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Paraquat as an Environmental Risk Factor in Parkinson's Disease Accelerates Age-Related Degeneration Via Rapid Influx of Extracellular Zn^{2+} into Nigral Dopaminergic Neurons

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Abstract

On the basis of the evidence that paraquat (PQ)-induced extracellular Zn^{2+} influx causes PQ-induced pathogenesis in the substantia nigra pars compacta (SNpc) of rats, we postulated that the transient receptor potential melastatin 2 (TRPM2) cation channels activated with PQ-induced reactive oxygen species (ROS) are linked with extracellular glutamate accumulation in the SNpc, followed by age-related intracellular Zn^{2+} dysregulation. Presynaptic activity (glutamate exocytosis), which was determined with FM4-64, was enhanced in the SNpc after exposure to PQ, and the enhancement was inhibited in the presence of N-(p-amylcinnamoyl)anthranilic acid (ACA), a blocker of TRPM2 cation channels, suggesting that PQ-induced ROS enhances presynaptic activity in the SNpc, probably via TRPM2 channel activation. Extracellular glutamate concentration in the SNpc was increased almost to the same extent under the SNpc perfusion with PQ of young and aged rats, and was suppressed by co-perfusion with ACA, suggesting that PQ-induced TRPM2 cation channel activation enhances glutamate exocytosis in the SNpc. Interestingly, PQ more markedly increased intracellular Zn^{2+} in the aged SNpc, which was also blocked by co-injection of ACA and CaEDTA, an extracellular Zn^{2+} chelator. Loss of nigrostriatal dopaminergic neurons was more severely increased in aged rats and completely blocked by co-injection of PQ and CaEDTA into the SNpc. The present study indicates that rapid influx of extracellular Zn^{2+} into dopaminergic neurons via PQ-induced TRPM2 cation channel activation accelerates nigrostriatal dopaminergic degeneration in aged rats. It is likely that vulnerability to PQ-induced pathogenesis in the aged SNpc is due to accelerated intracellular Zn^{2+} dysregulation.

Keywords Zn^{2+} · Dopaminergic neuron · Substantia nigra · Paraquat · TRPM2 cation channel · Parkinson's disease · Aging

Introduction

The etiology of idiopathic Parkinson's disease (PD) remains unknown, and the majority (~ 90%) of PD affecting more than 1% of the population over 60 years of age is sporadic. Several risk factors are identified, i.e., genetic background, environmental toxicant exposures, and aging. Aging is the well-known risk factor for the sporadic form of this disease [1–4]. PD is characterized by a selective loss of dopaminergic neurons in the sbstantia nigra pars compacta (SNpc) of the brain [5, 6]. However, the exact cause of the neuronal loss remains unclear.

Vulnerability to neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which has been widely used for PD model, is enhanced in aged animals [7, 8]. MPTP is not an environmental risk factor, while the herbicide paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride, PQ) has been implicated in PD pathogenesis because of its structural similarity to MPP+ (1-methyl-4-phenylpyridinium), a toxic metabolite of MPTP. Epidemiologic studies have indicated that chronic exposure to PQ is associated with the increased risk for developing PD [9, 10]. However, the molecular mechanisms of PQ toxicity in nigrostriatal dopaminergic neurons are still well not understood [11, 12]. PQ naturally exists as PQ^{2+}, a divalent cation and undergoes redox cycling with cellular diaphorases such as NADPH oxidase and nitric oxide synthase, which produces PQ^{+}, a monovalent cation that passes through dopamine transporters. Superoxide and reactive oxygen species (ROS) are produced from the redox cycling in both extracellular and intracellular compartments, followed by oxidative stress-mediated neurotoxicity [13, 14].

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On the other hand, PQ induces a transient increase in extracellular glutamate in the striatum of freely moving rats, followed by Ca^{2+} influx via N-methyl-D-aspartate (NMDA) receptor activation [15]. The possible involvement of glutamate excitotoxicity in PQ-induced pathophysiology leads to an idea for the pathogenesis. We have reported a unique mechanism of nigrostriatal dopaminergic degeneration, in which rapid influx of extracellular Zn^{2+} via extracellular glutamate accumulation causes PQ-induced PD in rats [16]. The rapid influx via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor activation causes intracellular Zn^{2+} dysregulation in nigral dopaminergic neurons, resulting in preferential neurodegeneration in the SNpc [17, 18]. However, the mechanism of extracellular glutamate accumulation in the SNpc remains to be clarified.

The basal (static) concentration of extracellular Zn^{2+}, which is estimated to be approximately 10 nM in the hippocampus [19, 20], may be increased age-dependently, based on the age-related increase in extracellular zinc concentration in the rat hippocampus determined by in vivo microdialysis [21]. The findings imply vulnerability to intracellular Zn^{2+} dysregulation in nigral dopaminergic neurons of aged rats after exposure to PQ. We postulated that the transient receptor potential melastatin 2 (TRPM2) cation channels activated with PQ-induced ROS are linked with extracellular glutamate accumulation in the SNpc, followed by age-related intracellular Zn^{2+} dysregulation. Because N-(p-amylcinnamoyl)anthranilic acid (ACA) directly blocks the transient receptor potential (TRP) channels including TRPM2 [22, 23], which are ROS-sensitive [24], in the present study, we examined whether ACA suppresses presynaptic glutamate release induced with PQ-induced ROS in the SNpc. Here, we report the mechanism on age-related intracellular Zn^{2+} dysregulation after exposure to PQ and that intracellular Zn^{2+} dysregulation accelerates age-related degeneration of nigral dopaminergic neurons.

Materials and Methods

Animals and Chemicals

Male Wistar rats (10–15 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan). Rats were housed under the standard laboratory conditions (23 ± 1 °C, 55 ± 5% humidity) and had access to tap water and food ad libitum. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka that refer to American Association for Laboratory Animals Science and the guidelines laid down by the NIH (NIH Guide for the Care and Use of Laboratory Animals) in the USA. The ethics committee of the University of Shizuoka has approved all experimental protocols.

ZnAF-2 and ZnAF-2DA, a membrane-impermeable and a membrane-permeable Zn^{2+} fluorescence probe, respectively, were kindly supplied from Sekisui Medical Co., LTD (Hachimantai, Japan). ZnAF-2DA is taken up into the cells through the cell membrane and is hydrolyzed by esterase in the cytosol to yield ZnAF-2, which cannot permeate the cell membrane [25, 26]. ZnAF-2 is selectively bound to Zn^{2+}, but not bound to other divalent cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, and Cu^{2+} [25]. FM4-64, an indicator of presynaptic activity, was purchased from Sigma-Aldrich (St. Louis, MO). Aminophenyl fluorescence (APF), a ROS fluorescence probe, e.g., hydroxyl radical and peroxynitrite, was purchased from Goryochemical (Sapporo, Japan). The indicators were dissolved in dimethyl sulfoxide (DMSO) and then diluted to Ringer solution containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, and 11 mM D-glucose (pH 7.3).

In Vitro Exocytosis Experiment

Rats were anesthetized with chloral hydrate (400 mg/kg) and decapitated. The brain was quickly removed and immersed in ice-cold choline-Ringer containing 124 mM choline chloride, 2.5 mM KCl, 2.5 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (pH 7.3) to suppress excessive neuronal excitation. Horizontal brain slices (400 μm) were prepared by using a vibratome ZERO-1 (Dosaka Kyoto, Japan) in an ice-cold choline-Ringer solution. Slices were then maintained in an ice-cold choline-Ringer solution. All solutions used in the experiments were continuously bubbled with 95% O_2 and 5% CO_2.

The brain slices were transferred to an incubation chamber filled with Ringer solution containing 5 μM FM4-64 and 45 mM KCl, allowed to stand at 25 °C for 90 s and transferred a chamber filled with Ringer solution to wash out extracellular FM4-64 and KCl for 15 min. Brain slices were transferred to a recording chamber filled with Ringer solution. FM4-64 fluorescence (excitation, 543 nm; emission, 640 nm) was measured for 30 s with a confocal laser-scanning microscopic system (Nikon A1 confocal microscopes, Nikon Corp.) at the rate of 1 Hz for 300 s through a × 10 objective. PQ (40 μM) and Trolox (1 mM) (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an antioxidative agent, were added to the brain slices, and FM4-64 fluorescence was measured for 300 s. Region of interest was set in the SNpc. In another experiment, ACA (40 μM), a blocker of TRP channels, was added to Ringer solution and FM4-64 fluorescence was measured for 30 s in the same manner and then treated as described above. Because FM4-64 fluorescence originates in vesicular membrane-bound FM4-64, FM4-64 fluorescence is attenuated by presynaptic activity [27, 28] (Fig. 1a). FM4-64 fluorescence immediately after addition of PQ and PQ + Trolox was expressed as 100%,

and the rate (%) of attenuated FM4-64 fluorescence was measured for 300 s.

In Vivo Microdialysis

The rats were anesthetized with chloral hydrate and individually placed in a stereotaxic apparatus. The skull was exposed; a burr hole was drilled, and a microdialysis probe (1-mm membrane, Eicom, Kyoto) was inserted into the right SNpc (5.3 mm posterior to the bregma, 2.0 mm lateral, 7.8 mm inferior to the dura) of anesthetized rats. The SNpc was preperfused with ACSF (127 mM NaCl, 2.5 mM KCl, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 21 mM $NaHCO_3$, and 3.4 mM D-glucose, pH 7.3) at 2.0 μl/min for 120 min to stabilize the region, perfused with ACSF for 60 min in the same manner to determine the basal concentration of glutamate and extracellular Zn^{2+}, and then perfused with 40 μM PQ in ACSF or 40 μM PQ + 50 μM ACA in ACSF for 120 min.

The perfusate was collected for 15 min and the basal levels and the levels during perfusion with PQ were averaged. ZnAF-2 (1 μM, 50 μl) was added to aliquot of the perfusate (10 μl) for measuring extracellular Zn^{2+} levels. The fluorescence of ZnAF-2 (Ex/Em; 485/535 nm) was measured using a plate reader ARVO sx (Perkin Elmer, USA). The perfusate samples (15 μl) were also analyzed for glutamate content by high-performance liquid chromatography (HPLC) [column, CAPCELL PAK C18 UG120A (1 mm × 150 mm) (Shiseido Co Ltd., Tokyo, Japan); mobile phase, 0.1 M potassium dihydrogen phosphate, 0.1 M di-sodium hydrogen phosphate,

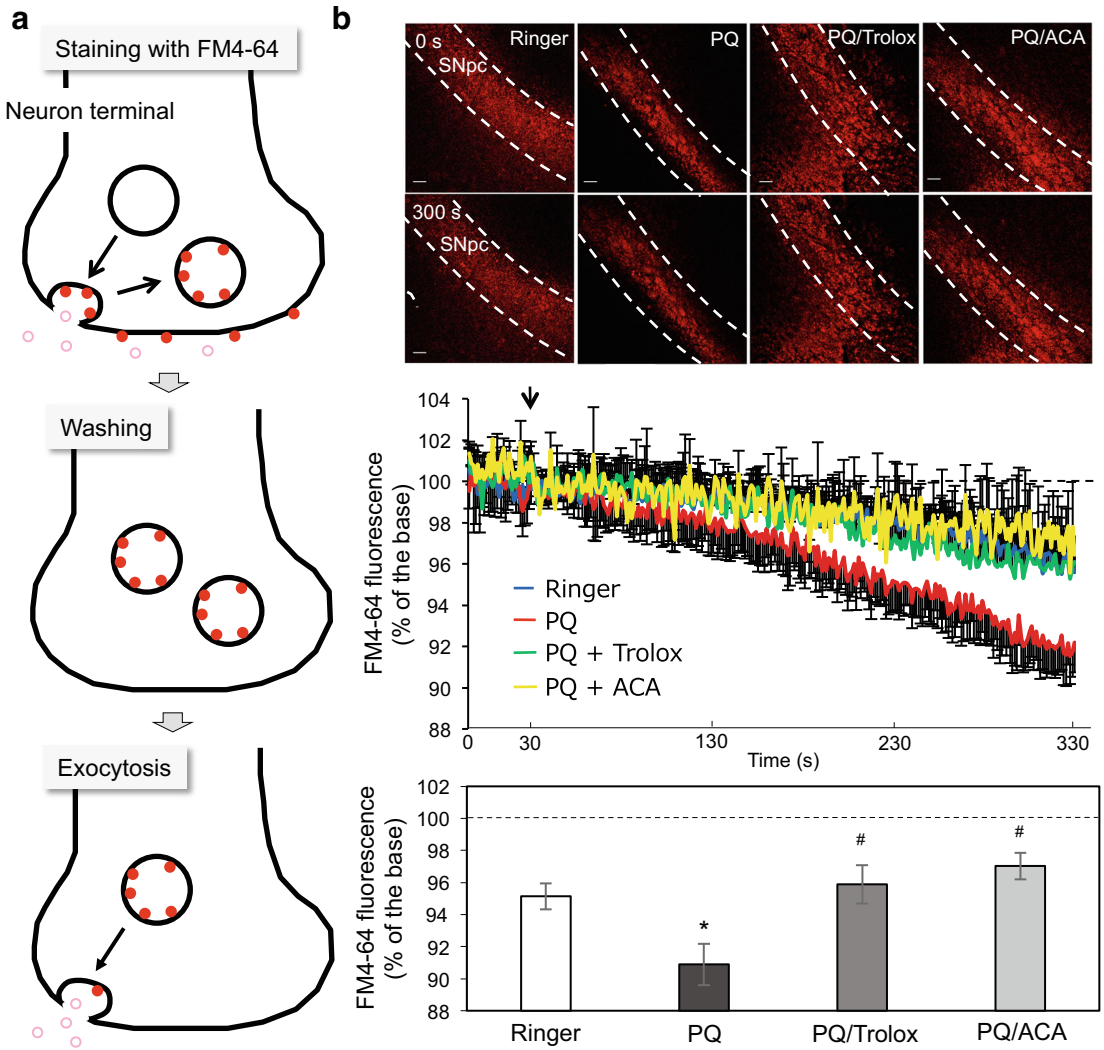

Fig. 1 Increase in ROS-induced exocytosis in the SNpc of young rats after exposure to PQ. **a** Schematic illustration of exocytosis assessed with FM4-64. **b** FM4-64 fluorescence in the SNpc of brain slices 0 s before exposure to 40 μM PQ ($n = 7$), 40 μM PQ + 1 mM Trolox ($n = 6$), or 40 μM PQ + 40 μM ACA ($n = 4$), and 300 s after exposure (upper). Each point and line represents the ratio of RM4-64 fluorescence in the SNpc surrounded by the white dotted line to each control FM4-64 fluorescence immediately after addition of PQ and PQ + Trolox, which was expressed as 100% (middle). Averaged FM4-64 fluorescence of the last 5 s (325–330 s) (lower). *, $p < 0.05$, vs. control (Ringer, $n = 11$), #, $p < 0.05$, vs. PQ (Tukey's test)

10% acetonitrile, 0.5 mM EDTA-2Na, 3% tetrahydrofuran, pH 6.0] using the pre-column derivatization technique with o-phthaldialdehyde and a fluorescence detector (NANOSPACE SI-2, Shiseido Co Ltd).

In Vitro Dynamics of ROS and Intracellular Zn^{2+}

Brain slices were prepared as described above and placed for 10 min in 3 μM APF in Ringer solution, rinsed in choline-Ringer solution for 15 min, and placed in a recording chamber filled with Ringer solution. The fluorescence of APF (laser, 490 nm; emission, 500–550 nm) was measured with a confocal laser-scanning microscopic system. Region of interest was set in the SNpc.

Brain slices were also placed for 30 min in 10 μM ZnAF-2DA in Ringer solution, rinsed in choline-Ringer solution for 20 min, placed in a chamber filled with 40 μM PQ, 40 μM PQ + 40 μM ACA, or 40 μM PQ + 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA receptor antagonist, in Ringer solution for 10 min, rinsed in choline-Ringer solution for 15 min, and transferred to a recording chamber filled with Ringer solution. The fluorescence of ZnAF-2 (laser, 488.4 nm; emission, 500–550 nm) was measured in the SNpc with a confocal laser-scanning microscopic system.

In Vivo Imaging of Intracellular Zn^{2+}

The rats were anesthetized with chloral hydrate and treated as described above. Injection cannulae (internal diameter, 0.15 mm; outer diameter, 0.35 mm) were carefully and slowly inserted into the right and left SNpc (5.3 mm posterior to the bregma, 2.0 mm lateral, 7.0 mm inferior to the dura) to avoid cellular damages. Thirty minutes after the surgical operation, 40 μM PQ, 40 μM PQ + 10 mM CaEDTA, an extracellular Zn^{2+} chelator, or 40 μM PQ + 50 μM ACA in saline containing ZnAF-2DA (100 μM), were bilaterally injected into the SNpc via cannulae at the rate of 0.2 μl/min for 5 min. Ten minutes after injection, the injection cannulas slowly pulled out the brain in about 3 min and the rats were decapitated. The brain was quickly removed and brain slices were prepared in the same manner. The brain slices were transferred to a recording chamber filled with Ringer solution. The fluorescence of ZnAF-2 was measured in the SNpc.

Behavioral Studies

An injection cannula was inserted into the right SNpc in the same manner, and 40 μM PQ or 40 μM PQ + 10 mM CaEDTA in saline were unilaterally injected into the SNpc via the cannula at the rate of 0.2 μl/min for 5 min. Ten minutes after injection, the injection cannula slowly pulled out the brain in about 3 min. Two weeks later, the rats were subcutaneously injected with apomorphine (0.5 mg/kg) and turning behavior was measured in response to apomorphine.

Tyrosine Hydroxylase (TH) Immunostaining

The rats were anesthetized and perfused with ice-cold 4% paraformaldehyde in PBS after the behavioral studies were finished, followed by removal of the brain and overnight fixation in 4% paraformaldehyde in PBS at 4 °C. Fixed brains were cryopreserved in 30% sucrose in PBS for 2 days and frozen in Tissue-Tek Optimal Cutting Temperature embedding medium. Coronal brain slices (30 μm) were prepared at − 20 °C in a cryostat, picked up on slides, and adhered at room temperature for 30 min. For immunostaining, slides were incubated in blocking solution (3% BSA, 0.1% Triton X-100 in PBS) for 1 h and rinsed with PBS for 5 min followed by overnight incubation with anti-tyrosine hydroxylase antibody (Abcam) at 4 °C. Slides were rinsed with PBS for 5 min and incubated in blocking buffer containing Alexa Fluor 633 goat anti-rabbit secondary antibody (ThermoFisher) for 3 h at room temperature. Following six rinses in PBS for 5 min, slides were mounted with Prolong Gold antifade reagent and placed for 24 h at 4 °C. Alexa Fluor 633 fluorescence was measured in the SNpc using a confocal laser-scanning microscopic system.

Data Analysis

For multiple comparisons, differences between treatments were assessed by one-way ANOVA followed by post hoc testing using the Tukey's test (the statistical software, GraphPad Prism 5). A value of $p < 0.05$ was considered significant. Data were expressed as means ± standard error. The results of statistical analysis are described in each figure legend.

Results

PQ-Induced ROS Production Enhances Presynaptic Activity (Glutamate Exocytosis)

To assess presynaptic activity under PQ-induced ROS production, the fluorescence of FM4-64, an indicator of presynaptic activity was measured in the SNpc of brain slices bathed in PQ (Fig. 1b). Attenuation of FM4-64 fluorescence, an index of exocytosis, was enhanced in the presence of PQ, while the enhancement was blocked in the co-presence of Trolox and ACA. On the other hand, dendritic release of dopamine in the

substantia nigra [29] indicates that FM4-64 fluorescence can be attenuated by somatodendritic dopamine exocytosis via postsynaptic dopaminergic activity. However, PQ induces extracellular glutamate accumulation in the SNpc [16] and this accumulation inhibits somatodendritic dopamine exocytosis [30, 31]. Therefore, it is likely that PQ-induced attenuation of FM4-64 fluorescence is due to presynaptic activity in the SNpc.

PQ-Induced ROS Production Increases Extracellular Zn^{2+} Influx Via AMPA Receptor Activation

To check glutamate neurotransmission under PQ-induced ROS production in vivo, extracellular glutamate level was determined under in vivo SNpc perfusion with PQ. Extracellular glutamate concentration was increased in the SNpc (Fig. 2). In contrast, extracellular Zn^{2+} level, which was measured with ZnAF-2, was decreased in the SNpc under the SNpc perfusion with PQ. It is estimated that PQ-induced increase in extracellular glutamate induces the influx of extracellular Zn^{2+} in the SNpc, resulting in the decrease in extracellular Zn^{2+} level. Interestingly, the bidirectional changes in extracellular levels of glutamate and Zn^{2+} were ameliorated under co-perfusion with ACA, suggesting that PQ-induced ROS production increases extracellular Zn^{2+} influx in the SNpc via extracellular glutamate signaling. To confirm this idea, intracellular Zn^{2+} dynamics was assessed in the SNpc of brain slices bathed in PQ in Ringer solution containing 10 nM Zn^{2+}, an estimated concentration of brain extracellular Zn^{2+} [19, 20]. Intracellular Zn^{2+} level, which was measured with ZnAF-2DA, was increased in the presence of PQ, while the increase was blocked in the co-presence of ACA and CNQX (Fig. 3), suggesting that PQ-induced ROS production increases intracellular Zn^{2+} in the SNpc via AMPA receptor activation.

PQ Accelerates Age-Related Degeneration Via Extracellular Zn^{2+} Influx

ROS production was compared in the SNpc of brain slices from young (10–15 weeks of age) and aged (> 60 weeks of age) rats. When brain slices were exposed to PQ, intracellular ROS level, which was determined by APF fluorescence, was increased almost to the same extent in the SNpc between young and aged slices (Fig. 4a). PQ-induced ROS production may increase glutamate exocytosis from neuron terminals via TRPM2 cation channel activation in the SNpc. When extracellular glutamate concentration was compared between young and aged rats, it was also increased almost to the same extent in the SNpc between young and aged rats (Fig. 4b).

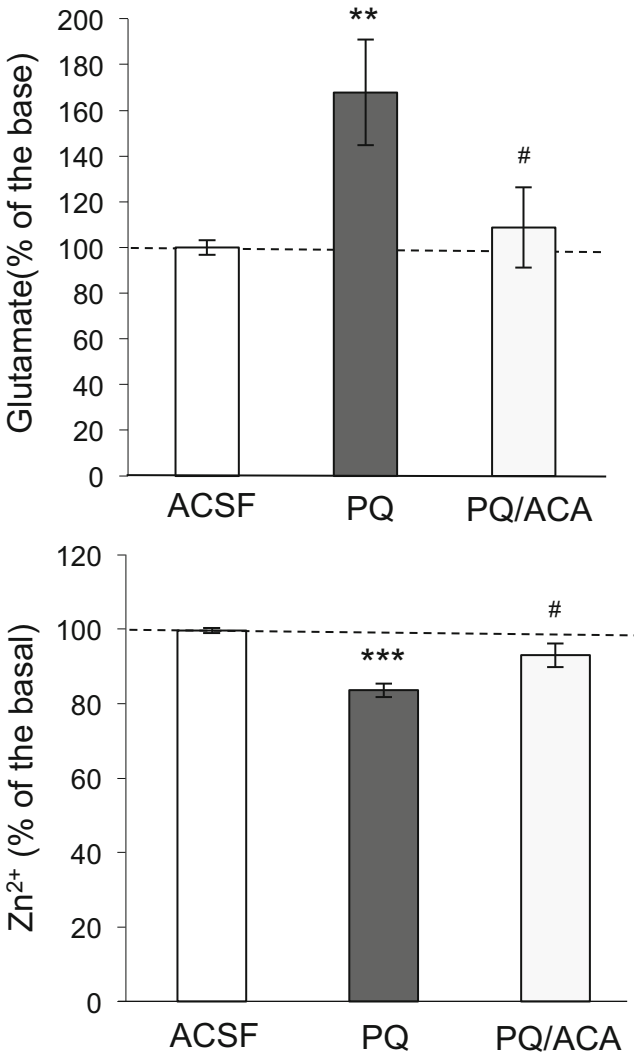

Fig. 2 PQ modifies extracellular concentrations of glutamate and Zn^{2+} in the SNpc of young rats. The SNpc was perfused with 40 μM PQ or 40 μM PQ + 50 μM ACA. Each bar and line represents the ratio of glutamate concentration in the perfusate with PQ ($n = 5$) or PQ + ACA ($n = 4$) (upper) and ZnAF-2 fluorescence in the perfusate with PQ ($n = 5$) or PQ + ACA ($n = 4$) (lower) to the basal glutamate concentration ($n = 9$) and the basal ZnAF-2 fluorescence ($n = 9$) in the perfusate, respectively, which was perfused with ACSF and expressed as 100%. **, $p < 0.01$, ***, $p < 0.001$, vs. the control (ACSF), #, $p < 0.05$, vs. PQ (Tukey's test)

When intracellular Zn^{2+} level was compared in the SNpc after PQ injection into the SNpc of young and aged rats, it was more markedly increased in the aged SNpc than the young SNpc (Fig. 5). The increase in intracellular Zn^{2+} was completely blocked by co-injection of ACA and CaEDTA.

Turning behavior in response to apomorphine, an index of movement disorder in PQ-induced PD in rats, was not observed at all after PQ injection into the SNpc of young and aged rats. PQ-induced loss of nigrostriatal dopaminergic neurons was determined by TH immunostaining after the

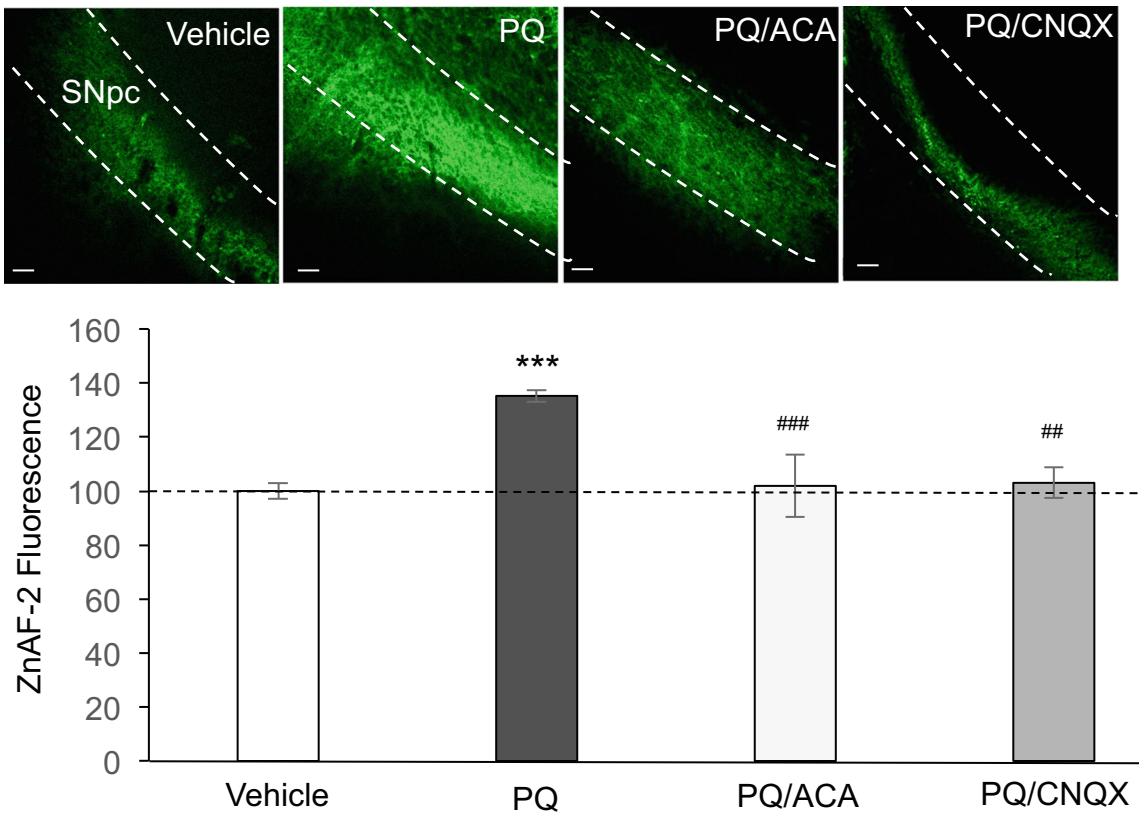

Fig. 3 PQ increases intracellular Zn^{2+} concentration in the SNpc of young rats. Brain slices loaded with ZnAF-2DA were bathed in Ringer (control, $n = 11$), 40 μM PQ ($n = 10$), 40 μM PQ + 40 μM ACA ($n = 5$), or 40 μM PQ + 10 μM CNQX ($n = 4$) for 10 min. Intracellular ZnAF-2 fluorescence was imaged in the SNpc (upper). Each bar and line represents the ratio of ZnAF-2 fluorescence to the control ZnAF-2 fluorescence, which was expressed as 100% (lower). ***, $p < 0.001$, vs. control (ACSF), ##, $p < 0.001$, vs. PQ (Tukey's test)

behavioral test was finished. Staining intensity in the ipsilateral SNpc was reduced to approximately 55% of the contralateral SNpc in young rats (Fig. 6). In contrast, staining intensity in the ipsilateral SNpc was more severely reduced in aged rats and reduced to approximately 30% of the contralateral SNpc. The reduction was completely rescued in aged rats by co-injection of CaEDTA.

Discussion

The high concentration of iron within the substantia nigra, which is likely to be associated with sporadic PD [32, 33], may act to catalyze the conversion of H_2O_2 produced during breakdown of dopamine to highly reactive hydroxyl radicals, resulting in increased oxidative damage in the region [34]. Iron and PQ as synergistic environmental risk factors in sporadic PD accelerate age-related degeneration of nigrostriatal dopaminergic neurons [35]. After chronic exposure to zinc, on the other hand, zinc accumulates in the nigrostriatal tissues and induces oxidative stress via the activation of NADPH oxidase and depletion of glutathione, which in turn activates the apoptotic machinery leading to dopaminergic degeneration [36]. Judging from age-dependent increase in the basal concentration of extracellular Zn^{2+} in the brain [21], it is possible that extracellular Zn^{2+} dynamics acts synergistically to cause neurodegeneration after exposure to PQ, which induces rapid extracellular Zn^{2+} influx into nigral dopaminergic neurons, and is involved in acceleration of age-related neurodegeneration.

Because intracellular Zn^{2+} concentration is estimated to be considerably low (~ 100 pM) in nigral dopaminergic neurons [37, 38], in the present study, we postulated that nigral dopaminergic neurons are sensitive to rapid influx of extracellular Zn^{2+} induced with PQ-induced ROS production and that the rapid influx is age-related. Presynaptic activity (glutamate exocytosis) in the SNpc, which was determined with FM4-64, was enhanced after exposure to PQ and the enhancement was inhibited in the presence of Trolox and ACA. The data suggest that PQ-induced ROS enhances presynaptic activity in the SNpc, probably via ROS-sensitive TRPM2 cation channels, which are calcium-permeable, non-selective cation channels [39]. The SNpc is innervated from the subthalamic nucleus and the amygdala via glutamatergic neurotransmitter system [40, 41]. Dopaminergic neurons express glutamate receptors in the SNpc [42]. It has been reported that excess

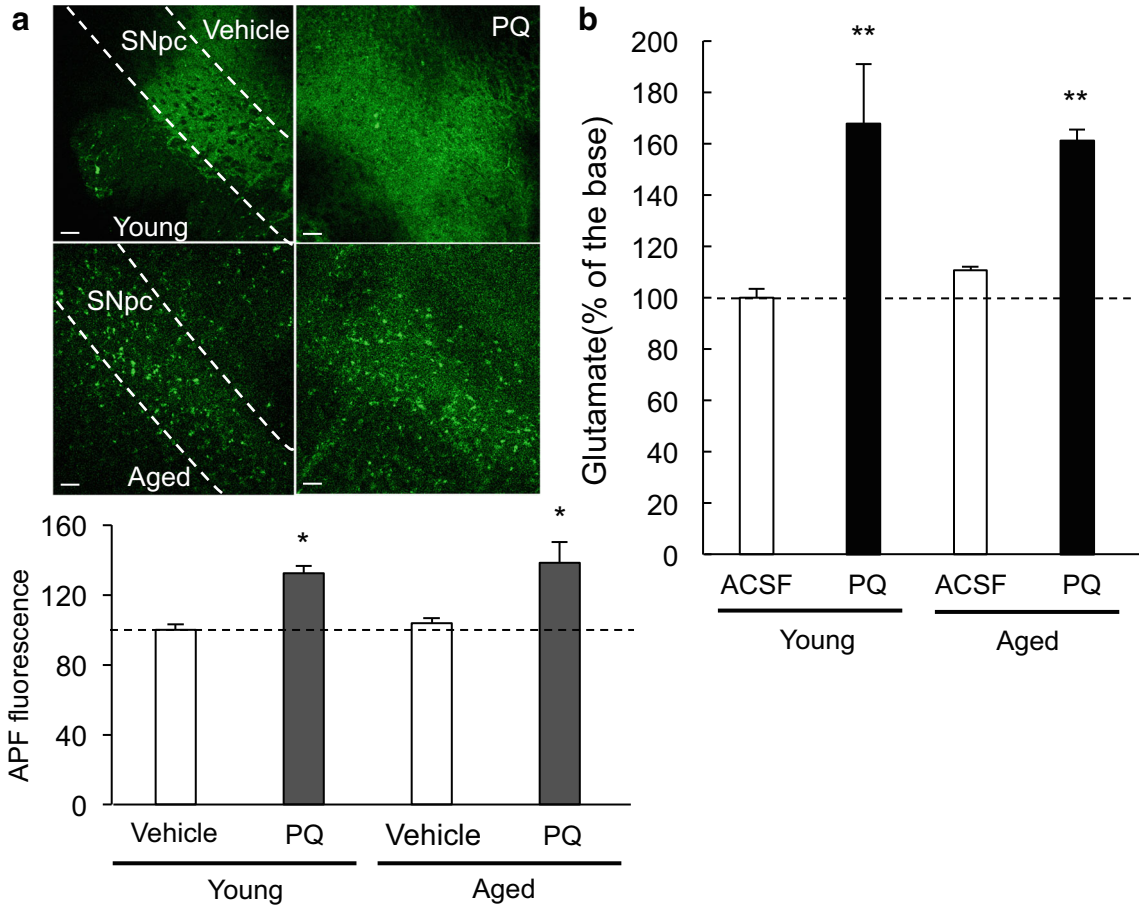

Fig. 4 Extracellular glutamate accumulation associated with PQ-induced ROS production in the SNpc of young and aged rats. **a** Brain slices from young and aged rats were bathed for 10 min in 40 μM PQ in Ringer solution containing 3 μM APF. APF fluorescence was imaged in the SNpc (upper). Note that APF fluorescence intensity was not significantly higher in the aged SNpc than the young SNpc. Each bar and line represents the ratio of APF fluorescence in the young ($n = 4$) and aged ($n = 4$) SNpc to the control APF fluorescence in the young SNpc ($n = 4$), which was expressed as 100%. *, $p < 0.05$, vs. each control (Ringer) (Tukey's test). **b** The SNpc of young and aged rats was perfused with 40 μM PQ. Each bar and line represents the ratio of glutamate concentration in the perfusate with PQ ($n = 5$) from young rats and that in the perfusate with ACSF ($n = 4$) and PQ ($n = 4$) from aged rats to the basal glutamate concentration ($n = 9$) in the perfusate from young rats, which was perfused with ACSF and expressed as 100%. **, $p < 0.01$ vs. each control (ACSF) (Tukey's test)

activation of glutamate receptors on dopaminergic neurons in the SNpc may be involved in pathophysiology of PD [43–47]. Recently, we have reported that PQ-induced ROS may induce extracellular glutamate accumulation in the SNpc [16]. It has been reported that neuron-specific TRPM2 cation channels may contribute to the pathology of cerebral ischemia and oxygen-glucose deprivation [24], which is linked with glutamate excitotoxicity. MPP^+ treatment increases the level of ROS that activates the function of TRPM2 cation channels and upregulates its expression. ROS-mediated activation of TRPM2 cation channels results in an increased intracellular Ca^{2+}, which in turn facilitates cell death in the human neuroblastoma SH-SY5Y cell line [48]. TRPM2 cation channel expression is also increased in substantia nigra of MPTP-induced PD mouse model and PD patients [48]. It is possible that TRPM2 cation channel activation in neuron terminals causes extracellular glutamate accumulation in the SNpc after exposure to PQ.

When extracellular glutamate accumulation in the SNpc was compared between young and aged rats, it was almost the same extent between them under SNpc perfusion with PQ. The increase in extracellular glutamate was suppressed by SNpc co-perfusion with ACA. When PQ-induced ROS production was also compared between young and aged rats, it was also almost the same extent in the SNpc of brain slices from young and aged rats. These data suggest that PQ-induced TRPM2 channel activation enhances glutamate exocytosis from neuron terminals in the SNpc. On the basis of PQ-induced ROS production in the aged SNpc, it is estimated that PQ-induced glutamate exocytosis is not enhanced in the aged SNpc. Extracellular glutamate signaling induces extracellular Zn^{2+} influx via AMPA receptor activation [49, 50]. As a matter of fact, in vitro PQ-induced increase in intracellular Zn^{2+} in the SNpc was inhibited in the presence of CNQX, an AMPA receptor antagonist. Furthermore, intracellular Zn^{2+} was increased in the SNpc after PQ injection into the SNpc

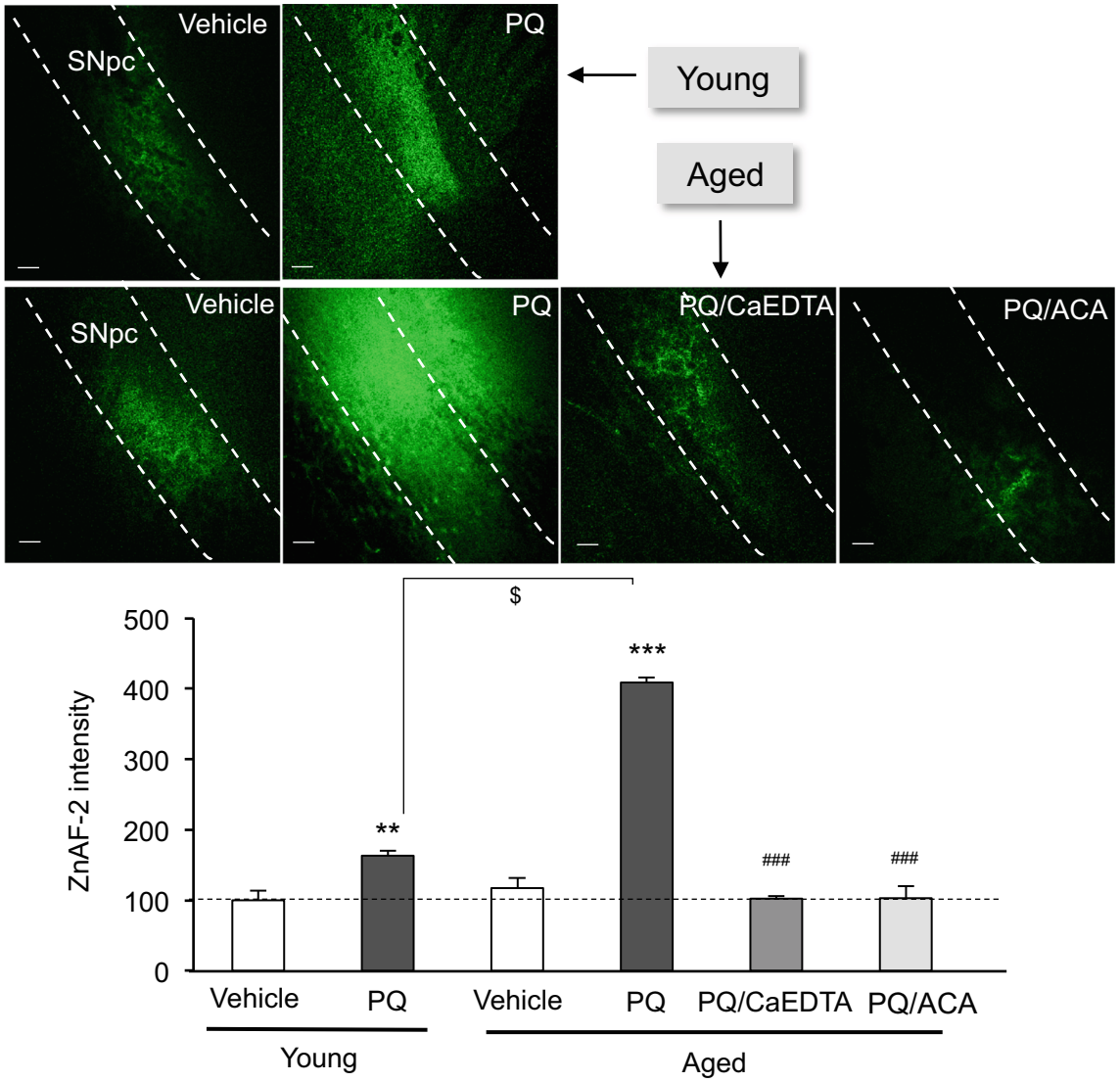

Fig. 5 Age-related increase in extracellular Zn^{2+} influx in the SNpc after injection of PQ into the SNpc of young and aged rats. Saline ($n = 5$) or 40 μM PQ in saline ($n = 4$), which contain ZnAF-2DA (100 μM), were bilaterally injected into the SNpc of young rats. Saline ($n = 6$), 40 μM PQ in saline ($n = 4$), 40 μM PQ + 10 mM CaEDTA in saline ($n = 4$), or 40 μM PQ + 50 μM ACA in saline ($n = 5$), which contain ZnAF-2DA (100 μM), were also bilaterally injected into the SNpc of aged rats. Ten minutes after injection, intracellular ZnAF-2 fluorescence was imaged in the SNpc (upper). Each bar and line represents the ratio of ZnAF-2 fluorescence in young and aged SNpc to the control ZnAF-2 fluorescence in young SNpc, which was expressed as 100% (lower). **, $p < 0.01$, ***, $p < 0.001$, vs. control (saline), $, $p < 0.05$, vs. young PQ, ##, $p < 0.01$, vs. aged PQ (Tukey's test)

in vivo, while the increase was blocked by co-injection of CaEDTA and ACA. Interestingly, intracellular Zn^{2+} was more markedly increased in the aged SNpc than the young SNpc, perhaps owing to aged-related characteristics (easiness) of extracellular Zn^{2+} influx via AMPA receptor activation, which is observed in the aged hippocampus [21, 51]. Furthermore, loss of nigrostriatal dopaminergic neurons was more severely increased in aged rats after exposure to PQ, consistent with more marked increase in intracellular Zn^{2+} in the aged SNpc. On the basis of the evidence that nigrostriatal dopaminergic degeneration, which does not induce any behavioral abnormality, is completely rescued by co-injection of PQ (the present dose) and ZnAF-2DA, an intracellular Zn^{2+} chelator into the SNpc of young rats [16], the rescuing effect was assessed in aged rats by co-injection of CaEDTA. Loss of nigrostriatal dopaminergic neurons was completely blocked by co-injection of PQ and CaEDTA into the SNpc of aged rats. The block of intracellular Zn^{2+} toxicity in the SNpc, which is induced by TRPM2 channel activation via PQ-induced ROS production, may be an effective strategy for defending PQ-induced pathogenesis.

The risk for developing PD can be increased in the process of aging after exposure to environmental neurotoxicants; age-related progressive nigrostriatal dopaminergic degeneration is

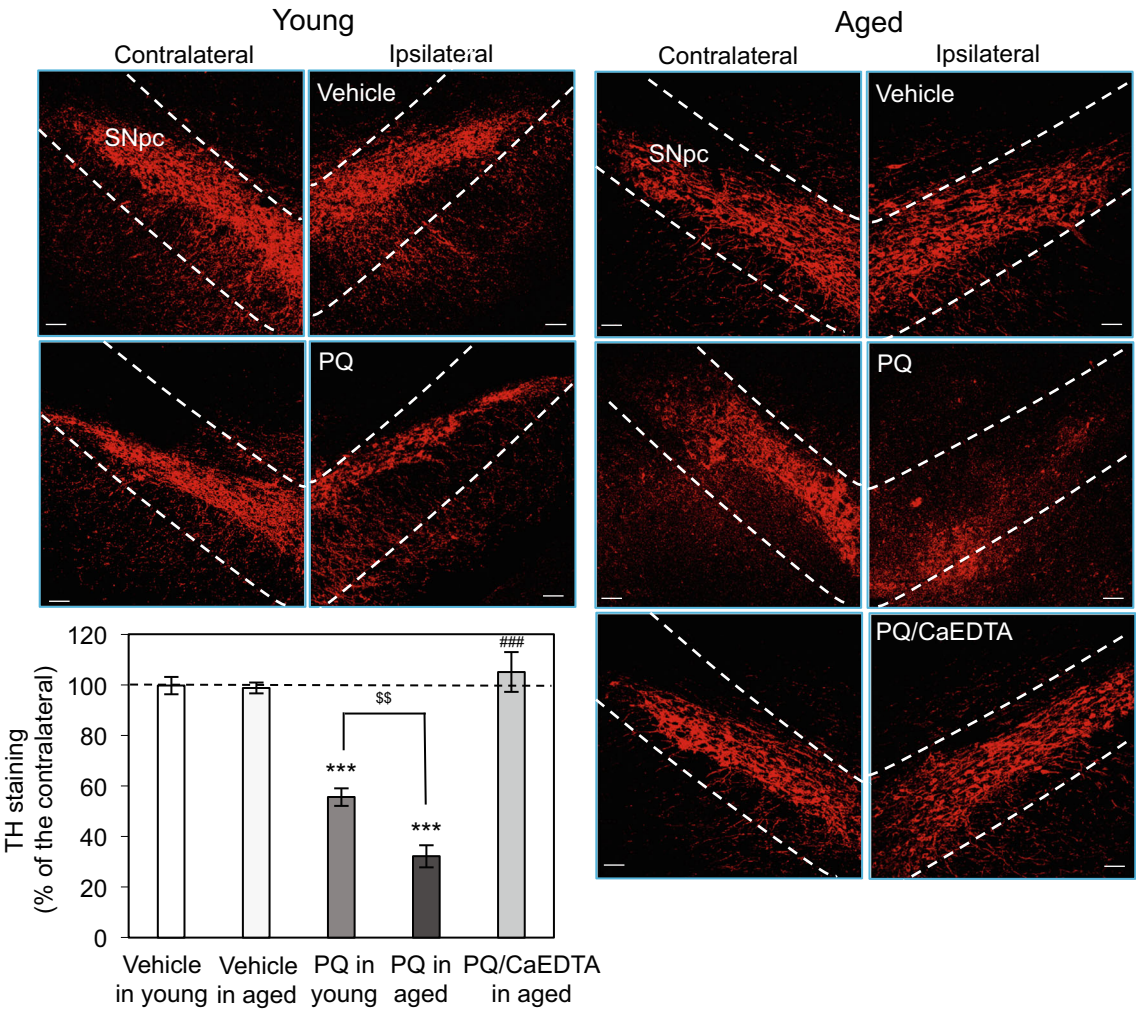

Fig. 6 Age-related neuronal loss in the SNpc after injection of PQ into the SNpc of young and aged rats. Saline ($n = 8$) or 40 μM PQ in saline ($n = 8$) were unilaterally injected into the SNpc of young rats. Saline ($n = 4$), 40 μM PQ in saline ($n = 5$), or 40 μM PQ + 10 mM CaEDTA in saline ($n = 4$) were also unilaterally injected into the SNpc of aged rats. Two weeks later, TH immunostaining with Alexa Fluor 633 fluorescence was performed in the SNpc. Each bar and line represents the ratio of Alexa Fluor 633 fluorescence in the ipsilateral SNpc to Alexa Fluor 633 fluorescence in the contralateral SNpc, which was expressed as 100%. ***, $p < 0.001$, vs. each contralateral side and each ipsilateral side injected with vehicle, $^{\$\$}$, ipsilateral side of young rats injected with PQ, $p < 0.01$, vs. $^{\#\#\#}$, $p < 0.001$, vs. ipsilateral side of aged rats injected with PQ (Tukey's test)

observed after exposure to fungicide maneb and PQ [52]. Age-related vulnerability to MPTP has been reported [7, 8], and Date et al. report that MPTP-treated aged mice provide a more useful model for studying anatomical and neurochemical characteristics of PD than young mice [53]. It is possible that metabolic disorder of synaptic dopamine, which leads to ROS production [54], induces age-related intracellular Zn^{2+} dysregulation, which is linked with pathogenesis of dopaminergic neurodegeneration in the SNpc. In the case of exposure to PQ, H_2O_2 produced from PQ in nigral dopaminergic neurons, which is retrogradely transported, might activate presynaptic TRPM2 channels of glutamatergic terminals [22].

In conclusion, the present study indicates that rapid influx of extracellular Zn^{2+} into dopaminergic neurons via PQ-induced TRPM2 cation channel activation accelerates nigrostriatal dopaminergic degeneration in aged rats. It is likely that vulnerability to PQ-induced pathogenesis in the aged SNpc is due to accelerated intracellular Zn^{2+} dysregulation.

Compliance with Ethical Standards

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

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