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Cortical glutamate levels decrease in a non-human primate model of dopamine deficiency

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Abstract

While Parkinson's disease is the result of dopaminergic dysfunction of the nigrostriatal system, the clinical manifestations of Parkinson's disease are brought about by alterations in multiple neural components, including cortical areas. We examined how 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration affected extracellular cortical glutamate levels by comparing glutamate levels in normal and MPTP-lesioned nonhuman primates (*Macaca mulatta*). Extracellular glutamate levels were measured using glutamate microelectrode biosensors. Unilateral MPTP-administration rendered the animals with hemiparkinsonian symptoms, including dopaminergic deficiencies in the substantia nigra and the premotor and motor cortices, and with statistically significant decreases in basal glutamate levels in the primary motor cortex on the side ipsilateral to the MPTP-lesion. These results suggest that the functional changes of the glutamatergic system, especially in the motor cortex, in models of Parkinson's disease could provide important insights into the mechanisms of this disease.

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1. Introduction

In idiopathic Parkinson's disease (PD), motor deficits are believed to be a result of dopamine deficiency in the motor circuit, comprised of the supplementary motor area, parts of the premotor cortex and the primary motor cortex (Alexander et al., 1990; Parent and Hazrati, 1995; Wichmann and DeLong, 1993; Wichmann and DeLong, 2003; Wichmann and DeLong, 2008). The cortical changes in these regions are considered to be some of the hallmark symptoms of neurodegenerative diseases including PD (Kuninobu et al., 1993; Lefaucheur, 2005; Sabatini et al., 2000). In PD, dopaminergic neurons in the substantia nigra undergo a loss of function that indirectly results in diminished activity through the basal ganglia-thalamo-cortical pathway that leads to a subsequent decrease in glutamatergic output from the motor cortex (Wichmann and DeLong, 1996).

Motor symptoms of PD are largely attributed to the imbalance of inhibitory and excitatory processes in the motor cortex and subcortical neuronal circuits after a nigrostriatal dopamine deficit (Ridding et al., 1995). Because glutamate is the principal excitatory neurotransmitter in the CNS, understanding the function of glutamate neurotransmission may provide important insight into understanding PD. Brain glutamate acts through ionotropic (NMDA, kainite and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, AMPA) receptors and G-protein-coupled metabotropic receptor subtypes (Bai et al., 2004; Hof et al., 2002). In the cerebral cortex, about 80% of neurons are glutamatergic pyramidal neurons and the remaining 20% of neurons are GABAergic nonpyramidal interneurons with axons confined to the cortex. Thus, all outputs of the cerebral cortex arise from glutamatergic pyramidal neurons located in a specific arrangement of layers and project to defined targets. Corticostriatal glutamatergic afferents and mesostriatal dopaminergic afferents commonly converge onto the same postsynaptic spines of medium projection neurons in which the vast majority of striatal neurons are GABAergic. Thus, this synaptic triad may be a crucial element in the striatum's regulation of glutamatergic cortical information flow (Bamford et al., 2004).

In PD, dopamine depletion leads to a complex cascade of events that produce an excessive inhibition of the thalamo-cortical glutamatergic pathway. Glutamatergic mechanisms are critically involved in determining both dendritic spine development and maintenance (Bloodgood and Sabatini, 2008; Korkotian and Segal, 2001; Lippman and Dunaevsky, 2005; McKinney, 2005; Passafaro et al., 2003). Presently, only a few studies have directly examined resting glutamate levels in a living nonhuman primate's brain, especially in one showing parkinsonian symptoms. To determine if cortical basal glutamate levels could be altered by dopaminergic dysfunction (MPTP-induced lesions), we employed electrochemical detection by using amperometry in conjunction with enzyme-based microelectrodes to characterize extracellular levels of glutamate in hemiparkinsonian monkeys.

2. Results

2.1. Verifying dopaminergic depletion

In addition to behavioral assessment with the nonhuman primate Parkinsonian rating scale, we used post-mortem analyses to verify the effectiveness of the MPTP lesion in depleting

dopaminergic neurons from the nigral-striatal pathway. After staining sections of the substantia nigra for tyrosine hydroxylase (TH), the enzyme that converts the dopamine precursor L-DOPA to dopamine, we identified 85% fewer TH positive (TH⁺) dopamine neurons in the MPTP-lesioned substantia nigra ($27,950 \pm 3,722$ TH⁺ cells) versus the unlesioned side ($186,877 \pm 2,472$ TH⁺ cells; $t(5) = 31.6$, $p < 0.0001$). Meanwhile, to determine the effect of dopamine denervation at the cortical level, we assessed the TH⁺ fiber density and found significantly lower levels in the MPTP-lesioned side than the unlesioned side (Fig. 1; $F_{(1,10)} = 17.68$; $p = 0.0018$). Sidak Multiple-comparison tests revealed significant less TH⁺ fiber density in the MPTP-lesioned side premotor cortex (1.4 ± 0.11 % of total fields) than the contralateral side (1.9 ± 0.14 ; $p < 0.05$). We found similar reductions in the motor cortex (1.3 ± 0.29 MPTP-lesioned side vs. 1.8 ± 0.32 contralateral side; $p < 0.05$). These results confirm that the MPTP lesion had produced a characteristic dopamine depletion of the nigrostriatal pathway.

2.2 Basal glutamate levels in the primary motor cortex

We measured basal glutamate levels in the motor cortex using glutamate biosensors (Fig. 2). Basal glutamate levels in the motor cortex on the side ipsilateral to MPTP administration were significantly lower than on the contralateral side (Fig. 3A). The average basal glutamate levels were 23% lower in the motor cortex on the lesioned side (6.3 ± 2.7 μ M) than in the same regions on the unlesioned side (8.1 ± 2.8 μ M; $t(5) = 2.97$, $p = 0.031$, [NHP #1: 1.0 μ M vs. 1.5 μ M, NHP #2: 5.0 μ M vs. 1.9 μ M, NHP #3: 8.9 μ M vs. 5.1 μ M, NHP #4: 20.6 μ M vs. 18.6 μ M, NHP #5: 9.8 μ M vs. 8.4 μ M, NHP #6: 3.2 μ M vs. 2.0 μ M, unlesioned vs. MPTP-lesioned side, respectively]). Next, when we examined glutamate levels from individual recording coordinates in the motor cortex, we observed that, again, glutamate levels were lower on the MPTP-lesioned side than in the unlesioned side, but not different from each other from medial to lateral (Fig. 3B, two-way repeated measures ANOVA, main effect of the lesion $F(1,4) = 23.33$, $p = 0.0085$, no main effect of the coordinate location $F(4,16) = 0.86$, $p = 0.50$ or interaction $F(4, 16) = 0.3545$, $p = 0.84$). When we compared basal glutamate levels in MPTP lesioned animals to basal glutamate levels in control animals, we found that administration of MPTP resulted in a non-significant reduction of basal glutamate levels (6.3 ± 2.7 μ M, $N=6$) in the cortex compared with normal animals (12.5 ± 5.0 μ M; $N=8$, $p = 0.34$, Fig. 4A). Finally, slightly higher basal glutamate levels were observed in the same-side hemisphere of normal age-matched controls than in the hemisphere contralateral to MPTP administration; however, the difference was not significant ($p=0.50$) (Fig. 4B).

3. Discussion

We found that pre-motor and primary motor cortical glutamate levels could be altered by MPTP-induced dopaminergic deficiency in hemiparkinsonian monkeys. The observed reduction in glutamate levels in this model helps provide a better understanding of these cortical changes that occur during dopamine deficiencies of the nigrostriatal system.

Since the discovery that MPTP is toxic to dopaminergic neurons in the substantia nigra pars compacta in humans (Langston et al., 1983), MPTP has been used to produce reliable

models of parkinsonism in nonhuman primates, which in turn are used to examine the functional consequences of dopamine loss in the basal ganglia. Studies in monkeys rendered parkinsonian by MPTP administration have greatly helped us to understand the pathophysiology of PD, and to develop new therapies directed at the motor dysfunction in parkinsonism (for reviews, see Petzinger et al., 2008; Schneider et al., 2008; Zhang and Gash, 2008). In this study, we used animals that had received an MPTP lesion approximately five years before we measured glutamate levels. Based on our previous studies (Ding et al., 2008), the MPTP lesion produces a stable dopamine deficiency model both at the cellular and behavioral levels. In addition, before recording glutamate levels, we confirmed the motor deficits of the animals while also verifying the TH⁺ cell loss in the substantia nigra post-mortem. Thus, we think the condition of the animal at the time of the glutamate recordings accurately reflects a model of dopamine deficiency.

The reduction in ipsilesional-side cortical levels of resting glutamate by MPTP administration support the theory that the deficiency of dopamine in the nigrostriatal system could lead to a complex cascade of events and eventually reduced neuronal activity in the motor cortex, which could be the result of inhibitory effects on the thalamo-cortical glutamate pathway. However, we did observe a change in TH⁺-fiber innervation of the pre motor and motor cortices, which may indicate direct cortical defects and could account, in part, for the changes in resting glutamate levels. While the source of the cortical glutamate level changes we observed is unclear, we observed a 26% decrease in TH⁺ fiber density in the premotor cortex and a 28% decrease in the motor cortex on the MPTP-lesioned side compared to the contralateral side. Previous studies have shown a decrease in dopamine innervation of the cortex in MPTP-treated monkeys (34% to 49% in dopamine content and 36 to 72% reduction in fibers) (Jan et al., 2003; Pifl et al., 1991) and PD patients (24% to 79% reduction in TH labeled fibers) (Gaspar et al., 1991). Inherent in that variability is the observation that regional areas of the cortex show variability in the susceptibility to MPTP toxicity such that in PD patients, cortical layers can have variability from 24% to 73% in TH-labeled fiber density in different layers within the cortex.

Previous studies have demonstrated that glutamate neurotransmission in the cortex is directly involved in brain activity in both resting and stimulated states (Duncan et al., 2011). In addition, decreased cortical activations (at rest) after MPTP administration were found decades ago. For example, the results from 2-deoxyglucose studies on MPTP-treated monkeys suggest that cortical activation is globally reduced (Schwartzman and Alexander, 1985). Also, electrophysiological studies in MPTP-treated monkeys found that activation of the motor cortex or the supplementary motor area was reduced (Watts and Mandir, 1992). Meanwhile, high-field MRI-based technologies indicate a decrease in glutamate levels in the brain of PD patients (Griffith et al., 2008). Results from these studies strongly indicate that cortical activation, which is considered linked with glutamatergic activity, could be reduced by MPTP administration. The lower resting levels we observed in the MPTP-lesioned side may be the result of a compensatory enhancement in glutamate uptake through an increase in transporter cell-surface expression or a decrease in tonic glutamate release that is likewise observed in aging (Nickell et al., 2006). Moreover, results from the present study support the concept that reduced primary motor cortex activation may directly lead to defective initiation of movement and slowed movement execution in PD (Watts and Mandir, 1992).

That said, while there may be changes in cortical glutamate levels, based on the size of the effect, the change may not be critical to the functional manifestations of the disease. In fact, the difference in glutamate levels between the lesioned side and unlesioned side cortex may indicate compensatory actions by other systems on the side ipsilateral to the lesion. However, more studies will be needed to confirm this conclusion as the effect on resting glutamate levels was significant but slight, the measurements were made under general anesthesia, and the sample size was small.

Compared to normal controls, the MPTP-lesioned and contralateral sides had non-significant decreases in resting levels of glutamate. The decrease in glutamate levels on the contralateral side compared to control normal could have been the result of MPTP leaking to the contralateral side through the Circle of Willis, and while some studies have reported that unilateral MPTP administration could produce a 10-15% neuronal loss on the contralateral side substantia nigra, data from our group shows that contralateral neuronal loss is small compared with aged matched controls because we used a much lower dose of MPTP with a rigorous surgical procedure. However, based on this present study, we do not have evidence to support the idea that unilateral MPTP administration could have a bilateral effect on pre-motor or motor cortical glutamate levels. Future studies will need to determine if other cortical areas show a compensatory change in glutamate levels in MPTP-treated monkeys, because an increasing number of reports have demonstrated that, given how dopamine depletion may disturb functions of the motor cortex that receive direct input from the basal ganglia (via the motor thalamus), a compensatory shift in activation toward other areas of the cortex may result (for review, see Galvan and Wichmann, 2008). Monitoring the bilateral alteration of glutamate neurotransmission may help shine light on the mechanisms of human PD progression from one hemisphere of the brain to the other.

In summary, the primary finding of the present study was that pre-motor and primary motor cortical glutamate levels could be altered by an MPTP-induced dopamine lesion. The current investigation provides additional critical information about the cortical changes that occur in a model of dopaminergic dysfunction. However, observations from the present study may only be a small piece of a larger picture illustrating the involvement of cortical glutamate in PD. Therefore, future studies are clearly needed, especially in assessing glutamate neurotransmission in awake, behaving conditions.

4. Experimental procedure

4.1. Animals

Data from a total of 14 female rhesus (*Macaca mulatta*) monkeys (including six hemiparkinsonian –17.5 – 23.5 years old, and eight normal, age-matched animals) is presented in the current study. Animals were not scientifically naïve, as they had been used in previous studies (e.g. Luan et al., 2008), but had not participated in a study for at least one year. The animals were housed in individual cages in a temperature-controlled room and maintained on a 12-hour light and 12-hour dark cycle. All experiments were conducted during the animal's daytime. Water was available *ad libitum*. Standard primate biscuits were supplemented daily with fresh fruits and vegetables. Approximately five years before the present study, the hemiparkinsonism was induced by a unilateral administration of

0.12mg/kg MPTP via the right carotid artery (Ding et al., 2008). All animals had stable, moderate hemiparkinsonian signs including bradykinesia (slowness), rigidity on both upper and lower limbs on the contralateral side of MPTP administration, stooped posture, mild postural instability, and responsive to levodopa (50mg- 75mg). The average total score of these animals was 5.25 ± 0.44 points on a nonhuman primate Parkinsonian rating scale to assess PD features (Ovadia et al., 1995). The eight normal animals were included as controls. Portions of the glutamate data collected from these eight normal animals was separately described elsewhere (Quintero et al., 2007). The animals' care was supervised by experienced veterinarians and all protocols used in the study were approved by the University of Kentucky's Animal Care and Use Committee following NIH and USDA guidelines.

4.2. Glutamate biosensors

The detailed procedures for measuring glutamate have been described elsewhere (Quintero et al., 2007). Briefly, ceramic-based multisite microelectrode arrays (MEAs) were assembled, enzyme coated with glutamate oxidase (GluOX), and used in self-referencing mode (Fig.1) (Burmeister and Gerhardt, 2001; Day et al., 2006; Quintero et al., 2007). The self-referencing approach employs two of the MEA sites, a GluOx coated site (+GluOx) and a site without GluOx (-GluOx) to quantify basal glutamate levels. Extracellular glutamate levels were measured using constant potential amperometry (+ 0.7 V vs. Ag/AgCl reference) controlled by a FAST16mkII electrochemical recording system (Quanteon, LLC, Nicholasville, KY). The morning of the recordings, all MEAs were calibrated with glutamate *in vitro* to generate a standard curve, determine the limit of detection of the MEA, and measure the selectivity of glutamate relative to the endogenous electroactive compound, ascorbic acid. MEA (N=6) parameters in this study showed similar responses to published results and had limits of detection = $0.7 \pm 0.3 \mu\text{M}$; sensitivity to glutamate (slope of standard curve) = $6.1 \pm 1.1 \text{ pA}/\mu\text{M}$; and selectivity to glutamate over ascorbic acid = $33 \pm 11: 1$.

4.3. Glutamate recordings

During surgery, trained surgery technicians monitored the following parameters: a) blood pressure, b) end tidal CO₂, c) breathing rate, d) pulse rate, e) pulse oximetry (SpO₂), f) temperature, and g) electrocardiography. Under general anesthesia (1-1.25% isoflurane), the skull directly over the pre- and primary motor cortex, based on T₁-weighted anatomical magnetic resonance imaging (MRI), of the MPTP-lesioned and unlesioned sides were removed (approx. 1.5 cm × 1.5 cm in size) and a reference electrode (a Ag/AgCl electrode, Model RE-5B, Bioanalytical Systems, West Lafayette, IN) was inserted into a pocket (5 mm × 50 mm) in the area above the occipital lobes. After initially advancing the center of the MEA to the cortical surface, the assembly was sequentially lowered to the recording depths (-2.5 to -3.0 mm below the surface of the brain) by a micromanipulator, which was attached to the stereotactic head frame. Five different points (2-3 mm apart) from the medial to the lateral primary cortex covering the trunk and upper body regions were used on each of the left and right sides of the cortex as illustrated in Fig. 1. MEAs equilibrated to a steady state for a minimum 10-15 min. at each implantation site before collecting basal glutamate levels; data were averaged for each self reference subtracted MEA channel. Basal glutamate values for the MPTP-lesioned side cortex and the unlesioned cortex were generated from an

average of the last 30 seconds of the equilibration period. Reported results are from hemisphere basal glutamate values. Hemisphere basal glutamate values for each animal were calculated by averaging the glutamate values from up to 5 location points in each hemisphere.

4.4. Post-mortem Morphology

Immediately after the glutamate recordings, the six MPTP-lesioned animals were deeply anesthetized with pentobarbital (20-25 mg/kg, IV) and euthanized via transcardial perfusion of 0.9% saline based on previously published procedures (Ding et al., 2008; Gash et al., 1996). The brains were immediately removed, immersion-fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 3 days and cryoprotected in 30% buffered sucrose until the brains sank. Then, 40 μ m-thick sections were cut on a sliding microtome through the substantia nigra. One of every six sections was processed for TH (monoclonal antibody, 1:1000; Chemicon International, Temecula, CA, USA) for assessment of MPTP-induced, TH-positive (TH⁺) neuronal loss in the midbrain, procedures described elsewhere (Ding et al., 2008; Gash et al., 1996; Grondin et al., 2002). The number of TH⁺ midbrain dopaminergic neurons was estimated bilaterally using an optical fractionator method for unbiased stereological cell counting. TH⁺ fiber density in the premotor cortex and motor cortex was quantified from 3-4 sections (excluding those sections where the biosensor had been inserted) of each animal as previously described (Gash et al., 1995; Grondin et al., 2002). A 1.2 $\times$ 1.2 mm grid was used to quantify the ratio of the TH⁺ fibers in the premotor cortex and motor cortex areas on the MPTP-lesioned side was compared to the same areas on the contralateral side.

4.5 Statistical analysis

Basal glutamate levels were analyzed from time-series recordings in individual animals using custom MatLab®-based analysis software and compared among animals at the group level using a Student's t-test, unless otherwise noted. Statistical comparisons between the MPTP-lesioned and unlesioned sides were treated as paired measurements. All statistical analyses were conducted using Prism 5/6 GraphPad Software (San Diego, CA). Differences of $p < 0.05$ were considered significant. Data displayed as mean $\pm$ standard error of the mean.

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Non standard abbreviations used

PD	Parkinson's disease
MEA	Microelectrode array
GluOx	glutamate oxidase
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

TH	tyrosine hydroxylase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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Research Highlights

- We examined cortical glutamate in a model of Parkinson's disease.
- The lesioned-side motor cortex had fewer tyrosine hydroxylase-positives fibers.
- Cortical glutamate levels were lower on the lesion-side than the contralateral.
- Cortical glutamate levels affected during dopaminergic dysfunction.

Figure 1.

TH⁺ fiber densities in the pre-motor and motor cortices. TH⁺ fiber densities were approximately 25% lower in the MPTP lesioned-side than the contralateral side in the pre-motor and motor cortex. * = $p < 0.05$.

Figure 2.

Basal glutamate levels were measured from five areas (2-3 mm apart) in the primary motor cortex from both the left and right hemispheres of MPTP-treated nonhuman primates. An enzyme-coated ceramic microelectrode array was used to differentially measure extracellular glutamate levels in five different points (e.g. blue circles) of the primary motor cortex. GluOx+: microelectrode site coated with protein matrix plus glutamate oxidase; GluOx-: microelectrode site coated with protein matrix not containing glutamate oxidase.

Figure 3.

Basal glutamate levels were lower in the motor cortex ipsilateral to the lesion than in the unlesioned side. A) Basal glutamate levels in the MPTP-lesioned side motor cortex were significantly lower than the contralateral motor cortex; ($p = 0.031$). B) Resting glutamate levels were not significantly different among the five separate coordinates where glutamate levels were measured ($p = 0.50$) as described in Fig. 2.

Figure 4.

Glutamate levels in MPTP-lesioned animals not significantly different than normal animals. A) Basal glutamate levels in the motor cortex were lower, but not significantly different, in the MPTP-lesioned side compared to glutamate levels in normal age-matched control animals. B) Basal glutamate levels in the motor cortex of the side contralateral to the MPTP lesion were not significantly different from levels in control, aged-matched animals.