infusion pump. The viral vectors (1μl of 1×10^{12} genomic particles/ml) were infused at a rate of 0.1μl/min for 10 min. Two weeks after AAV injection, the osmotic minipumps were then implanted subcutaneously and connected to the PVN cannulae for tBHQ (0.8μg/day)

administration for another 2 weeks. The SHR rats were randomly divided into four groups: (i) AAV-SCM-siRNA + vehicle; (ii) AAV-SCM-siRNA + tBHQ; (iii) AAV-NRF2-siRNA + vehicle; (iv) AAV-NRF2-siRNA + tBHQ. At the end of the experiment, tissue samples were collected for further analysis.

2.4. Measurement of mean arterial pressure (MAP)

Blood pressure (BP) was measured by using a noninvasive tail-cuff system (NIBP, AD Instruments, Australia) in conscious rats as previously described (Sun *et al.*, 2016b). In order to diminish the stress-induced BP fluctuations, the rats were trained by measuring MAP daily for at least 7 days before the operation. The MAP values were averaged from five measurements obtained from each rat.

2.5. Sympathetic neural recordings

Under anesthesia with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (ip), the left renal nerves of rat were isolated for recording sympathetic nerve activity (RSNA). The rectified and integrated RSNA were recorded using methods described as previously (Kang *et al.*, 2009; Li *et al.*, 2014a).

2.6. Collection of tissue samples

The bilateral PVN tissue was isolated according to Palkovits's microdissection procedure as previously described (Kang *et al.*, 2011). Tissue samples were then stored at -80℃ for future analysis.

2.7. Immunohistochemical and immunofluorescence studies

The PVN immunohistochemical and immunofluorescence staining were conducted as previously described (Qi *et al.*, 2016). We used the following antibodies: rabbit anti-Nrf2 (cat# ab31163, Abcam), rabbit anti-SOD1 (cat#ab16831, Abcam), rabbit anti-NOX2 (cat#ab-31092, Abcam), rabbit anti-Fra-LI (cat#sc253, Santa Cruz)， goat anti-IL-1β (cat#sc-1251, Santa Cruz)，

goat anti-GAD67 (cat#sc-7512, Santa Cruz), mouse anti-TH (cat#sc-25269, Santa Cruz), rabbit anti-HO-1 (cat#bs-2075R, Bioss), Immunofluorescent staining for Nrf2 was observed with a confocal laser-scanning microscope (Zeiss LSM 710, Carl Zeiss, Inc). Other immunohistochemistry or immunofluorescent stained sections were photographed with a conventional light microscopy (DP70, Olympus, Tokyo, Japan). For each animal, the numbers of positive staining cells in the bilateral PVN were manually counted in three consecutive sections and the average value was used.

2.8. Dihydroethidium staining

Superoxide generation was determined by fluorescent-labeled dihydroethidium (DHE; Molecular Probes). The method for DHE staining was conducted as previously described (Li *et al.*, 2014a). Briefly, Slices containing the PVN were incubated in DHE (0.05mM) for 25 min at 4 °C. Then, the sections were rinsed three times in PBS and observed using a conventional light microscopy. DHE staining was quantified by ImageJ software as described (Zimmerman *et al.*, 2004).

2.9. Western blotting

Western blot analysis was performed as described (Sun *et al.*, 2016b). The protein samples of PVN were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Electrophoresis and Blotting Apparatus (Bio-rad).Then they were transferred onto a PVDF membrane (Millipore). The membrane was incubated with the following antibodies: rabbit anti-Nrf2 (cat# ab31163, Abcam), rabbit anti-Cat (cat# ab16731, Abcam), rabbit anti-SOD1 (cat#ab16831, Abcam), rabbit anti-NOX2 (cat#ab-31092, Abcam), goat anti-IL-1β (cat#sc-1251, Santa Cruz)，mouse anti-IL-6 (cat#sc-32296, Santa Cruz)，goat anti-GAD67 (cat#sc-7512, Santa Cruz) , mouse anti-TH (cat#sc-25269, Santa Cruz), rabbit anti-lamninB1 (cat#12987-1-AP, Proteintech), rabbit anti-HO-1 (cat#bs-2075R, Bioss). The images were obtained by chemiluminescence using ChemiDoc XRS System (Bio-rad, USA) and quantified by ImageJ software.

2.10. Real-time quantitative PCR

Total RNA was isolated with TRIzol Reagent (TaKaRa, China). cDNA was synthesized using PrimeScript™ RT Master Mix (TaKaRa, China) according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was performed with SYBR® Premix Ex Taq™ II (TaKaRa, China) by Mx3005P qPCR system (Stratagene, La Jolla, CA). The mRNA expression was normalized to rat β-actin mRNA levels using the $2^{-\Delta\Delta CT}$ method. The information of genes, primer sequences are shown in Table 1 (Li *et al.*, 2015a).

2.11. ELISA for norepinephrine (NE) assessment

The plasma levels of NE were measured with the ELISA kits (Abnova, Taiwan) according to the manufacturer's protocols. The results were obtained using a microplate reader (Bio-Rad, Hercules, CA).

2.12. Statistical analysis

Values are expressed as mean ± SEM. Analysis of MAP data was performed by repeated-measures ANOVA. All other results were analyzed with ANOVA followed by Bonferroni's post test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PVN infusion of tBHQ or tempol attenuates MAP in hypertensive rats.

In our study, we chose SHR and WKY rats, for they are well-established animal model of essential hypertension. A noninvasive computerized tail-cuff system was used for the measurement of MAP. As shown in Fig. 1, the baseline blood pressure among SHR rats were ranging from 160 to 180mmHg, whereas, MAP in WKY group remained stable between 80 to 100 mmHg throughout the study. Blood pressure of SHR + tBHQ rats gradually reduced from Day 4, and it remained a lower level to the end of our study when compared with SHR + vehicle rats. Treatment with tBHQ at the dose of 0.8μg/day in PVN reduced MAP in SHR rats. Similarly,

chronic PVN infusion of tempol at the rate of 20μg/h reduced MAP from day 6 when compared with SHR rats (Fig. 1S).

3.2. PVN infusion of tBHQ or tempol decreases RSNA and plasma levels of NE in hypertensive rats.

As shown in Fig. 2, our electrophysiological results showed that there was an increase of renal sympathetic nerve activity (RSNA) in SHR compared with WKY rats. Administration of tBHQ in PVN decreased RSNA in hypertensive rats. Consistent with this, a significant reduction in circulating NE levels was observed in SHR + tBHQ group when compared with SHR + vehicle rats (Fig. 2C). In addition, the data from Fig. 2S showed that chronic administration of tempol in the PVN decreased RSNA and circulating NE levels in SHR rats.

3.3. PVN infusion of tBHQ activates Nrf2-dependent antioxidant responses in hypertensive rats.

tBHQ, a well-established Nrf2 agonist, can increase Nrf2 stability and nuclear accumulation. We next investigated whether the Nrf2 pathway in PVN was activated by observing the expression of Nrf2 protein in nuclear and cytoplasm, separately. The results presented in Fig. 3 showed that Nrf2 protein levels in cytoplasm as well as in nucleus were all increased in SHR + tBHQ group compared with SHR + vehicle rats. HO-1 is one of the most important cytoprotective enzymes directly regulated by Nrf2. As shown in Fig. 4, tBHQ was also found to upregulate HO-1expression in PVN of SHR rats. Taken together, our results indicated that tBHQ enhanced the nuclear accumulation of Nrf2 and upregulated the expression of its downstream antioxidants in PVN of hypertensive rats.

3.4. Chronic infusion of tBHQ decreases the production of superoxide anion and inhibits the excessive activation of neurons in PVN of hypertensive rats.

DHE, redox-sensitive and cell-permeable, has been extensively used to evaluate ROS production. As shown by Fig. 5A and C, our DHE staining results revealed that tBHQ infusion reduced the production of superoxide in PVN of hypertensive rats, whereas it failed to have any effects on WKY groups. Since ROS plays a critical role in the activation of neurons, we next observed the expression of Fra-like (fos family gene; a marker for chronic neuronal excitation) in the PVN. Immunohistochemical results (Fig. 5B and D) showed that SHR rats had increased expression of Fra-like in the PVN compared with WKY groups. After treatment with tBHQ, the expression of Fra-like in the PVN is reduced in hypertensive rats.

3.5. PVN infusion of tBHQ increases the antioxidase and decreases oxidase in hypertensive rats.

Superoxide dismutase (SOD) and catalase (CAT) are two important superoxide scavenging enzymes, which can eliminate the harmful substances in metabolism. Whereas, NAD(P)H oxidase family enzymes are the main sources of ROS in PVN. Then, we detected the expression of these enzymes in PVN. As shown in Fig. 6 and Fig. 7, SHR + vehicle rats had lower expression of SOD1 and CAT, higher levels of NOX2 (the catalytic subunit of NAD(P)H oxidase) compared with WKY rats. PVN infusion of tBHQ elevated the expression of SOD1 and CAT, inhibited the increase in NOX2 expression in hypertensive rats. However, tBHQ did not cause any significant difference in WKY groups.

3.6. Chronic infusion of tBHQ suppresses PVN inflammation in hypertensive rats.

Given the anti- inflammatory effects of Nrf2 signaling pathway (Innamorato *et al.*, 2008), we then investigated the expression of inflammatory cytokines at mRNA and protein levels in PVN. As shown in Fig. 8, our results suggested that SHR rats had markedly increased PICs levels compared with control group. After treatment with tBHQ, the expression of IL-1β and IL-6 at mRNA and protein levels were all down-regulated in SHR + tBHQ group compared with SHR + vehicle group. However, there was no significant difference in WKY groups.

3.7. PVN infusion of tBHQ improves the imbalance between the excitatory and inhibitory neurotransmitters.

GABA is a dominant inhibitory neurotransmitter whereas NE acts as an important excitatory neurotransmitter in PVN. They are closely related to the regulation of sympathetic activity in hypertension. Tyrosine hydroxylase (TH) and glutamate decarboxylase (GAD) are the key enzymes of NE and GABA synthesis pathway respectively. The expression of TH and GAD67 can indirectly reflect the levels of NE and GABA in PVN. The results from Fig. 9 showed that SHR rats had significant increase in the numbers of TH positive neurons and lower levels of GAD67 expression as compared to the control group. PVN infusion of tBHQ resulted in a decrease in the protein expression of TH but an increase in the expression of GAD67 in hypertensive rats. The western results (Fig. 10) were consistent with the immunohistochemical and immunofluorescence studies.

3.8. Effects of AAV Delivery of siRNA on Nrf2 expression in the PVN.

As shown in Fig. 11, two weeks after intra-PVN injection of AAV-NRF2-siRNA for 1.0×10^{9} genomic particles, robust eGFP expression was present in the PVN. There was no eGFP expression in the subfornical organ (SFO) or the supraoptic nucleus (SON). Western blot analyses were performed to evaluate the effects of Nrf2 silencing. The results in Fig. 11 revealed that Nrf2 protein level was reduced significantly by 50% to 70% in SHR rats treated with AAV-NRF2-siRNA compared with those treated with AAV-SCM-siRNA.

3.9. Knockdown of Nrf2 expression in the PVN abrogated the effects of tBHQ on hypertension.

Next, we evaluated whether tBHQ exerted its protective effects on hypertension through Nrf2 signaling. As shown in Fig. 12, chronic PVN infusion of tBHQ could reduce blood pressure in SHR + SCM-siRNA group but not in SHR + NRF2-siRNA group. The results indicated that down-regulation of Nrf2 expression in the PVN can abrogate the effects of tBHQ on hypertension.

4. Discussion

In the current study, we have for the first time investigated the effects of chronic administration of tBHQ in the PVN on hypertensive rats. Here are the novel findings: (i) PVN

administration of tBHQ reduces sympathetic activity and plasma NE levels, thus it could attenuate hypertension in SHR; (ii) chronic administration of tBHQ activates Nrf2 signaling in the PVN, increases the expression of HO-1, SOD1 and CAT, decreases oxidative stress and PICs in the PVN of SHR; (iii) tBHQ can restore the imbalance between excitatory and inhibitory neurotransmitters in the PVN of hypertensive rats.

It has been well-established that PVN is one of the most important central integration areas for the sympathetic nerve activity and blood pressure. A rich body of evidence has demonstrated that ROS can stimulate both central and peripheral sympathetic nervous system activity (Campese *et al.*, 2004). ROS production in the PVN is reported to be increased in several animal models of hypertension. Inhibition of ROS production in PVN can decrease the RSNA in hypertensive rats (Su *et al.*, 2014). Nrf2-ARE pathway provides a cellular defense against oxidative stress. Current information indicates that activation of Nrf2-ARE pathway has many protective effects especially in cardiovascular and neurodegenerative diseases (Steele *et al.*, 2013; Silva-Palacios *et al.*, 2016; Sukumari-Ramesh and Alleyne, 2016). tBHQ is commonly used as a general Nrf2 activator. Notably, when added to normal diet in a ratio of 1% (w/w) for 2 weeks, it has been shown to lower blood pressure in Ang II-incuded hypertension in mice (Xu *et al.*, 2016). Since tBHQ can cross the blood-brain barrier (Lu *et al.*, 2014), the anti-hypertensive effects of tBHQ can be partly attribute to its role in the central nervous system (CNS). The Nrf2 is widely expressed in the CNS and its activity is under tight regulation. Our group has found that activation of Nrf2-mediated signaling pathway can protect PVN from oxidative stress injury by improving mitochondrial function (Sun *et al.*, 2016a). In present study, we found that PVN administration of tBHQ or tempol can decrease sympathetic nerve activity and reduce blood pressure in hypertensive rats. tBHQ increased the nuclear and cytoplasmic Nrf2 levels and the expression of related antioxidants, such as HO-1, superoxide dismutase-1 (SOD-1) and catalase (CAT). Meanwhile, the PVN levels of ROS and NOX2 (the catalytic subunit of NAD(P)H oxidase) in SHR + tBHQ group was significantly decreased as compared to SHR + vehicle group. The protective effects of tBHQ on hypertension in SHR rats were abrogated when Nrf2 was down-regulated by Nrf2 siRNA. Therefore, we speculate that PVN administration of tBHQ is able to attenuate hypertension through activation of

Nrf2 antioxidant system.

Growing evidence has shown that Nrf2 can modulate immune response (Turley *et al.*, 2015). It is reported that Nrf2-null mice is more sensitive to inflammation and infection, and more likely to develop autoimmune disease (Innamorato *et al.*, 2008). Within the current study, we found that tBHQ administration suppressed interleukin-1β (IL-1β) and interleukin-6 (IL-6) production in PVN, which was consistent with the recent finding that activation of Nrf2 signaling has anti-inflammatory effects (Martin-de-Saavedra *et al.*, 2013; Qi *et al.*, 2016). In hypertensive rats, inflammation activation in PVN is associated with excess ROS generation. Thus, inhibition of ROS by tBHQ may restore the balance between pro- and anti-inflammatory cytokines in the PVN. Previous studies have also indicated that Nrf2 activation can inhibit NF-κB signaling pathway (Li *et al.*, 2008; Zheng *et al.*, 2015). However, the detailed interaction between these two transcription factors should be further explored in future studies.

It is well documented that there is an imbalance between the excitatory and inhibitory of the hypertensive rats. Glutamate and NE in the PVN are reported to increase the sympathetic outflow, whereas GABA is a dominant inhibitory neurotransmitter that down-regulate sympathetic activity (Li and Pan, 2007). In accordance with previous findings, our data suggest that the PVN expression of 67kDa isoform of glutamate decarboxylase (GAD67; a marker of GABAergic neurons) is decreased in SHR, while the tyrosine hydroxylase (TH) expression is elevated. PVN infusion of tBHQ can increase the expression of GAD67 and down-regulate the TH level. These results suggest that the anti-hypertension effects of tBHQ may attribute to its role in restoring the imbalance between the excitatory and inhibitory neurotransmitters.

Altogether, our study may suggest that PVN administration of tBHQ have beneficial effects against hypertension, this can be explained partly by its interaction with Nrf2 signaling pathway to inhibit ROS production, PICs and restore the imbalance between the excitatory and inhibitory neurotransmitters in PVN of hypertensive rats.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Author contribution statement

Y. K., X. Y. and J. B. designed the study. J. B., K. L., F. W., G. J. and Y. Z. performed all experiments. J. B., H. G. and H. L. also performed the data analysis and drafted the manuscript. C. H. and X. L. participated in data analysis. Y. K., X. Y. and J. Q. critically revised the manuscript. All authors reviewed the final manuscript.

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Figure legends：

Figure 1. Effects of PVN administration of tBHQ on mean arterial pressure (MAP) in SHR and WKY rats. SHR rats had significantly elevated MAP compared with WKY rats. PVN administration of tBHQ at the dose of 0.8ug/day for two weeks reduced MAP in SHR rats. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 2. Effects of PVN administration of tBHQ on renal sympathetic nerve activity (RSNA) and plasma norepinephrine (NE) levels in SHR and WKY rats. SHR and WKY rats were PVN administered either tBHQ (0.8ug/day) or vehicle for two weeks. (A) Representative images of RSNA in different groups. Statistical analysis of RSNA (B) and plasma NE levels (C) in different groups. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 3. Effects of PVN administration of tBHQ on Nuclear factor E2-related factor-2 (Nrf2) expression in cytoplasm and nucleus within PVN in SHR and WKY rats. (A) A representative immunoblot; and (B) densitometric analysis of protein expression of Nrf2 in cytoplasm and nucleus of different groups. (C) Confocal microscopic representative images of Nrf2 (red fluorescence) in coronal sections of the PVN in SHR and WKY groups. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 4. Effects of PVN administration of tBHQ on hemeoxygenase-1 (HO-1) in the PVN of SHR and WKY rats. (A) A representative immunofluorescence image; and (B) statistical analysis of HO-1 positive neurons in PVN section of SHR and WKY rats. (C) A representative immunoblot; and (D) densitometric analysis of HO-1 protein expression in different groups. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 5. Effects of PVN administration of tBHQ on superoxide anion and Fra-like positive neurons in the PVN of SHR and WKY rats. (A) A representative immunofluorescence image of superoxide production (red fluorescence) by dihydroethidium (DHE) staining of different groups. (B) A representative immunohistochemistry image for Fra-like positive neurons in SHR and WKY rats. Statistical analysis of dihydroethidium (C) and Fra-Like positive neurons (D) in the PVN of different groups. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 6. Effects of PVN administration of tBHQ on Superoxide dismutase (SOD1) and NAD(P)H oxidase subunit NOX2 in the PVN of SHR and WKY rats by immunofluorescence staining. (A) A representative immunofluorescence staining of SOD1 in the PVN section of SHR and WKY rats. (B) A representative immunofluorescence image of NOX2 in different groups. Statistical analysis of SOD1 (C) and NOX2 (D) positive neurons in coronal sections of the PVN in SHR and WKY groups. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 7. Effects of PVN administration of tBHQ on the protein expression of catalase (CAT), Superoxide dismutase (SOD1) and NAD(P)H oxidase subunit NOX2 in the PVN of SHR and WKY rats by western blot. (A) A representative immunoblot; and (B) densitometric analysis of protein expression of CAT, SOD1, NOX2 in the PVN of SHR and WKY rats. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 8. Effects of PVN administration of tBHQ on proinflammatory cytokines (PICs) in the PVN of SHR and WKY rats. (A) A representative immunofluorescence image showing IL-1β in the PVN section of different groups. (B) The mRNA expressions of IL-1β and IL-6 in the PVN of SHR and WKY rats. (C) A representative immunoblot; and (D) densitometric analysis of protein expression of IL-1β and IL-6 in the PVN of different groups. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* <

0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 9. Effects of PVN administration of tBHQ on the levels of GAD67 and TH in the PVN of SHR and WKY rats by immunohistochemical and immunofluorescence staining. (A) A representative immunohistochemistry staining showing GAD67 positive neurons in the section from PVN in different groups. (B) A representative immunofluorescence image of TH positive neurons in the PVN of SHR and WKY rats. Statistical analysis of GAD67 (C) and TH (D) positive neurons in coronal sections of the PVN in SHR and WKY groups. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 10. Effects of PVN administration of tBHQ on the expression levels of GAD67 and TH in the PVN of SHR and WKY rats by western blot. (A) A representative immunoblot; and (B) densitometric analysis of protein expression of GAD67 and TH in the PVN of SHR and WKY rats. Values are expressed as means ± SE. n=7 per group; **P* < 0.05 vs. WKY groups (WKY + tBHQ or WKY + vehicle); †*P* < 0.05, SHR + tBHQ vs. SHR + vehicle.

Figure 11. Effects of PVN viral delivery of SCM-siRNA, or NRF2-siRNA on Nrf2 expression in the PVN of SHR rats. (A) Representative fluorescence images of enhanced green fluorescent protein (eGFP) in PVN, subfornical organ (SFO) and supraoptic nucleus (SON). (B) A representative immunoblot; and (C) densitometric analysis of protein expression of Nrf2 in the PVN of SHR rats. Values are expressed as means ± SE. n=7 per group; †*P* < 0.05, SHR + NRF2-siRNA vs. SHR + SCM-siRNA.

Figure 12. Effects of PVN infusion of tBHQ on mean arterial pressure (MAP) in SHR rats treated with SCM-siRNA or NRF2-siRNA. PVN infusion of tBHQ reduced MAP in SHR + SCM-siRNA group but not in SHR + NRF2-siRNA group. Values are expressed as means ± SE. n=7 per group; †*P* < 0.05, SHR + SCM-siRNA + tBHQ vs. SHR + SCM-siRNA + vehicle.

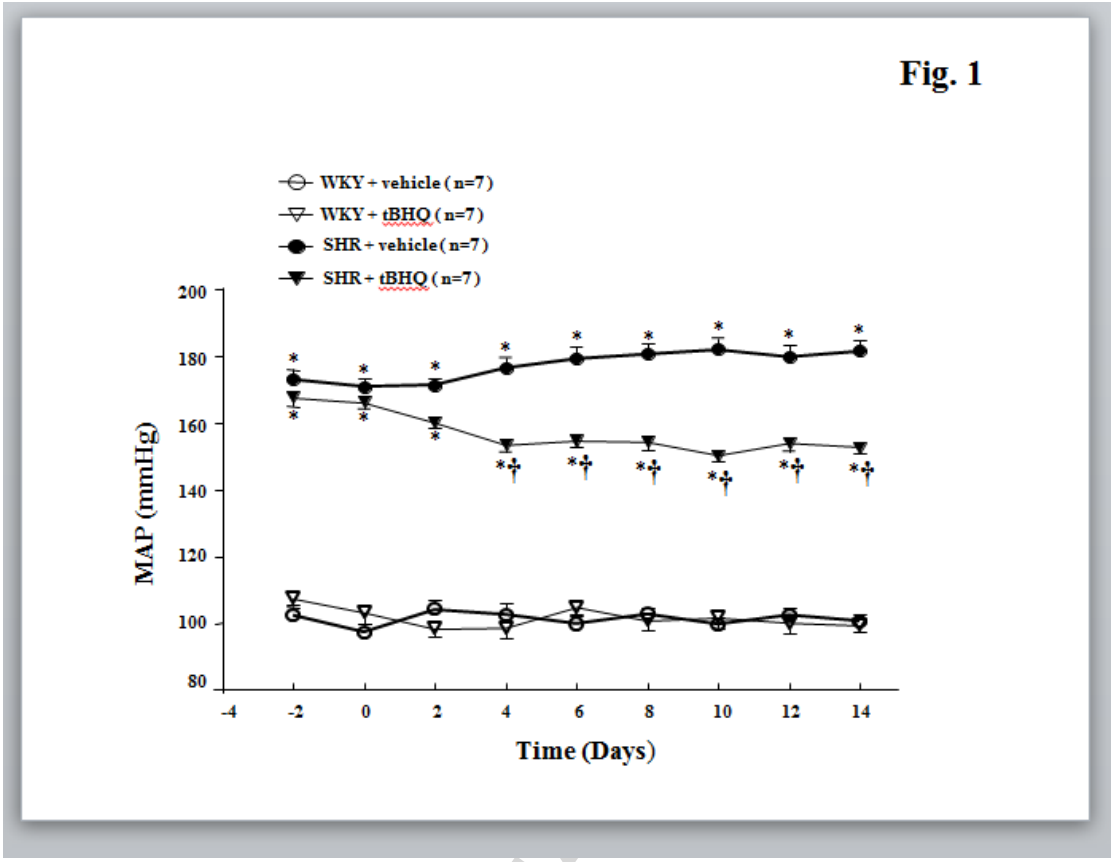
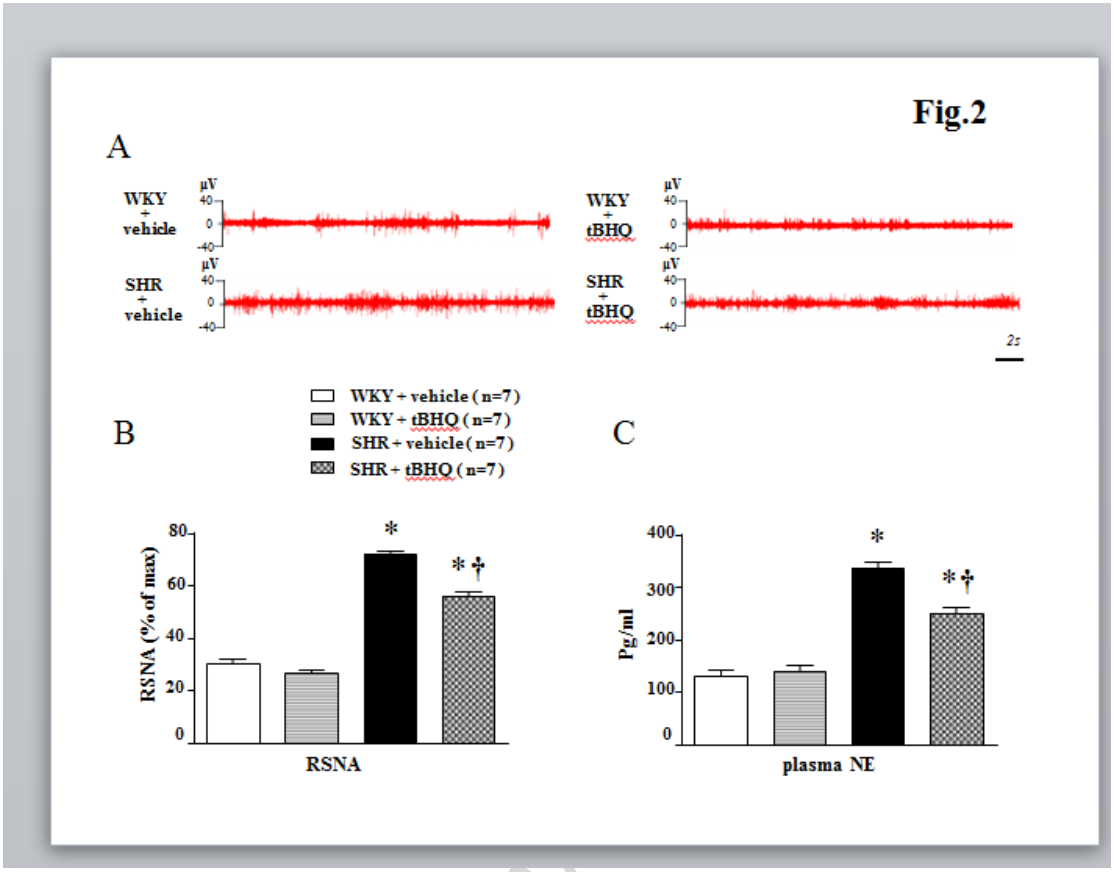


Figure 1



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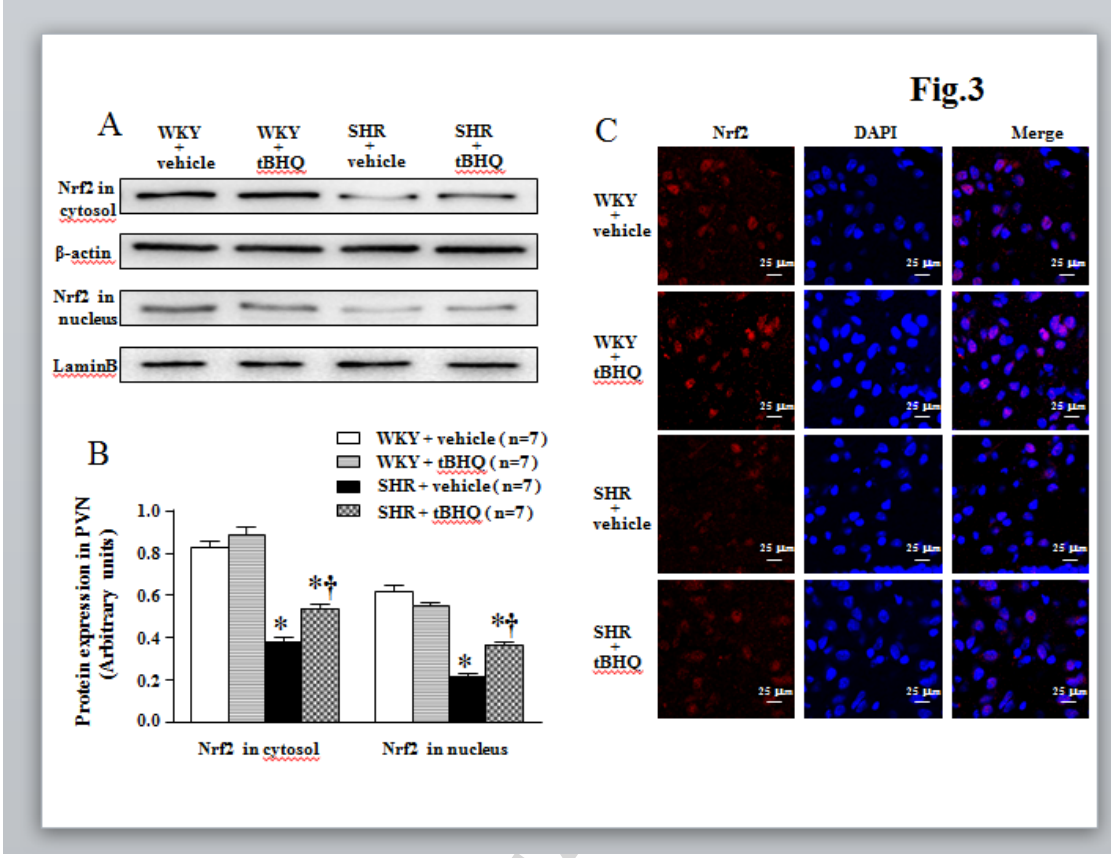

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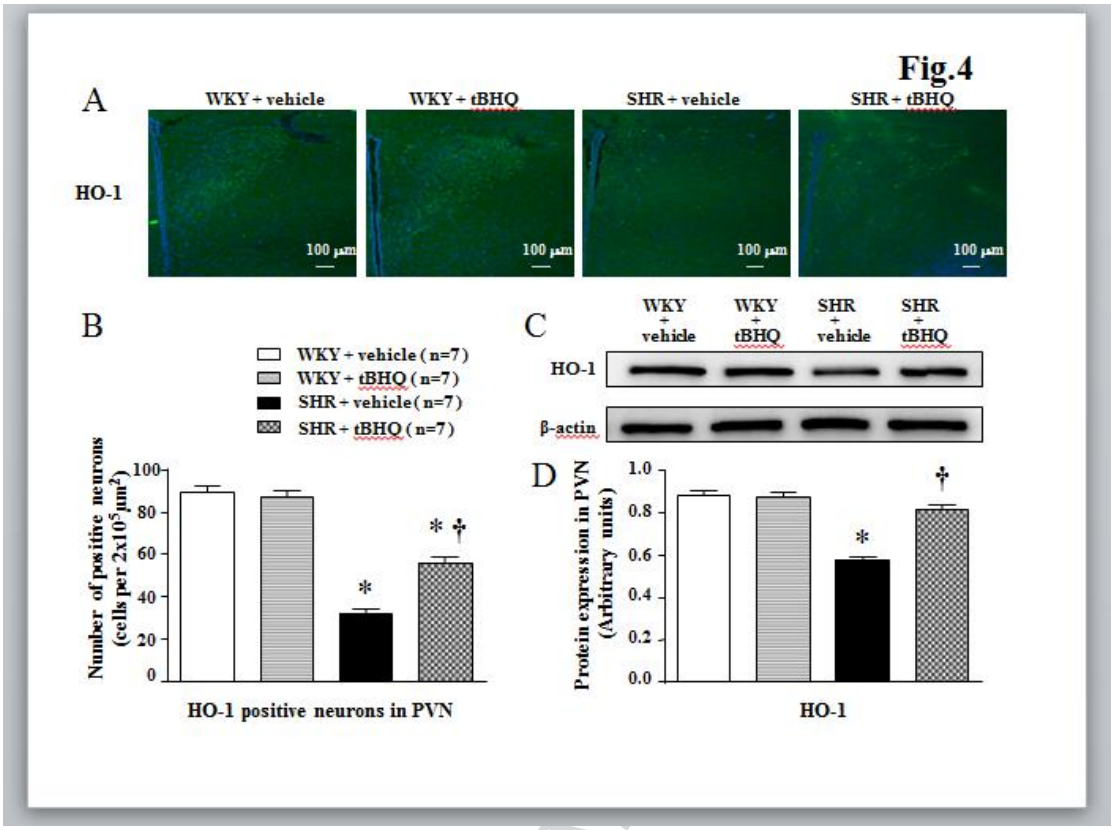

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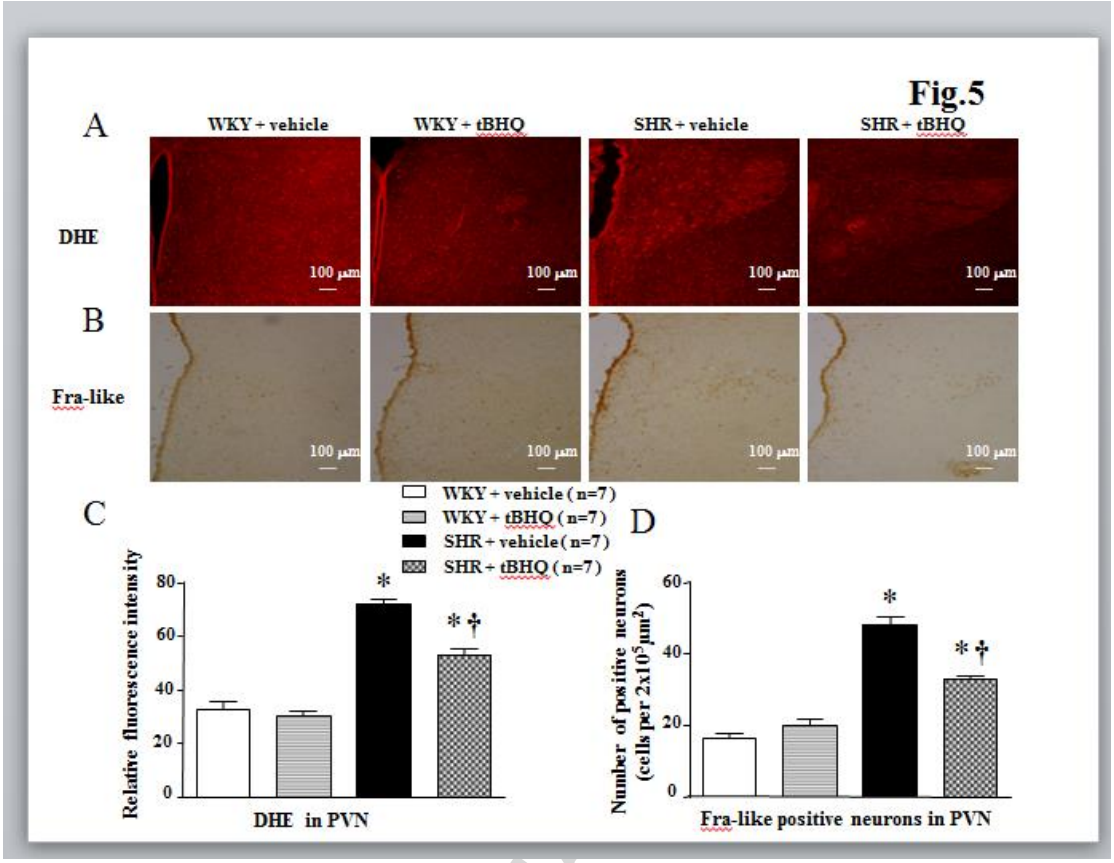

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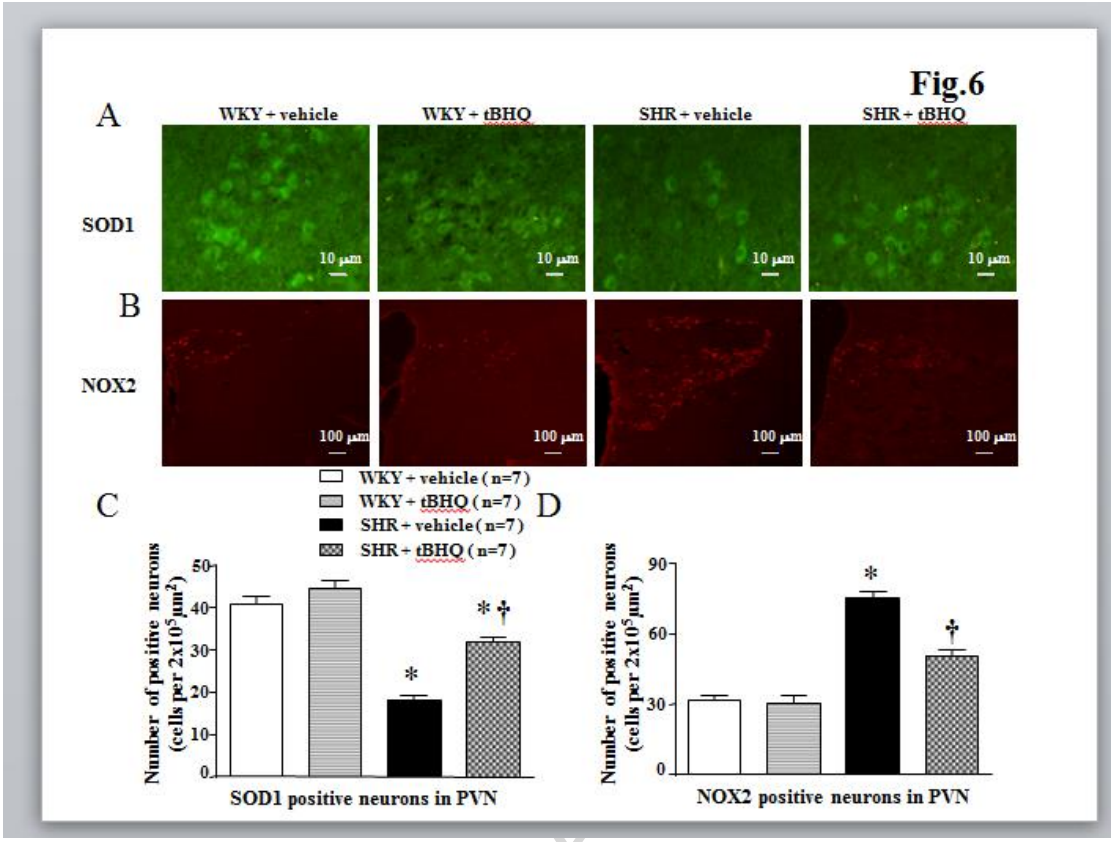

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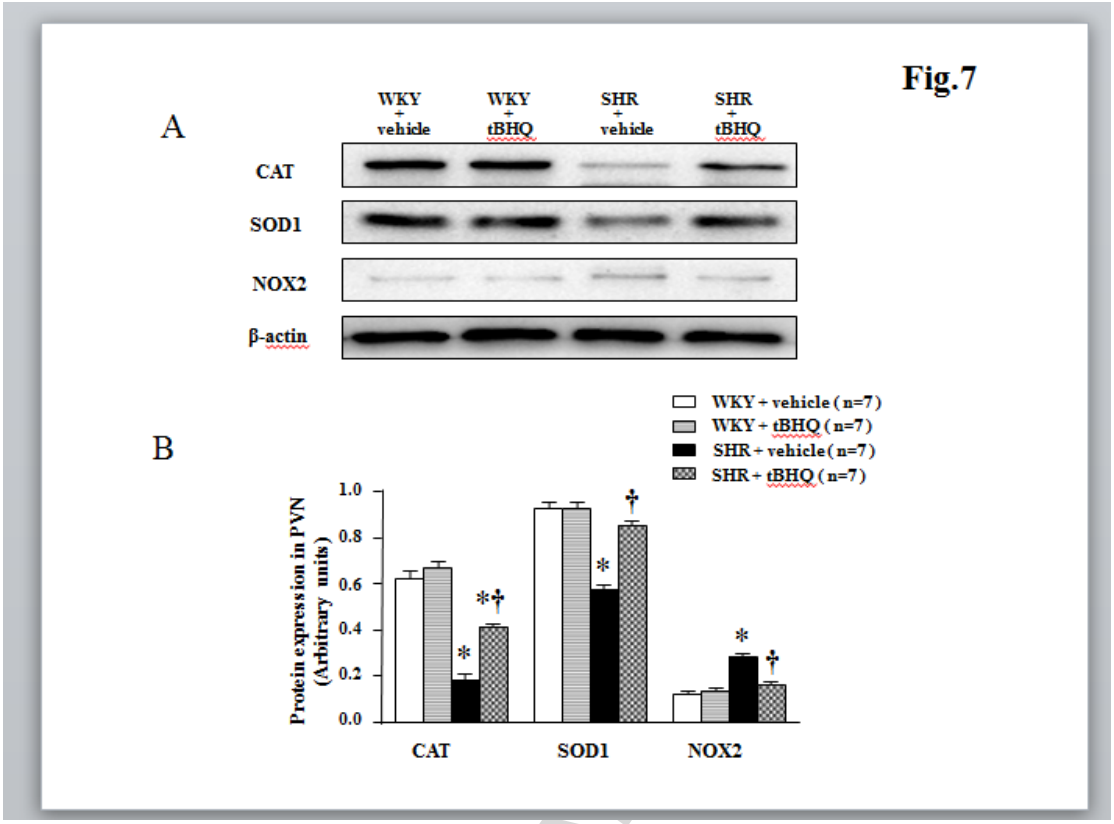

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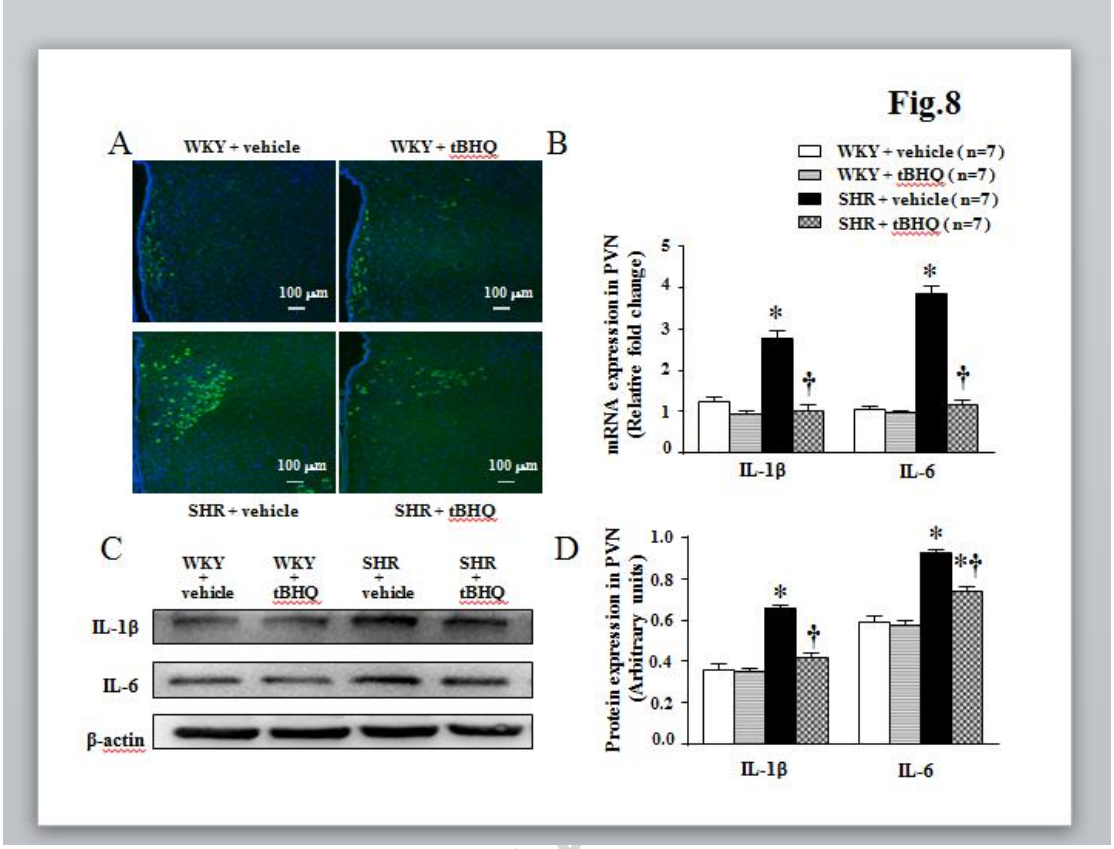

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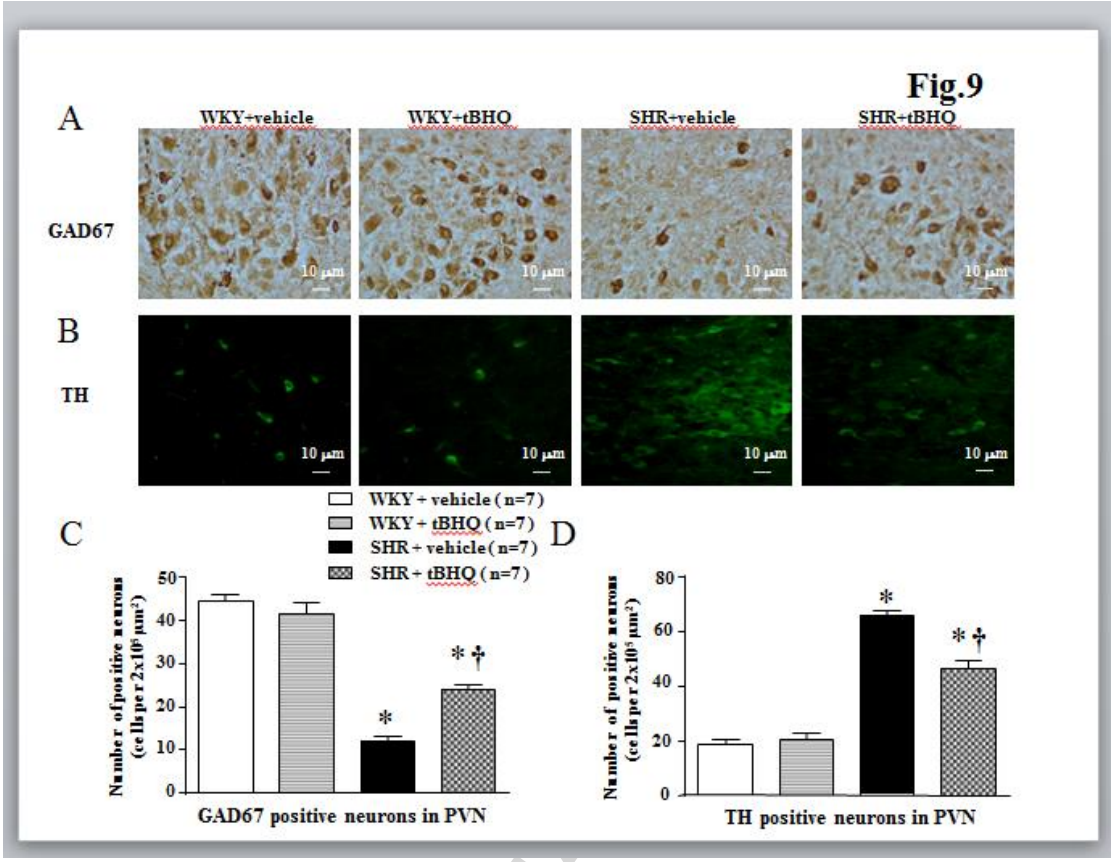
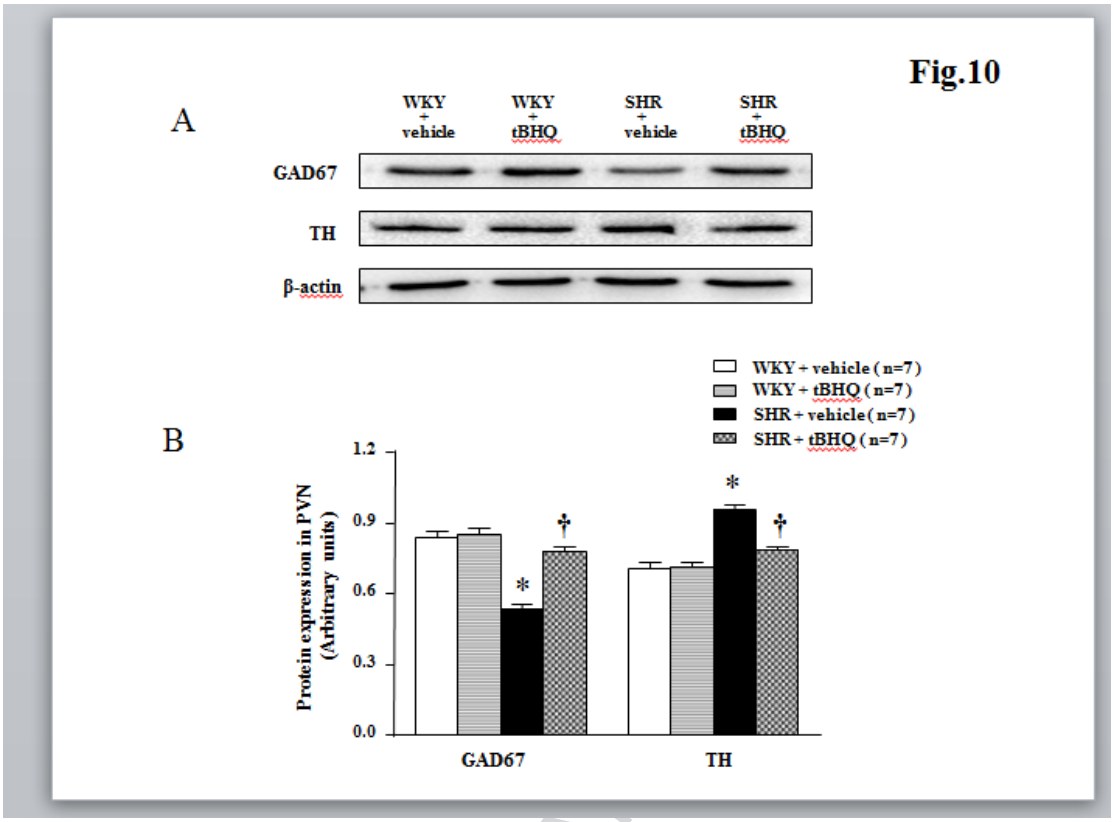


Figure 9



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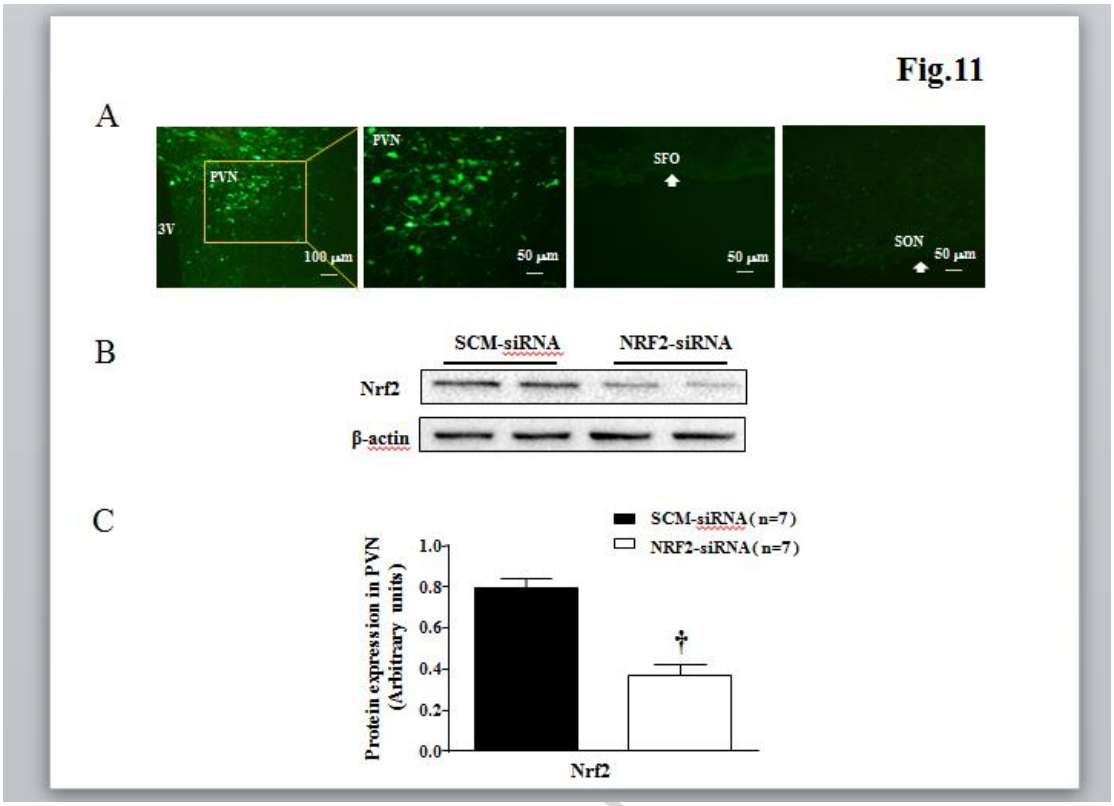

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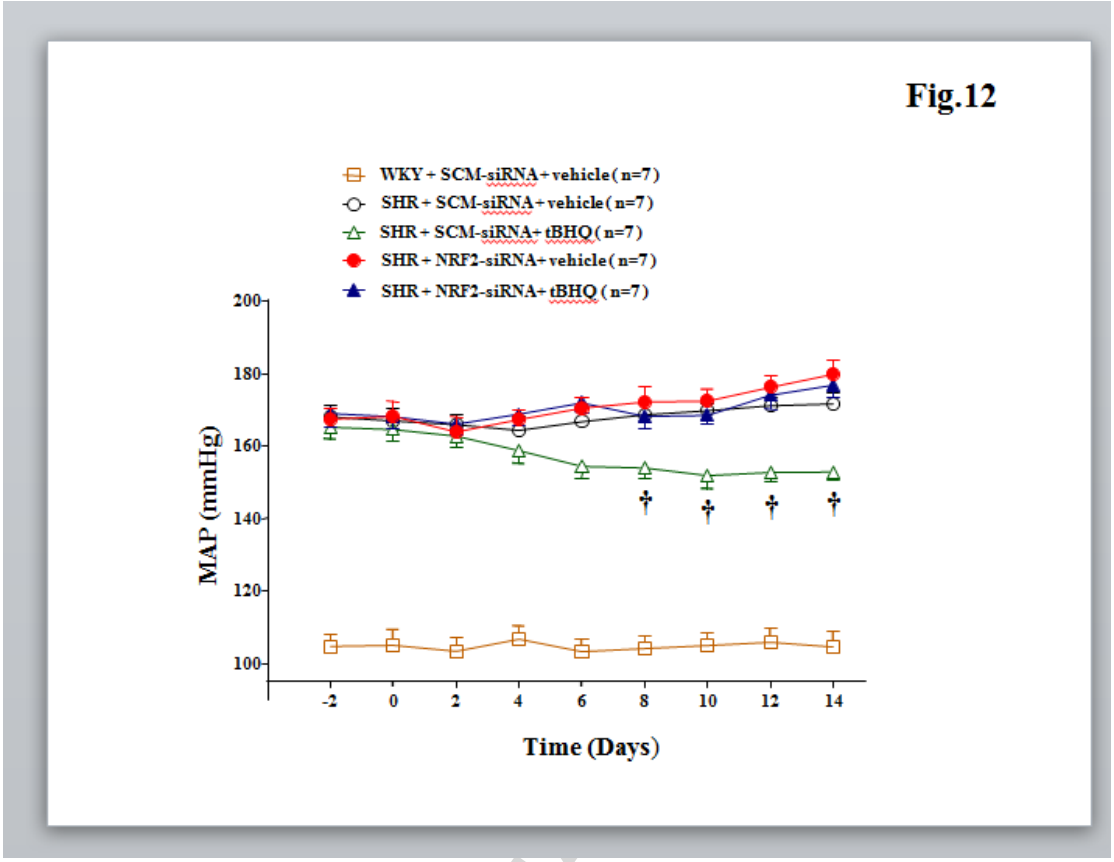

Figure 12

Table 1. Rat primers for RT- qPCR

Rat genes	GenBank accession number	Primer sequence	
		Forward(5'-3')	Reverse(5'-3')
IL-1β	NM_031512.2	CTGTGACTCGTGGGATGATGAC	CTTCTTCTTTGGGTATTGTTTGG
IL-6	NM_012589	AAGAAAGACAAAGCCAGAGTC	CACAAACTGATATGCTTAGGC
β-actin	NM_031144	CACCCGCGAG TACAACCTTC	CCCATACCCA CCATC ACACC

Highlights

- PVN administration of tBHQ attenuated hypertension in SHR
- tBHQ inhibited PVN oxidative stress in SHR via activation of the Nrf2 pathway.
- PVN infusion of tBHQ reduced proinflammatory cytokines in SHR.
- PVN infusion of tBHQ restored the imbalance of neurotransmitters in SHR.