BIOCHEMICAL PHARMACOLOGY 76 (2008) 1155–1164

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Paraquat inhibits postsynaptic AMPA receptors on dopaminergic neurons in the substantia nigra pars compacta

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ARTICLE INFO

Article history:
Received 14 July 2008
Accepted 5 August 2008

Keywords:
Paraquat
AMPA receptors
EPSC
Dopamine
Parkinson's disease

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease that mainly affects dopaminergic (DA-ergic) neurons in the substantia nigra pars compacta (SNc). Glutamate modulates neuronal excitability, and a high concentration of glutamatergic receptors is found on DA-ergic neurons in the SNc. Paraquat (PQ) is a putative causative agent for PD. Its effects on synaptic glutamate transmission in SNc DA-ergic neurons were evaluated using whole-cell voltage-clamp recording in brain slices from 7- to 14-day-old Wistar rats. In the presence of bicuculline (BIC), strychnine, and DL-aminophosphonovaleric acid, PQ reversibly suppressed AMPA receptor-mediated evoked excitatory postsynaptic currents (eEPSCs) in a concentration-dependent manner ($P < 0.05$). In the presence of tetrodotoxin (1 μM), PQ (50 μM) significantly reduced the amplitudes, but not the frequencies, of miniature EPSCs in the SNc, suggesting PQ inhibited eEPSCs through a postsynaptic mechanism. Exogenous application of AMPA to induce AMPA-mediated inward currents excluded involvement of a presynaptic response. The AMPA-induced currents in the SNc were significantly reduced by PQ (50 μM) to 74% of control levels ($P < 0.05$), supporting that PQ acts on postsynaptic AMPA receptors. No effect of PQ on eEPSCs was seen in the LD thalamic nucleus and hippocampus, showing PQ specifically inhibited DA-ergic neurons in the SNc. Our results demonstrate a novel mechanism of action of PQ on glutamate-gated postsynaptic AMPA receptors in SNc DA-ergic neurons. This effect may attenuate the excitability and function of DA-ergic neurons in the SNc, which may contribute to the pathogenesis of PD.

1. Introduction

Progressive loss of nigrostriatal dopaminergic (DA-ergic) neurons may elicit Parkinson's disease (PD). There is increasing evidence supporting the hypothesis that environmental factors contribute to PD development [1]. The discovery that administration of the chemical 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results in a syndrome resembling the clinical, biochemical, and pathological features of PD in humans and experimental animals stimulated a search for environmental chemicals resembling MPTP that might cause PD [2]. MPTP is converted by monoamine oxidase B to 1-methyl-4-phenylpyridinium ion (MPP^+), the effective neurotoxin [3]. Paraquat (1,1′-dimethyl-4,4′-bipyridium dichloride, PQ) is a widely used herbicide with striking structural similarity to MPP^+. Both PQ and MPP^+ are toxic for DA-ergic neurons may be similar [4]. Epidemiological studies have reported a strong correlation between the incidence of PD and PQ levels [5,6]. PQ has also been shown to induce nigrostriatal neuronal degeneration in rodents [7–10], supportive evidence for the close relationship between PQ and PD seen clinically.

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.08.006

The DA-ergic cells of substantia nigra pars compacta (SNc) release DA and transmit the signal from the SNc to striatum via D1 and D2 receptors and regulate the whole motor circuit [11]. It is known that glutamate modulates the neuronal excitability, and a high density of glutamatergic receptors is found on DA-ergic neuron cell bodies in the SNc [12]. The excitatory postsynaptic responses elicited in DA-ergic neurons by stimulation of excitatory afferents to the SNc have both fast AMPA receptor-mediated and slow NMDA receptor-mediated components [13]. These receptors play a critical role in the physiological function of DA-ergic neurons (control of excitability, pacemaker firing, and dendritic DA release) and are involved in some DA-ergic neuron-related diseases (neuronal death in PD, psychosis, and mechanism of action of drugs of abuse) [14]. In human postmortem studies, autoradiographic evaluation of glutamate binding sites in control and PD-diseased brains revealed a reduction in NMDA and AMPA receptors in the SNc in PD [15]. In addition, decreased platelet glutamate uptake has been demonstrated in patients with PD [16], support for a role of glutamate in this disease.

Activation of glutamate receptors on DA-ergic neurons triggers DA release and control motor functions. Although the biochemical studies have shown PQ is a free radical producer which may induce DA-ergic neuron degeneration, the effect of PQ on the excitability of DA-ergic neurons remains unclear. The electrophysiological approach could provide a direct observation of the effect of PQ on glutamate transmission system. To explore the pathophysiological mechanism of the effect of PQ in PD, we examined whether PQ affects synaptic glutamate neurotransmission in the SNc using brain slice whole-cell patch-clamp recording. Our results showed that PQ inhibited postsynaptic AMPA receptor-mediated glutamate transmission in SNc neurons, which may contribute to the neurodegenerative mechanism in PD. This finding is important in understanding the connection between environmental factors and the occurrence of PD.

2. Methods

2.1. Brain slice preparation

Neonatal Wistar rat pups (7–14 days old) of both genders were housed with the mother rat in polycarbonate boxes (four rats per box) in accordance with the *Guide for the Care and Use of Laboratory Animals* of the US National Institutes of Health. They were maintained in a controlled atmosphere with a 12 h dark/light cycle (lights off at 7:00 p.m.), a temperature of 22 ± 2 °C, and 50–70% humidity with free access to pelleted feed and fresh tap water. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experiments were approved by the Animal Ethics Committee of the National Taiwan University. The animals, under anesthesia with halothane, were killed by decapitation. Their brains were rapidly removed and placed in ice-cold (4 ± 2 °C) oxygenated solution consisting of (in mM): 125 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 5 $MgCl_2$, 26 $NaHCO_3$, 15 glucose aerated with 95% O_2–5% CO_2 (pH adjusted to 7.4 with 1 N HCl and NaOH). Three hundred micrometer horizontal slices (for the SNc and hippocampal dentate gyrus (DG)) and sagittal thalamic slices (for the lateral dorsal (LD) nucleus of the thalamus) were cut in the above solution using a vibratome (DTK-1000, Dosaka, Kyoto, Japan), then transferred to a holding chamber containing artificial cerebrospinal fluid (ACSF) consisting of (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 15 glucose aerated with 95% O_2–5% CO_2 (pH adjusted to 7.4 with 1 N HCl and NaOH). The slices were then maintained at room temperature (23 ± 2 °C) for at least 1 h before recording [17].

2.2. Electrophysiological recordings

The brain slice was transferred to the recording chamber, held submerged, and superfused continuously with ACSF at a flow rate of 1–2 ml min^{-1} for recording at room temperature (23 ± 2 °C). Evoked excitatory postsynaptic currents (eEPSCs) mediated by AMPA receptors was isolated pharmacologically by application of ACSF containing BIC (50 μM) and strychnine (0.5 μM) to block GABA and glycine receptors and Mg^{2+} (2 mM) and DL-aminophosphonovaleric acid (DL-APV, 50 μM) at a holding potential of −70 mV to inactivate NMDA receptors. Patch electrodes with a resistance of 3–8 MΩ were pulled from standard-walled borosilicate glass capillaries (CSF-150, Warner Instrument, USA) using a micropipette puller (P97, Sutter Instrument, USA), then filled with a CsCl-based internal solution consisting of (in mM): 140 CsCl, 9 NaCl, 1 $MgCl_2$, 1 EDTA, 10 HEPES, 5 QX-314, 2 Mg-ATP, 0.3 Na-GTP (pH adjusted to 7.3 with 1 N CsOH). DA-ergic neurons (in SNc) and non-DA-ergic neurons (granule cells in DG and thalamic neurons) were identified visually using an upright infrared microscope with a water-immersion lens (Olympus, Japan) [17]. After completion of the electrophysiological recordings, the brain slices were fixed and stained for further localization of the investigated neuronal cells (Fig. 1A). Whole-cell patch-clamp recordings were made using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Series resistance and whole-cell capacitance were monitored continuously during all experiments and estimated by compensating (70–80%) for the fast transients evoked at the onset and offset of 8 ms, −5 mV voltage-command steps. The data were discarded if the series resistance changed by more than 20%. After establishing a whole-cell patch, DA-ergic neurons were identified electrophysiologically on the basis of a prominent hyperpolarization-activated current (I_h) at negative voltage steps and a typical voltage-sag when negative current steps were applied in current-clamp mode [18].

To evoke the EPSCs, a stimulation glass pipette filled with 3 M NaCl solution, a stimulator (S-48, Grass-Telefactor, RI, USA), and an isolation unit (A.M.P.I., Jerusalem, Israel) were used. The position of the stimulation electrode was varied until a stable response was evoked in the recorded neuron. To evoke EPSCs in SNc DA-ergic neurons, the stimulation electrode was placed on the substantia nigra reticulata [13]; to evoke EPSCs in LD thalamic neurons, the stimulation electrode was placed nearby the recorded cell [17]; and to evoke EPSCs in DG granule cells, the stimulation electrode was placed on the inner molecular layer upon DG [19]. This setup endured an optimal evoking response from the recorded neurons as previous studies. The stimulus duration was modified from 10 to 50 μs and the strength from 5 to 20 V to attain a proper response, with a frequency of 0.2 Hz. After

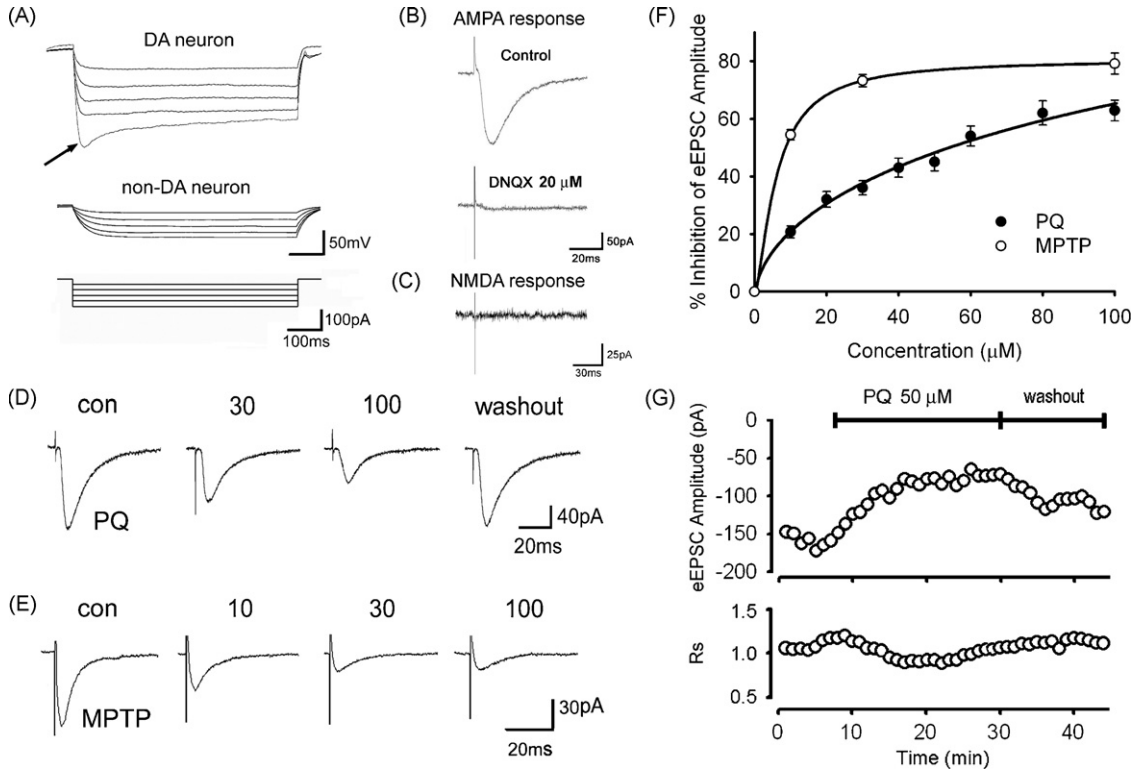

Fig. 1 – PQ or MPTP inhibits the evoked EPSCs in DA-ergic neurons in the SNc. (A) *Top panel*: Identification of DA-ergic neurons using a current-injection protocol. After injection of a negative current step, the membrane potential of a DA-ergic neuron shows a negative voltage-sag (indicated by the arrowhead). *Center panel*: After injection of a negative current step, a neuron lacking the voltage-sag was not recognized as a DA-ergic neuron and was discarded. *Bottom panel*: The current-injection protocol used to identify the electrophysiological characteristics of DA-ergic neurons. (B) The eEPSC is totally inhibited by DNQX (20 μM), indicating the component recorded is an AMPA receptor-mediated current. (C) No significant evoked NMDA-mediated response was observed during perfusion with BIC (50 μM), strychnine (0.5 μM), DNQX (50 μM), and Mg^{2+}-free solution at a holding potential of −70 mV. (D) eEPSCs were recorded by whole-cell patch clamp of DA-ergic neurons in the SNc held at −70 mV following focal afferent stimulation by a stimulating electrode. In ACSF solution containing BIC (50 μM) and strychnine (0.5 μM), a voltage pulse (0.05 Hz, 20 μs duration) of suprathreshold intensity was applied to elicit eEPSCs in the SNc. The amplitudes of eEPSCs in SNc in the presence of different concentrations (in μM) of PQ were recorded. Note that PQ induced a decline in the amplitude of eEPSCs in SNc neurons in a concentration-dependent manner. (E) MPTP has a similar inhibitory effect on eEPSCs in the SNc. The amplitudes of eEPSCs in the SNc in the presence of different concentrations (in μM) of MPTP were recorded. (F) Concentration-dependency of the effect of PQ and MPTP on the eEPSCs in SNc DA-ergic neurons. The IC_{50} for PQ is 53 μM. (G) A continuous recording from a DA-ergic neuron. The series resistance was continuously monitored.

attaining a regular evoking response, the stimulation duration and strength were kept constant across experiments. eEPSCs were quantified by measuring the peak amplitude of the averaged responses. Recordings were made for at least 3 min (about 36 events) to determine the average peak amplitude in control and drug experiments. Responses to drugs were collected and analyzed using the stable eEPSCs amplitude after more than 5 min of drug application.

To record AMPA receptor-mediated miniature EPSCs (mEPSCs) in isolation, tetrodotoxin (TTX, 1 μM), BIC (50 μM), strychnine (0.5 μM), and DL-APV (50 μM) were added to the bath solution. TTX was used to block voltage-gated Na^+ channel and action potential-dependent glutamate release which helped to isolate the non-action potential-dependent miniature glutamate release. Spontaneous synaptic currents were filtered at 5 kHz and digitized at 10 kHz and recorded directly on the computer hard disk using AxoScope 9.0 software (Axon Instrument). The access resistance was monitored at regular intervals throughout each study and recordings were discarded if it changed by >20%. The amplitude and frequency after drug application were analyzed.

In the experiment using exogenous application of AMPA, we applied AMPA (10 mM) directly to the surface of the SNc to induce inward currents. AMPA dissolved in perfusion solution was loaded into a patch pipette and ejected by back pressure every 1.5 min. A Picospritzer (General Valve, Fairfield, NJ, USA) was used to apply back pressure to the patch pipette (1–2 μm tip diameter, 10–50 ms duration, and pressure = 5–15 psi). TTX (1 μM), BIC (50 μM), strychnine (0.5 μM), and DL-APV (50 μM)

were included in the bath medium to isolate the AMPA receptor-induced inward current [19]. AMPA was applied by the patch pipette every 90 s. After five to six stable responses were acquired, PQ was administered in the bath solution and the response of PQ on exogenously applied AMPA-induced current was recorded and analyzed. In some experiments, we perfused ACSF containing BIC (50 μM), strychnine (0.5 μM), DNQX (6,7-dinitroquinoxaline-2,3-dione, 50 μM), and Mg^{2+}-free solution at a holding potential of −70 mV, to observe NMDA-mediated response.

2.3. Data analysis and statistical procedures

Data were acquired using a Pentium 3-based personal computer clone and a Digidata 1322A computer interface. (Axon Instruments, Foster City, CA, USA) and stored on the hard disk. eEPSCs were analyzed using Clampfit 9.0 (Axon Instruments, Foster City, CA, USA) and mEPSCs were analyzed using Mini Analysis Program 6.0 (Synaptosoft Inc., Fort Lee, NJ, USA). Statistical differences were examined using Student's *t*-test (to examine the eEPSCs between control and PQ treatment), and Kolmogorov–Smirnov's test (K–S test, to analyze the significance of mEPSCs). The overall analyses for comparison of mean values were performed by analysis of variance (ANOVA). Post hoc tests (i.e., Scheffe's method) were used to examine the specific difference between any of two groups while the results of the overall analysis reach statistical significance. The drugs were applied consecutively on the same cell and the IC_{50} of PQ and MPTP on eEPSCs were fitted by Hill's equation. The *n* number represented the number of patched cell. Data are expressed as the mean ± S.E.M. and $P < 0.05$ was taken to indicate statistical significance.

2.4. Drugs and chemical agents

The chemicals used for ACSF and internal solution (including $CaCl_2$, CsCl, CsOH, EDTA, glucose, HCl, HEPES, KCl, Mg-ATP, $MgCl_2$, NaCl, Na-GTP, $NaHCO_3$, NaOH, and QX-314) were purchased from Sigma (St. Louis, MO, USA). BIC, MPTP, PQ, strychnine, and TTX were also purchased from Sigma (St. Louis, MO, USA). DL-APV, DNQX, and AMPA were purchased from Tocris (Bristol, UK). All drugs used were prepared as stock solutions which were stored at 4 °C, diluted to the working concentration in extracellular solution immediately before use, and bath applied. DL-APV, MPTP, PQ, and TTX were dissolved in double-distilled water, while BIC, DNQX, and strychnine were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.3%.

3. Results

3.1. Identification of DA-ergic neurons in the SNc

Total 33 rat pups (18 males and 15 females) were used in these experiments and no gender differences ware observed in the present research. Fig. 1A shows that only those DA-ergic neurons in the SNc exhibiting a prominent I_h current showing voltage-sags after negative current injection [18] were included in our experiments (Fig. 1A). The holding current and conductance were continuously monitored during whole-cell patch-clamp recording. No significant shift of holding current shift or change of conductance was observed during all the experiments.

3.2. PQ or MPTP reduces the eEPSCs in the SNc

The effect of PQ on excitatory synaptic responses of DA-ergic neurons in the SNc and in thalamic neurons was examined using brain slice whole-cell voltage-clamp recording. The synaptic response mediated by AMPA receptors was isolated pharmacologically by application of a solution containing BIC (50 μM) and strychnine (0.5 μM) to block GABA and glycine receptors and Mg^{2+} (2 mM) and DL-APV (50 μM) at a holding potential of −70 mV to inactivate NMDA receptors. The AMPA-induced eEPSC was totally inhibited by DNQX (20 μM), indicating the component recorded was an AMPA receptor-mediated current (Fig. 1B). No significant NMDA-mediated response is found in our experimental preparation (Fig. 1C). PQ significantly inhibited the AMPA receptor-mediated eEPSCs in a concentration-dependent manner in DA-ergic neurons in the SNc (Fig. 1D). After application of PQ, the amplitude of the eEPSCs was reduced by 20.7 ± 2.1% (10 μM), 32.3 ± 2.7% (20 μM), 36.2 ± 2.5% (30 μM), 43.6 ± 3.3% (40 μM), 45.3 ± 3.1% (50 μM), 54.1 ± 3.5% (60 μM), 62.0 ± 4.2% (80 μM), and 62.9 ± 3.6% (100 μM) compared to control levels ($P < 0.05$, $n = 6$), respectively (Fig. 1D and F). This inhibitory effect of PQ could be partially reversed after 10 min washout with ACSF. Similar results were obtained by perfusion with MPTP. After application of MPTP, the amplitude of the eEPSCs was reduced by 54.4 ± 1.8% (10 μM), 73.2 ± 2.1% (30 μM), or 73.1 ± 3.6% (100 μM) compared to control levels ($P < 0.05$, $n = 5$), respectively (Fig. 1E and F). Fig. 1G shows a continuous recording from a DA-ergic neuron in which the series resistance was continuously monitored during the experiment.

3.3. PQ reduces the amplitude, but not the frequency, of AMPA-mediated mEPSCs in the SNc

In order to distinguish between presynaptic and postsynaptic effects of PQ, we recorded mEPSCs in the presence of TTX (1 μM) and DL-APV (50 μM) (Fig. 2A). The mean amplitude of the mEPSCs was 14.8 ± 0.6 pA before, and 12.1 ± 0.5 pA after, application of PQ (50 μM). The histogram of the amplitude of mEPSCs was shifted to the left with PQ perfusion (Fig. 2B). PQ also significantly shifted the cumulative probability plot of the mEPSCs amplitude ($n = 6$, $P < 0.05$ using the K–S test, Fig. 2C). Thus, PQ (50 μM) significantly reduced the mean amplitude of the mEPSCs ($n = 6$, $P < 0.05$ by the K–S test and Student's *t*-test, Fig. 2B–D). The mean frequency of the mEPSCs was 19.8 ± 3.6 Hz before, and 19.0 ± 5.4 Hz after, perfusion with PQ (50 μM). Thus, the frequency of the mEPSCs was not significantly altered by PQ (50 μM) ($n = 6$, $P > 0.05$ by the K–S test and Student's *t*-test, Fig. 2E). Since PQ reduced the amplitude, but not the frequency, of the mEPSCs, this suggests that its inhibitory effect is mediated by postsynaptic AMPA receptors.

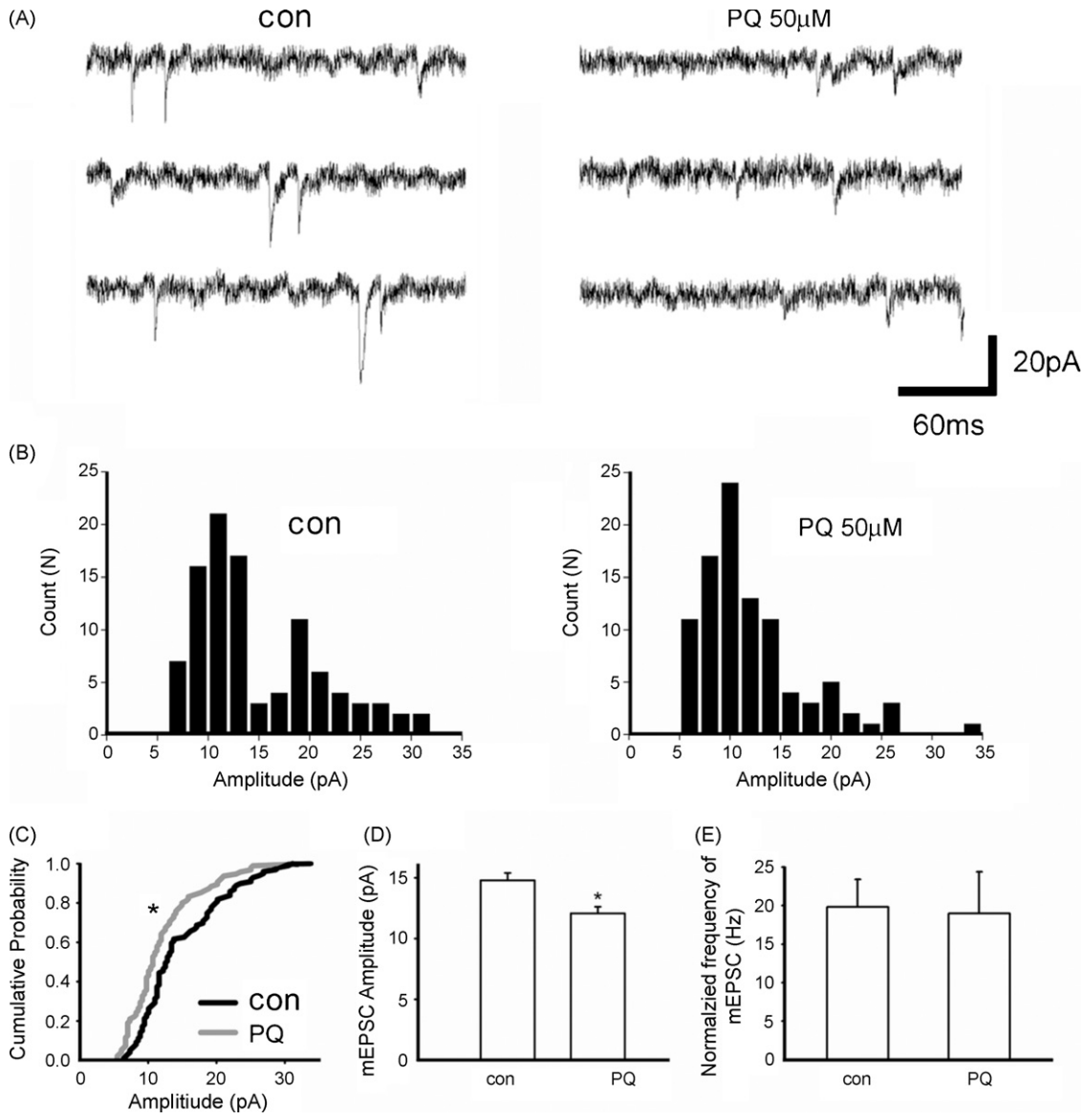

Fig. 2 – Effects of PQ on mEPSCs in the SNc. (A) PQ (50 μM) reduces the amplitude, but not the frequency, of mEPSCs in DA-ergic neurons in the SNc. The recording was performed in the presence of BIC (50 μM), DL-APV (50 μM), and TTX (1 μM) with a holding potential of −70 mV. (B) PQ (50 μM) left-shifts the amplitude histogram, showing that the amplitude of the mEPSCs is reduced by PQ (50 μM). (C) PQ (50 μM) significantly shifts the cumulative probability plot of the mEPSCs amplitude, again showing that PQ (50 μM) reduces the amplitude of the mEPSCs (*P < 0.05, K–S test). (D) Mean amplitude of the mEPSCs before and after PQ (50 μM) perfusion. The mean amplitude is significantly reduced by PQ (*P < 0.05, Student's t-test). (E) Mean frequency of the mEPSCs before and after PQ (50 μM) perfusion. The mean frequency is not reduced by PQ (P > 0.05, Student's t-test).

3.4. *PQ inhibits AMPA-induced currents*

To confirm the postsynaptic AMPA receptor inhibitory effect of PQ, exogenous application of AMPA (10 mM) was used in the presence of TTX (1 μM) to exclude presynaptic factors. After blocking GABA and NMDA receptors with BIC (50 μM) and DL-APV (50 μM), AMPA was applied to induce an AMPA-mediated inward current at a holding potential of −70 mV. PQ (50 μM) reduced the amplitude of the AMPA-induced inward current (Fig. 3A). The AMPA-induced inward current in the presence of PQ (50 μM) was 74.0 ± 6.0% of that in controls ($n = 4$, $P < 0.05$, Student's t-test, Fig. 3B), with partial reversal after washout.

3.5. *PQ did not alter the paired-pulse ratio*

Paired-pulse ratio (PPR) is a form of short-term plasticity contributed from presynaptic origin [20]. To confirm whether PQ differentially affected presynaptic or postsynaptic sites, we recorded PPR before and after application of PQ in the DA-ergic neurons of the SNc. Before PQ perfusion, the PPR of eEPSCs was

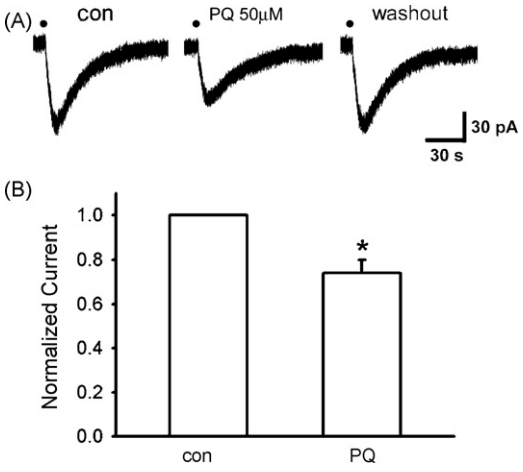

Fig. 3 – PQ inhibits the inward current induced by pipette application of AMPA. (A) The AMPA-induced inward current is inhibited by PQ (50 μM). The recording was performed in the presence of TTX (1 μM), BIC (50 μM), and DL-APV (50 μM) with a holding potential of −70 mV. The circles indicated the application of AMPA from pipette. (B) Summary data showing that the AMPA-induced inward current is inhibited after perfusion with PQ (50 μM) (*$P < 0.05$, Student's *t*-test).

1.43 ± 0.08 at an interval of 50 ms. After application of PQ (50 μM), the PPR was 1.35 ± 0.10, which was not significantly different from before PQ treatment (Fig. 4, $P > 0.05$, $n = 3$).

3.6. PQ selectively inhibits eEPSCs in the SNc, but not in the lateral dorsal nucleus of the thalamus and hippocampus

We investigated the DA-ergic neurons in SNc. The LD thalamic neuron is part of the motor circuit that is composed of GABA-ergic neurons and is not innervated by DA-ergic neurons [21]. DG granule cell contains glutamatergic neurons without significant DA innervation [19]. These two areas are used to compare the effect of PQ on the non-DA-ergic neurons. In the SNc, the amplitude of the eEPSCs was reduced by PQ by $32.3 \pm 2.7\%$ (20 μM), $43.6 \pm 3.3\%$ (40 μM), and $54.1 \pm 3.5\%$ (60 μM) (Fig. 1D and F, $P < 0.05$, $n = 6$). In contrast, in LD thalamic nucleus neurons, the same concentrations of PQ reduced the amplitude of the eEPSCs by only $2.4 \pm 1.5\%$ (20 μM), $5.2 \pm 1.5\%$ (40 μM), and $6.0 \pm 1.6\%$ (60 μM) (Fig. 5B, $P > 0.05$, $n = 6$). Similarly, in the hippocampal DG, and PQ reduced the amplitude of the eEPSCs by only $1.6 \pm 5.3\%$ (20 μM) and $13.5 \pm 5.5\%$ (60 μM) (Fig. 5B, $P > 0.05$, $n = 3$). Fig. 5C shows at the same concentration of PQ (20–60 μM), the inhibitory effect of PQ on LD and DG neurons was significantly lower than SNc ($P < 0.05$).

4. Discussion

The present study examined the effect of PQ on excitatory synaptic transmission in the SNc. Our three main findings were: (1) in the presence of BIC and strychnine (blocking inhibitory synaptic responses) and Mg^{2+} at a holding potential of −70 mV (blocking NMDA receptors), PQ reversibly suppressed AMPA receptor-mediated eEPSCs in DA-ergic neurons; (2) the mEPSCs is used as a measure of glutamatergic pre-/postsynaptic involvement and its amplitude is believed to depend on the number of postsynaptic receptors, while its frequency is often interpreted as the presynaptic glutamate release probability that may be dependent on activity changes in the active zones [22]. In the presence of TTX and DL-APV, PQ significantly reduced the amplitude, but not the frequency, of AMPA receptor-mediated mEPSCs in the SNc, suggesting PQ inhibited eEPSCs through a postsynaptic mechanism; and (3) exogenous application of AMPA to induce an AMPA-mediated inward current excludes involvement of a presynaptic response and allows direct observation of postsynaptic effects [23]. Under these conditions, PQ significantly reduced the currents induced in the SNc, showing that PQ inhibited postsynaptic AMPA receptors. Taken together, our results

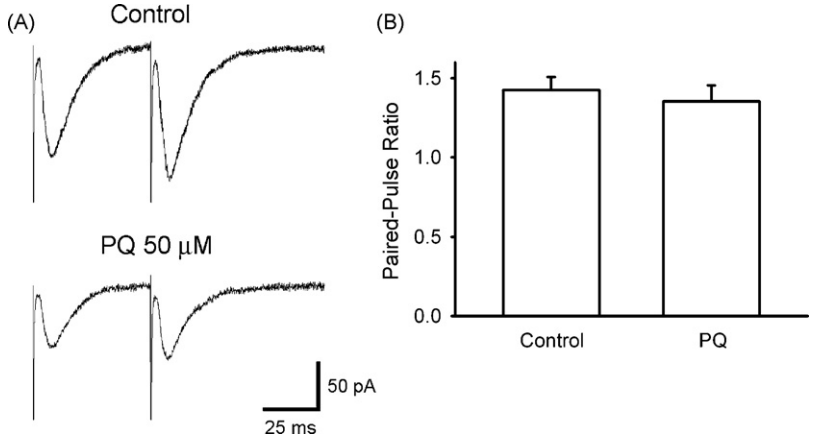

Fig. 4 – PQ did not affect the PPR of eEPSCs of DA-ergic neurons in SNc. (A) PQ did not alter the PPR of eEPSCs at 50 ms interval. The recording was performed in the presence of BIC (50 μM) and DL-APV (50 μM) with a holding potential of −70 mV. (B) Summary data show the average PPR of eEPSCs in the control and under PQ (50 mM) conditions ($P > 0.05$, Student's *t*-test).

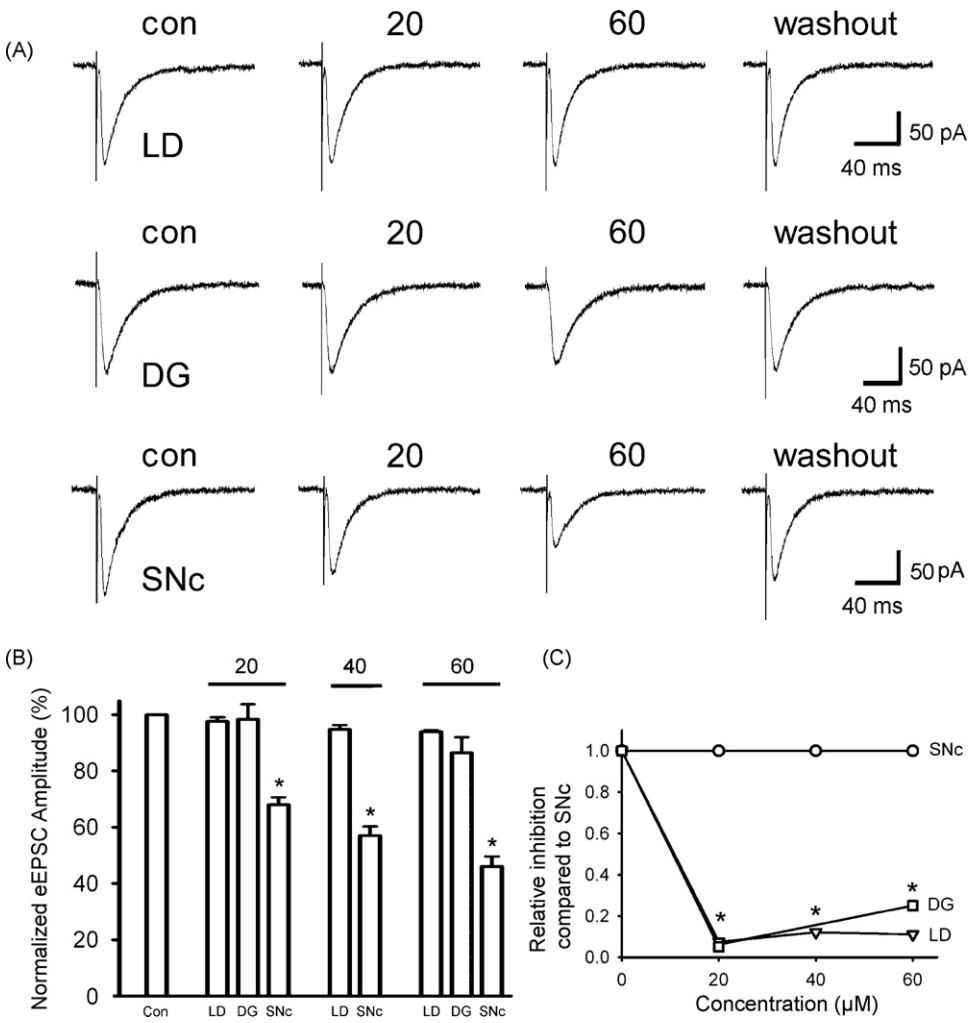

Fig. 5 – PQ inhibits DA-ergic neurons in the SNc, but not in the thalamus or hippocampus. (A) eEPSCs were recorded using whole-cell patch clamp in neurons in the LD thalamus nucleus, DG of the hippocampus, and SNc held at −70 mV following focal afferent stimulation by a stimulating electrode. In normal ACSF solution containing BIC (10 μM) and strychnine (0.5 μM), a voltage pulse (0.2 Hz, 10–50 μs duration) of suprathreshold intensity was applied to elicit eEPSCs. The amplitudes of the eEPSCs in the presence of different concentrations (in μM) of PQ were recorded. Note that PQ did not affect the amplitude of the eEPSCs in the LD and DG, but reduced the eEPSCs in a concentration-dependent manner in the SNc. (B) At the same concentration of PQ (20–60 μM), the inhibitory effect of PQ on LD and DG neurons was significantly lower than SNc. (C) Relative concentration–response curves for the effect of PQ on eEPSCs in the LD, DG, and SNc.

demonstrate a novel mechanism of action of PQ in inhibiting postsynaptic AMPA receptors to reduce DA-ergic neuron excitability in the SNc.

The excitatory input to DA-ergic cells in the SNc comes from the subthalamic nucleus, cortical regions [24], and pedunculopontine nucleus [25]. Activation of ionotropic glutamate receptors on SNc DA-ergic neurons causes membrane depolarization, typically accompanied by an increase in cell firing rate or burst firing [13,18,26], which triggers DA release from the nigrostriatal system and modulates motor functions [27]. In an *in vitro* bath application experiment, the rank order of potency for increasing the firing rate of DA-ergic neurons was AMPA > NMDA > glutamate [26]. Patients with PD have a lower content of glutamate receptors in various brain regions compared to age-matched controls, with a more severe deficit in the SNc [15]. This led to the hypothesis that insufficiency of glutamate receptors may cause abnormal release of dopamine and induce the clinical symptoms of PD [28]. Our results revealed that PQ inhibited postsynaptic AMPA receptors in SNc DA-ergic neurons and decreased DA-ergic neuronal excitation, thus adversely affecting downstream signaling in areas such as the caudate–putamen and contributing to the pathogenesis of PD.

PQ had an inhibitory effect on eEPSCs in the SNc, but not in hippocampal and thalamic neurons. Application of PQ (50 μM) reduced the amplitude of the AMPA receptor-mediated eEPSCs by 50% in the SNc, but had no significant effect on hippocampal and LD thalamic neurons. AMPA receptors are distributed throughout the central nerve system (CNS), with regional differences in density. High densities of AMPA

receptors are found in the hippocampus, while intermediate levels are found in the stratum, and lower levels in the brainstem and cerebellum [29]. The SNc contains AMPA receptor subunits GluR1-3 [30], the hippocampus contains GluR1-4, and thalamic neurons contain GluR2-4 [31]. These differences in AMPA subunit distribution may be involved in the different effects of PQ on SNc, hippocampal, and thalamic neurons. In addition, DA-ergic neurons have been shown to have an intrinsically greater susceptibility to PQ-induced degeneration [7–10]. Since the LD thalamic nucleus is composed of GABA-ergic neuron [21] while DG granule cell is the glutamatergic neuron [19] which are not innervated by DA-ergic synapses, we used these nuclei to examine the effects of PQ on non-DA-ergic neurons and found that they had no effect, supporting the previous evidence mentioned above. Furthermore, PQ inhibited exogenously applied AMPA-induced inward current provided a direct evidence of post-synaptic effect of PQ in brain slice preparation, which contains intact component of different proportions of AMPA subunits and is more closed to the physiological condition. To realize the underlying mechanism how PQ modulates AMPA receptor, additional experiments should be conducted with AMPA receptors expressed in an exogenous system to test the hypothesis that diversity of subunit composition underlying the differential response to PQ in SNc, hippocampus and thalamus in our future works.

PQ inhibited AMPA receptor responses was incomplete at concentrations up to 100 μM. It appears that PQ is a partial inhibitor of AMPA receptors. The present study was performed using a concentration of PQ which had reversible effects on the eEPSCs and mEPSCs. Under these experimental conditions, we found that PQ selectively inhibited the postsynaptic AMPA receptor, inferring that PQ may directly affects the physiological function of DA-ergic neurons in the SNc by reducing neuronal excitability. Despite the structural similarity of PQ, MPTP, and MPP^+, recent studies have suggested that the mechanism of toxicity of PQ is distinct from those of MPTP and MPP^+ [32]. PQ has been recognized to cause a reduction in EPSP in the rat spinal cord *in vivo* [33]. MPP^+ antagonizes postsynaptic glutamate receptor-mediated responses in hippocampal neurons [34]. Our present study showed that both MPTP and PQ significantly inhibited the eEPSCs in SNc DA-ergic neurons. Whether PQ and MPTP have a similar mode of action in glutamate transmission that contributes to the neurodegeneration that occurs following exposure to these compounds deserves further investigation.

Although NMDA receptor is a major type of glutamate receptor and plays an important role in regulating neuronal excitability, whether functional NMDA receptors exist in SNc in neonatal age remains controversial. Wu and Partridge used dissociated DA-ergic neurons from the SNc of 2-week-old Sprague–Dawley rats and revealed that AMPA induces an inward current that is similar to the glutamate-induced current, while NMDA fails to induce any current response in Mg^{2+}-free solution containing glycine at a clamp voltage of −60 mV [35]. Lui et al. reported that functional NMDA receptors could only be found in striatal neurons but not in nigral dopaminergic neurons *in vitro* by patch-clamp analysis on the age of postnatal day 1 in Sprague–Dawley rat [36]. In contrast, rotenone is reported to potentiate NMDA currents in dopaminergic cells leaving unaffected the AMPA-induced responses [37], while Lin and Lipski also found that the presence of NMDA-evoked responses in SNc neurons acutely dissociated from P4 to P16 Wistar rats, applying strict criteria for identification of these neurons as nigrostriatal and dopaminergic through whole-cell recordings [38]. These discrepancies may due to different developmental age, animal species, or experimental designs. In our present study, evoked NMDA-mediated current could not be successfully evoked and observed in brain slice preparation from 2-week-old Wistar rat. However, according to previous findings, we cannot totally exclude the possibility that NMDA receptors may be involved in this inhibitory effect of PQ in older rats or other species.

PPR facilitation is an enhancement of the synaptic response caused by presynaptic accumulation of Ca^{2+}. Changing PPR proposes a presynaptic mechanism [20]. As the result showed in Fig. 4, PQ inhibited the amplitude of eEPSCs but did not alter the PPR of eEPSCs in SNc, indicating that PQ did not affect presynaptic accumulation of Ca^{2+}. The discrepancy of the effect of PQ between eEPSCs and mEPSCs was not likely from additional effect of PQ on presynaptic accumulation of Ca^{2+}. Similar result is found in our previous study on lamotrigine, an effective anticonvulsant. The lamotrigine inhibition of eEPSCs is much greater than inhibition of mEPSCs or currents resulting from bath applied AMPA but did not alter PPR. The different extent of inhibition is due to the well-known Na^+ channel inhibitory effect of lamotrigine [19]. In the present study on PQ, we concluded a postsynaptic effect of PQ from AMPA receptor inhibitory effect which did not augment presynaptic accumulation of Ca^{2+}. However, we could not exclude the involvement of Na^+ channel inhibitory effect of PQ on nerve terminal which did not cause PPR change. In addition, it is noteworthy that presynaptic residual Ca^{2+} as the sole mechanism for PPR facilitation has been questioned [39,40]. Postsynaptic Ca^{2+}/calmodulin signaling pathways that alter AMPA receptor properties appear to regulate the magnitude of PPR [41,42], while certain reports argue against a role of affecting postsynaptic AMPA receptors in modulating synaptic facilitation, in which the partial block of AMPA receptor activity by CNQX did not change PPF [43,44]. Thus, the precise mechanism involved in regulating PPR facilitation and PQ remains to be further explored.

An epidemiological study revealed that the risk of PD is increased sixfold in individuals who have been exposed to PQ for more than 20 years [5]. Chronic low-level non-pulmonary toxic doses could produce parkinsonism. Little is actually known about actual human exposure levels to PQ and the routes by which they occur, although it is likely that they would include inhalation, per oral ingestion or through transdermal absorption. Low levels of PQ appear to be retained in tissue such as muscle after subcutaneous exposures from where it can then be slowly released into blood [45]. Bocchetta and Corsini reported two patients believed to suffer from PQ-induced parkinsonism [46]. Sánchez-Ramos et al. reported a young farmer who had been exposed to PQ and affected with PD [47]. Animal studies also showed that repeated systemic injection of PQ was sufficient to reduce locomotor activity and the decline of DA-ergic neurons in mice [7,10]. Intranigral injection of PQ (2.5–5 mM) causes a concentration-dependent

depletion of DA and motor deficit in rats [8,9]. High concentrations of PQ (75 μM to 0.2 mM) have been applied in cerebellar granule neurons culture to study the mechanism of PQ-induced toxicity [48]. We found that PQ (50 μM) inhibited 50% amplitude of AMPA-mediated eEPSCs in SNc. However, what concentration of PQ in the nervous system following lifetime exposure would induce PD is not clear.

Based on the structural similarity to MPP^+, PQ is doubted to penetrate blood–brain barrier and enter CNS. It is known that cation transporters help PQ to penetrate blood–brain barrier [49]. Several *in vivo* microdialysis experiments have estimated the concentration of PQ in CNS with different administration route. The results show the concentration of PQ in CNS ranging from 0.7 to 8 μM [50,51]. However, the exact concentration of PQ in CNS might be higher after long-term exposure and accumulation. Furthermore, several *in vitro* studies have used PQ up to 100 μM to test its neurotoxicity [32], the IC_{50} of PQ (~50 μM) in our present study was consistent with previous investigations.

In conclusion, our results demonstrate a novel mechanism of action of PQ on glutamate-gated postsynaptic AMPA receptors. This effect appears to be specific to DA-ergic neurons in the SNc. PQ-mediated inhibition of postsynaptic AMPA receptors may attenuate the firing rate and function of DA-ergic neurons in the SNc which may contribute to the pathogenesis of PD.

Acknowledgements

This work was supported by the National Science Council (96-2314-B-002-125), Taipei, Taiwan. We are indebted to Prof. Tomoyuki Takahashi and Dr. Kayako Onodera, Institute of Brain Science, Medical College, Toyoko University, Japan for their technical support and kind suggestions.

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