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Modulatory Role of Nurr1 Activation and Thrombin Inhibition in the Neuroprotective Effects of Dabigatran Etexilate in Rotenone-Induced Parkinson's Disease in Rats

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Abstract Recently, it has been shown that both decreased nuclear receptor-related 1 (Nurr1) expression and thrombin accumulation are involved in the degeneration of dopaminergic neurons in Parkinson's disease (PD). The new anticoagulant dabigatran etexilate (DE) is a direct thrombin inhibitor that owns benzimidazole group, which has been proposed to activate Nurr1. In the present study, we examined the neuroprotective effects of DE in rotenone model of PD. Rotenone was injected subcutaneously at a dose of 1.5 mg/kg every other day for 21 days. An oral regimen of DE (15 mg/kg) was started after the 5th rotenone injection following the manifestations of PD. Treatment of PD rats with DE mitigated rotenone-induced neuronal degeneration and restored striatal dopamine level with motor recovery. As well, DE enhanced Nurr1 expression in substantia nigra along with increasing transcriptional activation of Nurr1-controlled genes namely tyrosine hydroxylase, vascular monoamine transporter, glial cell line-derived neurotrophic factor, and its receptor gene c-Ret, which are critical for development and maintenance of dopaminergic neurons. DE also suppressed thrombin accumulation in substantia nigra. Both effects probably contributed to repressing neurotoxic proinflammatory cytokines, which was manifested by decreased level of nuclear factor kappa beta and tumor necrosis factor alpha. In conclusion, the present results suggest that DE could possess significant neuroprotective and regenerative effects in a rotenone-induced PD animal model as consequence of Nurr1 activation and thrombin inhibition.

Keywords Dabigatran etexilate · Nurr1 · Thrombin · Neuroprotection · Parkinson's disease · Rotenone

Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder [1]. It is characterized by progressive degeneration of dopaminergic neurons in the substantia nigra (SN) with subsequent loss of striatal dopamine (DA), which leads to the cardinal motor abnormalities [2]. The pathological features of PD include an abnormal accumulation of intracellular protein aggregates known as Lewy bodies throughout various brain regions, with α-synuclein comprising its major fraction [2, 3]. Prevailing therapeutic strategies for PD focus primarily on reducing the severity of symptoms by restoring the DA level in the nigrostriatal system, yet they have not addressed the progressive nature of the disease [4]. Thus, the need for a neuroprotective therapy in PD is obvious.

There is a great consideration about the nuclear receptor-related 1 (Nurr1) as a promising therapeutic target for halting PD progression [5]. Nurr1 is a transcription factor that belongs to the nuclear receptor superfamily and is widely distributed in the central nervous system (CNS) especially in the dopaminergic neurons in the SN [6]. Exclusively, Nurr1 is critical for

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development, maintenance, and survival of dopaminergic neurons [7]. It controls the expression of tyrosine hydroxylase (TH), aromatic amino acid decarboxylase, and vesicular monoamine transporter (VMAT) genes, which are involved in the synthesis and storage of DA in SN [8, 9]. Moreover, Nurr1 regulates the c-Ret gene, which is a ligand-binding component of the glial cell line-derived neurotrophic factor (GDNF) receptor complex in the midbrain dopaminergic neurons [10, 11]. GDNF is a neurotrophic factor that plays an essential role in development and survival of dopaminergic neurons through activation of several pathways including mitogen-activated protein kinase and phosphatidylinositol-3-kinase pathways that are required for neuronal persistence, plasticity, and neurite outgrowth [12, 13]. As well, GDNF has been revealed to protect dopaminergic neurons against apoptosis and neurotoxin-induced cell death [14, 15]. Interestingly, Nurr1 acts synergistically with other factors to promote the synthesis and secretion of neurotrophic factors including GDNF [16]. Furthermore, Nurr1 plays a vital role in repressing proinflammatory genes in glial cells and protecting dopaminergic neurons from inflammation-induced death [17]. It is worth mentioning that decreased expression of Nurr1 was observed in PD postmortem brains [18]. To this end, Nurr1 is not only essential for the development of dopaminergic neurons and maintenance of their functions but also plays a role in PD pathogenesis.

Recently, a number of studies revealed the impact of thrombin not only on the coagulation system but also on neuronal signaling, development, and plasticity [19, 20]. These declarations were based on the ability of the CNS to yield prothrombin, thrombin precursor, in developing brain [21] besides, the existence of thrombin receptors along with the tissue inhibitor of thrombin in CNS [20]. In this context, thrombin has been recognized within the CNS in rat and human samples under physiological conditions and has increasingly been involved in a wide variety of CNS functions including neuronal cell shape and development besides maintaining the blood brain barrier integrity and proper brain function [22–24]. However, elevated thrombin levels in the CNS were shown to contribute to neuroinflammation and neurotoxicity and have been linked to several disorders such as multiple sclerosis and other neurodegenerative diseases [20, 25, 26]. Remarkably, thrombin has been implicated in PD since it causes neurodegeneration in the basal ganglia [27] and it recapitulates the vital histopathological characteristics of PD, oxidative stress and neuroinflammation upon injection into the SN [28]. Moreover, a previous study showed that application of thrombin simultaneously with 6-hydroxydopamine, agent that induce PD, resulted in much pronounced neuronal damage and behavioral deficits [29]. Thrombin was proposed to exert its neurotoxic effects on dopaminergic neurons via protease-activated receptors, which are expressed throughout the brain in neurons and glial cells [20, 30]. Activation of protease-activated receptors resulted in microglial activation with subsequent release of proinflammatory cytokines including cyclooxygenase 2, inducible nitric oxide synthase, tumor necrosis factor alpha (TNF-α), and interleukins that endorse dopaminergic neurons death in the SN [30, 31]. Accordingly, the modulation of thrombin activity has become of increasing interest in the quest for new PD therapeutic targets.

Taken together, an agent that could both activate Nurr1 activity and inhibit thrombin release would greatly halt the progression of neurodegeneration. Even though the endogenous ligand for Nurr1 is unidentified, certain synthetic molecules were revealed to enhance Nurr1's transcriptional activity in vitro [32, 33]. Series of benzimidazole-based compounds have been proposed to activate Nurr1 [5]. The new anticoagulant dabigatran etexilate (DE) is a direct thrombin inhibitor that has shown to possess benzimidazole group [34]. Remarkably, DE has the advantage of being safe, convenient, and reliable drug with favorable biochemical and pharmacological profile [35]. Therefore, the aim of the present study was to explore the modulatory role of Nurr1 activation and thrombin inhibition in the possible neuroprotective effects of DE in rotenone-induced PD in rats.

Materials and Methods

Animals

Adult male Wistar rats (200–250 g) were obtained from the animal facility of the Faculty of Pharmacy, Cairo University, Egypt. Rats were housed at constant temperature (25 ± 2 °C), humidity (60 ± 10%), and a 12/12-h light/dark cycle and were allowed standard rat chow and water ad libitum. The study was approved by the Ethics Committee of Faculty of Pharmacy, Cairo University (Permit Number: 1832) and complies with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

Chemicals and Drugs

DE, rotenone, and dimethyl sulfoxide (DMSO) were purchased from Selleck Chemicals (Houston, TX, USA), Sigma–Aldrich (St. Louis, MO, USA), and Merck (Darmstadt, Germany), respectively. Fine chemicals and reagents, unless otherwise specified, were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

Experimental Design

Rats were randomly allocated into four groups, 15 rats each except for groups receiving rotenone, which started with 18 rats to compensate for lethality due to rotenone. The study

comprised of total 21 days, where group I received 11 subcutaneous injections of 1% DMSO (0.2 ml/kg) on rotenone corresponding days in addition to 0.5% carboxymethyl cellulose (CMC) (2.5 ml/kg, p.o.) daily starting from the 11th day to serve as the control group. Animals of group II received daily DE suspended in 0.5% CMC (15 mg/kg, p.o.) [36] starting from the 11th day to serve as the drug control group. Groups III & IV received 11 subcutaneous injections of rotenone (1.5 mg/kg) dissolved in 1% DMSO every other day [37]. In addition, group IV was daily treated 1 h before rotenone injection with DE starting from the 11th day as previously described (Fig. 1). This regimen was based on a preliminary study in which animals (n = 8 per group) received three and five subcutaneous injections of rotenone every other day at a dose of 1.5 mg/kg dissolved in 1% DMSO. Behavioral and histopathological evaluations as well as striatal DA level determination revealed significant PD manifestations after the 5th injection compared to the 3rd one (Figs. 2 and 3).

Behavioral Assessment

Twenty-four hours after the last rotenone injection, all rats were screened for motor impairments using the open field and rotarod tests.

Open Field

A square wooden box, with the measurements 80 × 80 × 40 cm, was used. The walls were painted with red and the floor was divided with white lines into 16 equal squares 4 × 4. Rats were placed individually in the central area of the open field and the locomotor behavior was video-recorded for 3 min. The floor and walls were cleaned after testing each rat to eliminate possible bias due to odors left by previous rat. The ambulation frequency (horizontal movement), rearing frequency (vertical movement), and immobility time were calculated for each animal [38].

Rotarod

A rotating rod (3 cm diameter, 90 cm height, and 25 rpm) was used to examine motor coordination and balance of the animals. Rats were acclimated on the rotarod by giving three training sessions of 5 min each for 3 days before the end of the experiment. After completing the open field test, rats were evaluated for a period of 5 min and the latency to fall off the rotarod within this period was recorded [39].

Brain Processing

After evaluation of the motor performance, rats in each group were divided into three sets and then were sacrificed by cervical dislocation under light anesthesia; subsequently, brains were rapidly dissected and washed with ice-cold saline. In the first set (n = 3 per group), brains were fixed in 10% (v/v) formalin for 24 h to perform histopathological staining with hematoxylin and eosin. In the other sets, the striata and SN tissues were excised from each brain on an ice-cold glass plate. Afterwards, in the second set (n = 6 per group), striata and SN were homogenized in ice-cold physiological saline as 10% (w/v), then the striatal homogenate was used for determination of DA level and the SN homogenate was employed for the estimation of nuclear factor kappa beta (NF-κB), TNF-α, and thrombin-antithrombin complex (TAT). In the third set (n = 6 per group), SN tissues were used for the assessment of gene expression of α-synuclein, Nurr1, TH, VMAT, GDNF, and corepressor for repressor element 1 silencing transcription factor (CoREST) as well as the protein expression of c-Ret.

Measured Parameters

Enzyme Linked Immunosorbent Assay of Levels of DA in the Striatum as well as NF-κB, TNF-α, and TAT in the SN

DA in the striatal tissues as well as NF-κB and TAT in the SN tissues were determined using ELISA assay kits (Cusabio, Wuhan, China). Likewise, TNF-α in the SN tissues was quantified using ELISA assay kit (Elabscience, Wuhan, China). All procedures were done according to the manufacturers' instructions. The results are expressed as nanograms per milligram protein for DA level and picograms per milligram protein for NF-κB, TNF-α, and TAT contents, where the protein content was quantified according to the method described by Lowry et al. [40].

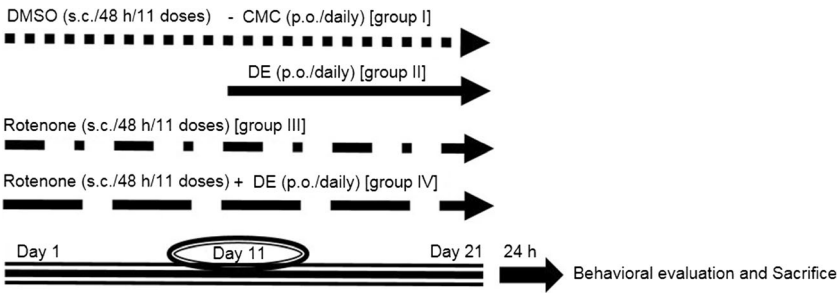

Fig. 1 Experimental design

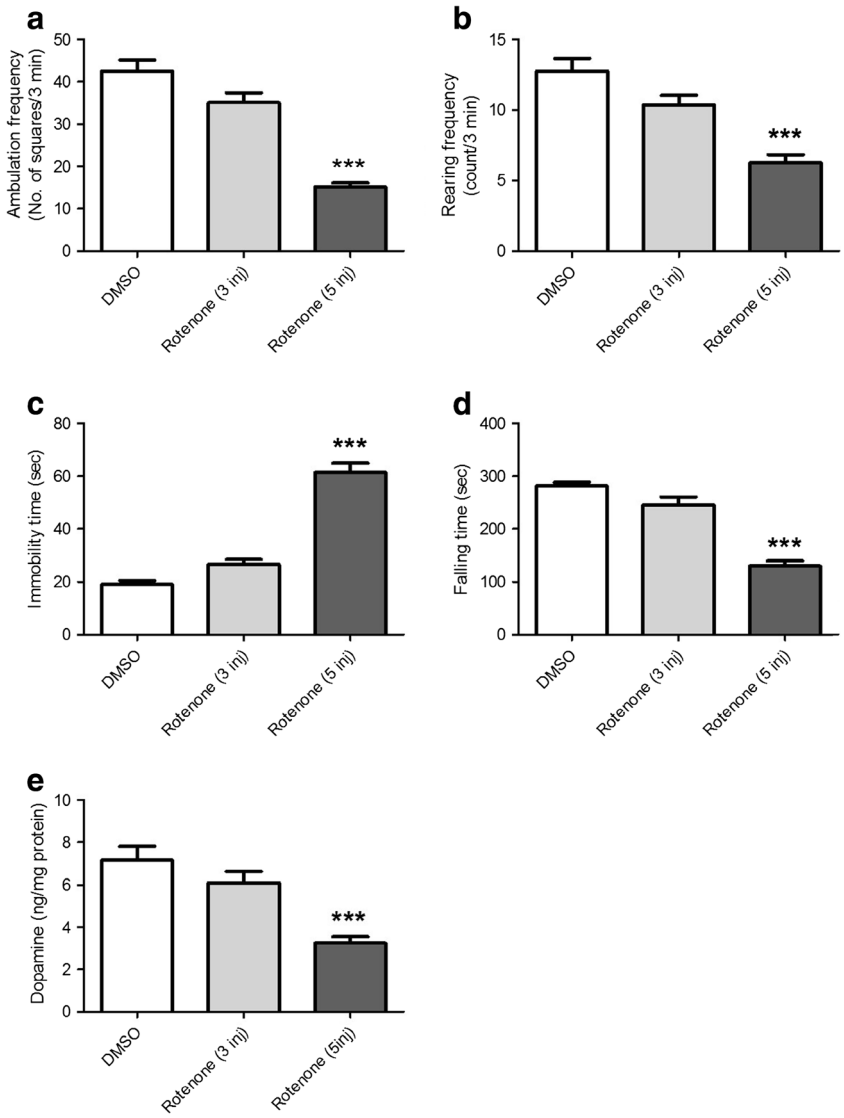

Fig. 2 Results of preliminary study. **a**–**d** Behavioral changes after administration of rotenone (1.5 mg/kg/48 h) for three and five injections ($n = 8$). **e** Striatal DA level after administration of rotenone (1.5 mg/kg/48 h) for three and five injections ($n = 5$). *Each bar* with vertical line represents the mean ± S.E.M., ***significantly different from DMSO group at $p < 0.001$

Quantitative Real-Time Polymerase Chain Reaction Analysis of α-Synuclein, Nurr1, TH, VMAT, GDNF, and CoREST Gene Expression in the SN

Total RNA was extracted from SN tissues using SV Total RNA Isolation system (Promega, Madison, WI, USA) and the pureness of obtained RNA was confirmed spectrophotometrically at OD 260/280 nm. Equal amounts of extracted RNA was then reverse transcribed into complementary DNA using RT-PCR kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. To access the expression of α-synuclein, Nurr1, TH, VMAT, GDNF, and CoREST genes, quantitative RT-PCR was accomplished using SYBR Green JumpStart Taq ReadyMix (Sigma–Aldrich, St. Louis, MO, USA) as described by the manufacturer. Briefly, in a 25 μl reaction volume, 5 μl of complementary DNA was added to 12.5 μl SYBR Green mixture, 5.5 μl RNase free water, and 2 μl of each primer (5 pmol/μl). The sequences of primers are described in Table 1. The PCR amplifications were performed with 40 cycles of denaturation for 15 s at 95 °C, annealing 60 s at 60 °C, and extension 60 s at 72 °C. After the quantitative RT-PCR run, the relative expression of target gene was obtained using the $2^{-\Delta\Delta CT}$ formula and β-actin as a housekeeping gene [41].

Western Blot Analysis of c-Ret Protein Expression in the SN

After protein solutions were extracted from SN tissues, equal amounts of proteins were loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis to be separated according to their molecular weight. Following electrophoresis, proteins were transferred to nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA) using a

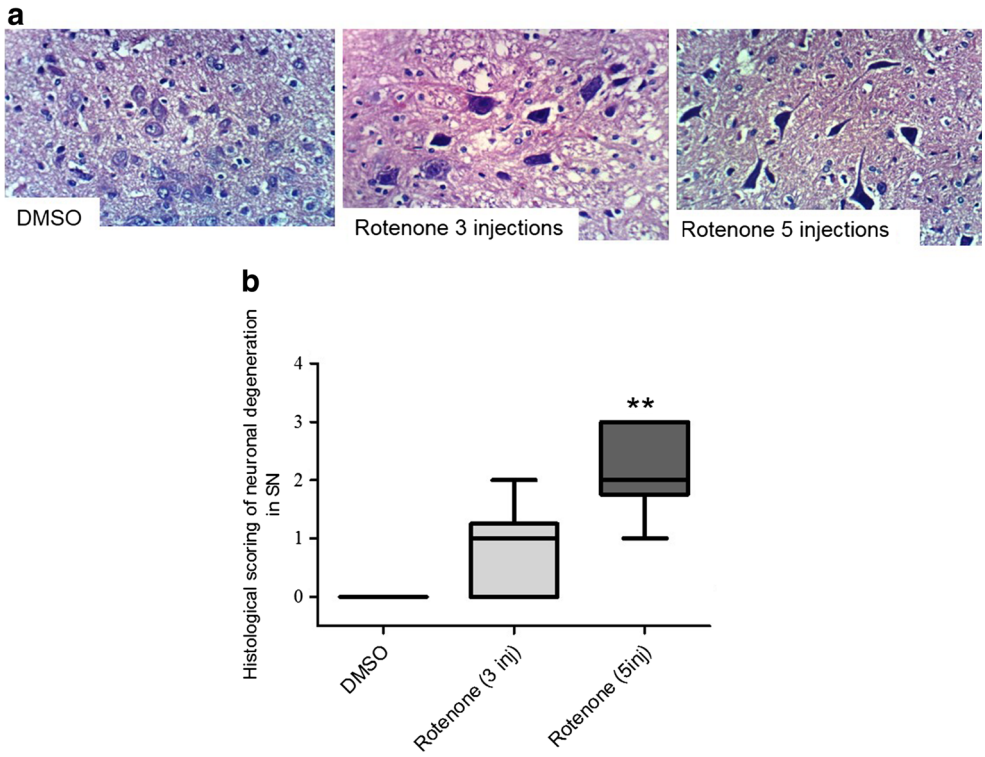

Fig. 3 Histological outcomes of preliminary study. **a** Histological sections in the SN of the experimental groups; where rat receiving DMSO showed normal intact nigral neurons with visible nuclei, while rat receiving rotenone for three injections showed apparent normal nigral neurons with few, scattered degenerative ones. On the other hand, rat receiving rotenone for five injections showed multifocal degenerative neurons with invisible nuclei (H&E ×400). **b** Histological scoring of neuronal degeneration in SN. Results are expressed as median and range of three rats per group; **significantly different from DMSO group at $p < 0.01$, using Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparisons test

semidry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were then soaked in a 5% skimmed milk to block non-specific binding sites. Afterwards, membranes were incubated with antibody against rat c-Ret (1:1000; Sigma–Aldrich, St. Louis, MO, USA) on a roller shaker at 4 °C overnight. Next, they were washed and incubated with the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (1:2000; Fluka, St. Louis, MO, USA). Finally, the blots were developed with enhanced chemiluminescence detection reagents (Amersham Biosciences, Arlington Heights, IL, USA). The amount of c-Ret protein was quantified by densitometric analysis using a scanning laser densitometer (GS-800 system, Bio-Rad, Hercules, CA, USA). Results are expressed as arbitrary units after normalization with β-actin protein expression.

Histopathological Examination

Histopathological assessment was performed on the midbrain sections of rats. Brains were carefully removed, rinsed with ice-cold saline, and immediately fixed in 10% formalin for 24 h. Coronal brain sections were processed for paraffin embedding and 5 μm sections were prepared. Sections were then stained with hematoxylin and eosin (H&E) and examined by the light electric microscope [Olympus CX21, Tokyo, Japan]. Histological sections were scored from 0 to 4 with regard to neuronal degeneration in SN, where 0 = normal; 1 = minimal or few degenerative neurons; 2 = moderate, multifocal groups of degenerative neurons; 3 = marked or large, multifocal degenerative neurons; and 4 = severe or conjoining groups of degenerative neurons [42].

Table 1 The primers sequences

Gene	Forward primer	Reverse primer
β-Actin	5′-CGTTGACATCCGTAAAGACCTC-3′	5′-TAGGAGCCAGGGCAGTAATCT-3′
α-Synuclein	5′-CACCATGGATGTATTCATGTTTCC-3′	5′-GGCTTCAGGTTCGTAGTCTTGAT-3′
Nurr1	5′-CGCGTCGCAGTTGCTTGACAC-3′	5′-TTGCCTGGAACCTGGAATAGT-3′
TH	5′-TGTCAGAGGAGCCCGAGGTC-3′	5′-CCAAGAGCAGCCCATCAAAG-3′
VMAT	5′-CTTTGGAGTTGGTTTTGC-3′	5′-GCAGTTGTGGTCCATGAG-3′
GDNF	5′-TATGAAGTTATGGGATGTCG TGGCT-3′	5′-TAGATACATCCACACCGTTT AGCGG-3′
CoREST	5′-TGAGCCTGAATCCTCCATTG-3’	5′-AGGCAGCCATTCCAGTCACA-3’

Statistical Analysis

All data were checked for normality as well as homogeneity of variance using Kolmogorov–Smirnov and Bartlett's tests, respectively. Datasets that met the assumptions for parametric analysis were analyzed using a one-way ANOVA followed by the Tukey multiple comparisons test and were expressed as mean ± S.E.M. The histopathological scores were analyzed using Kruskal–Wallis nonparametric one-way ANOVA followed by Dunn's multiple comparisons test and were expressed as median and range. For all the statistical tests, the level of significance was fixed at $p < 0.05$. Statistical analysis was performed using GraphPad Prism software version 5 (San Diego, CA, USA).

Results

Noteworthy, animals given DE alone did not show any significant alteration from control rats in all measured parameters.

DE-Amended Behavioral Changes Induced by Rotenone

Rotenone significantly deteriorated motor activity and coordination of rats as compared with the control group (Fig. 4). DE administration improved motor performance, which was revealed by significant increase in the ambulation and rearing frequencies as well as falling latency by 2.8-, 3- and 4.5-fold, respectively and marked decrease in immobility time by 53% as compared with the rotenone group.

DE-Reversed Rotenone-Induced Alterations in Striatal DA Level and α-Synuclein Gene Expression in SN

Rats receiving rotenone showed a massive decline in striatal DA level with increase in α-synuclein gene expression in SN as compared to their control counterparts (Fig. 5). Improvements in locomotion by DE were reflected as augmentation of striatal DA by 4.3-fold and downregulation of α-synuclein gene expression in SN by 72.4% as compared with the rotenone group.

DE-Mitigated Rotenone-Induced Alterations in Nurr1 and Nurr1-Regulated Genes

Repeated rotenone injection caused an obvious decrease in Nurr1 gene expression along with decline in gene expression of Nurr1-regulated TH, VMAT, GDNF, and protein expression of c-Ret in the SN as compared with the control group (Fig. 6). DE treatment significantly modulated the expression of Nurr1 by 3.4-fold as well as the abovementioned dopaminergic target genes after oral administration by threefold (TH), twofold (VMAT), 4.3 fold (GDNF), and 2.4 fold (c-Ret), respectively, as compared with the rotenone group.

DE-Attenuated Rotenone-Induced Inflammatory Changes

Rats receiving rotenone exhibited an immense reduction in CoREST gene expression together with elevation of NF-κB level and its downstream effector TNF-α in the SN as compared with the control group (Fig. 7). DE restored CoREST

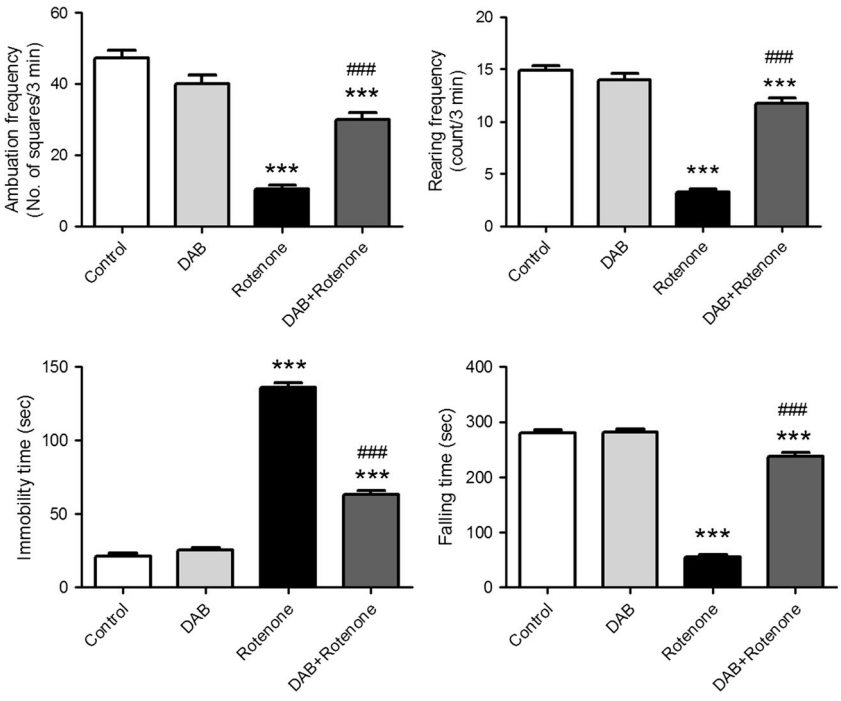

Fig. 4 DE amended behavioral changes induced by rotenone. *Each bar* with vertical line represents the mean ± S.E.M. of 15 rats per group; ***significantly different from control group at $p < 0.001$, ###significantly different from rotenone group at $p < 0.001$ using one-way ANOVA followed by Tukey multiple comparisons test

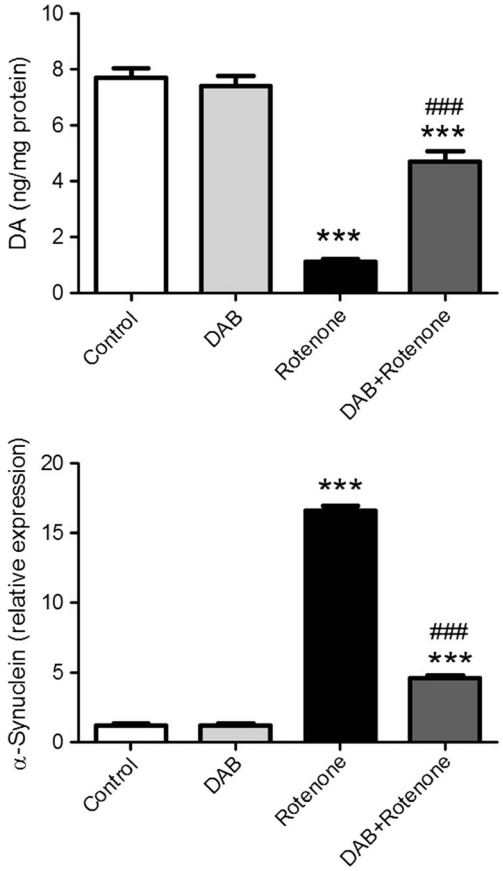

Fig. 5 DE reversed rotenone-induced alteration in striatal DA and α-synuclein in SN. *Each bar* with vertical line represents the mean ± S.E.M. of 6 rats per group; ***significantly different from control group at $p < 0.001$, ###significantly different from rotenone group at $p < 0.001$ using one-way ANOVA followed by Tukey multiple comparisons test

expression by 2.6-fold and suppressed NF-κB as well as TNF-α levels by 63.5 and 58.9%, respectively, as compared to rotenone-exposed rats.

DE-Alleviated Rotenone-Induced Alteration in Thrombin Generation

TAT striatal concentration was assessed and used as an indicator for thrombin generation. In rats receiving rotenone, TAT level was significantly high in comparison to control group, an effect that was ameliorated by DE which decreased the TAT level by 53.9% as compared with the rotenone group designating a drop in thrombin generation (Fig. 7).

DE-Preserved Dopaminergic Neurons in SN Against Rotenone-Induced Deterioration

Rotenone regimen resulted in a severe loss of dopaminergic neurons in the SN, indicated by abnormal histologic score (≥3) as compared with the control group (Fig. 8). On the other hand, treatment with DE conserved dopaminergic neurons against rotenone-induced degeneration.

Discussion

The purpose of the current investigation was to address the role of Nurr1 activation and thrombin inhibition in the possible protective effect of DE in rotenone-induced neurotoxicity in rat model of PD. Herein, it was revealed that DE can significantly improve motor impairments, prevent midbrain dopaminergic neuronal loss, restore striatal DA level, and lower the gene expression of the disease marker protein, α-synuclein, in PD animal model. Our experimental evidence supports that DE activates Nurr1 and enhances its functions by further inducing transcriptional activation of dopaminergic neuron-specific genes and further repressing neurotoxic proinflammatory cytokines. Moreover, DE suppresses thrombin accumulation in SN with subsequent decrease in neuroinflammation leading to significant neuroprotective and neurorestorative effects in a rotenone-induced PD animal model.

In the present study, we tried to explore the role of DE in a way similar to clinical real life, where the drug was given after the manifestations of PD. This regimen was planned conferring to the fact that prompt therapy from the point when declined motor activity was detected might rescue the dopaminergic neurons of PD rats to an optimum extent [43]. The rotenone-induced PD model is considered to adequately mimic the pathogenesis and progress of PD [44]. In this context, repeated exposure of rats to rotenone in the current study produced 17% mortality and the surviving rats showed most of the features of PD including α-synuclein accumulation with dopaminergic neuronal death and consequent striatal DA decline along with motor impairment, which are in line with former studies [43, 45, 46]. The molecular mechanisms triggering the degeneration of SN dopaminergic neurons in PD are ambiguous; however, deficiency of certain key molecules such as transcription factors can be implicated [8, 47]. Remarkably, paucity of Nurr1 transcription factor is established in autopsied PD midbrains, especially in α-synuclein overexpressing neurons [6, 48, 49]. In the current study, repeated rotenone injection resulted in upregulation of α-synuclein gene expression along with reduced Nurr1 gene expression. Furthermore, concomitant with the fall of Nurr1 expression, the levels of Nurr1-regulated genes (TH and VMAT) were also significantly diminished in rats' SN. The substantial decline of Nurr1-controlled genes further compromise the DA release, because these dopaminergic genes are critical for the synthesis of DA in the dopaminergic neurons [9]. Eventually, this drastic decrease in DA level resulted in the severe deterioration of motor activities of rats. These results are in line with previous studies that showed decline in Nurr1 along with Nurr1-related genes and DA in PD models

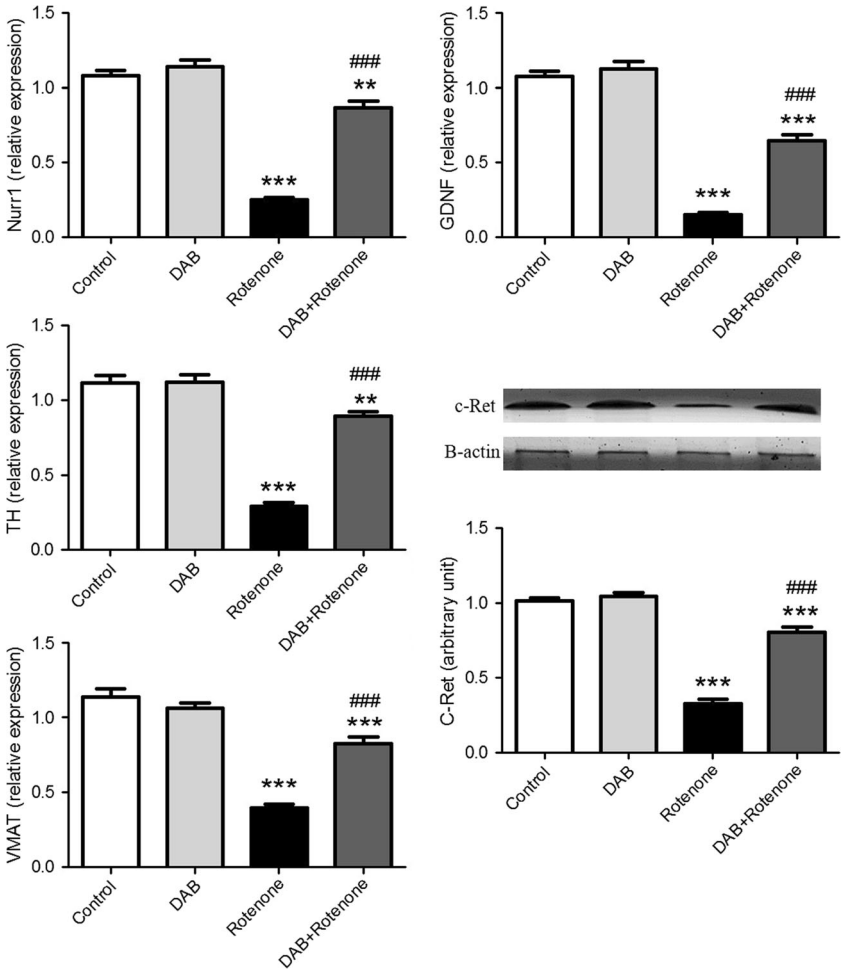

Fig. 6 DE mitigated rotenone-induced alteration in Nurr1 and Nurr1-regulated genes. *Each bar* with vertical line represents the mean ± S.E.M. of six rats per group; **significantly different from control group at $p < 0.01$,***significantly different from control group at $p < 0.001$, ###significantly different from rotenone group at $p < 0.001$ using one-way ANOVA followed by Tukey multiple comparisons test

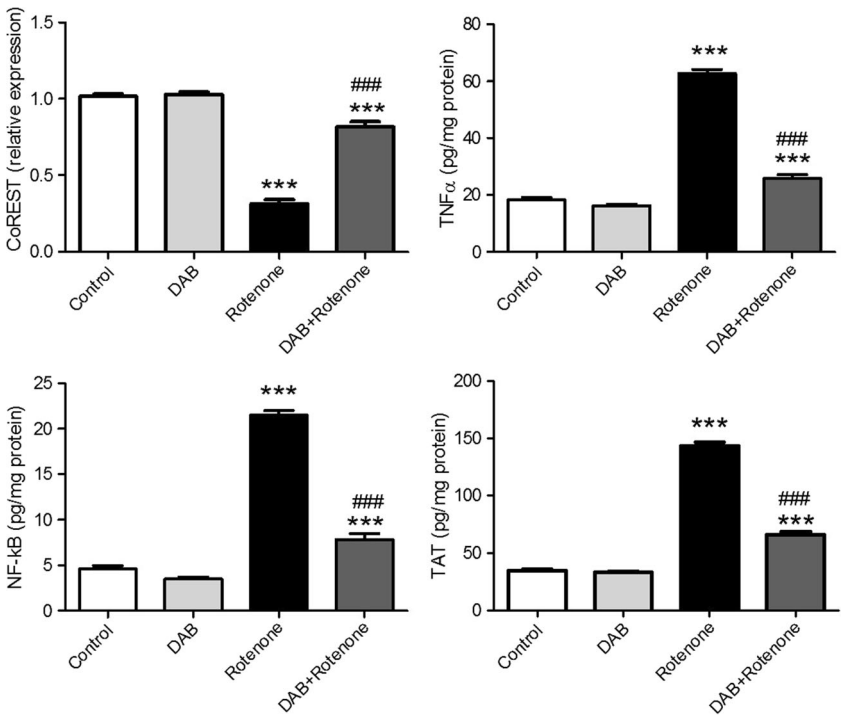

Fig. 7 DE attenuated rotenone-induced inflammatory changes and alteration in thrombin generation. *Each bar* with vertical line represents the mean ± S.E.M. of six rats per group; ***significantly different from control group at $p < 0.001$, ###significantly different from rotenone group at $p < 0.001$ using one-way ANOVA followed by Tukey multiple comparisons test

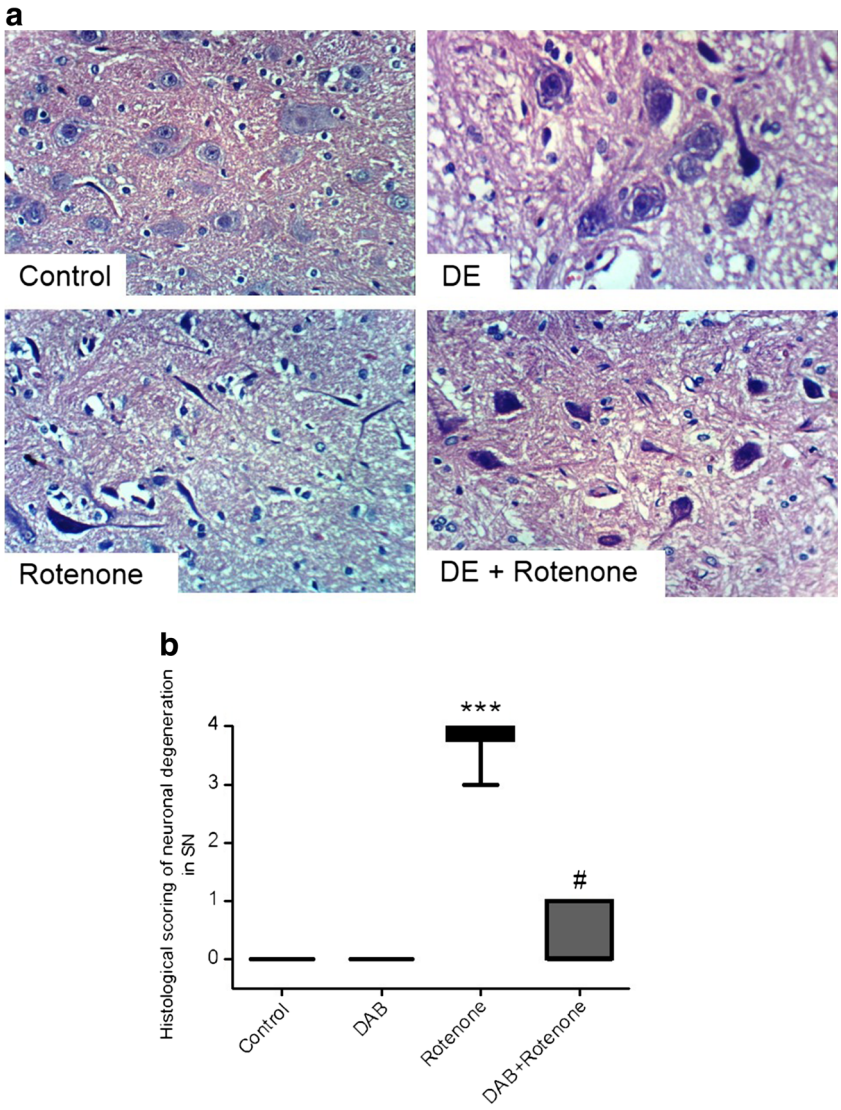

Fig. 8 DE preserved dopaminergic neurons in SN against rotenone-induced deterioration. **a** Histological sections in the SN of the experimental groups, where control rat as well as rat receiving DE alone showed normal intact nigral neurons with visible nuclei, while rat receiving rotenone showed necrosis and atrophy of nigral neurons with invisible nuclei. On the other hand, rat treated with DE showed apparent normal nigral neurons (H&E ×400). **b** Histological scoring of neuronal degeneration in SN. Results are expressed as median and range of three rats per group; ***significantly different from control group at $p < 0.001$; #significantly different from rotenone group at $p < 0.05$, using Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparisons test

[18, 33, 47, 50]. It is suggested that α-synuclein plays a vital role in regulating Nurr1 protein stability and that Nurr1 is a downstream molecular target of α-synuclein, where α-synuclein endorses a proteasome-dependent degradation of Nurr1 protein [50]. Furthermore, by entering the nucleus, α-synuclein exerts an impact on the transcriptional activity of Nurr1, which may be prompted by interactions with histones leading to inhibition of histone acetylation [51, 52]. In turn, reduced concentrations of Nurr1 make dopaminergic neurons more susceptible to α-synuclein-induced damage. Conversely, overexpression of Nurr1 can afford protection against the α-synuclein-induced toxic insult in nigral dopaminergic neurons in vitro [52, 53], which was observed here with DE treatment in vivo. DE enhanced Nurr1 expression, which was sufficient to activate the cell's neuroprotective machinery to preserve the nigral dopaminergic neurons. Consequently, the expression of Nurr1-controlled genes, TH and VMAT, was provoked along with restoration of striatal DA level. This favorable effect of DE on dopaminergic neurons was further evidenced by improved motor performance and coordination in open field and rotarod tests, respectively. Activation of Nurr1 by DE could be attributed to the benzimidazole scaffold, which is a part of DE's core structure. Previously, it was reported that benzimidazole-based compounds could activate Nurr1 with high efficacy [5, 54].

Besides controlling genes involved in the regulation of DA neurotransmission, Nurr1 modulates GDNF/c-Ret signaling, which is required for survival of nigral neurons [11, 55]. In this study, Nurr1 downregulation is accompanied by compromised GDNF/c-Ret survival signaling in SN of rats receiving rotenone. In agreement, previous studies showed that c-Ret was downregulated in nigral neurons of PD animals [50, 52]. Herein, enhanced expression of Nurr1 by DE was able to induce GDNF trophic response, as seen by the elevation of

the c-Ret receptor protein expression and GDNF gene expression, thus restoring the ability of neurons to access and respond to endogenous GDNF. Consistent with this observation, histological examination showed that the nigral neurons were preserved in DE-treated animals. Therefore, the interaction between Nurr1 and neurotrophic factors might play a significant role in the development and survival of dopaminergic neurons.

In addition to its vital roles in the development and maintenance of dopaminergic neurons, Nurr1 plays a dynamic role in protecting these neurons from inflammation-induced neuronal death [17, 56]. Inflammation is implicated in the pathogenesis of PD where the NF-κB pathway is of considerable interest because it regulates expression of many proinflammatory genes [57, 58]. Nurr1 exerts its anti-inflammatory activity via a Nurr1/CoREST transrepression pathway that functions in a feedback manner to restore the activated NF-κB gene transcription to a basal state [17, 59]. In this pathway, Nurr1 is recruited to NF-κB on inflammatory gene promoters after phosphorylation of p65 at serine by glycogen synthase kinase 3b (GSK3b), which provides the docking site necessary to recruit Nurr1 to p65 [60]. Nurr1 subsequently recruits the CoREST corepressor complex, which in turn mediates the clearance of NF-κB-p65 from the target promoters resulting in the transcriptional repression of inflammatory response [17, 59]. Consistent with these discoveries, reduced Nurr1 expression in rats receiving rotenone resulted in exaggerated inflammatory responses in SN, which was manifested by decreased gene expression of CoREST and elevated production of transcriptional factor NF-κB and its downstream effector TNF-α which participates in the death of nigral dopaminergic neurons. In the present study, DE suppressed NF-κB level in the SN leading to a dramatic decrease in TNF-α level. These data suggest that DE displayed its anti-inflammatory effect through modulation of Nurr1 activity resulting in the inhibition of NF-κB-dependent gene expression by recruiting corepressor protein, which would reduce the binding of p65 to inflammatory gene promoters.

Besides modulating Nurr1 expression, decreasing thrombin level may participate in the anti-inflammatory effect of DE recognized in rotenone-induced PD model as indicated by other studies [26, 61]. This assumption is further strengthened by the verdicts that thrombin is a potent proinflammatory mediator and that elevated thrombin level contributes to neuroinflammation [25, 26, 62]. In line with this, we observed an increased thrombin level in SN of rats receiving rotenone, which could partly account for the resulted inflammation and neuronal death. Therefore, thrombin inhibition could provide beneficial actions by reducing potentially damaging inflammatory events.

Taken together, the findings of the current investigation provide an evidence for the neuroprotective effect of DE against rotenone neurotoxicity mediated through Nurr1-and thrombin-dependent mechanisms. Distinctly, Nurr1 serves as a potential target for PD therapy by promoting the maintenance and survival of dopaminergic neurons and protecting them against inflammation-induced death. In addition, mitigating thrombin accumulation provides further protection against the detrimental inflammatory actions. Given the efficacy of DE in an animal model of rotenone-induced PD, it would be advisable to extend these experimental studies to clinical trials to aid in halting the progression of PD.

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Compliance with Ethical Standards The study was approved by the Ethics Committee of Faculty of Pharmacy, Cairo University (Permit Number: 1832) and complies with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

Conflict of Interest The authors declare that they have no conflict of interest.

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