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Paraquat induces long-lasting dopamine overflow through excitotoxic pathway in the striatum of freely moving rats

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Abstract

The herbicide paraquat is an environmental factor that could be involved in the etiology of Parkinson's disease (PD). We have previously shown that paraquat penetrates through the blood-brain barrier and is taken up by neural cell. In this study, we examined the *in vivo* toxic mechanism of paraquat to dopamine neurons. GBR-12909, a selective dopamine transporter inhibitor, reduced paraquat uptake into the striatal tissue including dopaminergic terminals. The subchronic treatment with systemic paraquat significantly decreased brain dopamine content in the striatum and slightly in the midbrain and cortex, and was accompanied by the diminished level of its acidic metabolites in rats. When paraquat was administered through a microdialysis probe, a transitory increase in the extracellular levels of glutamate, followed by long-lasting elevations of the extracellular levels of NOx^- (NO_2^- plus NO_3^-) and dopamine were detected in the striatum of freely moving rats. This dopamine overflow lasted for more than 24 h after the paraquat treatment. Dopamine overflow was inhibited by N^G-nitro-L-arginine methyl ester, dizocilpine, 6,7-dinitroquinoxaline-2,3-dione and L-deprenyl. The toxic mechanism of paraquat involves glutamate induced activation of non-NMDA receptors, resulting in activation of NMDA receptor-channels. The influx of Ca^{2+} into cells stimulates nitric oxide synthase. Released NO would diffuse to dopaminergic terminals and further induce mitochondrial dysfunction by the formation of peroxynitrite, resulting in continuous and long-lasting dopamine overflow. The constant exposure to low level of paraquat may lead to the vulnerability of dopaminergic terminals in humans, and might potentiate neurodegeneration caused by the exposure of other substances, such as endogenous dopaminergic toxins.

Keywords: Paraquat; Glutamate; Nitric oxide; Dopamine; Striatum; Neurotoxicity; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, affecting almost 1% of the population over 65 years old [17]. The prominent pathological feature of the PD brain is the selective deterioration of dopaminergic neurons in the substantia nigra pars compacta of the midbrain and a resultant decrease in dopamine levels in the striatum, the main target innervated by this neuronal population. Increasing evidence from epidemiological studies has implicated the possible involvement of environmental chemicals or the endogenous substances, such as ß-carboline [30], tetrahydroisoquinoline [23] and salsolinol [29], in the selective dopaminergic cell loss in the substantia nigra of non-familial PD. Non-familial PD represents over 90% of all PD cases and its onset typically occurs after 50 years of age [24,33,39,55]. Identified PD risk factors include herbicide and pesticide usage, farming and well-water consumption [14,48,49]. A recent study of over 19,000 white male twins has confirmed that genetic heritability is not the basis of sporadic PD with onset over age 50 [56]. These findings lead to the hypothesis that PD may be initiated or precipitated by environmental or endogenous toxins in genetically-predisposed individuals [5,6,10,29,30,44,59]. An environmental factor hypothesis has been given credence by the identification of the pre-toxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a chemical contaminant of synthetic heroin, that produces an acute parkinsonian syndrome in humans, similar to idiopathic PD [25]. After the conversion of MPTP to 1-methyl-4-phenyl pyridinium (MPP^+) in the brain, MPP^+ blocks the mitochondrial respiration at complex I of the electron transport chain [21,36]. The selective toxicity of MPP^+ for dopaminergic neurons is due to the fact that it is an excellent substrate for the dopamine transporter, and is thereby accumulated preferentially in cells that transport dopamine. MPTP, however, is not found in the natural environment. Paraquat (1,1'-dimethyl-4,4'-bipyridinium), widely used as a non-selective herbicide, bears structural similarity to MPP^+. This herbicide adversely impacts on dopamine systems, and a strong geographical overlapping has been found between the incidence of the disease and the

amount of paraquat used [26,28,42,43]. Paraquat induces brain damage in fatal poisoning cases [18,20] and dopaminergic neuronal damage in experimental animals [8,9]. It has been suggested that the mixture of paraquat and dithiocarbamate induces the selective dopaminergic neurotoxicity [59]. However, the impact of paraquat on dopamine systems is equivocal, because its neurotoxic mechanism has not been well documented. We have already demonstrated the blood-brain barrier permeability of paraquat; this herbicide is translocated into the brain by the neutral aminoacid transporter and then is taken up in brain tissues by a certain sodium-dependent transporter [51].

It is considered that the excitotoxicity induced by NMDA receptor activation, associating Ca^{2+} penetration into cells by activation of non-NMDA receptor, is a major mechanism of neurodegeneration in various neurological diseases [12]. There is increasing evidence that excitotoxic injury also plays a critical role in progressive degeneration of dopamine neurons in PD [3,11,22]. The influx of Ca^{2+} induced by the activation of NMDA triggers the mobilization of Ca^{2+}-dependent intracellular processes including the activation of neuronal nitric oxide synthase (NOS). Nitric oxide (NO) produced by NOS is thought to play an important role in excitotoxicity, probably through the formation of peroxynitrite anion by reaction with superoxide radical [7,15,27,38]. The series of radicals act as mitochondrial toxins inducing selective dopaminergic cell death [3,35,52].

The aim of this study was to reveal the *in vivo* neurotoxic mechanism of paraquat. We suggest that paraquat activated the excitotoxic pathway to lead to vulnerability on dopaminergic neurons and terminals in the midbrain and the striatum, respectively, which could be involved in the pathogenesis of PD.

2. Materials and methods

2.1. Chemicals

Paraquat dichloride was obtained from Tokyo Chemical Industry (Tokyo, Japan). MPP^+ iodide, L-deprenyl and dizocilpine (MK-801) were purchased from Research Biochemicals International (Natick, MA, USA). 1-Octanesulfonic acid was obtained from Nacalai Tesque (Kyoto, Japan). GBR-12909, dopamine, norepinephrine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), serotonin (5-HT) were obtained from Sigma (Saint Louis, MO, USA). The other reagents were of analytical or high performance liquid chromatographic (HPLC) grade, and were from Wako (Osaka, Japan).

2.2. Effects of systematic paraquat administration on brain monoamine and their metabolite contents

Paraquat dichloride (10 mg/kg, equal to ca. 40 μmoles) was subcutaneously given to male Wistar rats (8 weeks old, 210-260 g; SLC, Shizuoka, Japan) once a day for 5 days. The rats were decapitated 2 days after the last injection, and the brain was removed immediately and dissected into eight regions (frontal cortex, rear cortex, striatum, midbrain, hippocampus, hypothalamus, cerebellum and pons & medulla). The quantitative analysis of monoamines in brain tissue was performed according to the method of Matsubara et al [31]. Briefly, the tissue was sonicated (50% duty cycle for 1 min) in an ice-cold polyethylene tube containing 0.5 ml of 0.2 M $HClO_4$ and 100 μl each of 0.1 M EDTA and 0.1 M $NaHSO_3$. The samples were allowed to stand in an ice bath for 30 min and centrifuged at 20,000 g for 15 min at 4˚C. An aliquot of 0.5 ml of the supernatant, 0.5 pmoles of isoproterenol (internal standard), and 100 μl of 1 M sodium acetate were mixed in a plastic tube. After filtration, an aliquot of 50 μl of the sample was injected into an HPLC apparatus. Norepinephrine, DOPAC, dopamine, 5-HIAA, HVA and 5-HT were separated on a reversed-phase C18 column (15 cm X 4.6 mm i.d.; Eicompak MA-5ODS; EICOM, Kyoto, Japan) and detected electrochemically with a glassy carbon working electrode, set at 700 mV versus Ag/AgCl (EICOM). The mobile phase consisted of 90 mM sodium acetate-100

mM citric acid buffer (pH 3.5, containing 25 µM disodium EDTA and 1 mM sodium octanesulfonate)/methanol (80:20, v/v), and was delivered at a flow rate of 0.8 ml/min. The animal experiments were done in accordance with the guidelines for care and use of laboratory animals by the Committee of Asahikawa Medical College.

2.3. Brain microdialysis

The rats were housed under conditions of constant temperature, humidity and dark/light (12/12h) cycle. Anesthetized (50 mg/kg i.p., sodium pentobarbital) rats were stereotaxically implanted with 22-gauge cannulae in the left striata at AP + 0.48 mm, L + 3.0 mm from bregma, and - 3.7 mm from the surface of the skull, according to the stereotaxic atlas of Paxinos and Watson [40]. Dummy probes were then placed inside the cannulae. The rats were housed in plastic cages (30 x 30 x 35 cm) with free access to food and water, and a greater than 5-day recovery period was allotted. The microdialysis probes with a dialysis area of 3 mm length were of the I-shaped type prepared according to the method of Nakahara et al. [34]. After insertion through the guide cannula, the probe was connected to a microinfusion pump and perfused with Ringer's solution (146 mM Na^+, 1.26 mM Ca^{2+}, 4 mM K^+, 1.0 mM Mg^{2+} and 154.5 mM Cl^-) at a flow rate of 2 µl/min.

2.4. Paraquat uptake by the dopamine transporter

As our previous report indicated that paraquat uptake into the brain cells is mediated by a sodium exchange transporter [51], we evaluated the involvement of the dopamine transporter in the uptake of paraquat into the striatal cells by the microdialysis technique. After 4 h pre-perfusion with Ringer's solution to recover from brain injury due to the probe insertion, 50 µM paraquat with or without 50 µM GBR-12909, a specific inhibitor for dopamine transporter, was administered to the striatum through the microdialysis probe for 60 min, followed by a sequential 180-min washout with Ringer's solution. Then, the

animal was decapitated, and ipsi- and contra-lateral striata were removed immediately. The quantitative analysis of striatal paraquat was performed according to the method of Shimizu et al [51].

2.5. Striatal extracellular glutamate release

Paraquat (0.5, 5 or 50 µM) was administered to the striatum through the microdialysis membrane for 60 min, followed by a sequential 180-min washout with Ringer's solution. The dialysate in the striatum was collected every 20-min. Three samples just before the perfusion of paraquat were averaged as 100% for the basal glutamate efflux. Glutamate level was determined by HPLC/fluorometric detection according to the method of Shimizu et al [50] with a slight modification. In brief, an aliquot of 40 µl of the dialysate was automatically mixed with 15 µl of the reacting reagent, which consisted of 4 mM o-phthalaldehyde and 5 mM 2-mercaptoethanol in 0.1 M carbonate buffer (pH 9.5), for 15 min at 12˚C. Then, an aliquot of 45 µl of the mixture was immediately and automatically injected into an HPLC apparatus (Shimadzu LC-10A) with a fluorescence detector (Shimadzu RF-10A). Derivatized glutamate was separated on a reverse phase C18 column (Cosmosil 5C18, 150 x 4.6 mm, Nacalai Tesque). The mobile phase was composed of a mixture (30:70, v/v) of methanol with 0.1 M phosphate buffer (pH 6.0, containing 27 µM disodium EDTA), and the flow rate was 1.0 ml/min. The fluorescence intensity at 445 nm was measured with an excitation wavelength of 340 nm.

2.6. Striatal extracellular NO release

After the stabilization for 3 h, 4 consecutive dialysate samples were analyzed to measure the baseline NOx^- (NO_2^- plus NO_3^-) level. Paraquat (50 µM) was administered to the striatum through the microdialysis probe for 60 min, followed by a sequential 180-min washout with Ringer's solution. Three samples just before the perfusion of paraquat were

averaged as 100% for the basal NOx^- efflux. An every 10-min dialysate was automatically delivered to an HPLC/NO detection system (ENO-20, Eicom). NO_2^- and NO_3^- were separated on a packed column (NO-PAK, 4.6 x 50 mm; Eicom), followed by the reduction of NO_3^- to NO_2^- in a subsequent cadmium reduction column (NO-RED, Eicom). NO_2^- was mixed with a Griess reagent (Eicom) in a reaction coil. The absorbance of the resultant product was measured at 540 nm using a flow-through spectrophotometer. The mobile phase (NORCAR; Eicom) and the Griess reagent were pumped at the rates of 0.33 and 0.11 µl/min, respectively. The reliability of the reduction column was checked by injecting a standard solution before and after each experiment.

2.7. Involvement of glutamate receptors and NOS in the long-term dopamine overflow

Paraquat (5 or 50 µM) was administered to the striatum through the microdialysis probe for 60 min, followed by a sequential 180-min washout with Ringer's solution at a flow rate of 2 µl/min. The dopamine and its metabolites levels in the striatum dialysate were measured at every 20-min for 4 h. Three samples just before the perfusion of paraquat were averaged as 100% for the basal dopamine efflux. Then, the striatum was continuously perfused with Ringer's solution at a flow rate of 0.5 µl/min for overnight. On the next experimental day, 24 h after the paraquat treatment, three dialysate samples, which were averaged as a 24 h dopamine level, were collected with a flow rate of 2.0 µl/min Ringer's solution. The striatal specimen was also perfused with N^G-nitro-L-arginine methyl ester (L-NAME), MK-801, 6,7-dinitroquinoxaline-2,3-dione (DNQX) or L-deprenyl in Ringer's solution (1000, 100, 50 or 10 µM, respectively) for 240 min. Subsequently, paraquat (50 µM) in Ringer's solution containing L-NAME, MK-801, DNQX or L-deprenyl was perfused for 60 min followed by sequential perfusion without paraquat for 24 h.

The striatal dialysate was automatically injected into the HPLC system with electrochemical detection. The mobile phase, 0.1 M citric acid/sodium acetate buffer (pH

3.9) containing 0.63 mM 1-octanesulfphonic acid, 16 µM EDTA and 14% methanol (v/v), was delivered at a flow rate of 0.8 ml/min using a Shimadzu LC-10AD HPLC pump.

2.8. Statistical Analysis

The data were analyzed by two-way ANOVA with repeated measurement or one factorial ANOVA, followed by a post hoc Dunnett *t*-test. A *P* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of subchronic treatment with paraquat on the concentration of monoamines in various brain regions

Monoamine concentrations in eight regions of brain were determined 2 days after the 5-day treatment with paraquat and are shown in Fig. 1. Paraquat affected dopamine and its acidic metabolites in the striatum, midbrain and cortex. Dopamine content in the striatum was reduced to 70% of that in control rats ($P < 0.05$). The 5-HT level was 80% of control value in the striatum ($P < 0.01$). Paraquat reduced DOPAC and HVA contents to 40-60% and 25-50% compared with the control in the midbrain and cortex ($P < 0.05$).

3.2. Paraquat uptake by dopamine transporter

When paraquat was administered to the striatum through a microdialysis probe, a significant amount of paraquat was detected in the ipsi-lateral but not contra-lateral striata after a sequential 180-min washout with Ringer's solution (Table 1). Treatment with 50 µM GBR-12909 significantly inhibited the striatal uptake of paraquat ($P < 0.02$).

3.3 Glutamate efflux induced by paraquat

The average basal concentration of glutamate in the dialysate did not differ among the experimental groups. The mean basal level of glutamate for all animals was 26.1 ± 7.1 pmoles in 40 µl dialysate (mean ± SEM). The perfusion of paraquat increased the extracellular levels of glutamate in the striatum in a dose-dependent manner ($F(3,17) = 3.331$, $P < 0.05$ by the two-way ANOVA). The perfusion of 50 µM paraquat elevated the striatal levels of glutamate to 180% of the basal value. The extent of glutamate elevation was small, and the increased level returned to the basal level shortly after the termination of paraquat perfusion (Fig. 2).

3.4. Changes in extracellular NO_X^- efflux induced by paraquat

The average basal concentration of NOx^- in the dialysate was not different between control and paraquat treatment groups. The mean basal value of NOx^- in all animals was 0.63 ± 0.10 pmol/10 min in the dialysate (mean ± SEM). Paraquat (50 µM) increased the extracellular levels of NOx^- in the striatum ($F(1,9) = 11.787$, $P < 0.01$ by the two-way ANOVA). The extent of elevation of NOx^- levels was small; however, this increase continued at least 3 h after termination of paraquat perfusion (Fig. 3).

3.5. Long lasting dopamine overflow after paraquat perfusion

The average basal concentration of dopamine in the dialysate did not differ among the experimental groups. The mean basal level of dopamine in all animals was 103.0 ± 14.4 fmoles in 40 µl dialysate (mean ± SEM). Paraquat increased the extracellular levels of dopamine in the striatum in a dose-dependent manner ($F(2,11) = 12.546$, $P < 0.01$ by the two-way ANOVA). Although the extent of dopamine efflux induced by paraquat, was small, it is noteworthy that dopamine overflow was continuously evoked more than 24 h

after the paraquat perfusion (Fig. 4). The perfusion with paraquat did not affect extracellular DOPAC level (data are not shown).

3.6. Effect of DNQX, MK-801, L-NAME and L-deprenyl on the dopamine overflow induced by paraquat in the striatum of freely moving rats

The blockade of AMPA/kinate receptors with DNQX (50 μM) inhibited the 50 μM paraquat-induced increase in extracellular dopamine levels in the striatum (Fig. 5A). An antagonist of NMDA receptor, MK-801 (100 μM), attenuated the late phase of dopamine overflow induced by paraquat, however, the initial phase of the dopamine overflow was not inhibited (Fig. 5B). L-NAME (1 mM) completely inhibited the dopamine overflow induced by 50 μM paraquat (Fig. 5C). The L-deprenyl (10 μM) also decreased the paraquat-induced increase in the striatal extracellular dopamine levels (Fig. 5D).

3.7. Effect of DNQX, MK-801, L-NAME and L-deprenyl on the long-lasting dopamine overflow in the striatum induced by paraquat

Paraquat induces a long-lasting dopamine overflow, that can be observed at 24 h after its perfusion for 1 h. This effect of paraquat was inhibited by treatment with L-NAME, MK-801, DNQX and L-deprenyl ($P < 0.01$). The extent of dopamine overflow is expressed as a percent value of that in control animals. These data are summarized in Fig. 6.

4. Discussion

Recently, various studies have increased interest in the possibility that environmental chemicals, including paraquat, may be related to the development of PD [10,41,53,57].

Although controversial, paraquat is one of the possible herbicides that are involved in PD, since a strong correlation has been found between the incidence of the disease and the amount of paraquat used [26,28,42,43]. The concurrent exposure to paraquat and some other chemicals could perturb the balance of homeostatic defense mechanisms leading to neuronal degeneration, even if exposure to each pesticide may be insufficient to induce overt effects [59]. However, the mechanism of the neuronal toxicity occurring under continuous exposure to low levels of paraquat for a long period has not been determined. In the present study, we investigated the *in vivo* mechanisms of paraquat induced toxicity in the striatum by using the microdialysis technique. Our results indicate that paraquat would stimulate glutamate efflux to initiate excitotoxicity of NOS containing neurons, resulting in evoked depolarization of NMDA receptor channels and Ca^{2+} penetration into cells by activation of non-NMDA receptor channels. The influx of Ca^{2+} into cells stimulates NOS. Subsequently, released NO would diffuse to dopaminergic terminals and induce mitochondrial dysfunction, causing continuous and long-lasting dopamine overflow.

Degeneration of dopaminergic neurons following administration of MPTP to mammals has been used as an animal model for PD [16,47]. This neurotoxin produces many (but not all) of the changes seen in human idiopathic PD, including reductions in the levels of dopamine and its metabolites, and a loss of dopaminergic neurons in the nigrostriatum [39]. This discovery led to a hypothesis that other neurotoxins, similar to MPTP, may produce PD. Recently, the involvement of pesticides, such as paraquat, has been proposed to contribute the etiology of idiopathic PD [5,19,43,59]. In this hypothesis, paraquat must be accumulated in dopaminergic terminals via the dopamine transporter to induce dopaminergic neurotoxicity. The subchronic treatment with paraquat reduced dopamine and its acidic metabolite content in the striatum and midbrain, suggesting that paraquat mainly damaged dopamine neurons, although the 5-HT level was also slightly reduced in the striatum. In PD patients, it is well known that 5-HT and norepinephrine systems are also slightly damaged. The dose of paraquat used in this study was relatively high, but was not fatal. A decrease in the level of dopamine and its acidic metabolite was also observed in the cortex,

as well as in the nigrostriatum. A similar region non-specific toxicity of dopaminergic neurons is also detected in animals subchronically treated with MPTP [1,2]. Treatment with the dopamine transporter inhibitor significantly reduced paraquat uptake into the striatal tissue, indicating that paraquat was taken into the dopaminergic terminals by the dopamine transporter. This result supports the comparatively specific damage of dopaminergic neurons by subchronic paraquat treatment, and is consistent with our previous finding of a significant reduction of paraquat uptake under a low Na^+ condition [51]. This paraquat accumulation into striatal tissue is not influenced by putrescine, which is a substrate of polyamine transporters mediating paraquat accumulation into lung tissues [51]. However, the inhibition of paraquat uptake was not completely blocked by the treatment with GBR-12909. Therefore, there was the possibility that paraquat might be partially taken up by cellular mechanisms independent of the dopamine transporter.

Glutamate and reactive oxygen species (ROS) including NO have been postulated to play pivotal roles in the pathogenesis of the neuronal cell loss that is associated with several neurological disease states including PD. MPP^+ is also suggested to induce the glutamate-NO mediated cytotoxicity against dopaminergic neurons in the cultured rat mesencephalon [45,46]. The elevation of extracellular glutamate concentration evoked by MPP^+, particularly under conditions of reduced dopaminergic ATP production, is expected to result in excessive stimulation of NMDA and AMPA/kinate receptors leading to an influx of Ca^{2+} and excitotoxicity [19,37]. In similar fashion, paraquat could cause excitotoxicity of dopaminergic neurons. Paraquat is known to be an inhibitor of mitochondrial respiration chain [58]. In the present study, paraquat dose-dependently elevated extracellular glutamate levels. This phenomenon was observed immediately and shortly after paraquat treatment. This result indicates that paraquat stimulates striatal neurons to release glutamate. The action of synaptic glutamate is terminated by a high-affinity uptake process via the plasma membrane glutamate transporter on the presynaptic nerve terminals and glial cells. Since this transporter is a Na^+-dependent ion-channel, it was also possible the transient elevation of extracellular glutamate would be due to the inhibition of the glutamate

re-uptake system by paraquat. The activity of NMDA receptor channels is regulated not only by the concentration of glutamate in the synaptic space, but also by a voltage-dependent blockade of the channel by Mg^{2+}. Thus, entry of Ca^{2+} into neurons through NMDA receptor-channels requires binding of glutamate to NMDA receptors as well as depolarization of the neuron by the activity of glutamate at AMPA/kinate receptors [54]. Indeed, in the present study, the blockade of AMPA/kinate receptors with DNQX inhibited paraquat-induced dopamine overflow more potently than MK-801. Activated NMDA receptor-channels allow an influx of Ca^{2+}, which in excess can activate a variety of potentially destructive cascades. The high intracellular Ca^{2+} levels also lead to activation of NOS. NO can diffuse to the dopaminergic terminals and then interact with ROS from other sources to generate highly reactive peroxynitrite [4], which uncouples mitochondrial electron transport enhancing mitochondrial production of free radicals [13]. Indeed, a significant elevation of extracellular NOx^- level was observed right after paraquat perfusion. Although the extent of this elevation was small, this increase in NOx^- levels continued for a long-time, at least 3 h after termination of paraquat perfusion. Because of the limitation of the present experimental protocol for NOx^- detection, the level at 24 h after termination of paraquat exposure could not be determined.

A long-lasting elevation of dopamine levels which was observed sequentially after the elevation of glutamate and NOx^-. This long-lasting elevation of dopamine induced by paraquat would be the last step of the excitotoxic events. This conclusion is based on the observations that long-lasting dopamine release was inhibited by treatment with glutamate receptor antagonists, by a NOS inhibitor and by L-deprenyl. L-Deprenyl is a therapeutic drug for PD. These results suggest that glutamate-mediated NO cytotoxicity plays an important role in the toxic effect of paraquat on dopaminergic terminals. L-Deprenyl prevents the decrease in mitochondrial membrane potential caused by withdrawal of trophic support, resulting in the prevention of apoptosis [60], and may rescue neurons from dopaminergic neurotoxins, including MPP^+, by directly influencing mitochondrial electron transport [32]. The long-lasting increase in extracellular dopamine levels caused by

paraquat could result from a delayed dopaminergic neuronal cytotoxicity. This neural damage, at least, should lead to dopaminergic neuronal vulnerability, and might potentiate neurodegeneration caused by exposure to other neurotoxic substances, such as endogenous dopaminergic toxins [23,29,30]. The present findings should be important in considering the pathogenesis of PD.

In conclusion, paraquat stimulated glutamate efflux from neural cells or inhibited glutamate uptake system, and initiates a cascade of excitotoxic reaction leading to damage of dopaminergic terminals. The mechanism involves glutamate induced activation of non-NMDA receptors, resulting in activation of NMDA receptor. The activation of NMDA receptor channels results in massive influx of Ca^{2+} into the cells. The entry of Ca^{2+} into cells stimulates NOS. Released NO would diffuse to dopaminergic terminals and induce mitochondrial dysfunction by the formation of peroxynitrite, resulting in continuous and long-lasting dopamine overflow. Paraquat might trigger production of ROS from the mitochondrial electron transport chain in dopamine neurons. The ROS easily react with NO to generate peroxynitrite, which could be a major substrate underlying paraquat neurotoxicity. This neurotoxic event could cause a continuous and long-lasting overflow of dopamine. The present findings indicate that paraquat could be considered an exogenous neurotoxin involved in the etiology of PD, or at least, exposure to low levels of paraquat for a long time would make dopaminergic neurons vulnerable to oxidative stress and cell death.

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Table 1

Effect of GBR-12909 on paraquat accumulation in striatal tissue

	n	Ipsilateral (pmol)	Contralateral (pmol)
Paraquat	9	172.5 ± 43.0	ND[a]
Paraquat with GBR-12909	10	53.5 ± 11.82*	ND

Accumulation of paraquat in hemi-striatal tissue (35 - 55 mg) after a 60-min perfusion with a 50 µM paraquat solution containing vehicle or 50 µM GBR-12909 through a microdialysis probe, followed by a 180-min washout with Ringer's solution. Data are represent means ± SEM.

[a] Not detectable. * $P < 0.02$ vs accumulation of paraquat perfused under Ringer's solution by the Dunnett *t*-test for the one factorial ANOVA.

Legends for figures

Fig. 1. Effects of subchronic treatment with paraquat on monoamines and their metabolites in the striatum (A), midbrain (B), frontal cortex (C), rear cortex (D), hypothalamus (E), hippocampus (F), cerebellum (G) and pons & medulla (H) of Wistar rats. Paraquat (10 mg/kg) or vehicle was given subcutaneously once a day for 5 days. The monoamines and their metabolite content were analyzed 2 days after the last injection. The number of experimental rats used is given in parentheses. Data represent means ± SEM. NE, norepinephrine and DA, dopamine. $*P < 0.5$, $**P < 0.01$ compared with control by the Dunnett *t*-test for the one factorial ANOVA.

Fig. 2. The time-course of extracellular glutamate level after paraquat administration in the striatum of freely moving rats. Paraquat (0.5, 5 or 50 μM) was administered through a dialysis membrane for 60 min followed by a 180-min washout with Ringer's solution. Control rats were performed with normal Ringer's perfusion medium throughout the experiment. The basal concentration of glutamate in the dialysate was calculated by averaging those in the 3 samples collected before drug perfusion, and was expressed as a basal value of 100%. The number of experimental rats used is given in the parentheses. Data represent means ± SEM, expressed as percentages of the basal value.

Fig. 3. The time-course of extracellular NOx^- (NO_2^- plus NO_3^-) level after paraquat administration in the striatum of freely moving rats. Paraquat (50 μM) was administered through a dialysis membrane for 60 min, followed by a 180-min washout with Ringer's solution. Control rats were performed with normal Ringer's perfusion medium throughout the experiment. Three samples before the perfusion of paraquat were averaged as 100% for the basal NOx^- efflux. The number of experimental rats used is given in the parenthesis. Data represent means ± SEM, expressed as percentages of the basal value.

Fig. 4. Long lasting dopamine overflow after paraquat perfusion. Paraquat (5 or 50 μM) was administered through a dialysis probe for 60 min and then continuously perfused with Ringer's solution for 24 h. Control rats were performed with normal Ringer's perfusion medium throughout the experiment. The basal concentration of dopamine in the dialysates was calculated by averaging those in the 3 samples collected before drug perfusion, and was expressed as a basal value of 100%. The number of experimental animals used is given in the parentheses. Data represent means ± SEM, expressed as percentages of the basal value.

Fig. 5. Effects of DNQX (A), MK-801 (B), L-NAME (C) and L-deprenyl (D) on the dopamine overflow induced by paraquat in the striatum of freely moving rats. The striatal specimen was perfused with L-NAME, MK-801, DNQX or L-deprenyl in Ringer's solution (1000, 100, 50 or 10 μM, respectively) for 240 min. Subsequently, paraquat (50 μM) in Ringer's solution containing L-NAME, MK-801, DNQX or L-deprenyl was perfused for 60 min followed by sequential perfusion without paraquat for 24 h. Control rats were performed with Ringer's perfusion medium containing L-NAME, MK-801, DNQX or L-deprenyl throughout the experiment. The basal concentration of dopamine in dialysates was calculated by averaging those in the 3 samples collected before drug perfusion, and was expressed as a basal value of 100%. The number of experimental rats used is given in the parenthesis. Data represent means ± SEM, expressed as percentages of the basal value.

Fig. 6. Effects of DNQX, MK-801, L-NAME and L-deprenyl on the long-lasting dopamine overflow 24 h after paraquat perfusion in the striatum of freely moving rats. The striatum was perfused with L-NAME, MK-801, DNQX or L-deprenyl in Ringer's solution (1000, 100, 50 or 10 μM, respectively) for 240 min. Subsequently, paraquat (50 μM) in Ringer's solution containing L-NAME, MK-801, DNQX or L-deprenyl was perfused for 60 min followed by sequential perfusion without paraquat for 24 h. Control rats were performed with normal Ringer's solution or Ringer's perfusion medium containing L-NAME, MK-801, DNQX or L-deprenyl throughout the experiment. The extent of dopamine overflow is

expressed as a percent value of that in control animals (mean ± SEM). The number of experimental rats used is given in the parentheses. *$P < 0.01$ compared with the value in the animal treated with paraquat alone by the Dunnett *t*-test for the one factorial ANOVA.

FIG. 1

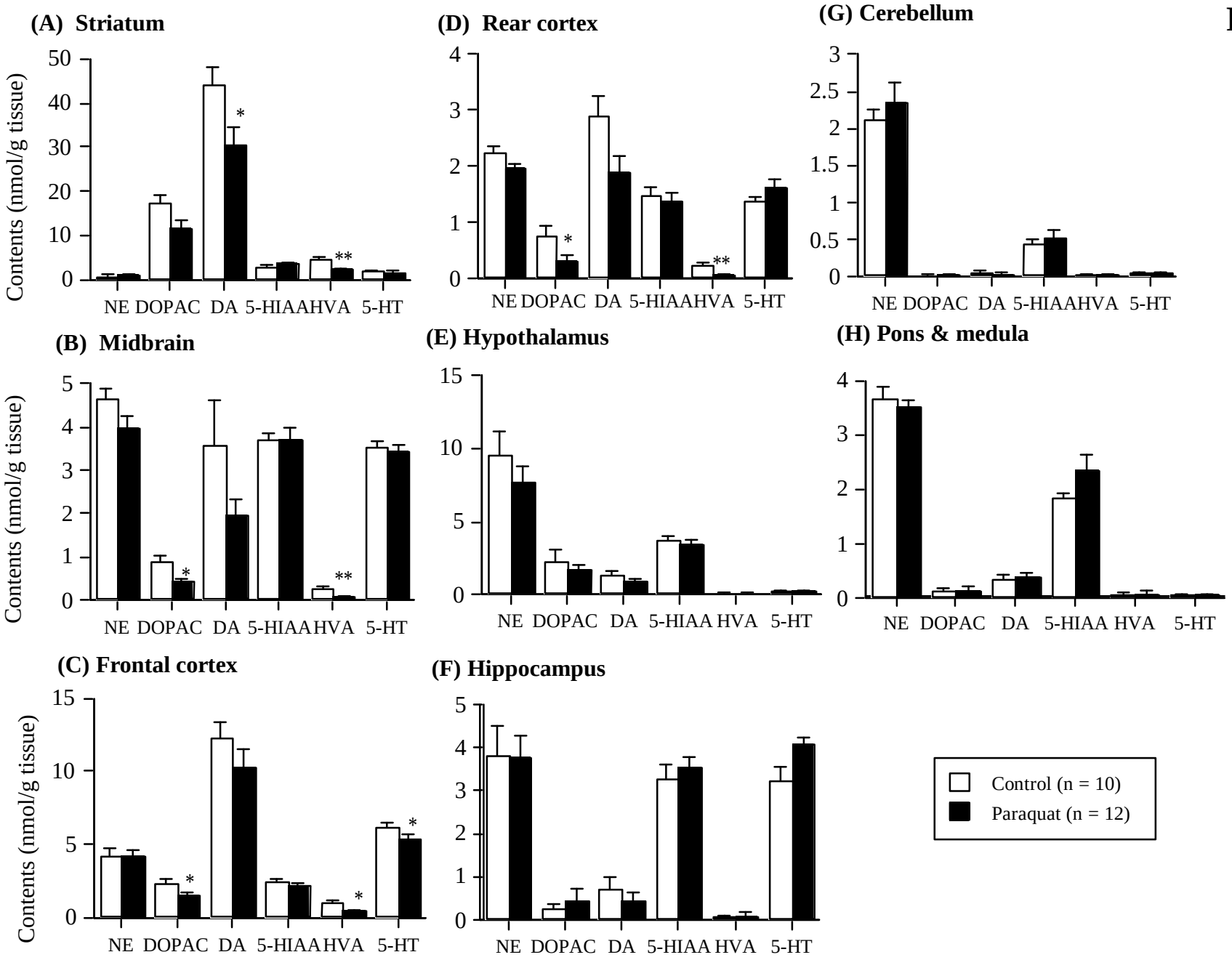

(A) Striatum
(B) Midbrain
(C) Frontal cortex
(D) Rear cortex
(E) Hypothalamus
(F) Hippocampus
(G) Cerebellum
(H) Pons & medula
Contents (nmol/g tissue)
NE DOPAC DA 5-HIAA HVA 5-HT
Control (n = 10)
Paraquat (n = 12)

FIG. 2

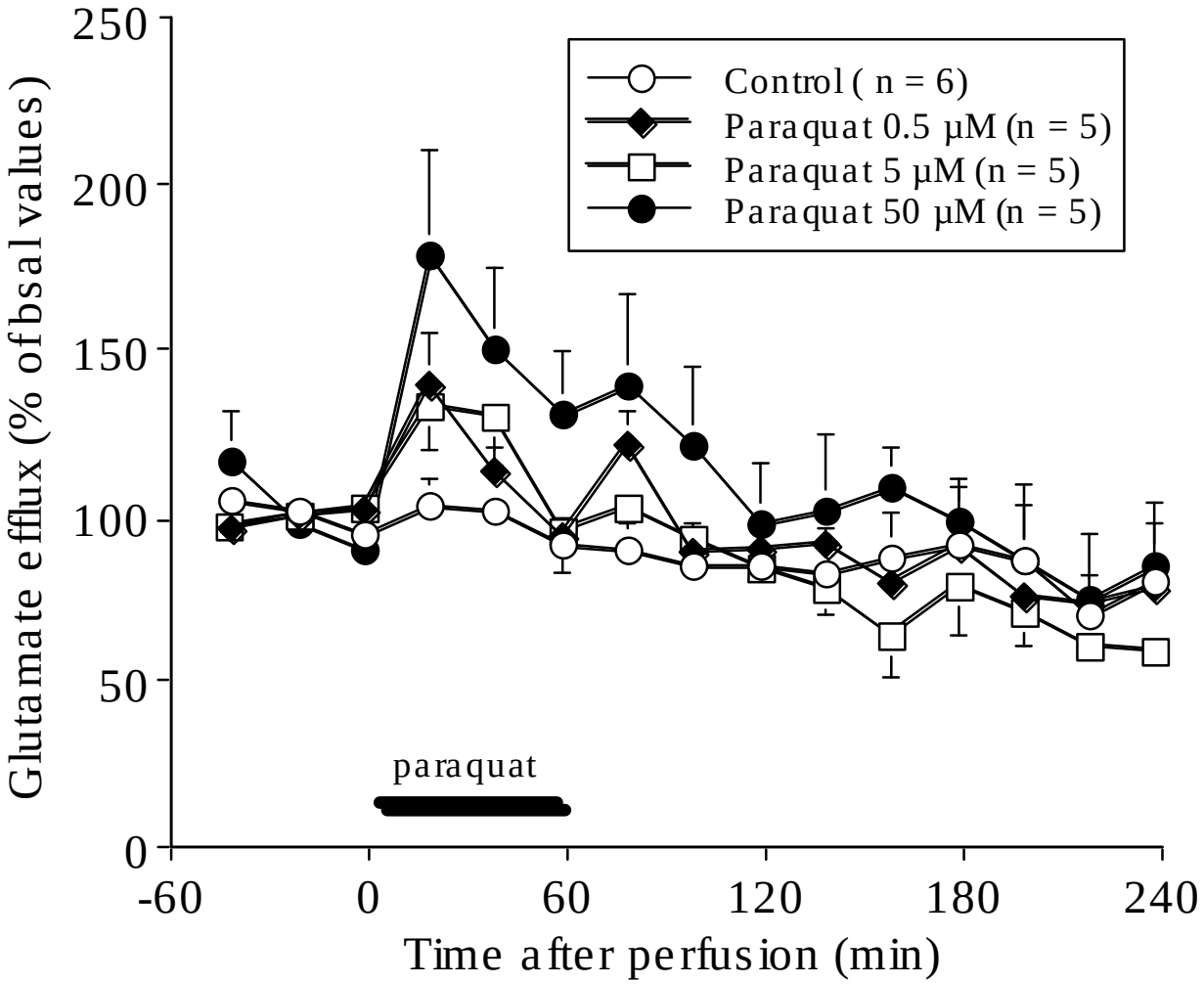

Control (n = 6)
Paraquat 0.5 µM (n = 5)
Paraquat 5 µM (n = 5)
Paraquat 50 µM (n = 5)
Glutamate efflux (% of bsal values)
250
200
150
100
50
0
paraquat
-60
0
60
120
180
240
Time after perfusion (min)

FIG. 3

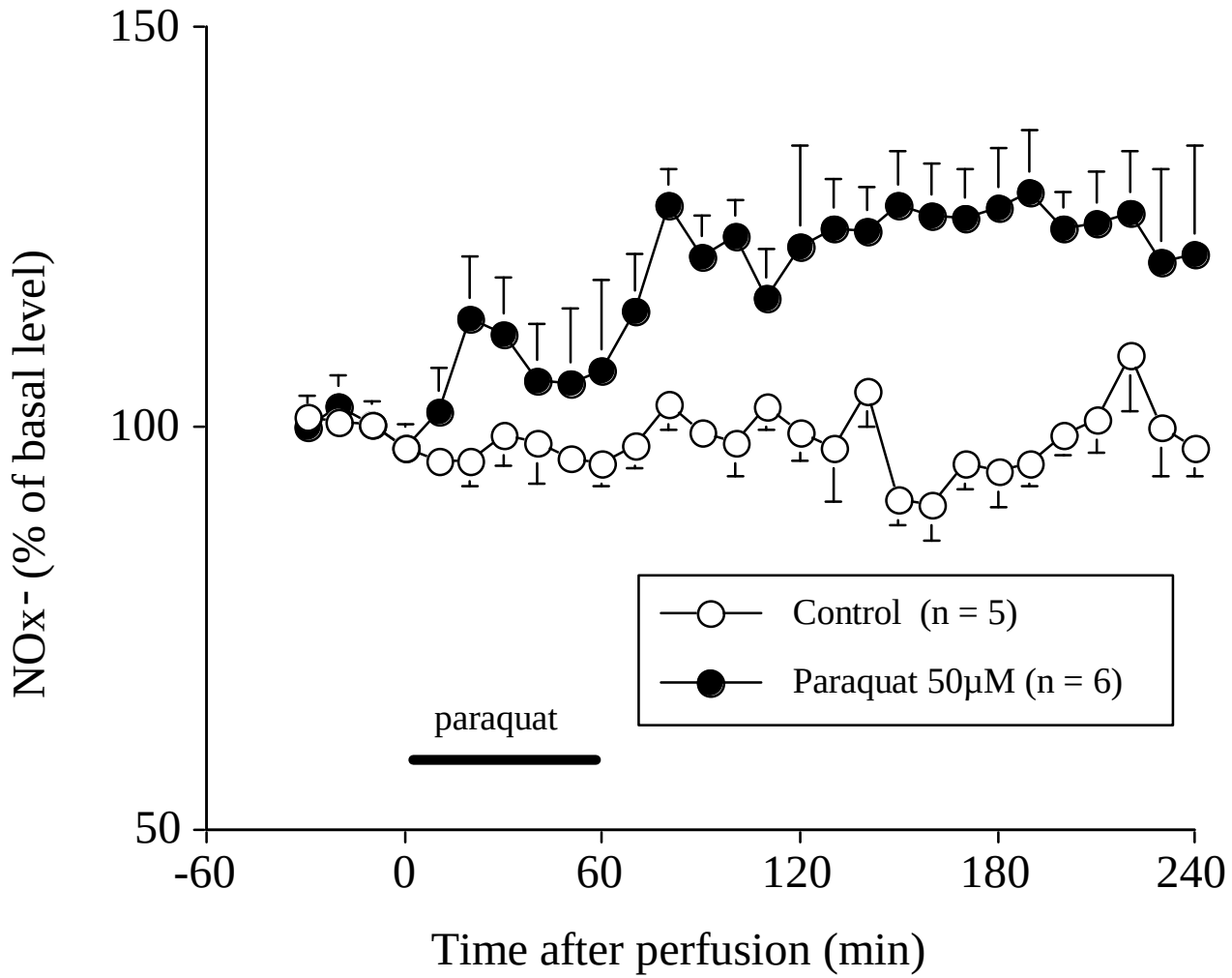

150
100
50
NOx- (% of basal level)
-60
0
60
120
180
240
Time after perfusion (min)
paraquat
Control (n = 5)
Paraquat 50µM (n = 6)

FIG. 4

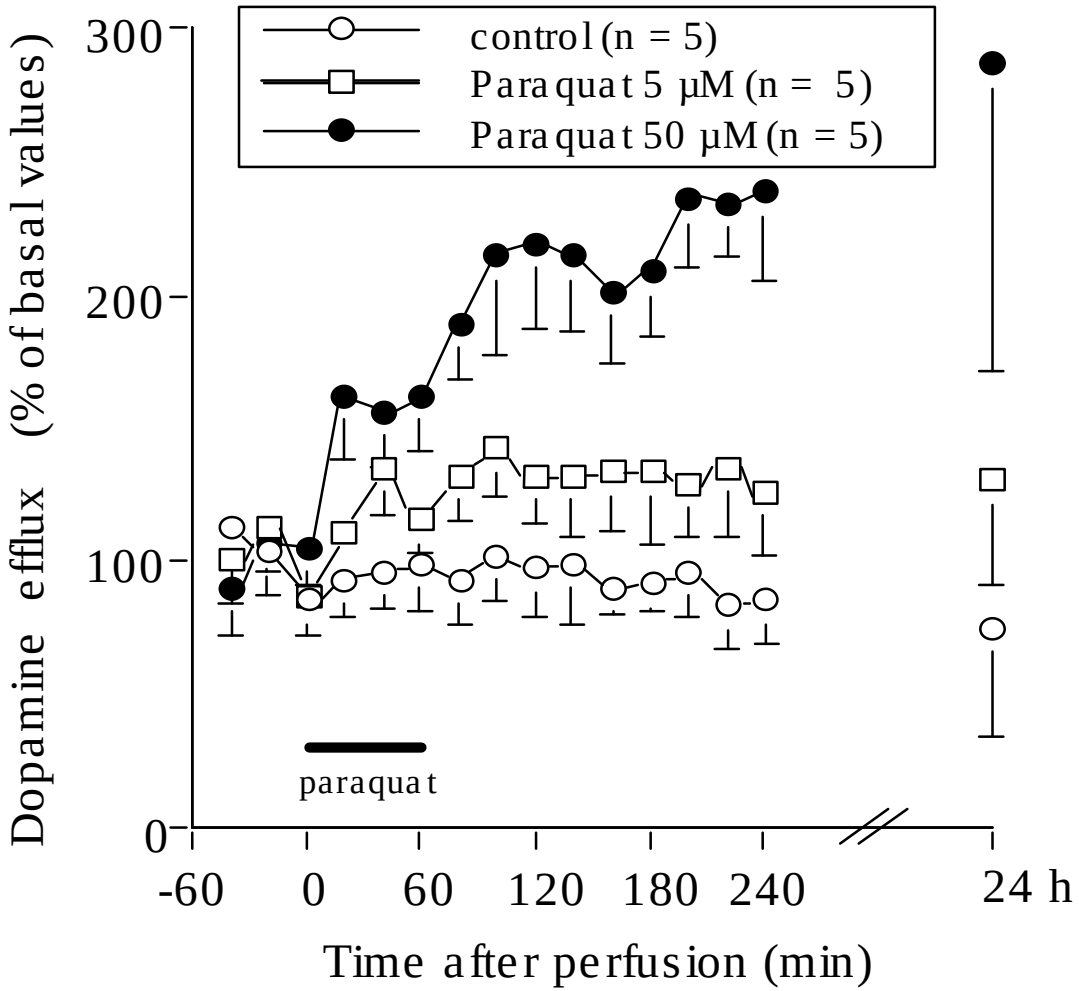

control (n = 5)
Paraquat 5 µM (n = 5)
Paraquat 50 µM (n = 5)
Dopamine efflux (% of basal values)
300
200
100
0
paraquat
-60
0
60
120
180
240
24 h
Time after perfusion (min)

FIG. 5

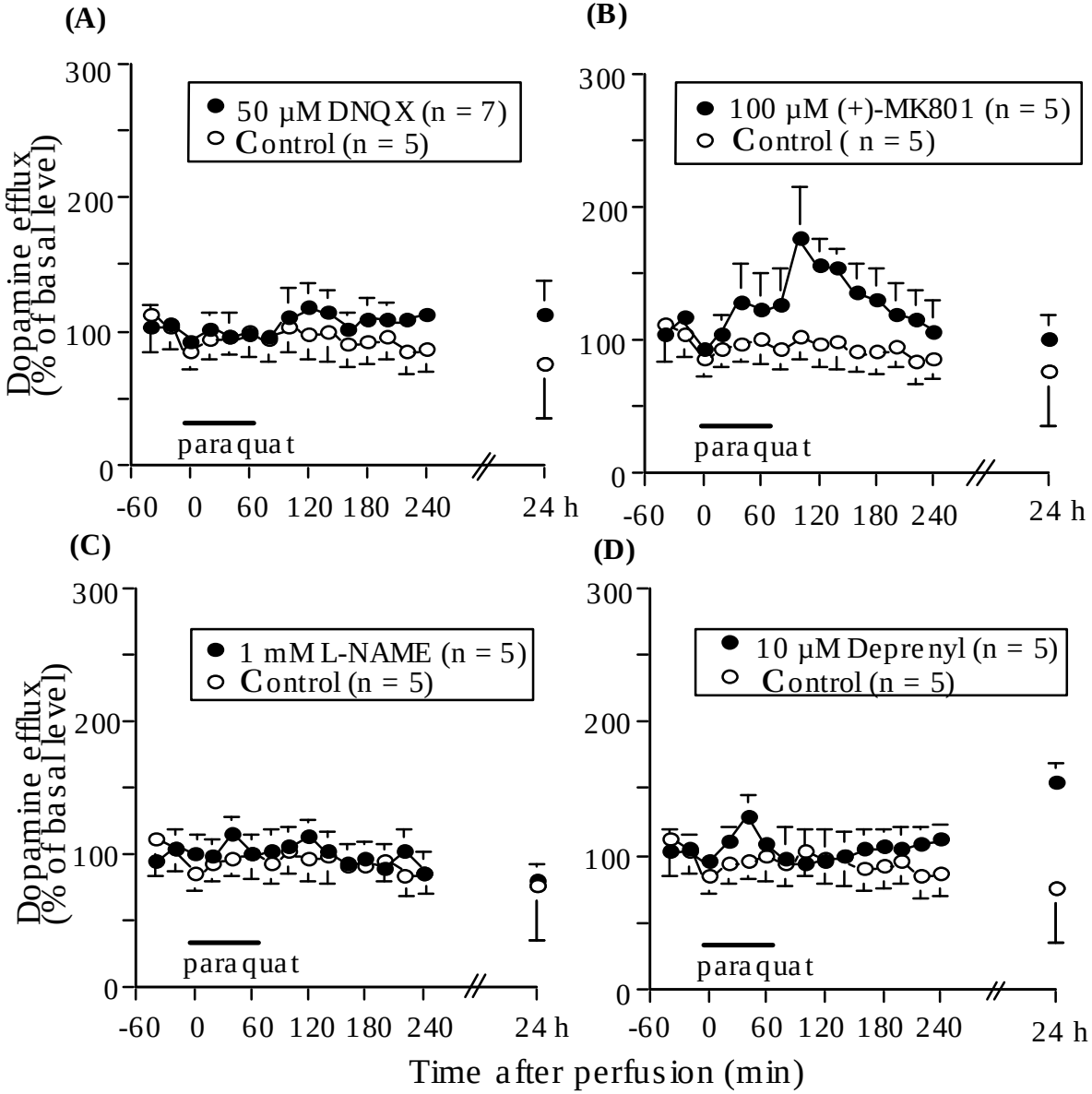
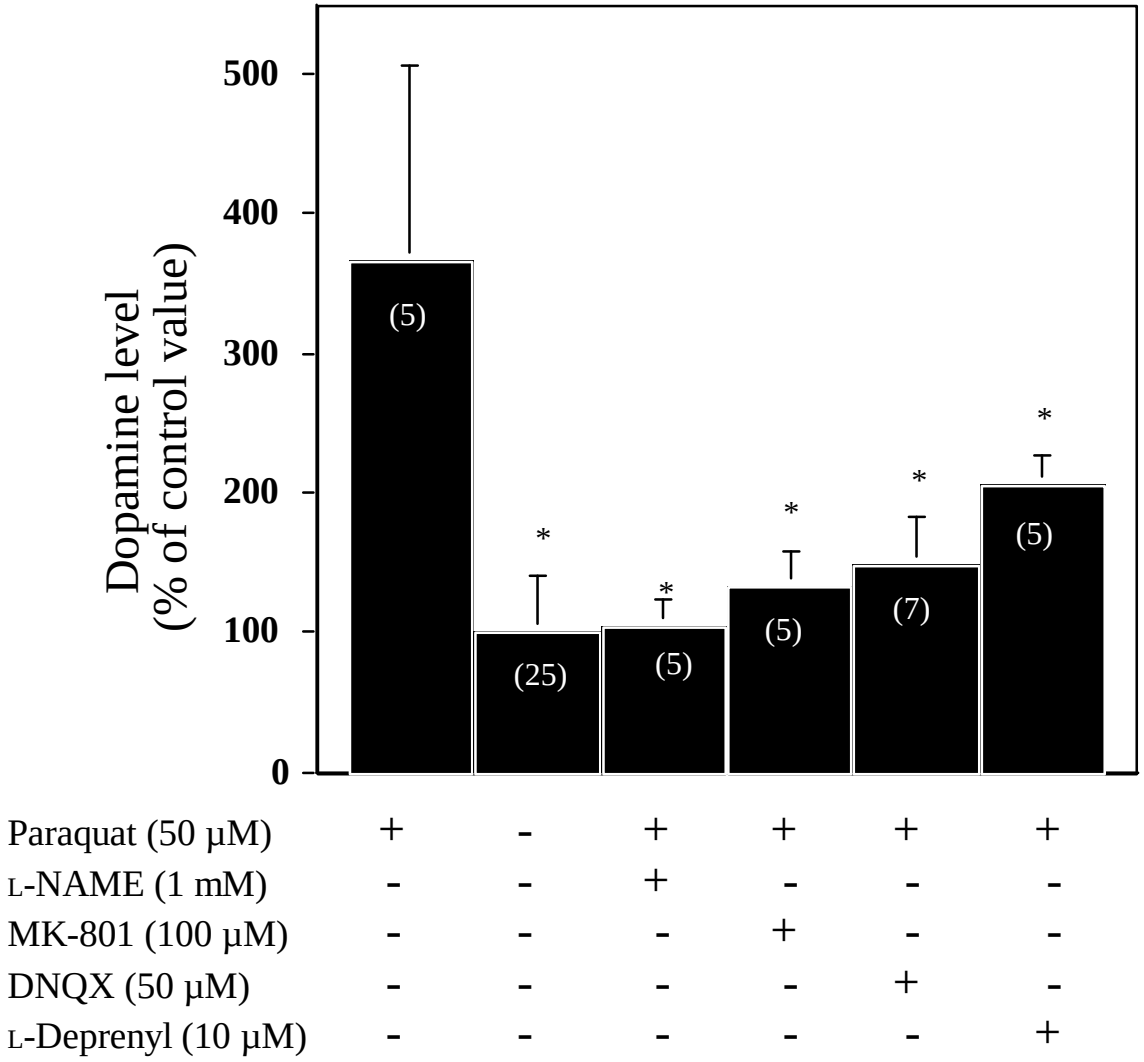

(A)
(B)
(C)
(D)
50 μM DNQX (n = 7)
Control (n = 5)
100 μM (+)-MK801 (n = 5)
Control (n = 5)
1 mM L-NAME (n = 5)
Control (n = 5)
10 μM Deprenyl (n = 5)
Control (n = 5)
paraquat
Dopamine efflux (% of basal level)
300
200
100
0
-60
0
60
120
180
240
24 h
Time after perfusion (min)

FIG. 6


Dopamine level
(% of control value)
500
400
300
200
100
0
(5)
(25)
(5)
(5)
(7)
(5)
*
*
*
*
*
Paraquat (50 μM) + - + + + +
L-NAME (1 mM) - - + - - -
MK-801 (100 μM) - - - + - -
DNQX (50 μM) - - - - + -
L-Deprenyl (10 μM) - - - - - +