

Biochemical and Behavioral Recovery in a Rodent Model of Parkinson's Disease following Stereotactic Implantation of Dopamine-Containing Liposomes

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We have developed and characterized dopamine-containing liposomes which exhibited *in vitro* sustained release of dopamine for over 40 days. These liposomes were stereotactically implanted into the partially denervated corpus striatum of rats subjected to unilateral lesions of the substantia nigra. *In vivo* release of dopamine into striatal extracellular fluid was monitored by microdialysis and behavior was assessed by quantifying apomorphine-induced asymmetric rotation. Extracellular dopamine levels in the partially denervated striatum of the dopamine liposome-treated rats were greater than the levels in the lesioned rats which received control liposomes and these levels remained elevated for 25 days. In parallel, those rats which received dopamine liposomes exhibited partial behavioral recovery, with attenuation of asymmetric rotation following systemic apomorphine administration. These results suggest that dopamine-containing liposomes can partially ameliorate the deficits associated with a rodent model of Parkinson's disease, and demonstrate the potential of this technology as a method for the controlled delivery of therapeutic agents into discrete areas of the brain. © 1992 Academic Press, Inc.

INTRODUCTION

Parkinson's disease (PD) has served as a prototypic neurodegenerative disease for the development of novel therapeutic strategies as it has perhaps the simplest neurochemical signature and is responsive to therapies

which facilitate striatal dopamine neurotransmission, and animal models of PD are well established.

The mainstay of current PD treatment is the use of L-dopa (7, 22, 23, 36). However, inconstant clinical effects, in part due to wide fluctuations in plasma and brain concentrations of the drug (25, 30), limit its efficacy. A number of alternative strategies have been developed to obtain more stable enhancement of dopaminergic neurotransmission including controlled release L-dopa preparations (5, 6, 29); subcutaneous (26), intravenous (28), and intracerebroventricular (14) infusions of L-dopa or dopaminergic agonists; as well as the direct infusion of dopamine into the striatum (12). Perhaps the most dramatic attempt at restoring the dopamine deficit has been the transplantation of tissue into the brain (1, 20, 21). However, the early promising results from autologous, adrenal transplantation have not been confirmed (19), and the role and efficacy of fetal tissue transplants remains unclear (15, 31).

An alternative to these approaches includes the use of controlled release technology to deliver therapeutic agents directly to specific areas of the brain. We have recently reported on the development and use of a dopamine-ethylenevinylacetate (EVA) co-polymer with sustained zero-order release characteristics (9, 11). However, in the rodent, the size of the implant necessitated the resection of overlying cortex for placement adjacent to the corpus striatum. An alternative, potentially less traumatic, controlled delivery system is polymer-based microspheres. However, the *in vitro* release kinetics of such polymeric microspheres are not satisfactory for chronic delivery: 90% of the dopamine content released *in vitro* within 24 h from a polylactide polymer (24).

Another potential delivery system for controlled release of dopamine is the use of liposomes. Liposomes are lipid vesicles in which drugs may be encapsulated either in their aqueous compartment and/or within the

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free of gliotic infiltration, and cellular preservation was excellent.

These results suggest that replacement of extracellular dopamine in the striatum using liposomes may partially restore the biochemical and behavioral deficits in an animal model of Parkinson's Disease. The mode of action of brain tissue implants (either of adrenal or fetal origin) imbedded in or overlying the corpus striatum continues to be controversial. Accumulating evidence suggests that tonic release of dopamine is not necessarily the only mode of action of such transplants. Release of growth and/or trophic factors with sprouting of surviving neurons may also be responsible for recovery (15, 31). Nonetheless, this study, and several others in which dopamine has been either directly infused via pump (12) or injected via microcapsules (24) or polymer (4, 35), suggests that replacement of dopamine *per se* may be adequate for behavioral recovery.

Although we have demonstrated release and partial behavioral recovery for only several weeks, it is possible that the membrane composition of the liposomes can be altered, for example by increasing the cholesterol content or alternatively by microencapsulating the liposome (17, 18), to produce liposomes which may be able to release an amine, or other pharmacological agent, for extended periods of time, thereby rendering this technology suitable for long-term replacement therapy in a number of neurological diseases. Moreover, in some disorders including brain tumors, pain, and cerebrovascular accidents, controlled and stereotactically localized release of pharmacological agents for short periods of time (ranging from days to weeks) may prove sufficient for optimal response.

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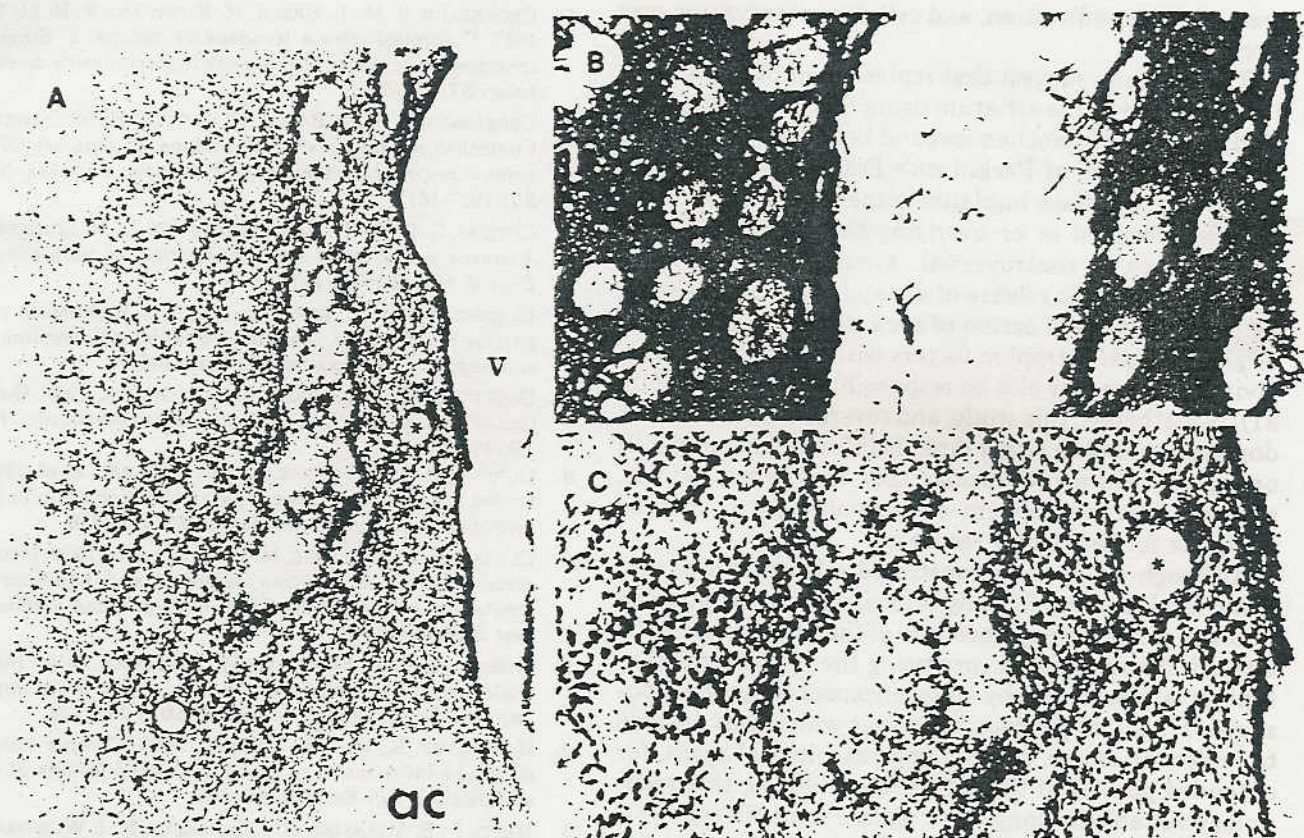


FIG. 4. Histological assessment of injection site. (A) Photomicrograph showing maximal extent of tissue loss induced by liposome injection. The central cavity is surrounded by an area of gliotic infiltration, which rapidly merges with an intact neuronal area. The small gliotic area and the adjacent intact neurons (arrow) can be seen more clearly in C. ac, anterior commissure; v, ventricle; *, blood vessels. (B) Acetylcholinesterase staining. Not only are neurons evident in the area immediately adjacent to the cavity, but in addition the neuropil is intact as reflected by no loss of acetylcholinesterase staining in the area immediately surrounding the central core of the injection.

mine concentration in the immediate vicinity of the implant is likely to have reached even higher, nonphysiological levels. Diffusion of dopamine from a point source through intact striatum is markedly limited; however, in the denervated striatum, with reduction in process density and neurotransmitter reuptake, diffusion is much greater (8a).

The lag in the behavioral response compared to the biochemical recovery is likely to reflect the mechanism by which apomorphine-induced asymmetric rotation occurs. This rotation is believed to result from asymmetric postsynaptic receptor sensitivity, with the partially denervated side having a compensatory supersensitivity of postsynaptic receptors (34). The time course of the development of these receptor changes is in the order of 7 to 10 days, so despite achieving high ECF DA levels on Day 4, the normalization of postsynaptic receptor sensitivity would not be expected to occur until 7 to 10 days postimplantation. The significant reduction in rotations on Day 13 is likely to be due to this mechanism. Although such normalization of receptor sensitivity would be expected to persist so long as ECF DA levels

remained within a "normal" range, it is apparent that the DA levels achieved on Day 26, despite being significantly elevated above the levels found in the lesioned animals with control liposomes, were inadequate to maintain the normal postsynaptic receptor sensitivity. However, there was a trend for reduced rotation which did not achieve significance because of the large variation in rotational behavior.

Prior to liposome implantation, there was an inverse correlation between extracellular levels of dopamine (obtained on Day 0 dialysis) and rotational behavior. This relationship was maintained following implantation with those rats having the greatest restoration of ECF dopamine having the most attenuated rotational response to apomorphine.

The histological examination of the striatum was marked by a limited necrotic central core at the injection site. This cavity was orientated dorsoventrally (i.e., along the needle tract), and its extent was consistent with the injection volume of 3 μ l. Gliosis was essentially confined to a zone within 750 μ m of the injection site. Despite central cavitation, tissue beyond this zone was

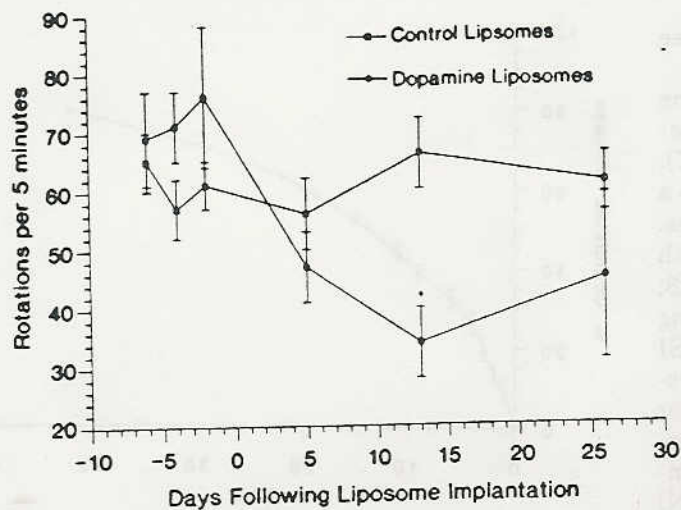


FIG. 2. Apomorphine-induced rotations in Nigrostriatal-lesioned rats: Effect of intrastriatal implantation of dopamine-containing liposomes. On three occasions prior to implantation and on Days 5, 13, and 26 postsurgery, rats were administered apomorphine (1 mg/kg ip) and placed in the rotometers with complete rotations quantitated in the 5-min interval, 15 to 20 min post drug administration. In the group of rats which received DA-containing liposomes ($n = 5$), there was a $53 \pm 9\%$ attenuation of the rotational behavior on Day 13, which remained attenuated on Day 26 by $40 \pm 11\%$. Vertical bars represent the SEM, * $P < 0.05$, ANOVA with repeated measures.

rotational behavior in the DA-containing liposome group ($P < 0.05$).

In Vivo Release

The basal dopamine release (Day 0) in the left, unlesioned striatum of the control liposome rats was 2.27 ± 0.24 fmol/min (uncorrected for probe recovery), whereas on the right it was reduced by 88.6% to 0.26 ± 0.04 fmol/min. In the DA-containing liposome rats, the left striatum basal release was 2.22 ± 0.15 fmol/min and on the lesioned side it was 0.32 ± 0.06 fmol/min. These values were not significantly different from control liposome animals. Following liposome implantation there was no significant change in the DA release in the control liposome-treated rats. However, in those rats which received the DA liposomes, the ipsilateral ECF DA increased to 4.58 ± 1.1 fmol/min on Day 4 and remained significantly elevated above the control animals, although levels had fallen to less than those in the contralateral striatum by Day 12 (Fig. 3).

Histology

The injection site for the liposomes was marked by cavitation (Fig. 4A). The cavity was filled with glial elements (Fig. 4C). However, adjacent to the core of the injection site the tissue was remarkably well preserved. Excellent neuronal preservation and very limited gliosis

was noted in the tissue surrounding the injection site (Figs. 4A and 4B). The intact nature of the neuropil surrounding the injection could also be seen using acetylcholinesterase staining (Fig. 4B).

DISCUSSION

The *in vitro* data suggest that controlled dopamine release obtained from the liposomes can extend beyond 40 days and that such release approximates kinetics between zero- and first-order. The *in vivo* release of dopamine which was quantified by microdialysis at a site 1.5 mm from the implantation site suggests that extracellular dopamine levels were reached which exceeded the control values, and that these levels declined over 25 days, but remained elevated above the baseline concentrations.

The restoration of extracellular dopamine via release from dopamine-containing liposomes was associated with behavioral recovery as determined by the apomorphine-induced rotation on Day 13. The significant behavioral recovery on Days 4 through 13 persisted despite a drop in ECF dopamine concentrations. The supra-elevated dopamine levels on Day 4 (obtained 1.5 mm from the implantation site) suggest that the dopa-

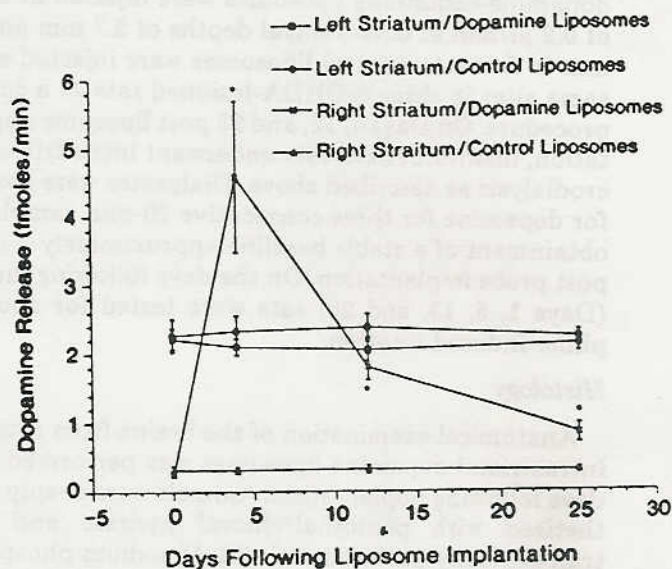


FIG. 3. Dopamine release in the striatum of right nigrostriatal-lesioned rats: Effect of intrastriatal implantation of dopamine-containing liposomes. *In vivo* release. Striatal extracellular fluid levels of dopamine are reported as femtomoles per minute of dialysate, uncorrected for recovery, at baseline (Day 0) and at the four time points postimplantation (Days 4, 12, and 25). There is a significant increase in dialysate dopamine concentration in the lesioned striatum of the rats who received intrastriatal implantation of the dopamine-containing liposomes ($n = 5$), whereas there was no change in the rats who received control liposomes ($n = 3$). Vertical bars represent the SEM, * $P < 0.05$, ANOVA with repeated measures.

hemispherical and Plexiglas rotometers, allowing free movement.

Dialysate samples were directly assayed for dopamine at 20-min intervals using high-pressure liquid chromatography with electrochemical detection (HPLC-EC). Twenty microliters of the dialysate was injected onto a 10×2.1 -mm column packed with C_{18} 3- μ m particles. The mobile phase was a 70 mM phosphate buffer with 2.5 mM sodium octyl sulphate, 0.1 mM EDTA, pH 5.8; MeOH 15% v/v. A nonpulsatile flow was achieved using an Altex 110 double piston pump with an in-line SSI pulse dampener at a rate of 0.25 ml/min. With this system dopamine is selectively retained and the major acidic metabolites elute within the solvent front. Chromatograms were completed within 6 min. A LC3 potentiostat (Bioanalytical Systems, West Lafayette, IN) with a low-volume electrochemical cell and a voltage of 0.7 V vs a Ag/AgCl reference electrode was used for electrochemical detection.

Following injury release of dopamine in the first three to four collections, a stable baseline was recorded over the next 2 h, approximately 3 to 5 h following probe implantation. Rats were then anesthetized with 3 ml/kg of equithesin and a Hamilton 10- μ l syringe with a 28-gauge needle implanted into the right corpus striatum through the anterior skull hole. Two 3- μ l volumes of dopamine-containing liposomes were injected at a rate of 0.2 μ l/min at dorsoventral depths of 5.7 mm and 4.0 mm in five rats; control liposomes were injected at the same sites in three 6-OHDA-lesioned rats as a control procedure. On Days 4, 12, and 25 post liposome implantation, unanesthetized rats underwent intrastriatal microdialysis as described above. Dialysates were assayed for dopamine for three consecutive 20-min samples on obtainment of a stable baseline approximately 2 to 3 h post probe implantation. On the days following dialysis (Days 1, 5, 13, and 26) rats were tested for apomorphine-induced rotation.

Histology

Anatomical examination of the brains from rats with intrastriatal dopamine liposomes was performed at 30 days following implantation. Animals were deeply anesthetized with pentothal/chloral hydrate and then transcardially perfused with 0.1 M sodium phosphate-buffered saline, followed by 4% paraformaldehyde in the same buffer. Frozen sections were cut at 40- μ m intervals through the striatum. Sets of sections were stained with cresyl violet, and adjacent sections stained for demonstration of acetylcholinesterase (using isomylpyridinium inhibition of butyryl cholinesterase) following the method of Karnovsky and Roots (16).

Statistical Analysis

A total of 8 animals were studied (dopamine-containing liposome group, 5; control liposome group, 3). The

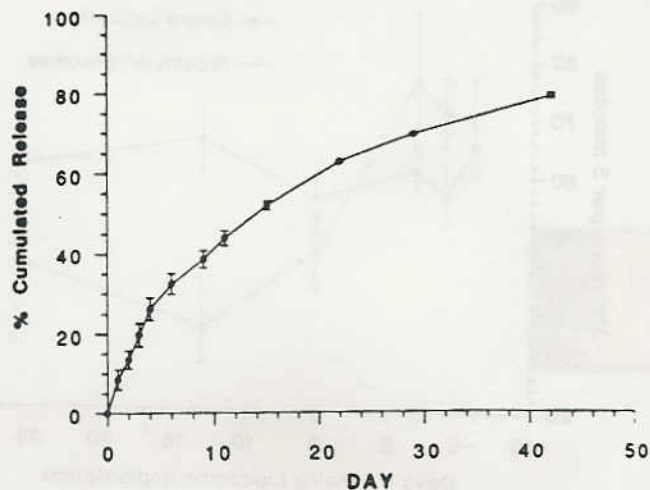


FIG. 1. *In vitro* release of dopamine from liposomes. The *in vitro* experiments revealed a controlled release of dopamine from the liposomes. The release was followed for 42 days at which stage $79.5 \pm 0.6\%$ of the total content of liposomal dopamine (560 μ g) had been released. Data represent the mean cumulated release of dopamine over time with vertical bars representing the standard error.

behavioral rotational data for each group of animals and the microdialysis data from control and lesioned striata in both groups were analyzed by repeated measures analysis of variance with Fisher's post hoc test. Values were deemed significant at the $P < 0.05$ level.

RESULTS

In Vitro Release

The cumulative *in vitro* release of dopamine from the liposomes is represented in Fig. 1. Release kinetics were between zero- and first-order. Release from liposomes usually closely parallels first-order kinetics; the more linear release kinetics of these liposomes may in part be due to the use of the hydrogenated phospholipid which formed a "gel state" bilayer at 37°C. This bilayer provides a significant barrier to dopamine release. In addition, the charge of the liposome membrane is negative. Since dopamine at physiological pH is positively charged, the electrostatic repulsion may have also acted to delay release.

Rotational Behavior: Apomorphine-Induced Rotation

The data obtained from the baseline and postimplantation rotation experiments are represented in Fig. 2. In the control liposome-treated group of lesioned rats there was a nonsignificant trend toward a reduced number of rotations, whereas in the rats implanted with DA-containing liposomes, there was a significant attenuation of the rotational behavior on Day 13. In addition, the overall ANOVA showed a significant reduction in

lipid membrane (2). We have therefore engineered dopamine-containing liposomes which release dopamine for over 40 days *in vitro* with kinetics between zero- and first-order. These liposomes were stereotactically implanted within the partially denervated striata of rats, who had received unilateral, intranigral injections of the neurotoxin, 6-hydroxydopamine (6-OHDA), a well-established model of PD (33). *In vivo* intrastriatal dopamine release and behavioral correlates were monitored at repeated intervals for 25 days following liposome implantation.

MATERIALS AND METHODS

Preparation of Dopamine-Containing Liposomes

The liposomes were prepared by a modification of the reverse phase evaporation (REV) technique (32). Briefly, 132 μmol of hydrogenated soylcithin (PPC, Nattarman, Cologne, FRG), cholesterol (CH, Sigma), and dicetylphosphate (DCP, Sigma), (molar ratio of 4.5:4.5:1) were dissolved in 12 ml of chloroform/diisopropyl ether (1:2, v/v) in a 100-ml round bottom flask at 60°C. To this organic phase, a 2-ml aliquot of 50 mg/ml dopamine hydrochloride (Sigma) in buffer (10 mM phosphate-buffered saline, pH 4.5, containing 0.4% EDTA) was added. The mixture was sonicated for 1 min at 60°C with a bath sonicator. The organic phase was evaporated (Rotavapor R110, Brinkman) until a gel formed and further continued until the liposome formation was complete. Unencapsulated dopamine was removed by repeated centrifugation (Sorvall RC-5B, DuPont Instruments) at 20,000g for 30 min and replacement of the supernatant with fresh buffer. The final pellet was resuspended in 1 ml of buffer. The dopamine concentration was determined by adding an equal volume of *n*-butanol to a diluted sample of the liposome preparation. After thorough vortexing, the emulsion was separated into two phases by centrifugation and absorbance was measured in the aqueous phase at 269 nm. The dopamine concentration was calculated to be 14 mg/ml in the final liposome preparation.

In Vitro Release Experiments

One milliliter of liposomes was diluted to 10 ml with 10 mM phosphate-buffered saline (PBS), pH 7.4, containing 0.4% EDTA. Four milliliters of this suspension was transferred into dialysis tubing (Spectraphor 7, MW cutoff 50,000 Da Spectraphysics) and sealed with closures. Duplicate samples were placed into 25-ml bottles and 20 ml of buffer (PBS, pH 7.4) was added. Release experiments were performed by shaking the bottle at 150 rpm at 37°C in an air gravity incubator. At specified time intervals, the buffer was removed and replaced with fresh buffer. To maintain sink conditions, dopamine concentrations in the buffer were never allowed to

exceed 100 μM . To prevent oxidation of dopamine, air was removed from the buffer and the bottles by flushing with argon. Dopamine release was quantitated spectrophotometrically at 269 nm.

Unilateral Nigral 6-OHDA Lesions

Unilateral substantia nigra lesions were produced as previously described (8). In brief, 6-OHDA was injected into the right anteromedial substantia nigra (AP -5.5, L 1.5, V 8.1 (flat skull) (27)) of male Sprague-Dawley rats (250-280 g, Charles River). Eight micrograms (free base) of 6-OHDA (Sigma, St. Louis, MO) was dissolved in 4 μl of ice-cold saline containing 0.2 mg/ml ascorbic acid and was injected into each animal over an 8-min interval using a 10- μl Hamilton syringe. The syringe was removed 5 min after the injection was terminated.

Behavioral Testing

Two to three weeks postoperatively, lesion adequacy was tested by measuring apomorphine-induced rotation. Rats were administered apomorphine (1 mg/kg) intraperitoneally (ip) and placed in a hemispherical Plexiglass rotometer. In rats with >90% striatal dopamine depletion, this dose of apomorphine results in greater than 20 rotations/5-min period (15 to 20 min post apomorphine administration) (13).

Lesioned rats with greater than 7 rotations/min were anesthetized with equithesin (Nembutal, 81 ml; chloral hydrate, 21.25 g; MgSO_4 , 10.63 g; propan-1-2-diol, 198 ml; absolute alcohol, 50 ml made up to 500 ml in H_2O) (3 ml/kg) and placed in a Kopf stereotaxic frame. The skull was exposed and holes were drilled above the right and left striatum (coordinates: AP + 0.5, L 2.5) with an additional hole drilled above the right striatum at coordinates AP + 2.0, L 2.5. Eighteen gauge guide cannulae were cemented in place and the animals allowed to recover from surgery. Over the following 2 weeks, animals were repeatedly tested for rotational behavior and a stable baseline was obtained when the number of rotations in three consecutive tests varied by less than 25%.

Intracerebral Microdialysis

Unanesthetized rats, accustomed to handling, had bilateral dialysis probes implanted through the guide cannulae. The probes were of concentric design (2, 9) with 4 mm exposed surface and an outer diameter of 300 μm . The probes were perfused with a microperfusion pump (Model 22, Harvard Instruments, Sth Natick, MA) at 1.2 $\mu\text{l}/\text{min}$ with an artificial extracellular fluid (Na+ 135 mM, Ca^{2+} 1.2 mM, Mg^{2+} 1.0 mM, K+ 3.0 mM, ascorbate 100 μM , 2.0 mM phosphate buffered to pH 7.4) via a 2-channel liquid swivel (BAS, West Lafayette, IN). During the period of dialysis, rats were placed in the

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