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Blockade of $mGluR_5$ Reverses Abnormal Firing of Subthalamic Nucleus Neurons in 6-Hydroxydopamine Partially Lesioned Rats

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Abstract

Activation of metabotropic glutamate receptor 5 ($mGluR_5$) in the subthalamic nucleus (STN) results in burst-firing activity of STN neurons, which is similar to that observed in Parkinson's disease (PD). We examined the effects of chronic and systemic treatment with 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a selective $mGluR_5$ antagonist, in firing activity of STN neurons in partially lesioned rats by 6-hydroxydopamine (6-OHDA). In 6-OHDA-lesioned rats treated with vehicle, injection of 6-OHDA (4 μg) into the medial forebrain bundle produced a partial lesion causing 36% loss of tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the substantia nigra pars compacta (SNpc). The 6-OHDA lesion in vehicle-treated rats showed an increasing firing rate and a more irregular firing pattern of STN neurons. Whereas chronic, systemic treatment of MPEP (3 mg/kg/day, 14 days) produced neuroprotecive effects on the TH-ir neurons and normalized the hyperactive firing activity of STN neurons in 6-OHDA partially lesioned rats. These data demonstrate that partial lesion of the nigrostriatal pathway increases firing activity of STN neurons in the rat, and chronic, systemic MPEP treatment has the neuroprotective effect and reverses the abnormal firing activity of STN neurons, suggesting that MPEP has an important implication for the treatment of PD.

Key Words: MPEP, partial lesion, tyrosine hydroxylase, Parkinson's disease, electrophysiology

Introduction

It has been suggested that dopamine (DA) depletion and reactive increase in glutamatergic drive in the basal ganglia (BG) are primary for the expression of Parkinson's disease (PD) motor symptoms. Abnormal 'bursting' pattern of neuronal activity in the subthalamic nucleus (STN) is a crucial manifestation of glutamatergic systems hyperactivity (3). Electrophysiological studies have shown that activation of Group I metabotropic glutamate receptors (mGluRs) in the STN results in direct excitatory effects (1), activation of $mGluR_5$, specifically, on STN neurons increases their burst-firing activity which is similar to that observed in PD (1). Hyperactivity of STN might lead to the increased glutamatergic innervation of the substantia nigra pars compacta (SNpc) contributing to the degeneration of dopaminergic neurons through excitotoxicity (27). Therefore, reducing STN hyperactivity may produce neuroprotective effects in PD. Indeed, STN lesions have been shown to normalize firing rate and firing pattern of the substantia nigra pars reticulata (SNr) neurons in unilateral nigrostriatal tract lesioned rats by 6-hydroxydopamine (6-OHDA) (7, 30) and also shown to reduce the increased activation of the globus pallidus pars medialis and SNr by electrophysiological studies in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkeys (2). The $mGluR_5$ subtype is particularly expressed in the STN where it is located postsynaptically on glutamatergic

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output neurons and mediates direct excitatory effects on those neurons (1). Behavioral studies have also found that blockade of $mGluR_5$ in the STN without SNr or the entopeduncular nucleus (EP) improves motor asymmetry in 6-OHDA-lesioned rats (23). Metabolic studies have also suggested that the expression of the cytochrome oxidase mRNA, an index of neuronal activity, is increased in the STN and SNr of 6-OHDA-lesioned rats, which is prevented by a selective $mGluR_5$ antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) treatment (6). A recent work in our laboratory has also shown that chronic and systemic treatment with MPEP indeed produces neuroprotective effects on preventing loss of dopaminergic neurons and reverses the abnormal firing activity of dopaminergic neurons in the SNpc (9). We inferred that MPEP normalized the firing activity of dopaminergic neurons in the SNpc of partially lesioned rats mostly through blocking the $mGluR_5$ located in the STN. However, the action pathway underlying these neuroprotective effects produced by MPEP is unclear.

The aim of the present study was to verify the effect of MPEP on STN neurons in the medial forebrain bundle (MFB) lesioned model which mimics early stage of PD (31) by using immunocytochemical and electrophysiological methods. In particular, we examined changes in the firing rate and firing pattern of STN neurons after chronic treatment with MPEP.

Materials and Methods

Animals

Male Sprague-Dawley rats, weighing 230-250 g, were used in this study. Rats were kept under artificial conditions of light and temperature with food and water available *ad libitum*. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and according to the guideline of the Institutional Animal Care Committee of the University. All efforts were made to minimize the number of animals used and their suffering.

Chemicals

The drugs used in this study were desipramine hydrochloride, 6-OHDA hydrochloride, MPEP hydrochloride and apomorphine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA). Desipramine was prepared in saline; 6-OHDA and apomorphine were prepared in saline containing 0.01% ascorbic acid and MPEP was prepared in sterile water. These drugs were prepared on the day of the experiment.

Surgery and Drug Treatment

Rats were divided into three groups: (i) 6-OHDA-lesion+MPEP treated group (n = 6), unilaterally 6-OHDA injection in the MFB and chronic, systemic injection of MPEP; (ii) 6-OHDA lesion+vehicle-treated group (n = 8), saline treatment instead of MPEP; (iii) sham-operated group (n = 8), saline injection instead of 6-OHDA in the MFB. Unilateral 6-OHDA lesion was carried out as previously described (9). Briefly, rats were anesthetized with 4% chloral hydrate (300 mg/kg, i.p.), pretreated with desipramine (25 mg/kg, i.p.) to prevent the noradrenergic neurons from 6-OHDA toxicity, mounted in a stereotaxic instrument and injected with 6-OHDA (4 μg/4 μl) into the right MFB according to the following stereotaxic coordinates: AP 3.6-3.8 mm posterior to bregma, L 1.1-1.3 mm from the midline, D 8.2-8.4 mm from the dura. The injection was made at a rate of 0.5 μl/min using a 5 μl Hamilton microsyringe. After each injection, the micropipette was left in place for an additional 5 min and then slowly withdrawn. In the same manner, control rats received an injection of 4 μl of saline containing 0.01% ascorbic acid into the right MFB. The micropipette was remained in place for an additional 5 min following the injection and then slowly withdrawn. After the surgery, MPEP-treated rats received daily single injections of MPEP (3.0 mg/kg, i.p.) for fourteen consecutive days. The vehicle-treated rats received an equal volume of saline. In treatment groups, the initial injection of saline or MPEP was administrated 1 h after 6-OHDA.

Electrophysiological Recording

The electrophysiological recording of neurons in the STN was performed 2 weeks after surgery. Extracellular, single unit recordings were undertaken in rats anesthetized with 4% chloral hydrate (300 mg/kg, i.p.) and mounted on a stereotaxic instrument as described previously (15). Body temperature was maintained at 37 ± 0.5°C and heart rate was monitored throughout the experiment. Glass microelectrodes (10-20 MΩ) filled with 1% Pontamine Sky Blue in 0.5 M sodium acetate were directed stereotaxically to the right STN (AP 3.6-3.8 mm posterior to bregma, L 2.4-2.8 mm from the midline, D 6.5-8.5 mm from the dura). Electrical signals were amplified, bandpass-filtered and displayed on an oscilloscope and audio monitor. The data were collected on a computer equipped with Spike 2 analysis system (Cambridge Electronic Design, Cambridge CB4 0FE, England) for off-line analysis. The isolated units were monitored for at least 5 min to reach the stable firing activity, and then 2-5 min of spontaneous firing activity was

recorded. The analysis of the firing patterns of each STN neuron was processed by the firing rate and mean interspike interval (ISI) coefficient of variation associated with 1,000 consecutive spikes events (15). According to the ISI histogram (ISIH), the mean ISI coefficient of variation and spike trains, firing patterns were classified into the following types: (i) regular firing, characterized by a nearly symmetrical distribution of the ISIH; (ii) irregular firing, characterized by an asymmetrical distribution of the ISIH; (iii) bursting firing pattern with ISIH exhibiting an obviously positive skewness with a long progressive decline (10).

Histology and Immunocytochemistry

At the end of extracellular single unit recording the recording site was marked by the ejection of Pontamine Sky Blue (-20 μA, 15 min). The rat was given an overdose of urethane and perfused with saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS). The brain was immediately removed to post-fix in the same fixative for 4 h and then was placed in PBS with 20% sucrose overnight. Thirty micrometer sections were cut using a cryotome. Cresyl violet staining and immunocytochemical staining of tyrosine hydroxylase (TH) were used to determine the location of the recording sites and the extent of SNpc dopaminergic degeneration, respectively.

Free-floating TH immunohistochemistry was carried out as previously described (20). Briefly, sections were preincubated with 3% bovine serum albumin in PBS containing 0.3% Triton X-100 for 30 min at room temperature and then incubated at 4°C for 48 h with anti-TH polyclonal antibodies (1:800; Chemicon, Temecula, CA, USA). Next, sections were incubated for 2 h with biotinylated anti-rabbit IgG (1:200; Chemicon) and incubated for 2 h with the avidin-biotin-peroxidase complex (1:200; Vector Laboratories, Burlingame, CA, USA) at room temperature. Finally they were exposed for 10-15 min at room temperature to a solution of 0.05% 3,3'-diaminobemidine (Sigma-Aldrich) containing 0.01% H_2O_2 which served as a chromogen in the subsequent visualization reaction. Procedures of rinsing sections were performed between each step excluding the blocking solution step and the addition of the primary antibody. Sections were rinsed, mounted onto gelatin-coated slides, dehydrated and cleared in xylene and coverslipped.

After TH staining, counting of TH immunoreactive (TH-ir) neuron bodies in the SNpc was carried out on representative 3 sections per animal. A neuron was considered when intact and round with clear nucleus and/or cytoplasm. The number of TH-ir neurons was expressed as the average of the counts obtained from the representative 3 sections. The full extent of the structure in each section was examined in all groups. Only sections in which the medial and lateral parts of the SNpc and ventral tegmental area were clearly separated by the medial terminal nucleus of the accessory optic tract level were selected for analysis of TH-ir neuron number. This approach has been used by others to ensure that comparable rostrocaudal levels of the SNpc are sampled between animals (9). Only rats with a moderate (< 50%) loss of TH-ir neurons were used to analyze the TH immunocytochemical and electrophysiological data.

Statistical Analysis

The number of TH-ir neurons in lesioned side of each slice (a) was compared with that of intact side (b) to obtain a percentage of TH-ir loss ($[(b\text{-}a)/b] \times 100\%$). Comparisons of the TH-ir neuron lost and the firing rates of STN neurons between groups were statistically evaluated using one-way ANOVA. *Post-hoc* multiple comparisons were made using the Dunnett's test as appropriate. The mean ISI coefficient of variation was analyzed using the non-parametric analysis Mann-Whitney U test. The proportions of different firing patterns between different groups were compared using the χ^2 test. All data were expressed as means ± SEM, statistical analyses were performed using SPSS 13.0 for Windows and the level of significance was determined as $P < 0.05$.

Results

TH Immunocytochemistry Confirmed Partial Lesion

In sham-operated, vehicle-treated and MPEP-treated rats, the percentages of TH-ir neurons surviving in the SNpc on the injected side were 85.4 ± 1.7%, 63.7 ± 2.1% and 81.7 ± 2.6%, respectively, compared with the normal side. In 6-OHDA-lesioned rats treated with vehicle, the percentage of TH-ir neuron loss was significantly higher than that of sham-operated rats ($P < 0.01$, n = 8; Dunnett's test; Fig. 1, A, B and D), similar to a partial lesion of the nigrostriatal pathway as seen in patients with preclinical PD. In 6-OHDA-lesioned rats treated with MPEP, the percentage of TH-ir neuron loss was not different compared with sham-operated rats (n = 6; Fig. 1, A, C and D) indicating that the administrated MPEP had a neuroprotective action *in vivo* against 6-OHDA toxicity.

Firing Properties of STN Neurons

All STN neurons recorded in sham-operated,

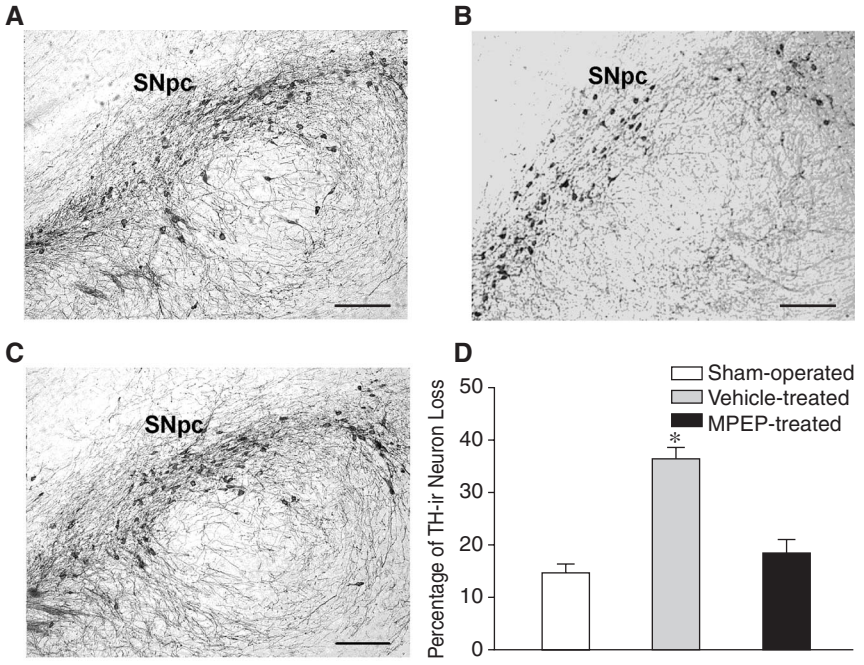

Fig. 1. Representative photomicrographs of TH immunostaining showing the injected side of the SNpc in sham-operated rats (A), 6-OHDA lesion with vehicle-treated rats (B) and 6-OHDA lesion with MPEP-treated rats (C). Histogram showing the number of TH-ir neurons loss in the SNpc (D) of sham-operated (n = 8), vehicle-treated (n = 8) and MPEP-treated rats (n = 6). Scale bars, A-C = 100 μm. *$P < 0.01$, in comparison with the values of sham-operated rats.

vehicle-treated and MPEP-treated rats exhibited a biphasic extracellular waveforms (17; Fig. 2 C), and the recorded neurons location was histologically confirmed from the STN.

In sham-operated rats, the firing rate of STN neurons ranged from 7.2 to 14.8 spikes/s with a mean of 9.5 ± 0.3 spikes/s (n = 51; Fig. 2A) and the mean ISI coefficient of variation had a mean value of 0.42 ± 0.03 (Fig. 2B). A majority of STN neurons (82%) exhibited a regular firing pattern, only 14% and 4% of the neurons displayed irregular and burst firing pattern, respectively (Fig. 2D). In partially lesioned rats treated with vehicle, the firing rate of STN neurons varied from 4.1 to 18.3 spikes/s; the mean firing rate increased significantly to 12.3 ± 0.6 spikes/s compared with the sham-operated rats ($P < 0.01$, n = 47; Dunnett's test; Fig. 2A). The mean ISI coefficient of variation significantly increased to 0.66 ± 0.05 ($P < 0.001$; Mann-Whitney *U* test; Fig. 2B). The percentage of STN neurons displaying regular firing pattern decreased to 59%, but those irregular and burst firing neurons increased to 26% and 15%, respectively ($P < 0.01$; χ^2 test; Fig. 2D). These data show that 6-OHDA lesion led to a more irregular firing pattern of STN neurons than sham-operated rats. In 6-OHDA-lesioned rats treated with MPEP, STN neurons had a firing rate ranging from 3.4 to 14.6 spikes/s, the mean firing rate decreased slightly to 8.7 ± 0.5 spikes/s, but not significantly compared with sham-operated rats (n = 42; Fig. 2A), the mean ISI coefficient of variation was 0.49 ± 0.03, similar to the sham-operated rats (Fig. 2B). The percentages of neurons exhibiting regular, irregular and burst firing pattern were 79%, 14% and 7% (Fig. 2D), respectively, indicating that the firing rate of STN neurons and degree of regularity of the neuronal firing were normalized after MPEP treatment.

Discussion

The main results of the present study show that: (i) injection of 6-OHDA into the MFB produced a small loss of dopaminergic neurons in the SNpc (36%), which is similar to that by Truong *et al*. (2006) who showed an average loss of 35% of dopaminergic neurons in the SNpc of the lesioned rats (29). Therefore, this model allowed us to test the efficacy of the treatment with $mGluR_5$ antagonist MPEP; (ii) chronic and systemic treatment with MPEP reversed the abnormal firing activity of STN neurons induced by partial lesion with 6-OHDA.

In this study, 6-OHDA injection into the MFB increased the firing rate and produced a more irregular firing pattern of STN neurons, consistent with previous studies that the mean firing rate of STN neurons and amount of random and burst firing neurons showed increasing events in rats with 6-OHDA lesions of the SNpc or MFB *in vivo* (5, 17). Metabolic studies also support the concept of hyperactivity in the STN in

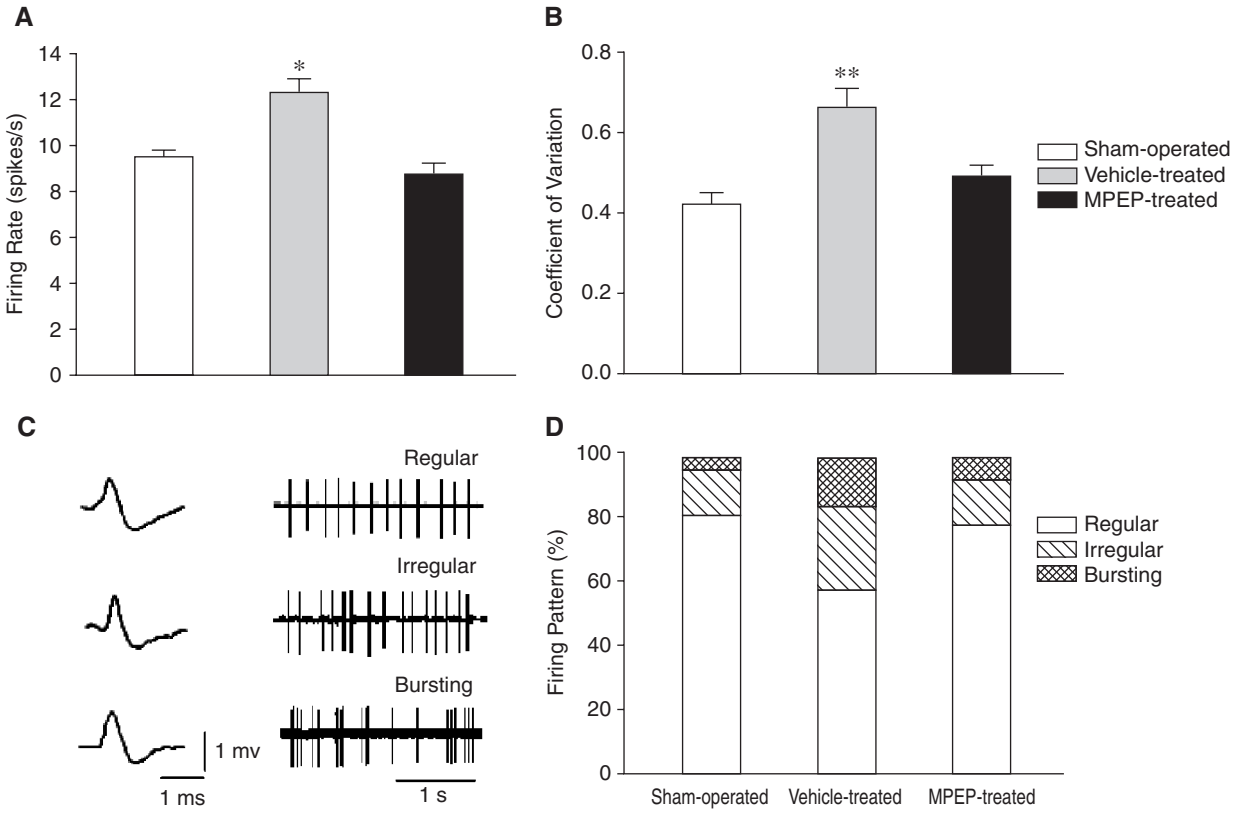

Fig. 2. Histograms showing the effect of MPEP treatment on firing activity of STN neurons. Shown in the figure are the firing rate (A), mean ISI coefficient of variation (B), the representative sample of the spontaneous activity showing the extracellular action potential waveform and pattern of regular, irregular and burst-firing (C), and the distribution of firing patterns (D) of STN neurons recorded in sham-operated (n = 51), vehicle-treated (n = 47) and MPEP-treated (n = 42) rats. *$P < 0.01$, **$P < 0.001$ in comparison with the values in sham-operated rats.

parkinsonian patients and in animal models of parkinsonism (22). In contrast, our results do not reproduce those of other studies which showed that the mean firing rate of STN neurons was decreased in rats with 6-OHDA lesions than in normal animals (21, 31). One factor may underline this discrepancy is that the experimental method may be important for the results of electrophysiological recording. Recording the firing activity of STN neurons in slice eliminated the innervation of input area to the STN and weakened the signal integration in the STN. The pedunculopontine nucleus (PPN) is a mesopontine structure in the basal ganglia which plays an important role in motor control. PPN receives excitatory inputs from the STN (13) and sends excitatory signals towards several regions, including the SNpc (8), STN (13), SNr (25) and others. The hyperactivity of the STN-induced hyperactivity of the basal ganglia output structures, which is considered a characteristic of parkinsonism, the STN and PPN were reciprocally connected by excitatory projections and both structures were shown to be hyperactive in PD animal models (5). Both polysynaptic pathways originating in the excitatory projection from the PPN to the SNpc (8) and propagating through the indirect basal ganglia pathway and the monosynaptic PPN-STN excitatory projection are responsible for the effect of PPN on the STN neurons firing activity (13). Modulation of STN firing rate by the PPN depends on the state of the nigro-striatal pathway, and PPN modulates the activity of the STN predominantly through the polysynaptic indirect pathway under the normal nigro-striatal projection, whereas the direct monosynaptic projection from the PPN to the STN becomes the main factor when the nigro-striatal pathway was impaired (5). Hence, the increased PPN neuronal activity (14) makes the STN neurons more active in the 6-OHDA rat model (4). On the other hand, GABAergic neurons of the globus pallidus (GP) also influences the response of STN neurons by a feedback inhibition from the GP to the STN (16) and a dis-inhibitory mechanism involving corticostriatal and striatopallidal pathways (28). Loss of DA in the striatum reduces the activity of the inhibitory GABAergic pallido-subthalamic pathway (19) and consequently decreases the activity of GP neurons in the lesioned animal (11), which might lead to an increased firing rate of the STN neurons in the DA-depleted state (17). Moreover, the

activity of STN neurons was enhanced after lesion of the external GP (26). As mentioned above, the firing activity of STN neurons modulated by the PPN and GP, increased PPN activity and decreased GP control on the STN resulting in an increase of firing rate of STN neurons and more irregular firing pattern in nigro-striatal lesioned rats. However, both *in vitro* studies with slices (31) and *in vivo* method by intra-subthalamic injection of 6-OHDA (21) disconnected the pathway between STN and projection regions including the PPN and GP, which might lead to the contrary results in 6-OHDA lesioned rats. In our results, the firing rate and firing pattern of the STN neurons were observed *in vivo*, and partial nigro-striatal lesion was realized by 6-OHDA injection on the MFB, which remained the intact pathway of the STN. Therefore, our study showed that STN neurons displayed an increased firing rate and a more irregular firing pattern in 6-OHDA lesion with vehicle-treated rats than that in sham-operated rats, consistent with previous studies (5, 17).

Both *in situ* hybridization and immunohistochemical studies have shown that mRNA and protein for $mGluR_5$ subtype are significantly expressed in several structures within the basal ganglia, particularly in the STN and its output targets, including caudate, SNpc, SNr, GP and EP (18). More and more evidence have shown that the STN is an important site of action for the beneficial effects of $mGluR_5$ antagonist in a rat model of PD (6, 23), and the activation of $mGluR_5$ receptors on STN neurons increases their burst firing activity (1). MPEP, a selective non-competitive $mGluR_5$ antagonist (12), produces anti-parkinsonian effects in animal models (6). Systemically administered MPEP was shown to normalize the firing rate of dopaminergic neurons in the SNpc (9) and to reverse the overactive neuronal metabolic activity in the STN induced by partial DA depletion lesions (6). Because selective activation of $mGluR_5$ produces a direct excitation of STN neurons (1), several reports have suggested that the STN is a preferential target of MPEP treatment regardless of whether MPEP acts directly on STN neurons or indirectly through its afferents (6). A study by Phillips *et al*. supports the selective action of MPEP on the STN showing that intracerebral injection of MPEP directly in the STN was able to alleviate the motor deficit by a unilateral DA denervation (23). Furthermore, loss of DA in the striatum decreases the activity of GP neurons and consequently increases the firing rate of STN neurons by the inhibitory GABAergic pallido-subthalamic pathway (19). It is well documented that application of MPEP may enhance the activity of GP neurons though strengthening of the $mGluR_1$-mediated depolarization in the GP (24) and MPEP also inhibits mGluR agonist 3,4-dihydroxyphenylglycol-induced depolarization in the STN in parkinsonism (1). Our results showed that the firing rate and firing pattern of STN neurons in 6-OHDA lesion with MPEP-treated rats was not statistically different compared with the sham-operated rats. We infer that MPEP reverses the firing activity of STN neurons through direct blockade $mGluR_5$ located on STN neurons, or through blockade $mGluR_5$ expressed on the GP neurons to recover the inhibitory modulation of GP to the STN, and ultimately normalize the firing activity of STN neurons.

In summary, this study indicates that chronic and systemic treatment with MPEP produces significant neuroprotective effects in partially lesioned rats. It also provides the cellular evidence that a partial, unilateral 6-OHDA lesion of the nigrostriatal pathway induces an increase in firing rate of STN neurons of rats, and the firing activity of the neurons becomes more irregular, suggesting that excitatory PPN-STN connection and inhibitory GABAergic pallido-subthalamic pathway may play an important role in the modulation of the firing activity of STN neurons, and effects of MPEP normalizing the firing activity of STN neurons suggest that the $mGluR_5$ blockade on STN or GP neurons may be crucial in the functional neuroprotective effects in the lesioned rats. These findings further support the potential of $mGluR_5$ antagonist, such as MPEP, as a promising anti-parkinsonian strategy.

Acknowledgments

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