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Acute and subchronic MPTP administration differentially affects striatal glutamate synaptic function

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Abstract

We previously reported that 1 month following unilateral loss (>95%) of striatal dopamine, there is an increase in striatal glutamate function as measured by in vivo microdialysis and quantitative immuno-gold electron microscopy (Meshul, C.K., et al., 1999, *Neuroscience* 88, 1–16). The goal of this study was to determine the effect of bilateral loss of striatal dopamine on striatal glutamate function following acute or subchronic administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to C57/B6J mice. Animals were administered either single injections (ip) of 30 mg/kg/day for 7 days (subchronically treated group) or 20 mg/kg × 4 doses every 2 h (acutely treated group) of the toxin or saline. One month following the first injection, there was a 44 and 65% loss in the relative density of tyrosine hydroxylase (TH) immunolabeling within the dorsolateral striatum in the subchronically and acutely MPTP-treated groups compared to the saline group, respectively. There was a decrease in the basal level of extracellular glutamate within the striatum in the subchronically MPTP-treated animals compared to an increase in the acutely treated group in relationship to the saline group. Ultrastructurally, only in the acutely MPTP-treated group was there a decrease in the density of glutamate immunolabeling within nerve terminals associated with an asymmetrical synaptic contact in the dorsolateral striatum compared to either the subchronic or saline groups. In addition, there was a decrease in the relative density of GluR-2/3 subunit immunolabeling within the dorsolateral striatum in the acute MPTP compared to the saline group. These data indicate that differences in striatal glutamate function appear to be associated with the dosing interval of MPTP administration and the variable loss of striatal TH immunolabeling.

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Keywords: MPTP; Immunocytochemistry; Parkinson's disease; Electron microscopy; Glutamate synapses; In vivo microdialysis; Striatum

Introduction

Administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to C57/B6J mice results in degeneration of nigrostriatal dopamine neurons (Jackson-Lewis et al., 1995). The dosing and timing of MPTP administration determines the extent of striatal terminal degeneration and substantia nigra pars compacta cell body loss

(Sonsalla and Heikkila, 1986). The biochemical and behavioral deficits that occur following a substantial lesion from MPTP treatment closely resemble the clinical symptoms of idiopathic Parkinson's disease which are observed after a >80% loss of striatal dopamine (Kopin and Markey, 1988). The efficacy of potential therapeutic agents has been tested in this animal model by monitoring if, for example, the behavioral deficits are decreased with treatment or if the onset of symptoms can be delayed (Fredriksson et al., 1994).

Dopamine/glutamate interactions within the striatum have been well documented and these neurotransmitters are known to influence each others' release (Starr, 1995b). Dopaminergic input from the nigrostriatal pathway and glu-

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tamatergic input from the corticostriatal tract converge on the same output neurons within this region of the basal ganglia and therefore cooperatively modulate the activity of striatal output neurons (Bouyer et al., 1984; Dube et al., 1988; Morari et al., 1994, 1996; Smith et al., 1994; Yamamoto and Davy, 1992). Dynamic changes in striatal glutamate levels following a 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway, resulting in a >95% loss of striatal dopamine, have been measured in tissue homogenates and in extracellular microdialysate samples (Lindfors and Ungerstedt, 1990; Meshul et al., 1999, 2002) as well as immunocytochemically with electron microscopy (Meshul et al. 1999, 2002, Meshul and Allen, 2000). These reports suggest an increase in corticostriatal glutamate synapse function as measured by an increase in tissue and extracellular glutamate levels as well as a decrease in the density in glutamate immunolabeling in nerve terminals associated with the corticostriatal pathway.

Pharmacologically, the therapeutic potential of administering glutamate receptor antagonists to parkinsonian non-human primates (Papa and Chase, 1996) and mice (Fredricksson et al., 1994, 1999) has been shown to decrease dyskinesias produced by *l*-dopa treatment. Although clinical manifestations of Parkinson's disease can appear with as little as 60–70% dopamine loss, dynamic changes in glutamate synapse function, as a result of decreased striatal dopaminergic tone, may be evident well in advance of clinical symptoms and may provide an avenue for early therapeutic intervention.

Using the MPTP-treated mouse as a model to study dopamine/glutamate interactions following bilateral loss of striatal dopamine, the goal of this study was to investigate changes in striatal glutamate synapse function using two different doses and dosing intervals. It has been reported that administration of MPTP either subchronically for 7 days (30 mg/kg/day) or acutely (20 mg/kg \times 4 doses every 2 h) results in varying degrees of striatal dopamine loss (Heikkila et al., 1984; Ricaurte et al., 1986; Sonsalla and Heikkila, 1986). Following subchronic treatment, the loss of striatal dopamine ranges from 60 to 75%, while following acute MPTP treatment dopamine loss is more severe, ranging between 74 and 95%. These losses of striatal dopamine are suggestive of the decrease in dopamine levels found as Parkinson's disease continues its progression. In addition, with a daily dose of 30 mg/kg of MPTP, there is a more gradual loss of striatal dopamine compared to the acutely treated animals (Heikkila et al., 1984). This loss of striatal dopamine remains for up to 1 month following the last dose of the neurotoxin (Ricaurte et al., 1986). Although it has been reported that an acute injection of MPTP (40 mg/kg) results in a transient (~4 h) decrease in striatal tissue glutamate content (Chan et al., 1994), there have been no reports on the long-term effects of MPTP treatment on striatal glutamate function. In the current study, striatal glutamate function was assessed by measuring the extracellular levels of glutamate using *in vivo* microdialysis and

quantifying the density of nerve terminal glutamate immunolabeling using quantitative immuno-gold electron microscopy. In a separate group of acutely treated animals, the density of several glutamate receptor subunits was quantified using immunohistochemical staining as a measure of postsynaptic changes following these two dosing regimens.

Materials and methods

Animals

Male C57/B6J mice (8 weeks old, Jackson Laboratories, Bar Harbor, ME) were allowed to acclimate in a climate-controlled room with a constant light/dark cycle (12 h on, 12 h off) for at least 2 weeks prior to the start of injections. Water and food were available *ad libitum*. Animals were housed four per cage prior to implantation of the dialysis guide cannulas, whereby they were housed individually. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1978). All efforts were made to minimize animal suffering and to reduce the number of animals used.

MPTP administration

Beginning no earlier than 10 weeks of age (range, 10–11 weeks), animals were injected either subchronically (30 mg/kg/day as the free base, ip) or acutely [20 mg/kg \times 4 (as the free base) doses every 2 h, ip] with MPTP or vehicle (saline, 1 ml/kg). In the acutely treated group, 35% of the animals died. One month following the first injection, immunolabeling or *in vivo* microdialysis was carried out (see below). For both the light/electron microscopic studies and the microdialysis procedure, 8–10 animals were used for each of the three treatment groups.

Surgical procedures

Three weeks after the first injection, animals were anesthetized (1 ml/kg of 2.5% ketamine, 1% xylazine, and 0.5% acepromazine in normal saline), their heads were shaved, and they were placed in a Kopf stereotaxic apparatus fitted with a small rodent bite plate. The skin above the skull was cut and the top of the skull exposed. A small hole was drilled and the dura was punctured at the following coordinates from bregma (Franklin and Paxinos, 1997): anterior, +1.2 mm; lateral, +1.8 mm. A stainless-steel guide cannula (5 mm long, 21-gauge; Small Parts, Miami Lakes, FL) was lowered 1.5 mm from the surface of the skull. The guide cannula was held in a fixed position by three stainless-steel screws attached to the skull and encompassed by cranio-plastic (Plastics One, Inc., Roanoke, VA). The animals were allowed to recover for 1 week prior to the start of the microdialysis experiment.

In vivo microdialysis

Dialysis probes were prepared as described by Robinson and Wishaw (1988), with modifications (Meshul et al., 1999). The probes were 210 μm in diameter and 2 mm in length. One day prior to use, the efficiency of transmitter recovery by the probe was determined by collecting three 10-minute samples (perfusing flow rate of 2 $\mu\text{l}/\text{min}$) after placing the probe in a solution of glutamate (200 $\text{pg}/\mu\text{l}$) in artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 3.4 mM KCl, 1.5 mM CaCl_2 , 1.0 mM MgCl_2 , 1.4 mM NaH_2PO_4 , and 4.85 mM NaHPO_4 , pH 7.4).

Following the collection of the probe recovery samples and the day prior to the start of the actual dialysis procedure, the probe was lowered into the guide cannula with the entire length of the dialysis probe in the caudate nucleus. The tip of the guide cannula was positioned at the level above the corpus callosum. The probe was secured to the guide cannula with epoxy. The aCSF flowed through the probe overnight at a rate of 0.2 $\mu\text{l}/\text{min}$. The following morning, the pump speed was increased to 2 $\mu\text{l}/\text{min}$ for 1 h and then four samples were collected every 15 min to determine the basal level of extracellular glutamate. We have previously verified that changes in the basal extracellular level of striatal glutamate are dependent on the presence of calcium within the aCSF. Replacement of calcium with the divalent chelating agent EGTA and increasing the aCSF concentration of magnesium resulted in a significant decrease in the basal level of glutamate (Meshul et al., 2002). This suggests that at least a portion of the resting level of striatal glutamate is of neuronal origin. Both the saline- and MPTP-treated groups were then challenged with MPTP (30 mg/kg, ip) and an additional six 15-min samples were collected. Therefore, we report data on the following groups: saline (SAL), MPTP given either subchronically or acutely (MPTP), saline-treated animals challenged with a single dose of MPTP (SAL/MPTP), and subchronically or acutely MPTP-treated animals given a single challenge dose of MPTP (MPTP/MPTP). At the conclusion of the experiment, the animals were perfused with glutaraldehyde fixative (see below), vibratome sections (100 μm) were cut and stained with thionin and the site of the probe placement within the caudate was verified histologically. Probe placement extended 2 mm along the lateral quadrant of the striatum. If the placement was not correct (i.e., outside the striatum), the data from that animal were discarded. The four baseline data points and the six MPTP-challenged data points were separately averaged at each time point and then a grand mean was determined for either the baseline or the MPTP-challenged samples. The values are expressed as means \pm SEM in picomoles per microliter extracellular level of striatal glutamate. The mean probe recovery was $10.4 \pm 1.2\%$. All the data between groups were analyzed using a one-way ANOVA. Significant main effects were further characterized using Peritz' *f* test for comparison of multiple means.

HPLC detection of dialysate glutamate levels

Glutamate concentration in dialysate was determined using a Hewlett–Packard HPLC 1090 interfaced with a Hewlett–Packard 1046A programmable fluorescence detector. Dialysates were derivatized with *o*-phthalaldehyde (OPA) and chromatographed according to a modification of the method of Schuster (1988), as previously reported (Meshul et al., 1999, 2002). Dialysates were derivatized by adding 1 μl of sample, 5 μl of borate buffer (pH 10.4), and 1 μl of OPA. The reaction mixture was injected into a reverse-phase C18 column (HP No. 79916AA) and OPA derivatives were separated using a linear gradient. Solvent A contained 0.018% (v/v) TEA, 0.3% (v/v) tetrahydrofuran, and 20 mM sodium acetate buffer, pH 7.2. Solvent B contained 40% (v/v) acetonitrile, 40% (v/v) methanol, and 20% (v/v) 100 mM sodium acetate, pH 7.4. The OPA derivatives of glutamate were detected using an excitation wavelength of 340 nm and an emission wavelength of 450 nm. Assay sensitivity was in the subpicomole range.

Light microscopic immunocytochemistry

A separate series of mice was used for the light microscopic immunocytochemical localization of tyrosine hydroxylase (TH), NMDA receptor subunits (NMDAR-1, -2A and -2B), and AMPA receptor subunits (GluR-1 and -2/3). Thirty days after the first injection of either MPTP or saline, mice were perfused with 3 ml of heparin [1000 units/ml in phosphate buffer (PB), pH 7.3] followed immediately by 40 ml of 1% acrolein/2% paraformaldehyde in 0.1 M PB as previously detailed (Meshul and Allen, 2000). The brains were removed, washed overnight in cold PB, and then cut with a vibratome. All the tissues from each treatment group were cut and immunolabeled with a given antibody on the same day in order to limit the variables that may occur by cutting and labeling the tissue on different days. Free-floating 100- μm sections were washed in 1% sodium borohydride in PB for 30 min at room temperature, followed by four rinses in PB. Sections were preincubated in blocking solution (10% normal goat serum in PB with 0.1% Triton X-100) at 4°C for 1 h immediately followed with overnight incubation with the following antibodies: tyrosine hydroxylase (TH, monoclonal, Diasorin, Minneapolis, MN; 1:40,000); NMDAR-1 (monoclonal, NR1, which recognizes all the splice variants of the NMDA-R1 subunits, Upstate Biotechnology, Lake Placid, NY, 1:100); NMDAR-2A (polyclonal, Chemicon, 1:1000); NMDAR-2B (polyclonal, Chemicon, 1:1500); GluR-1 (polyclonal, Chemicon, 1:1000); and GluR-2/3 (polyclonal, Chemicon, 1:1000). The antibodies were diluted in blocking solution and incubated overnight with the striatal sections at 4°C on a shaker. The tissue was then rinsed in blocking solution and exposed to the secondary antibody [0.44% goat anti-mouse (monoclonal) or goat anti-rabbit (polyclonal) IgG, biotinylated, in PB (Vector, Burlingame, CA)] for 45 min at room temper-

ature. The tissue was washed in buffer, incubated in ABC (Vector, according to the manufacturer's directions) for 45 min at room temperature, and washed in buffer, and the reaction product was visualized using nickel-enhanced diaminobenzidine (DAB kit, Vector, 2-min exposure). The slices were then washed in buffer, mounted on gelatin-coated slides, air-dried, and coverslipped. As a control, elimination of the primary antibody resulted in a complete lack of tissue immunolabeling (data not shown).

Optical density measurements were carried out by first capturing the image taken directly from the immunoreactive material using a Zeiss Axioplan light microscope (Carl Zeiss, Inc., West Germany) and transporting the image using a Polaroid Digital Microscope Camera (Cambridge, MA) at 1.25 \times magnification. Relative optical density measurements were calculated using Image-Pro Plus software (Version 3.01, Media Cybernetics, Silver Springs, MD). Six slices from each group were analyzed (alternate sections throughout the rostral–caudal extent of the striatum). Lower values of optical density correlated with areas of lighter immunoreactivity on the tissue. A computer-generated mean optical density for the dorsolateral striatum of both sides was collected. The relative optical density of both the left and the right dorsolateral striatum was determined and averaged for each slice. The dorsolateral striatum was analyzed since this is the major area of input of the corticostriatal pathway (McGeorge and Faull, 1989). The relative density of the overlying corpus callosum was taken as a background measure and subtracted from the value generated from the dorsolateral striatum. The data were analyzed using a one-way ANOVA followed by a post-hoc analysis using Peritz' *f* test for comparison of multiple means (Meshul et al., 1999). Analysis of the immunolabeled tissue was carried out by an individual blinded to the experimental group.

Immuno-gold electron microscopy

Thirty days after the first injection of MPTP, the mice were anesthetized, their chest cavities were opened, and the mice were perfused transcardially with 3 ml of 1000 units/ml heparin in 0.1 M Hepes buffer (pH 7.3) followed immediately by 40 ml of 2.5% glutaraldehyde/0.5% paraformaldehyde in 0.1 M Hepes, pH 7.3, containing 0.1% picric acid. After the perfusion, the entire brain was then removed and placed in cold (4°C) fixative overnight. All animals from each treatment group were perfused on the same day in order to limit the variables from perfusing mice on different days. These animals were not challenged with a single dose of MPTP prior to perfusing them so that a comparison could be made between the density of glutamate immunolabeling and the basal extracellular level of glutamate.

Following vibratome sectioning (200 μ m) and dissection of the left dorsolateral striatum (equivalent to +1.0 mm anterior; Franklin and Paxinos, 1997), the tissue was pro-

cessed as previously described (Meshul et al., 1994). All tissue from each of the treatment groups was cut and processed on the same day in order to limit the variables that may occur by cutting and processing tissue on different days.

Postembedding immuno-gold electron microscopy was performed according to the method of Phend et al. (1992), as modified by Meshul et al. (1994). The glutamate antibody (non-affinity-purified, rabbit polyclonal; Biogenesis, Brentwood, NH), as previously characterized by Hepler et al. (1988), was diluted 1:400,000 in TBST 7.6. Aspartate (1 mM) was added to the glutamate antibody mixture 24 h prior to incubation with the thin-sectioned tissue to prevent any cross-reactivity with aspartate within the tissue. Photographs (10/animal) were taken randomly at a final magnification of $\times 40,000$ throughout the neuropil using a digital camera (AMT, Boston, MA). The images were directly captured and stored on the computer by an individual blinded to the experimental groups. The glutamate immunolabeling technique was carried out for all of the treatment groups on the same day in order to limit the variables that may occur by carrying out this procedure on different days.

The number of gold particles per nerve terminal associated with an asymmetrical synaptic contact was counted and the area of the nerve terminal was determined using Image Pro Plus software (Media Cybernetics, Silver Springs, MD, Version 3.01). Glutamate-containing nerve terminals were typically photographed making a synaptic contact on a dendritic spine, indicating that they most likely originated from the motor cortex (Dube et al., 1988; Smith et al., 1994). The gold particles contacting the synaptic vesicles within the nerve terminal were counted and were considered part of the vesicular or neurotransmitter pool as previously established (Meshul et al., 1999). In addition, the mitochondrial pool of glutamate was analyzed within nerve terminals to determine if the nonvesicular pool of glutamate was affected by the toxin treatment. The density of gold particles per square micrometer of nerve terminal area was determined for each animal and the mean density for each treatment group calculated (mean density \pm SEM). The differences between treatment groups were analyzed with a one-way ANOVA and significant main effects were further characterized using the Fisher post hoc test for comparison of multiple means. The specificity of the immunolabeling for the glutamate antibody was previously established by incubating the antibody overnight with 3 mM glutamate (Meshul et al., 1994). This mixture was then applied to the sections as detailed above, with the final results showing a lack of tissue immunolabeling.

The total number of synapses for each of the treatment groups making an asymmetrical synaptic contact that were analyzed are as follows: subchronic MPTP group, 184 synapses; acute MPTP group, 185 synapses; and saline group, 177 synapses.

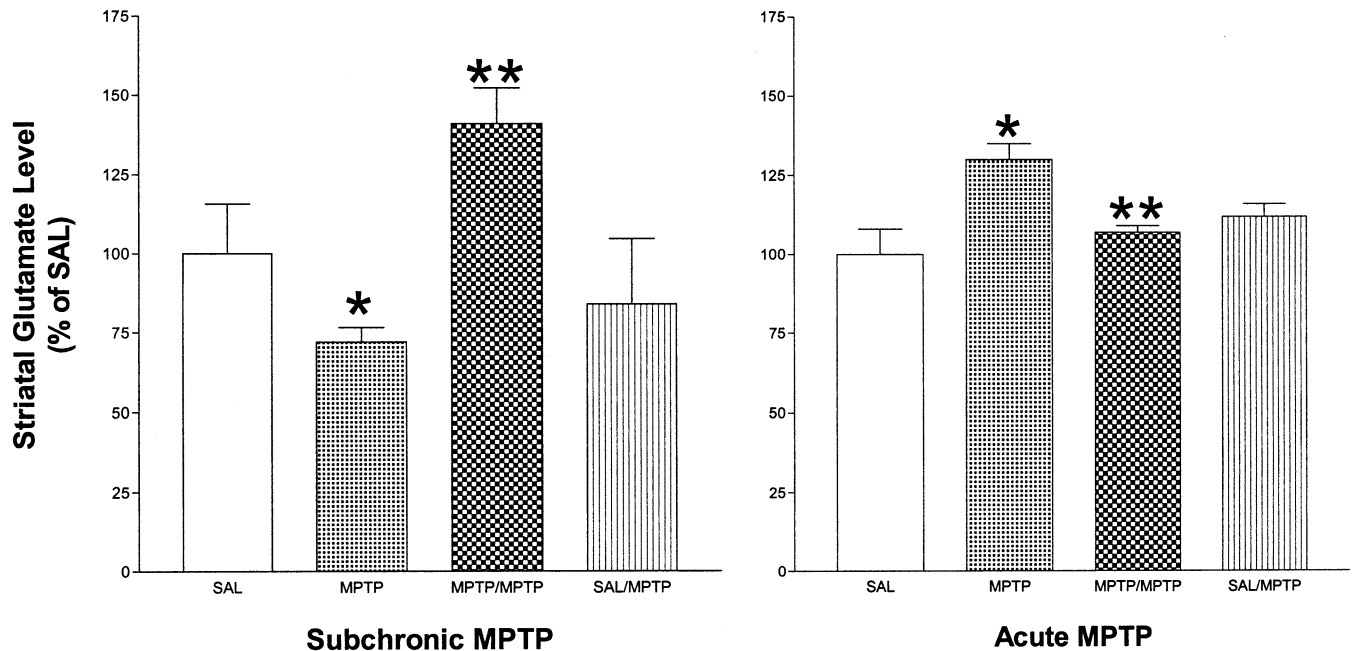


Fig. 1. In vivo microdialysis of the striatum. Subchronic MPTP treatment (left figure). Mice were treated daily for 7 days with MPTP (30 mg/kg, ip) or saline (SAL, 1 ml/kg, ip) and dialysis was carried out 30 days after the first injection. Four baseline samples were first collected (SAL or MPTP) and then an acute challenge dose of MPTP (30 mg/kg, ip) was given to both groups of animals (MPTP/MPTP or SAL/MPTP) and an additional six samples were collected. Values are means \pm SEM. * $P < 0.05$ compared to the SAL group as determined by Peritz' f test for comparison of multiple means. ** $P < 0.05$ compared to the other subchronically treated groups as determined by Peritz' f test for comparison of multiple means. Acute MPTP treatment (right figure). Mice were treated with four doses of MPTP (20 mg/kg, ip) or saline (SAL, 1 ml/kg, ip) every 2 h for a total of four doses. Dialysis was carried out 30 days after the injections. Four baseline samples were first collected (SAL or MPTP) and then an acute challenge dose of MPTP (30 mg/kg, ip) was given to both groups of animals (MPTP/MPTP or SAL/MPTP) and an additional six samples were collected. Values are means \pm SEM. * $P < 0.05$ compared to the other acutely treated groups as determined by Peritz' f test for comparison of multiple means. ** $P < 0.05$ compared to the MPTP group as determined by Peritz' f test for comparison of multiple means.

Results

In vivo microdialysis

Subchronically treated

There was a significant decrease (32%) in the basal extracellular level of glutamate within the striatum in the subchronically MPTP-treated compared to the saline (SAL)-treated group ($P < 0.05$; Fig. 1). After collecting the four 15-min basal samples, all animals were injected with a challenge dose of MPTP. In the MPTP-treated group (MPTP), the challenge dose resulted in a significant increase in the extracellular level of striatal glutamate (MPTP/MPTP) compared to either the basal levels of glutamate from the MPTP group or the saline group challenged with MPTP (SAL/MPTP) ($P < 0.05$). In animals only treated with saline (SAL) on days 1–7, a challenge dose of MPTP (SAL/MPTP) 30 days later resulted in no change in the extracellular level of glutamate.

Acutely treated

In contrast, 30 days following acute treatment in animals with four doses of MPTP (20 mg/kg) or saline every 2 h, there was nearly a 30% increase in the basal extracellular

level of striatal glutamate compared to the saline-treated group (Fig. 1). Following a challenge dose of MPTP to the MPTP-treated group (MPTP/MPTP), there was a small (18%) but significant decrease ($P < 0.05$) in the extracellular level of striatal glutamate which restored extracellular glutamate levels back to that observed in the saline-treated group. There was no change in the extracellular level of striatal glutamate in the saline-treated group following a challenge dose of MPTP (SAL/MPTP).

Electron microscopic glutamate immunolabeling

To determine if changes in the basal level of striatal glutamate were associated with alterations in the density of glutamate immunolabeling in nerve terminals making an asymmetrical synaptic contact, quantitative immuno-gold electron microscopy was carried out. An example of nerve terminal glutamate immunolabeling in a saline- and MPTP (acute)-treated group is illustrated in Fig. 2. There is a higher density of gold labeling within the nerve terminal as compared to the adjacent dendritic spine, illustrating the specificity of the technique. Following subchronic administration of MPTP for 7 days, the dorsolateral striatum was dissected and processed for immunolabeling 30 days after

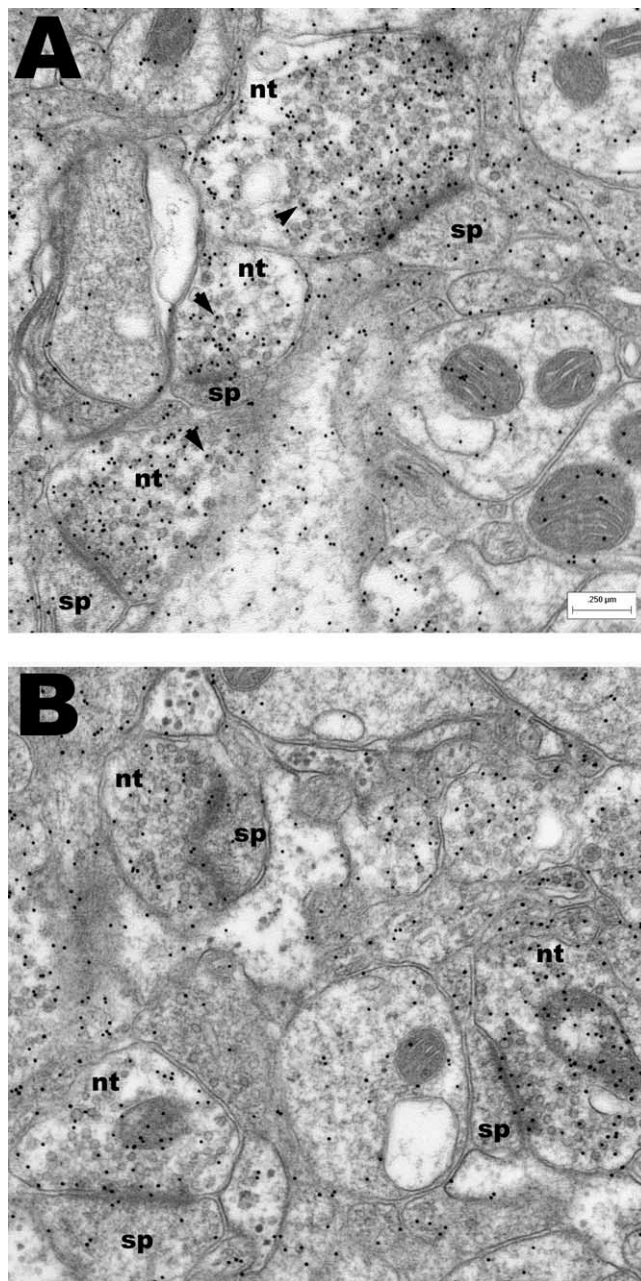


Fig. 2. Electron photomicrographs using the immuno-gold technique to localize an antibody against the neurotransmitter glutamate within the dorsolateral striatum. (A) Saline group. Within the nerve terminal (nt), there is an accumulation of small round synaptic vesicles and 10-nm gold particles indicating the location of the antibody (arrowhead). All three nerve terminals are making synaptic contact with an underlying dendritic spine (sp). (B) MPTP-treated group given an acute injection (20 mg/kg \times 4 every 2 h) and then perfused with fixative 30 days later. Note the decrease in the density of immuno-gold particles in all three terminals compared to that observed in the control preparation presented in Panel A. Calibration bar, 0.25 μ m.

the first dose of the neurotoxin. This subchronic dosing schedule resulted in a small but insignificant decrease (12.5%; $P > 0.05$) in the density of nerve terminal glutamate immunoreactivity within the dorsolateral striatum (Fig. 3).

However, 30 days following acute administration of MPTP, there was a significant decrease (32.8%; $P < 0.05$) in the density of glutamate immunolabeling within striatal nerve terminals making an asymmetrical synaptic contact (Fig. 3). There was no change in the density of glutamate immunolabeling within the mitochondrial pool after acute MPTP treatment (values are mean number of gold particles/ μ m² \pm SEM: saline, 27.1 \pm 1.6; MPTP, 22.5 \pm 3.1, $P > 0.05$). This suggests that alterations in the density of nerve terminal glutamate immunoreactivity were specific for the neurotransmitter pool (Meshul et al., 1999).

Tyrosine hydroxylase immunohistochemistry

To quantify the extent of nigrostriatal damage caused by subchronic and acute treatment with MPTP, the relative density of TH (an enzyme involved in the synthesis of dopamine) immunolabeling within the striatum was determined. An example of TH immunolabeling within the dorsolateral striatum of a saline- and acutely MPTP-treated animal is illustrated in Fig. 4. Thirty days after the first dose of the neurotoxin or saline, there was a significant decrease in the relative density of striatal TH immunolabeling in both the subchronically and acutely MPTP-treated groups compared to the saline-treated group. There was a 44% decrease in striatal TH labeling after subchronic treatment, while there was a 65% decrease in labeling following acute administration of MPTP compared to the saline group (Fig. 5; $P < 0.05$). There was no significant difference between the two MPTP-treated groups.

Glutamate receptor subunit immunolabeling

Due to alterations in both the basal extracellular level of striatal glutamate as measured by in vivo microdialysis and in the density of presynaptic glutamate immuno-gold labeling following acute MPTP treatment, the relative density of several glutamate receptor subunits was carried out. The relative density of the NMDAR-1 (all splice variants), NMDAR-2A and -2B, and GluR-1 subunits was unchanged between the saline and the acutely MPTP-treated group (data not shown). An example of GluR-2/3 subunit immunolabeling within the dorsolateral striatum of the saline and the acutely MPTP-treated group is illustrated in Fig. 6. There was a small, but significant, decrease (26%) ($P < 0.05$) in the relative density of GluR-2/3 subunit immunolabeling within the dorsolateral striatum in the acutely MPTP-treated group compared to the saline group (Fig. 7).

Discussion

We report in the current study that subchronic administration of the neurotoxin MPTP for 7 days (30 mg/kg/day) results in a decrease in the basal level of extracellular striatal glutamate when measured 30 days after MPTP ad-

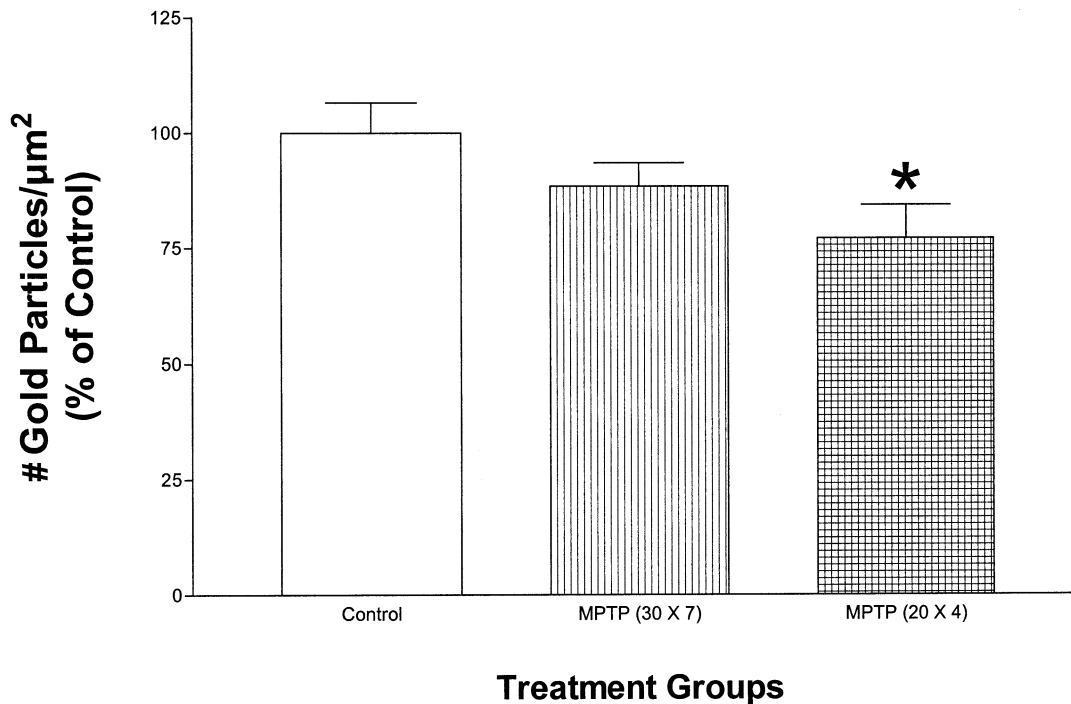


Fig. 3. Subchronic (30 mg/kg/d \times 7 days) or acute (20 mg/kg \times 4 every 2 h) administration of MPTP to C57/B6J mice compared to the saline-treated (control) group. Using quantitative immuno-gold electron microscopy, there was a significant decrease in the density of striatal nerve terminal glutamate immunolabeling following the acute administration of MPTP compared to control treatment. There was no difference between the two MPTP-treated groups. * $P < 0.05$ compared to the saline-treated (control) group as determined by the Fisher post hoc test for comparison of multiple means.

ministration. This is in contrast to the effects of acutely administered MPTP (20 mg/kg \times 4 doses every 2 h), which led to an increase in the basal extracellular level of striatal glutamate. Ultrastructurally, there was a corresponding decrease in the density of glutamate immunolabeling within nerve terminals associated with an asymmetrical (excitatory) synaptic contact only in the acutely MPTP-treated animals compared to the saline group. Also, in the acutely treated group, there was a decrease in the relative density of labeling of the AMPA receptor subunit GluR-2/3 within the dorsolateral striatum compared to the saline group.

In vivo microdialysis

Both subchronic and acute MPTP treatment resulted in significant decreases in the relative density of TH immunolabeling within the dorsolateral striatum (44 vs 65%, respectively) compared to the saline group. In response to different degrees of loss of TH labeling, we report a differential effect on the basal extracellular level of striatal glutamate. Similarly, it has been reported that identical subchronic and acute regimens of MPTP administration also produce differential amounts of striatal tissue dopamine loss; subchronic MPTP treatment does not deplete striatal tissue levels of dopamine to the same extent as acute drug administration (Heikkila et al., 1984; Ricaurte et al., 1986; Son-salla and Heikkila, 1986). Therefore, our results with TH

immunolabeling are consistent with these biochemical findings.

The observed decrease in the basal level of striatal glutamate following subchronic MPTP treatment could be due to a decrease in the release/synthesis or an increase in the uptake of glutamate. The decrease reported in this study may be explained by the observation that following the administration of either MPP⁺ or MPTP through the dialysis probe, there is an increase in the extracellular striatal level of dopamine (Wu et al., 2000). We speculate that this increase in extracellular dopamine could feed back onto the glutamate terminal, resulting in a decrease in the extracellular levels of glutamate. In support of this hypothesis, presynaptic dopamine D-2 receptors have been localized within the presynaptic terminal of synapses making an asymmetrical (excitatory) synaptic contact (Sesack et al., 1994) and it has been established that dopamine negatively affects glutamate synapse function, in that dopamine agonists are known to have an inhibitory effect on either release or basal levels of striatal glutamate (Godukhin et al., 1984; Maura et al., 1988; Mitchell and Doggett, 1980; Rowlands and Roberts, 1980; Yamamoto and Davy, 1992).

We hypothesized that the decrease in basal extracellular glutamate levels observed following subchronic MPTP treatment might be accentuated after an MPTP challenge due to the augmented loss of striatal dopamine. However, it has been reported that the active metabolite of MPTP,

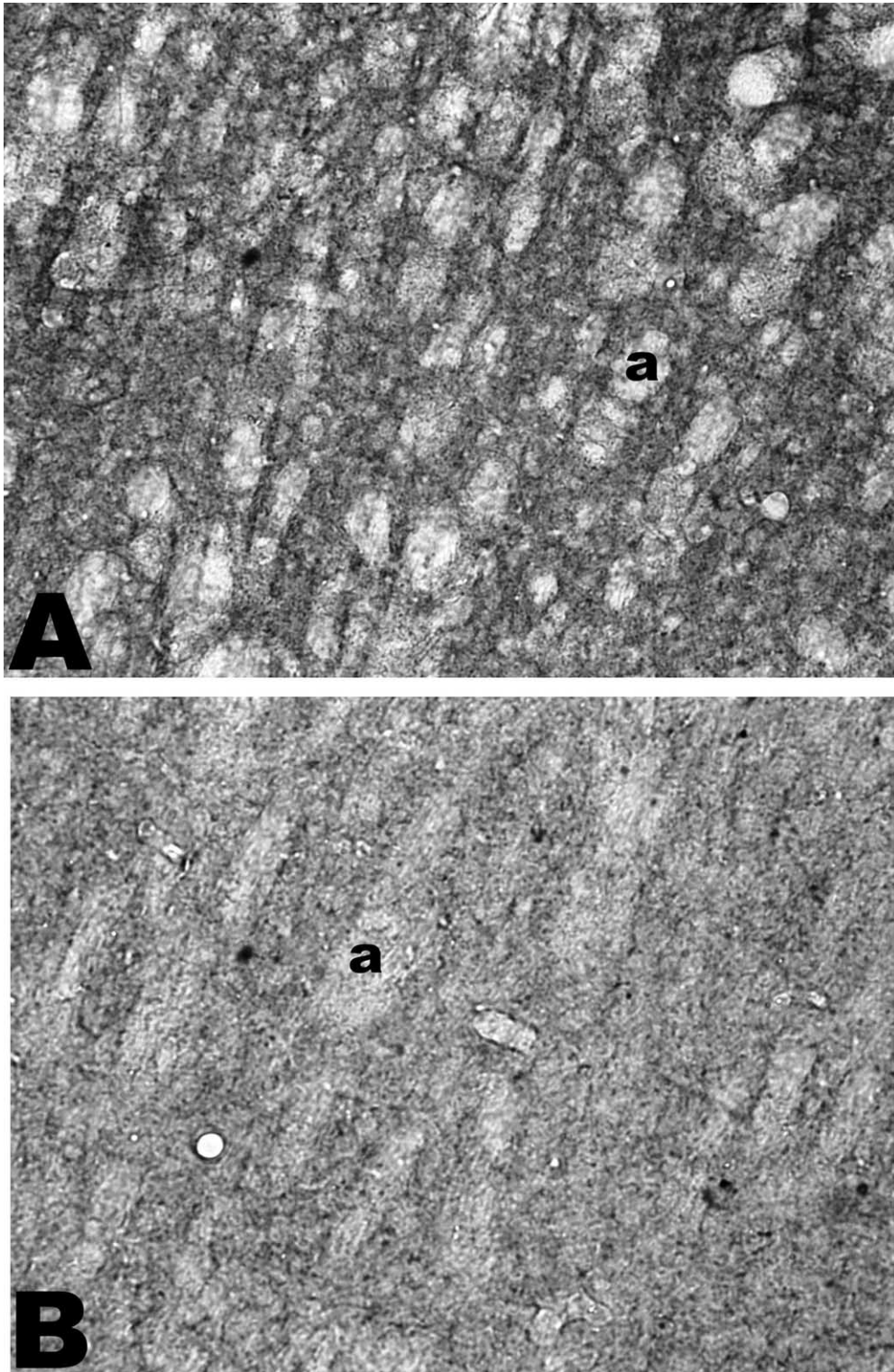


Fig. 4. Tyrosine hydroxylase (TH) immunolabeling within the dorsolateral striatum 30 days following acute administration of either MPTP (20 mg/kg \times 4 every 2 h) or saline. (A) This figure is TH labeling within a saline-treated animal. (B) This figure is from an acutely MPTP-treated animal. Note the significant decrease in TH labeling within the dorsolateral striatum in the MPTP-treated animal (B) compared to the saline-treated animal (A). a = bundle of myelinated axons.

namely, MPP^+ , blocks the uptake of dopamine into aminergic nerve terminals (Wright et al., 1998) and significantly impairs glutamate clearance in astrocytic cultures (Di

Monte et al., 1999). Dopamine is also known to block high-affinity glutamate uptake (Kerkerian et al., 1987). This should result in an increase in the extracellular level of

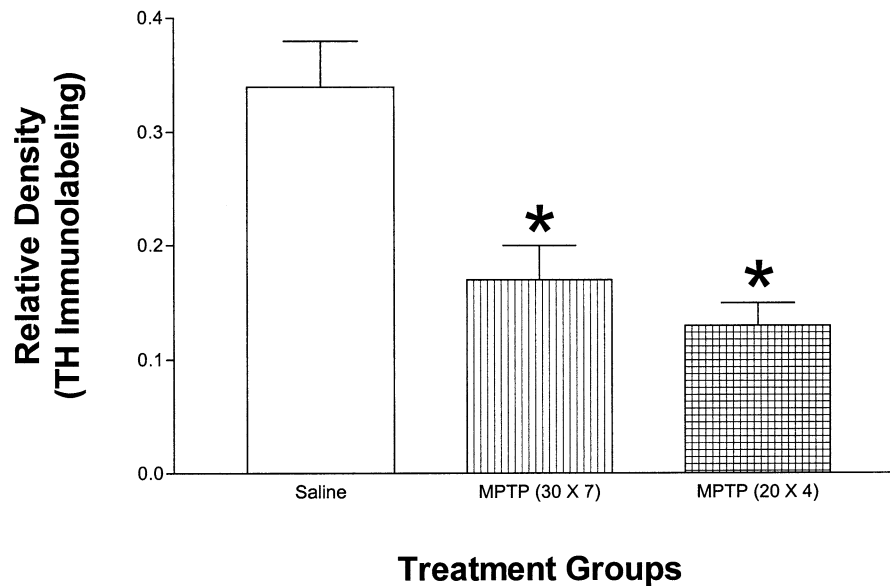


Fig. 5. Relative density of tyrosine hydroxylase (TH) immunolabeling within the dorsolateral striatum 30 days after saline (1 ml/kg) or subchronic (30 mg/kg/day \times 7 days) or acute (20 mg/kg \times 4 injections every 2 h) treatment with MPTP. There was a significant decrease in the density of TH immunolabeling in both the subchronic and acute MPTP-treated groups compared to the saline group. There was no difference between the two MPTP-treated groups. * $P < 0.05$ compared to the saline-treated group as determined by Peritz' f test for comparison of multiple means.

glutamate, a result consistent with the findings of the current study.

In contrast, following acute MPTP treatment, there is a significant loss of striatal dopamine tissue levels (up to 95%; Sonsalla and Heikkila, 1986). We speculate that this decreased the level of the negative feedback on the glutamate nerve terminal, resulting in the observed increase in the extracellular level of glutamate.

Given the reversal of extracellular glutamate levels following a challenge dose of MPTP as observed in the subchronically treated group compared to the decrease in extracellular glutamate levels in the acutely treated group following such a challenge, we speculate that there may be a greater number of dynamic changes in the striatum in response to moderate dopamine depletion compared to the changes following a greater percentage of dopamine loss. For example, even though there is a 44% decrease in TH immunoreactivity in the striatum of subchronically MPTP-treated mice, the balance between dopamine and glutamate levels in the striatum may be offset such that, overall, there is greater dopaminergic tone on striatal glutamate terminals, resulting in the observed decrease in basal extracellular glutamate. When the subchronically treated group is then challenged with MPTP, this balance may once again be perturbed, resulting in an increase in glutamate extracellular levels as reported in this study. Future studies will investigate alterations in striatal dopamine extracellular levels following a challenge dose of MPTP in order to further investigate the effects of this neurotransmitter on striatal glutamate function.

Depending on the dose and time course, MPTP administration results in either necrotic (acute MPTP) or apoptotic

(subchronic MPTP) cell death (Jackson-Lewis et al., 1995; Tatton and Kish, 1997). This distinction is of importance when investigating the acute effects of MPTP and the differential role that oxidative stress may play during these two processes of cell death needs to be taken into consideration. However, since the microdialysis and electron microscopic data was collected 30 days following MPTP treatment, a time point well beyond when both necrotic and apoptotic cell death has long ceased to be active, it is unclear as to the role of either of these processes in terms of influencing glutamate synapse function.

Differential alterations in activity of the thalamocortico-striatal pathway could account for the increase or decrease in striatal glutamate levels following acute or subchronic MPTP administration, respectively. We have reported time-dependent changes in extracellular levels of striatal glutamate 1 and 3 months following a 6-OHDA lesion of the nigrostriatal pathway (Meshul et al., 1999). It was suggested that the increase in basal extracellular levels 1 month after the lesion was due to an increase in corticostriatal activity and this reversed 3 months later. Changes in the firing rate/pattern of the excitatory input from the subthalamic nucleus to either the substantia nigra pars reticulata or the internal segment of the globus pallidus following the loss of dopamine neurons may account for this differential effect on striatal glutamate function (Bergman et al., 1994; Bevan and Wilson, 1999; Magil et al., 2001). The same differential alteration in activity of the subthalamic nucleus or globus pallidus following acute or subchronic MPTP treatment could account for the findings in the present study.

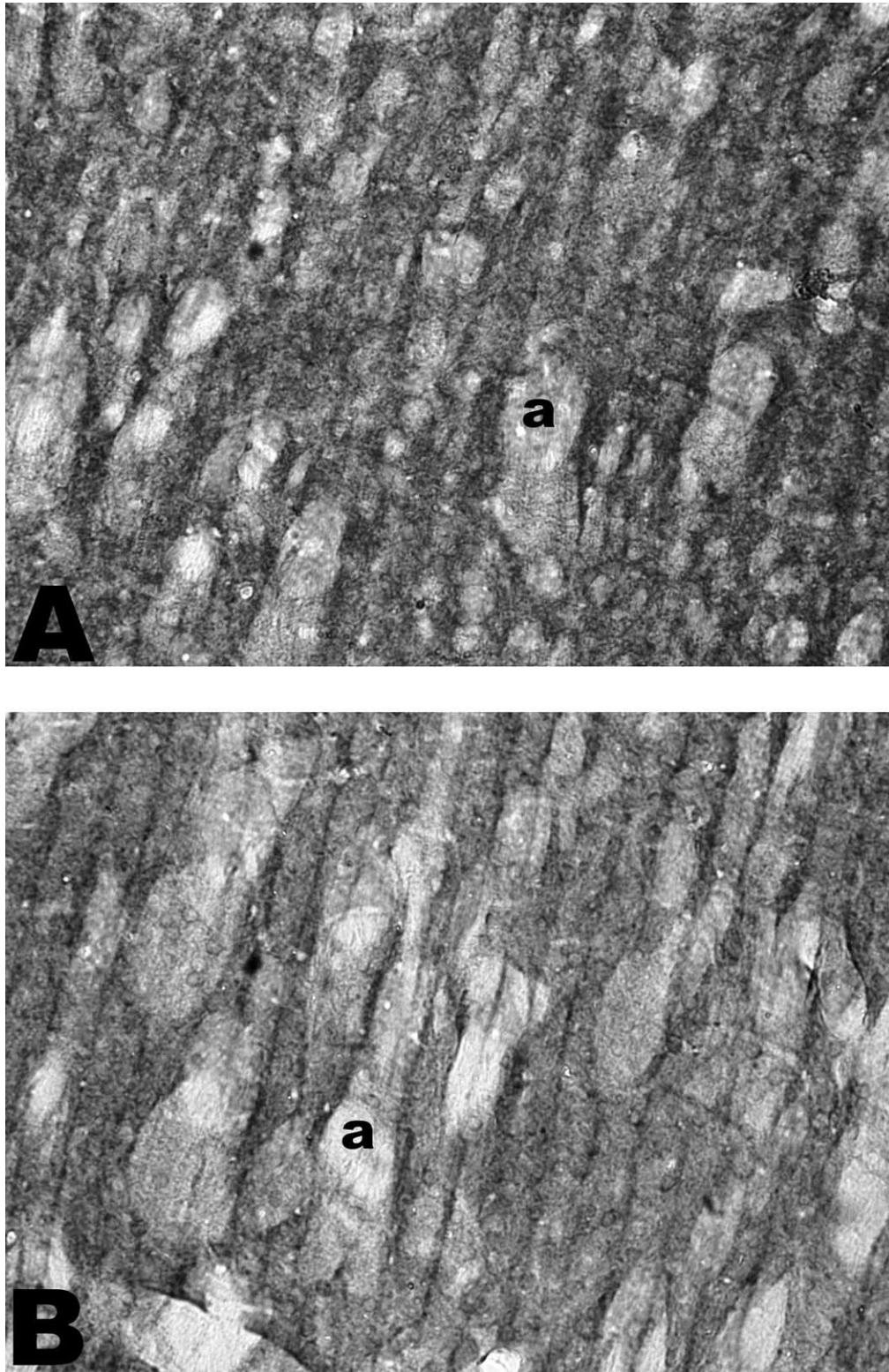


Fig. 6. Immunolabeling of the dorsolateral striatum for the GluR-2/3 receptor subunit 30 days following acute administration of either MPTP (20 mg/kg \times 4 every 2 h) or saline. (A) This figure is GluR-2/3 subunit labeling within the saline-treated group. Note that the labeling is evenly dispersed within the neuropil and shows no somal staining. (B) This figure is from an acutely MPTP-treated mouse. Note the decrease in GluR-2/3 subunit labeling within the dorsolateral striatum in the MPTP-treated group (B) compared to the saline group (A). a = bundle of myelinated axons.

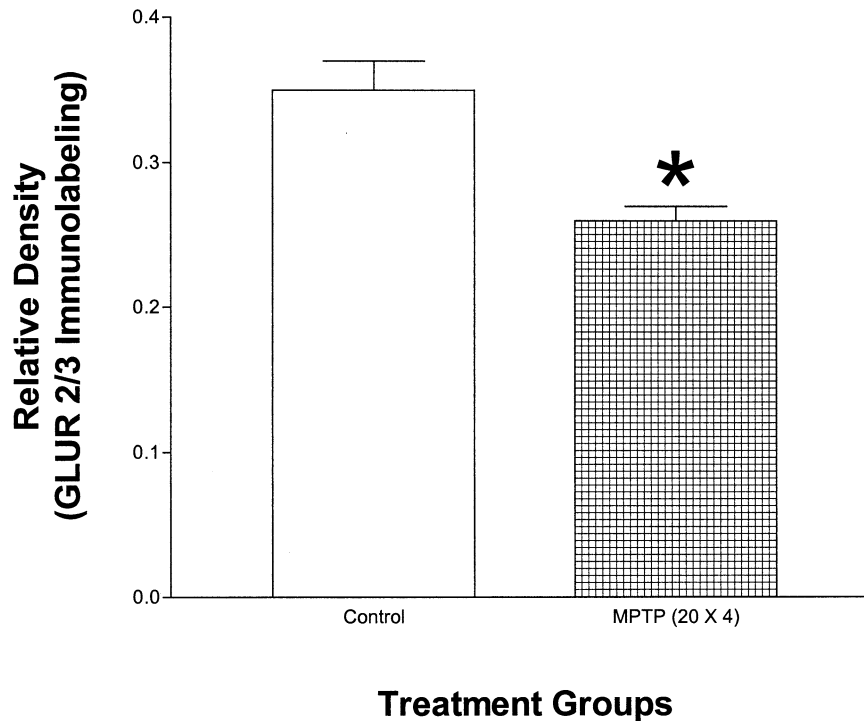


Fig. 7. Relative density of GluR-2/3 subunit immunolabeling within the dorsolateral striatum 30 days after saline (1 ml/kg) or acute (20 mg/kg \times 4 injections every 2 h) treatment with MPTP. There was a significant decrease in the density of GluR-2/3 subunit immunolabeling in the acutely MPTP-treated group compared to the saline group. * $P < 0.05$ compared to the saline group using the Student t test.

Nerve terminal glutamate immunolabeling

The decrease in the density of striatal glutamate immunolabeling within nerve terminals associated with an asymmetrical synaptic contact 30 days following acute MPTP administration is directly associated with an increase in the extracellular level of glutamate as measured by *in vivo* microdialysis. This is consistent with our earlier finding that 1 month following a unilateral lesion of the nigrostriatal pathway (>95% loss of striatal dopamine) the increase in the basal extracellular level of striatal glutamate was associated with a decrease in the density of nerve terminal glutamate immunolabeling (Meshul et al., 1999). It has been reported that acute MPTP can result in as great as a 95% bilateral loss of striatal dopamine (Sonsalla and Heikkila, 1986), although it appears to range from 74 to 95% (Ricaurte et al., 1986; Sonsalla and Heikkila, 1986). It is of interest that a unilateral lesion of the nigrostriatal pathway with the neurotoxin 6-OHDA results in a similar loss of striatal dopamine (> 95%; Meshul et al., 1999), suggesting that both a unilateral and bilateral loss of striatal dopamine (~90%) results in an increase in striatal glutamate function. This finding is important since it has been reported that a unilateral versus bilateral partial lesion of the nigrostriatal pathway results in a differential effect on mRNA expression for enkephalin compared to substance P, such that there were no changes in enkephalin mRNA expression following

a bilateral versus a unilateral nigrostriatal lesion (Salin et al., 1996).

Although there was a decrease in the extracellular level of striatal glutamate following subchronic MPTP treatment, there was no change in the density of nerve terminal glutamate immunolabeling. It is possible that the decrease in the extracellular glutamate level could be due to alterations in the release of glutamate, resulting in the nerve terminal compensating by decreasing the synthesis/uptake or increasing the breakdown of this neurotransmitter in order to maintain a steady-state pool of glutamate. This hypothesis would be consistent with our results showing a lack of change in the density of nerve terminal glutamate immunolabeling following subchronic MPTP treatment. As an interesting comparison, we have reported that subchronic administration of cocaine for 7 days results in no change in the density of glutamate immuno-gold labeling within the nucleus accumbens (Kozell and Meshul, 2001), although it has been reported that basal levels of extracellular glutamate are decreased in similarly treated animals (Pierce et al., 1996). This suggests that in some instances, there is not an exact association between changes in the level of extracellular glutamate as measured by *in vivo* microdialysis and quantitative immuno-gold electron microscopy. In this case, other factors, such as alterations in uptake or synthesis of glutamate that serve to restore the system back to a baseline level, need to be taken into consideration.

Glutamate receptor subunit immunolabeling

Excessive glutamatergic tone is considered a pathophysiological feature of Parkinson's disease. Given that we observed an increase in extracellular glutamate levels after acute MPTP treatment, the relative density of several glutamate receptor subunits was determined using light microscopic immunolabeling within the dorsolateral striatum, which receives a major glutamatergic input from the motor cortex (McGeorge and Faull, 1989). We chose to analyze the relative density of glutamate receptor immunolabeling in only the acutely treated group for the following reasons: this group had the greatest loss of TH immunoreactivity (and presumably the greatest loss of dopamine) and a significant decrease in the density of nerve terminal glutamate immunolabeling, corresponding to an increase in extracellular striatal glutamate levels. Since acute MPTP treatment results in an increase in striatal glutamate synapse function presynaptically, we predicted that alterations in the density of postsynaptic glutamate receptors may also be taking place. In this particular MPTP model, the only decrease found was in the relative density of GluR-2/3 subunit immunolabeling within the dorsolateral striatum compared to the saline group. This finding suggests that an increase in glutamate neurotransmitter release, as measured by *in vivo* microdialysis and immuno-gold electron microscopy, may be responsible for the decrease in the relative density of this specific glutamate receptor subunit. These results in both pre- and postsynaptic glutamate function are in agreement with previous findings from our group (Meshul et al., 1999) and others (O'Dell and Marshall, 1996; Porter et al., 1994), respectively, using the 6-OHDA lesion model.

Although there were no changes in any of the other glutamate receptor subunits we examined, it has been reported that several months after MPTP treatment in monkeys there is an increase in GluR1 protein expression within the caudate/putamen (Betarbet et al., 2000), no change in MK-801, AMPA, or NMDA binding (He et al., 2000; Calon et al., 2002; Silverdale et al., 2001), and no change in either glutamate or AMPA binding sites 2 weeks after MPTP treatment in C57/B6 mice (Wullner et al., 1993). In a 6-OHDA lesion model there was a small but significant decrease in the density of [³H]glutamate receptor binding (O'Dell and Marshall, 1996; Porter et al., 1994) in both rats and in patients with Parkinson's disease (Gerlach et al., 1996). There are variable reports of changes in NMDA receptor subunit expression and levels following a unilateral 6-OHDA nigrostriatal lesion (Dunah et al., 2000; Ganguly and Keefe, 2001). We conclude from these studies that although the basal glutamatergic tone in the striatum is elevated following acute MPTP administration or near-complete 6-OHDA lesioning, this alteration does not necessarily result in a decrease in several glutamate receptor subunits 1 month following reduction in striatal dopamine concentration.

Role of glutamate synapses in MPTP-induced dopamine loss

There appears to be no definitive role for the excitatory neurotransmitter glutamate in the loss of dopamine within the nigrostriatal pathway following MPTP administration. It was first reported that a single dose of MPTP was associated with a transient, 4-h decrease in the tissue content of striatal glutamate which returned to the control level after 24 h (Chan et al., 1994). It is apparent from the current study that multiple doses of MPTP can result in prolonged changes in striatal glutamate function.

There are numerous conflicting reports regarding the protective effect of the NMDA glutamate receptor antagonist MK-801 against the loss of dopamine within the striatum or dopamine cell bodies within the substantia nigra pars compacta. It has been reported that MK-801 provides either full protection (Srivastava et al., 1993; Storey et al., 1992; Turski et al., 1991), partial or temporary protection (Brouillet and Beal, 1993; Chan et al., 1993; Santiago et al., 1992; Tabatabaei et al., 1992), or no protection (Chan et al., 1997; Kupsch et al., 1992; Michel and Agid, 1992; Sonsalla et al., 1992). However, where glutamate antagonists may be of benefit is in reducing the side effects of *l*-dopa treatment in Parkinson's disease. There is some evidence (but see Domino and Sheng, 1993) in the various animal models of Parkinson's disease that coadministration of NMDA or AMPA receptor antagonists in conjunction with *l*-dopa has seen some success (Brotchie et al., 1991; Carlsson and Carlsson, 1989; Engber et al., 1994; Greenamyre et al., 1994; Jonkers et al., 2002; Klockgether et al., 1991; Loschmann et al., 1991; Papa and Chase, 1996; Starr, 1995a). Although increases in striatal glutamate release may play a role in the loss of dopamine within the striatum and/or substantia nigra following MPTP administration, it is clear from the current study that the dosing regiment used to induce various degrees of striatal dopamine loss clearly influences glutamate function within this brain region.

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