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RESEARCH

The Effects of Carvacrol on Transient Receptor Potential (TRP) Channels in an Animal Model of Parkinson's Disease

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Abstract

In this study, we aimed to investigate the effects of carvacrol (CA), a widely used phytochemical having anti-oxidant and neuroprotective effects, on transient receptor potential (TRP) channels in an animal model of Parkinson's disease (PD). A total of 64 adult male Spraque-Dawley rats were divided into four groups: sham-operated, PD animal model (unilateral intrastriatal injections of 6-hydroxydopamine (6-OHDA), 6 µg/µl), PD + vehicle (dimethyl sulfoxide (DMSO)) treatment, and PD + CA treatment (10 mg/kg, every other day, for 14 days). Half of the brain samples of substantia nigra pars compacta (SNpc) and striatum (CPu) were collected for immunohistochemistry and the remaining half were used for molecular analyses. CA treatment significantly increased the density of dopaminergic neurons immunolabeled with tyrosine hydroxylase and transient receptor potential canonical 1 (TRPC1) channel in the SNpc of PD animals. In contrast, the density of astrocytes immunolabeled with glial fibrillary acetic acid and transient receptor potential ankyrin 1 (TRPA1) channel significantly decreased following CA treatment in the CPu of PD animals. RT-PCR and western blot analyses showed that 6-OHDA administration significantly reduced TRPA1 and TPRPC1 mRNA expression and protein levels in both SNpc and CPu. CA treatment significantly upregulated TRPA1 expression in PD group, while TRPC1 levels did not display an alteration. Based on this data it was concluded that CA treatment might protect the number of dopaminergic neurons by reducing the reactive astrogliosis and modulating the expression of TRP channels in both neurons and astrocytes in an animal model of PD.

Keywords Parkinson's disease · Carvacrol · TRPA1 · TRPC1 · Calcium signaling

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Introduction

Parkinson's disease (PD) is considered as the most common movement disorder and the second most common neurodegenerative disease in present. Currently, more than 6 million people have PD worldwide and this number will be expected to exceed 12 million by 2050 (GBD 2016 Parkinson's Disease Collaborators 2018; Walter 2018). Although the typical motor symptoms are tremor, rigidity, bradykinesia/akinesia, and postural instability, the clinical manifestation may be accompanied by other motor and non-motor symptoms. PD is characterized by progressive loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the misfolded alpha-synuclein accumulation in cytoplasmic inclusions called as Lewy bodies (Balestrino and Schapira 2020). So far, a number of molecular mechanisms have been underlined to be responsible for the specific loss of dopaminergic neurons in the SNpc, but the role of calcium ions has become a novel interest due to its critical role in neuronal survival. Intracellular calcium (Ca^{2+}) dynamics,

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especially in the storage organelles such as the endoplasmic reticulum and mitochondria, have been shown to play role in the pathophysiology of complex neurodegenerative diseases including PD (Kazuno et al. 2006). While intracellular Ca^{2+} stores are necessary to stimulate dopamine release from the SNpc (Patel et al. 2009), unfolded protein response can disrupt intracellular Ca^{2+} homeostasis by altering the activity of cation channels, mainly the transient receptor potential (TRP) channels (Lee et al. 2021).

TRP channels are expressed in the central nervous system and critically involved not only in normal physiological but also in pathophysiological responses through regulation of membrane potentials and calcium signaling (Hong et al. 2020; Wang et al. 2020). These non-selective cation-permeable channels are divided into six subfamilies based on their significant sequence homology: transient receptor potential canonical (TRPC), transient receptor potential melastatin (TRPM), transient receptor potential vanilloid (TRPV), transient receptor potential ankyrin (TRPA), transient receptor potential polycytin (TRPP), and transient receptor potential muculipin (TRPML). Various TRP channels, such as TRPC1, TRPM2, and TRPV1, have been shown to be involved in PD (Vaidya and Sharma 2020). For instance, treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes reduction in the protein expression of TRPC1, but not TRPC3, and mitochondrial functioning in SNpc (Selvaraj et al. 2009). Similarly, decreased level of TRPC1 has been detected in brain lysates from the SNpc of PD patients (Sun et al. 2017). MPP^+ (1-methyl-4-phenylpyridinium)-induced oxidative stress also promotes dopaminergic cell death in the SNpc by increasing intracellular Ca^{2+} influx via TRPM2 channel activity (Sun et al. 2018). On the other hand, astrocytic TRPV1 has been shown to mediate production of endogenous ciliary neurotrophic factor, which prevents MPP^+-induced degeneration of dopaminergic neurons (Nam et al. 2015). Therefore, targeting TRP channels might have a therapeutic potential to prevent the progressive degeneration of dopaminergic neurons in PD.

Carvacrol (CA) is a monoterpenic phenol found in many aromatic plants having anti-microbial, anti-inflammatory, anti-oxidant and cytoprotective effects in organisms (Baser 2008). Previous studies have shown that pretreatment with CA reduces the neurotoxicity by reducing malondialdehyde and nitrite levels and enhancing the catalase activity in hemi-Parkinsonian rats (Baluchnejadmojarad et al. 2014). It also induces neuroprotective effects by downregulating caspase-3 following unilateral 6-hydroxydopamine (6-OHDA) injection possibly by blockage of TRPM7 channels in mice (Dati et al. 2017). In both in vitro and in vivo models of PD, CA increases cell viability via reduction in intracellular reactive oxygen species (ROS) and lipid peroxidation (Manouchehrabadi et al. 2020). Since the mechanisms of CA's action have not been completely elucidated yet and relatively little is known about the role of CA on TRP channels, we aimed to investigate the effects of CA specifically on TRPC1 as the main Ca^{2+} channel of dopaminergic neurons and TRPA1 present in the astrocytes, by using immunohistochemical and molecular approaches in an animal model of PD.

Material and Method

Animals

A total of 64 adult male Spraque-Dawley rats weighing 200–250 g were used in the present study. All procedures were carried out in accordance with the guidelines of Central Ethics Committee for Animal Experiments (CECAE) in Turkey and approved by the Animal Experiments Local Ethics Committee of Eskişehir Osmangazi University (HADYEK protocol number: 2016/525). Experimental animals were maintained in the Medical and Surgical Experimental Research Center (TICAM) with an ambient temperature of 21 ± 1 °C with 12:12-h light/dark cycle and given free access to standard laboratory chow diet and water. Animals were randomly assigned to one of the following groups: (1) sham (placebo surgery), (2) PD animal model (6-OHDA group 6 µg/µl), (3) PD + vehicle (6-OHDA + 0.5% dimethyl sulfoxide (DMSO)), and (4) PD + CA treatment (6-OHDA + CA 10 mg/kg every other day, for 14 days). Each group was consisted of 16 animals and further divided into two groups for different experimental procedures.

Parkinson's Disease Animal Model

The rats were anesthetized with a combination of ketamine (75 mg/kg i.p.) + xylazine (10 mg/kg i.p.) (Bayer, Germany) before placing into the stereotaxic instrument (David Kopf Instruments — Model 5000). The corpus striatum (CPu) was entered unilaterally in the rat brain with the help of the stereotaximetric system (using the stereotaximetric coordinates for the striatum according to Watson-Paxinos Atlas, with the Bregma as a reference point: anterior–posterior (AP): 1.60, lateral (L): 2.6, and vertical (V): 5.1). The neurotoxin 6-OHDA was used to promote dopaminergic neuronal cell death. Twelve micrograms of 6-OHDA (6 µg/µl; Sigma-Aldrich, USA) prepared in 0.3% ascorbic acid (Merck, Germany) in 2 µl volumes for PD groups and only 0.3% ascorbic acid for sham groups were injected in a volume of 2 µl at 0.5 µl/min to the right CPu (unilateral lesion) with a needle attached to a Hamilton syringe. The needle was left about 5 more minutes before being slowly retracted to avoid reflux. The body temperatures of the animals were kept constant (37 ± 0.5 °C) with a rectal thermometer and heating pad during the surgery. In vivo procedures were performed at the same time of the day for each rat by considering intraday

rhythm changes in the animals. After the surgery, the incision on the skull was carefully sutured under sterile conditions, and the animals were kept individually in different cages and observed until complete recovery. Following the intrastriatal administration, 0.9% saline solution (2 µl) was administered i.p. to Groups 1 and 2 and DMSO (2 µl) to Group 3.

Immunohistochemical Procedures

Fifteen days after the stereotaxic surgery, animals ($n = 8$ per group) were submitted to transcardiac perfusion with 0.1 M phosphate buffer saline (PBS; pH 7.4) followed by 4% paraformaldehyde solution under ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia. After overnight post-fixation in 4% PFA, brains were dissected and embedded in paraffin, and then 4-µm-thick serial sections were taken from the prepared paraffin blocks. Following deparaffinization sections were run through distilled water to remove the alcohol, and 1/10 diluted citrate buffer (AP-9003–999 Thermo Scientific) was applied in the microwave oven for antigen retrieval. After washing with distilled water, endogenous peroxidase activity was blocked with 3% hydrogen peroxide (TA-125-HP Thermo Scientific) for 10 min. Then, first successive set of serial sections was incubated with anti-Glial Fibrillary Acidic Protein (GFAP; Calbiochem, USA; 1:100 dilution) and anti-TRPA1 primary antibodies (Millipore, USA; 1:100 dilution) in the same humid environment for 60 min. Another successive set of serial sections was incubated with anti-TH (Sigma, USA; 1:100 dilution) and anti-TRPC1 (Santa Cruz, USA; 1:50 dilution) primary antibodies under the same conditions in the oven. After the incubation, the sections stained with GFAP and TRPA1 were incubated with goat anti-mouse secondary antibody (BA1001-Boster Bio, USA), and sections stained with tyrosine hydroxylase (TH) and TRPC1 were incubated with goat-anti-rabbit secondary antibody (BA1003-Boster Bio, USA) conjugated with 3,3′-diaminobenzidine (DAB) for 30 min. For quantitative evaluations, persons blind to the treatment took the digital photomicrographs of successive sections and after superimposing them using Nikon Nis Elements 4.2 image analysis program, the number of TRPC1-expressing TH-positive neurons and the number of TRPA1-expressing GFAP-positive astrocytes in the SNpc were quantified. Measurements from four to six sections per brain were averaged to obtain one value per subject in each group.

Gene Expression Analyses

Fifteen days after stereotaxic surgery, animals ($n = 8$ per group) underwent cervical dislocation and brains were removed for molecular analyzes (RT-PCR and WB). SNpc and striatal areas were carefully separated from each brain tissue sample with the aid of a brain blocker and stored at − 80 °C in liquid nitrogen. For ribonucleic acid (RNA) isolation, tissues were first brought to room temperature and then homogenized. The RNA concentrations of samples were determined in a fluorometer device (Qubit® 2.0 Fluorometer, Life Technologies Corp., CA, USA) Qubit® RNA BR Assay Kit (Life Technologies Corp., CA, USA) by isolating the RNA of dopaminergic neuron and astrocyte cells from tissues of SN and CPu brain regions. The high-capacity complementary deoxyribonucleic acid (cDNA) reverse transcription kit (Life Technologies Corp., CA, USA) was used for cDNA synthesis from the RNA samples that had equal concentrations. The cDNAs obtained by reverse transcription were amplified with real time-polymerase chain reaction (RT-PCR) in the presence of sequence-specific primers and probes. In the RT-PCR protocol, the kits which included Taq polymerase enzyme kit and primer–probe mixtures designed for the amplification of target genes TRPA1 and TRPC1 and the internal positive control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used in this respect. The kits that contained primer–probe mixtures designed for the amplification of the target genes TRPA1 and TRPC1 and the internal positive control gene GAPDH were used along with the enzyme kit that contained polymerase. The threshold cycle (CT) value of each sample was obtained after the reaction was performed in the RT-PCR device. The calculation of the target gene expression level was done comparatively according to the $2^{-\Delta\Delta CT}$ method (Pfaffl 2001). Each series of experiments was performed twice.

Western Blot Analysis

The protein amount was measured with the Qubit 2.0 Fluorometer (Life Technologies Corp., CA, USA) by using the Qubit Protein Assay Kit (Life Technologies Corp., CA, USA). The obtained samples were loaded onto the gel, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis was applied. Then, the protein samples were transferred to a nitrocellulose membrane and blotting was performed. For blocking, the iBind Western Device (Thermo Fisher Scientific Inc., MA, USA) system; for antibody treatment, the Anti-TRPC1 (ab75322, Abcam plc., Cambridge, UK), Anti-TRPA1 (ab58844, Abcam plc., Cambridge, UK), and Anti-GFAP (ab7260, Abcam plc., Cambridge, UK) primary antibodies; and Goat Anti-Rabbit IgG H&L (ab6722, Abcam plc., Cambridge, UK) secondary antibody that was diluted 1:1000 were used. For imaging, Kodak Gel Logic 1500 Molecular Imaging System (Eastman Kodak Company, NY, USA) chemiluminescence imaging device was used, and the obtained data were evaluated with densitometry analysis by using ImageJ (https://imagej.nih.gov/ij/) program. The band intensities obtained with the densitometry analysis were normalized with GFAP.

Statistical Analysis

The Shapiro–Wilk test was used to test whether the data fit the normal distribution. One-way ANOVA analysis of variance was performed in groups that had normal distribution, and Kruskal–Wallis *H* test was used in groups that did not comply with normal distribution. The results are given as mean ± standard error (SEM). The $p < 0.05$ value was determined as the level of significance. The Statistical Package for the Social Sciences (SPSS) 22.0 was used for statistical analysis.

Results

Immunohistochemical analyses of tyrosine hydroxylase (TH)-positive neurons in the SNpc revealed that 6-OHDA application significantly ($F(3, 28) = 44{,}33$, $p < 0.001$) reduced the density of dopaminergic neurons compared to the **sham**-operated group (Fig. 1A, B).

Interestingly, both vehicle (DMSO) and CA treatment caused a significant increase in the number of TH-immunostained neurons compared to the PD group. However, in terms of the morphological integrity of dopaminergic neurons, CA-treated group displayed better neuronal appearance than those treated with the vehicle (6-OHDA + DMSO). We also compared the density of TRPC1-expressing cells to investigate their co-localization with TH-positive neurons and found a very similar distribution among groups suggesting that they might play a role in modulating dopaminergic neurons (Fig. 2A, B). On the other hand, CA treatment significantly reduced the mean density of GFAP-positive and TRPA1-expressing astrocytes in the SNpc in comparison to both sham-operated and vehicle-treated group (Fig. 2A).

In PD group, too, the density of GFAP-immunolabeled astrocytes seems to be decreased although there was no significant difference in the TRPA1-expressing astrocytes in comparison to sham- or vehicle-treated groups (Fig. 3A, B).

We then measured the TRPA1 and TRPC1 expressions in the substantia nigra (SN) and the CPu regions using RT-PCR technique. While 6-OHDA application significantly ($p < 0.05$) reduced the TRPA1 expression level in the SN region, CA application recovered the TRPA1 expression level close to those of sham group (Fig. 4A). In PD animals, a significant decrease was also observed in the TRPC1 expression level. However, CA application did not cause an alteration in the TRPC1 expression level. In the CPu region, TRPA1 expression results were similar to those of SN region, whereas CA application did not cause a significant change in the expression level of TRPC1 (Fig. 4B).

We also wanted to confirm gene expression results with the amount of the protein by using western blot analysis approach. We found that in both SN and CPu regions, 6-OHDA administration significantly decreased the TRPA1 protein level, but CA administration increased the TRPA1 protein to even higher level than those of the sham group (Fig. 5A–C). On the other hand, we were not able to detect a significant change in the TRPC1 protein levels in both SN and CPu regions following 6-OHDA or CA administration (Fig. 5A).

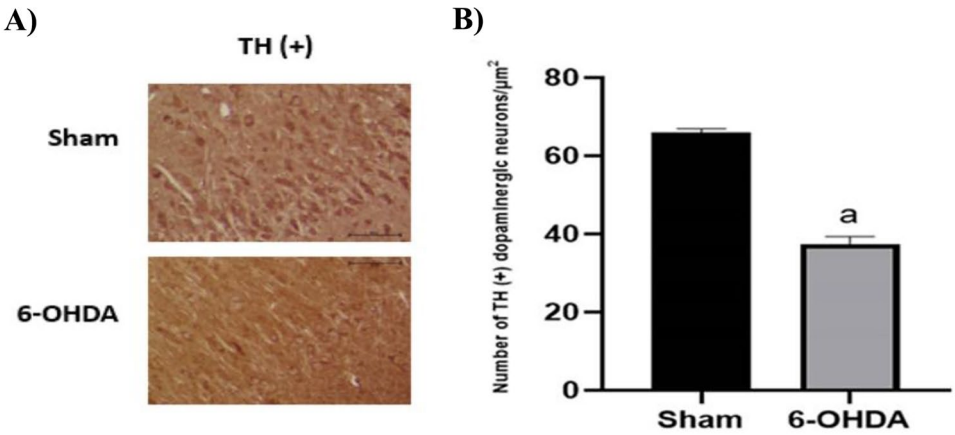

Fig. 1 Photomicrograph images and histogram graphic showing SNpc region dopaminergic neurons immunohistochemically labeled with TH of the sham group and the 6-OHDA Parkinson's model group. **A** Histological images of TH(+) marked dopaminergic neurons obtained from sections in the SNpc region of the experimental Parkinson model group created with sham group and 6-OHDA. Scale bar = 50 µm (×400 magnification). **B** Number of TH(+) dopaminergic neurons/µm^2. Results are presented as mean ± standard error of the mean (SEM) ($n = 8$ for each group). $^a p < 0.05$ compared to sham group

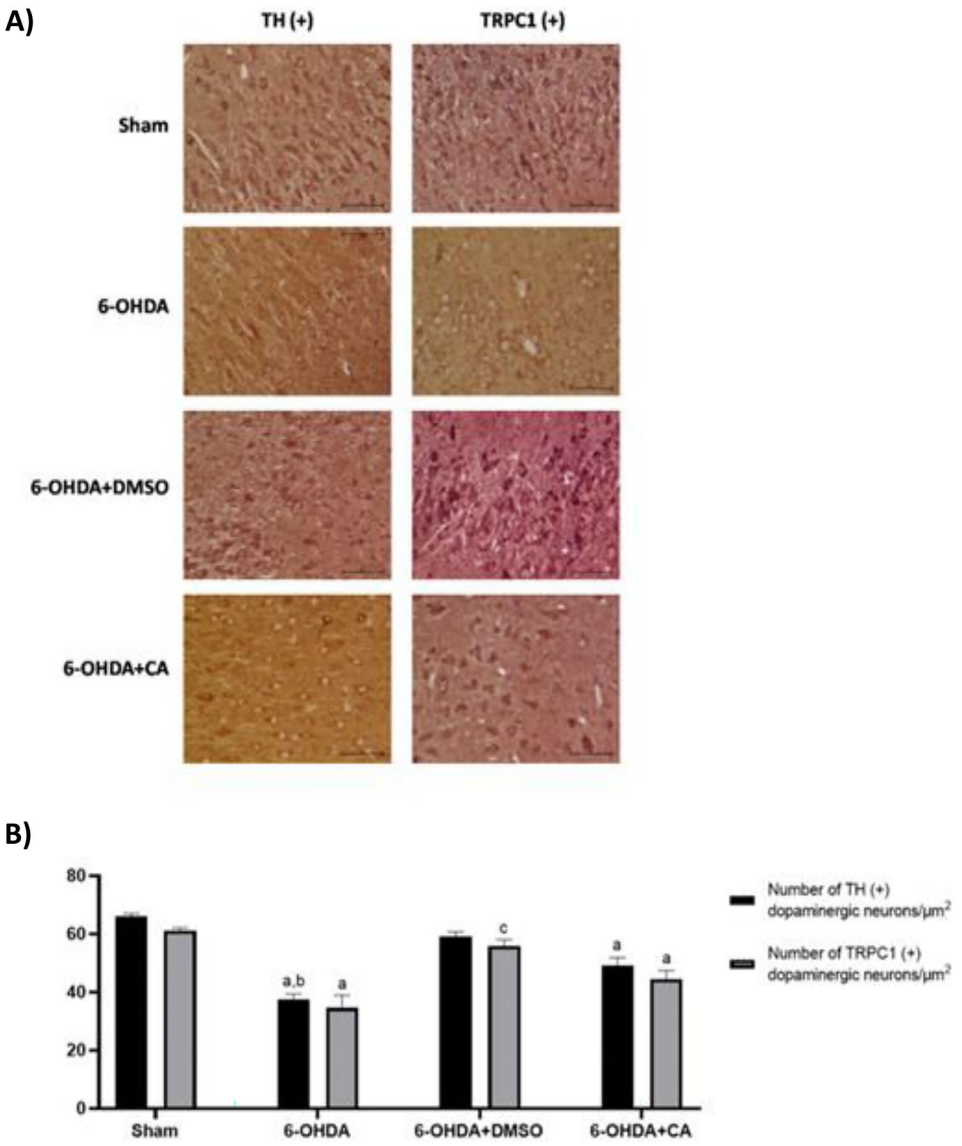

Fig. 2 Representative photomicrographs showing TH(+)-TRPC1(+)-labeled dopaminergic neurons immunohistochemically obtained from serial sections in the SNpc region of CA treatment in an experimental Parkinson's model created with 6-OHDA in rats and dopaminergic neuron count/μm^2. **A** Histological images of TH(+)-TRPC1(+)-labeled dopaminergic neurons obtained from serial sections in the SNpc region of CA treatment in an experimental Parkinson's model created with 6-OHDA in rats. Scale bar = 50 μm (×400 magnification). **B** Number of TH(+) and TRPC1(+) dopaminergic neurons /μm^2. Results are presented as mean ± standard error of the mean (SEM) ($n = 8$ for each group). [a]$p < 0.05$ compared to **sham** and 6-OHDA + DMSO groups. [b]$p < 0.05$ compared to 6-OHDA + CA group. [c]$p < 0.05$ compared to 6-OHDA groups

Discussion

As the most common neurodegenerative movement disorder, PD does not have an effective treatment modality for reversing the progressive loss of dopaminergic neuron in the SNpc. Recent studies emphasized the importance of Ca^{2+} overload on neurodegeneration in PD pathology and suggested a possible role for TRP channels in the management of motor and non-motor symptoms of PD, due to their interactions with oxidative stress, apoptosis, and excitotoxicity signaling pathways (Vaidya and Sharma 2020; Zheng 2013; Samanta et al. 2018). In this study, using an animal model of PD, we investigated the effects of CA emerging as the regulator of TRP cation channels as well as displaying anti-oxidant and neuroprotective properties in certain areas of the brain in both in vivo and in vitro models (Suntres et al. 2015).

We focused on the TRPC1, the major TRP channel responsible for Ca^{2+} entry into SNpc dopaminergic neurons and TRPA1, due to its neuromodulatory functions and substantial involvement in Ca^{2+} regulation of astrocytes. Our results showed that 6-OHDA treatment significantly decreased TRPA1 and TPRPC1 mRNA expression and protein levels in both SNpc and striatum. While CA administration significantly enhanced TRPA1 expression in PD animals, TRPC1 protein levels did not display an alteration. It has been shown that in SH-SY5Y human neuroblastoma cell line, which displays dopaminergic activity, salsolinol-induced cytotoxicity leads to 60% reduction in TRPC1 protein level (Bollimuntha et al. 2006). Overexpression of TRPC1 inhibits apoptotic complex and enhances survivability of these neurons, whereas addition of TRPC1 blockers can prevent protective effects. Systemic administration of another neurotoxin MPTP, which crosses the blood–brain

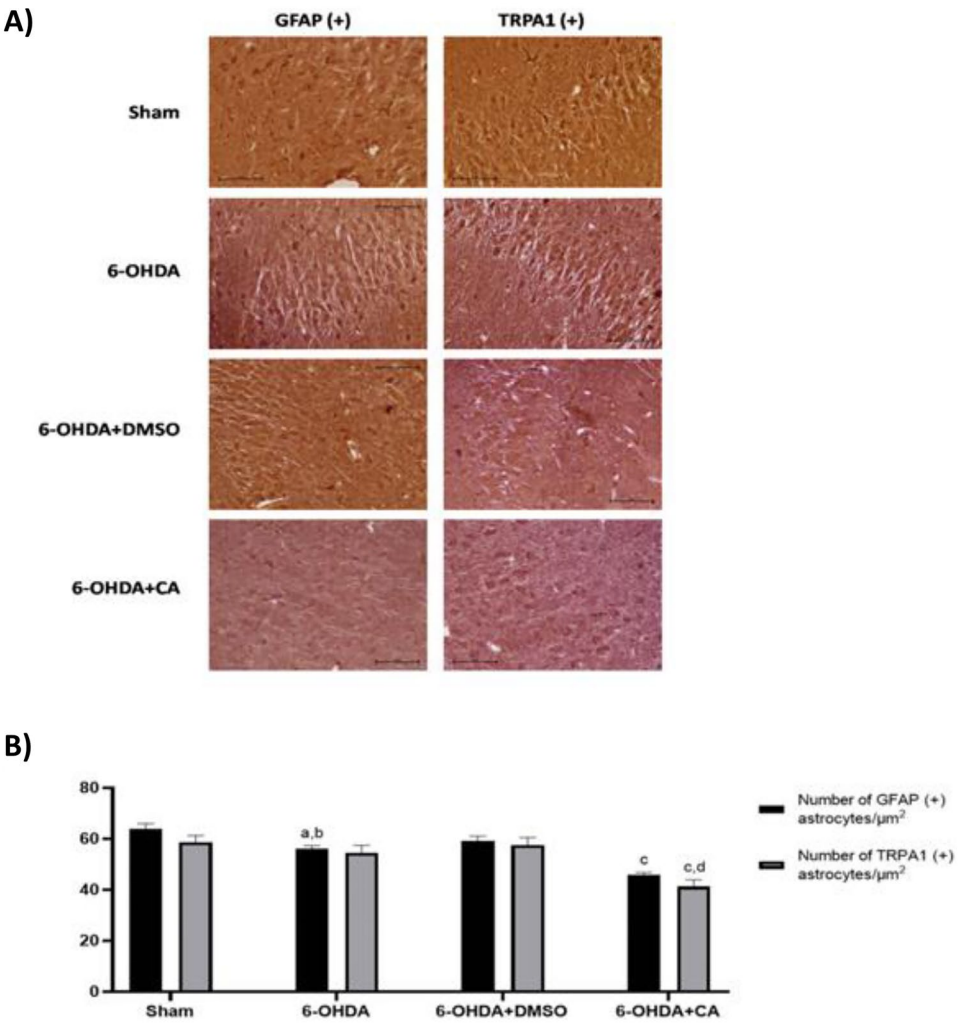

Fig. 3 Representative photomicrographs showing GFAP(+)-TRPA1(+)-labeled astrocytes immunohistochemically obtained from serial sections in the SNpc region of CA treatment in an experimental Parkinson's model created with 6-OHDA in rats and dopaminergic neuron count/μm^2. **A** Histological sections of GFAP(+)-TRPA1(+)-labeled astrocyte cells obtained from serial sections in the SNpc region of CA treatment in an experimental Parkinson's model created with 6-OHDA in rats. Scale bar = 50 μm (×400 magnification). **B** Number of GFAP(+) and TRPA1(+) astrocytes/μm^2. Results are presented as mean ± standard error of the mean (SEM) ($n = 8$ for each group). [a]$p < 0.05$ compared to sham group. [b]$p < 0.05$ compared to 6-OHDA + CA group. [c]$p < 0.05$ compared to sham and 6-OHDA + DMSO groups. [d]$p < 0.05$ compared to 6-OHDA group

barrier, is taken up by glial cells to be metabolized to MPP^+ and then released to be specifically taken up by dopaminergic neurons to induce cell loss (Przedborski et al. 2004), causes functional loss in TRPC1 channels, and thereby induces ER stress (Selvaraj et al. 2012). Interestingly, in PD models it has also been shown that inhibition of endogenous store-operated Ca^{2+} entry (SOCE) triggers unfolded protein response (UPR), ER stress, and dysfunction/degeneration of dopaminergic neurons (Secondo et al. 2018). In this context, overexpression of TRPC1 protected against MPTP-induced loss of SOCE and UPR, while knocking down the gene in mice increased UPR. Activation of TRPC1 initiates Ca^{2+} entry that regulates the AKT/mTOR pathway, which is essential for the protection of dopaminergic neurons against neurotoxins that induce PD-like symptoms. Consistent with these findings, in vitro experiments showed that silencing of TRPC1 enhanced the UPR similar to TRPC1 knockout mice and reduced the number of dopaminergic neurons. In brain lysates of patients with PD, too, reduction in TRPC1 levels and enhanced UPR were reported (Secondo et al. 2018; Selvaraj et al. 2012). Overall, these results suggest that TRPC1 is remarkably involved in regulating Ca^{2+} homeostasis in dopaminergic neurons and contributes to dopaminergic neuronal survival via inhibiting the UPR.

In our immunohistochemical staining, CA administration significantly increased the density of TRPC1-positive neurons and reversed the effects of 6-OHDA-induced neurotoxicity. Morphologic appearance of healthier TH-positive dopaminergic neurons, displaying regular contours and symmetrical round-shape nuclei, were more prominent in these animals compared to the vehicle-treated ones who displayed neurodegenerative changes. Accordingly, Lins et al. (2018) showed that daily i.p. injections of CA, at both 12.5- and 25-mg/kg doses for 30 days, prevent the reduction of the TH-immunoreactivity in the SNpc and dorsal striatum in reserpin-induced rat model of PD. Yet, these responses in dopaminergic neurons cannot be attributed solely to TRPC1 channels, since other TRP channels expressed in the SN might also be involved in excitotoxicity (Chung et al. 2011; Thapak et al. 2020). In SH-SY5Y cells, MPP^+ treatment has

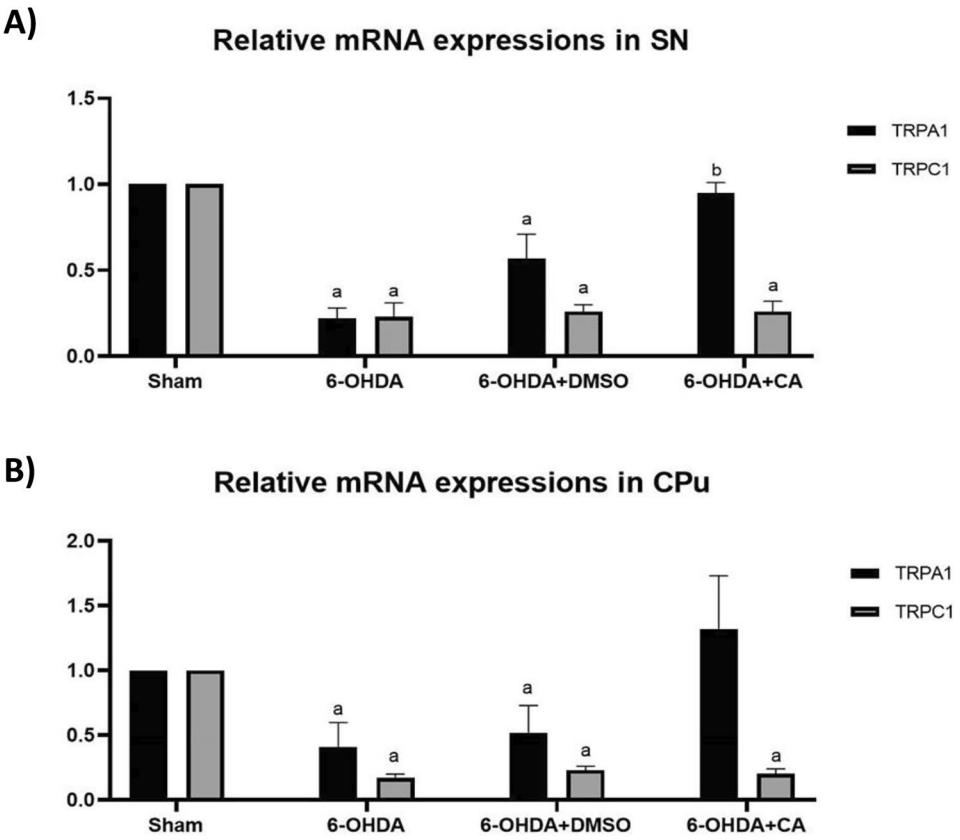

Fig. 4 Gene expression results of TRPA1 and TRPC1 proteins in SNpc and CPu brain regions of rats obtained by RT_PCR method. **A** Relative mRNA expressions of TRPA1 and TRPC1 in SN. **B** Relative mRNA expressions of TRPA1 and TRPC1 in CPu. Results are presented as mean ± standard error of the mean (SEM) ($n = 8$ for each group). [a]$p < 0.05$ compared to sham group. [b]$p < 0.05$ compared to 6-OHDA group

been shown to increase intracellular Ca^{2+} influx via TRPM2 channel activity and promote cell death mainly due to an increased ROS level, caspase 3 activity, and the activation of downstream apoptotic pathway (Sun et al. 2018). Furthermore, pharmacological inhibition or knockdown of TRPM2 using siRNA showed an increased protection by preventing MPP^+-induced Ca^{2+} increase and inhibited apoptosis. TRPM7 channels also regulate Mg^{2+} homeostasis in cells and an increase in the concentration of Mg^{2+} led to significant improvement in the survival of TH-positive dopaminergic neurons and neurite length following MPP^+ toxicity (Hashimoto et al. 2008). In a recent study, it has been shown that TRPC1 functions as a negative regulator of TRPC4 and TRPC5, since heterodimers of TRPC1/4 and TRPC1/5 suppress inward current causing decrease in the Ca^{2+} influx and Ca^{2+}-dependent apoptosis in neurons (Kim et al. 2019). In the present study, cytotoxic effects (Yuan et al. 2014) of DMSO, which we used to prepare the injection form of CA, were also observed besides its anti-oxidant, analgesic, and anti-inflammatory effects (Lu and Mattson 2001). According to our immunohistochemical results, the TRPC1(+) cell count was found to be higher in the 6-OHDA + DMSO group than in the treatment group. However, the appearance of the cells was in an apoptotic and degenerated condition. DMSO, which is used as a solvent, initiated the apoptotic process; CA provided the survival and healthy appearance of some neurons with its neuroprotective effect. Taken together, these findings suggest that the reduction in TRPC1 protein level following neurotoxin exposure is one strong contributory factor in the survival of dopaminergic neurons and plant-derived compounds like CA might be helpful in overcoming the effects of toxins by modulating TRP channels.

Majority of the studies focused on the emerging roles of Ca^{2+} related signaling pathways in neurons, but the role of the astrocytes in the pathogenesis of PD have been ignored. Astrocytes play active and critical roles in many aspects of neurodegeneration processes in PD by expressing ion channels and specific transporters that might positively or negatively mediate Ca^{2+} signaling in cells (Rappold and Tieu 2010). Increasing evidence indicates that the ion channel proteins of astrocytes, including TRP, aquaporins, K-ATP channels, and P2X7 receptors, are strongly associated with oxidative stress and neuroinflammation seen in the etiopathogenesis of many neurodegenerative diseases (Wang et al. 2022). In astrocytes, TRP channels participate in SOCE-mediated Ca^{2+} homeostasis, but in pathological states, TRP channels in astrocytes can be activated by ROS, inflammatory factors, and pathological markers of neurodegenerative diseases, such as Aβ which disrupts Ca^{2+} homeostasis (Lim et al. 2016; Lee et al. 2019). TRPA1 is one of the non-selective transmembrane cation channels, mainly expressed in primary sensory neurons and non-neuronal

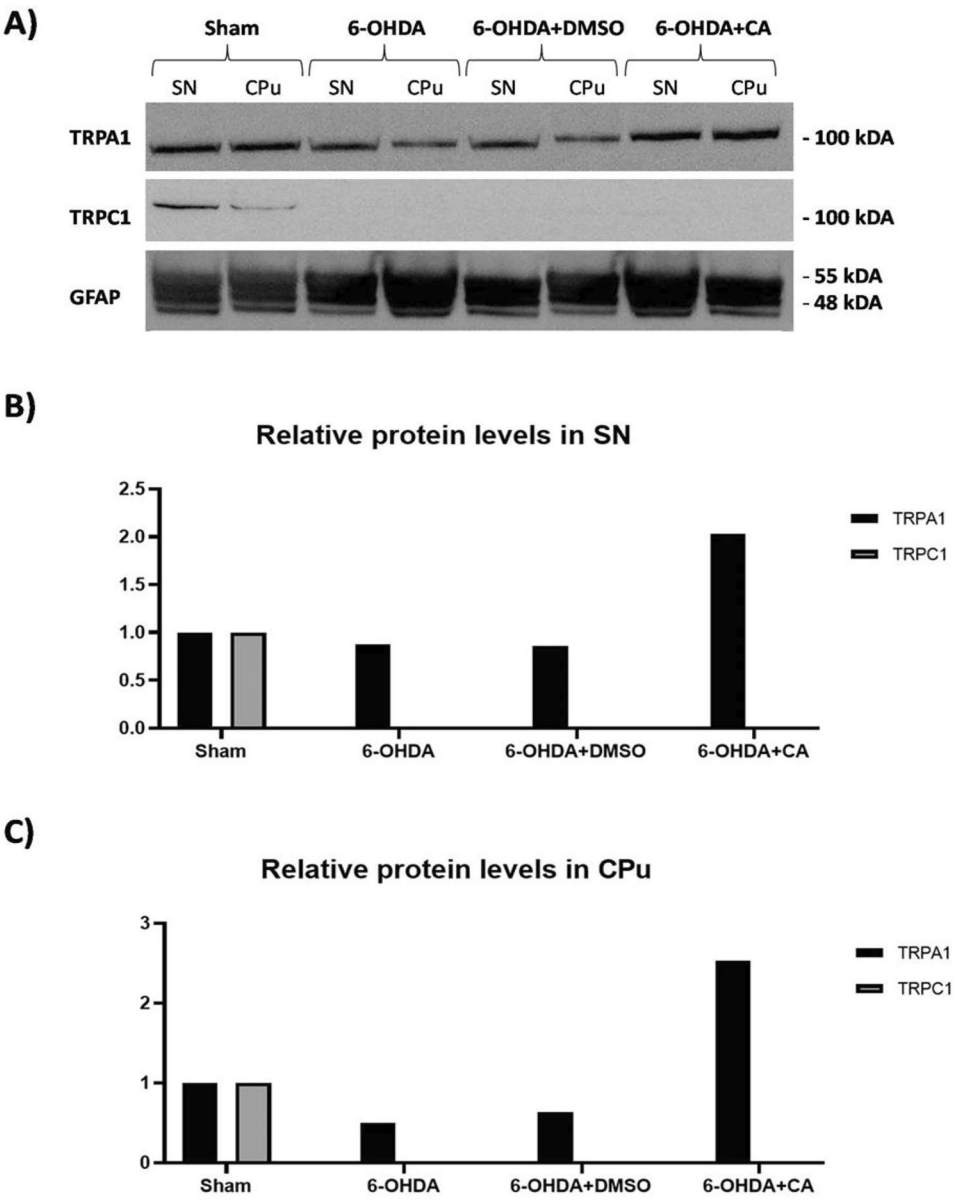

Fig. 5 Western blot results of TRPA1 and TRPC1 proteins in SNpc and CPu brain regions of rats. **A** Representative western blots for TRPA1, TRPC1, and GFAP in the SN and CPu. **B** Densitometric quantification of TRPA1 and TRPC1 in the SN. **C** Densitometric quantification of TRPA1 and TRPC1 in the CPu were normalized to total GFAP expression level and calculated as fold change to the sham group

cells including astrocytes (Shigetomi et al. 2012). This channel plays important roles in the brain development and the physiological functions of astrocytes (Lee et al. 2016). In a transgenic mouse model of Alzheimer's disease, expression of TRPA1 in astrocyte was found significantly higher than in that of controls (Lee et al. 2016). Ablation of TRPA1 channel function positively affected the behavioral tests and decreased proinflammatory cytokine production, but exacerbated astrogliosis.

CA is among the best-known chemical compounds that act as TRPA1 channel activators (Stueber et al. 2017). The activation of channels by CA in dorsal root ganglion neurons triggers Ca^{2+} flow from extracellular sources and intracellular stores, and thereby caused an increase in intracellular Ca^{2+} release. In our study, RT-PCR and western blot analyses showed that CA treatment significantly increased TRPA1 gene expression and protein levels both in the SN and corpus striatum of PD animals. When astrocytes become reactive in response to injury and inflammation, they typically become hypertrophic with thicker processes and upregulate GFAP expression (Shigetomi et al. 2019). In our immunohistochemical analyses, the density of GFAP-labeled and TRPA1-positive astrocytes in SNpc decreased following CA administration compared to 6-OHDA groups suggesting a role for CA in preventing reactive astrogliosis which then possibly can lead to neuroinflammation. In response to injury and/or hyperexcitability astrocytes rapidly generate Ca^{2+} signals having harmful effect in neuronal tissue. Anatomical, functional, and molecular changes occurring in the

reactive astrocytes vary greatly depending on the type of pathological condition, degree of disease progression, and many other factors (Escartin et al. 2021). Yet, loss of ion buffering functions and aberrant Ca^{2+} signals in reactive astrocytes seems to be a common characteristic of astrocyte-mediated brain diseases even in their presymptomatic phase (Koizumi et al. 2022). Since excessive activation of astrocytes can greatly enhance astrocytic Ca^{2+} overload, ROS, and other pro-inflammatory factors, it is possible that CA treatment in our study might counteract these effects by reducing the density of GFAP-labeled astrocytes in PD animals. However, the pathological significance of TRPA1 in PD pathologies needs further investigation.

In a recent study, acrolein scavenger dimercaprol has been shown to reverse the upregulation of TRPA1 channels not only in the SNpc and striatum but also in the sensory cortex of 6-OHDA rats (Shi et al. 2021). This observation is consistent with a previous report confirming the functional activation of TRPA1 in pain sensation and neurogenic inflammation (Kheradpezhouh et al. 2017). Since neuropathic pain is a very common non-motor symptom detected in up to 85% of PD patients, further research is necessary for understanding the role of TRP channels in alterations observed in different parts of the brain following 6-OHDA administration.

Conclusion

In conclusion, results of this study showed that natural compounds such as CA have multiple therapeutic potentials in alleviating symptoms of neurodegenerative diseases and regulation of Ca^{2+} homeostasis in neurons and astrocytes might be a novel and promising strategy to prolong the survival of dopaminergic neurons in animal models of PD.

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Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Tülay Akan], [Orhan Tansel Korkmaz] and [Faruk Saydam]. The first draft of the manuscript was written by [Tülay Akan], [Yasemin Aydın] and [Emel Ulupınar]. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of Data and Materials Data available on request from the authors.

Declarations

Ethical Approval This study was performed in line with the principles of the Declaration of Helsinki. All procedures were performed in accordance with the guidelines of the Animal Experiments Center Ethics Committee (CECAE) in Turkey and approved by the Eskişehir Osmangazi University Animal Experiments Local Ethics Committee (HADYEK protocol number: 2016/525).

Competing Interests The authors declare no competing interests.

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